

Intranasal Treatment with a Recombinant Hypoallergenic Derivative of the Major Birch Pollen Allergen Bet v 1 Prevents Allergic Sensitization and Airway Inflammation in Mice

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Key Words

Type I allergy · Birch pollen · Bet v 1 · Recombinant hypoallergenic fragments · Tolerance/suppression · Mucosa · Animal model, in vivo · BALB/c

Abstract

Background: The major birch pollen allergen Bet v 1 represents one of the most prevalent environmental allergens responsible for allergic airway inflammation.

Objective: In the present study we sought to compare the complete recombinant Bet v 1 allergen molecule with genetically produced hypoallergenic fragments of Bet v 1 regarding mucosal tolerance induction in a mouse model of allergic asthma. **Methods:** BALB/c mice were intranasally treated with recombinant Bet v 1 or with two recombinant Bet v 1 fragments (F I: aa 1–74; F II: aa 75–160) prior to aerosol sensitization with birch pollen and Bet v 1. **Results:** Intranasal application of F II, containing the major T cell epitope, led to significant reduction of IgE/IgG1 antibody responses, in vitro cytokine production (IL-5, IFN- γ , IL-10) and negative immediate cutaneous hypersensitivity reactions comparable to the pre-

treatment with the complete rBet v 1 allergen. Moreover, airway inflammation (eosinophilia, IL-5) was inhibited by the pretreatment with either the complete Bet v 1 or F II. However, for prevention of airway hyperresponsiveness the complete molecule was required. The mechanisms leading to immunosuppression seemed to differ in their dependence on the conformation of the molecules, since tolerance induced with the complete Bet v 1, but not with F II, was transferable with spleen cells and associated with increased TGF- β mRNA levels. **Conclusion:** We conclude that mucosal tolerance induction with recombinant allergens and genetically engineered hypoallergenic derivatives thereof could provide a convenient and safe intervention strategy against type I allergy.

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Introduction

The prevalence of type I allergy has constantly increased within the last decades, leading to the fact that presently 25% of the population in industrialized countries are afflicted by allergic diseases. Clinical symptoms

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such as allergic rhinitis, conjunctivitis or bronchial asthma are mainly elicited by airborne allergens, among which birch pollen (BP) represents an important source. The major allergen of BP is Bet v 1, a 17-kD molecule [1], to which 95% of the BP-allergic patients react [2, 3].

Bet v 1 is one of the best-characterized allergens, which has been produced as recombinant allergen in *Escherichia coli* [4, 5]. The natural and recombinant (r) Bet v 1 molecules possess equivalent immunological and biological properties [6]. Studies on T cell-activating potency and IgE-binding properties revealed the existence of hypoallergenic isoforms of natural Bet v 1, characterized by a low IgE-binding capacity but a strong T cell-activating property [7]. By genetic engineering hypoallergenic recombinant molecules were produced [8, 9]. One approach was to disrupt the three-dimensional structure and thereby the conformational B cell epitopes by producing two halves of the Bet v 1 molecule [8]. These fragments, comprising aa 1–74 (F I) and aa 75–160 (F II), displayed a more than 100-fold reduced capacity to induce basophil histamine release and immediate type I skin reactivity in BP-allergic patients [8, 10].

Allergen avoidance or prolonged breast feeding are recommended prophylactic measures in atopics [11, 12]. The only curative treatment in certain cases of pollinosis is specific immunotherapy (SIT), performed by injecting increasing doses of allergen to induce a state of hyporesponsiveness [13, 14]. Even though this treatment is effective in the majority of patients it has several disadvantages. One is that the administration of the allergenic molecules may cause severe and life-threatening anaphylactic reactions. Hypoallergenic molecules have therefore been suggested to improve the safety of SIT. Another disadvantage of conventional SIT is the long duration of the treatment and the frequent injections, which can lead to low compliance of the patients. Thus, treatment strategies combining clinical efficacy with safety and improved acceptance of the patient need to be developed. One strategy for improvement of conventional measures may be the change of the conventional parenteral route of administration to application of allergens, in particular of hypoallergenic molecules, via mucosal surfaces.

In experimental animal models it has been demonstrated that mucosal administration of soluble antigens leads to a state of antigen-specific unresponsiveness termed oral/mucosal tolerance [15]. Mucosal tolerance has therefore been suggested as a treatment strategy against diseases mediated by immunological hyperreactivity, such as autoimmune diseases [16, 17] and allergies [18–20].

We have previously established a mouse model of allergic sensitization to BP and its major allergen Bet v 1 [20, 21]. The present study was performed to compare the effectiveness of mucosal tolerance induction with the complete Bet v 1 molecule and the two recombinant non-anaphylactic fragments of Bet v 1 in preventing allergic immune responses as well as airway inflammation.

Material and Methods

Animals

Female, 7-week-old BALB/c mice were obtained from Charles River (Sulzfeld, Germany). All experiments were approved by the Animal Experimentation Committee of the University of Vienna and the Federal Ministry of Education, Science and Culture.

Antigens

rBet v 1 was obtained from Biomay GesmbH (Linz, Austria). rBet v 1 fragments, comprising aa 1–74 (F I) and aa 75–160 (F II) of Bet v 1a, were expressed in *E. coli* strain BL21 (DE3) and purified as described [8]. Pure BP was purchased from Allergon (Välinge, Sweden) and BP extract was prepared as previously described [20].

Sensitization and Tolerization

Sensitization was performed by one intraperitoneal (i.p.) injection of 1 µg of Al(OH)₃-adsorbed rBet v 1 followed by a 10 days' aerosol exposure to 0.1 % w/v BP extract as previously described [20]. The sensitization was followed by an aerosol challenge with a 1 % w/v BP extract on 2 consecutive days.

For tolerance induction rBet v 1, F I, F II or an equimolar mixture of fragments I+II (F I+II) were applied 3 times intranasally (i.n.) at a concentration of 10 µg in 30 µl 0.9% NaCl prior to sensitization. Control mice were treated i.n. with 30 µl of 0.9% NaCl followed by sham immunization with phosphate-buffered saline (PBS) or sensitization with rBet v 1/BP as described above.

Experimental Design

Group 1: Group 1 contained untreated, naive mice.

Group 2: Sham Sensitization. Mice were 3 times i.n. pretreated with NaCl (days 0, 7, 14). After another 10 days (day 24) the mice were injected i.p. with PBS/Al(OH)₃ and 14 days later they were aerosol-exposed to PBS during 10 days (days 38–42, 45–49). Seven days later the mice were challenged with 1% BP extract on 2 consecutive days (days 57, 58). Sampling and analyses were performed 24 h after the aerosol challenge (day 59).

Group 3: Sensitization. NaCl was i.n. applied 3 times (days 0, 7, 14) (sham pretreatment). Ten days later the mice received one i.p. injection of Al(OH)₃-adsorbed rBet v 1. Fourteen days later an aerosol treatment with 0.1% BP solution was performed during 10 days (days 38–42, 45–49). Aerosol challenge and sampling were performed as described for group 2.

Groups 4–7: Pretreatment. Mice were 3 times i.n. pretreated with rBet v 1 (group 4), F I (group 5), F II (group 6) or with an equimolar mixture of F I+II (group 7). Sensitization, antigen challenge and sampling were performed as described for group 3.

Sampling

Blood samples were taken before and 7 days after sensitization by tail bleeding. Twenty-four hours after the last aerosol challenge (day 56) bronchoalveolar lavage (BAL) fluid was collected as previously described [21].

Spleens and lungs with associated lymph nodes were removed under sterile conditions. The organs were homogenized, filtered through sterile filters and cell suspensions prepared as described [20].

Allergen-Specific Antibody Levels and Type I Skin Tests in vivo

Microtiter plates were coated with rBet v 1 (2 µg/ml). Serum samples were diluted 1/1,000 for IgG1, 1/500 for IgG2a and 1/10 for IgE antibodies. Rat anti-mouse IgG1, IgG2a and IgE antibodies (1 µg/ml, Pharmingen, San Diego, Calif.) and thereafter peroxidase-conjugated mouse anti-rat IgG antibodies (1/1,000, Jackson Immuno Lab, West Grove, Pa.) were used. Results reflect optical density (OD) values after subtraction of baseline levels from preimmune sera as described [21].

Intradermal skin tests were performed according to previous description [20]. rBet v 1 (2.5 µg/ml) and BP extract (2.5 µg/ml) were intradermally injected and the skin reactions were evaluated as described [20].

T Cell Epitope Mapping and Cytokine Production in Lung and Spleen Cell Cultures

Overlapping dodecapeptides, covering the whole Bet v 1 sequence, were used for epitope mapping experiments as previously described [22]. Proliferation assays were performed with spleen cells from Bet v 1-sensitized mice (2×10^5 /well) incubated with 2 µg/well peptides, F I, F II and rBet v 1 as described [22].

IL-5, IFN-γ and IL-10 were measured in supernatants of spleen and lung cell cultured (10^7 cells/0.5 ml/well), stimulated with or without BP extract (10 µg/well), using mouse ELISA kits (Endogen, Cambridge, Mass.) as previously described [20]. Results reflect the measured levels after subtraction of baseline levels of unstimulated cultures.

Airway Eosinophilia

BAL samples were spun onto microscope slides, air-dried, stained with hematoxylin and eosin (Hemacolor; Merck, Darmstadt, Germany) and total number of eosinophils/ 10^3 cells was determined as previously described [23].

Lung Histology

Lungs were inflated in situ endotracheally with 15% sucrose and 25% OCT compound (Tissue-Tek OCT compound, Sakura Finetek Europe BV, Zoeterwoude, The Netherlands) in PBS. Following ligation of the right main stem bronchus and vessels the right lung was removed, frozen at -20°C in OCT compound and stored at -70°C . The left lung was fixed with 7.5% neutral-buffered formalin, removed and embedded in paraffin. Five micrometer paraffin and cryostat sections were stained with Giemsa and graded regarding the intensity of the inflammatory infiltration on a semiquantitative scale from 0 to 3 (0 = no clustering of inflammatory cells around bronchi or vessels; 1 = one or two centrally located inflammatory cell aggregates; 2 = inflammatory cell aggregates around a majority of central bronchi and vessels; 3 = extension of inflammation to the lung periphery). The grading of slides was performed blinded as to the treatment.

Determination of Airway Responsiveness

Measurements of airway responsiveness were performed 24 h after the last challenge (day 59) by head-out body plethysmography as previously described [24]. Airflow signals were recorded in response to methacholine (0, 12.5, 25, 50, 100, 200 mg/ml) and aerosolized with a nebulizer. The concentration of methacholine that caused a 50% reduction in expiratory air flow (MCh_{50} , mg/ml) was determined.

Detection of TGF-β mRNA

TGF-β mRNA levels were determined as previously described [20]. Twenty-four hours after the last aerosol exposure spleen cell suspensions were prepared, the cells within groups 3, 4 and 6 were pooled and 2×10^7 cells were used for RNA preparation. TGF-β primers were as follows: 5' TGF-β: ACC GCA ACA ACG CCA TCT AT and 3' TGF-β: GTA ACG CCA GGA ATT GTT GC. Semiquantitation of the RT-PCR results was achieved by amplification of β-actin using the following primers: 5' β-actin: TGG AAT CCT GTG GCA TCC ATG AAA C and 3' β-actin: TAA AAC GCA GCT CAG TAA CAG TCC G. The intensities of the TGF-β bands were normalized to β-actin values.

Adoptive Cell Transfer

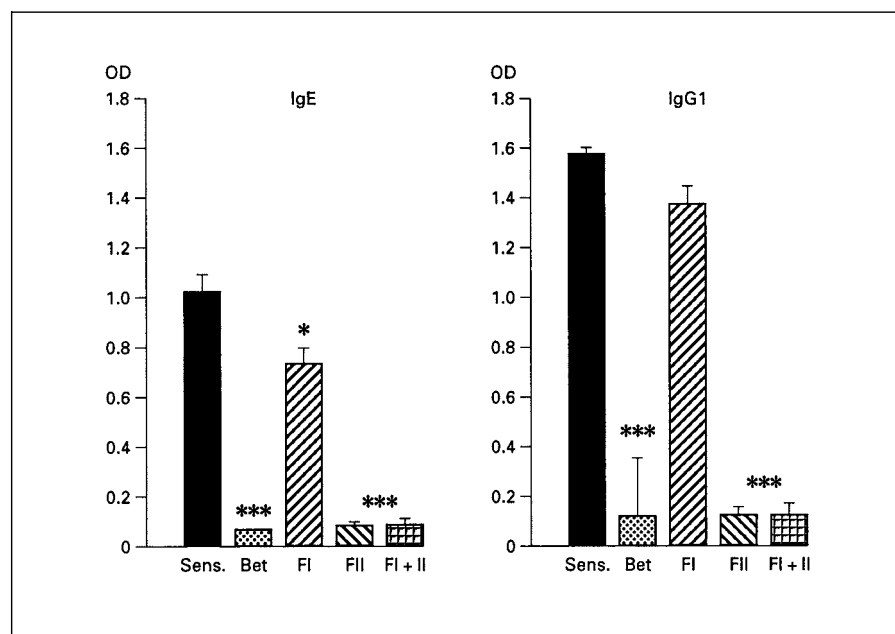
Mice were 3 times i.n. treated with rBet v 1 or F II or sham-treated with NaCl as described above. Seven days after the last i.n. antigen application mice were sacrificed and spleen cell cultures prepared. Cells were separated by depletion using anti-CD4, anti-CD8 and anti-B220 Dynabeads antibodies according to the manufacturer's description (Dyna, Oslo, Norway). The different cell fractions were analyzed by FACS using anti-CD4-FITC, anti-CD8-PerCP, anti-B220-FITC, anti-α/β-TCR-PE and anti-γ/δ-TCR-PE-conjugated antibodies (Pharmingen, San Diego, Calif.). The purity of the α/βTCR+/CD4+ or α/βTCR+/CD8+ cell populations was >90%. A contamination with CD4+ and CD8+ T cells in the respective cell fraction was excluded.

1×10^7 total spleen cells/100 µl PBS or 2×10^6 separated cells/100 µl PBS were intravenously injected into naive recipients. Four hours after the cell transfer the recipients were sensitized according to the standard sensitization scheme as described for group 3. At sacrifice (day 59) blood samples were taken and serum was analyzed for allergen-specific IgE antibody levels.

Results

In pilot experiments we investigated whether rBet v 1 fragments (F I: aa 1–74; F II: aa 75–160) induce type I skin reactions and T cell proliferation in rBet v 1-sensitized mice. In contrast to complete rBet v 1, neither F I nor F II induced positive skin reactions in already sensitized mice and they are thus not allergenic in our mouse model (data not shown). Spleen cells from rBet v 1-sensitized mice were stimulated with rBet v 1, F I and F II. rBet v 1 and F II induced comparable proliferative responses (stimulation index (SI): rBet v 1: 4.25; SI: F II: 4.40), whereas lymphocyte proliferation upon stimulation with F I was markedly lower (SI: F I: 2.19). T cell epitope mapping, using overlap-

Fig. 1. Allergen-specific IgE and IgG1 antibody levels. Bet v 1-specific IgE and IgG1 antibody levels measured in sera from group 3 (sensitized), group 4 (Bet), group 5 (F I), group 6 (F II) and group 7 (F I+II). The OD values were measured in sera from 6 mice of each group from which the values of the preimmune sera were subtracted. Bars represent the mean ODs and the error bars indicate SEMs. * $p < 0.05$; *** $p < 0.001$ as determined by the Mann-Whitney U test.



ping synthetic dodecapeptides spanning the complete Bet v 1 sequence, showed that F II contains the major T cell epitope of Bet v 1 (aa 139–152 [22]).

Bet v 1-Specific Antibody Production and Type I Skin Reactions in vivo

rBet v 1 and rBet v 1 fragments were i.n. applied prior to sensitization. The i.n. pretreatment with the complete rBet v 1 allergen (group 4) and with F II (group 6) led to a significant reduction of Bet v 1-specific IgE and IgG1 antibody levels ($p < 0.001$) compared to the sensitized controls (group 3; fig. 1). In contrast, i.n. pretreatment with F I (group 5) did not inhibit the production of Bet v 1-specific IgG1 and only moderately reduced ($p < 0.05$) allergen-specific IgE. Simultaneous application of both fragments (group 7) had the same suppressive effects on the production of Bet v 1-specific antibodies as the pretreatment with the complete molecule or with F II (fig. 1). IgG2a antibodies, which were low in the sensitized mice ($OD 0.129 \pm 0.35$), were not substantially changed by the pretreatment with the complete Bet v 1 molecule ($OD 0.08 \pm 0.13$) or with the Bet v 1 fragments (F I: $OD 0.112 \pm 0.032$; F II: $OD 0.09 \pm 0.03$; F I+II: $OD 0.145 \pm 0.126$).

In accordance with the reduced IgG1/IgE antibody levels, none of the mice (0/6) pretreated with the complete rBet v 1 or with F II displayed positive type I skin reactions, whereas all sensitized mice (6/6) exhibited strong

skin reactions to rBet v 1 and BP extract. I.n. pretreatment with F I failed to inhibit allergen-specific cutaneous anaphylactic reactions in all animals tested (data not shown).

In vitro Cytokine Production

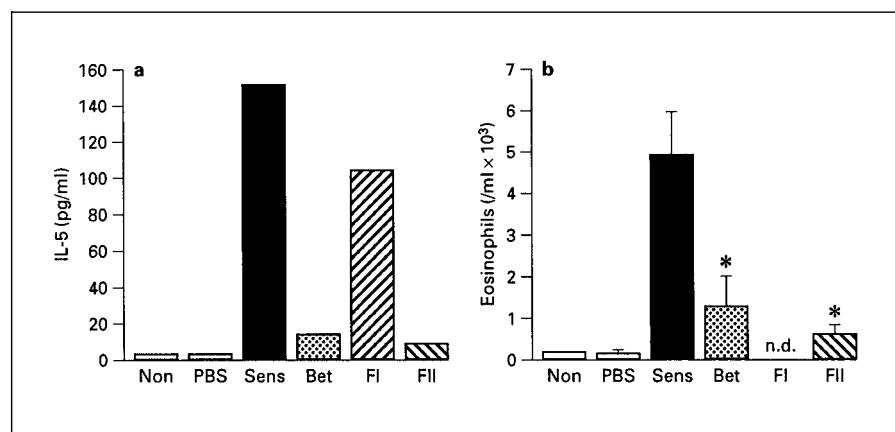
IL-5, IFN- γ and IL-10 levels were measured in supernatants of antigen-stimulated spleen cell cultures. Sensitized mice displayed 12 times higher IL-5 levels (15.3 pg/ml) than mice tolerized either with the complete rBet v 1 molecule (0.96 pg/ml) or with F II (1.3 pg/ml), but only 2 times higher than the group of F I-pretreated mice (7.2 pg/ml). IFN- γ levels were 2 times lower in the rBet v 1-tolerized group (74.6 pg/ml) and 1.7 times lower in the F II-pretreated group (86.7 pg/ml) compared to the sensitized controls (150.3 pg/ml). No differences in IFN- γ levels were seen between the sensitized (150.3 pg/ml) and the F I-pretreated groups (122.4 pg/ml). Also IL-10 levels were lower in the supernatants of the rBet v 1-pretreated animals (180.2 pg/ml) and of mice pretreated with F II (34.9 pg/ml) as compared to the sensitized group (251.4 pg/ml).

Airway Inflammation

To assess the effects of mucosal pretreatment on airway inflammation, eosinophilic infiltration and IL-5 production within the airways were determined.

IL-5 production of antigen-stimulated lung cell suspensions (fig. 2a) as well as eosinophilic infiltration in the

Fig. 2. IL-5 levels in cultured lung cells and eosinophils in BAL. **a** Levels of IL-5 were determined in pooled lung cell cultures from each group. **b** The total number of eosinophils/ 10^3 cells in BAL was determined (group 1 = non; group 2 = PBS; group 3 = sensitized; group 4 = Bet; group 5 = F I; group 6 = F II). Error bars indicate SEMs. * $p < 0.05$ as determined by the Mann-Whitney U test. n.d. = Not done.



BAL (fig. 2b) were equally reduced in animals i.n. pretreated with the complete rBet v 1 molecule or with F II. IL-5 was less reduced after pretreatment with F I, and no IL-5 or eosinophils could be detected in naive animals or in PBS mice (fig. 2a, b).

Additionally, lung inflammation was evaluated by histopathological analyses (fig. 3). Sensitized mice showed dense cuffs of inflammatory cells surrounding bronchi and medium-sized vessels, which was scored as a grade 3 inflammatory reaction. The inflammatory infiltrates consisted of a mixture of lymphocytes and eosinophils (fig. 3a). In contrast, mice which were pretreated with the complete molecule (fig. 3b) or with F II (fig. 3d) or sham-sensitized with PBS (fig. 3c) displayed no or only occasionally inflammatory cell aggregates without eosinophilic infiltrates (grade 1). Lungs of naive mice did not contain any inflammatory infiltrates and were uniformly scored as grade 0 (fig. 3e).

Airway Hyperresponsiveness

The sensitized animals developed airway hyperresponsiveness (AHR), reflected by a significantly lower MCh_{50} concentration as compared to naive or PBS control animals (fig. 4). Mucosal pretreatment with rBet v 1 inhibited development of AHR, since the MCh_{50} concentration in the rBet v 1-tolerized mice was significantly higher than that of the sensitized mice ($p < 0.05$; fig. 4). In contrast to the Bet v 1-tolerized mice, there was no difference in the MCh_{50} concentration between the F II-pretreated and the sensitized mice (fig. 4).

TGF- β mRNA Expression

TGF- β mRNA levels were compared for groups 3, 4 and 6. The ratio of β -actin to TGF- β was 8 times higher

($p < 0.05$) in the group of mice i.n. pretreated with the complete rBet v 1 molecule (group 4) compared to the sensitized control animals (group 3). In contrast, cells from mice pretreated with the F II (group 6) did not display a difference in the β -actin/TGF- β ratio compared to the sensitized controls (fig. 5a).

Adoptive Cell Transfer

Spleen cells from rBet v 1 or F II-tolerized mice were transferred into naive mice, which were subsequently sensitized with rBet v 1/BP. Mice receiving spleen cells from rBet v 1-tolerized mice displayed significantly lower allergen-specific IgE levels ($\text{OD } 0.44 \pm 0.22$) than control mice receiving naive spleen cells when thereafter sensitized to rBet v 1 ($\text{OD } 0.80 \pm 0.19$, $p < 0.05$) (fig. 5b). In contrast, transfer of spleen cells from F II-tolerized mice was not associated with suppressed allergen-specific IgE production following sensitization (sensitization: $\text{OD } 0.54 \pm 0.06$, F II transfer: $\text{OD } 0.59 \pm 0.42$) (fig. 5b). Spleen cells from rBet v 1-tolerized mice were thereafter separated into CD4+, CD8+ T cells, B220+ or CD4-CD8-B220- cells and injected into naive animals prior to sensitization. Allergen-specific IgE antibody levels were suppressed after transfer of both CD4+ and CD8+ T cells compared to those of sensitized controls (sensitization: $\text{OD } 0.80 \pm 0.19$; CD4+ transfer: $\text{OD } 0.36 \pm 0.16$; CD8+ transfer: $\text{OD } 0.39 \pm 0.07$). IgE antibody levels in mice receiving B220+ or CD4-CD8-B220- cells did not differ as compared to the sensitized controls (fig. 5c).

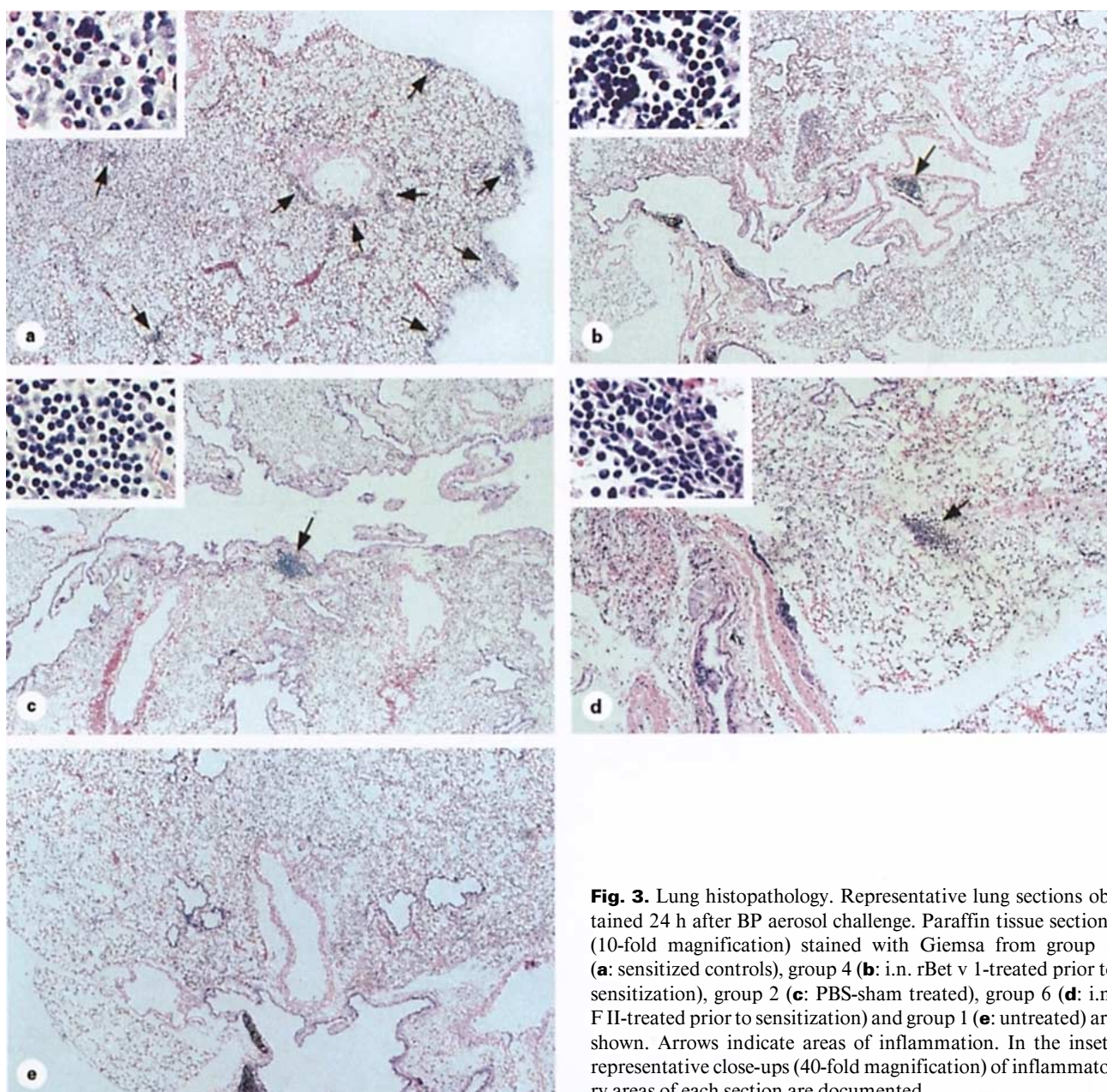


Fig. 3. Lung histopathology. Representative lung sections obtained 24 h after BP aerosol challenge. Paraffin tissue sections (10-fold magnification) stained with Giemsa from group 3 (**a**: sensitized controls), group 4 (**b**: i.n. rBet v 1-treated prior to sensitization), group 2 (**c**: PBS-sham treated), group 6 (**d**: i.n. F II-treated prior to sensitization) and group 1 (**e**: untreated) are shown. Arrows indicate areas of inflammation. In the insets representative close-ups (40-fold magnification) of inflammatory areas of each section are documented.

Discussion

In the present study we used the rBet v 1 fragments for mucosal tolerance induction, because they did not – in contrast to the complete Bet v 1 molecule – elicit cutaneous anaphylactic reactions in Bet v 1-sensitized mice. Similarly as it has been described in a mouse model of house dust mite allergy [25], we demonstrate that the presence of the immunodominant peptide was necessary

for tolerance induction, since the pretreatment with the dominant T cell epitope-containing F II, but not with the F I, led to significant suppression of allergen-specific immune responses. Both IgG1 and IgE antibody responses were reduced to the same extent in animals treated with the complete rBet v 1 molecule or F II. A recent study demonstrated that i.n. tolerance induction with a bee venom allergen and overlapping peptides covering the sequence of this allergen led to a reduction of

Fig. 4. Airway responsiveness. The concentration of MCh₅₀ was determined for group 1 (untreated mice), group 2 (PBS-sham treated and BP-challenged), group 3 (■ = sensitized controls), group 4 (▨ = rBet v 1-treated prior sensitization) and for group 6 (▩ = F II-treated prior sensitization). Means of 12 individual measurements per group are displayed. Error bars indicate SEMs. * *p* < 0.05 as determined by the Mann-Whitney U test.

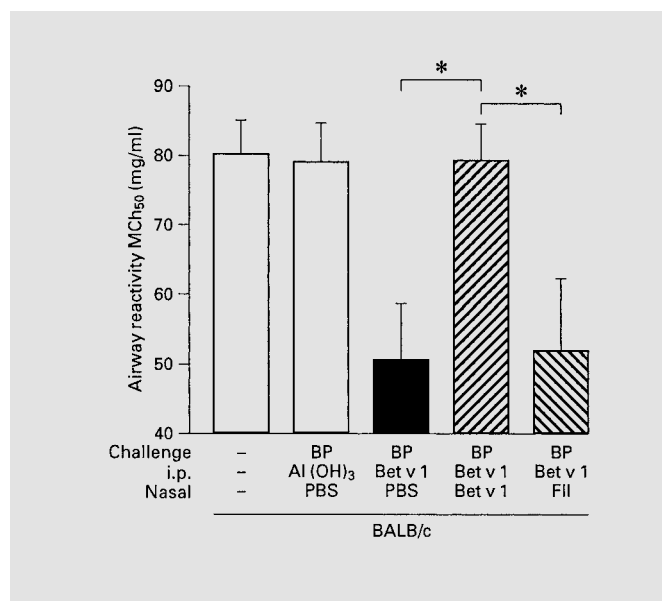
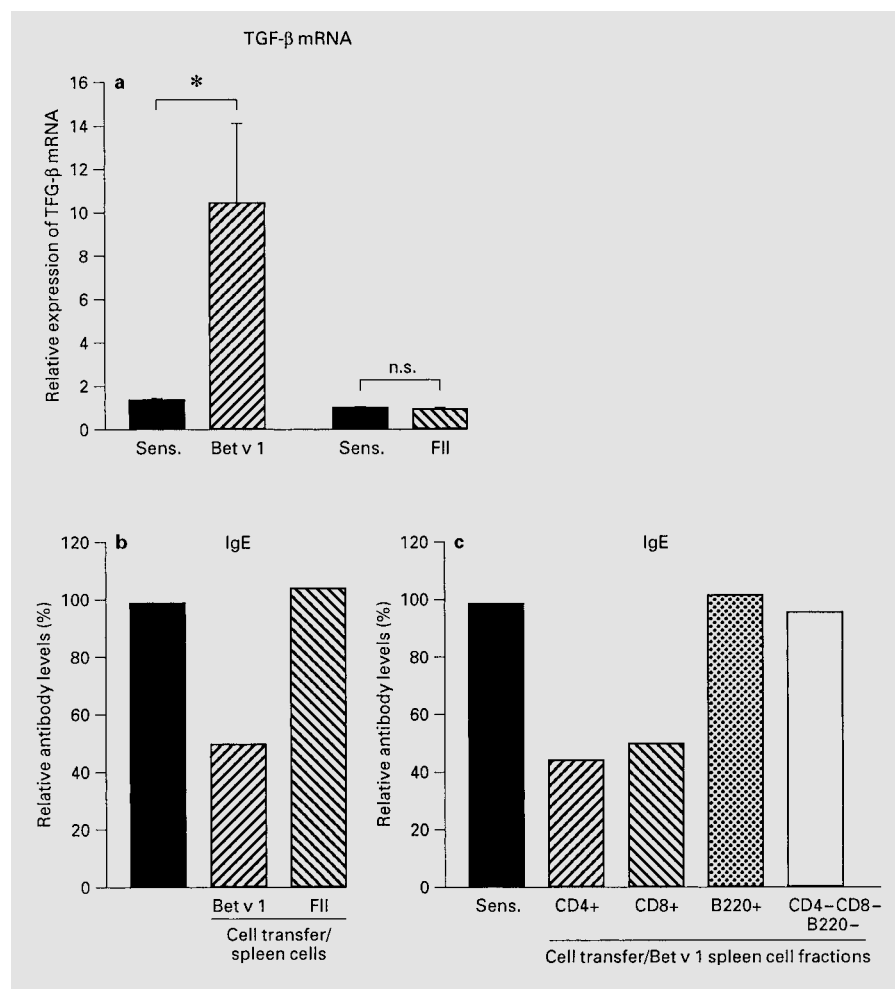


Fig. 5. TGF- β mRNA expression (**a**) and adoptive cell transfer experiments with whole spleen cells (**b**) or spleen cell fractions (**c**). **a** TGF- β mRNA expression in spleen cell pools from 6 sensitized (group 3; ■), rBet v 1-pretreated (group 4; ▨) or F II-pretreated (group 5; ▩) mice. Bars indicate the ratio of β -actin/TGF- β as mean values of 4 individual PCR experiments. * *p* < 0.05; n.s. = nonsignificant. **b** Relative IgE levels in sera of mice which received spleen cells from rBet v 1-tolerized (▨) or F II-tolerized mice (▩) prior to sensitization. **c** Relative IgE levels in sera of mice which had received spleen cell fractions from rBet v 1-tolerized mice prior to sensitization. The IgE levels of sensitized mice (■) were set to 100%, the IgE levels in sera of mice receiving cells from pretreated mice were expressed as relative values.



IgE, but not IgG1, and an increase of IgG2a antibodies and IFN- γ production [26]. In our study tolerance induction was not associated with a shift towards Th1-like immune responses, since we did not detect an increase in IgG2a antibody levels. Moreover, IFN- γ production in vitro – similar to IL-5 and IL-10 – was reduced after i.n. treatment with rBet v 1 or F II. In accordance with the antibody responses pretreatment with the complete rBet v 1 molecule and F II, but not with F I, also inhibited allergen-specific cutaneous hypersensitivity reactions in vivo. We therefore interpret our results as a consequence of a general antigen-specific immunosuppression.

As the pretreatment with F I did not have the same potency to suppress humoral and cellular systemic immune responses, studies on airway inflammation and AHR were only performed in animals pretreated with the complete molecule or F II. Both, the complete rBet v 1 molecule and F II inhibited the development of airway inflammation demonstrated by significant reduction of eosinophils in BAL as well as markedly reduced IL-5 production within the lungs. Also the histological analysis of the lung tissues confirmed that eosinophilic and lymphocyte infiltrations were inhibited by the pretreatment with either of the molecules. Thus, i.n. tolerance induction with the hypoallergenic fragment F II had the same potency as the complete allergen in preventing immunological and pathophysiological parameters relevant for type I allergic manifestations.

AHR is a hallmark of allergic asthma in humans associated with increased levels of total and allergen-specific IgE, eosinophils and IL-5 production in airways [27]. Even though experimental studies provide a rather diverse picture on the pathogenesis of allergic asthma in mouse models [28–30], we were also interested to investigate the effects of mucosal tolerance induction on AHR. In our model of allergic sensitization development of AHR correlated well with the presence of allergen-specific IgE, eosinophils, IL-5 production and lung inflammation. As the i.n. pretreatment with the complete Bet v 1 molecule prevented also the development of AHR, it is suggested that both IL-4-dependent IgE production as well as IL-5-induced eosinophilia were involved in the development of AHR. In agreement with a recent study [31] our results also show that pathophysiological parameters of AHR can be influenced by mucosal tolerance induction. Interestingly, i.n. pretreatment with F II, which also resulted in reduced allergic immune responses and airway inflammation, did not influence AHR. This finding may support a recent report by Hogan et al. [32] postulating that AHR can develop independently of IL-4

and IL-5, probably due to a third, yet undefined, T cell-dependent pathway.

It was recently demonstrated with the nonatopic bee venom allergen phospholipase A2 that antigen presentation, T cell activation and cytokine production depend on its structural features [33]. In terms of the atopic allergen Bet v 1 it might also be possible that the unfolded, major T cell epitope containing fragment and the complete folded molecule, including B as well as T cell epitopes, are presented differently by APCs, thus differentially regulating the immune responses and perhaps even lung pathophysiology. Indeed, cell transfer experiments supported the idea of distinctly operating mechanisms, since spleen cells from rBet v 1-pretreated mice could transfer tolerance with respect to IgE levels, whereas spleen cells from F II-treated mice could not. Moreover, only pretreatment with rBet v 1, but not F II, enhanced the mRNA production of TGF- β , a major cytokine involved in suppressive mechanisms during mucosal tolerance induction [34]. These results may indicate that mucosal tolerance induction with the complete molecule was mediated by active suppression [17, 20], whereas mucosal tolerance induction with the fragment rather led to mechanisms such as anergy of allergen-specific T cells, as it has also been reported for mucosal tolerance induction with other immunodominant T cell epitopes [26, 35]. Based on a recent study by Hansen et al. [36], demonstrating that TGF- β producing Th cells downregulated airway inflammation and airway hyperreactivity, it is tempting to speculate that the different effects of mucosal pretreatment with the complete molecule or F II on AHR were mediated by the presence or the absence of TGF- β in our model. However, since it has been described that TGF- β is produced by CD4+ regulatory T cells [37, 38], this cytokine might not be the only suppressive mediator in our model, since tolerance was not only transferable with CD4+ but also with CD8+ cells, as described by others as well [39, 40].

In conclusion, our study demonstrates that mucosal application of recombinant allergens and their hypoallergenic derivatives prevented key parameters involved in the development of allergic asthma. The immunosuppressive potential of this intervention might therefore be considered as a prophylactic measure in atopic patients. Moreover, the relevance of this strategy lies in testing recombinant allergens and their derivatives of interest to function as potent tolerogens to be used for future immunotherapy based on mucosal tolerance induction.

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