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Airway inflammation induced after allergic poly-sensitization can be prevented by mucosal but not by systemic administration of poly-peptides

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Clinical and Experimental Allergy

Summary

Background Patients with multiple sensitizations require alternative forms of treatment, as the efficacy of conventional immunotherapy is unsatisfactory.

Objective In the present study, we sought to compare the efficacy of a subcutaneously (s.c.) and a mucosally applied polyvalent vaccine to reduce allergic immune responses within airway and lung tissues.

Methods Female BALB/c mice were intraperitoneally immunized with recombinant (r)Bet v 1, rPhl p 1 and rPhl p 5, followed by an aerosol challenge of birch and phleum pollen extract. For tolerance induction, either a mixture of the immunodominant peptides or a hybrid peptide of the respective antigens was s.c. injected or intranasally applied before poly-sensitization.

Results Mucosal but not systemic pre-treatment with poly-peptides led to significant suppression of eosinophils and IL-5 production in bronchoalveolar lavages, as well as IL-5, IL-4, IL-13 and eotaxin levels in lung cell cultures. Lung histology showed a clear reduction of cellular infiltration and mucus production only in intranasally pre-treated mice. In accordance, also the systemic immune response, characterized by IgE-dependent basophil degranulation and IL-4 levels *in vitro*, was significantly reduced by mucosal antigen application, but only marginally influenced by subcutaneous pre-treatment. Both treatment routes led to up-regulated CTLA4 expression in splenocytes, whereas only after mucosal pre-treatment Foxp3 expression levels were enhanced in lung CD3⁺ T cells. Furthermore, intranasal but not subcutaneous application of the peptides enhanced IL-10 levels in the lungs, indicating regulatory mechanisms operating in local tolerance induction.

Conclusion Mucosal application of peptides is superior to systemic application in preventing both local and systemic poly-allergic T helper2 immune responses, suggesting mucosal tolerance induction as an attractive strategy for the primary and secondary prevention of allergic multi-sensitization and lung pathology.

Keywords airway inflammation, Foxp3, hybrid, IL-10, mucosal tolerance, polypeptides, poly-sensitization, polyvalent vaccine, type I allergy

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Introduction

Type I allergy is commonly caused by sensitization to environmental allergens, such as tree or grass pollen, house dust mite (HDM) and animal dander, and leads to clinical manifestations that include rhinoconjunctivitis, atopic dermatitis and allergic asthma. The underlying immunological mechanism implies unbalanced T helper (Th1)/Th2 immune responses characterized by an in-

creased number of IL-4-producing Th2 cells and production of IgE to these normally harmless antigens.

Allergen-specific immunotherapy (SIT) administered via the parenteral route has been used for almost a century to affect such Th2-prone immune responses and is still a state-of-the-art treatment for allergic rhinitis or insect venom hypersensitivity [1, 2]. Nevertheless, frequent injections and the non-neglectable risk of anaphylactic reactions may limit the patient's compliance to the

treatment. Thus, over the past years, research focused on the establishment of non-invasive forms of immunotherapy by the application of allergenic extracts via mucosal routes (i.e. oral, nasal, sublingual) in order to improve the safety profile but yet preserve the immunological effects mediated by the induction of tolerance [3, 4]. Additional improvement could be achieved by the use of recombinant allergens or allergen peptides designed to match the sensitization profile of the respective allergic patient [5, 6]. Along with these alternative treatment strategies, new approaches of primary prevention vaccines before initial sensitization are considered for patients at risk [2, 5]. In this respect, we previously demonstrated in a mouse model of birch pollen (BP) allergy that intranasal administration of Bet v 1, the major BP allergen, suppressed allergic sensitization in naïve and in primed mice [7, 8].

Another drawback of SIT involves the effectiveness of this treatment, which is limited to a certain group of allergic patients, namely young and mono-sensitized individuals. For the growing number of patients who display sensitizations to several different allergens, mainly with increasing age, SIT is not recommended due to a low efficacy and an increased risk of anaphylactic side-effects [9]. In order to develop new treatment strategies, especially for patients with multiple sensitizations, we have recently established a mouse model of allergic poly-sensitization to the major birch and grass pollen allergens Bet v 1, Phl p 1 and Phl p 5. Using this murine model, we successfully prevented poly-sensitization by intranasal application of the immunodominant peptides of the respective allergens, whereas treatment with the allergenic proteins did not have this effect [10].

In the current study, we compared the subcutaneous and the mucosal route of antigen application concerning their potency to prevent allergic poly-sensitization. Particular attention was drawn to the prevention of airway inflammation and lung pathology by either of the treatment regimes, because the lungs are the primary target organ in respiratory allergy. Our results show that intranasal but not subcutaneous pre-treatment with the immunodominant peptides of Bet v 1, Phl p 1 and Phl p 5, applied either as a mixture or as a hybrid peptide, could prevent the establishment of both systemic and local Th2 immune responses in poly-sensitized mice.

Material and methods

Animals

Female inbred 7-week-old BALB/c mice ($n = 8$ per group) 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.

Recombinant allergens and natural allergen extracts

rBet v 1, rPhl p 1 and rPhl p 5 were obtained from Biomay AG (Vienna, Austria). BP (*Betula verrucosa*) and timothy grass pollen (*Phleum pratense*) were purchased from Allergon (Välinge, Sweden), and extracts were prepared as previously described [11].

Peptide synthesis

Identification of the immunodominant regions of Bet v 1, Phl p 1 and Phl p 5 was performed by epitope mapping studies, and the respective peptides for intranasal pre-treatment were synthesized using the Fmoc strategy with HBTU activation on the Applied Biosystems (Foster City, CA, USA) peptide synthesizer model 433A as previously described [10]. The identity of the produced peptides was checked by mass spectrometry and the peptides were purified to > 90% purity by preparative HPLC (piCHEM, Graz, Austria).

The amino acid sequence of the peptides was as follows: Bet v 1: MGETLLRAVESY; Phl p 1: AGELELQFRRVKCKY; Phl p 5: TVATAPEVKYTVFETALK; hybrid: MGETLLRAVESYAGELELQFRRVKCKYTVATAPEVKYTVFETALK.

Poly-sensitization, intranasal and subcutaneous pre-treatment

Poly-sensitization was performed by three intraperitoneal (i.p.) immunizations (days 22, 36 and 50) of a mixture of rBet v 1, rPhl p 1 and rPhl p 5 (5 µg each) adsorbed to aluminium hydroxide ($\text{Al}(\text{OH})_3$; Serva, Heidelberg, Germany) at 14-day intervals (group 1). For mucosal pre-treatment, a mixture of Bet v 1 peptide, Phl p 1 peptide and Phl p 5 peptide (5 µg each) was applied intranasally (i.n.) in 30 µL of 0.9% NaCl three times at 7-day intervals (days 0, 7 and 14) before poly-sensitization (group 2). Intranasal pre-treatment with the hybrid peptide was performed using a concentration of 20 µg construct in 30 µL 0.9% NaCl per application (group 3). The same application regime was used for systemic pre-treatment, where a mixture of the peptides (5 µg each; group 4) or the hybrid (20 µg; group 5) was subcutaneously (s.c.) applied in 100 µL of 0.9% NaCl. Control mice were i.n. sham-treated with 30 µL of 0.9% NaCl or s.c. sham-treated with 100 µL of 0.9% NaCl before poly-sensitization (group 1, sham).

One week after the last i.p. immunization, an aerosol challenge with 1% w/v BP and phleum extract was performed on 2 consecutive days as previously described [7].

Sampling

Blood samples were taken before treatment and 2 days after aerosol challenge (day 60) by tail bleeding. After the

bleeding, the mice were killed, bronchoalveolar lavages (BAL) were collected, and cell suspensions from spleen, lung and bronchial lymph nodes were prepared as previously described [7, 11].

Isolation of CD3⁺ T cells from lung and lymph nodes

CD3⁺ T cells from pooled lung cell suspensions and from pooled bronchial lymph node suspensions were isolated by positive selection with MACS anti-CD90 (Thy1.2) magnetic beads (Myltenyi Biotec, Auburn, CA, USA), according to the manufacturer's instructions. Isolated lymphocytes were stained with fluorescein isothiocyanate-, phycoerythrin-, peridinin chlorophyll protein- or allophycocyanin-conjugated monoclonal antibodies to mouse B220, CD3, CD4 or CD8 (BD Biosciences Pharmingen, San Diego, CA, USA) and analysed by flow cytometry on an FACS Calibur using CellQuest software (BD Biosciences Pharmingen). For control staining, isotype-matched control antibodies were used. The lymphocyte purity was >80% from lung and >95% from bronchial lymph nodes.

Lung histology

Forty-eight hours after the last aerosol challenge, lungs were collected, fixed in 10% neutral-buffered formalin and embedded in paraffin. Sections (5 µm thick) were cut and stained with haematoxylin and eosin (H&E) as well as periodic acid/Schiff (PAS) as described previously [7]. The intensity of the inflammatory infiltration was graded on a semi-quantitative 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 the majority of central bronchi, 3 = scale 2 plus inflammation in the lung periphery). The grading of slides was performed by individuals blinded to the experimental protocol.

Airway eosinophilia

BAL samples were spun onto microscope slides and stained with H&E (Hemacolor; Merck, Darmstadt, Germany). The number of macrophages, lymphocytes and eosinophils is expressed as percentage of the total cell number counted as previously described [7].

Rat basophil leukaemia cell mediator release assay

Rat basophil leukaemia (RBL)-2H3 cells were incubated with the sera obtained from i.n. or s.c. pre-treated and poly-sensitized mice at dilutions of 1/30–1/300. Degranulation of RBL cells was induced by adding 0.03 µg of each allergen diluted in 100 µL Tyrode's buffer. Supernatants were analysed for β-hexosaminidase activity as

previously described [12]. Results are reported as percentages of total β-hexosaminidase release after adding 1% Triton X-100 and are shown after subtraction of baseline release levels obtained with pre-immune sera.

Detection of allergen-specific immunoglobulin G2a levels in serum

Microtitre plates (Nunc, Roskilde, Denmark) were coated with each of the recombinant allergens (5 µg/mL) before incubation with the sera. Rat anti-mouse IgG2a antibody (1/500; Pharmingen) was used, followed by peroxidase-conjugated mouse anti-rat IgG antibody (1/2000; Jackson Immuno Lab, West Grove, PA, USA) [11]. Results show the optical density (OD) values after subtraction of baseline levels from pre-immune sera.

Cytokine production

Spleen cell cultures (5×10^6 cells/well) and pooled lung cell cultures (5×10^6 cells/well) were incubated for 40 h with BP (25 µg/well) or phleum extract (25 µg/well) as described [7]. Levels of IL-4, IL-5, IL-10, IL-13, IFN-γ and eotaxin were measured with mouse ELISA kits (sensitivity < 5 pg/mL; Endogen, Cambridge, MA, USA) [7]. IL-5 was measured in BAL fluids as previously described. Cytokine levels are given in pg/mL after subtraction of baseline levels of unstimulated cultures.

Quantification of mRNA expression by real-time reverse transcriptase polymerase chain reaction

Total RNA was isolated from pooled spleen cell suspensions, from pooled isolated pulmonary CD3⁺ T cells and from pooled isolated bronchial lymph node CD3⁺ T cells on the day of killing using an RNeasy Minikit (Quiagen, Valencia, CA, USA), treated with DNase (Quiagen) and then reverse-transcribed into cDNA using random hexamers (GeneAmp RT-PCR kit, PE, Boston, MA, USA).

Gene expression was determined by quantitative real-time PCR on a LightCycler[®] instrument 1.2 using LightCycler[®] FastStart kits without (TaqMan) or with SYBRGreen according to the manufacturer's instructions (Roche, Mannheim, Germany). Amplification of the housekeeping gene 5-aminolevulinic acid synthase (ALAS1) (Universal ProbeLibrary probe #64; Roche) was performed in order to standardize the amount of sample cDNA added. Data are presented as the relative ratio of the target genes to the housekeeping gene ALAS1, and the efficiency of all target and reference genes was determined by serial cDNA dilution.

For quantification of TGF-β, IL-10 and Fox p 3 mRNA, pre-designed TaqMan[®] assays were used. An initial activation step for 10 min at 95 °C was followed by 50 cycles of 10 s at 95 °C, 30 s at 60 °C and 1 s at 72 °C. Primers for IFN-γ, T-bet, GATA-3, ICOS and CTLA4 were designed

using the Primer3 software from the Whitehead Institute for Biomedical Research. The amplification was performed as described [13] by an activation step for 10 min at 95 °C, which was followed by 50 cycles of 5 s at 95 °C, 5 s at 65 °C and 15 s at 72 °C and melting curve analysis (SYBRGreen).

Statistics

Data are expressed as means $\pm$ SEMs from three independent experiments. Differences between groups were tested by Kruskal–Wallis tests. *Post hoc* comparisons for all pairs of groups were performed by applying Tukey–Kramer tests. *P*-values below 0.05 were considered significant. Pair-wise comparison of sham-treated sensitized vs. pre-treated groups was carried out using the Mann–Whitney *U*-test.

Results

Mucosal but not systemic pre-treatment prevents airway inflammation

Eosinophils and cytokine levels in bronchoalveolar lavage and lung cell culture. Airway inflammation was determined by the number of inflammatory cells (macrophages, lymphocytes, eosinophils) and IL-5 levels in BAL fluids. Both eosinophils and IL-5 were significantly reduced after intranasal application of the peptide mixture or the hybrid (Table 1). The number of lymphocytes was significantly reduced in both groups, while macrophage numbers were increased (Table 1). Additionally, Th2 cytokines IL-4, IL-5 and IL-13 and the eosinophil-specific chemotactic factor eotaxin were decreased in lung cell cultures from i.n. pre-treated mice compared with sham-treated poly-sensitized controls (Fig. 1). Systemic administration of antigen led to a significantly reduced number of eosinophils and lymphocytes in BALs from peptide mixture, but not from hybrid pre-treated mice (Table 1). However, IL-5 levels in BALs were unchanged in all s.c. pre-treated groups compared with control animals (Table

1). Th2 cytokine levels and eotaxin in lung cell cultures were unaffected after subcutaneous application of the hybrid (Fig. 1). Systemic peptide mixture pre-treatment led to reduced IL-5 and IL-13 levels in lungs, but no influence on IL-4 or eotaxin production was found (Fig. 1). The original data to Fig. 1 are summarized in Table 2.

Histological analysis of lung inflammation. Forty-eight hours after the last airway challenge, lung tissue was obtained from all experimental groups to analyse the effect of mucosal and systemic pre-treatment on airway inflammation (Figs 2A and B). Sham-treated poly-sensitized mice showed massive cellular infiltration around bronchi and blood vessels, which was scored as grade 3 inflammatory reaction. Eosinophils and lymphocytes were the predominant infiltrating cells (Fig. 2A: a). In the i.n. pre-treated mice, with either the peptides or the hybrid, cellular infiltration in the lung was significantly reduced with occasional mononuclear cell infiltrates but not eosinophils (grade 1; Fig. 2A: c, d). In contrast, lung sections of s.c. peptide or hybrid pre-treated mice showed similar cellular infiltrations as the poly-sensitized sham-treated controls (grade 2–3; Fig. 2A: e, f). No inflammatory infiltrates were found in the lungs of naïve mice (grade 0; Fig. 2A: b). PAS staining revealed mucus hypersecretion and occlusion of the airways as dominant histopathological features of the poly-sensitized control animals (Fig. 2B: g). These features were drastically reduced in lung sections from i.n. pre-treated mice (Fig. 2: i, k), but not in sections from s.c. pre-treated animals (Fig. 2B: l, m). Lung sections from naïve mice showed no mucus secretion (Fig. 2b: h).

Systemic T helper2 immune responses to all three allergens are inhibited by mucosal pre-treatment

Intranasal pre-treatment with the mixture of peptides or a synthetic hybrid led to significantly reduced IgE-dependent basophil degranulation to all three allergens compared with sham-treated poly-sensitized controls (Fig. 3). According to the reduction of IgE reactivity, allergen-specific IL-4 levels

Table 1. Number of macrophages, lymphocytes and eosinophils (cells/mL $\times 10^3$) and IL-5 levels (pg/mL) in bronchoalveolar lavage (BAL) fluid from sham-treated poly-sensitized (sham) and from intranasally (i.n.) or subcutaneously (s.c.) pre-treated mice (peptide, hybrid)

	i.n. pre-treatment			s.c. pre-treatment		
	Sham	Peptide	Hybrid	Sham	Peptide	Hybrid
Macrophages	22.03 $\pm$ 3.85	26.14 $\pm$ 3.28	28.04 $\pm$ 4.14	22.03 $\pm$ 3.85	26.54 $\pm$ 2.48	22.85 $\pm$ 6.36
Lymphocytes	2.12 $\pm$ 0.95	1.18 $\pm$ 0.42*	0.73 $\pm$ 0.59*	2.12 $\pm$ 0.95	0.88 $\pm$ 0.44*	1.06 $\pm$ 0.74
Eosinophils	5.90 $\pm$ 2.84	1.56 $\pm$ 0.70*	1.41 $\pm$ 0.91*	5.90 $\pm$ 2.84	2.60 $\pm$ 2.04*	5.87 $\pm$ 2.64
IL-5	38.02 $\pm$ 14.5	16.95 $\pm$ 6.9**	18.31 $\pm$ 5.1**	38.02 $\pm$ 14.5	29.25 $\pm$ 4.04	41.31 $\pm$ 14.8

**P* < 0.05: peptide or hybrid vs. sham-treated as determined by Mann–Whitney *U*-test.

***P* < 0.01: peptide or hybrid vs. sham-treated as determined by Mann–Whitney *U*-test.

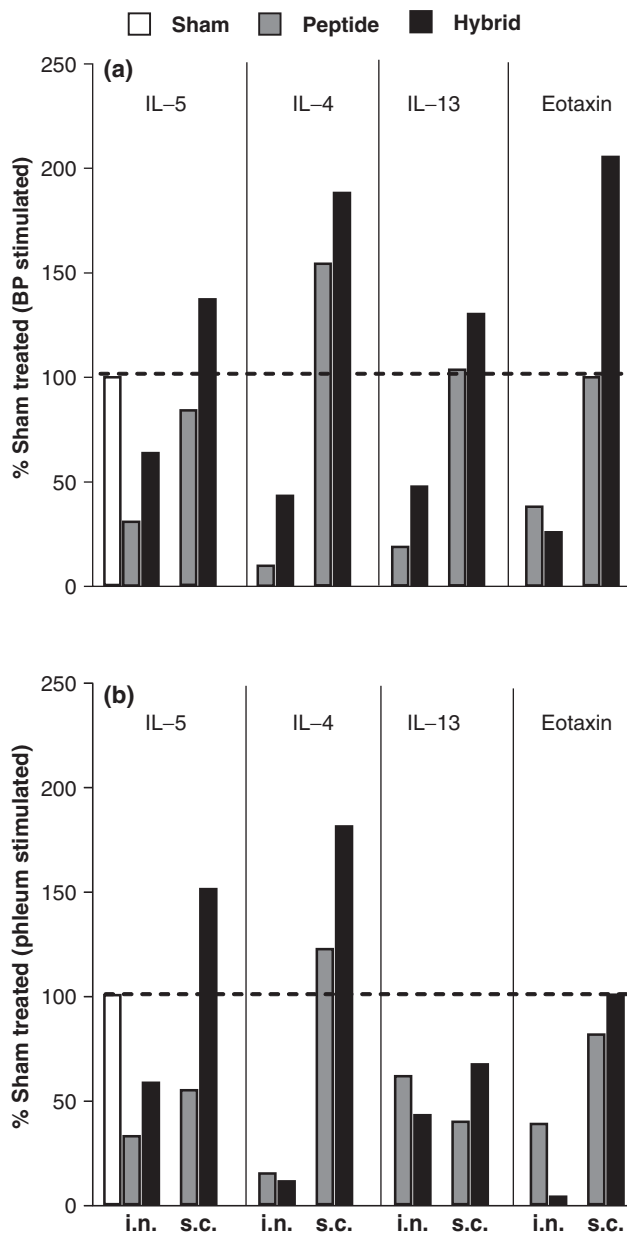


Fig. 1. IL-5, IL-4, IL-13 and eotaxin levels in pooled lung cell cultures after *in vitro* stimulation with (a) birch pollen or (b) phleum extract from sham-treated poly-sensitized mice (white bars), from intranasal (i.n.) or subcutaneous (s.c.) peptide mixture (grey bars) and from i.n. or s.c. hybrid (black bars) pre-treated mice. Data from one representative experiment out of three are shown as percentage of the sham-treated controls (100% of pg/mL).

in vitro were significantly lower in spleen cell cultures of all i.n. pre-treated groups (Table 3). Subcutaneous peptide or hybrid pre-treatment led to a significant reduction of Phl p 1-specific but not Bet v 1- or Phl p 5-specific basophil degranulation (Fig. 3). IL-4 production was significantly reduced after a subcutaneous pre-treatment, except for phleum-stimulated splenocytes from mice s.c. pre-treated with the peptide mixture (Table 3).

Table 2. IL-5, IL-4, IL-13 and eotaxin levels (pg/mL) in pooled lung cell cultures from sham-treated poly-sensitized (sham) and from intranasally (i.n.) or subcutaneously (s.c.) pre-treated mice (peptide, hybrid) after *in vitro* stimulation with birch pollen (BP) and phleum extract (phleum)

	i.n. pre-treatment			s.c. pre-treatment		
	Sham	Peptide	Hybrid	Sham	Peptide	Hybrid
IL-5						
BP	508.51	163.25	336.14	545.38	444.25	723.42
Phleum	598.36	220.52	394.63	755.75	370.58	1022.87
IL-4						
BP	48.11	5.52	24.05	62.71	85.53	104.37
Phleum	106.03	34.71	25.7	365.82	288.14	426.52
IL-13						
BP	809.52	152.48	387.4	292.35	303.14	381.25
Phleum	1409.56	864.59	600.79	1223.12	482.85	818.47
Eotaxin						
BP	48.36	18.41	12.52	5.45	5.89	11.13
Phleum	35.35	13.53	1.35	14.36	11.67	14.35

Th1 immune responses, characterized by allergen-specific IgG2a antibody production and IFN- γ levels *in vitro*, were enhanced in all i.n. pre-treated groups (Table 4). In contrast, subcutaneous pre-treatment with the peptide mixture or the hybrid resulted in decreased IgG2a antibody levels (not significant) and unchanged IFN- γ production compared with sham-treated poly-sensitized controls (Table 4).

Intranasal but not subcutaneous pre-treatment leads to enhanced interleukin-10 levels in the lungs

IL-10 levels of *in vitro*-stimulated splenocytes from i.n. or s.c. pre-treated groups were decreased or remained unchanged compared with sham-treated poly-sensitized controls (Fig. 4). In contrast, IL-10 levels in the lungs showed a marked increase after intranasal pre-treatment with the peptide mixture or the hybrid (Fig. 4). Subcutaneous pre-treatment did not alter IL-10 production in the lung, with the exception of elevated levels in phleum-stimulated cells after subcutaneous application of the hybrid (Fig. 4).

In splenocytes, mRNA expression levels of IL-10, TGF- β and Foxp3 were unmodified (data not shown), but CTLA4 was up-regulated after mucosal hybrid (relative expression sham/i.n. peptide/i.n. hybrid: 1/0.96/1.9) and subcutaneous peptide mixture (relative expression sham/s.c. peptide/s.c. hybrid: 1/2.14/1.35) pre-treatment.

Expression of T helper1, T helper2 and regulatory pathway molecules in bronchial CD3⁺ T cells

Isolated CD3⁺ T cells from lungs and from bronchial lymph nodes were analysed by real-time RT-PCR for mRNA expression profiles of molecules involved in Th1

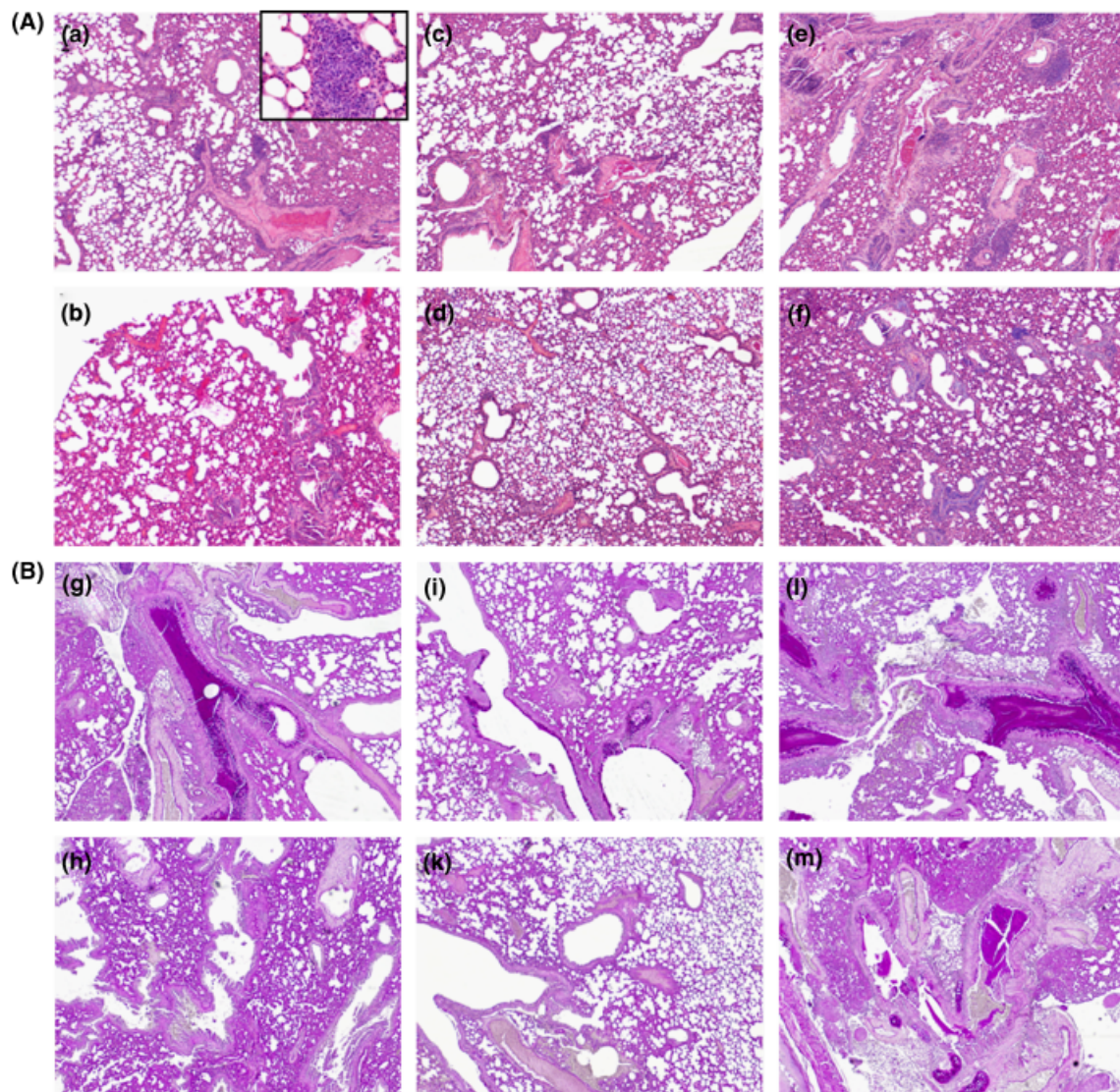


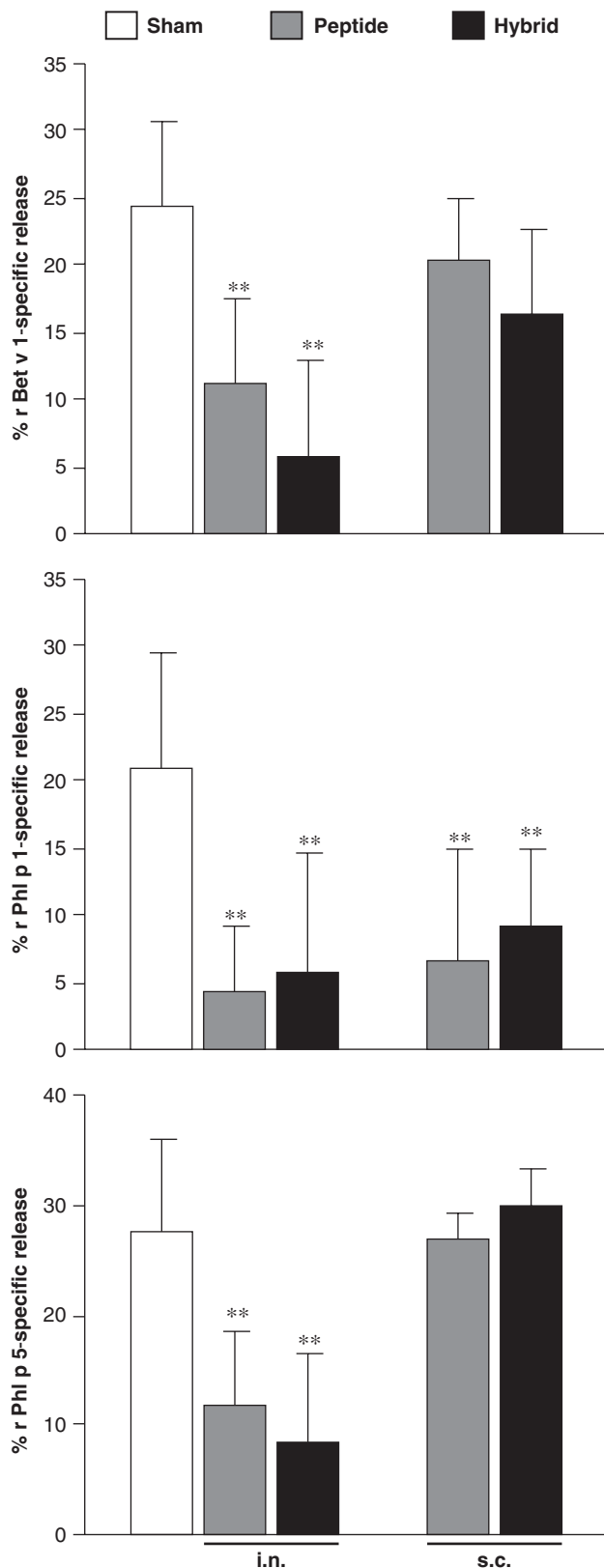
Fig. 2. Haematoxylin and eosin (2A) and periodic acid/Schiff (PAS) staining (2B) of representative lung sections from sham-treated poly-sensitized mice [a: haematoxylin and eosin (H&E), g: PAS], intranasal (i.n.) peptide (c: H&E, i: PAS) and i.n. hybrid (d: H&E, k: PAS) pre-treated mice, s.c. peptide (e: H&E, l: PAS) and subcutaneous (s.c.) hybrid (f: H&E, m: PAS) pre-treated mice and naïve mice (b: H&E, h: PAS). Magnification $\times 4$, representative close-up in 2A: a $\times 40$.

(IFN- γ , T-bet), Th2 (ICOS, GATA-3) and regulatory T cell differentiation (IL-10, TGF- β , Foxp3, CTLA4).

In pulmonary CD3⁺ T cells, down-regulation of mRNA levels of IFN- γ and the transcription factor T-bet was most pronounced in intranasal peptide mixture pre-treated mice (Table 5A). Subcutaneous pre-treatment with the peptide mixture or the hybrid led to moderate reduction of IFN- γ and T-bet expression. Relative expression of the transcription factor GATA-3 was markedly reduced in lung CD3⁺ T cells from animals treated with the intranasal peptide mixture and moderately reduced after mucosal pre-treatment with the hybrid and after subcutaneous pre-treatment (Table 5A). Expression levels of the co-stimulatory molecule ICOS and of the regulatory cytokine TGF- β remained unchanged in all treated groups compared with

sham-treated poly-sensitized controls (Table 5A). IL-10 expression levels were enhanced in pulmonary CD3⁺ T cells from intranasal peptide mixture-treated mice. Up-regulation of Foxp3 expression was found in mucosally pre-treated mice, whereas systemically treated mice showed up-regulation of CTLA4 expression (Table 5A).

In bronchial lymph node CD3⁺ T cells from i.n. pre-treated mice, mRNA expression of both Th1 and Th2 molecules was reduced (Table 5B). Subcutaneous application of the peptide mixture but not of the hybrid led to reduction of IFN- γ , T-bet and GATA-3 mRNA. Foxp3 mRNA was not significantly changed after subcutaneous and intranasal pre-treatment (Table 5B). Expression levels of regulatory cytokines and CTLA4 were unaltered after mucosal and systemic pre-treatment (Table 5B).



Discussion

The most characteristic feature of respiratory allergy is the development of airway inflammation characterized by inflammatory cell infiltrates in the lungs, such as Th2 lymphocytes and eosinophils, and the release of inflammatory mediators, contributing to mucus hypersecretion and airway hyperresponsiveness [14, 15]. Human studies revealed that the prevalence of asthma, the most common chronic inflammatory disorder of the airways, was significantly higher in poly-sensitized patients compared with mono-sensitized patients [16, 17]. Several murine models of allergy have shown that the administration of linear peptides induced immunotolerance to the protein containing the peptide and influenced airway inflammation [18–21]. Still, an important issue remains to be answered, namely which treatment route has the most promising effect on lung allergic pathology. Using a mouse model of poly-sensitization to tree and grass pollen allergens, our studies focused on the comparison of mucosal and systemic tolerance induction to several different allergens, the latter route being the standard application route of SIT in the clinics. Based on our previous studies, we chose the intranasal route of antigen application rather than the oral route, because intranasal antigen application preferentially targets the respiratory tract, lower dosages can be used and degradation of antigen is less likely to occur [11, 22].

Histological analysis of lung sections revealed that in the i.n. poly-peptide pre-treated mice, inflammatory cellular infiltration and mucus hypersecretion were significantly reduced compared with sham-treated animals. In contrast, subcutaneous antigen application did not prevent these signs of acute airway inflammation (Fig. 2). Along these lines, eosinophilia and IL-5 levels in BAL of poly-tolerized mice were significantly suppressed by mucosal pre-treatment, but only marginally influenced by systemic application of the peptide mixture or the hybrid (Table 1). Additionally, birch and grass pollen-stimulated lung cell suspensions of i.n. – but not of s.c. – pre-treated mice showed markedly suppressed Th2 cytokine (IL-4, IL-5, IL-13) and eotaxin (CCL11) levels, a major chemoattractant for eosinophils and Th2 cells (Fig. 1; Table 2). The systemic Th2 immune response, characterized by IgE-dependent basophil degranulation and IL-4 production *in vitro*, was significantly suppressed towards all three allergens in the mucosally pre-treated

Fig. 3. IgE-dependent Bet v 1-, Phl p 1- and Phl p 5-specific basophil degranulation (Rat basophil leukaemia cell mediator release assay) from sham-treated poly-sensitized mice (white bars), from intranasal (i.n.) or subcutaneous (s.c.) peptide mixture (grey bars) and from i.n. or s.c. hybrid pre-treated mice (black bars) ($n = 8$ mice per group showing one representative experiment out of three; ** $P < 0.01$ pre-treated vs. sham-treated).

groups (Fig. 3; Table 3). Subcutaneous peptide application was effective in suppressing the IL-4 production, but at the humoral level only Phl p1-specific basophil degranulation was significantly reduced (Fig. 3; Table 3). Our results demonstrate clearly that mucosal antigen delivery can prevent airway inflammation in poly-sensitized mice, while subcutaneous antigen application only affects systemic Th2 immune responses. These results are in line with studies conducted in ovalbumin-sensitized mice, showing that intranasal/oral and intradermal antigen application had similar effects in modulating systemic immune parameters, whereas local Th2 immune responses in the lungs were primarily affected by the mucosal route [23, 24]. Using three important aeroallergens, our results might have even more clinical impact than those obtained with the prototype allergen ovalbumin.

SIT protocols rely on the use of an adjuvant such as aluminium hydroxide, which is associated with the induction of Th2 immune responses [25]. To ensure that inefficient tolerance induction via the subcutaneous route was not due to inappropriate antigen delivery, we additionally s.c. injected the peptides and the hybrid adsorbed to aluminium hydroxide before poly-sensitization. How-

ever, we could not find any improvement concerning the suppression of systemic or local Th2 immune responses compared with subcutaneous peptide administration without adjuvant (data not shown).

The mechanisms underlying tolerance induction seem to depend on the conformation of the antigen, as we recently demonstrated in a mouse model of BP allergy, where mucosal treatment with Bet v 1 protein but not with Bet v 1 peptide induced the formation of IL-10- and TGF- β -producing regulatory T cells [8]. Along these lines, we have shown in poly-sensitized mice that mucosal tolerance induction using an allergen chimera, consisting of the whole Bet v 1 protein linked to the major T cell epitopes from Phl p 1 and Phl p 5, was associated with elevated TGF- β and IL-10 expression in splenocytes [26]. These data confirm that only proteins in their conformational structure were able to induce regulatory cytokines. Accordingly, we did not find enhanced IL-10 (Fig. 4) and TGF- β levels (data not shown) in spleen cells from i.n. or s.c. peptide-tolerized mice, indicating a role for cytokine-independent mechanisms in peripheral systemic tolerance to the three allergens. Recent studies described regulatory T cells expressing CTLA4 to be important in allergy development and mucosal tolerance [27, 28]. In this respect, we found enhanced CTLA4 expression in splenocytes from i.n. and s.c. desensitized mice (see 'Results', paragraph 4).

Although IL-10 does not seem to play a major role at the systemic level of poly-tolerance induction, we found up-regulated IL-10 levels in lung cell cultures from mucosally pre-treated mice (Fig. 4). Our data are in line with a study in HDM-allergic mice showing enhanced IL-10 production in bronchial lymph nodes, but not in splenocytes after peptide-induced intranasal tolerance [20], and indicate differential regulatory mechanisms underlying local and systemic tolerance induction. The expression levels of both transcription factors T-bet [29] and GATA-3 [30], key regulators for Th1 and Th2 cell development, were decreased in lungs of mucosal peptide-tolerized mice, in

Table 3. IL-4 levels (pg/mL) in splenocytes from sham-treated poly-sensitized (sham) and from intranasally (i.n.) or subcutaneously (s.c.) pre-treated mice (peptide, hybrid)

	i.n. pre-treatment		s.c. pre-treatment	
	BP	Phleum	BP	Phleum
Sham	20.5 $\pm$ 11.8	78.5 $\pm$ 32.5	20.5 $\pm$ 11.8	78.5 $\pm$ 32.5
Peptide	< 5**	10.1 $\pm$ 4.7**	6.5 $\pm$ 3.9**	104.9 $\pm$ 88.7
Hybrid	< 5**	24.3 $\pm$ 6.1**	6.6 $\pm$ 2.7**	33.1 $\pm$ 16.2**

IL-4 levels were measured in spleen cell cultures after stimulation with BP or phleum extract.

** $P < 0.01$: peptide or hybrid vs. sham-treated as determined by Mann-Whitney *U*-test.

BP, birch pollen.

Table 4. Allergen-specific IgG2a antibody levels and IFN- γ production *in vitro* in sham-treated poly-sensitized (sham) and in intranasally (i.n.) or subcutaneously (s.c.) pre-treated mice (peptide, hybrid)

	IgG2a (OD) [†]			IFN- γ (pg/mL) [‡]	
	rBet v 1	rPhl p 1	rPhl p 5	BP	Phleum
Sham	0.34 $\pm$ 0.12	0.43 $\pm$ 0.11	0.77 $\pm$ 0.41	305.27 $\pm$ 80.87	181.41 $\pm$ 92.11
i.n. peptide	0.93 $\pm$ 0.61	1.03 $\pm$ 0.67	1.66 $\pm$ 0.49	528.78 $\pm$ 308.17	440.62 $\pm$ 135.11*
i.n. hybrid	1.03 $\pm$ 0.82	1.16 $\pm$ 0.85	1.17 $\pm$ 0.65	496.93 $\pm$ 297.32	464.61 $\pm$ 263.51*
s.c. peptide	0.17 $\pm$ 0.18	0.014 $\pm$ 0.01*	0.52 $\pm$ 0.28	251.86 $\pm$ 109.83	214.06 $\pm$ 127.35
s.c. hybrid	0.26 $\pm$ 0.17	0.026 $\pm$ 0.01*	0.28 $\pm$ 0.15*	303.17 $\pm$ 266.48	204.55 $\pm$ 103.51

[†]IgG2a antibody levels in the sera were measured by ELISA.

[‡]IFN- γ levels were measured in spleen cell cultures after stimulation with BP or phleum extract.

* $P < 0.01$: peptide or hybrid vs. sham-treated as determined by Mann-Whitney *U*-test.

BP, birch pollen.

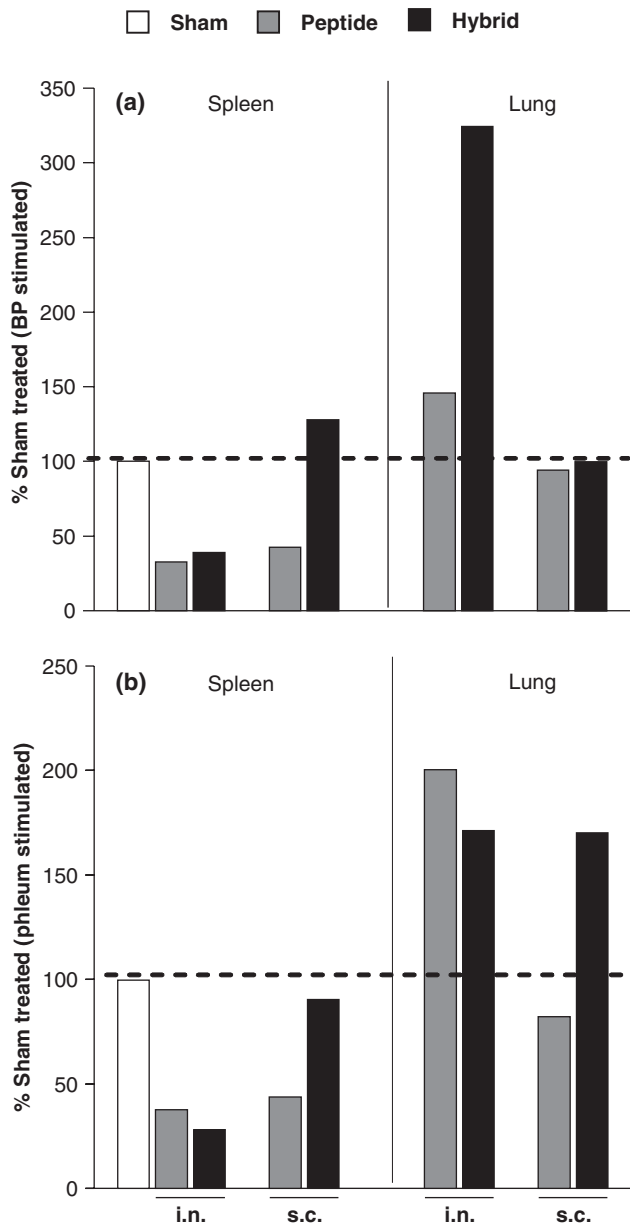


Fig. 4. IL-10 levels in spleen cell cultures and in pooled lung cell cultures after *in vitro* stimulation with (a) birch pollen or (b) phleum extract from sham-treated poly-sensitized mice (white bars), from i.n. or s.c. peptide mixture (grey bars) and from i.n. or s.c. hybrid (black bars) pre-treated mice. Data from one representative experiment out of three are shown as percentage of the sham-treated controls (100% of pg/mL).

accordance with reduced lung Th1 and Th2 cytokine levels (Fig. 1; Table 5). A possible role for regulatory T cells in local tolerance induction might be indicated by enhanced Foxp3 expression in lung CD3⁺ T cells of mucosally pre-treated mice (Table 5A). Recent publications support the involvement of this T cell population in the regulation of airway inflammation by showing increased numbers of T cells expressing Foxp3 in lung-draining lymph nodes after local inhalation tolerance in ovalbumin-sensitized mice

Table 5. Th1-, Th2- and regulatory pathway related mRNA expression of (A) pulmonary CD3⁺ T cells and (B) CD3⁺ T cells from bronchial lymph nodes

Table 1. Effect of peptide and hybrid immunization on the expression of Th1 and Th2 cytokines and regulatory T-cells												
			i.n. pre-treatment					s.c. pre-treatment				
			Peptide			Hybrid		Peptide			Hybrid	
			Sham	A	B	A	B	Sham	A	B	A	B
Th1												
	IFN- γ	1	0.27	0.87	0.73	0.84	1	0.73	0.45	0.83	0.81	
	T-bet	1	0.52	0.47	0.84	0.92	1	0.96	0.50	0.57	0.78	
Th2												
	GATA-3	1	0.13	0.71	0.80	0.76	1	0.76	0.59	0.74	1.11	
	ICOS	1	1.12	0.65	1.14	0.75	1	1.28	0.97	1.40	0.82	
Regulatory T-cells												
	IL-10	1	1.50	0.91	1.08	0.70	1	0.72	1.23	0.64	1.41	
	TGF- β	1	1.12	1.22	1.15	0.98	1	0.75	1.16	0.77	1.07	
	Foxp3	1	1.52	1.09	1.84	1.27	1	1.32	1.41	1.03	1.32	
	CTLA4	1	0.95	0.71	1.18	0.93	1	1.79	0.75	1.86	1.06	

Relative expression of the target genes to the housekeeping gene ALAS1 was determined by quantitative real-time RT-PCR in isolated CD3⁺ T cells from pooled lung and bronchial lymph node cells.

[31]. Another group reported that resolution of airway inflammation is mediated by CD4⁺ CD25⁺ Foxp3⁺ regulatory T cells in lungs of Der p 1-challenged mice [32]. The fact that we could not substantiate the enhanced IL-10 production at the mRNA level in lung CD3⁺ T cells from mucosally treated animals indicates the involvement of cells other than T cells in local immunosuppression. In this respect, Akbari et al. demonstrated that respiratory exposure to antigen resulted in IL-10 production by pulmonary dendritic cells and stimulation of regulatory T cells, probably by the involvement of the ICOS-ICOS-ligand-mediated co-stimulatory pathway [33, 34]. In addition to a defined cell population, the identification of the main production site of IL-10 is most important; apart from the lungs as effector organs, we have recently observed that IL-10, at the mRNA and protein level, is particularly induced in the nasal-associated lymphoid tissue as well as the lung-draining lymph nodes, representing the induction sites of immunity or tolerance (K. Hufnagl, unpublished data). Therefore, our data suggest IL-10 to be a key factor in the prevention of local airway inflammation, which could not be induced by the subcutaneous administration of peptides (Fig. 4).

Taken together, we have demonstrated that mucosal application of the immunodominant peptides of the major birch and grass pollen allergens is superior to systemic administration in preventing poly-allergic Th2 immune responses and airway inflammation in poly-sensitized mice. The use of such polyvalent allergy vaccines via the mucosal route might be suitable for application in the clinics to prevent the establishment of multiple sensitizations and airway/lung pathology.

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