

Intranasal tolerance induction with polypeptides derived from 3 noncross-reactive major aeroallergens prevents allergic polysensitization in mice

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Background: Specific immunotherapy is less effective in patients with multiple allergic sensitizations compared with monosensitized patients.

Objective: We therefore established a mouse model of polysensitization to the major birch and timothy grass pollen allergens to test whether allergic polysensitization can be prevented by multiple allergen application via the mucosal route.

Methods: Female BALB/c mice were immunized intraperitoneally with recombinant (r) Bet v 1, rPhl p 1, and rPhl p 5. For intranasal tolerance induction, a mixture of the complete allergens was compared with allergen-derived immunodominant peptides applied either as a mixture or as a synthetic hybrid peptide composed of the T-cell epitopes of the 3 allergens.

Results: Intranasal application of the mixture of the complete allergen molecules did not prevent polysensitization to the same allergens. In contrast, pretreatment with a mixture of the immunodominant peptides or the hybrid peptide led to significantly reduced allergen-specific IgE responses in sera, IL-4 production *in vitro*, and suppressed airway inflammation. TGF- β mRNA levels did not change, and IL-10 production was significantly suppressed after the pretreatment. The fact that the reduction of IL-10 was not abrogated after IL-10 receptor neutralization and that tolerance was not transferable with splenocytes indicates that the suppression of T_H2 responses in polysensitized mice might not be mediated by immunosuppressive cytokines.

Conclusion: Our study demonstrates that it is possible to suppress allergic immune responses simultaneously to several clinically important allergens. Thus, mucosal coapplication of selected peptides/hybrid peptides could be the basis of a

mucosal polyvalent vaccine to prevent multiple sensitivities in atopic patients. (*J Allergy Clin Immunol* 2005;116:370-6.)

Key words: Animal model, type I allergy, polysensitization, mucosal tolerance, polypeptides, hybrid, IL-10, TGF- β

About 25% of the population in industrialized countries has type I allergy, a genetically determined immunological disorder. In Europe, the most common seasonal airborne allergens are derived from white birch (*Betula verrucosa*) and timothy grass (*Phleum pratense*).¹ More than 90% of the patients allergic to birch pollen (BP) react to the major allergen Bet v 1, and in patients allergic to grass pollen, Phl p 1 and Phl p 5 represent major allergens.²⁻⁴

There is substantial clinical evidence that many patients with allergy are cosensitized to several unrelated airborne allergens. In this context, recent studies demonstrated that BP allergy is frequently associated with sensitization to other inhalant allergens, including grass pollen, and that multiallergies often develop with increasing age.^{5,6}

One of the most effective treatments for type I allergy, especially in young and monosensitized patients, is specific immunotherapy, performed by repeated subcutaneous injections of increasing amounts of allergen extracts.⁷ In patients with multiple sensitivities, specific immunotherapy is known to be of low efficacy and is associated with an increased risk of anaphylactic side reactions.^{7,8} Thus, there is a clear necessity to improve or develop new treatment strategies particularly for polysensitized individuals.

Such improvements could be achieved by the use of recombinant allergens or allergen-derived peptides according to the patient's sensitization profile,⁹⁻¹¹ instead of natural allergen extracts containing a variety of allergenic molecules. Recently the possibility of using prophylactic allergy vaccines has been discussed.¹² Following the concept of vaccination against many infectious diseases, prophylactic treatment to most common allergens could be a possible strategy for early allergy prevention. In addition, the change to a less invasive route of administration, such as application of allergens via the mucosal surfaces, might even enhance

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Supported by grants from the Austrian Science Fund (F01814, P14634-PAT, Y0784GEN, FWF T163).

Received for publication July 9, 2004; revised March 10, 2005; accepted for publication April 1, 2005.

Available online May 24, 2005.

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0091-6749/\$30.00

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doi:10.1016/j.jaci.2005.04.002

Abbreviations used

aa: Amino acid
BP: Birch pollen
r: Recombinant
RBL: Rat basophil leukemia

the patient's compliance to treatment. In several experimental animal studies, it has been shown that mucosal tolerance is highly effective in preventing allergic disease.¹³⁻¹⁷ A recent study in mice demonstrated that the intranasal route of desensitization can be even more effective than the intradermal route.¹⁸ We previously demonstrated in a murine model of allergic sensitization to BP that mucosal administration of recombinant (r) Bet v 1 suppressed allergic sensitization and airway inflammation in naive and in sensitized mice.^{16,17,19} In the current study, we established a murine model of polysensitization to the noncross-reactive major birch and grass pollen allergens Bet v 1, Phl p 1, and Phl p 5. By using this model, we tested whether it is possible to induce tolerance simultaneously against these different pollen allergens by intranasal application of either the allergen proteins or the allergen-derived peptides composed of the immunodominant epitopes thereof. Our results show that intranasal application of polypeptides successfully prevented allergic polysensitization.

METHODS

Animals

Female inbred 7-week-old BALB/c mice ($n = 5$ 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). Birch pollen (*B verrucosa*) and timothy grass pollen (*P pratense*) were purchased from Allergon (Välinge, Sweden), and extracts were prepared as previously described.¹⁶

Epitope mapping studies

For T-cell epitope mapping, a panel of 50 peptides of the Bet v 1 molecule (Cambridge Research Biochemicals Limited, Cambridge, United Kingdom), 77 peptides of Phl p 1 (Cambridge Research Biochemicals Limited), and 92 peptides of Phl p 5 (provided by Dr Helmut Fiebig, Reinbek, Germany) were used. Spleen cell suspensions from Bet v 1, Phl p 1, and Phl p 5 immunized mice were incubated with each of the dodecapeptides (2 $\mu\text{g}/\text{well}$), spanning the whole amino acid (aa) sequence of the respective antigens. The dodecapeptides overlapped by 9 residues. Proliferative responses were measured according to a previous description.¹⁶

Synthesis, purification, and characterization of peptides for intranasal pretreatment

Subsequent to the identification of the immunodominant regions of Bet v 1, Phl p 1, and Phl p 5 by epitope mapping, the respective peptides (see Results and Tables E1-E3 in the Journal's Online Repository at www.mosby.com/jaci) for intranasal pretreatment were synthesized by using a 9-fluorenylmethoxycarbonyl strategy with 2-(1H-benzotriazol-1-yl)-1,3,3-tetramethyluronium hexafluorophosphat activation (0.1-mmol small-scale cycles) on the Applied Biosystems (Foster City, Calif) peptide synthesizer Model 433A.²⁰ The identity to the peptides was checked by mass spectrometry, and the peptides were purified to >90% purity by preparative HPLC (Pichem, Graz, Austria).

Dose-finding and kinetic studies for intranasal pretreatment

Dose-finding experiments and kinetic studies with Bet v 1, Phl p 1, and Phl p 5 proteins were performed by using 0.1 to 100 $\mu\text{g}/\text{antigen}$ applied 3 times at 7-day intervals or every third day for 3 weeks. In line with previous studies in monosensitized mice, a treatment regimen with 10 μg applied 3 times every 7 days was chosen.^{16,17} Dose-finding experiments with the immunodominant peptides (1-100 $\mu\text{g}/\text{peptide}$) revealed that low-dose application (ie, 5 $\mu\text{g}/\text{peptide}$) was optimal for intranasal tolerance induction.

Polysensitization and intranasal pretreatment

Polysensitization was performed by 3 intraperitoneal injections (days 22, 36, and 50) of a mixture of 5 μg rBet v 1, 5 μg rPhl p 1, and 5 μg rPhl p 5 adsorbed to aluminium hydroxide ($\text{Al}(\text{OH})_3$; Serva, Heidelberg, Germany) at 14-day intervals (group 1). For intranasal pretreatment, a mixture of the 3 allergens, Bet v 1, Phl p 1, and Phl p 5 (10 μg each), was applied intranasally in 30 μL 0.9% NaCl 3 times at 7-day intervals (days 0, 7, and 14) before polysensitization (group 2). Peptide pretreatment was performed by applying a mixture of 5 μg Bet v 1 peptide, 5 μg Phl p 1 peptide, and 5 μg Phl p 5 peptide 2 in a volume of 30 μL (group 3). Intranasal pretreatment with the hybrid peptide was performed by using a concentration of 20 μg construct in 30 μL per application (group 4). Control mice were intranasally sham-treated with 30 μL 0.9% NaCl before polysensitization (group 1).

One week after the last intraperitoneal immunization, an aerosol challenge with 1% wt/vol BP and *Phleum* extract was performed on 2 consecutive days, as previously described.¹⁷

Sampling

Blood samples were taken before treatment and 2 days after the aerosol challenge (day 60) by tail bleeding. After the bleeding, the mice were sacrificed, spleen cell suspensions were prepared, and bronchoalveolar lavages were collected, as previously described.^{16,17}

Airway eosinophilia

Bronchoalveolar lavage samples were spun onto microscope slides and stained with hematoxylin and eosin (Hemacolor; Merck, Darmstadt, Germany). The number of eosinophils was expressed as percentage of the total counted cell number, as previously described.¹⁹

Detection of allergen-specific antibody levels in serum

Microtiter plates (Nunc, Roskilde, Denmark) were coated with each of the recombinant allergens (5 $\mu\text{g}/\text{mL}$) before incubation with sera. Rat antimouse IgG1, IgG2a, and IgE antibodies (1/500; Pharmingen, San Diego, Calif) were used, followed by peroxidase-conjugated mouse antirat IgG antibodies (1/2000; Jackson Immuno

TABLE I. Allergen-specific antibody levels, T-cell proliferation, and cytokine production in polysensitized mice

	rBet v 1	rPhl p 1	rPhl p 5		BP	Phleum
IgG1*	1.98 ± 0.28	1.92 ± 0.05	1.61 ± 0.27	IFN- γ †	1501.9 ± 621.1	1307.9 ± 372.7
IgE*	0.85 ± 0.21	0.48 ± 0.05	0.53 ± 0.02	IL-4‡	28.6 ± 14.6	65.8 ± 17.3
IgG2a*	0.51 ± 0.35	0.31 ± 0.12	0.75 ± 0.37	IL-5‡	51.7 ± 42.52	246.7 ± 105.1
Stimulation index†	2.97 ± 0.51	2.63 ± 1.02	4.41 ± 2.38			

*Serum antibody levels (OD) were measured by ELISA.

†Spleen cells from polysensitized mice were cultured with the respective antigens for 4 days (stimulation index, background medium values 5820.2 ± 1519.3 cpm).

‡Cytokine levels (pg/mL) were measured by ELISA. All results are mean values (±SDs) from 3 independent experiments with 5 animals per experiment.

Lab, West Grove, Pa).¹⁶ Results show the OD values after subtraction of baseline levels (0.058 ± 0.025) from preimmune sera.

Rat basophil leukemia cell mediator release assay

Rat basophil leukemia (RBL)-2H3 cells were incubated with sera obtained from pretreated and polysensitized mice at dilutions of 1/30 to 1/300. Degranulation of RBL cells was induced by adding 0.03 μ g of each allergen diluted in 100 μ L Tyrode's buffer. Supernatants were analyzed for β -hexosaminidase activity as previously described.²¹ Results are reported as percentages of total β -hexosaminidase released after addition of 1% Triton X-100 and are shown after subtraction of baseline release levels (1.13 ± 0.81) obtained with preimmune sera.

Lymphocyte proliferation and cytokine production

Proliferation of splenocytes (2×10^5 cells/well) after stimulation with recombinant allergens (2 μ g/well) was measured as previously described.¹⁶

IFN- γ , IL-4, IL-5, and IL-10 production was measured in spleen cell suspensions incubated for 40 hours with BP (25 μ g/well) or *Phleum* extract (25 μ g/well) as described.¹⁶ IL-5 was measured in bronchoalveolar lavage fluids as previously described.¹⁷ For neutralization of the IL-10 receptor *in vitro* pooled splenocytes were cultured in the presence of rat antimouse IL-10 receptor mAb (60 μ g/mL; BD Biosciences, Heidelberg, Germany) or control rat IgG1, κ isotype antibody (BD Biosciences).²² Cytokine levels are shown in pg/mL after subtraction of baseline levels (IFN- γ , 147.23 ± 61.6 pg/mL; IL-4/5, 4.8 ± 4.1 pg/mL; IL-10, 27.75 ± 23.3 pg/mL) of unstimulated cultures.

Quantification of TGF- β mRNA expression by real-time RT-PCR

Total RNA was isolated from pooled spleen cell suspensions on the day of sacrifice by using RNeasy Minikit (Quiagen, Valencia, Calif), treated with DNase (Quiagen), and then reverse-transcribed into cDNA by using random hexamers (GeneAmp RT-PCR kit; Perkin Elmer, Boston, Mass). Gene expression was determined by quantitative real-time PCR by using predesigned TaqMan Gene Expression Assays according to the manufacturer's protocol on an ABI 7700 sequence detection system (Applied Biosystems, Foster City, Calif). Amplification of the endogenous control 18S rRNA (Applied Biosystems) was performed to standardize the amount of sample cDNA added, and relative quantitation was performed by using the standard curve method (Applied Biosystems). The thermal cycle conditions were 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles of amplification at 95°C for 15 seconds and 60°C for 1 minute.

Adoptive cell transfer

Pretreatment with the peptide mixture or the hybrid peptide was performed as described. Eight days after the last intranasal application, donor spleen cells (1×10^7) were intravenously injected into naive recipients.¹⁷ Four hours after the cell transfer, the recipients were polysensitized as described, and immune responses were evaluated 7 days after the last immunization.

Statistics

Data are expressed as means ± SEMs from 3 independent experiments. Differences between groups were tested by Kruskal-Wallis tests. Post hoc comparisons for all pairs of groups were performed applying Tukey-Kramer tests. *P* values below .05 were considered significant. Pairwise comparison of sensitized versus pretreated groups was performed by using the Mann-Whitney *U* test.

RESULTS

Polysensitized mice displayed comparable immune responses to each of the 3 allergens

Immunization with rBet v 1, rPhl p 1, and rPhl p 5 induced comparably high IgG1, IgE, and IgG2a levels and strong lymphocyte proliferative responses to each of the allergens (Table I). In addition, similar levels of IL-4, IL-5, and IFN- γ were detected after stimulation of spleen cell suspensions from polysensitized mice with BP and *Phleum* extract (Table I). Naive splenocytes stimulated with rBet v 1, rPhl p 1, rPhl p 5, BP, or *Phleum* extract did not differ in their proliferative responses or in cytokine levels from medium control levels (data not shown).

Epitope mapping studies for characterization of the immunodominant peptides of Bet v 1, Phl p 1, and Phl p 5

In Bet v 1 immunized mice, 1 immunodominant T-cell epitope, MGETLLRAVESY, was located at the C-terminus corresponding to the aa sequence position 139-150.²³ One immunodominant region of Phl p 1, AGELELQF-RRVKCKY, corresponding to the aa sequence position 127-141, was identified, also described as a T-cell-reactive region in human beings.²⁴ Concerning Phl p 5, 2 immunodominant regions, KVDAAFKVAATAANA corresponding to the aa sequence 166-180 (peptide 1) and TVATAPEVKYTVFETALK corresponding to the aa

sequence 226–243 (peptide 2), were identified. Comparison of the tolerogenicity of these peptides revealed that peptide 2 led to the highest immunosuppression in Phl p 5–sensitized mice (data not shown). Peptide 2 was therefore selected for tolerance induction in polysensitized mice.

Synthesis of peptides for intranasal tolerance induction

According to the epitope mapping studies, we synthesized single peptides from Bet v 1 (aa: SKEMGETLLRAVESYLLAHSDE), Phl p 1 (aa: LRSAGEL-ELQFRRVKCKYPEG), and Phl p 5 (aa sequence: YAATVATAPEVKYTVFETALKKAI) for intranasal application as peptide mixture, or a hybrid peptide composed of the immunodominant regions of these 3 allergens (aa: MGETLLRAVESYAGELELQFRRVK-CKYTVATAPEVKYTVFETALK; see Tables E1–E3 in the Online Repository at www.mosby.com/jaci).

Intranasal tolerance induction with the peptide mixture and the hybrid peptide, but not with the protein mixture, suppressed T_H2 immune responses and airway inflammation in polysensitized mice

Sera from peptide mixture as well as from hybrid peptide–pretreated mice, but not from mice pretreated with the complete proteins, induced significantly lower IgE-induced basophil degranulation *in vitro* than sera from polysensitized mice (Fig 1, A). In accordance with this reduction, IL-4 levels were significantly suppressed in spleen cell cultures from mice pretreated with the peptide mixture or the hybrid peptide. In contrast, IL-4 levels were enhanced or remained unchanged in the protein-pretreated group (Fig 1, B). Unlike the allergen-specific T_H2 responses, IgG2a antibody production was not significantly changed by either of the pretreatments (Table II). Similarly, IFN- γ levels *in vitro* remained unchanged except for a significant enhancement in BP-stimulated splenocytes from the hybrid peptide–pretreated mice (Table II).

Airway inflammation, characterized by eosinophils and IL-5 production in bronchoalveolar lavages, was significantly reduced after pretreatment with the peptide mixture or the hybrid peptide (Fig 2).

Polytolerance induction is not regulated by TGF- β or IL-10

No significant changes in TGF- β mRNA expression were detected after pretreatment with the peptide mixture or the hybrid peptide compared with polysensitized controls (Fig 3, A). IL-10 levels were significantly suppressed in peptide mixture as well as hybrid peptide–pretreated mice (Fig 3, B). Neutralization of IL-10 receptor *in vitro* did not abrogate the suppression of IL-10 levels in the pretreated groups (Table III). Moreover, adoptive cell transfer experiments showed that tolerance was not transferable by spleen cells, because IgE-induced basophil

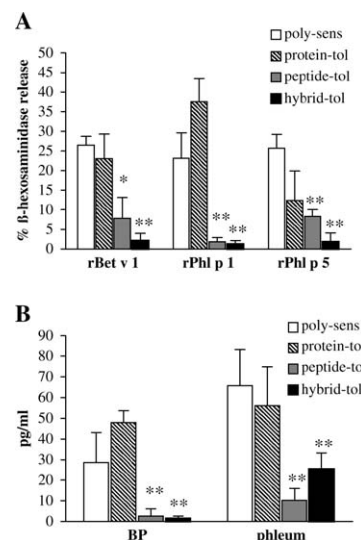


FIG 1. IgE-dependent allergen-specific basophil degranulation (RBL assay) (A), and IL-4 production (B) *in vitro* from protein mixture (hatched bars), peptide mixture (gray bars), and hybrid peptide–pretreated mice (black bars) compared with polysensitized controls (white bars). * $P < .05$, ** $P < .01$, pretreated vs polysensitized.

degranulation remained unchanged in polysensitized recipients (Fig 3, C).

DISCUSSION

In the current study, we demonstrate that it is possible to prevent the development of multiple sensitivities to birch and grass pollen allergens by intranasal antigen application. Successful tolerance induction was achieved by intranasal administration of the immunodominant peptides of Bet v 1, Phl p 1, and Phl p 5, but not by the mixture of the allergen proteins.

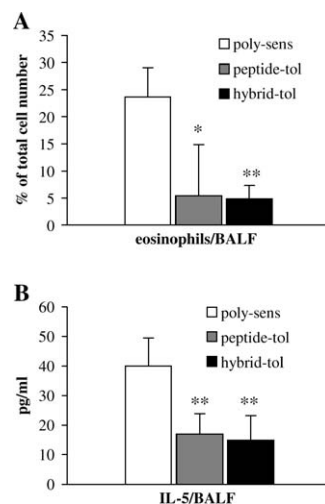
It was recently shown that the dose of coadministered antigens is most important for the establishment of a balanced T_H2 immune response.²⁵ Accordingly, in the current study, we demonstrated that immunization with the optimal doses of Bet v 1, Phl p 1, and Phl p 5 (ie, 5 μ g each) led to comparable humoral and cellular immune responses to all 3 allergens/antigens (Table I). Moreover, the immunodominant epitopes recognized by T cells from polysensitized mice were identical to some of the T-cell epitopes in patients allergic to birch and grass pollen.^{24,26,27} Thus, our murine model of multiple allergen sensitivity showed similar immunological characteristics to those of human pollinosis.

From previous studies we know that rBet v 1 acts as a potent mucosal tolerogen in monosensitized mice.^{16,17,19} However, when rBet v 1 was intranasally applied before polysensitization with the 3 allergens, the tolerizing effects of Bet v 1 toward itself were considerably impaired.²⁸ In addition, in polysensitized mice, mucosal pretreatment with Bet v 1 alone did not alter the immune response toward the coapplied grass pollen allergens, indicating that no bystander or linked suppression can be

TABLE II. Allergen-specific IgG2a antibody levels and IFN- γ production *in vitro* in polysensitized and pretreated mice

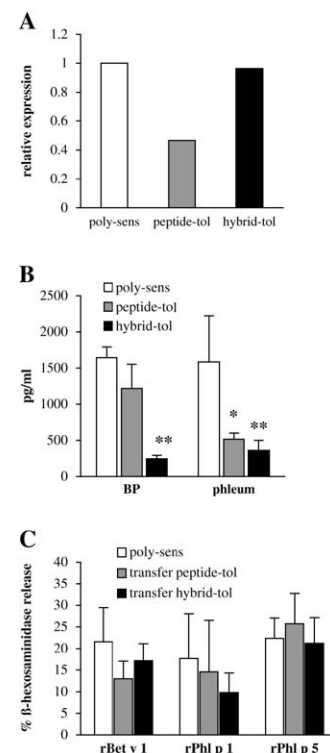
	IgG2a (OD)*			IFN- γ (pg/mL)†	
	rBet v 1	rPhl p 1	rPhl p 5	BP	Phleum
Polysensitized	0.51 $\pm$ 0.35	0.31 $\pm$ 0.12	0.75 $\pm$ 0.37	1501.9 $\pm$ 621.1	1307.9 $\pm$ 372.7
Protein-tolerized	0.29 $\pm$ 0.19	0.29 $\pm$ 0.26	0.71 $\pm$ 0.51	2160.7 $\pm$ 752.1	1885.6 $\pm$ 553.3
Peptide-tolerized	0.93 $\pm$ 0.71	1.02 $\pm$ 0.77	1.56 $\pm$ 0.46	2305.7 $\pm$ 798.5	1753.5 $\pm$ 657.1
Hybrid-tolerized	1.25 $\pm$ 1.03	1.07 $\pm$ 0.82	1.38 $\pm$ 0.61	3486.9 $\pm$ 184.1**	1869.7 $\pm$ 204.1

*IgG2a antibody levels were measured by ELISA.

†IFN- γ levels were measured in spleen cell cultures after stimulation with BP or *Phleum* extract.** $P < .01$ hybrid-tolerized vs polysensitized as determined by Mann-Whitney U test.**FIG 2.** Number of eosinophils (**A**) and IL-5 levels (**B**) in bronchoalveolar lavage fluids from polysensitized mice (white bars), from peptide mixture-pretreated mice (gray bars), and from mice pretreated with the hybrid peptide (black bars). * $P < .05$, ** $P < .01$, pretreated vs polysensitized. BALF, Bronchoalveolar lavage fluid.

achieved in mice with multiple sensitivities (data not shown).^{22,29} Therefore, a mixture of all 3 allergen proteins was used for intranasal pretreatment. However, it was not possible to reduce IgE-dependent basophil degranulation, allergen-specific antibody responses, or cytokine production with the mixture of the proteins in polysensitized mice. These results are in line with other studies showing that the tolerogenic efficacy was impaired when more than 1 protein antigen was ingested via the nasal or oral route.^{30,31}

Several studies have demonstrated that allergen-derived immunodominant peptides applied via mucosal surfaces have a high tolerogenicity in naive and in primed animals.^{15,32,33} It was further shown that treatment of mice with a peptide containing a single immunodominant epitope led to inhibition of immune responses directed to the other epitopes on the natural allergen, a phenomenon called *intramolecular suppression*.³³ Therefore, to enhance the efficacy of tolerance induction in the polysensitized mice, we applied a mixture of the immunodominant peptides or a synthetic hybrid composed of the major T-cell epitopes of Bet v 1, Phl p 1, and Phl p 5.

**FIG 3.** **A**, TGF- β mRNA expression from peptide mixture (gray bar) and hybrid peptide-pretreated mice (black bar) shown as relative values in comparison with polysensitized controls (white bar). **B**, IL-10 production *in vitro* from polysensitized (white bars), peptide mixture (gray bars), and hybrid peptide-pretreated (black bars) mice. * $P < .05$, ** $P < .01$, pretreated vs polysensitized. **C**, RBL assay after adoptive transfer of spleen cells from peptide mixture (transfer, gray bars) or hybrid peptide-pretreated mice (transfer, black bars) compared with polysensitized controls (white bars).

Indeed, intranasal administration of either of the peptide mixture or the hybrid peptide led to a highly significant reduction of allergen-specific T_H2 responses (Fig 1) and airway inflammation (Fig 2). Other studies showing that application of a panel of peptides suppressed T_H2 immune responses were directed only against single allergens,^{32,34} whereas this is the first report showing that peptide-induced tolerance can be directed against 3—and probably more—different antigens/allergens.

Our observation that the mixture of protein allergens was unable to induce tolerance, whereas the polypeptides

TABLE III. IL-10 levels after neutralization of IL-10 receptor *in vitro**

	Control Ab		α IL-10 receptor Ab	
	BP	Phleum	BP	Phleum
Poly-sens	241.82	940.66	283.94	1095.78
Peptide-tol	138.63	472.41	158.91	661.14
Hybrid-tol	165.84	630.72	283.74	790.85

*Pooled splenocytes from polysensitized (poly-sens), peptide mixture (peptide-tol), or hybrid peptide (hybrid-tol) pretreated mice were stimulated with BP or *Phleum* extract in the presence of a neutralizing anti IL-10 receptor antibody or an isotype control. IL-10 levels (pg/mL) in supernatants were measured by ELISA.

were able to do so, leads to the speculation that because of the different conformation of the allergens, distinct antigen-presenting cells were targeted.³⁵ It might further be speculated that the reduced/nontolerogenic capacity of the protein mixture is a result of an intermolecular competition among a higher number of epitopes for the accessibility of MHC molecules than might be the case with only 3 (or few) immunodominant peptides.³⁶ In studies on secondary prevention, the efficacy of the peptide treatment depending on their length was evaluated: whereas short peptides (20 aa or less) have the advantage that they cannot cross-link IgE on mast cells, they may require characterization of major HLA-restricted T-cell epitopes on a patient basis.³⁷ Long peptides (50 aa or more) might overcome this problem, but at the cost of safety.³² The obvious advantage of primary prevention, as performed in this study, lies in the risk-free application of selected peptides, but limitations might still be based on the polymorphisms of the HLA molecules. However, it is also known that peptides capable of binding multiple HLA types and of being immunogenic in context with different HLA molecules do exist.³⁸ This has been also shown for several allergens.^{27,39} The fact that the immunodominant peptides of the 3 allergens—representing major T-cell epitopes in a high number of patients allergic to birch and grass pollen^{24,26,27}—were also tolerogenic in another mouse strain, ie, C57Bl/6J (data not shown), may indicate that tolerance induction was not restricted to a particular MHC haplotype.

Concerning the underlying mechanisms, we previously demonstrated in Bet v 1-monosensitized mice that tolerance induction depends on the conformation of the antigen: whereas tolerance induction with the whole Bet v 1 molecule led to enhanced TGF- β and IL-10 mRNA levels and tolerance was transferable with spleen cells, tolerance induction with a fragment of Bet v 1—including the immunodominant T-cell epitope—was not mediated by regulatory mechanisms.^{16,17} Similarly, in this study of polypeptide-induced tolerance, we found significantly reduced IL-10 levels along with unchanged TGF- β mRNA levels. Neutralization of the IL-10 receptor *in vitro* did not abrogate the reduction of IL-10 (Table III). Moreover, because tolerance was not transferable with splenocytes, it seems unlikely that regulatory cytokines mediated tolerance in polysensitized mice. Thus, mecha-

nisms such as clonal anergy, described in a model of peptide-induced tolerance,³² or cytokine-independent mechanisms, such as cell-cell contact interaction via the Notch signalling pathway⁴⁰ or via cytotoxic T lymphocyte-associated antigen 4 expression on antigen-specific regulatory T cells,⁴¹ might rather play a role in our model of polytolerance induction.

In conclusion, this is the first report showing that mucosal application of polypeptide constructs inhibited polysensitization to several antigens/allergens. Thus, these data could be the basis for the development of a mucosal polyvalent allergy vaccine for primary prevention of multiple sensitivities in atopic individuals. It remains to be investigated whether polytolerance induction can be used for treatment of already established multisensitivities.

We thank Karin Baier for technical assistance, Dr Michael Kundi for statistical analysis, and Dr Helmut Fiebig for providing the Phl p 5 peptides.

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TABLE E1. Bet v 1, Phl p 1, and Phl p 5 amino acid sequences from the epitope mapping studies: Identification of immunodominant regions of Bet v 1, Phl p 1, Phl p 5 by epitope mapping*

Bet v 1	MGETLLRAVESY
Phl p 1	AGELELQFRRVKCKY
Phl p 5 (peptide 1)	KVDAAFKVAATAANA
Phl p 5 (peptide 2)	TVATAPEVKYTVFETALK

*In epitope mapping experiments, using overlapping peptides, 1 immunodominant region of Bet v 1, 1 of Phl p 1, and 2 of Phl p 5 were identified. With respect to the immunodominant regions of Phl p 5, peptide 2 revealed a higher tolerogenicity than peptide 1 and was therefore selected for subsequent intranasal application.

TABLE E2. Bet v 1, Phl p 1, and Phl p 5 amino acid sequences from the peptides used for *in vivo* pretreatment: synthesis of peptides used for intranasal pretreatment as peptide mixture*

Bet v 1	SKEMGETLLRAVESYLLAHSDE
Phl p 1	LRSAGELELQFRRVKCKYPEG
Phl p 5 (peptide 2)	YAATVATAPEVKYTVFETALKKAI

*The peptides of Bet v 1, Phl p 1, and Phl p 5 which were used for intranasal pretreatment as a peptide mixture included amino acids flanking the respective N-terminal and C-terminal endings of the immunodominant regions to achieve a comparable length of these 3 peptides.

TABLE E3. Bet v 1, Phl p 1, and Phl p 5 amino acid sequences from the peptides used for *in vivo* pretreatment: synthesis of the hybrid peptide used for intranasal pretreatment*

1	12	27	45
MGETLLRAVESYAGELELQFRRVKCKYTVATAPEVKYTVFETALK			
aa	1-12	Bet v 1	
aa	13-27	Phl p 1	
aa	28-45	Phl p 5 (peptide 2)	

*For construction of the hybrid peptide, the immunodominant regions of Bet v 1, Phl p 1, and Phl p 5 were sequentially synthesized to form 1 peptide.