

Original article

A recombinant allergen chimer as novel mucosal vaccine candidate for prevention of multi-sensitivities

Background: As conventional immunotherapy is less efficacious in patients with allergic multi-sensitivities compared with mono-sensitized subjects, new intervention strategies are needed. Therefore, an allergen chimer was genetically engineered for treatment of multi-sensitization with birch and grass pollen on the basis of mucosal tolerance induction.

Methods: The major birch pollen allergen Bet v 1 served as a scaffold for N- and C-terminal linkage of the immunodominant peptides of the grass pollen allergens Phl p 1 and Phl p 5 and this new construct was cloned and expressed in *Escherichia coli*. After purification, physicochemical and immunological characterization the chimer was used for intranasal tolerance induction prior to poly-sensitization with Bet v 1, Phl p 1 and Phl p 5.

Results: The immunological characterization revealed that the conformation of Bet v 1 within the chimer was comparable to that of natural as well as recombinant Bet v 1. The chimer was immunogenic in mice for T and B cell responses to the three allergens. Intranasal application of the chimer prior to poly-sensitization significantly suppressed humoral and cellular allergen-specific Th2 responses and prevented development of airway inflammation upon allergen challenge. Moreover, local allergen-specific IgA antibodies were induced by the chimer. The mechanisms of poly-tolerance induction seemed to be mediated by regulatory cytokines, since TGF- β and IL-10 mRNA in splenocytes were upregulated and tolerance was transferable with these cells.

Conclusion: The data indicate that such allergen chimer harboring several unrelated allergens or allergen peptides could serve as mucosal polyvalent vaccines for prevention of multi-sensitivities.

C. Wild¹, M. Wallner², K. Hufnagl¹,
H. Fuchs³, K. Hoffmann-Sommergruber³,
H. Breiteneder³, O. Scheiner³,
F. Ferreira², U. Wiedermann¹

¹Department of Specific Prophylaxis and Tropical Medicine, Center for Physiology and Pathophysiology, Medical University of Vienna, Vienna, Austria; ²Department of Allergy and Immunology, Institute of Molecular Biology, University of Salzburg, Austria; ³Department of Pathophysiology, Center for Physiology and Pathophysiology, Medical University of Vienna, Vienna, Austria

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Ursula Wiedermann, MD, PhD
Department of Specific Prophylaxis and Tropical Medicine
Center for Physiology and Pathophysiology
Medical University of Vienna
Kinderspitalgasse 15
A-1090 Vienna
Austria

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Allergen-specific immunotherapy (SIT) represents the only curative treatment strategy against type I allergy. SIT is based on the administration of increasing amounts of natural allergen preparations on a regular basis to substantially reduce allergic symptoms (1). Even though SIT is often effective, particularly in young and mono-sensitized patients, several drawbacks have to be taken into account which may limit the application of this treatment: presently, SIT is performed with allergen extracts containing a variety of allergenic, non-allergenic or even toxic proteins which are difficult to standardize and do not always follow the patient's individual allergen profile. Moreover, the frequent injections during a period of 2–3 years, as well as the risk for anaphylactic side reactions which may occur during allergen application into a sensitized organism, often limit the patient's compliance. Thus, within recent years efforts have been undertaken to develop approaches that address these

problems, suggesting the use of only the disease eliciting allergens in recombinant form instead of crude allergen extracts (2), the change to a less invasive route, i.e. mucosal application (3, 4), or even prophylactic vaccines for subjects at risk (5, 6).

Along these lines, we previously demonstrated in a mouse model of birch pollen allergy that mucosal (intranasal or oral) application of the major birch pollen allergen Bet v 1 suppressed allergic immune responses in naïve and sensitized mice (7, 8), suggesting mucosal tolerance induction with recombinant allergens as a feasible concept for prophylactic and therapeutic allergy treatment (6).

Based on clinical data showing that many mono-sensitized patients acquire additional allergies with increasing age (9) and that SIT is not recommended for these poly-sensitized patients (10), our current research has focused on the development of novel treatment

strategies based on mucosal tolerance induction for patients with multi-sensitivities or at risk to develop additional allergies. In this regard, we recently established a mouse model of poly-sensitization to the major birch and grass pollen allergens Bet v 1, Phl p 1 and Phl p 5, to test if mucosal tolerance can be simultaneously induced to several allergens (11).

The three-dimensional structure of an antigen and its distinct treatment by antigen-presenting cells, in particular dendritic cells, has been shown not only to be crucial for the development of certain T cell cytokine pattern during the sensitization process (12, 13), but also during mucosal tolerance induction (13). In the latter respect, we demonstrated that the presence of the immunodominant peptides is essential for mucosal tolerance induction (14) but for maintenance of immunosuppression and for induction of regulatory mechanisms the complete molecule in its native conformation is required (15).

The aim of the current study was to create an allergen chimera for simultaneous treatment of birch and grass pollen allergy, consisting of a host allergen in its native conformation anchoring the immunodominant peptides of two or more non cross-reactive guest allergens. As Bet v 1 proved to be a potent tolerogen inducing long-term immunosuppression via regulatory mechanisms (7,

15), this molecule served as a scaffold for linkage of the immunodominant T cell epitopes of Phl p 1 and Phl p 5. In a mouse model of poly-sensitization (11), this allergen chimera was used for mucosal pretreatment to evaluate if such recombinant constructs could serve as mucosal vaccine candidates for prevention of allergic multi-sensitivities.

Material and methods

Construction of the birch–grass–pollen chimera expression plasmid

For construction of the birch–grass–pollen chimera (Fig. 1A) Bet v 1 was PCR amplified (primers listed in Table 1) from its original cloning vector. At the 5' end of Bet v 1 the restriction sites *Nco*I and *Eco*RI and at the 3' end the restriction sites *Hind*III and *Xho*I were added to the nucleotide sequence. The gene was cloned into the plasmid pHis Parallel 2 using the restriction sites *Nco*I and *Xho*I (16).

For PCR amplification (primers listed in Table 1) of the immunodominant peptides of Phl p 1 and Phl p 5 (11), a Phl p cDNA library served as template. Phl p 5 was amplified using primers introducing *Nco*I and *Eco*RI restriction sites. The primers used for amplification of Phl p 1 introduced the restriction sites *Hind*III and *Xho*I. The chimera was constructed by inserting the two peptides into the pHis Parallel 2 – Bet v 1 construct using the respective restriction sites. Correct sequence and integrity of open reading frame was confirmed by DNA sequence analysis. The expression construct was

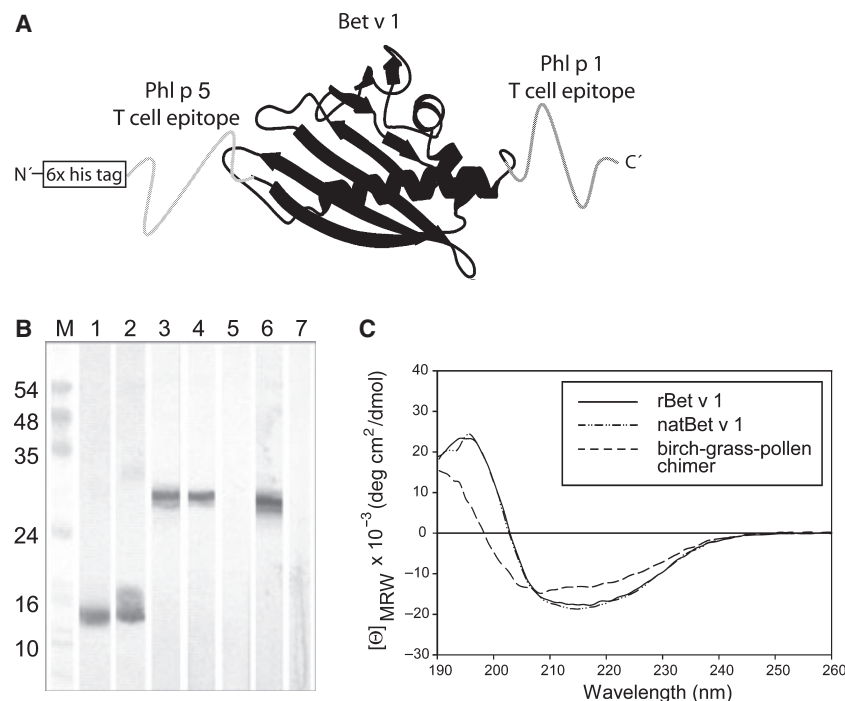


Figure 1. Production of the birch–grass–pollen chimera. (A) Scheme of the pHis parallel 2–chimer. Bet v 1 was chosen as scaffold and the immunodominant peptides of Phl p 1 and Phl p 5 were linked to the N and C-terminus of Bet v 1. (B) Immunoblot analysis showing the binding of BIP 1, a mouse monoclonal anti-Bet v 1 IgG antibody, to Bet v 1 (lane 1), natural birch pollen (lane 2) and the chimera (lane 3) and the binding of BIP 4 (another mouse monoclonal anti-Bet v 1 IgG antibody, lane 4) and human anti-Bet v 1-IgE antibodies (lane 6) to the chimera. As controls buffer (lane 5) and human control serum (lane 7) were used. (C) Far UV CD spectra of natural (dashed/dotted line) and recombinant (solid line) Bet v 1 and birch–grass–pollen chimera (dashed line). Data are baseline subtracted, normalized and presented as mean residue molar ellipticity [θ]MRW.

Table 1. Primers used for construction of the birch–grass–pollen chimer

Primer	Sequence	Restriction sites
Bet v 1 fwd	5'-CATGCCATGGGAGAATTCGGTGTTCATTACGAAACTG-3'	<i>NcoI</i> , <i>EcoRI</i>
Bet v 1 rev	5'-CACACTCCGATGCCTACAACAAGCTTGGACTCGAGCGG-3'	<i>HindIII</i> , <i>XhoI</i>
Phl p 5 fwd	5'-CATGCCATGGCCTACGCCGCCACCGTC-3'	<i>NcoI</i>
Phl p 5 rev	5'-ATCACCGCCATGTCCGAAGAATTCAT-3'	<i>EcoRI</i>
Phl p 1 fwd	5'-ACCAAGCTTGAGCAGAAGCTGCCGAGC-3'	<i>HindIII</i>
Phl p 1 rev	5'-CCCGGAGGGCACCAAGGTGTAACCTCGAGCGG-3'	<i>XhoI</i>

Restriction sites are underlined.

electrotransformed into competent BL 21 (DE 3) *Escherichia coli* cells.

Expression and purification of the birch–grass–pollen chimer

A single transformant colony of the birch–grass–pollen chimer expressing *E. coli* clone was picked and grown in LB/1 mM ampicillin under constant shaking at 37°C to an OD₆₀₀ of 0.7. The recombinant protein was expressed and harvested as previously described (17).

Inclusion bodies were solubilized in lysisbuffer (50 mM NaPO₄, pH 8.0; 300 mM NaCl; 10 mM imidazol; 6 M urea) containing 10% glycerol and 10 mg lysozyme. The recombinant birch–grass–pollen chimer was purified by Ni–NTA–affinity chromatography under denaturing conditions as described (18). The fractions were examined on sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), pooled and dialysed against 20 mM NaPO₄, pH 8.0 reducing the urea concentration stepwise (4 M urea, 2 M urea, without urea) and finally against 10 mM NaPO₄, pH 7.2. Purified proteins were lyophilized and stored at –20°C.

Protein concentrations were determined using the bicinchonic acid (BCA) protein assay (Pierce, Rockford, IL, USA) according to the user manual.

Contamination with bacterial endotoxin was determined using the LAL ENDOCHROME test (Charles River Endosafe, Wilmington, MA, USA) according to the manufacturer's instructions.

Physicochemical characterization of the birch–grass–pollen chimer

Amino acid composition of purified recombinant allergens and the allergen standard was determined using the Pico Tag method (Waters, Milford, MA, USA). Phenylthiocarbamyl amino acid derivatives were analyzed by reversed phase–HPLC (HP 1100; Agilent Technologies, Palo Alto, CA, USA) using a 3.9 × 150 mm, 4 µM Nova-Pak C18 column (Waters, Milford, MA, USA). UV absorbance was monitored at 254 nm.

Far UV circular dichroism (CD) spectra were recorded in aqueous solutions with a JASCO J-810 spectropolarimeter (Jasco, Essex, UK) fitted with a Neslab RTE-111 M temperature control system (Thermo Neslab Inc., Newington, NH, USA) as described (19). The final spectra were obtained after subtraction of the baseline recorded under identical conditions. All measurements were performed in 10 mM sodium phosphate buffer pH 7.4. Protein concentrations were exactly determined for each protein by amino acid analysis. All data are presented as mean residue molar ellipticity [Θ]_{MRW} at a given wavelength.

For peptide mapping protein samples were heat denatured, reduced, alkylated and finally digested with trypsin (Boehringer Ingelheim, Ingelheim, Germany). Proteolytic digests were terminated by drying the samples in a vacuum centrifuge.

Mass data were acquired using electrospray ionization coupled to a Quadrupole Time of Flight Mass Spectrometry (ESI-QTOF MS)

(Micromass QToF Global Ultima mass spectrometer; Waters, Milford, MA, USA). Samples were dissolved in 50% acetonitrile in 0.2% aqueous formic acid at a concentration of approximately 100 fmol/µl and ionized by direct infusion at a speed of 5 µl/min. Proteolytic fragments were analyzed using the MaxEnt3 software (Waters).

Immunoblot studies

Bacterial extracts or purified proteins were separated by SDS–PAGE and transferred to nitrocellulose according to standard protocols (20). Monoclonal mouse anti-Bet v 1 IgG antibodies (1/10; BIP 1 and BIP 4) (20) or a pool of Bet v 1 specific IgE positive patients sera (1/10), followed by rat anti-mouse IgG 1 (1/500; Pharmingen, San Diego, CA, USA) and an alkaline phosphatase-conjugated anti-rat IgG (1/2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or rabbit anti-human IgE (1/500; Dako, Glostrup, Denmark) followed by alkaline phosphatase-conjugated swine anti-rabbit antibodies (1/200; Dako) were used for detection. Controls were performed using buffer only or non-allergic human serum.

Animals and antigens

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

The recombinant allergens Bet v 1, Phl p 1 and Phl p 5 were purchased from Biomay GmbH (Vienna, Austria). Birch pollen (*Betula verrucosa*) and timothy grass pollen (*Phleum pratense*) were purchased from Allergon (Välinge, Sweden) and extracts prepared for allergen challenge experiments as previously described (7).

Animal experiments – treatment protocols

Systemic immunization for characterization of the birch–grass–pollen chimer in vivo. To evaluate the immunogenicity of the birch–grass–pollen chimer mice were immunized four times intraperitoneally (i.p.) in 2-week intervals (days 0, 14, 28 and 42) with either 1 µg Bet v 1, 1 µg Phl p 1, 1 µg Phl p 5 or 1 µg birch–grass–pollen chimer adsorbed to 2 mg Al(OH)₃ in a total volume of 150 µl. Samples were taken 7 days after the last immunization (day 66).

Dose finding experiments. For tolerance induction with the birch–grass–pollen chimer, dose finding experiments were performed using 1–100 µg of the chimer for intranasal (i.n.) application. According to previously established protocols i.n. treatment was performed three times in 7-day intervals. The results revealed that 15 µg of the birch–grass–pollen chimer, which is equimolar to 10 µg Bet v 1, was the optimal dose for poly-tolerance induction.

Intranasal pretreatment with the birch-grass-pollen chimera and allergen challenge experiments. The birch-grass-pollen chimera, 15 µg in 30 µl 0.9% NaCl (tol with birch-grass-pollen chimera), was i.n. applied three times in 7-day intervals (days 0, 7 and 14) before poly-sensitization with Bet v 1, Phl p 1 and Phl p 5. Ten days later (day 24), the mice were three times i.p. immunized with a mixture of 3 µg Bet v 1, 3 µg Phl p 1 and 3 µg Phl p 5 adsorbed to Al(OH)₃ in 15-day intervals. Control mice were i.n. treated either with 10 µg Bet v 1 in 30 µl 0.9% NaCl (tol with Bet v 1) or sham treated with 30 µl 0.9% NaCl (sham-pretreated) prior to poly-sensitization with the allergen-mixture. The experiment was finalized at day 60.

In a second experimental setup, mice were pretreated with the birch-grass-pollen chimera and i.p. sensitized as described above. One week after the last i.p. sensitization with the three allergens an aerosol challenge with 1% birch pollen (BP) and 1% *Phleum* extract was performed on two consecutive days, as previously described (14). Three-days later, the mice were killed, serum and bronchoalveolar lavages (BAL) were collected for measurement of systemic and local immune responses and lung inflammation.

Adoptive cell transfer. For adoptive cell transfer experiments 15 µg of the birch-grass-pollen chimera was three times i.n. applied to donor mice (days 0, 7 and 14). Seven days (day 21) after the last i.n. antigen application the mice were killed and spleen cell cultures were prepared. As much as 1×10^7 total spleen cells/100 µl PBS were then intravenously (i.v.) injected into naïve recipients (tol. via cell transfer of birch-grass-pollen chimera tol. mice). Four hours after the cell transfer, the recipients were poly-sensitized as described above. Control mice were either i.n. treated with the chimera (tol. directly with birch-grass-pollen chimera) or sham treated with NaCl (sham-pretreated) before poly-sensitization with Bet v 1, Phl p 1 and Phl p 5 adsorbed to Al(OH)₃. The experiment was finalized 7 days after the last immunization (day 56).

Sampling

Blood samples were taken by tail bleeding before the first administration and before to kill. Sera were prepared for detection of allergen-specific antibody levels and stored at -20°C until analysis.

Seven days after the last immunization animals were killed, spleens were removed under sterile conditions and cell suspensions were prepared as previously described (7). BALs were collected as previously delineated elsewhere (8).

Determination of allergen-specific antibody levels in sera and BAL

Allergen-specific antibody levels in mouse sera (IgE and IgG2a) or BAL (IgA) were determined by ELISA. Microtiter plates (Nunc-Immuno Plate) were coated with 2 µg/ml of either Bet v 1, Phl p 1, Phl p 5 or the birch-grass-pollen chimera. Serum samples were diluted 1/500 for IgG2a and 1/10 for IgE. BAL samples were applied undiluted for IgA measurement. Rat anti-mouse IgG2a, IgE and IgA antibodies (1 µg/ml, Pharmingen, San Diego, CA, USA) were applied and peroxidase-conjugated mouse anti-rat IgG antibodies (1/2000; Jackson, Immuno Labs Inc., West Grove, PA, USA) were used for detection (7).

Cytokine production

In vitro cytokine production upon allergen re-stimulation was measured in spleen cell cultures of the different experimental groups (7). Spleen cells were cultured in 48-well flat bottom plates

at a concentration of 5×10^6 cells/500 µl with or without Bet v 1, Phl p 1 or Phl p 5 (10 µg/well). Supernatants were taken after 48 h for measurement of interleukin (IL)-5 and interferon (IFN)-γ.

Interleukin-5 was measured with a commercially available mouse ELISA kit (Endogen, Woburn, MA, USA), with sensitivity < 5 pg/ml. IFN-γ was measured as previously described (8).

Airway inflammation

BAL 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 total counted cell number, as previously described (8). BAL supernatants were used for measurement of IL-5 levels by ELISA.

Rat basophil leukaemia cell mediator release assay

Rat basophil leukaemia (RBL)-H2H3 cells were incubated with mouse sera (1/30). Degranulation of RBL cells was induced by adding 0.03 µg of each allergen diluted in 100 µl 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.

TGF-β mRNA and IL-10 mRNA RT-PCR

Total RNA was isolated from pooled murine splenocytes using RNeasy Minikit (Qiagen, Valencia, CA, USA), treated with DNase (Qiagen) and thereafter reverse transcription into cDNA using oligo dT primers (GeneAmp RT-PCR kit; PE, Boston, MA, USA) was performed. Gene expression of TGF-β and IL-10 was determined by semi-quantitative RT-PCR as previously described (22). The intensities of the TGF-β and IL-10 bands were normalized to β-actin values.

Statistics

Data are expressed as means ± SEMs. Statistical comparison of sensitized vs pretreated groups was performed using the Mann-Whitney U test. P-values below 0.05 were considered significant.

Results

Production and physicochemical characterization of the birch-grass-pollen chimera

Construction of the birch-grass-pollen chimera expression plasmid. The allergen chimera was designed such that it successively coded for a 6 × His-tag the immunodominant peptide of Phl p 5, the whole sequence of Bet v 1 and the immunodominant peptide of Phl p 1 (Fig. 1A). The insertion of the Bet v 1 sequence and the insertion of the two peptides were confirmed by DNA sequence analysis.

Expression and purification of the birch-grass-pollen chimera. The expressed allergen chimera was detected by SDS-PAGE and appeared as a single band corresponding to the calculated mass (27.8 kD) of both Phl p peptides

and Bet v 1. The recombinant protein was purified under strong denaturing conditions by Ni-NTA affinity chromatography and refolded by dialysis with stepwise reduction of urea concentration. Endotoxin levels were <0.05 EU/ μ g purified protein, which corresponds to baseline levels of commercially available proteins (15).

Physicochemical characterization of the birch-grass-pollen chimer. The protein was digested with trypsin and the resulting peptides were analyzed by ESI Q TOF. The measured mass values were compared with the theoretical ones which resulted in a sequence coverage of 97.6%. Thereby, the primary structure of the birch-grass-pollen chimer was verified.

Comparison of the CD spectra of the chimer, the natural and the recombinant Bet v 1 (Fig. 1C) revealed a slight shift of the chimer-curve in comparison with those of the Bet v 1 allergens. The CD spectrum of the chimer represents the distinct shape only observed with folded proteins, verifying the presence of secondary structure elements.

Immunological characterization of the birch-grass-pollen chimer

Immunological characterization of the birch-grass-pollen chimer *in vitro*. Immunoblot analysis revealed that the chimer was recognized by both monoclonal antibodies, BIP 1 (lane 3) and BIP 4 (lane 4) as well as by the serum pool of human sera from allergic patients (lane 6). This binding pattern was comparable with that obtained with natural (lane 2) or recombinant Bet v 1 (lane 1). Sera of non-allergic donors did not elicit any IgE binding to the chimer (lane 7). The buffer control (lane 5) also gave a negative signal (Fig. 1B).

Immunological characterization of the birch-grass-pollen chimer *in vivo*. Mice immunized with the birch-grass-pollen chimer displayed comparably high allergen-specific antibody levels (IgG) against Bet v 1, Phl p 1 and Phl p 5 as mice immunized with the single antigens Bet v 1, Phl p 1 or Phl p 5 (Fig. 2).

Use of the birch-grass-pollen chimer for mucosal tolerance induction in poly-sensitized mice

Allergen-specific antibody levels and cytokine production. Mice intranasally treated with the chimer before poly-sensitization displayed markedly reduced IgE antibody responses to all the three allergens Bet v 1, Phl p 1 and Phl p 5. Moreover, IL-5 production *in vitro* was significantly reduced by pretreatment with the allergen chimer (Fig. 3).

In contrast, no significant differences of allergen-specific IgG2a levels and IFN- γ production *in vitro* were detected in comparison with sham-pretreated controls (Table 2), indicating that pretreatment with the allergen chimer selectively reduced Th2 responses.

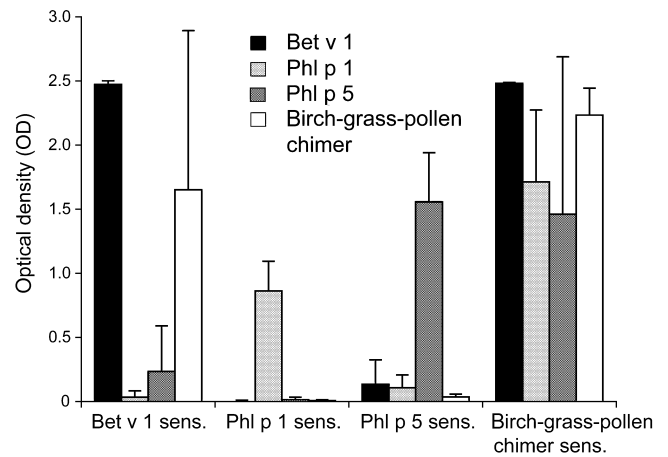


Figure 2. Immunological characterization of the birch-grass-pollen chimer *in vivo*. IgG antibody responses in sera of mice immunized with the chimer, Bet v 1, Phl p 1 or Phl p 5. Allergen-specific IgG antibodies were detected by ELISA. Bars indicate mean antibody titers $\pm$ the standard error of the mean (α -Bet v 1-IgG: black bars, α -Phl p 1-IgG: light gray bars, α -Phl p 5-IgG: dark gray bars, α -chimer-IgG: white bars).

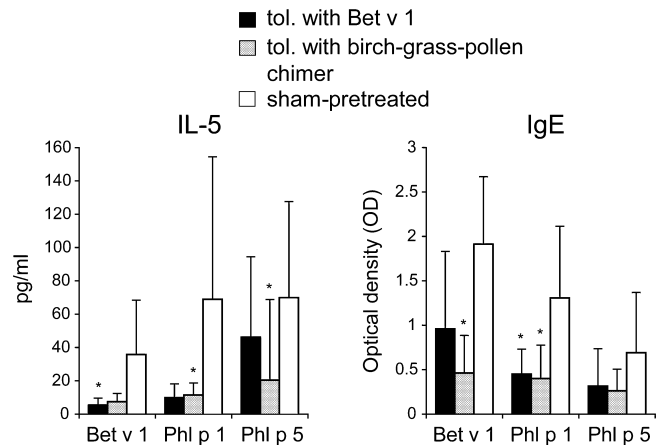


Figure 3. Systemic immune responses after intranasal pretreatment of poly-sensitized mice with the birch-grass-pollen chimer. IL-5 levels and serum IgE antibody responses in mice i.n. treated with the birch-grass-pollen chimer three times with 7-day intervals before poly-sensitization with Bet v 1, Phl p 1 and Phl p 5. Allergen-specific IgE antibodies were detected by ELISA. IL-5 levels were measured in supernatants of splenocytes after *in vitro* stimulation with Bet v 1, Phl p 1 or Phl p 5 using an ELISA kit. Bars indicate means of optical density (OD) of antibody levels or IL-5 concentrations (pg/ml) $\pm$ the standard error of the mean (black bars: Bet v 1 pretreatment, gray bars: chimer pretreatment, white bars: sham-pretreatment). * $P < 0.05$ tolerized vs sham-pretreated as determined by Mann-Whitney *U*-test.

Local immune responses, airway inflammation and functional IgE-assay after allergen challenge. Intranasal pretreatment with the chimer prevented the development of airway inflammation upon aerosol provocation.

Table 2. Tolerance induction with the birch-grass-pollen chimer

	IgG2a (OD)			IFN- γ (pg/ml)		
	α -Bet v 1	α -Phl p 1	α -Phl p 5	Bet v 1	Phl p 1	Phl p 5
tol Bet v 1	0.61 $\pm$ 0.71	1.75 $\pm$ 0.65**	1.18 $\pm$ 0.60	598.4 $\pm$ 379.6	105.8 $\pm$ 53.7	314.1 $\pm$ 178.8
tol Chimer	1.31 $\pm$ 0.64*	1.06 $\pm$ 0.69	1.19 $\pm$ 0.38	467.6 $\pm$ 355.3	123.2 $\pm$ 146.5	268.6 $\pm$ 141.5
Sham-pretreated	0.57 $\pm$ 0.39	0.51 $\pm$ 0.24	0.73 $\pm$ 0.28	363.1 $\pm$ 213.1	81.1 $\pm$ 66.0	353.6 $\pm$ 195.4

IgG2a and IFN- γ after i.n. pretreatment with the allergen chimer. Mice were pretreated with either Bet v 1 (tol Bet v 1), the allergen chimer (tol Chimer) or NaCl (sham-pretreated).

Antibody levels were measured by ELISA. Cytokines were measured in supernatants of spleen cell culture stimulated with either Bet v 1, Phl p 1, or Phl p 5 for 48 h.

* $P < 0.05$; ** $P < 0.01$ pretreated vs poly-sensitized as determined by Mann-Whitney U -test.

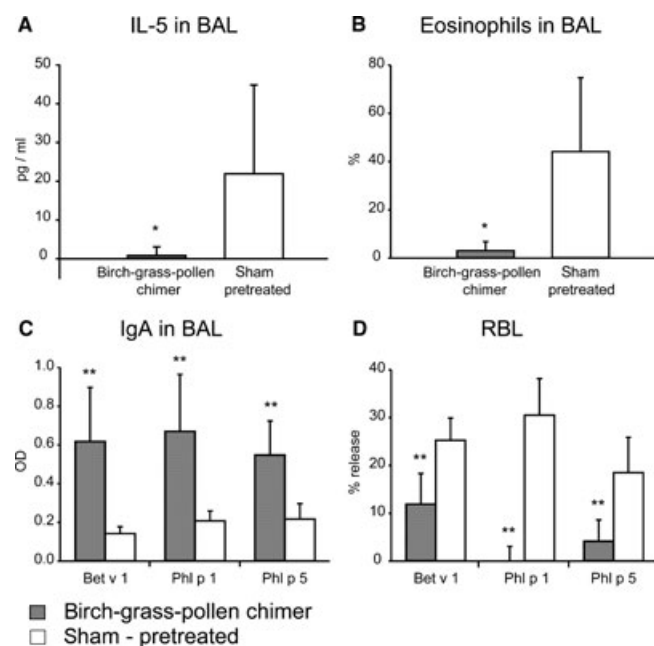


Figure 4. Airway inflammation, local IgA production and IgE-mediated rat basophile assay after allergen challenge. Mice, i.n. treated with the birch-grass-pollen chimer before poly-sensitization, were aerosol challenged with 1% birch- and grass-pollen extract. Allergic airway inflammation, characterized by IL-5 levels (A) and number of eosinophils (B), as well as allergen-specific IgA levels (C) were measured in BAL fluids. IgE dependent degranulation of rat basophiles (D: RBL) was measured with sera from chimer pretreated or sham-pretreated mice. Bars indicate means $\pm$ the standard error of the mean (gray bars: chimer pretreatment, white bars: sham-pretreatment). * $P < 0.05$; ** $P < 0.01$ tolerized vs sham-pretreated as determined by Mann-Whitney U -test.

The number of eosinophils as well as IL-5 production in BAL was significantly lower in chimer pretreated than in sham-pretreated controls (Fig. 4A,B). On the other hand, allergen-specific IgA production in BAL was markedly enhanced in the chimer pretreated animals compared with the controls (Fig. 4C). In line with the local responses also systemic allergic responses remained reduced after the allergen challenge: sera from chimer pretreated mice elicited significantly lower IgE-induced basophil degran-

ulation *in vitro* than sera from sham-pretreated mice (Fig. 4D).

Mechanisms of tolerance induction with the birch-grass-pollen chimer. For adoptive cell transfer experiments spleen cells of chimer pretreated mice were transferred into naïve recipients before poly-sensitization. Tolerance was transferable with splenocytes, illustrated by a suppression of IgE antibodies by 46 % against Bet v 1, 69% against Phl p 1 and 65% against Phl p 5 in poly-sensitized recipients compared with sham-pretreated sensitized controls (Fig. 5). As also described in Fig. 3, directly tolerized mice displayed significantly reduced IgE antibody levels as well (76%: Bet v 1, 87%: Phl p 1, 62%: Phl p 5), indicating regulatory mechanisms to operate (Fig. 5). Accordingly, elevated transcription levels of TGF- β and IL-10 mRNA were detected in spleen cells of chimer pretreated (pretreated) or recipient mice (recipient) compared with the poly-sensitized controls (sensitized). The relative transcription levels were constituted of RT-PCRs and assigned as integrated

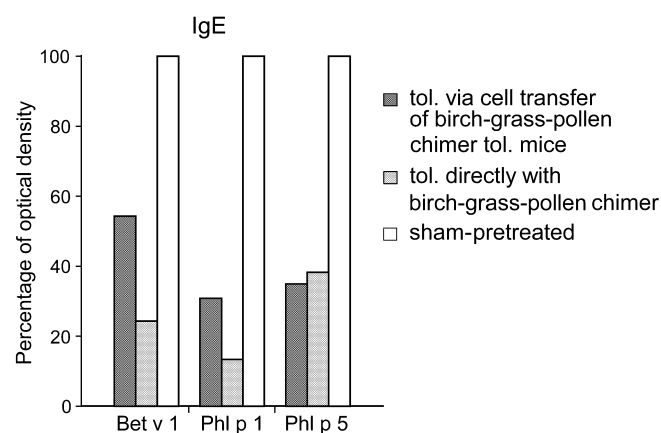


Figure 5. Adoptive cell transfer after poly-tolerance induction with the birch-grass-pollen chimer. Data are shown as percentage optical density (OD) of IgE antibodies. Mice tolerized via adoptive transfer of splenocytes from chimer pretreated mice (black bars) or tolerized directly with the chimer (gray bars) were compared with sham-pretreated controls (white bars). OD of sham-pretreated mice was set to 100%.

density values (IDV) and normalized to β -actin IDV. IL-10 mRNA was 1.7/1.4/1.0 IDV (recipient/pretreated/sensitized mice) and TGF- β mRNA was 1.6/1.7/1.0 IDV (recipient/pretreated/sensitized mice).

Discussion

A key issue in the prevention of multiple sensitizations is the generation of vaccines that comprise several major allergens in one molecule. Several approaches have been undertaken to combine portions of homologues proteins to form new hybrid molecules or chimera with defined features (23, 24), either leading to enhanced immunogenicity with induction of so called blocking IgG antibodies (25), or preventing allergic immune responses by injecting hybrid/chimeric molecules before sensitization with the natural allergen (26–28). In the latter case, this was achieved by destruction of conformational B-cell epitopes and preservation of the entire T-cell epitope repertoire of bee venom allergens in an approach to combine two or three bee venom allergens in one protein (27, 28).

Our approach of constructing a chimeric molecule differs from the existing ones in three important aspects: first, we use the mucosal route of application, since our previous studies revealed that mucosal tolerance induction with allergens is of high efficacy in preventing or treating allergic immune responses (7, 8, 22) and, most probably, will be better accepted compared with parenteral application of vaccines. Secondly, the backbone of our construct consists of a complete molecule with retained T- and B-cell epitopes. The presence of both linear and conformational epitopes seems of particular importance for enhanced tolerogenic properties, as we recently demonstrated for the Bet v 1 molecule in contrast to Bet v 1 peptides (15). Thirdly, the rationale for the construction of a chimeric molecule is to use such a construct for prevention of allergic sensitization against different allergen species to prevent the establishment of multiple sensitizations. Based on these aspects and the fact that combined tree and grass pollen allergies are the most common inhalant allergies within Europe (29), the allergen chimer was constructed of the whole Bet v 1 molecule as one of the most potent tolerogens among inhalant allergens, and two major T-cell epitopes of the non-cross reactive grass pollen allergens Phl p 1 and Phl p 5, which have been recently used for mucosal tolerance induction in poly-sensitized mice (11). Notably, the selected peptides were identified as major T-cell epitopes in grass pollen allergic patients (30, 31).

After expression and purification of the chimer (Fig. 1A) analysis of the amino acid sequence by ESI Q TOF revealed that the primary structure of the chimer correlated to the deduced amino acid sequence. By CD spectroscopy secondary structure elements could be verified. Differences in signal amplitude and a slight shift of the curve compared with those of the Bet v 1

allergens (Fig. 1C) might be explained by the fact that after incorporation of the two grass pollen peptides Bet v 1 only represents 64% of the total chimer molecule. Despite the linkage of the grass pollen peptides Phl p 1 and Phl p 5 to the Bet v 1 scaffold, the three-dimensional structure of the Bet v 1 chimer remained essentially unchanged, as the monoclonal anti-Bet v 1 antibodies and human anti-Bet v 1-IgE antibodies bound equally well to the natural allergen as well as to the chimer (Fig. 1B). In accordance to the stable structure the immunogenicity of the chimer was verified by immunization studies in mice, showing that all animals developed specific antibody levels to the three allergens (Fig. 5). Mice, which were immunized with Phl p 1 or Phl p 5 only elicited antibodies against the respective allergens, but not against the peptides within the chimer, whereas mice immunized with Bet v 1 mounted antibody responses against both Bet v 1 and the chimer (Fig. 2).

To test the tolerogenic capacity of the allergen chimer in our mouse model of poly-sensitization, different doses of the molecule were intranasally applied before poly-sensitization. These dose finding studies revealed that 15 μ g of the allergen chimer was the optimal amount for intranasal application (data not shown). Intranasal pretreatment with this chimer led to significant reduction of allergen-specific Th2 responses to all three allergens (Fig. 3). These data are in line with our recent findings demonstrating that intranasal pretreatment with polypeptides also prevented allergic co-sensitization to the birch and grass pollen allergens (11). According to the previously described phenomenon of intramolecular suppression (32), only one immunodominant peptide of either of the grass pollen allergens needed to be incorporated into the chimer to induce suppression against the complete grass pollen allergens. With respect to a broad usage in humans, though, tolerance induction with the linked peptides might have limitations because of individual recognition by a large diversity of MHC and T-cell receptors (33). However, using the tolerogenic Bet v 1 molecule as scaffold, the MHC restriction towards the linked peptides might be overcome by the induction of bystander suppression, which has been shown to develop to antigens presented to the immune system in context with an antigen to which tolerance is induced (34, 35).

To evaluate the efficacy of the new vaccination concept after allergen challenge, mice were exposed to an aerosol with whole birch and grass pollen extract. Intranasal pretreatment with the chimer prevented the development of airway inflammation, reflected by a significant reduction of IL-5 levels and eosinophils in BAL of treated compared with control mice (Fig. 4A,B). Additionally, IgE-mediated degranulation of rat basophiles, equivalent to allergic sensitization *in vivo*, was significantly suppressed after the challenge in the chimer treated mice (Fig. 4D). Another indication for the efficacy of mucosal vaccination with the chimer, which has not been previously observed after

pretreatment with Bet v 1 alone or after peptide treatment, was the induction of local allergen-specific IgA antibodies (Fig. 4C). These IgA antibodies might be of importance to promote protection at the site of allergen exposure, since it has been described that secretory IgA can act as blocking antibodies in nasal secretions and inhibit IgE mediated histamine release (36, 37).

Regarding the mechanisms of poly-tolerance induction, immunosuppression with the allergen chimera was transferable with spleen cells from treated mice (Fig. 5), which exhibited enhanced mRNA levels of TGF- β and IL-10 mRNA. These results are in line with our previous findings in mono-tolerized mice, showing that Bet v 1, but not Bet v 1 peptides, induced the formation of TGF- β and IL-10 producing regulatory cells (15). Accordingly, we assumed that tolerance induction with the allergen chimera in poly-sensitized mice was mediated by regulatory cells, which was in contrast to tolerance induction with polypeptides mediated by cytokine independent mechanisms (11). As TGF- β and IL-10 are also important for switching and upregulation of IgA production, simultaneous production of allergen-specific IgA antibodies might reflect the previously described

phenomenon of split tolerance (38). As concomitant development of secretory IgA responses and systemic tolerance has been described to occur after mucosal application of more complex antigens with lower solubility (39), which may apply for allergen chimeras rather than single antigens, this could explain why we have not previously observed split tolerance after mucosal application of single allergens or allergen peptides.

Taken together, we demonstrate the construction of a novel allergen chimera capable of preventing allergic multiple sensitization and inducing potential protective local immune responses when applied via the mucosal route. This prototype chimera may thus serve as a basis for further construction of allergen chimeras comprising different combinations of common seasonal and perennial allergens.

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