

Analysis of Epitope-Specific Immune Responses Induced by Vaccination with Structurally Folded and Unfolded Recombinant Bet v 1 Allergen Derivatives in Man¹

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Previously, we have constructed recombinant derivatives of the major birch pollen allergen, Bet v 1, with a more than 100-fold reduced ability to induce IgE-mediated allergic reactions. These derivatives differed from each other because the two recombinant Bet v 1 fragments represented unfolded molecules whereas the recombinant trimer resembled most of the structural fold of the Bet v 1 allergen. In this study, we analyzed the Ab (IgE, IgG subclass, IgA, IgM) response to Bet v 1, recombinant and synthetic Bet v 1-derived peptides in birch pollen allergic patients who had been vaccinated with the derivatives or adjuvant alone. Furthermore, we studied the induction of IgE-mediated skin responses in these patients using Bet v 1 and Bet v 1 fragments. Both types of vaccines induced a comparable IgG1 and IgG4 response against new sequential epitopes which overlap with the conformational IgE epitopes of Bet v 1. This response was 4- to 5-fold higher than that induced by immunotherapy with birch pollen extract. Trimer more than fragments induced also IgE responses against new epitopes and a transient increase in skin sensitivity to the fragments at the beginning of therapy. However, skin reactions to Bet v 1 tended to decrease one year after treatment in both actively treated groups. We demonstrate that vaccination with folded and unfolded recombinant allergen derivatives induces IgG Abs against new epitopes. These data may be important for the development of therapeutic as well as prophylactic vaccines based on recombinant allergens. *The Journal of Immunology*, 2007, 179: 5309–5316.

Allergen-specific immunotherapy is the only Ag-specific treatment for IgE-mediated allergies (1, 2). It is based on the administration of the disease-eliciting allergens to patients and has been shown in numerous clinical studies to improve the quality of life in patients with respiratory allergy and to change the course of allergic disease (3, 4).

However, the administration of allergens to patients in the course of specific immunotherapy involves the risk of inducing side effects, which limits its broad application (5–7). The latter problem has been addressed by several strategies to modify allergens with the goal to improve the safety, specificity and efficacy of specific immunotherapy (reviewed in 8–10). These

approaches include the use of allergen-derived T cell peptides without IgE reactivity to induce allergen-specific tolerance (11), the coupling of CpG oligonucleotides to allergens to reduce their allergenic activity and redirect the allergen-specific immune response (12–14) and the use of recombinant allergens, in particular the genetic engineering of allergen derivatives with reduced allergenic activity and retained T cell epitope repertoires (9).

We have developed two types of recombinant derivatives of the major birch pollen allergen Bet v 1 (rBet v 1 fragments, rBet v 1 trimer) for immunotherapy of birch pollen allergy (15, 16). The two rBet v 1 fragments differed substantially from the natural Bet v 1 allergen by the lack of structural fold and IgE reactivity (15), whereas the Bet v 1 trimer resembled much of the structural fold of Bet v 1 and reacted with Bet v 1-specific IgE Abs (16). However, both Bet v 1 derivatives (i.e., fragments and trimer) exhibited a >100-fold reduced allergenic activity as tested in basophil activation assays and by in vivo provocation testing (skin testing, nasal provocation testing) in birch pollen allergic patients (17–19). When birch pollen allergic patients were treated by injection immunotherapy with the two types of recombinant Bet v 1 derivatives we found that both (i.e., Bet v 1 fragments and trimer) induced IgG Abs against the natural Bet v 1 allergen and even to Bet v 1-related allergens from pollens of related trees and plant food (20, 21). These IgG Abs inhibited Bet v 1-induced basophil degranulation (20), rises of Bet v 1-specific IgE Ab levels due to natural birch pollen exposure (20) and Bet v 1-induced nasal reactivity (22), suggesting that the induction of blocking Abs which compete with the IgE recognition of the allergen may be an important mechanism underlying immunotherapy with the genetically modified Bet v 1 derivatives (20, 22). Since the recombinant Bet v 1 fragments and the recombinant Bet

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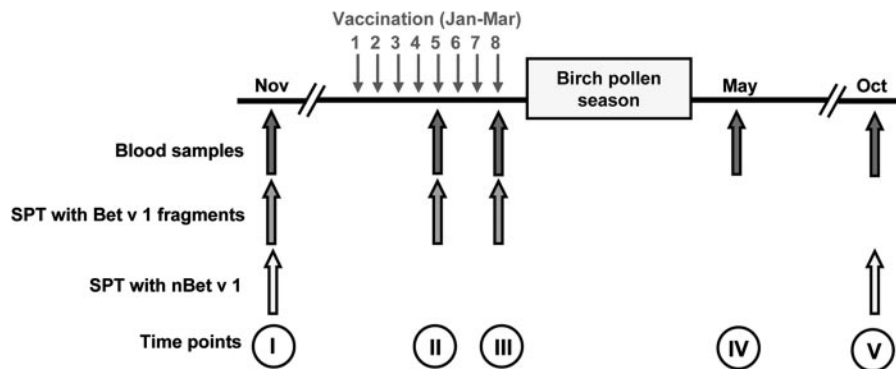


FIGURE 1. Timeline—course of the study. The study was conducted between November 2000 and October 2001. Between November 2000 and March 2001, patients received typically eight injections with either Bet v 1 trimer, an equimolar mixture of Bet v 1 fragments, or placebo. Blood samples were obtained before therapy (time point I), after the 5th injection (time point II), at the 8th injection (time point III, i.e., before or at the beginning of birch season), after the birch pollen season 2001 (time point IV), and 1 year after the start of the trial (Oct. 2001, time point V). Skin prick tests with a birch pollen extract containing a defined Bet v 1 content were performed at the beginning of the study (time point I) and after 1 year (time point V). Skin prick tests with the Bet v 1 fragments were performed before therapy (time point I), before injection 5 (time point II), and at injection 8 (time point III).

v 1 trimer differed substantially in their molecular structure, IgE reactivity and capacity to induce Th1 versus Th2 cytokine responses (Th1: trimer > fragments) we were interested to study the subclass-specific immune responses and their epitope specificity (15, 16, 23).

In this study, we performed a detailed analysis of the IgE, IgG subclass, IgA and IgM response to recombinant Bet v 1 fragments and to a panel of Bet v 1-derived synthetic peptides to study the epitope specificity of the Ab responses induced by the vaccination with the genetically modified Bet v 1 derivatives in patients. Furthermore, we performed an analysis of the skin reactivities to Bet v 1 fragments and natural Bet v 1 in the treated patients to determine whether the vaccines may induce skin reactivities to new Bet v 1-derived epitopes and to analyze the evolution of Bet v 1-specific skin responses.

Materials and Methods

Protocol of the clinical study

In a double-blind, placebo controlled, multicenter immunotherapy study (20), birch pollen allergic patients received vaccinations with aluminum hydroxide-adsorbed derivatives of Bet v 1 (an equimolar mixture of two hypoallergenic Bet v 1 fragments (15); hypoallergenic Bet v 1 trimer (16); or aluminum hydroxide alone (placebo)). The study protocol is depicted in Fig. 1. In a single preseasonal treatment course study, participants received one to two weekly s.c. injections with increasing doses (1–80 μ g) of the hypoallergenic Bet v 1 derivatives. Blood sampling and skin prick testing was done at defined time points as shown in Fig. 1. In this substudy, we analyzed serum samples and skin test results from the 60 patients who were treated in the Vienna centre and who had received a cumulative dose of at least 30 μ g of the active preparation. The study was approved by the local ethical committee and written informed consent was obtained from each patient. For a comparison of the magnitudes of therapy-induced IgG Ab levels, sera from 6 patients who had received one preseasonal course of specific immunotherapy with standard quality (SQ) natural birch pollen extract adsorbed to aluminum hydroxide gel (Alutard SQ; ALK-Abello) were included in the analysis.

Skin prick tests with Bet v 1 fragments and natural Bet v 1

To monitor the induction of clinical sensitivity to the hypoallergenic preparations, skin prick tests with an equimolar mixture of the two Bet v 1 fragments (6.4 μ g/ml, 64 μ g/ml), histamine hydrochloride (1%, positive control) and 0.9% sodium hydrochloride (negative control) (Allergopharma) were performed before therapy and before the fifth and eighth vaccination (Fig. 1, time points I, II, and III). Reactions were recorded 20 min after testing by transferring the ballpoint pen surrounded weal reaction with a scotch tape to paper. The size of the skin reactions was measured by an independent investigator and weal diameters were calculated as follows: (maximal longitudinal + maximal transversal diameter)/2.

Double-blind skin prick tests were performed with birch pollen extract containing standardized Bet v 1 concentrations (Allergopharma) before therapy (Autumn 2000) and after 1 year (Autumn 2001) (Fig. 1). Triplicate skin prick tests were performed with Bet v 1 concentrations of 0.25, 1, 4, 16, and 64 μ g/ml and histamine concentrations of 1 and 0.1% (positive controls). Duplicate skin prick tests were performed with 0.9% sodium chloride (negative control). Reactions were recorded 20 min after testing by transferring the ballpoint pen-surrounded weal reaction with a scotch tape to paper. The size of weal reactions was determined by an independent investigator by computerized planimetry. Results are displayed as means.

Recombinant Bet v 1, Bet v 1 fragments, and Bet v 1 peptides

Recombinant Bet v 1 was obtained from Biomay. The two Bet v 1 fragments (F1: aa 1–73, F2: aa 74–159) were expressed in *Escherichia coli* and purified as described (15). Six linear peptides (P1: aa 1–24; P2: aa 30–59; P3: aa 50–79; P4: aa 110–139; P5: aa 130–160; P6: aa 75–104) (25–32 amino acids length) containing surface-exposed amino acids spanning the Bet v 1 sequence were synthesized using Fmoc strategy with HBTU activation (0.1 mmol small-scale cycles) on the Applied Biosystems peptide synthesizer model 433A and purified to >90% purity by preparative HPLC on a reversed phase C-18 column using an acetonitrile gradient (PisChem) (24).

Detection of IgE, IgG subclass, IgM, and IgA Ab levels by ELISA

ELISA plates (Nunc Maxisorp) were coated with rBet v 1 (3 μ g/ml), each of the two Bet v 1 fragments (F1 or F2, 3 μ g/ml), or each of the six Bet v 1 peptides (5 μ g/ml) diluted in 100 mM sodium carbonate-bicarbonate buffer overnight at 4°C on a shaker. Plates were washed with PBS, 0.05% v/v Tween 20 and blocked in 1% w/v BSA (overnight, 4°C).

For the detection of IgE Abs sera were diluted 1/3 (peptides) or 1/10 (Bet v 1, Bet v 1 fragments) in buffer A (TBS, 0.05% v/v Tween 20, 0.5% w/v BSA). IgE levels were measured using of an alkaline phosphatase-conjugated mouse mAb to human IgE (555859; BD Pharmingen) diluted 1/1000 in buffer A. Para-nitrophenylphosphate substrate tablets (N9389; Sigma-Aldrich) were used for the enzymatic color reaction. Plates were read at 405 nm.

For the detection of IgG1, IgG2, and IgG4 Ab levels, serum samples were diluted 1/50 in PBS, 0.05% v/v Tween 20, 0.5% w/v BSA. ELISA were performed as described (22).

For the measurement of IgA and IgM Ab levels sera were diluted 1:50 in buffer A. Alkaline phosphatase-conjugated mouse mAbs to human IgA₁/IgA₂ (G20–359; BD Pharmingen) or IgM (A2189; Sigma-Aldrich) in a dilution of 1/1000 in buffer A were used for detection. Para-nitrophenylphosphate substrate tablets (N9389; Sigma-Aldrich) were used for the enzymatic color reaction. Plates were read at 405 nm.

All ELISA plates included at least four negative control wells without serum addition. For standardization of ELISA measuring rBet v 1 and Bet v 1 fragment specific Ab levels, three reference sera with previously evaluated ELISA OD values were used on each plate (25). IgG Ab levels of

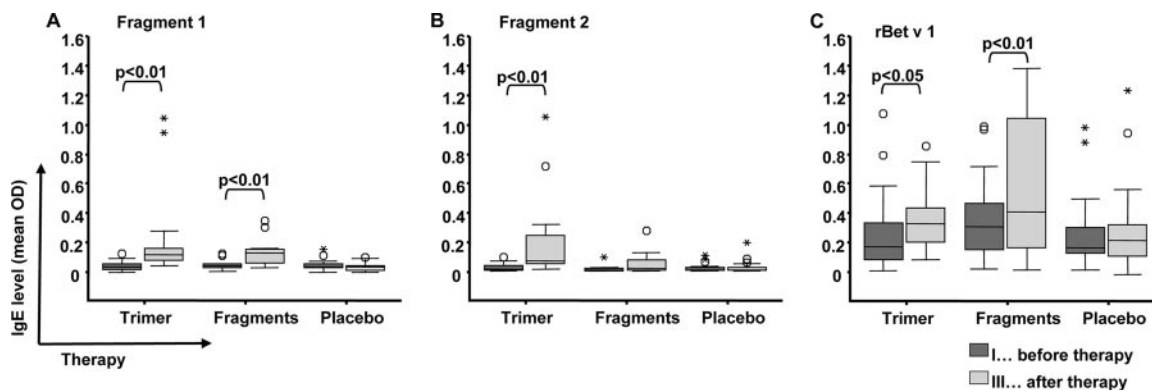


FIGURE 2. IgE levels to Bet v 1 fragments and to recombinant Bet v 1 before and after therapy. Serum IgE levels to fragment 1 (A), fragment 2 (B), and rBet v 1 (C) were measured for each group (trimer, fragments, and placebo) by ELISA (y-axis: OD values). Dark gray bars indicate IgE levels before therapy (time point I), light gray bars indicate IgE levels after therapy (time point III). In Fig. 3C, two outliers that exceeded an OD of 3.0 at time point III are not displayed (trimer patients 44 and 45); p values of <0.05 are displayed; $\circ$, outlier; *, extreme value.

patients treated with recombinant allergen derivatives and of extract treated patients were compared by testing 2-fold serial dilutions (1:50–1:800) of the sera. Experiments with both groups of sera were done on the same plate to avoid plate-to-plate variabilities. Measurement of peptide-specific Ab responses were performed for each peptide by including all sera from all three time points in one single experiment using defined assay conditions.

All ELISA results are displayed as mean values of duplicates with $<5\%$ deviation.

Statistical analysis

Wilcoxon signed rank tests were used to compare IgG1–4, IgE, IgA, and IgM levels before and after therapy within groups. Increases before and after therapy were considered significant only if OD values differed by at least 0.050 and p values were <0.05 .

Results

Vaccination and analysis of patients

Patients ($n = 60$) from the Vienna study center who had received at least 30 μg of the study preparations (Bet v 1 trimer, Bet v 1

fragments) or placebo were included in this substudy. Twenty of these patients had received Bet v 1 trimer, 13 patients had been treated with a mixture of the two fragments, and 27 patients had received placebo treatment.

Patients treated with Bet v 1 trimer received on average 8.4 injections (range: 7–10) and an average cumulative injected dose of 171 μg (range: 69–265 μg) of the active preparation. Patients receiving Bet v 1 fragments were given an average injection course of 8.5 injections (range: 7–9) and an average cumulative dose of 168 μg (range: 85–245 μg). Serum samples were taken from the patients before treatment (November 2000, time point I), during and after treatment/before the birch pollen season (time point II and III), after the birch season (time point IV) and one year after time point I (October 2001, time point V) (Fig. 1). Skin prick tests with Bet v 1 fragments and trimer were performed at time point I, II and III and with standardized birch pollen extract at time point I and V.

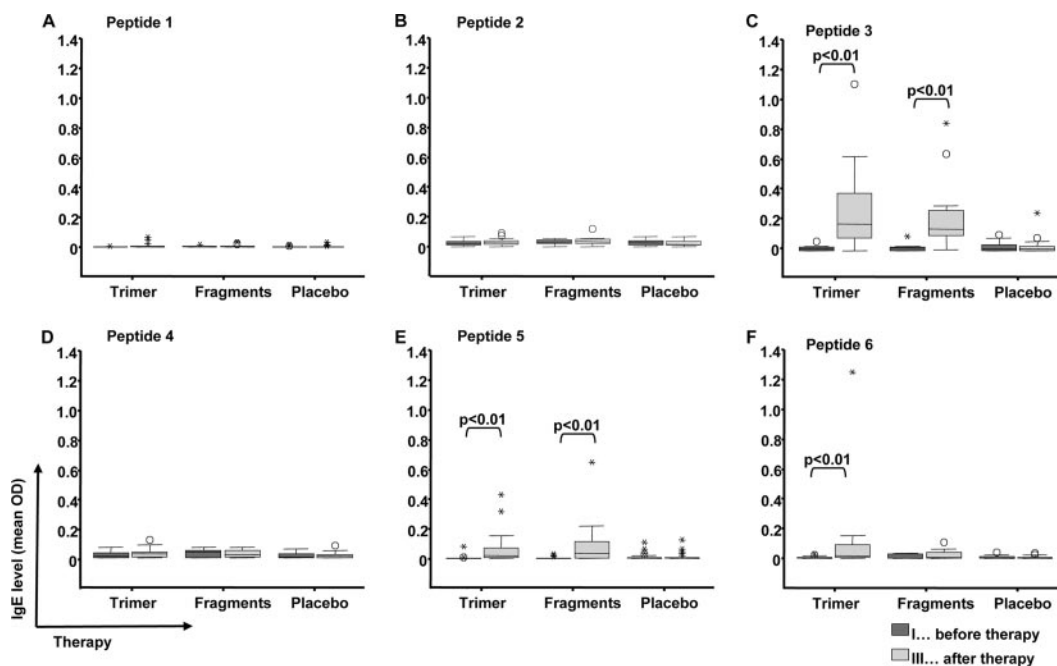


FIGURE 3. IgE levels to six Bet v 1 peptides before and after therapy. IgE Ab levels directed against peptides 1–6 (A–F) were measured in each group (trimer, fragments, placebo) by ELISA (y-axis: OD values). Bars in dark gray indicate IgE levels before therapy (time point I), light gray bars indicate IgE levels after therapy (time point III); p values of <0.05 are displayed; $\circ$, outlier; *, extreme value.

TABLE I. Mean increases of IgE, IgG1, IgG4, IgA, and IgM levels^a

		Bet v 1	F1	F2	P1	P2	P3	P4	P5	P6
IgE	T	0.40	<i>0.17</i>	0.16	0.01	0.01	0.25	0.01	0.06	0.10
	F	0.22	0.08	0.04	0.00	0.01	0.22	0.00	0.10	0.01
	P	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
IgG ₁	T	0.68	0.42	0.42	0.02	0.05	0.14	0.06	0.16	0.11
	F	0.54	0.43	0.41	0.03	0.04	0.14	0.05	0.23	0.11
	P	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00
IgG ₄	T	0.47	0.18	0.23	0.00	0.01	0.02	0.01	0.04	0.04
	F	0.27	0.09	0.10	0.00	0.00	0.00	0.01	0.03	0.02
	P	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
IgA	T	0.13	0.03	0.12	0.00	0.00	0.00	0.00	0.01	0.01
	F	0.10	0.11	0.03	0.00	0.00	0.00	0.00	0.00	0.00
	P	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
IgM	T	0.09	0.04	0.11	0.00	0.00	0.03	0.00	0.03	0.01
	F	0.00	0.00	0.00	0.03	0.03	0.01	0.02	0.03	0.00
	P	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00

^a Ab levels are displayed for patients who received trimer therapy (T), *n* = 20; fragment therapy (F), *n* = 13; and placebo (P), *n* = 27. Statistically significant increases of OD values (time point I—time point III) of >0.050 are marked (*p* < 0.05).

Active treatment induced a transient rise of IgE Abs to new epitopes

Before treatment patients in the trimer, fragments and placebo group had comparable levels of Bet v 1-specific IgE Abs but showed no IgE reactivity to fragment 1, fragment 2 (Fig. 2) or the six Bet v 1-derived peptides (Fig. 3). Only active but not placebo treatment induced a significant rise of IgE levels to Bet v 1, to both Bet v 1 fragments (Fig. 2) and to three of the six sequential peptides (peptide 3, 5 and 6; Fig. 3). A rise of peptide-specific IgE Abs was observed in 13 of the 20 trimer-treated patients, in nine of the 13 fragments-treated patients and only in one of the 27 placebo patients.

Absolute increases of IgE Ab levels to rBet v 1, fragment 1, fragment 2, and to the six peptides are shown in Table I. Trimer treatment induced higher increases of Bet v 1 specific IgE Ab levels than fragment treatment (Table I). No IgE increases were noted for the placebo group.

The induction of IgE to the two fragments and to peptide 3, 5 and 6 was transient and decreased during the birch pollen season. After one year (time point V), IgE levels to the fragments had on average fallen to the same levels as measured before treatment with Bet v 1 fragments (Fragment 1: before treatment: OD =

0.049, after treatment: OD = 0.042; Fragment 2: before treatment: OD = 0.015, after treatment: OD = 0.022; data not shown).

Likewise, in the trimer-treated group IgE levels to the fragments fell substantially during and after the birch pollen season, and after one year were only mildly elevated as compared with before treatment (Fragment 1: before treatment: OD = 0.043, after treatment: OD = 0.080; Fragment 2: before treatment: OD = 0.024, after treatment: OD = 0.077). The IgE increase to the peptides was equally transient, and IgE levels decreased during and after the birch pollen season in all patients (data not shown).

De novo induction of IgG1 and IgG4 Abs to several sequential epitopes in the actively treated patients

Immunotherapy with Bet v 1 fragments and Bet v 1 trimer induced an IgG1 response to several sequential epitopes on the surface of the Bet v 1 molecule. We measured significant increases of IgG1 to Bet v 1 (Fig. 4C), both Bet v 1 fragments (Fig. 4, A and B) and to each of the six peptides (Fig. 5, A–F) in the trimer and fragment group but not in the placebo group. Sixteen of the trimer-treated patients, 10 of the fragment-treated patients but none of the 27 placebo patients developed peptide-specific IgG1 Abs. The absolute increases of IgG1 Ab levels to rBet v 1, Bet v 1 fragments and the peptides were comparable in the trimer- and fragment-treated group but no relevant increases occurred in the placebo group (Table I).

Significant rises of IgG4 Ab levels to Bet v 1 and the fragments were also observed in both active treatment groups (Fig. 6). A detectable increase of IgG4 Abs to certain peptides was observed only in 4 of the 33 actively treated patients (Fig. 7). Absolute increases of rBet v 1-, fragment- and peptide-specific IgG4 Ab levels were higher in the trimer-treated group than in the fragment-treated group (Table I). No increases were detected in the placebo group (Table I).

Bet v 1-specific IgG1- and IgG4-levels were determined on the same ELISA plate for sera from six patients who had received a four-month preseasonal treatment with SQ birch pollen extract and two randomly picked trimer- and two fragment-treated patients. Mean IgG1 and IgG4 titers were 4- to 5-fold higher in fragment- and trimer-treated patients than in birch pollen extract-treated patients.

We noted a mild increase of IgA Ab levels to Bet v 1 in the trimer- and fragment-treated groups (Table I), but no relevant de novo induction of IgA to the linear peptides was observed (Table I). Furthermore, no relevant rises of peptide-specific IgG2 (data

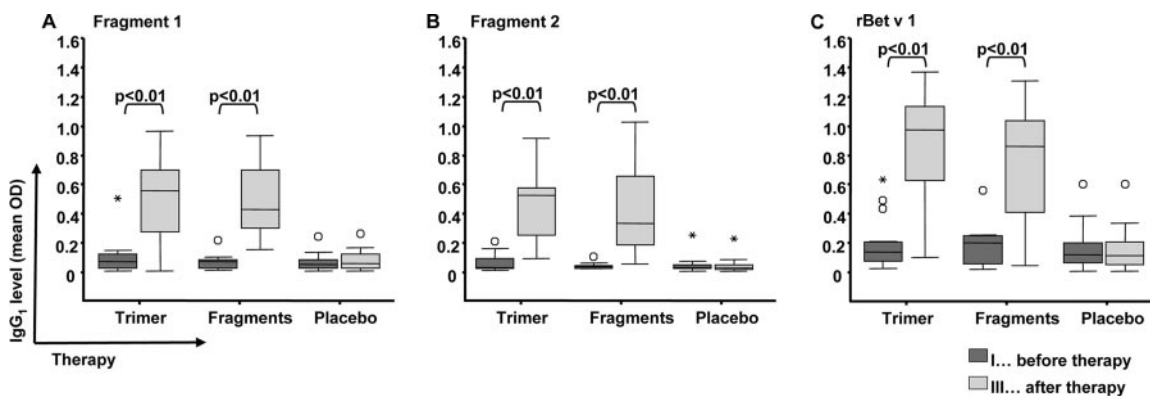


FIGURE 4. IgG1 levels to Bet v 1 fragments and to recombinant Bet v 1 before and after therapy. Serum IgG1 levels to fragment 1 (A), fragment 2 (B), and rBet v 1 (C) were measured for each group (trimer, fragments, placebo) by ELISA (y-axis: OD values). Dark gray bars indicate IgG1 levels before therapy (time point I), light gray bars indicate IgG1 levels after therapy (time point III); *p* values of <0.05 are displayed; ○, outlier; *, extreme value.

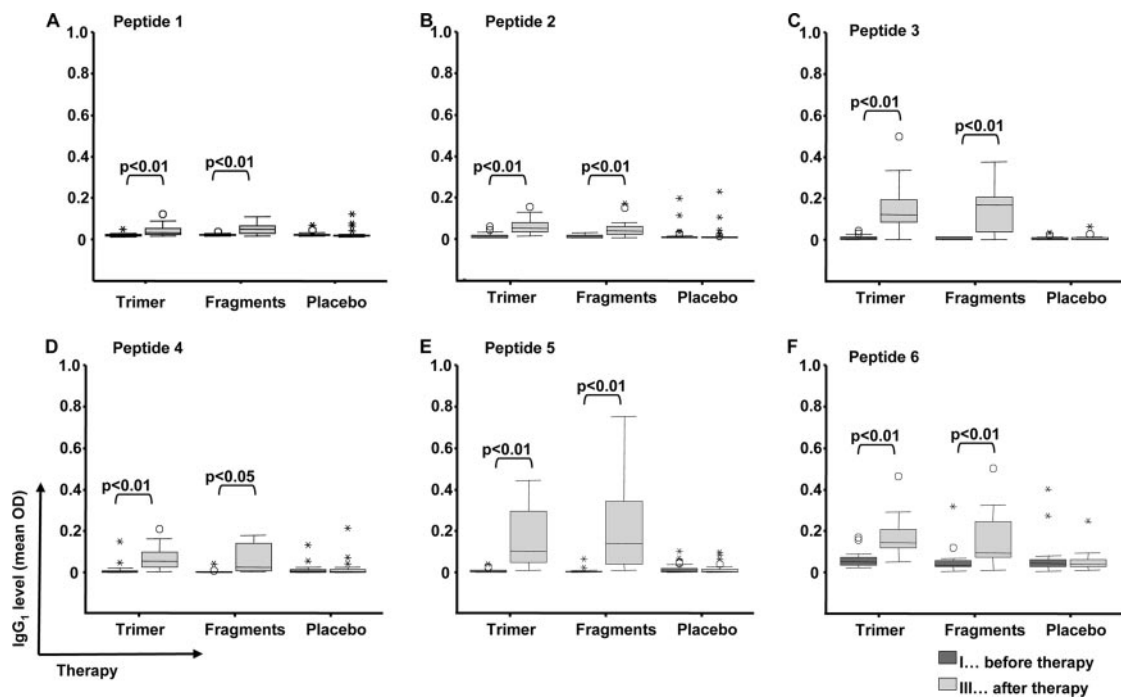


FIGURE 5. IgG1 levels to six Bet v 1 peptides before and after therapy. IgG1 Ab levels directed against peptide 1–6 (A–F) were measured in each group (trimer, fragments, placebo) by ELISA. Bars in dark gray indicate IgG1 levels before therapy (time point I), light gray bars indicate IgG1 levels after therapy (time point III); p values of <0.05 are displayed; ○, outlier; *, extreme value.

not shown) and IgM Abs was noted in any of the three groups (Table I). In almost all actively treated patients IgG1 was induced to more sequential epitopes than IgG2, IgG4 and IgE.

Evolution of skin reactivity to Bet v 1 fragments and natural Bet v 1 in actively and placebo-treated patients

Seventeen Bet v 1 trimer-, 12 fragments- and 25 placebo-treated patients were pricked with 6.4 $\mu\text{g}/\text{ml}$ (Fig. 8, A–C) and 64 $\mu\text{g}/\text{ml}$ (Fig. 8, D–F) of an equimolar mixture of Bet v 1 fragments before, during and after immunotherapy to study the evolution of immediate type skin responses to the fragments. The hypoallergenic nature of the fragments is demonstrated by the fact that only seven patients (trimer group $n = 1$, fragment group $n = 3$, placebo group $n = 3$) exhibited detectable skin reactivities to an equimolar fragments mixture of 64 $\mu\text{g}/\text{ml}$ fragments before therapy (time point I, Fig. 8).

After four injections (time point II) skin reactivity to 64 $\mu\text{g}/\text{ml}$ fragments developed in eight trimer-treated patients but in only one fragment- and two placebo-treated patients. At the time of the eighth injection (end of treatment, time point III), additional six trimer and nine fragment-treated patients and one placebo patient mounted skin reactions to 64 $\mu\text{g}/\text{ml}$ of the fragment mix. Each of the skin reactivities to the 64 $\mu\text{g}/\text{ml}$ of the fragment mix were <8 mm mean weal diameter in all but one patient (Fig. 8, D–F) and on average smaller than the positive control with 1% histamine (data not shown).

A similar trend in the development of skin reactivities was observed with the 6.4 $\mu\text{g}/\text{ml}$ dose of fragments but the reactions never exceeded 5 mm mean weal diameter (Fig. 8, A–C).

The evolution of skin responses to Bet v 1 trimer was also assessed before, during and after immunotherapy. No relevant changes were observed in the trimer group (average mean weal

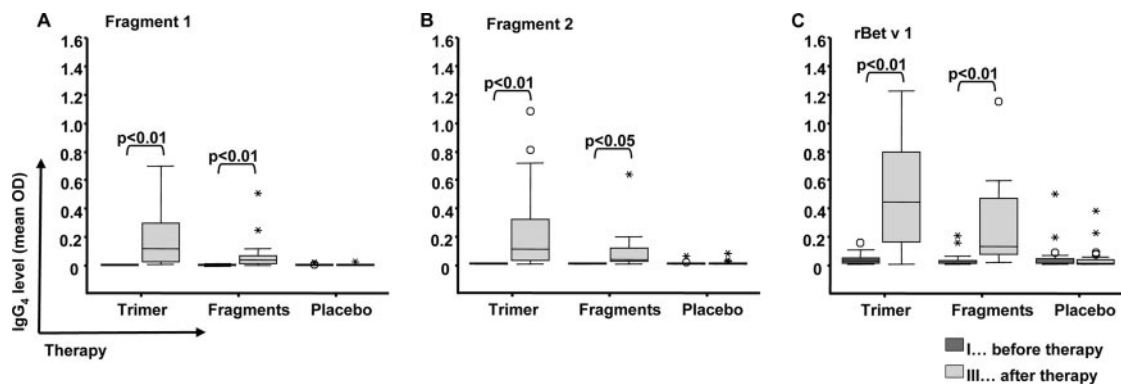


FIGURE 6. IgG4 levels to Bet v 1 fragments and to recombinant Bet v 1 before and after therapy. Serum IgG4 levels to fragment 1 (A), fragment 2 (B) and rBet v 1 (C) were measured for each group (trimer, fragments, placebo) by ELISA (y-axis: OD values). Dark gray bars indicate IgG4 levels before therapy (time point I), light gray bars indicate IgG4 levels after therapy (time point III); p values of <0.05 are displayed; ○, outlier; *, extreme value.

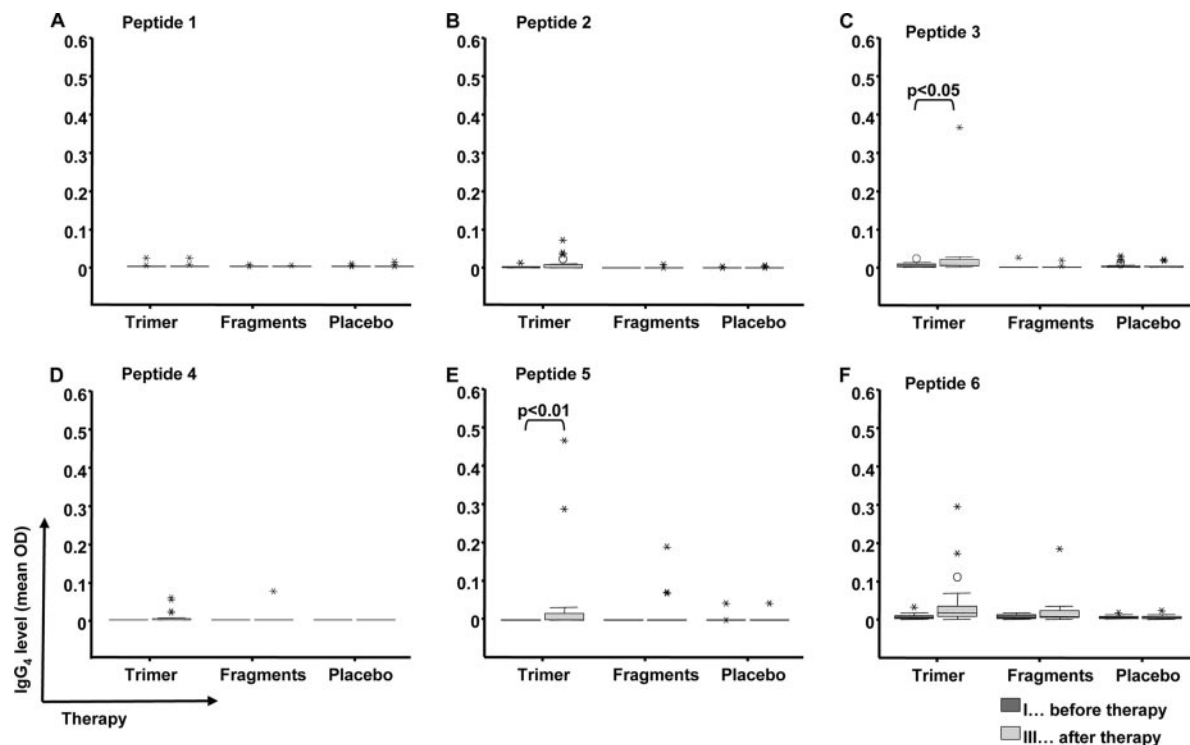


FIGURE 7. IgG4 levels to six Bet v 1 peptides before and after therapy. IgG4 Ab levels directed against peptide 1–6 (A–F) were measured in each group (trimer, fragments, placebo) by ELISA (y-axis: OD values). Bars in dark gray indicate IgG4 levels before therapy (time point I), light gray bars indicate IgG4 levels after therapy (time point III); ○, outlier; *, extreme value.

diameters I: 3.01 mm; II: 3.41 mm; III: 3.17 mm), in the fragment group (average mean weal diameters I: 2.97 mm; II: 3.04 mm, III: 2.97 mm) and in the placebo group (average mean weal diameters I: 2.84 mm; II: 2.92 mm; III: 2.91 mm).

In addition, we studied evolution of skin reactivity to natural Bet v 1 comparing patients before therapy (time point I) and after one

year (time point V) (Fig. 9). There was a tendency of a stronger decrease of skin reactions in both treatment groups as compared with the placebo group which however failed to reach statistical significance. On average mean weal areas at 16 $\mu\text{g/ml}$ Bet v 1 decreased 36 mm^2 in the trimer group, 29 mm^2 in fragments-treated patients compared with 16 mm^2 in the placebo group (Fig. 9).

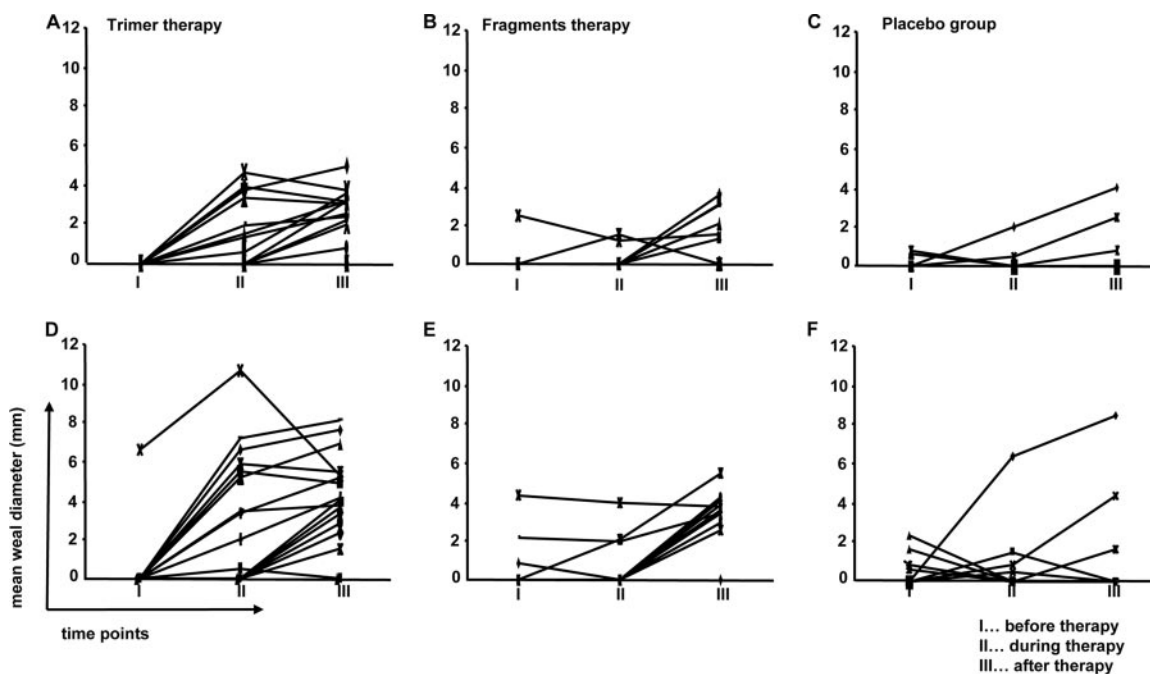


FIGURE 8. Development of skin reactions to Bet v 1 fragments in the individual patients. Patients receiving Bet v 1 trimer ($n = 17$), fragments ($n = 12$) or placebo treatment ($n = 25$) were pricked before therapy (time point I), during (time point II) and after therapy (time point III) with two concentrations of an equimolar mixture of Bet v 1 fragments: 6.4 $\mu\text{g/ml}$ (A–C) and 64 $\mu\text{g/ml}$ (D–F). Weal mean diameters are displayed for each group.

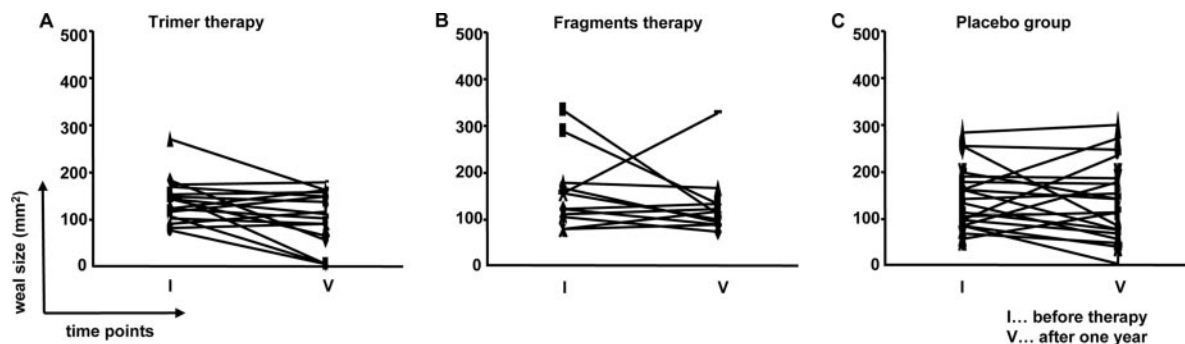


FIGURE 9. Development of Bet v 1-specific skin reactions in each patient before and after 1 year of therapy. Trimer- (A) ($n = 17$), fragment- (B) ($n = 12$), or placebo- (C) ($n = 23$) treated patients were pricked with a birch pollen extract with defined Bet v 1 contents ($16 \mu\text{g/ml}$) before therapy and 1 year after the trial. Skin reactions (weal areas in mm^2) were measured by computerized planimetry.

Discussion

In this study we investigated the development and epitope specificities of Ab responses after vaccination with genetically modified derivatives of the major birch pollen allergen versus placebo (i.e., adjuvant alone) in birch pollen allergic patients. The recombinant Bet v 1 derivatives, i.e., two rBet v 1 fragments and a recombinant trimer, differed substantially from each other regarding their structural characteristics. rBet v 1 fragments had lost the three-dimensional structure of the Bet v 1 allergen and represented unfolded polypeptides (15) whereas the recombinant Bet v 1 trimer retained much of the fold of the Bet v 1 allergen (16). rBet v 1 fragments showed almost no reactivity with IgE Abs of birch pollen allergic patients, whereas Bet v 1 trimer showed IgE reactivity (15, 16). rBet v 1 trimer had induced preferential activation of Th1 cytokines in PBMCs of birch pollen allergic patients and seemed to induce Th1 responses *in vivo*, whereas rBet v 1 fragments showed a different behavior in these assays (Th1: F2 > F1) (16). The major common property of the two recombinant Bet v 1 preparations was their >100-fold reduced allergenic activity with respect to the induction of IgE-mediated effector cell degranulation and immediate type inflammation *in vivo* as studied by skin and nasal provocation testing (17–19).

Interestingly, rBet v 1 fragments as well as trimer induced strong IgG1 and IgG4 Ab responses against the Bet v 1 allergen which yielded 4- to 5-fold higher Bet v 1-specific titers than a preseasonal injection course using standard immunotherapy with natural birch pollen extract. Trimeric Bet v 1 appeared to be more potent in inducing Bet v 1-specific Ab responses than rBet v 1 fragments, at least as far as the induction of IgG4 responses was concerned but also the induction of Bet v 1-specific IgE was stronger in the trimer treated group. The induction of Ab responses was highly specific for the patients who had received active treatment and was not found in the placebo group.

The analysis of the epitope specificity of the therapy-induced IgE and IgG1,4 Ab response with unfolded fragments and linear peptides showed that vaccination had induced an immune response against new epitopes. Although these epitopes are not recognized by IgE Abs from birch pollen allergic patients it has been shown earlier that fragment- and peptide-specific IgG Abs can compete with allergic patients IgE binding to Bet v 1 which may explain the reduction of Bet v 1-induced histamine release from basophils (20) and Bet v 1-induced nasal sensitivity (22) observed in patients who had developed such blocking IgG Abs.

Since both preparations of genetically modified Bet v 1 derivatives induced basically the same type of IgG Ab responses, they appear both suited for immunotherapy treatment of birch pollen allergic patients. Since we noted an induction of IgE responses

toward new epitopes in both actively treated groups, we were interested to study whether this *de novo* induction of IgE responses may lead to increased sensitivity in the patients. The newly formed IgE response was basically directed against new epitopes similar as the IgG response. Interestingly, the IgE response induced against the sequential epitopes was not boosted by seasonal exposure to folded natural Bet v 1 because it declined when treatment was stopped. However, the newly induced IgE response was associated with the induction of mild skin responses to the Bet v 1 fragments preferentially in the trimer treated group already after few injections. A development of mild cutaneous sensitivity to the Bet v 1 fragments was noted also in fragment- and placebo-treated patients and may be rather related to increased IgE production and sensitivity induced by pollen contact with the beginning of the pollen season.

There are at least two possible explanations why the trimer induced higher IgE responses and cutaneous sensitivity. First it is possible that the trimer preparation was more immunogenic and hence induced besides higher IgG also higher *de novo* IgE responses. Second and perhaps more likely, the trimer resembled much of the fold of the Bet v 1 allergen and hence may induce a stronger IgE response than the fragments.

The induction of IgE responses and skin reactions was mild and transient and obviously was overwhelmed by the induction of IgG responses because both actively treated groups showed a reduced skin response to natural Bet v 1 when compared with the placebo group although this did not reach statistical significance.

In summary our data demonstrate that genetically modified Bet v 1 derivatives induced irrespective of the preservation of the fold of the natural allergen a *de novo* Ab response against new epitopes which can inhibit allergic patients IgE binding to the natural allergen. We therefore believe that vaccines based on hypoallergenic allergen derivatives have a potential to inhibit allergic inflammation and IgE production via the effects of IgG Abs which compete with allergen-specific IgE responses in patients. As yet we could not demonstrate that these vaccines induce a tolerogenic T cell response or a relevant induction of allergen-specific IgA responses but this may be achieved by other forms of administration of the vaccines. It is also possible that such responses can be obtained via alternative routes such as mucosal application (26, 27) or the use of adjuvants which foster such responses, similar as has been demonstrated for CpG-conjugated allergens (14).

At present both types of genetically modified allergens (unfolded and folded) protein derivatives seem to be suited for therapeutic applications but the unfolded fragment preparation may have advantages over the trimeric form regarding a potential use as

prophylactic vaccine because the induction of IgE responses was lower than that of the trimer.

In summary the investigation of immune responses achieved in humans with genetically modified allergens should contribute to the understanding of the mechanisms of this treatment and may conceivably provide information for a further development of such vaccines for therapy and prophylaxis of allergy.

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Disclosures

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