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Clinical effects of immunotherapy with genetically modified recombinant birch pollen Bet v 1 derivatives

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Clinical and Experimental Allergy

Summary

Background Birch pollen and pollen from related trees of the Fagales order are a major cause of allergic rhinitis, conjunctivitis, and asthma through the spring season in northern and central Europe.

Objective To investigate the clinical effects of injection immunotherapy with genetically modified derivatives of major birch pollen allergen Bet v 1 on pollen-induced allergic symptoms.

Methods A three-arm double-blind placebo-controlled immunotherapy study was conducted with one pre-seasonal course of treatment using two derivatives of Bet v 1, namely a recombinant Bet v 1 trimer and an equimolar mixture of two recombinant Bet v 1 fragments together representing the whole protein sequence. Analysis of local and systemic adverse events was performed for 124 patients who had received at least one dose of medication. Clinical efficacy was monitored by symptom medication scores and interval scoring in the per protocol-treated population ($n = 84$). In addition, skin and nasal provocation responses and allergen-specific antibodies were assessed.

Results There were trends towards improvement in the subjects' well-being and clinical symptoms (nasal scores), although comparisons with a placebo group did not show statistical significance in the main end-point, the combined symptom medication score. Reductions in skin and nasal sensitivity were observed for some subjects with a trend for the Bet v 1 trimer to be more effective than the fragments. Treatment induced strong IgG1 and IgG4 allergen-specific antibody responses. Local injection-site reactions were most frequent in the trimer group affecting 59.5% of patients as opposed to 37.8% and 30.6% in the fragment and placebo groups, respectively. Systemic reactions were elicited more frequently by fragments. A large proportion of adverse side-effects appeared hours following injections, and might be attributable to concurrent exposure to related pollens.

Conclusion Single courses of injection immunotherapy with Bet v 1 allergen derivatives showed trends towards improved well-being and reduced reactivity to specific allergen provocation, but did not yield significant improvement in the combined symptom medication score in this study.

Keywords allergen variants, allergic rhinitis, allergy, Bet v 1, birch pollen, immunotherapy, recombinant allergens

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Introduction

Pollens from wind-pollinated plants including trees are among the most frequent and potent elicitors of IgE-mediated allergy. The *Betulaceae* family is widespread in

middle and northern Europe and North America, and includes the genera *Alnus* (alder), *Betula* (birch) and *Corylus* (hazel) [1]. The pollen season for one genus

seldom lasts for more than a few weeks, but the well-documented cross-reactivity between tree pollens of the Fagales order contributes to a protracted season of symptoms for many allergic patients [2–5]. A considerable proportion of birch pollen allergic patients also suffers allergic symptoms after the consumption of fruits, vegetables and spices which contain allergens that share epitopes with the pollen-derived allergens [6, 7].

Various studies have concluded that specific immunotherapy conducted with pollen preparations is effective in alleviating the symptoms of seasonal allergic rhinitis (SAR) [8]. Birch pollen has been shown to contain several different allergens, but Bet v 1 represents by far the most important [5, 9]. Therefore immunotherapy with Bet v 1 alone may well suffice to ameliorate symptoms of sensitization to the entire allergen composition of birch pollen. Bet v 1 binds most of the tree pollen-specific IgE antibodies and shares epitopes with corresponding allergens from Fagales pollens and plant-derived foods [5, 10]. Bet v 1 exists in several different isoforms, which differ in their amino-acid composition, in their IgE and T cell reactivity and allergenic activity [11, 12].

The cDNA coding for Bet v 1 has been isolated and the recombinant protein expressed in *Escherichia coli* and purified [13, 14].

Hypoallergenic derivatives of the major isoform Bet v 1a have been created with recombinant DNA technology either by expressing three copies of the gene in sequence to produce a trimer [15, 16] or by cleaving the gene in order to create two recombinant peptides (fragments) that together represent the whole sequence of Bet v 1 [17]. The Bet v 1 fragments and trimer are characterized by retained T cell reactivity and strongly reduced allergenic activity, as has been demonstrated by skin and provocation testing [18–20], and therefore present a reduced risk for inducing

IgE-mediated side-effects while retaining therapeutic potential.

A multicentre, double-blind, placebo-controlled study has now been undertaken in birch pollen allergic subjects to assess the clinical efficacy of a single course of pre-seasonal injection immunotherapy with either the trimer, an equimolar mixture of the two fragments or a placebo. Here we report the analysis of the clinical results obtained in this study.

Methods

Study participants

The study was performed in three centres (Vienna, Strasbourg, Stockholm) with the approval of the Local Ethics Committees and respective national authorities. Patients provided informed written consent, and the study was conducted in accordance with the Guidelines for Good Clinical Practice [21]. A total of 125 subjects were enrolled on the basis of a history of moderate to severe SAR with or without mild/moderate asthma attributable to birch pollen, as confirmed by nasal provocation and skin prick test (SPT), and 124 (safety population) were randomized to receive treatment and received at least one injection (Table 1). Sensitization to the co-seasonal allergen ash (*Fraxinus excelsior*) and clinically relevant sensitizations to perennial allergens were exclusion criteria, as were unstable bronchial asthma or GINA steps 3 and 4 [22], generalized eczema, severe atopic dermatitis, any other severe acute or chronic diseases, or SIT with birch pollen within the last 3 years. A total of 84 subjects completed 12 months of the study, and this per protocol-treated population (PP) was used for the primary analysis of efficacy.

Table 1. Demographic data of patients who participated in the study

Centre Treatment	Vienna			Strasbourg			Stockholm		
	Fragments	Trimer	Placebo	Fragments	Trimer	Placebo	Fragments	Trimer	Placebo
Randomized (<i>n</i> = 124)	22	21	28	6	8	12	10	8	9
Analysis set (<i>n</i> = 84)	8	14	20	4	7	10	7	7	7
M/F	5/3	7/7	8/12	1/3	4/3	6/4	2/5	2/5	5/2
Mean age	34.8	40.4	36.6	46.5	45.1	44.9	35.6	35.3	38.7
(minimum/maximum)	23–48	27–56	23–56	35–56	32–54	29–57	23–52	25–54	22–53
	Fragments			Trimer			Placebo		
Eye symptoms	19/19			27/28			37/37		
Nose symptoms	19/19			28/28			37/37		
Lung symptoms	6/19 (31.6%)			16/28 (57.1%)			22/37 (59.5%)		
Food related symptoms	5/19			8/28			3/37		

Numbers of patients who entered the trial ('Randomized') and who could be completely analysed ('Analysis set') are displayed. The number of patients who reported eye, nose and lung related symptoms in connection with birch pollen exposure are listed in the lower part of the table.

The demographic and clinical characterization of the PP population is shown in Table 1.

Immunotherapy preparations

Bet v 1 was cloned from a cDNA expression library derived from birch (*Betula verrucosa*) pollen [13]. cDNAs corresponding to the N-terminal (aa1–74) and C-terminal (aa75–159) peptide fragments [17] and three copies of the Bet v 1 cDNA linked by nine nucleotide bridges encoding three amino acids (trimer) [16] were sub-cloned into appropriate expression vectors and expressed in *E. coli*. The recombinant proteins were purified using various combinations of chromatographic techniques including hydrophobic-interaction, anion-exchange and size-exclusion [23]. The proteins were analysed to confirm identity and purity, and adsorbed to aluminum hydroxide. The final study preparations were supplied in two concentrations containing 100 and 10 µg protein per litre, the fragment preparation containing equimolar amounts of the N- and C-terminal peptides, in physiological-buffered solution with 0.25% phenol and 1 mg Al³⁺. The highest dose administered was 0.8 mL/80 µg protein. A matching placebo contained all components with the exception of the protein.

Procedures

SPT was conducted during the first screening visit with saline (negative control), histamine (1% histamine dihydrochloride, positive control), and birch, hazel, alder, hornbeam, oak, grass, and mugwort pollens, *Dermatophagoides farinae* and *pteronyssinus*, dog, cat, *Alternaria alternata*, and *Cladosporium herbarum* (Allergopharma, Reinbek, Germany). Nasal provocation tests with birch pollen extract (Allergopharma) were performed before immunotherapy and at the end of the study as described previously [24] using 10-fold increasing concentrations of a birch pollen extract standardized with respect to Bet v 1.

A quantitative SPT (QPT) was performed in triplicate with standardized natural birch pollen extract in four-fold dilutions (0.25, 1, 4, 16, 64 µg/mL) Bet v 1, saline and histamine dihydrochloride controls. Testing was conducted with a randomized double-blind protocol before therapy and again 12 months later. Weal responses were recorded after 15 min by delineating the perimeter with a fine ball-point pen and transferring an impression to paper with adhesive tape. Weal areas were measured using computerized digital planimetry. Corticosteroids and antihistamines were discontinued 3 days before each test.

Patients documented the nature and severity of symptoms and drug usage on a daily basis in study diaries over an 8-week period encompassing the pollen season. Symptoms were recorded specifically for eyes (itching, tear flow, conjunctival redness), nose (sneezing, runny nose,

blocked nose), and lungs (cough, wheezing, asthma with respiratory distress). Intensity of symptoms was scored as 0 = none, 1 = mild, 2 = moderate and 3 = heavy. The daily drug consumption was recorded and subsequently scored as follows: disodium cromoglycate, local α - or β -mimetics, 1; local antihistamines, 2; oral antihistamines or local steroids, 3; and oral steroids, 4. The combined symptom medication score served as the basis for a primary end-point. A combined symptom-medication score is in fact the primary target variable favoured by the licensing authorities as confirmed by the new draft Guideline on the Clinical Development of Products for Specific Immunotherapy (CHMP/EWP/18504/2006) published by the European Medicines Agency (EMA). Data were analysed for a 35-day period starting with the day when birch pollen counts reached 60 grains/m³ and calculated as a mean daily score for each subject. Before the first injection and at the end of the pollen season, patients assessed their general well-being for the previous birch pollen season relative to a 10 point visual rating scale. Investigators assessed the success of treatment in terms of a patient's condition together with their use of symptomatic medication.

Blood samples were collected before and after immunotherapy, in May and after 12 months. Bet v 1-specific IgE antibodies were measured using the Allervance system (Allergopharma). Allergen-specific IgG1 antibodies were detected using microtitre plates coated with either monoclonal anti-IgG1 antibody JDC-1 (BD Biosciences, Heidelberg, Germany; 10 µg/mL in carbonate buffer pH 9) or Bet v 1 pollen extract (Allergopharma; 5 µg/mL). Antibody-coated wells were incubated with purified IgG1 (Sigma-Aldrich, Taufkirchen, Germany) as reference, with concentrations of 4–2000 µg/L, and allergen-coated wells with serum samples diluted at least 1 : 2. Biotinylated anti-IgG1 antibody G17-1 (BD Biosciences; 1 µg/mL) and alkaline phosphatase labelled streptavidin (Sigma; 1 µg/mL) with para-nitrophenylphosphate as substrate were used for detection. Samples were tested in duplicate with dilutions as necessary to fall within the working-range of the assays. Plates were read at 405 nm after 15 min substrate incubation. The same assay format was used to detect IgG4 antibodies with anti-IgG4 antibody JDC-14 (BD Biosciences; 2 µg/mL), purified IgG4 (Sigma) as reference, and biotinylated anti-IgG4 antibody G17-4 (BD Biosciences; 1 µg/mL) for detection.

Randomization was made on a per centre basis, and patients were allocated to treatment sets previously randomized and labelled by the producer using computer generated random number tables to ensure concealment of the allocation. Treatment commenced between November 2000 and January 2001 and continued up to the beginning of the 2001 birch pollen season. Dosage was increased from 1 to 80 µg in two-fold concentration steps with subcutaneous injections given at 7–14 day intervals.

Once the maximum or maintenance dose had been achieved the interval was increased stepwise to 14 and then 28 days.

Assessment of adverse reactions

Undesirable side-effects were graded according to the Position Paper of the German Society for Allergology and Clinical Immunology [25], based on the criteria defined by Tryba *et al.* [26]. Grade 0: local reaction, restricted cutaneous reaction; grade 1: mild general reaction, disseminated cutaneous reaction (e.g. flush, generalized urticaria, pruritus), mucosal membrane reaction (nasal, conjunctival), general reaction (e.g. agitation, headache); grade 2: pronounced general reaction, circulatory dysregulation (change in blood pressure/pulse), shortness of breath (mild dyspnea, beginning of bronchospasm); grade 3: severe general reaction, shock (e.g. severe hypotension, paleness), bronchospasm with severe dyspnea, loss of consciousness, faecal and urinary incontinence; grade 4: vital organ failure, apnea, circulatory arrest.

Statistical analysis

A blind data review was performed before decoding, and decisions concerning the handling of drop-outs or missing data were made on the basis of blinded results. The assessment of efficacy was based on the PP group including all patients who were examined after 12 months. The assessment of safety and tolerance included all patients who received at least one injection of the trial medication. The homogeneity of success rate in the three study groups was evaluated by means of a χ^2 -test and the differences in the symptom-medication-score between the study groups by means of the Kruskal-Wallis *H*-test. Additional evaluations of differences between the study groups were made with the Wilcoxon-Mann-Whitney *U*-test and changes within the groups were examined with the Wilcoxon's signed rank test.

Results

Variability in pollen exposure and development of immunoglobulin E responses

A *post hoc* analysis indicated that there was considerable variation in the date of onset of the pollen season and pollen exposure in the three study centres reflecting different geographical locations and prevailing climate. Patients from Strasbourg were exposed as early as February 2001 to substantial amounts of alder pollen, and birch pollen exposure started at the end of March, 1 week earlier than in Vienna. In contrast, the birch pollen season started 1 month later (beginning of May) in Stockholm. Only patients in Vienna and Strasbourg were exposed to

relevant amounts of birch pollen with peak counts of 1050 and 894 grains/m³, respectively, whereas counts in Stockholm remained below 200 grains/m³. Levels of exposure were reflected in increases in Bet v 1-reactive IgE in the placebo groups of the three study centres. All placebo subjects in Vienna showed strong responses during the birch pollen season (Fig. 1: difference between

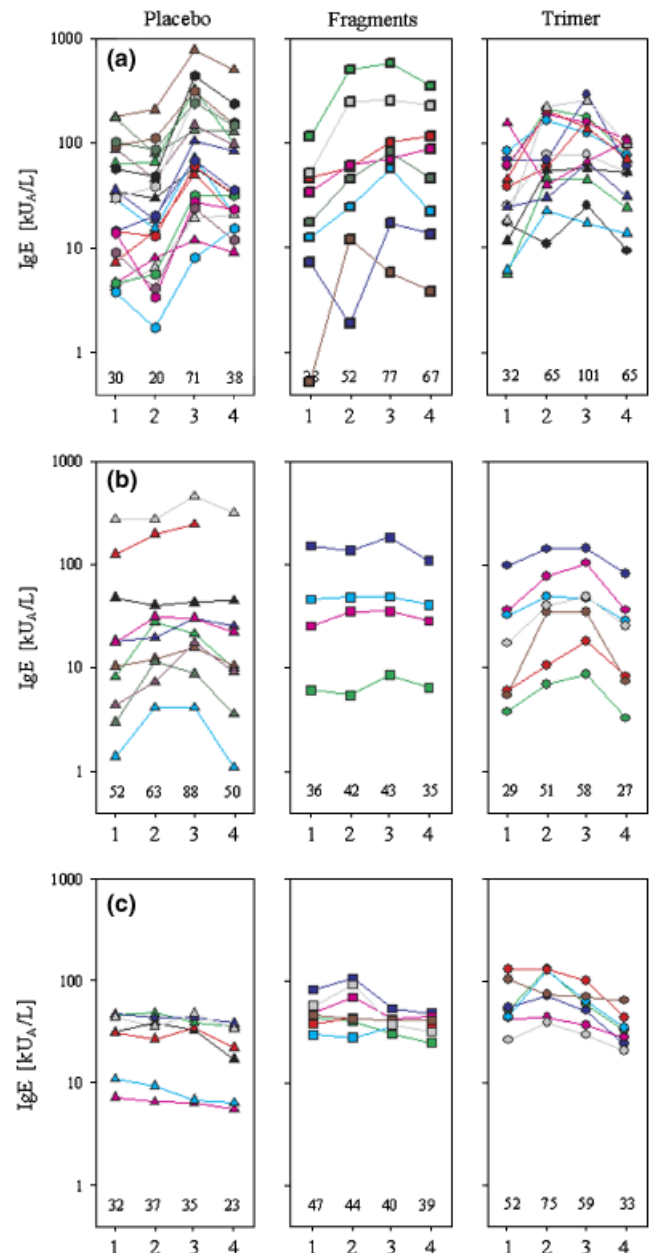


Fig. 1. Bet v 1-specific IgE antibody responses during the course of the study. Evolution of Bet v 1-specific IgE antibody levels (kU_A/L) in patients treated with placebo, fragments or trimer in Vienna (a), Strasbourg (b) and Stockholm (c) measured at 4 time-points, 1: November 2000, before immunotherapy; 2: March 2001, after immunotherapy; 3: May 2001, and 4: October 2001, after 12 months. Values within the graphs at each time-point denote the median antibody concentrations.

samples 2 and 3), while increases seen in the Strasbourg group occurred somewhat earlier and there were no increases among the Stockholm subjects. Therapy-associated rises in Bet v 1-specific IgE levels (Fig. 1: difference between samples 1 and 2) were seen in the actively-treated patients.

Magnitude and development of immunoglobulin G1 (IgG1) and immunoglobulin G4 (IgG4) responses in actively treated and placebo subjects

Both regimens (fragments and trimer) induced strong IgG1 (Fig. 2) and IgG4 (Fig. 3) responses against the Bet v 1 allergen in all the treated subjects. The specific antibody levels peaked at the end of treatment and were substantially reduced (i.e. almost returned to baseline levels) 7 months after discontinuation of injections. In most subjects the specific antibody concentrations increased more than 100-fold compared with baseline levels, whereas no relevant increases were observed in the placebo groups from the three centres.

Demonstration of clinical efficacy in pollen-exposed patients by visual rating scale and symptom/medication scores

As another part of *post hoc* analysis, subjects' self-assessment of their well-being on a 10 point visual rating scale failed to show any significant differences between the groups, both when considering all centres together and when excluding the Stockholm centre because of no clinically relevant pollen exposure (Fig. 4). However, comparisons within the treatment groups of scores for the pollen season before and following immunotherapy showed significant differences within all the groups when considering all three centres together. When the Stockholm centre was excluded the trimer-treated group showed a significant improvement ($P=0.044$) while the fragment-treated and placebo groups showed no significant differences. Exclusion of those subjects that had undergone an unsuccessful course of immunotherapy more than 3 years previously enhanced the difference seen in the fragment-treated group although just failing to achieve statistical significance ($P=0.055$).

The analyses of the symptom and medication scores are shown in Table 2. Reductions in eye and nasal symptoms were noted for both actively-treated groups by comparison with placebo. The combined mean daily symptom scores (lung, eyes, nose) for Vienna and Strasbourg together showed median values of 4.66, 3.13, and 2.39 for placebo, trimer, and fragments respectively and corresponding medication scores were 1.73, 2.14, and 2.10. But there were no significant differences between the groups (Kruskal–Wallis test).

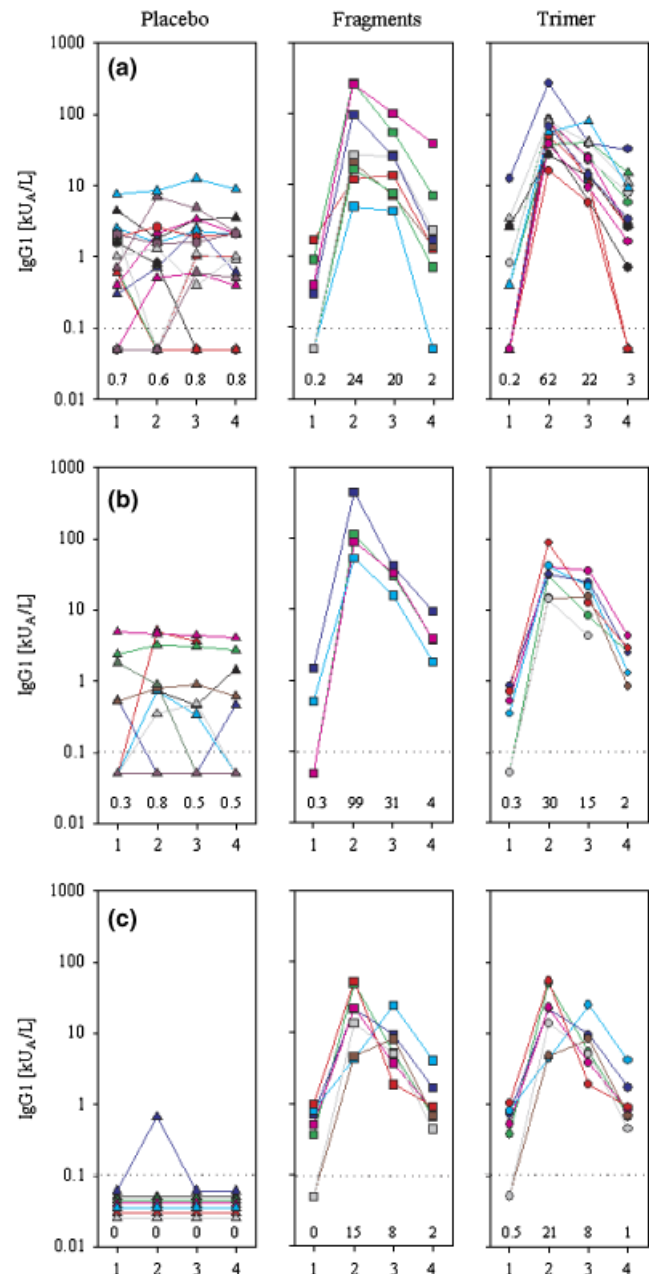


Fig. 2. Bet v 1-specific IgG1 antibody responses during the course of the study. Bet v 1-specific IgG1 antibody levels (arbitrary units/mL) for the individual patients in each of the three study centres Vienna (a), Strasbourg (b) and Stockholm (c). Time-points and color/symbol designations are the same as in Fig. 1, and values within the graphs at each time-point denote the median antibody concentrations.

Reduction in nasal and skin sensitivity in actively-treated patients

Objective assessments of nasal and skin sensitivity were performed by nasal provocation testing and QPT before immunotherapy and after 12 months. The analysis of the nasal sensitivity to a series of five increasing concentrations of Bet v 1 before and after immunotherapy showed

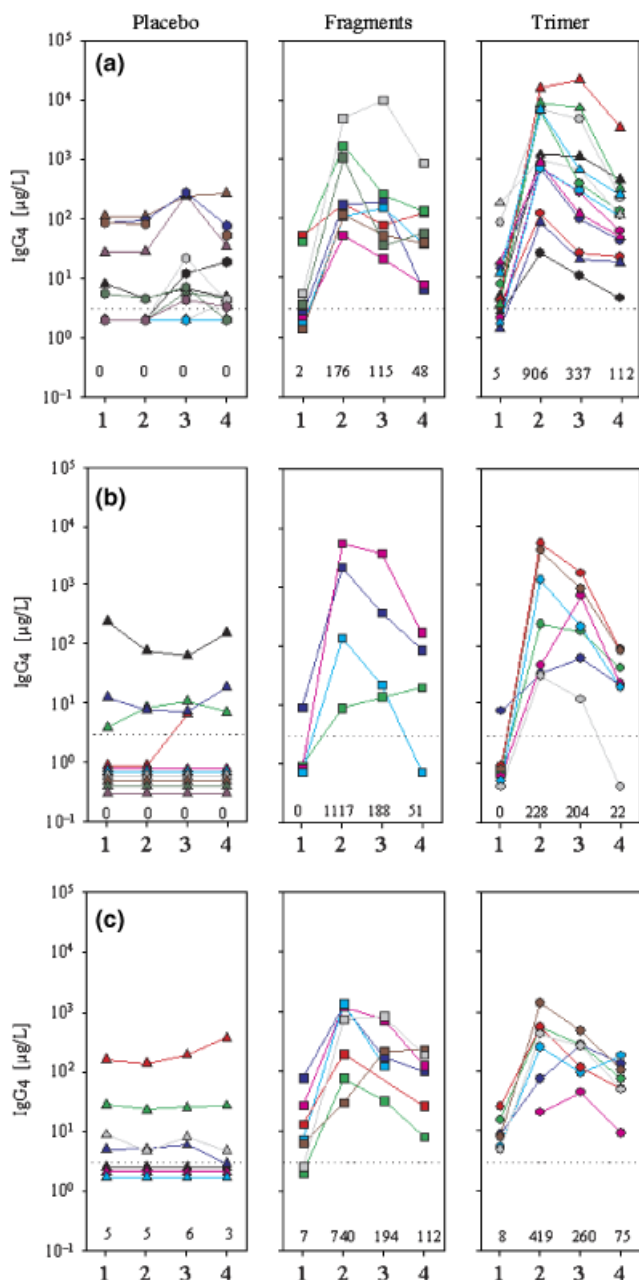


Fig. 3. Bet v 1-specific IgG4 antibody responses during the course of the study. Bet v 1-specific IgG4 antibody levels ($\mu\text{g/L}$) for the individual patients in each of the three study centres Vienna (a), Strasbourg (b) and Stockholm (c). Time-points and color/symbol designations are the same as in Figs 1 and 2, and values within the graphs at each time-point denote the median antibody concentrations.

decreases in nasal sensitivity for a majority of subjects tested in the trimer-treated group, with 15 improving, 11 unchanged, and only one deteriorating (Fig. 5). In the fragment-treated group seven of the 17 subjects tested showed decreased sensitivity. In both cases the differences were statistically significant. In the larger placebo group there was no significant difference although 14 of 36 subjects showed decreased sensitivity. There were no

statistically significant differences between the groups either before immunotherapy or after 12 months (χ^2 test, $P = 0.19$).

The QPT threshold responses also showed no statistically significant differences between the groups either before immunotherapy or after 12 months. Significant decreases in sensitivity were seen within all three groups (Fig. 6). The trimer-treated group had the largest number of subjects showing increased tolerance, with 16 (67%) showing an increase while six remained unchanged and only two showed increased skin sensitivity. In the fragment and placebo groups, increased tolerance was seen in 59% and 44% of subjects, respectively.

Analyses of local and systemic adverse events

Table 3 summarizes the nature and features of local and systemic reactions in the 124 subjects who commenced treatment. The reactions were classified according to Tryba [25, 26] in five grades.

Grade 0 reactions, involving swelling at the injection site, were seen in all three study groups, but more frequently in the trimer-treated subjects. Local pain and/or itching were reported by subjects in all study groups, with itching alone being more frequently associated with the Bet v 1 preparations. The large majority of the local reactions with fragment therapy occurred several hours after the injections, whereas those associated with the trimer appeared somewhat sooner.

The majority of systemic side-effects were assigned to grade 1 and resolved either spontaneously or under appropriate medication. Conjunctivitis (percentages per injection; fragments: 5.3%; trimer: 3.1%; placebo: 1.9%) and rhinitis (fragments: 16.1%; trimer: 10.0%; placebo: 6.3%) were observed in the actively treated and the placebo groups and the majority were late in onset (i.e. after several hours), occurring during a period when other tree pollen, particularly alder (*Alnus*), were prevalent. This raises the distinct possibility that they were attributable to pollen exposure rather than to the therapy. Urticaria was noted exclusively in the actively-treated groups, and was more frequently associated with fragments than with trimer (percentages per injection, fragments: 3.0%; trimer: 1.7%). Four of eight urticarial reactions in the fragment group were associated with the 80 μg maximum dose, as were four from six reactions in the trimer group.

Grade 2 reactions (asthma, dyspnoea, gastro-intestinal reaction, and circulatory dysregulation) were only found in the actively-treated subjects. Cases of asthma (percentages per injection; fragments: 3.0%; trimer: 1.1%) and dyspnoea were rapidly controlled with inhaled β_2 agonist. The reaction involving circulatory dysregulation was observed 5 min after injection of 60 μg of the fragment preparation in a 35-year-old female subject. The patient developed a drop in blood pressure, which was

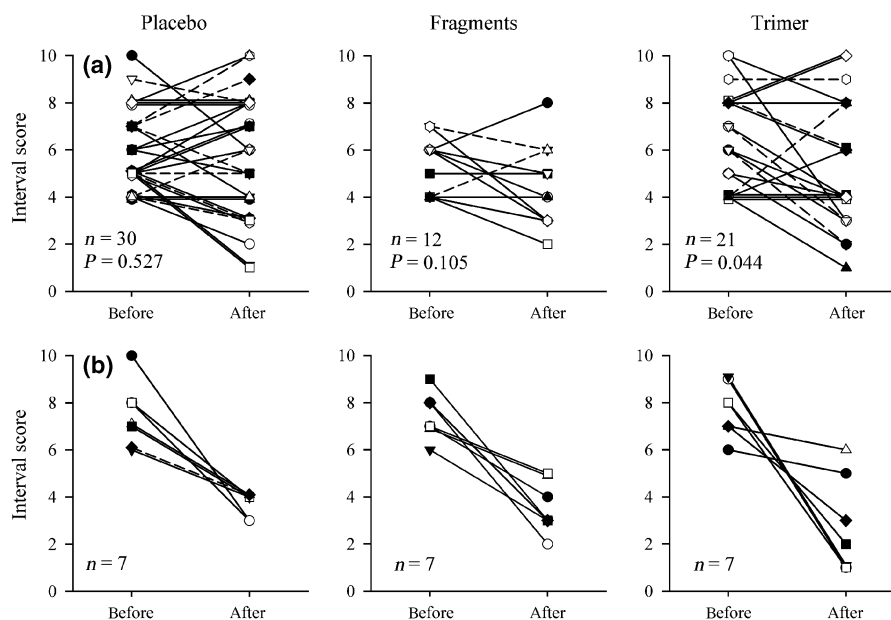


Fig. 4. Subjects assessment of their well-being on a visual rating scale. Subjects assessed their state of well-being on a ten-point scale for the pollen seasons prior to and immediately following immunotherapy. (a) Subjects in Vienna and Strasbourg, and (b) subjects in Stockholm. Broken lines indicate those subjects who had undergone an unsuccessful course of immunotherapy more than three years prior to this study.

Table 2. Symptom and Medication Scores (SMS) from patients' diaries

	All three centres			Vienna and Strasbourg		
	Fragments	Trimer	Placebo	Fragments	Trimer	Placebo
Number of scores	19	28	37	12	21	30
Nasal	1.29	1.75	2.37	1.59	1.71	2.69
Eyes	0.54	0.73	1.09	0.64	0.91	1.23
Lung	0.03	0.30	0.26	0.06	0.31	0.53
Symptoms	1.84	2.91	4.40	2.39	3.13	4.66
Medication	2.14	2.11	1.46	2.10	2.14	1.73
Combined SMS	4.20	5.83	5.80	5.16	6.52	6.43

Median symptom/medication scores for the 2001 pollen season for all three centres together and for Vienna and Strasbourg.

successfully treated with adrenaline, methylprednisolone, and dexchlorpheniramine. The Bet v 1-specific IgE concentration was 26 kU_A/L, but no IgE antibody against the fragments was detectable. The subject withdrew from the study. A 53-year-old female developed generalized urticaria and a gastrointestinal reaction, but no drop in blood pressure, 2 h and 45 min after an injection of 20 µg of the trimer. She was treated successfully with methylprednisolone and dexchlorpheniramine. The Bet v 1-specific IgE concentration was 39 kU_A/L. This subject continued in the study after appropriate dose adjustment. Most of the reactions occurred at doses smaller than or equal to 20 µg protein or the equivalent 200 µL strength B placebo. However, 7/15 cases of general urticaria were associated with the highest dose (80 µg), four in the trimer group and three in the fragment group. In general, systemic adverse

events were elicited more frequently by fragments. Applying the Kruskal–Wallis test for several independent samples reveals significant differences between the three study groups for the number of subjects experiencing local reactions ($P=0.022$), grade 1 reactions ($P=0.000$), and grade 2 reactions ($P=0.002$). Applying the Wilcoxon–Mann–Whitney U -test for two independent samples showed no significant difference between the numbers of subjects experiencing reactions in the two active treatment groups (local reactions $P=0.052$; grade 1 $P=0.069$; grade 2 $P=0.144$). Numbers did not differ significantly between fragments and placebo in respect of local reactions ($P=0.543$), but there were significant differences ($P=0.000$) in respect of both grade 1 and 2 reactions. There were significant differences between trimer and placebo-treated subjects in all three reaction categories, local ($P=0.008$), grade 1 ($P=0.001$), and grade 2 ($P=0.019$).

Four subjects withdrew from the study due to adverse events, three subjects in the Bet v 1 fragment group and one in the placebo group.

Discussion

Here we report the clinical assessment of immunotherapy with genetically modified variants of the major birch pollen allergen Bet v 1 in birch pollen allergic patients. Clinical parameters and objective measurements for sensitivity (i.e. quantitative skin prick and nasal provocation testing) were assessed in the per protocol-treated group ($n=84$), for which full documentation and fulfillment of

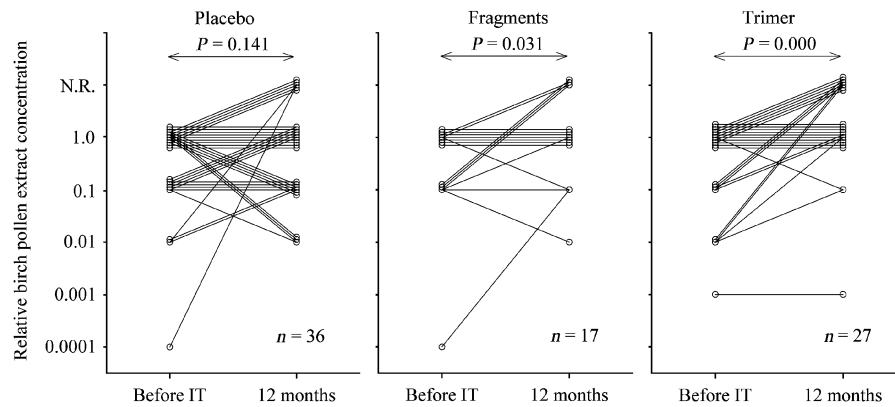


Fig. 5. Differences in nasal provocation responses before treatment and after 12 months. Nasal provocation was performed using a series of 10-fold dilutions of a freshly prepared stock solution (1.0) containing the equivalent of 7.0 µg/mL Bet v 1, starting with the lowest concentration and continuing until a positive response was obtained or no response (N. R.) was seen with the stock solution. There were no statistically significant differences between the groups, Chi-square test $P = 0.19$. The significance of differences within the groups was judged using a two-tailed Wilcoxon's signed ranks test.

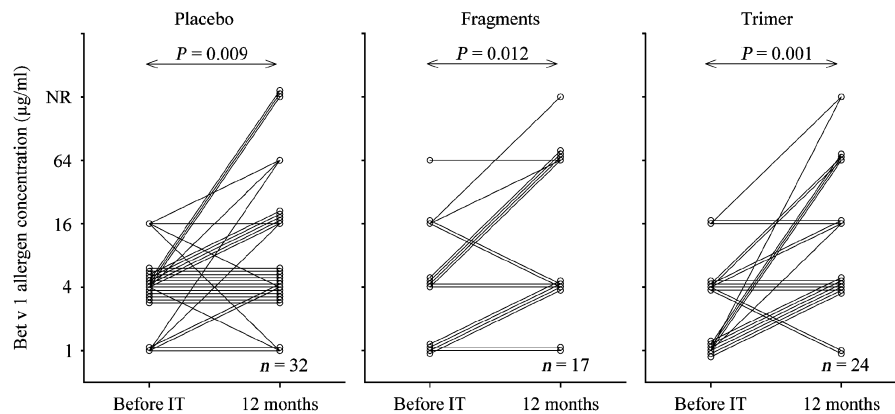


Fig. 6. Differences in threshold skin prick test responses before treatment and after 12 months. Five allergen concentrations as well as histamine and negative control solutions were tested in triplicate. The threshold dose was defined as the lowest allergen concentration for which the geometric mean reaction was larger than that to 1.0% histamine dihydrochloride. There were no statistically significant differences between the groups either before immunotherapy or 12 months later (Chi-square test $P = 0.77$). The significance of differences within the groups was judged using a two-tailed Wilcoxon's signed ranks test.

all study requirements were achieved. The analysis of self-assessment with a visual rating score and symptom-medication scores failed to show significant differences between the treatment groups. However, results may have been confounded for several reasons. One may have been the very low pollen counts in Stockholm which contributed to significant differences between pre- and post-treatment within the groups. The lack of pollen exposure was confirmed by the absence of a boost in allergen-specific immunotherapy IgE during the pollen season and the marked improvement in the placebo group.

When the Stockholm subjects were excluded from the analysis, a statistically significant improvement in well-being was observed in the trimer-treated patients and a trend toward significance in the fragment-treated subjects when comparing pre- and post-treatment. Another factor which may have confounded results has been the inclusion of patients who had undergone an unsuccessful

course of immunotherapy more than 3 years previously. Such subjects may constitute a group of non-responders. The small number of subjects (i.e. 84 out of 124) included in the final analysis rendered the study under-powered, and this was also a contributory factor in the analysis of symptom and medication scores. In fact there were nine drop-outs in the true sense: two subjects moved away, one lost contact, one withdrew consent, one withdrew through lack of time, and four withdrew following adverse events, one in the placebo and three in the fragment groups. The reason for the exclusion of the other subjects was the unavailability of adequately completed diaries which were the basis for assessing the primary target variable.

Symptom scores were lower in the treated groups than in the placebo group, but it has to be noted that small increases in the use of symptomatic medication may have contributed to these differences.

Table 3. Nature and frequency of local and systemic adverse events

	Bet v 1 fragments	Bet v 1 trimer	Placebo	Total
Subjects (n)	38	37	49	124
Grade 0		Reactions/subjects		
Swelling 5–10 cm	47/14	78/20	40/14	163
Swelling >10 cm	2/2	23/8	7/5	32
Grade 1		Reactions/subjects		
Conjunctivitis	16/12	11/7	9/5	36
Rhinitis	49/22	35/14	30/11	114
Cough	24/8	12/4	3/3	39
Wheezing	9/4	6/2	1/1	16
General urticaria	9/8	6/6	0/0	15
General itching	4/4	4/3	2/1	10
General erythema	0/0	2/1	0/0	2
Grade 2		Reactions/subjects		
Asthma	9/7	4/3	0/0	13
Dyspnea	3/2	1/1	0/0	4
Circulatory dysregulation	1/1	0/0	0/0	1
Gastrointestinal reaction	0/0	1/1	0/0	1

Previous studies using recombinant allergens had shown a good correlation between results from skin testing and nasal provocation testing indicating that skin-test results reflect nasal sensitivity [27]. In fact, trends towards reduced skin and nasal sensitivity were observed in the present study with the trimer-treated subjects showing more favourable increases in threshold responses than obtained with fragments, but improvements in the placebo group detracted from these results. It has been suggested that the reduction of IgE-mediated reactivity in the skin and nose may be attributed to the induction of Bet v 1-specific IgG antibody responses [24, 28], and if this is the case then the large increases in concentrations of both IgG1 and IgG4 antibodies would explain this reduced organ responsiveness.

However, the treatment had been started relatively close to the beginning of the pollen season in the Vienna and Strasbourg centre where patients experienced relevant pollen exposure. It is therefore possible that the IgG antibody responses were not fully developed during pollen exposure and better clinical outcomes might have been achieved if the treatment could have been initiated earlier. Moreover, skin and nasal provocation testing had been done at a time when, due to the exclusively pre-seasonal treatment, the IgG antibodies had already returned towards baseline which may explain the relatively low protective effect observed in these assays. In fact, protective effects were observed in a subgroup of patients tested after the pollen season when the IgG antibodies were high [24]. Furthermore, we had found that treatment with fragments as well as with trimer, but not with placebo, suppressed the seasonal boosts of allergen-

specific IgE production suggesting that the active treatment had disease-modifying character [28]. In this context it should also be mentioned that three other immunotherapy trials with recombinant allergen molecules (i.e. one performed with recombinant grass pollen allergens [29], one with a recombinant Bet v 1-folding variant [30] and one with recombinant Bet v 1 [31]) which were started well ahead of the respective pollen seasons and which were designed to maintain high allergen-specific IgG responses, achieved significant clinical improvements [reviewed in 32].

Besides the comparison of verum- vs. placebo-treated patients regarding clinical effects, an assessment of the symptoms within the verum- and placebo-treated patients before and after treatment would have been another possibility for measuring the effects of treatment. In theory, and for single centre trials, this might have been achieved by obtaining a baseline assessment in the season before treatment. However, it may be difficult to establish a uniform baseline if pollen exposure varies in different study centres as in fact has been observed during treatment in our study. A possible solution to this problem may be an objective evaluation of symptoms before and after treatment by controlled allergen exposure e.g. by provocation testing in a pollen chamber [33].

In addition to the induction of Bet v 1-specific IgG antibodies, we have also observed alterations in allergen-specific T cell reactivity in trimer-treated patients in the form of increased expression of T-helper type 2 cytokines, but no induction of tolerance related cytokines i.e. IL-10 [34].

The side-effects were analysed in all 124 patients who received study medication. The recombinant Bet v 1 allergen variants have been designed to reduce their IgE-reactivity and thus their capacity to induce immediate-type reactions [15–17]. Indeed most of the side-effects observed appeared later than 1 h after injection. The majority of systemic side-effects were mild and of grade 1 [26]. However, grade 2 reactions such as asthma, dyspnoea, circulatory dysregulation, and gastrointestinal disturbance were observed several hours after injection. We consider that these reactions are most likely non-IgE-mediated, because most of the patients did not have detectable IgE-antibodies against the fragments and little or no skin reactivity was found when patients were skin tested with the trimer and fragments before the treatment. The release of calcitonin-related peptide, a T cell-derived vasoactive substance, may be considered a possible mechanism behind these reactions [35]. Most side-effects occurred with low doses of active preparations or placebo, and the majority of the subjects tolerated the highest doses (80 µg) of fragments or trimer, receiving cumulative doses in excess of 150 µg in 51/75 cases. In this context it should also be noted that immunotherapy with T cell-reactive peptides of the major cat allergen Fel d 1, which lacked

IgE-reactivity, induced side-effects of grade 2, i.e. asthma in a majority of the treated patients [36]. It is possible that the lower rate of side-effects observed in the present study is due to the fact that the proteins were adsorbed to aluminium hydroxide and thus not released systemically as would be the case with the injection of soluble peptides.

Side-effects reported for injection immunotherapy studies performed with aluminium hydroxide-adsorbed birch pollen extracts seem rather low but cannot be compared with the adverse events recorded in our study for several reasons [37, 38].

The vaccines used in earlier studies performed with birch pollen extracts were reported to contain 100 000 SQ-U in the maintenance dose which should correspond to 12 µg of Bet v 1, but it is not clear how the adsorption protocol used to generate these vaccines might have modified the allergen and how much allergen were bound to alum in the final formulation administered to the patients. The 80 µg maintenance dose administered in our study is thus at least six-fold higher than in the preparations used in the former extract-based studies, which is also reflected by the fact that the recombinant Bet v 1 preparations used in our study induced approximately four- to five-fold higher Bet v 1-specific IgG antibody levels compared with extract-based vaccines after the same period of administration [39].

Furthermore, we chose to report all adverse events, whereas certain of the extract-based studies [38] specifically exclude the reporting of large local reactions. It is also worth noting that the earlier extract-based studies were conducted in Scandinavia where cross-reacting species such as alder and hazel do not present a problem as they did in Vienna and Strasbourg.

Increases in birch pollen-specific IgE in the placebo subjects in the Vienna centre were attributed to natural birch pollen exposure. The increases seen before the birch pollen season in the placebo subjects from Strasbourg are consistent with exposure to cross-reacting tree pollens which were noted to be prevalent and which may have contributed to several of the reactions documented during therapy.

Although the study was clearly under-powered, and a pollen season failed to materialize in one of the three centres, the results may be seen as encouraging for two reasons. First, patients were treated with variants of a single Bet v 1 isoform and showed clinical and objective improvement to birch pollen exposure while developing IgG antibody responses against the natural, unmodified allergen. Second, clinical improvement was achieved with only one course of pre-seasonal immunotherapy. Other possible advantages of this treatment over allergen extract-based treatment may be the following. First, we did not observe any therapy-induced *de-novo* sensitization to new allergens in our patients (V. Niederberger & R. Valenta, unpublished) whereas Moverare *et al* reported

that more than 20% of patients receiving birch pollen SIT developed IgE antibodies against cross-reactive birch pollen allergens [40]. Second, we did not find evidence for IgE-mediated side-effects as are observed with aqueous extracts and which could lead to fatalities [41]. Third, we found that the genetically modified allergens induced four- to five-fold higher Bet v 1-specific IgG antibodies than conventional allergen extract-based injection immunotherapy [39].

In conclusion, we believe that the data encourage further investigation into the potential of such recombinant allergen derivatives for specific immunotherapy.

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