

Comparison of genetically engineered hypoallergenic rBet v 1 derivatives with rBet v 1 wild-type by skin prick and intradermal testing: results obtained in a French population

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Summary

Background Bet v 1, the major allergen in birch pollen, is recognized by more than 90% of patients allergic to birch in northern and central Europe. Immunotherapy is commonly performed with birch pollen extracts. Recently, hypoallergenic derivatives of Bet v 1 (rBet v 1 fragments, rBet v 1 dimer and trimer) were constructed and purified.

Objective Our aim was to compare the allergenic activity of wild-type rBet v 1 with recombinant Bet v 1 derivatives (rBet v 1 fragments, dimer and trimer) with potentially reduced anaphylactic activity by skin testing in a French population.

Methods Among the 36 birch pollen allergic patients included in the study, 29 were tested by skin prick testing and 30 by intradermal injections with purified monosubstances: rBet v 1 fragments (F1: aa1–74 and F2: aa75–160), Bet v 1 dimer and trimer. Intradermal tests were performed by the end-point intradermal titration method. Eight of the intradermally-tested patients were previously hyposensitized. Tests were performed over a period of 6 months (before, during and after birch pollen season); Bet v 1-specific IgE and IgG₄ subclass responses were measured by immunoblotting and ELISA.

Results All patients showed lower reactivity with the modified rBet v 1 allergens, both in skin prick and intradermal tests. In 25 and 23 out of 29 patients the lowest concentration of fragment 1 and 2, respectively, resulting in a positive prick test was 100-fold higher than the lowest concentration of monomer resulting in a positive prick test. For dimer it was 100-fold or more in 25 out of 29 patients, and for trimer it was 100-fold or superior in 26 out of 29 patients. By intradermal testing, the end-point concentration was 160-fold higher for trimer than for monomer in 24 patients and 40-fold higher in five patients. For the two fragments the end-point concentration was 160-fold higher in 20 out of 22 patients.

Conclusion Genetically modified hypoallergenic derivatives of the major birch pollen allergen, Bet v 1 showed reduced capacity to induce immediate type skin reactions. They may represent candidate molecules for immunotherapy of birch pollen allergy with reduced risk of anaphylactic side-effects.

Keywords: Bet v 1, birch pollen allergy, intradermal testing, recombinant hypoallergenic Bet v 1 derivatives, skin prick testing

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Introduction

The prevalence of birch pollen allergy as well as the sensitization against certain birch pollen allergens (Bet v 1 and Bet v 2) depends on geographical variation and is estimated to affect between 20 and 40% of patients with allergic rhinitis in Europe [1–3]. More than 90% of birch pollen allergic patients react to the major birch pollen allergen Bet v 1 in the middle and northern part of Europe [4–6]. Almost 60% react exclusively to Bet v 1 with most of their immunoglobulin (Ig) E antibodies directed against this molecule [7]. Recombinant Bet v 1 which shares immunological and biological properties with natural Bet v 1 has been produced in *Escherichia coli* [8,9]. It has been shown to cross-react extensively with the homologous proteins present in pollen of trees of the *Fagales* order (birch, alder, hazel hornbeam and oak) as well as with allergens present in plant-derived food, vegetables and spices [7,10–12]. Therapy of birch and related allergies is currently performed by immunotherapy with natural birch pollen extract [13–15].

In other, more complex allergen/epitope systems several approaches have so far been pursued to obtain hypoallergenic allergen derivatives for immunotherapy: They include synthetic peptides carrying T-cell epitopes (e.g. Fel d 1-, bee venom phospholipase-derived peptides) [16,17] or orally-administered allergen hydrolysates [18], which were thought to induce tolerance. Other approaches used chemically modified allergens (e.g. allergoids) to induce blocking antibodies and modulate T-cell functions [19].

Here we report the clinical evaluation of hypoallergenic derivatives of the major allergenic component of birch pollen, Bet v 1, which were obtained by genetic engineering of the active recombinant allergen. We have constructed two recombinant Bet v 1 fragments comprising aa1–74 (F1) and aa75–160 (F2) as well as recombinant Bet v 1 oligomers (dimer, trimer) which showed greatly reduced capacity to induce basophil histamine release [20,21]. As demonstrated by circular dichroism analysis, both recombinant Bet v 1 fragments had lost their three dimensional structure and thus their IgE-binding capacity [20]. Recombinant oligomeric forms of Bet v 1 (dimer, trimer) were produced by ligation of two or three copies of the Bet v 1 cDNA in plasmid pET-17b and expression of the constructs in *Escherichia coli* [21, Vrtala and Valenta, unpublished results]. The recombinant Bet v 1 oligomers retained IgE-binding capacity but, unexpectedly, exhibited a greatly reduced capacity to induce basophil histamine release. Circular dichroism indicated that microaggregation and thus reorientation of IgE epitopes may be responsible for this phenomenon (Valenta and Vrtala, unpublished results). Based on the *in vitro* data indicating reduced allergenic activity of the genetically modified Bet v 1 derivatives, we consider to prepare vaccines against birch pollen allergy

which are either composed of an equimolar mixture of the rBet v 1 fragments or the rBet v 1 trimer. However, before recombinant Bet v 1 derivatives can be used for specific immunotherapy, extensive skin test studies need to be performed to compare their capacity to induce immediate type reactions with wild-type Bet v 1. Here we present a comparison of the skin reactivity measured both by skin prick and intradermal testing of rBet v 1 monomer (wild-type) with genetically engineered hypoallergenic Bet v 1 fragments and oligomers. Birch pollen allergic patients, either previously hyposensitized or nonhyposensitized, of a French population were tested with the antigens in a period of 6 months (before, during and after birch pollen season 1998).

Methods

Allergen extracts and recombinant allergen preparations

Birch pollen extract used for immunoblotting was prepared from *Betula verucosa* pollen purchased from Allergon (Valinge, Sweden) as described [22]. Recombinant Bet v 1 monomer (wild-type), Bet v 1a was expressed in *Escherichia coli* and purified to homogeneity [23]. Recombinant Bet v 1 fragments (aa 1–74, aa 75–160) were generated by PCR using the Bet v 1a cDNA as a template, expressed in *E. coli* BL21 (DE3) and purified to homogeneity [20]. Recombinant Bet v 1 oligomers (rBet v 1 dimer and rBet v 1 trimer) were produced by PCR and ligation of two or three copies of Bet v 1a [24] cDNA into plasmid pET-17b (Novagen, Madison, WI). Recombinant proteins were overexpressed in *E. coli* BL21 (DE3) and purified to homogeneity [21].

Allergic patients and nonatopic individuals

Thirty-six patients between 18 and 48 years of age (mean age 32.1, 11 men) were included in the study. The patients were included on the basis of a case history indicative for birch pollen allergy, a positive skin test to commercial birch pollen extract (Stallergène Laboratories, France), and IgE antibodies to birch pollen extracts. Twenty-four of the 36 patients suffered from birch pollen-associated food allergy. Twelve of the patients had previously received birch pollen immunotherapy and 24 were untreated. Patients having severe asthma as defined by International Consensus, intercurrent infectious or neoplastic disease, AIDS, pregnant or lactating women and patients on beta-blocker were excluded from the study. Symptomatic patients taking antihistamines as their treatment were tested only if they could withdraw the drug for 5 days if it was cetirizine or loratidine and, for 8 weeks, if it was astemizole. Two atopic patients without birch pollen allergy and eight nonatopic subjects were included in the study as controls. The study was approved by the Consultative Committee for Protection of the Persons for Biomedical Research

(CCPPRB) of Strasbourg, France. A detailed explanation of the study was given to all participants and their written consent was obtained before testing.

Serology

Total IgE levels in sera were measured by CAP-RAST (Pharmacia, Uppsala, Sweden). Specific IgE and IgG₄ antibodies to rBet v 1 monomer (wild-type) were determined by ELISA as described [5]. In brief, ELISA plates (Nunc, Roskilde, Denmark) were coated with 5 µg/mL of purified allergens. Patients' sera were diluted 1 : 10 for the detection of specific IgE and 1 : 100 for the measurement of IgG₄. Bound human antibodies were detected with 1 : 1000 diluted mouse monoclonal antihuman IgE and antihuman IgG₄ antibodies (Pharmingen, San Diego, CA, USA), respectively. Bound mouse antibodies were then detected with a horseradish peroxidase-coupled sheep antimouse antiserum (Amersham, Buckinghamshire, UK). Results represent means of duplicate determinations with an error of less than 5%. Serum IgE and IgG₄ reactivity to natural birch pollen proteins was also tested by immunoblotting. Nitrocellulose-blotted birch pollen extract was blocked in buffer A (50 mmol/L Naphosphate pH 7.5, 0.5% w/v BSA, 0.5% v/v Tween 20, and 0.05% w/v NaN₃) and incubated with sera diluted 1 : 10 (IgE) or 1 : 100 (IgG₄) in buffer A overnight at 4 °C. After washing in buffer A, bound IgE antibodies were detected with ¹²⁵I-labelled antihuman IgE antibodies diluted 1 : 10 in buffer A (RAST; Pharmacia, Uppsala, Sweden). Bound IgG₄ antibodies were detected with a mouse monoclonal antihuman IgG₄ antibody (Pharmingen) and an ¹²⁵I-labelled sheep antimouse Ig antiserum (Amersham).

Skin prick testing

Skin prick testing was done in 29 birch pollen allergic patients, two atopic persons without birch pollen allergy and eight nonallergic individuals with histamine (10 mg/mL) as a positive control, physiological saline as negative control, birch pollen extract (Stallergènes Laboratories), rBet v 1 (BIOMAY, Linz, Austria) at concentrations of 10 and 50 µg/mL, fragments rBet v 1 (aa 1–74) (F1) and rBet v 1 (aa 75–160) (F2) at concentrations of 10, 100 and 1000 µg/mL, rBet v 1 dimer at concentrations of 10, 100 and 1000 µg/mL and rBet v 1 trimer at concentrations of 10, 100 and 1000 µg/mL.

Thirty µL of each solution was placed 3 cm apart using the volar side of both the patient's forearms and the skin was then pricked with Prick-Lancettes (Dome Hollister Stier Laboratories, Puteaux, France). The prick test as read 10 min later was considered positive if mean diameter of the weal induced by the allergen was at least 75% of that induced by histamine. Weal reactions were documented by photography and by transferring the ball point pen

surrounded weal and flare with a tape to paper. The mean diameter was calculated by measuring the maximal longitudinal diameter (D) and the maximal transverse diameter (d) according to the formula (D + d)/2. Mean diameter of the weal obtained with rBet v 1 monomer was compared to that obtained with oligomers and fragments. The choice of dilutions for rBet v 1 was based on a previous study [4]. The choice of dilutions for fragments was based on preliminary skin tests performed in eight patients and on *in vitro* histamine release studies, both showing 100-fold less reactivity as compared to monomer [20,21].

Intradermal tests

Intradermal (ID) tests were performed on the lateral sides of the patients arms with (1) rBet v 1 (*n* = 30); (2) rBet v 1 trimer (*n* = 30); (3) rBet v 1 F1 (*n* = 22) and; (4) rBet v 1 F2 (*n* = 22). Forty µL of a 0.01-µg/mL dilution of rBet v 1 and 0.1 µg/mL dilution of the hypoallergenic Bet v 1 derivatives were injected intradermally in order to obtain an initial weal, which was delineated. Reaction was considered positive if 10 min later the initial weal increased in size. In that case the next ID injections were performed with fourfold diluted solutions and the procedure was repeated until the reaction remained negative, i.e. initial weal did not increase in size or the most diluted solution was injected (0.00003 µg/mL for rBet v 1 and 0.0003 µg/mL for the hypoallergenic Bet v 1 derivatives). On the other hand, if initial dilutions gave negative tests, further injections were performed with fourfold higher concentrated solutions and the procedure was repeated until the reactions were positive or the highest concentrations were reached (2.56 µg/mL for rBet v 1 and 25.6 µg/mL for the hypoallergenic Bet v 1 derivatives).

Results

Clinical and serological characterization of the patients

Positive cutaneous tests to rBet v 1 wild-type were found in all patients. Twenty-three patients out of 29 (79%) were found monosensitized to Bet v 1 when tested by immunoblot (Fig. 1a). IgG₄ against Bet v 1 wild-type and fragments were preferentially detected in hyposensitized patients: six out of 10 tested hyposensitized as opposed to only one out of 21 tested nonhyposensitized patients had IgG₄ against Bet v 1 and rBet v 1 fragment 2 (Table 1, Fig. 1b). The mean IgE and IgG₄ levels against rBet v 1 and rBet v 1 fragments were always higher in the group of patients who had received immunotherapy (Table 1).

Recombinant hypoallergenic Bet v 1 derivatives have a reduced capacity to elicit skin prick reactions.

rBet v 1 monomer elicited comparable weal reactions to the

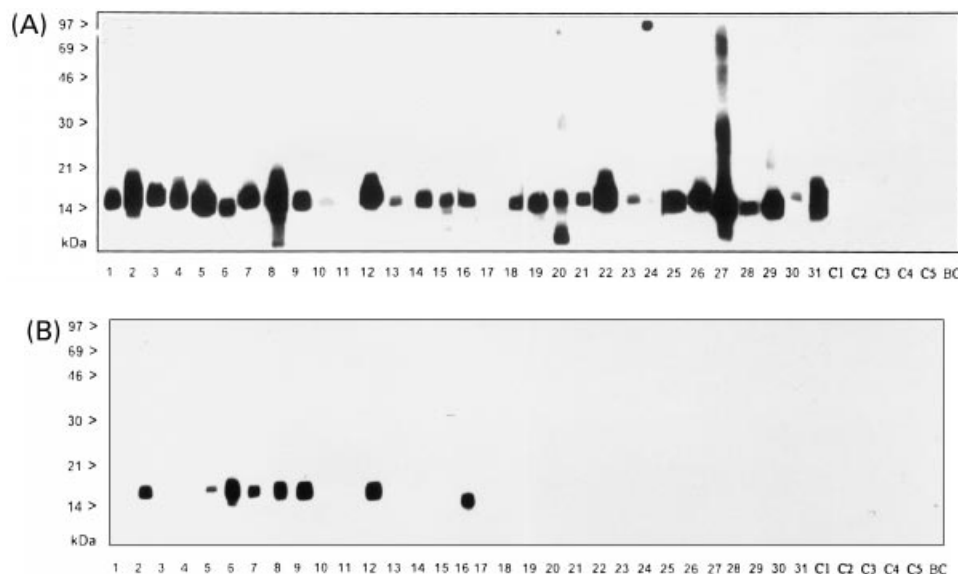


Fig. 1. IgE and IgG₄ reactivity to natural birch pollen allergen. Nitrocellulose-blotted birch pollen extract was exposed to serum IgE (a) or serum IgG₄ (b) of birch pollen allergic patients (lanes 1–31) and controls (C1–C5; BC: buffer). Bound IgE and IgG₄ antibodies were detected with ¹²⁵I-labelled antibodies. Molecular weights are displayed on the left margin in kDa.

natural commercial birch pollen extract at a concentration of 10 µg/mL in 21 patients and at a concentration of 50 µg/mL in the remaining eight patients. Neither the two atopics without birch pollen allergy, nor the eight nonatopic individuals displayed immediate-type skin reactions to rBet v 1 wild-type (data not shown). No patient reacted with the 10 µg/mL concentration of rBet v 1 dimer or trimer whereas three patients reacted with 10 µg/mL for fragment 1 and 4 with 10 µg/mL for fragment 2. Four patients did even not react to 1000 µg/mL of the trimer, so did also eight patients for fragment 1 and 2.

The comparison of skin prick reactivity of genetically modified rBet v 1 allergens with skin reactivity of rBet v 1 monomer was done in the form of a ratio of the lowest concentrations giving a positive skin prick test. As displayed in Fig. 2, the majority of the patients reacted 100- or more than 100-fold less to the engineered recombinant derivatives (25 out of 29 for fragment 1, 23 out of 29 for fragment 2, 25 out of 29 for dimer, 26 out of 29 for trimer).

The 2 rBet v 1 fragments and 2 oligomers did not induce weal or flare reactions up to a concentration of 1000 µg/mL in the control individuals (two atopics without birch pollen allergy and eight nonatopic individuals) (data not shown).

Results from the intradermal testing of rBet v 1 wild-type and hypoallergenic rBet v 1 derivatives

(a) Birch pollen-allergic patients exhibit different sensitivities to rBet v 1 wild-type and rBet v 1 derivatives. Overall, we observed a large intersubject variability regarding

the end-points of intradermal tests performed with rBet v 1 monomer, the lowest concentration of rBet v 1 resulting in a positive reaction was 3×10^{-5} µg/mL, whereas the highest concentration was 6×10^{-1} µg/mL. The sensitivities to rBet v 1 trimer ranged between 0.1 µg/mL and 25.6 µg/mL. The sensitivities to rBet v 1 fragment 1 ranged between 0.4 µg/mL and more than 25.6 µg/mL and for recombinant Bet v 1 fragment 2 between 0.1 µg/mL and 25.6 µg/mL. In conclusion, the broadest intersubject variability was observed for rBet v 1 wild-type.

Figure 3 (a-d) displays the number of patients reacting to different end point concentrations of rBet v 1 wildtype (Fig. 3a), rBet v 1 trimer (Fig. 3b), rBet v 1 fragment 1 (Fig. 3c) and rBet v 1 fragment 2 (Fig. 3d). Interestingly, positive reactions to very low concentrations of rBet v 1 wild-type and modified derivatives were preferentially observed when we tested during and after the *Fagales* pollen season as evidenced by local pollen counts.

(b) Birch pollen-allergic patients exhibit stronger immediate type skin reactivity to rBet v 1 wild-type compared to hypoallergenic derivatives during and after birch pollen season.

The comparison of intradermal reactivity of trimers and fragments with rBet v 1 monomer is displayed in Fig. 4 (a,b,c) as a ratio of their positive end-point concentrations. Regarding trimer, five patients had a ratio of 40 and 24 had a ratio of 160 or more; seven patients out of those 24 had a ratio of more than 2000 (Fig. 4a). In one patient, as the intradermal tests remained positive to the lowest

Table 1. Mean IgE and IgG4 responses to rBet v 1 and rBet v 1 fragments

No.	Age	Sex	Prick	IT	Date of ID test	Total IgE kUA/L	Specific IgE_OD Bet V1	IgG4_OD Bet V1	IgE WB	IgG4 WB
1	43	F	Yes	+	ND	51.9	0168	0257	+	–
2	44	F	Yes	+	30-Jan	69.2	0801	1	+	+
3	29	F	Yes	–	ND	599	0407	0	+	–
4	40	M	Yes	+	ND	321	0229	0511	+	–
5	29	F	Yes	+	30-Jan	727	0517	0307	+	+
6	48	F	Yes	+	ND	8.5	0	1445	+	+
7	39	M	Yes	+	09-Feb	53.1	0195	0,97	+	+
8	41	M	Yes	+	09-Feb	393	0,61	1416	+	+
9	37	M	Yes	+	06-Feb, 10-Jul	50	0097	1155	+	+
10	41	F	Yes	+	ND	7.9	0093	0	+	–
11	30	F	No	–	09-Feb	ND	ND	ND	ND	ND
12	36	M	Yes	–	12-Feb	55	0136	1294	+	+
13	26	F	Yes	–	16-Feb	428	0104	0	+	–
14	28	F	Yes	–	17-Feb	44.7	0166	0	+	–
15	19	F	Yes	–	23-Feb	416	0139		+	–
16	24	M	Yes	–	23-Feb	43.5	0	0	+	+
17	24	F	Yes	–	02-Mar	32.6	0	0	–	–
18	46	F	Yes	–	02-Mar	20	0		+	–
19	22	F	Yes	+	03-Mar	37.4	0161	0086	+	–
20	25	F	Yes	–	04-Mar	319	0,1	0	+	–
21	47	F	Yes	–	06-Mar	11	0,1	0	+	–
22	21	F	Yes	–	09-Mar	515	1208	0102	+	–
23	26	F	Yes	–	13-Mar	75.5	0085	0	+	–
24	26	M	Yes	–	16-Mar	62.5	0081	0	+	–
25	32	F	Yes	–	16-Mar	44.3	0212	0107	+	–
26	46	F	Yes	–	18-Mar	356	0332	0096	+	–
27	29	M	Yes	–	19-Mar	369	1031	0	+	–
28	23	F	Yes	–	23-Mar	<3	0	0	+	–
29	20	F	Yes	–	23-Mar	65	0528	0106	+	+
30	22	M	Yes	–	ND	ND	0082	0	+	–
31	24	F	No	–	10-Jul	1766	ND	ND	ND	ND
32	34	F	No	–	10-Jul	642	ND	ND	ND	ND
33	38	F	No	–	10-Jul	90	ND	ND	ND	ND
34	31	M	No	+	10-Jul	153	ND	ND	ND	ND
35	24	F	No	–	10-Jul	705	ND	ND	ND	ND
36	43	M	No	+	24-Aug	500	ND	ND	ND	ND
C1 (A)	21	M	Yes		ND	39.5	0276	0	–	–
C2 (A)	22	F	Yes		ND	39.2	0	0	–	–
C3 (NA)	52	F	Yes		ND	<3	0	0	–	–
C4 (NA)	46	F	Yes		ND	15.7	0	0	–	–
C5 (NA)	41	F	Yes		ND	26.5	0	0	–	–

concentration tested, the ratio could not be calculated. For fragment 1, all 22 patients had a ratio of 40 or more; in 13 among them it was of 10000 or more (Fig. 4b). Concerning fragment 2, two patients tested before pollen season had a ratio between 10 and 40. Remaining 20 patients had a ratio of 160 or more; in nine of these patients it was 10000-fold or more (Fig. 4c). The highest ratio of the positive end-point

concentrations of trimer and fragments with rBet v 1 was observed during or after the pollen season (Fig. 3).

(c) Hyposensitized and nonhyposensitized patients display comparable sensitivities to hypoallergenic rBet v 1 derivatives

Among 13 patients tested intradermally before pollen

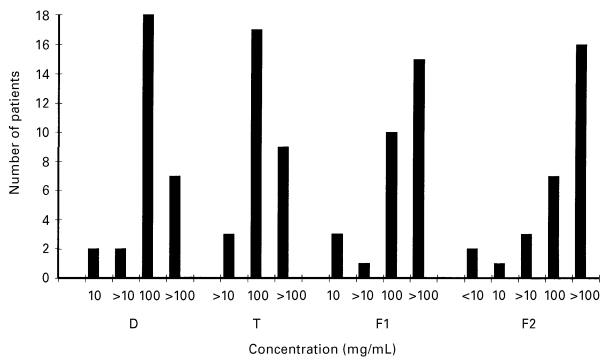


Fig. 2. Number of patients (ordinate) according to the ratio (abscissa) of positive skin prick test concentration of rBet v 1 dimer (D), trimer (T), fragment 1 (F1) and fragment 2 (F2) with rBet v 1 wild-type.

season, five were hyposensitized. End-point concentrations for all allergens tested did not differ substantially from those in nonhyposensitized patients during the same period. Only one patient, tested after the birch pollen season, was hyposensitized and had a high cutaneous reactivity ($3 \times 10^{-5} \mu\text{g/mL}$ for Bet v 1 monomer, $0.1 \mu\text{g/mL}$ for trimer, $>25.6 \mu\text{g/mL}$ for F1 and $6.4 \mu\text{g/mL}$ for F2).

(d) Lack of adverse reactions to genetically engineered hypoallergenic Bet v 1 derivatives

None of the patients had either immediate systemic reaction or delayed cutaneous reaction when observed 24 h later. By intracutaneous injections we could show that the majority of

patients tolerated cumulative doses of $5 \times 10^{-4} \mu\text{g}$ of rBet v 1 (11 patients), the highest dose injected was $0.298 \mu\text{g}$ in one patient. Much higher doses were tolerated for trimer ($>1.374 \mu\text{g}$ in seven patients) as well as for fragment 1 ($>1.364 \mu\text{g}$ in 18 patients) and fragment 2 ($>1.364 \mu\text{g}$ in 10 patients).

Discussion

The application of molecular biological techniques has not only allowed to produce recombinant allergens which closely mimic their natural counterparts for *in vitro* and *in vivo* diagnosis, but also to engineer hypoallergenic variants suitable for immunotherapy [25–27]. The purpose of our study was to compare the skin test reactivity of rBet v 1 (the major birch pollen allergen) with genetically engineered hypoallergenic Bet v 1 derivatives. We demonstrated that the majority of the patients tested had comparable reactivity to Bet v 1 monomer at concentrations more than 100-fold higher than rBet v 1 fragments and oligomers. The reduced allergenic activity of the rBet v 1 fragments can be attributed to their lack of IgE-binding capacity caused by disruption of the molecules conformation [21]. By contrast, the oligomeric forms of Bet v 1 (rBet v 1 dimer and trimer) retained IgE-binding capacity and fold but spectroscopical analysis indicated microaggregation which could lead to a reorientation of IgE epitopes and thus cause reduced effector cell activation (Vrtala and Valenta, unpublished data). Although, one has to consider that the absence of local

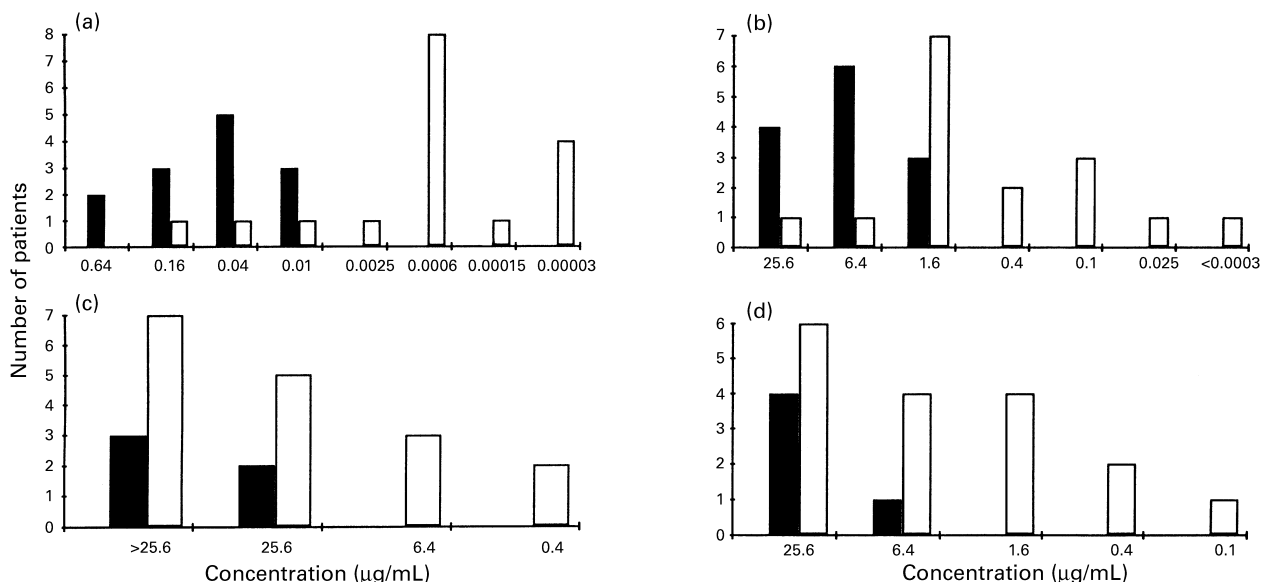


Fig. 3. Number of patients (ordinate) tested by intradermal tests using end-point concentration ($\mu\text{g/mL}$, abscissa), before (black columns) and during or after (white columns) the *Fagales* season. (a) = rBet v 1 wild-type (b) = rBet v 1 trimer (c) = rBet v 1 fragment 1 and (d) = rBet v 1 fragment 2.

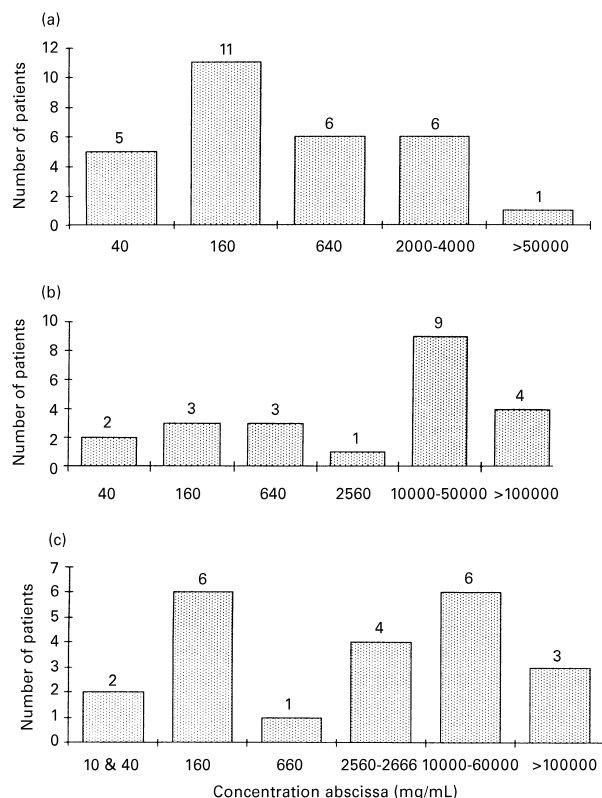


Fig. 4. Number of patients (ordinate) according to the ratio (abscissa) of positive end-point concentration of trimer (a), fragment 1 (b) and fragment 2 (c) with rBet v 1 wild-type.

reactions during intradermal testing cannot predict the absence of systemic effects during immunotherapy, we think that the skin prick test data support the assumption that the recombinant hypoallergenic Bet v 1 derivatives may represent safe candidate vaccines for the treatment of birch pollen allergic patients. Moreover, it is planned to prepare vaccines using either the hypoallergenic recombinant Bet v 1 fragments or the rBet v 1 trimer adsorbed to $\text{Al}(\text{OH})_3$ as adjuvant. The coupling of the recombinant allergen derivatives to an adjuvant will prevent spreading of the molecules and thus decrease the likelihood of systemic reactions.

In order to more precisely investigate (1) the capacity of these molecules to elicit immediate or late phase skin reactions after intradermal injection (2) the ratios of hypoallergenic derivatives eliciting immediate-type skin reactions comparable to the rBet v 1 wild-type and (3) the sensitivities before, during and after the birch pollen season, we performed intradermal end-point titration tests. We did not include Bet v 1 dimer in intradermal tests because it proved to have a higher skin test reactivity than Bet v 1 trimer in the skin prick test. The end-point titration method has been shown in previous studies to estimate allergenic activities more accurately and with good reproducibility and

it correlates with parallel line bioassay *in vivo* and RAST inhibition measurements [28]. Also by intradermal testing we were able to confirm the greatly reduced skin test reactivity of genetically engineered Bet v 1 fragments and trimers. Intradermal testing also showed a large interindividual variation of cutaneous sensitivities to rBet v 1 monomer in our study group. This is in accordance with a previous study using intradermal testing in 87 patients with allergic pollinosis in whom skin sensitivity to ragweed pollen extract varied from $1 \times 10^{-5} \mu\text{g/mL}$ to $1 \times 10^{-1} \mu\text{g/mL}$ [28]. The intersubject variation of the ratio between rBet v 1 wild-type and the rBet v 1 derivatives could be linked to higher cutaneous sensitivities to rBet v 1 in patients tested during or after the pollen season. Seasonal variation in skin reactivities was lower for trimer and fragments than for rBet v 1. Although the testing before and during or after the season could be done only in one patient, for reasons of the time consuming intradermal methods (average 15–20 intradermal tests per patient requiring 2–3 h), we believe that the increase of sensitivity to Bet v 1 monomer during and after season results from seasonal antigen contact. This contention was supported by our finding that after seasonal exposure, Bet v 1-specific IgE levels had increased in this patient and by previous studies showing increases of specific IgE antibody levels and cutaneous reactivities associated with seasonal exposure in pollen sensitive patients [29–31].

That hypoallergenic molecules can be injected intradermally at higher doses than rBet v 1 wild-type, even in the birch pollen season, allows us to hope that immunotherapy with the modified allergens can be performed with lower risk of anaphylactic side effects even during the peak season.

Although, the number of hyposensitized patients included in this study was small, it was interesting to note that preferentially, individuals who had received conventional immunotherapy with birch pollen extract before, contained IgG₄ antibodies to rBet v 1 monomer and also to recombinant Bet v 1 fragments. The latter may be due to the induction of antibody responses against denatured or fragmented Bet v 1 injected during treatment with birch pollen extracts. However, we noted no differences in the skin sensitivity of these patients compared to nonhyposensitized individuals. This finding indicates that a previous treatment with birch pollen extract will not induce sensitization towards the recombinant hypoallergenic Bet v 1 derivatives, which could mean that they can be used to treat even patients who had received unsuccessful conventional birch pollen extract-based immunotherapy.

In conclusion, our study shows that recombinant hypoallergenic Bet v 1 derivatives can be administered in high doses, which is known to favour the development of allergen-specific Th1 responses [32–34]. It is planned to prepare

vaccines which consist of the two rBet v 1 fragments or the rBet v 1 trimer adsorbed to Al(OH)₃ similar as in current extract-based formulations for injection immunotherapy. Future therapy studies will now have to be performed to evaluate safety and efficacy of the adsorbates and to elucidate the basic mechanisms of immunotherapy on a component based level.

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