

Hypoallergenic derivatives of the major birch pollen allergen Bet v 1 obtained by rational sequence reassembly

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Background: At least 100 million patients suffer from birch pollen allergy.

Objective: Rational design of recombinant derivatives of the major birch pollen allergen, Bet v 1, characterized by reduced IgE reactivity, preservation of sequences relevant for the induction of allergen-specific blocking IgG, and maintenance of T-cell epitopes for immunotherapy of birch pollen allergy.

Methods: Three recombinant mosaic proteins derived from Bet v 1 were generated by reassembly of codon-optimized genes coding for Bet v 1 fragments containing the elements for the induction of allergen-specific blocking IgG antibodies and the major T-cell epitopes. The proteins were expressed in *Escherichia coli* as recombinant mosaic molecules and compared with the Bet v 1 wild-type protein by chemical and structural methods, regarding IgE-binding and IgG-binding capacity, in basophil activation assays and tested for the *in vivo* induction of IgG responses.

Results: Three recombinant Bet v 1 (rBet v 1) mosaic proteins with strongly reduced IgE reactivity and allergenic activity were expressed and purified. Immunization with the recombinant hypoallergens induced IgG antibodies that inhibited IgE reactivity of patients with allergy to Bet v 1 comparable to those induced with the rBet v 1 wild-type allergen.

Conclusion: We report the generation and preclinical characterization of 3 hypoallergenic rBet v 1 derivatives with suitable properties for immunotherapy of birch pollen allergy. (J Allergy Clin Immunol 2010;■■■:■■■-■■■.)

Key words: Birch pollen allergy, immunotherapy, rBet v 1, recombinant hypoallergenic derivative molecules

Allergy to pollen from birch and related trees (eg, alder, hazel) is one of the most important allergies in Northern and Middle Europe, North America, and certain parts of Australia and Asia.¹ Recombinant Bet v 1 (rBet v 1) assembling the structural, immunologic, and allergenic properties of natural Bet v 1 has been expressed in *Escherichia coli*.²⁻⁶ In a recently reported double-blind, placebo-controlled immunotherapy trial, it was shown that rBet v 1 treatment was as effective as treatment with birch pollen extract or purified natural Bet v 1, demonstrating that rBet v 1 can replace a complex birch pollen extract.⁷ In attempts to minimize the risk of IgE-mediated side effects, several hypoallergenic derivatives of Bet v 1 have been made by recombinant technology.⁸ Two hypoallergenic rBet v 1 derivatives (ie, 2 rBet v 1 fragments, rBet v 1 trimer)^{9,10} have been tested for immunotherapy of patients with birch pollen allergy.¹¹ The clinical outcome of the study performed with the hypoallergenic Bet v 1 derivatives was less successful than the study conducted with rBet v 1, which may have been a result of factors related to the study design and the lack of a pollen season in one of the study centers.¹² However, patients treated with the recombinant hypoallergenic Bet v 1 derivatives tolerated more than a 5-fold increase in maintenance dose compared with the doses given in the rBet v 1 study and showed a blunting of the rises of the specific IgE responses caused by seasonal allergen exposure.¹¹ Moreover, there was a clear reduction of nasal symptoms in a subgroup of patients who had nasal provocation testing performed.¹³ Further clinical studies with a hypoallergenic derivative made by chemical denaturation of rBet v 1 yielded similar immunologic effects to the study performed with the recombinant hypoallergenic Bet v 1 derivatives and demonstrated clinically efficacy in phase III studies.¹⁴⁻¹⁶

We report the rational design, structural, immunologic, and preclinical characterization of 3 recombinant hypoallergenic Bet v 1 derivatives without chemical denaturation steps. These derivatives were prepared by reassembly of the complete Bet v 1 sequence within single molecules by using codon-optimized synthetic genes in which the sequences relevant for the induction of blocking IgG antibodies and the dominant T-cell epitopes were preserved.

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Abbreviations used

HSA: Human serum albumin
 PBS-T: PBS + 0.05% Tween 20
 rBet v 1: Recombinant Bet v 1

METHODS**Patients' sera, plasmids, and recombinant allergen**

Patients with birch pollen allergy were characterized by case history and positive skin prick testing. Serum IgE Abs specific to birch pollen extract and rBet v 1 were determined by ImmunoCAP measurements (Phadia, Uppsala, Sweden) as described³ (see this article's [Table E1](#) in the Online Repository at [www.jacionline.org](#)). Control sera were taken from 2 volunteers without allergy.

The plasmid pET 17b (Novagen Inc, Madison, Wis), used for the expression of rBet v 1 and of the rBet v 1 derivatives, is described.¹⁷ The recombinant *E coli*-expressed (BL 21-DE3) (Stratagene, La Jolla, Calif) birch pollen allergen Bet v 1 (batch 21) was obtained from Biomay (Vienna, Austria).

mAbs

Bip 1, a mAb with specificity for the major birch pollen allergen Bet v 1, was previously described.¹⁸ The mouse mAb 4A6 was raised against purified recombinant birch pollen profiling.¹⁹ Anti-IgE mAb E-124.2.8 was purchased from Immunotech (Marseille, France).

Mouse IgG mAbs against peptide 2 (mAb#2; aa 30-59) and against peptide 6 (mAb#12; aa 74-104) of Bet v 1 were obtained by immunization of mice by using Keyhole limpet haemocyanin-coupled synthetic peptides (peptide 2: LFPKVAPQAISSEVENIEGNGGPPTIKKISF; peptide 6: EDVHTNFKYNYN VIEGGPIGDTLEKISNEIK).

Construction of hypoallergenic Bet v 1 derivatives

On the basis of the Bet v 1 sequence,² synthetic genes were generated, giving rise to 3 different recombinant Bet v 1 derivatives: restructured Bet v 1 #1 (Bet v 1-rs1), composed of amino acids 75-160 + 1-74; restructured Bet v 1 #2 (Bet v 1-rs2), composed of amino acids 110-160 + 1-109; and Bet v 1 mosaic, composed of amino acids 110-160 + 60-109 + 1-59 ([Fig 1, A](#)).

The synthetic genes of each of the recombinant Bet v 1 derivatives were cloned into the pET17b (Novagen Inc) cloning vector via *NdeI* and *EcoRI* restriction sites. The correct sequence of the derivative molecules was confirmed by double-stranded DNA sequencing (Eurofins Medigenomix GmbH, Ebersberg, ATG Biosynthetics GmbH, Merzhausen, Germany).

Expression and purification of the recombinant hypoallergenic Bet v 1 derivatives

Batch fermentation of *E coli* BL 21 (DE3) transformed with pET-17b-Bet v 1-rs1, rBet v 1-rs2, or mosaic was carried out at 37°C in a 10-L fermenter (New Brunswick Scientific, Bioflow 3000, New Brunswick, NJ) in LB medium with the addition of 0.05% (vol/vol) glycerol, 0.25% (wt/vol) MgSO₄ × 7 H₂O, and 0.18% w/v Na₂HPO₄ × 2 H₂O for 8 hours at 37°C until a cell density (OD_{600nm}) of 0.4 to 0.6 was reached. Protein expression was induced by adding 0.5 mmol/L isopropyl-β-thiogalactopyranoside (Calbiochem, Merck, Darmstadt, Germany). Recombinant proteins were produced and characterized as described in this article's "Purification and characterization of recombinant hypoallergens" section in the Methods in the Online Repository at [www.jacionline.org](#).

Detection of IgG binding capacity of rBet v 1 and rBet v 1 derivatives

Purified rBet v 1 and rBet v 1 derivative molecules were tested for reactivity with specific antibodies. Five micrograms of each protein/slot was separated by SDS-PAGE²⁰ and blotted onto nitrocellulose.²¹ Nitrocellulose-blotted proteins were incubated with either a 1:2000 dilution of a rabbit anti-rBet v 1 or

the corresponding preimmune serum, or with a 1:1000 dilution of mouse monoclonal IgG antibodies. Bound IgG antibodies were detected with a 1:1000 dilution of ¹²⁵I-labeled goat antirabbit antibodies or with a 1:1000 dilution of ¹²⁵I-labeled goat antimouse antibodies (NEN Life Science Products, Inc, Boston, Mass) and visualized by autoradiography.⁹

IgE reactivity of dot-blotted rBet v 1 and Bet v 1 derivatives

Two-microliter aliquots containing 1 μg purified rBet v 1, each of the rBet v 1 derivatives, BSA, and human serum albumin (HSA; negative control proteins; Roth, Karlsruhe, Germany) were dotted onto nitrocellulose. Nitrocellulose strips were incubated with sera from 19 individuals with birch pollen allergy, 2 individuals without allergy, or buffer without addition of serum. Bound IgE antibodies were detected with a 1:20 dilution of ¹²⁵I-labeled antihuman IgE antibodies (RAST RIA; Demeditec Diagnostics, Kiel-Wellsee Germany). The presence of rBet v 1 and rBet v 1 derivatives on the nitrocellulose membrane was shown with rabbit anti-rBet v 1 antibodies, which were detected with 1:1000 dilution of ¹²⁵I-labeled donkey antirabbit antibodies (NEN Life Science Products).

Immunization of rabbits and determination of IgG antibody levels

Rabbits were immunized twice, at study day 0 and at day 28, with 200 μg purified rBet v 1, rBet v 1-rs1, rBet v 1-rs2, or rBet v 1-mosaic initially adsorbed to CFA and followed by booster injection using incomplete Freund's adjuvant, or with 100 μg of the proteins adsorbed to Al(OH)₃. Preimmune sera were obtained from the rabbits before immunization (Charles River Breeding Laboratories, Kisslegg, Germany).

ELISA plates (Greiner, Kremsmünster, Austria) were coated with rBet v 1, rBet v 1 derivatives, or BSA (negative control; 5 μg/mL diluted in PBS) at 4°C overnight. After washing 3 times with PBS + 0.05% Tween 20 (PBS-T) and blocking with 2% BSA (Roth, Karlsruhe, Germany) in PBS-T for 6 hours, plates were incubated with rabbit antisera in 5 different dilutions (PBS-T, 0.5 wt/vol % BSA; 1:1000, 1:5000, 1:10,000, 1:100,000, and 1:1,000,000) for antibodies generated with CFA-adsorbed proteins or in 8 different dilutions (1:500, 1:1000, 1:2000, 1:4000, 1:8000, 1:16,000, 1:32,000, and 1:64,000) for antibodies induced with Al(OH)₃-adsorbed proteins. Controls were performed with normal rabbit antibodies. Plates were washed 5 times with PBS-T, and bound rabbit IgG antibodies were detected with a 1:1000-diluted antirabbit IgG horseradish peroxidase-linked whole antibody from donkey (GE Healthcare, UK Limited, Chalfant, St Gilles) for 1 hour at 37°C and 4°C. After washing with PBS-T (5 times), the color development was performed by addition of staining solution 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (100 μL/well; Sigma-Aldrich, St Louis, Mo). The optical density was measured using an ELISA Reader (Dynatech, Denkendorf, Germany) at 405 nm.

Allergenic activity of allergen derivatives

The allergenic activity of allergen derivatives was compared with that of the Bet v 1 wild-type allergen using CD203c assays as described in this article's "Allergenic activity of allergen derivatives" section in the Methods in the Online Repository at [www.jacionline.org](#).

Inhibition of IgE binding of patients with allergies to Bet v 1 by IgG antibodies

The inhibition of IgE binding of patients with allergies to Bet v 1 by IgG antibodies was performed as described in this article's "ELISA competition assay" section in the Methods in the Online Repository at [www.jacionline.org](#).

RESULTS**Rational construction of hypoallergenic rBet v 1 derivatives**

It has been shown that 2 recombinant fragments of Bet v 1 composed of amino acids 1-74 and 75-160 preserved the Bet v

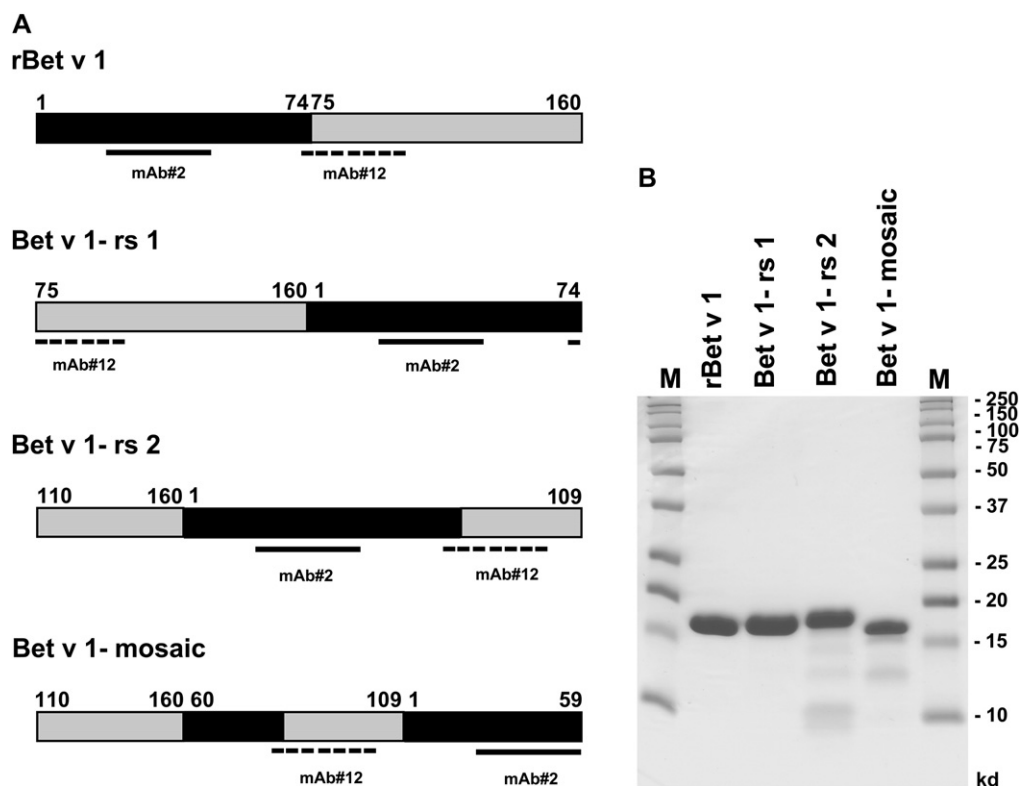


FIG 1. Hypoallergenic rBet v 1 derivatives. **A**, Construction scheme of the rBet v 1 derivatives. The amino acids at the borders of the protein segments and the binding sites of 2 mAbs, mAb#2 and mAb#12, are indicated. **B**, SDS-PAGE of purified rBet v 1 and rBet v 1 derivatives. *M*, Molecular mass markers (kd).

l-specific T-cell epitopes but exhibited an approximately 100-fold reduced allergenic activity compared with rBet v 1 as shown *in vitro* by basophil activation testing and in several *in vivo* provocation studies.^{9,22-25} Each of these fragments contains a peptide defined by 2 mAbs—mAb#2 (aa 30-59) and mAb#12 (aa 74-104)—that induced Bet v 1-specific IgG antibodies inhibiting the binding of IgE of patients with birch pollen allergy to Bet v 1²⁶ (Fig 1, A). The first Bet v 1 derivative, designated Bet v 1-rs1, was made by reassembling the hypoallergenic fragments aa 1-74 and aa 75-160 within 1 molecule as a tail-to-head construct as described for a Phl p 12 derivative²⁷ (Fig 1, A). The second Bet v 1 derivative, designated Bet v 1-rs2, was prepared by reassembling aa 1-109, which contains 3 peptides (P2, aa 30-59; P3, aa 50-79; and P6, aa 74-104) that had induced strong blocking IgG antibody responses against Bet v 1²⁶ at the C-terminus and a portion composed of aa 110-160 at the N-terminus. Because of the possibility that the fragment aa 1-109 might regain allergenic activity, it was broken into 2 pieces, aa 60-109 containing P6 and aa 1-59 containing P2, and a mosaic was constructed from these pieces. This mosaic, designated Bet v 1-mosaic, consists of Bet v 1 portions aa 110-160, aa 60-109, and aa 1-59 from the N-terminus to the C-terminus (Fig 1, A).

High-level expression, purification, and structural and immunochemical characterization of rBet v 1 derivatives

High-level expression of the recombinant proteins yielding more than 20% of the total *E coli* proteins was obtained. Each of

the recombinant proteins could be purified from the inclusion body fraction of the bacteria via several chromatography steps to more than 90% purity (Fig 1, B). When the proteins were analyzed by gel filtration after 22 and 23 months of storage at -20°C , rBet v 1 occurred as monomer (see this article's Fig E1, A, in the Online Repository at www.jacionline.org). Bet v 1-rs2 and Bet v 1-mosaic appeared as stable peaks, which may correspond to monomers with a higher hydrodynamic radius because of their unfolded condition or to dimers (Fig E1, B and C). Bet v 1-rs1 showed signs of degradation (Fig E1, D). The calculated mass for rBet v 1 and the 3 derivatives without methionine was (17439.6 Da), which corresponds with the Mass determined by matrix-assisted laser desorption/ionization time-of-flight analysis (ie, Bet v 1-rs1, 17420.3 D; Bet v 1-rs2, 17421.6 D; Bet v 1-mosaic, 17452.5 D; Bet v 1, 17381.6 D; data not shown). In contrast with rBet v 1, which exhibited the typical fold of a mixed α -helical and β -sheet-containing protein, all 3 rBet v 1 derivatives were unfolded in CD analysis (data not shown).

Next we used Bet v 1-specific antibody probes to test IgG reactivity of the Bet v 1 derivatives. Nitrocellulose-blotted rBet v 1 and rBet v 1 derivatives reacted with the polyclonal rabbit antibodies that had been raised against rBet v 1 (Fig 2, A). Interestingly, the mAb Bip 1, which recognizes conformational epitopes of Bet v 1, reacted with the folded rBet v 1 wild-type protein but not with the unfolded rBet v 1 derivatives (Fig 2, B). The mAbs specific for P2 (mAb#2; aa 30-59) and for P6 (mAb#12; aa 74-104) reacted with rBet v 1 and each of the 3 rBet v 1 derivatives (Fig 2, C and D). The rabbit preimmune serum and an isotype-

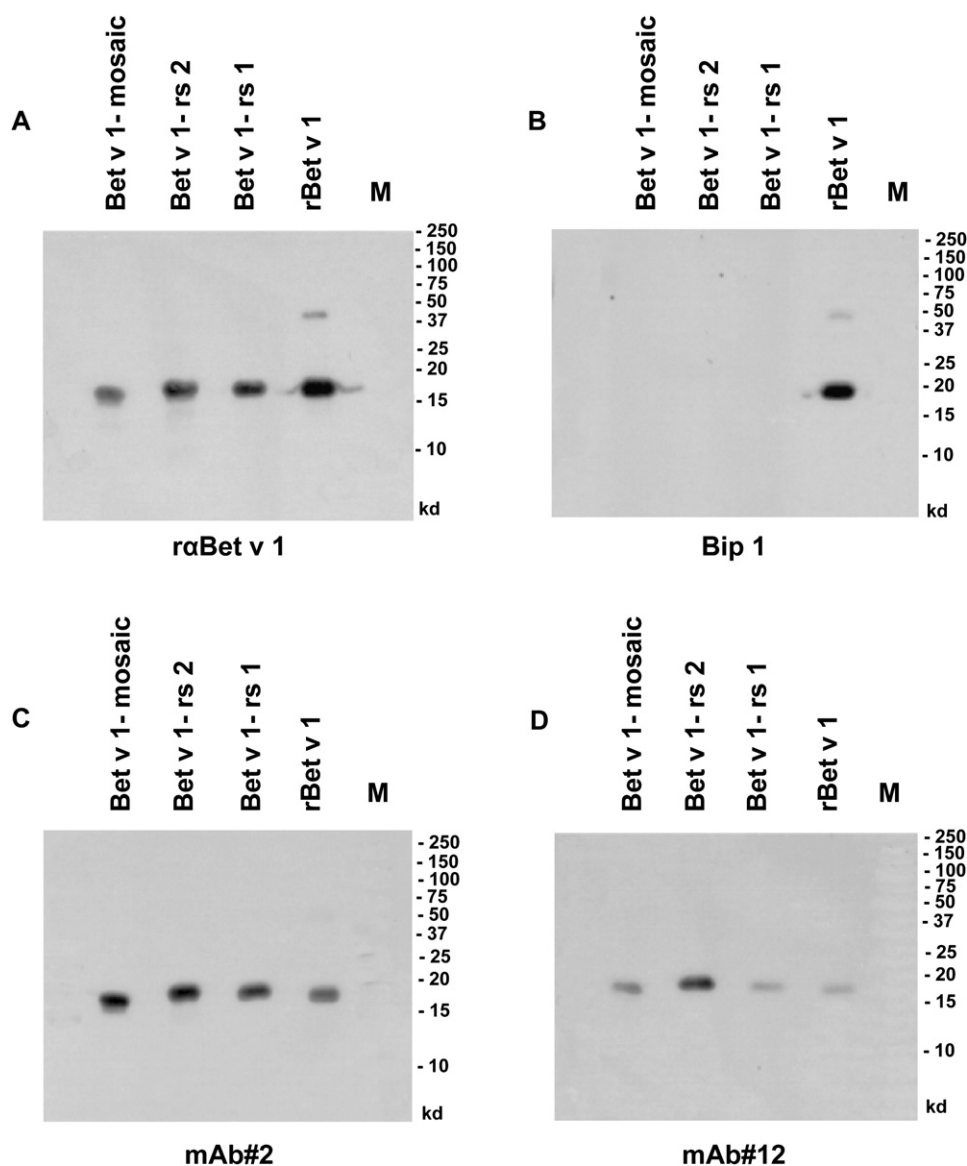


FIG 2. IgG reactivity of rBet v 1 and rBet v 1 derivatives with Bet v 1-specific antibodies. Nitrocellulose-blotted rBet v 1 and rBet v 1 derivatives were probed with rabbit anti-Bet v 1 antibodies (αBet v 1) (A), mAb Bip 1 (B), mAb#2 (C), or mAb#12 (D). Molecular weights are displayed in the *right margins* in kilodaltons. M, Marker.

matched mouse mAb without specificity for Bet v 1 did not show any binding (data not shown).

Comparison of the IgE reactivity of rBet v 1 and rBet v 1 derivatives

We found that none of the 19 patients with birch pollen allergy tested exhibited any detectable IgE reactivity to the rBet v 1 derivatives, whereas they showed IgE binding to rBet v 1 (Fig 3, 1-19). No IgE reactivity to the control proteins HSA and BSA was found. Serum IgE from individuals without allergy and buffer showed no reactivity to any of the proteins (Fig 3, 20-21, 0). The presence of rBet v 1 and rBet v 1 derivatives on the membrane was confirmed by testing with rabbit anti-rBet v 1 antibodies (Fig 3, 22).

Reduced allergenic activity of rBet v 1 derivatives

In a first set of experiments, we compared rBet v 1-rs1 with rBet v 1 and an equimolar mix of rBet v 1 fragments for their allergenic activity by using basophils from patients with birch pollen allergy. rBet v 1-rs1 did not cause any upregulation of the CD203c expression up to the maximum concentration (ie, 50 pmol/L) tested in the 6 patients, whereas rBet v 1 started to induce basophil activation at 0.5 pmol/L, and each of the patients responded to 50 pmol/L. rBet v 1-rs1 exhibited even lower allergenic activity than the rBet v 1 fragment mix that induced CD203c upregulation at 5 pmol/L in 1 patient and at 50 pmol/L in 2 of the 6 patients (Fig 4, A). We then compared the 3 rBet v 1 derivatives with rBet v 1 regarding allergenic activity by using basophils from additional 4 patients (Fig 4, B). In 3 patients, rBet v 1 derivatives did not induce any CD203c upregulation up to the maximum concentration

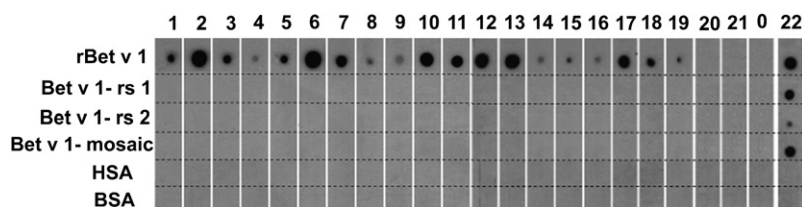


FIG 3. IgE reactivity of rBet v 1 and rBet v 1 derivatives. Nitrocellulose-dotted rBet v 1, rBet v 1-rs1, rBet v 1-rs2, rBet v 1-mosaic, HSA, and BSA were exposed to sera from patients with birch pollen allergy (1-19), individuals without allergy (20-21), buffer (0), or anti-rBet v 1 antibodies (22). Bound IgE and rabbit IgG antibodies were detected and visualized by autoradiography.

of 50 pmol/L, whereas rBet v 1 caused activation at 5 pmol/L. In the fourth patient, the rBet v 1 derivatives showed a 100-fold reduction of allergenic activity compared with rBet v 1 (Fig 4, B). This article's Fig E2 in the Online Repository at www.jacionline.org shows the reproducibility of the CD203c assay when performed in triplicate determinations in 3 additional patients with birch pollen allergy. It confirms the strongly reduced allergenic activity of the rBet v 1 derivatives.

Immunization with rBet v 1 derivatives induces IgG antibodies that recognize Bet v 1 and strongly inhibit patients' IgE binding to Bet v 1

As shown in this article's Fig E3 in the Online Repository at www.jacionline.org, rabbits immunized with the CFA-adsorbed rBet v 1 derivatives showed an almost comparable IgG response to rBet v 1 wild-type to the rabbit immunized with rBet v 1 (Fig E3, A). Rabbit anti-rBet v 1 antibodies reacted with the rBet v 1 derivatives, although somewhat less than the anti-rBet v 1 derivative antibodies (Fig E3, B-D). Immunization with Al(OH)₃-adsorbed rBet v 1 and rBet v 1-mosaic gave an almost comparable IgG response to rBet v 1 wild-type, whereas immunization with rBet v 1-rs1 and rBet v 1-rs2 resulted in lower titers of rBet v 1-specific antibodies (see this article's Fig E4 in the Online Repository at www.jacionline.org).

We then investigated whether rBet v 1 derivative-induced rabbit IgG antibodies can inhibit the binding of patients' serum IgE to the wild-type rBet v 1 in an ELISA competition assay. In case of immunization with CFA-adsorbed proteins, the anti-rBet v 1-rs1 antiserum inhibited the binding of IgE of patients with birch pollen allergy to rBet v 1 between 56.5% and 98% (85% mean inhibition). The anti-rBet v 1-rs2 antiserum showed inhibition rates between 59.5% and 98.5% (87% mean inhibition), and the anti-rBet v 1-mosaic inhibited between 58% and 99.5% (82% mean inhibition). Interestingly, the rabbit antiserum against rBet v 1 derivatives showed higher average inhibition rates than the anti-rBet v 1 antibodies, which yielded only 62% mean inhibition of IgE binding to rBet v 1 (Table I). Immunization with Al(OH)₃-adsorbed rBet v 1 derivatives resulted also in Bet v 1-specific IgG responses that blocked IgE binding of patients with allergy to Bet v 1 (Fig E4; see this article's Table E2 in the Online Repository at www.jacionline.org).

DISCUSSION

In this study, we constructed by rational molecular reassembly 3 recombinant hypoallergenic derivatives of the major birch pollen allergen, Bet v 1, for immunotherapy. The recombinant Bet v 1 fragments that have been reassembled as a tail-to-head construct for the first derivative, Bet v 1-rs1, have been evaluated

regarding their allergenic activity in several skin test and provocation test studies in patients with birch pollen allergy, showing a consistent reduction of allergenic activity.^{5,7,9,11,23,24} rBet v 1-rs1 also exhibited a strongly reduced allergenic activity. Similar results were obtained for the second reassembled molecule, although we maintained in Bet v 1-rs2 the portions necessary for the induction of IgG antibodies against the IgE binding sites of Bet v 1 in a large fragment composed of aa 1-109, which was preceded by a smaller portion composed of aa 110-160 containing the major T-cell epitope of Bet v 1.²⁸ The third derivative, designated Bet v 1-mosaic, was made similar to Bet v 1-rs2. The C-terminal portion aa 110-160 carrying the major T-cell epitope was placed at the N-terminus, but the portion aa 1-109 responsible for the induction of blocking antibodies was disrupted again to eliminate possible IgE reactivity to such a large fragment. The fragment aa 1-109 was therefore reassembled by 2 fragments containing 2 peptides that have been shown to induce blocking IgG responses.²⁶

All 3 rBet v 1 derivatives could be expressed at high levels in an *E coli* expression system and purified via several chromatography steps close to homogeneity. They were recognized by antibodies raised against those peptides that induced blocking antibody responses against Bet v 1. A mAb, Bip 1, which recognizes a conformational epitope on Bet v 1, did not react with the 3 derivatives, indicating that their 3-dimensional structure is altered compared with Bet v 1. The last assumption was confirmed by CD measurements demonstrating that the 3 derivatives were unfolded molecules.

The 3 derivatives had greatly reduced IgE reactivity as already observed for the unfolded rBet v 1 fragments and exhibited an even greater reduction of allergenic activity than a mix of the 2 fragments.⁹ They show important advantages over the previously characterized rBet v 1 fragments. They combine both fragments within 1 protein, which can be easily expressed in large amounts in *E coli*. Furthermore, their allergenic activity seems to be lower than that of the fragments.

Several *in vitro* results as well as immunotherapy studies indicate that blocking IgG antibodies are involved in protection against allergic immune responses. It has been shown that allergen-specific blocking IgG inhibits immediate and late-phase responses. Moreover, allergen-specific blocking IgG antibodies inhibit the boosts of IgE production induced by allergen contact.^{11,29}

A recent immunotherapy study performed with rBet v 1 demonstrated that the reduction of clinical symptoms and skin sensitivity was associated with the production of allergen-specific IgG responses.⁷

Therefore, immunization experiments with the 3 rBet v 1 derivatives were performed, and it was shown that the derivatives

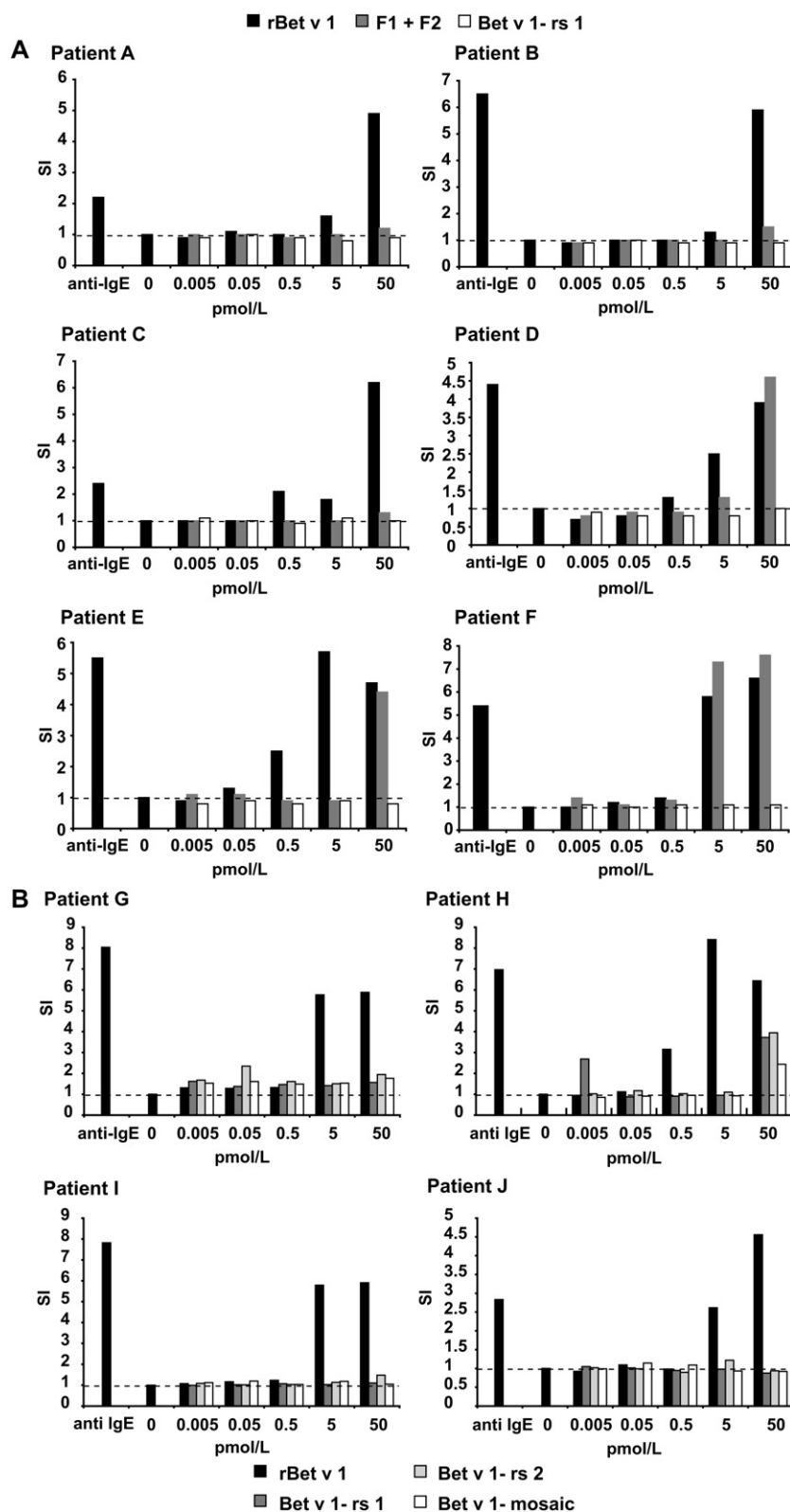


FIG 4. Allergenic activity of rBet v 1 and rBet v 1 derivatives as determined by CD203c upregulation. Blood samples from birch-allergic patients (A-F) were exposed to increasing concentrations (0.005 to 50 pmol/L) of rBet v 1, an equimolar mixture of rBet v 1 fragments (F1+F2) or rBet v 1-rs1 (x-axes; **A**), and in additional patients (G-J) with rBet v 1, rBet v 1-rs1, rBet v 1-rs2, and rBet v 1-mosaic (x-axes; **B**). Anti-IgE served as a positive control. The stimulation indices (SI; y-axes) reflect the upregulation of CD203c expression compared with buffer (0).

TABLE I. Inhibition of IgE binding of patients with allergy to rBet v 1 by IgG antibodies raised with CFA-adsorbed proteins

OD values	OD values		% Inhibition	OD values		% Inhibition	OD values		% Inhibition	OD values		% Inhibition
Patient	nrs	α Bet v 1	α Bet v 1	nrs	α Bet v 1-rs1	α Bet v 1-rs1	nrs	α Bet v 1-rs2	α Bet v 1-rs2	nrs	α Bet v 1-mosaic	α Bet v 1-mosaic
1	0.111	0.066	40.5	0.121	0.037	69.5	0.13	0.036	72	0.131	0.044	66.5
2	0.353	0.15	57.5	0.404	0.082	79.7	0.433	0.071	83.5	0.477	0.117	75.5
3	1.055	0.269	74.5	1.193	0.06	95	1.248	0.049	96	1.445	0.085	94
4	0.306	0.093	69.6	0.336	0.038	89	0.357	0.035	90	0.391	0.047	88
5	0.508	0.14	72.5	0.612	0.052	91.5	0.657	0.049	92.5	0.674	0.061	91
6	3.211	0.466	85.5	3.335	0.095	98	3.471	0.047	98.5	3.601	0.408	89
7	1.662	0.483	71	1.902	0.074	96	2.026	0.063	97	2.098	0.154	93
8	0.125	0.086	31	0.14	0.033	76.5	0.142	0.033	77	0.149	0.045	70
9	0.991	0.374	62	0.442	0.128	71	0.452	0.092	79.6	0.466	0.171	64
10	1.571	0.315	80	1.546	0.063	96	1.817	0.04	98	1.977	0.219	89
11	0.582	0.245	58	0.655	0.039	94	0.689	0.027	96	0.704	0.074	89.5
12	1.32	0.154	88.5	1.641	0.063	96	1.833	0.048	97	2.051	0.109	95
13	1.424	0.361	74.7	1.573	0.048	97	1.682	0.038	98	1.705	0.12	99.5
14	0.158	0.127	19.6	0.16	0.06	62.5	0.177	0.061	65.5	0.198	0.068	66
15	0.098	0.057	41.8	0.106	0.046	56.5	0.111	0.045	59.5	0.111	0.047	58
16	0.617	0.073	88	0.738	0.049	93	0.805	0.047	94	0.805	0.086	89
17	0.523	0.134	74.4	0.546	0.048	91	0.586	0.041	93	0.602	0.071	88
18	0.118	0.083	29.7	0.126	0.035	72	0.135	0.033	75.5	0.144	0.04	72
% Mean inhibition			62			85			87			82

nrs, Rabbit preimmune sera; *α Bet v 1*, rabbit IgG antibody raised against rBet v 1; *α Bet v 1-mosaic*, rabbit IgG antibody raised against rBet v 1-mosaic; *α Bet v 1-rs1*, rabbit IgG antibody raised against rBet v 1-rs1; *α Bet v 1-rs2*, rabbit IgG antibody raised against rBet v 1-rs2.

1-18, Patients with birch pollen allergy. OD values correspond to the amounts of bound IgE antibodies. Percentages (%) of inhibition of patients' IgE binding to rBet v 1 obtained with each antiserum versus the preimmune sera as well as the mean inhibition values are shown.

induced IgG responses comparable to the Bet v 1 wild-type allergen.

Moreover, we found that the IgG antibodies induced with each of the 3 rBet v 1 derivatives inhibited the binding of IgE of patients with birch pollen allergy to the Bet v 1 allergen similar to those induced with the folded rBet v 1 wild-type molecule. This result was unexpected, but it may be attributed to the fact that rBet v 1 derivatives contained those peptides that are involved in the induction of blocking antibodies.

We believe that the strategy of allergen fragmentation and consecutive systematic recombination in the form of mosaic molecules has advantages over the use of small hypoallergenic allergen fragments because small protein fragments may exhibit lower immunogenicity and induce lesser amounts of allergen-specific IgG antibodies. In addition, one reassembled molecule is more easy to produce and purify compared to several isolated fragments.⁸ We did not have the chance to compare the 3 rBet v 1 hypoallergens with the recently described rBet v 1 derivative obtained by the shuffling of DNA sequences from Bet v 1, Aln g 1, and Cor a 1, but it is likely that their allergenic activity is lower because the IgE reactivity of the shuffled constructs was reduced only 8-fold to 20-fold,³⁰ whereas we could not detect any IgE binding to the 3 derivatives in the patients we tested.

The 3 recombinant derivatives characterized here may also have several advantages over a hypoallergenic rBet v 1 folding variant that has been successfully used for immunotherapy of patients.^{14,16} The folding variant exhibited residual IgE reactivity and needed to be produced by chemical modification¹⁴ of the rBet v 1 molecules, whereas the hypoallergenic derivatives described here can be obtained directly by recombinant expression. The mosaic approach we used also seems to have the advantage of being generally applicable to allergenic molecules from different sources (eg, grass pollen).^{31,32} Whereas for immunotherapy of grass

pollen allergy, several molecules may be necessary, only 1 of the rBet v 1 derivatives is needed for clinical studies of efficacy.

In conclusion, the hypoallergenic Bet v 1 derivatives, and in particular Bet v 1-mosaic, described here may be considered promising candidate molecules for active vaccination but also for the induction of tolerance.^{33,34} Moreover, their genes may be useful for gene therapy and genetic immunization protocols^{35,36} because the resulting proteins exhibit reduced allergenic activity.

Clinical implications: Three recombinant hypoallergenic Bet v 1 derivatives were engineered as vaccines for the treatment of birch pollen and related allergies.

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METHODS

Purification and characterization of recombinant hypoallergens

Inclusion bodies were isolated from the cells using lysozyme (0.1 mg/g cell wet weight) (Sigma-Aldrich, St Louis, Mo) and repetitive freezing and thawing in buffer I (50 mmol/L TRIS base, 1 mmol/L EDTA, and 0.1% Triton X-100) for Bet v 1-rs1 and Bet v 1-mosaic, or buffer II (25 mmol/L NaH₂PO₄, pH 7.4, and 0.1% Triton X-100) for Bet v 1-rs2 (5 mL/g cell wet weight). NaCl was added to a final concentration of 200 mmol/L, and the suspensions were centrifuged (10,000g for 30 minutes at 4°C), leaving the protein-containing inclusion body in the pellet.

Bet v 1-rs1 and Bet v 1-rs2 pellets were washed with 1% Triton X-100, 2 mmol/L EDTA, 2 mmol/L β-mercaptoethanol, 20 mmol/L TRIS/HCl pH 8.0 (3 times), and afterward with 50% ethanol, 20 mmol/L TRIS/HCl pH 8.0 (2 times). The Bet v 1-mosaic pellet was washed once with 1% Triton X-100, 20 mmol/L NaH₂PO₄ pH 7.4 and once with 25% ethanol, 20 mmol/L NaH₂PO₄ pH 7.0. Inclusion bodies were suspended and stirred for 30 minutes in buffer A (6 mol/L urea, 10 mmol/L TRIS/HCl, 1 mmol/L EDTA, pH 7.0) in case of Bet v 1-rs1, or in buffer B (5 mol/L urea in 20 mmol/L sodium acetate buffer, pH 5.0) in case of Bet v 1-rs2 and mosaic. The suspensions were centrifuged (10,000g for 30 minutes at 4°C), and the final supernatant was used for purification.

Recombinant Bet v 1-rs1 was first purified by anion exchange chromatography on a Q-Sepharose FF column (GE Healthcare, UK Limited, Chalfant, St Gilles) by applying a linear gradient from 0 to 250 mmol/L NaCl in buffer A. Fractions containing Bet v 1-rs1 were dialyzed against buffer C (6 mol/L urea, 20 mmol/L NaH₂PO₄, 1.5 mol/L NaCl, pH 4.5) and subjected to hydrophobic interaction chromatography (HIC) by using a Phenyl Sepharose FF column (GE Healthcare, UK Limited) equilibrated with buffer C. Purified Bet v 1-rs1 was eluted with a linear gradient from 0% to 100% buffer D (6 mol/L urea, 20 mmol/L TRIS base, pH 9.3), and pure fractions were dialyzed first against 6 mol/L urea and then against 1 mmol/L acetic acid. Finally, the protein was subjected to 0.2-μm filtration and stored at -20°C.

Recombinant Bet v 1-rs2 and Bet v 1-mosaic were purified by cation exchange chromatography using a SP-Sepharose FF column (GE Healthcare, UK Limited) equilibrated with buffer B and eluted with a linear gradient from 0 to 400 mmol/L NaCl in the same buffer. To the fractions containing Bet v 1-rs2, NaCl was added to a final concentration of 1.7 mol/L. Protein was centrifuged (10,000g for 30 minutes at 4°C) and the supernatant subjected to HIC by using a Phenyl Sepharose FF column (GE Healthcare, UK Limited) equilibrated with buffer E (6 mol/L urea, 1.7 mol/L NaCl in a 20-mmol/L sodium acetate buffer, pH 5.0). Bet v 1-rs2 was then eluted with a linear gradient from 1.7 to 0 mol/L NaCl in buffer E, and fractions containing >90% pure rBet v 1-rs2 were dialyzed against buffer F (6 mol/L urea, 20 mmol/L NaH₂PO₄ pH 7.0) and subjected to anion exchange chromatography by using a Q-Sepharose FF column (GE Healthcare, UK Limited) equilibrated with the buffer F. To the fractions containing Bet v 1-mosaic, urea and NaCl were added to a final concentration of 6.5 mol/L and 3.5 mol/L, respectively. The protein was centrifuged (10,000g for 30 minutes at 4°C) and the supernatant subjected to HIC by using a Phenyl Sepharose FF column (GE Healthcare, UK Limited), equilibrated buffer G (6.5 mol/L urea, 3.5 mol/L NaCl in a 20-mmol/L sodium acetate buffer, pH 5.0). Bet v 1-mosaic was eluted with a linear gradient from 3.5 to 0 mol/L NaCl in buffer G.

Finally, Bet v 1-rs2 or Bet v 1-mosaic fractions containing >90% purity were pooled, dialyzed against 5 mmol/L NaH₂PO₄ pH 7.4, subjected to 0.2-μm filtration, and stored at -20°C.

Purified rBet v 1 and each of the rBet v 1 derivatives (5 μg protein/slot) were resolved on 12.5% sodium SDS-PAGE in the presence or absence of 2-mercaptoethanol.^{E1} Proteins were visualized by staining with Coomassie brilliant blue. The presence of endotoxin was determined in a limulus amoebocyte lysate chromogenic assay (QCL-1000 Chromogenic Limulus Amoebocyte Lysate Endpoint Assay; Bio-Whittaker, Walkersville, Md).

Laser desorption mass spectra of the proteins were acquired in a linear mode with a matrix-assisted laser desorption/ionization time-of-flight instrument (Microflex; Bruker, Billerica, Mass). Samples were dissolved in 10% acetonitrile (0.1% trifluoroacetic acid), and *sinapinic acid* (dissolved in 60% acetonitrile, 0.1% trifluoroacetic acid) was used as a matrix. For sample

preparation, a 1:1 mixture of protein and matrix solution was deposited onto the target and air-dried.

CD spectra were acquired on a JASCO (Tokyo, Japan) J-810 spectropolarimeter. CD measurements were performed with purified rBet v 1 and rBet v 1 derivatives at room temperature at protein concentrations of 0.1 mg/mL by using a rectangular quartz cuvette with a 0.1-cm path length. Far UV spectra were recorded in the wavelength ranges between 190 and 260 nm with a resolution of 0.5 nm at a scan speed of 50 nm/min and resulted from averaging of 3 measurements. The final spectra were baseline-corrected, and results were expressed as the mean residue ellipticity (Θ) at a given wavelength. The secondary structure content of rBet v 1 and Bet v 1 derivative molecules was calculated by using the secondary structure estimation program CDSSTR.^{E2}

For gel filtration, rBet v 1 derivatives that had been stored for 22 months at -20°C were loaded onto a Superdex 200 5/150 GL column (GE Healthcare, Uppsala, Sweden), equilibrated with 10 mmol/L sodium phosphate buffer, pH 7.4, containing 150 mmol/L NaCl. Bovine γ-globulin (158 kd), chicken ovalbumin (44 kd), and horse myoglobin (17 kd) from Gel Filtration Standard (BIO-RAD, Richmond, Calif) were used for calibration. Recombinant Bet v 1 that also had been stored for 22 months at -20°C was applied to the column under the same conditions to show the elution characteristics of the wild-type protein. The flow rate was 0.3 mL/min. The molecular masses were calculated by using linear regression of the logarithm of molecular mass versus elution volumes derived from UV measurements at 280 nm. Recombinant Bet v 1 derivatives were reapplied to gel filtration with the Superdex 200 5/150 GL column to check the stability of the proteins after further storage at -20°C.

Allergenic activity of allergen derivatives

Heparinized peripheral blood samples were obtained from individuals with birch pollen allergy after informed consent was given. Blood aliquots (100 μL) from 6 patients were incubated (unique) with serial dilutions (0.005-50 pmol/L) of rBet v1, an equimolar mix of the rBet v 1 fragments (F1 + F2), or rBet v 1-rs1 for 15 minutes at 37°C. Blood aliquots (100 μL) from 4 additional patients were incubated (unique) with serial dilutions (0.005-50 pmol/L) of rBet v1, rBet v 1-rs1, rBet v 1-rs2, or rBet v 1-mosaic as described. A monoclonal anti-IgE antibody E-124.2.8 (1 μg/mL; Immunotech, Marseille, France) and PBS (control buffer) were used as controls. Thereafter, samples were washed in PBS containing 20 mmol/L EDTA (Gibco, Carlsbad, Calif), and cells were incubated with 10 μL phycoerythrin-conjugated CD203c mAb 97A6 (Immunotech) for 15 minutes at room temperature. After erythrocyte lysis using FACS Lysing Solution (Becton Dickinson Biosciences, San Jose, Calif), cells were washed and resuspended in PBS and then analyzed by 2-color flow cytometry on a FACSScan (Becton Dickinson Biosciences) using Flowjo Software (Tree Star Inc, Ashland, Ore). Anti-IgE-induced upregulation of CD203c was calculated from mean fluorescence intensities (MFIs) obtained with stimulated (MFI_{stim}) and unstimulated (MFI_{control}) cells and is expressed as stimulation index (MFI_{stim}:MFI_{control}).^{E3} Three patients (Fig E2) were tested in triplicate to analyze the reproducibility of the assay. For those patients, results are shown as mean values of triplicate determinations with SDs.

ELISA competition assay

To analyze the ability of rBet v 1 derivatives-induced rabbit IgG to inhibit the binding of IgE of patients with birch pollen allergy to rBet v 1, an ELISA competition assay was performed. ELISA plates (Greiner, Kremsmünster, Austria) were coated with 100 μL rBet v 1 (5 μg/mL diluted in PBS) overnight at 4°C. Plates were blocked with 2% BSA (Roth, Karlsruhe, Germany) in PBS-T for 6 hours at 4°C and then preincubated overnight at 4°C with 1:50 dilutions (in PBS 0.5% BSA/0.05% Tween) of the rabbit sera anti-rBet v 1, anti-rBet v 1-rs1, anti-rBet v 1-rs2, or anti-rBet v 1-mosaic raised with CFA, or 1:10 dilutions for rabbit sera raised with Al(OH)₃, and for control purposes with the corresponding rabbit preimmune sera. Plates were washed 3 times with PBS-T and incubated with 1:10 diluted sera from 18 patients with birch pollen allergy sensitized to Bet v 1. Bound human IgE antibodies were detected by using a 1:2500 diluted alkaline phosphatase-conjugated mouse monoclonal antihuman IgE antibody (BD Pharmingen, San Diego, Calif). Color development was performed by addition of staining solution 2,2'-azino-bis (3-

ethylbenzthiazoline-6-sulfonic acid) diammonium salt (100 μ L/well; Sigma-Aldrich, St Louis, Mo), and the OD was measured in an ELISA Reader (Dynatech, Denkendorf, Germany) at 405 nm. The percentage of inhibition of IgE binding was calculated using the OD values obtained, as follows:

$$\% \text{ inhibition of IgE binding} = 100 - (OD_s/OD_p) \times 100,$$

where OD_s is the extinction coefficient after preincubation with the rabbit serum, and OD_p is the extinction coefficient after preincubation with the preimmune serum.

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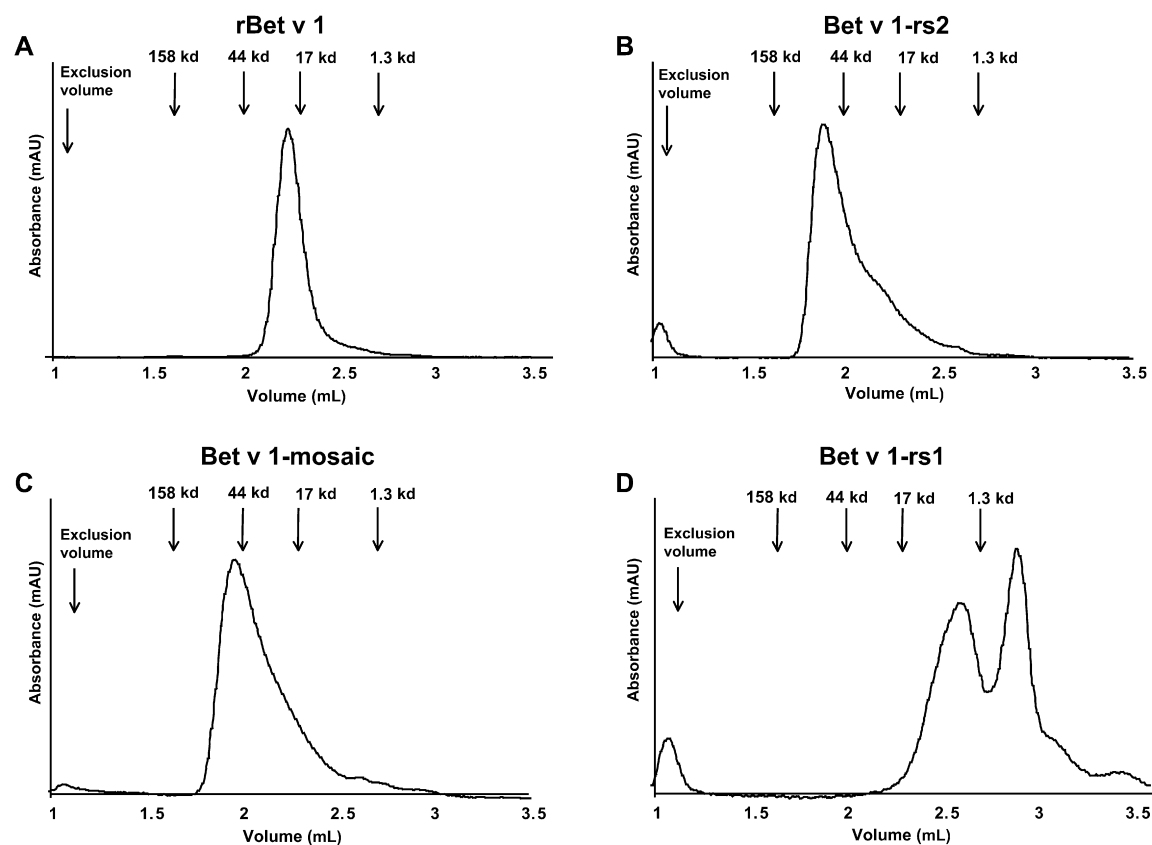


FIG E1. Gel filtration profiles of rBet v 1 (**A**), Bet v 1-rs2 (**B**), Bet v 1-mosaic (**C**), and Bet v 1-rs1 (**D**). The x-axes show the elution volumes (mL), and the y-axes the adsorbance of the proteins at 280 nm. Arrows indicate the approximate molecular masses (kd) as determined with the standards.

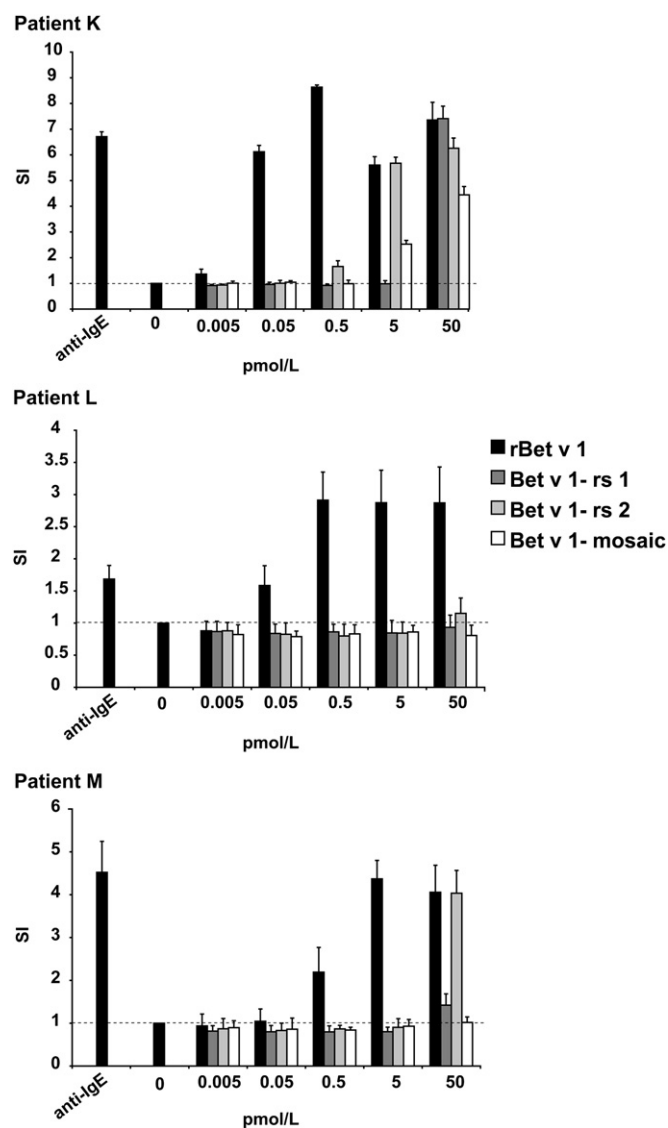
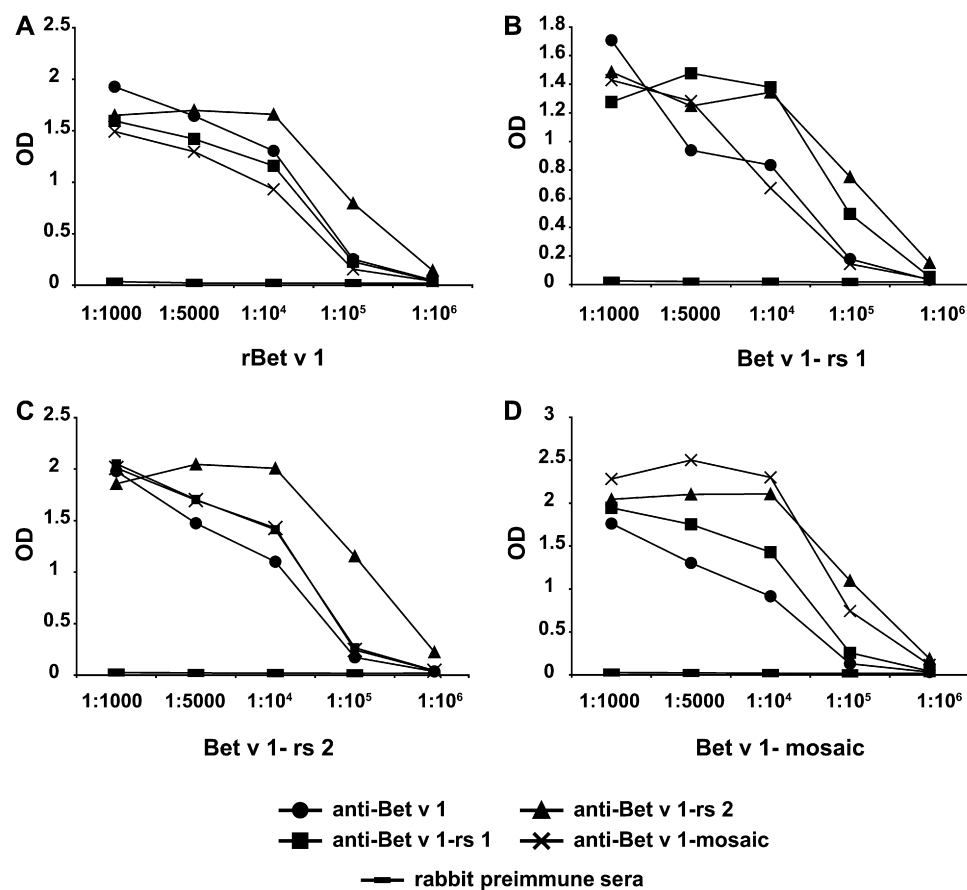


FIG E2. Allergenic activity of rBet v 1 and rBet v 1 derivatives as determined by CD203c upregulation. Blood samples from 3 patients with birch allergy (K-M) were exposed to increasing concentrations (0.005-50 pmol/L) of rBet v 1, rBet v 1-rs1, rBet v 1-rs2, and rBet v 1-mosaic (x-axes). Anti-IgE served as a positive control. The mean stimulation indices (SIs; y-axes) reflect the upregulation of CD203c expression compared with buffer (0). SDs are indicated.



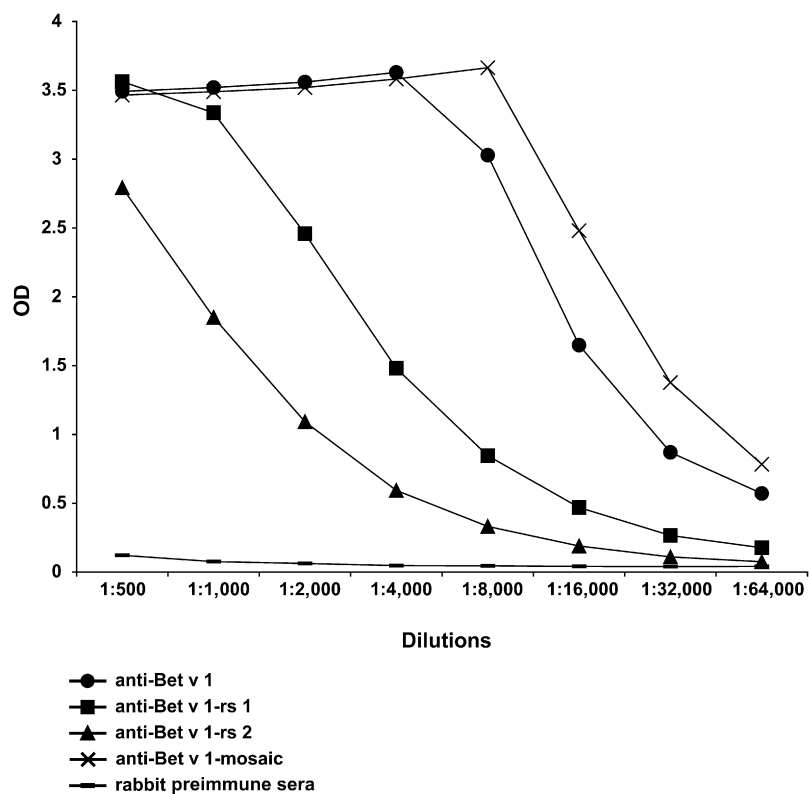


FIG E4. Reactivity of rabbit antibodies raised with aluminum hydroxide-adsorbed proteins by ELISA. Different dilutions (x-axes) of rabbit anti-rBet v 1 (anti-Bet v 1: ●), anti-rBet v 1—derivative antibodies (anti-Bet v 1-rs1: ■; anti-Bet v 1-rs2: ▲ anti-Bet v 1-mosaic: ×) or antibodies from a normal rabbit (—) reacted with rBet v 1. OD values (y-axes) correspond to the amounts of bound antibodies.

TABLE E1. Demographic, serologic, and clinical characterization of the individuals

Subjects with allergy	Sex/age (y)	Birch pollen-related symptoms	Type of treatment	IgE CAP (kUA/L)		Total IgE	Other allergies
				rBet v 1	(kU/L)		
1	M/56	RC, AD, AS, OAS	AH, TCt	15.7	14.6	860	a, g, pf, npf, mo, mi
2	M/44	RC, AD	AH	27.4	27.1	69.3	pf
3 (C)	M/28	RC, AD	AH, TCt, SIT	76.4	16.84	>5,000	a, g, pf, npf, mo, mi, w
4 (H)	M/27	RC, AD	AH, TCt	15.4	10.1	281	a, pf, mi
5	M/47	RC, AD, OAS	no	25.6	20.2	114	a, g, pf, mi
6	M/38	RC, AD	AH, TCt	>200	>200	>10,000	a, g, pf, npf, mo, mi, w
7	F/46	RC, AD	TCt	>100	26	>5,000	a, g, pf, npf, mo, mi, w
8	M	RC, AD	AH, TCt, UV	12	13.66	3,674	a, g, pf, npf, mi, w
9	F/42	RC, AD, AS	AH, SIT	145.4	47.8	>10,000	a, g, pf, npf, mi, w
10 (F)	M/29	RC	AH	98.1	>100	760	a, g, pf, npf, mo, mi, w
11	F	RC, AD	no	170.6	85.2	6,192	a, g, pf, npf, mo, mi, w
12	F/46	RC, AS, OAS	no	57.5	60.5	466	a, g, pf, mi
13 (A)	F/30	RC, OAS	AH	>100	>100	>5,000	a, g, pf, npf, mo, mi, w
14 (B)	M/29	RC	no	12.4	10.4	285	pf
15 (E)	M/42	RC	no	1.01	1	36.1	mi
16 (D)	F/30	RC	no	4.87	3.41	144	a, g, pf, npf, mi
17	M/47	RC, OAS	no	37.1	28.7	144	a, g, pf, mo, w
18	M/52	RC	AH, BD, TCt	36.9	36	115	pf
19	M/51	RC	AH, TCt	8.45	7.41	29.7	g, pf, mi
(G)	M/32	RC	AH, SIT	6.61	3.88	102	a, g, pf, npf, mi,mo, w
(I)	M/23	RC	AH	24.8	21.7	233	a, g, pf, npf, mo, mi, w
(J)	F/30	RC, AD,AS, OAS	AH, BD	58.7	47	4,886	a, pf, mo, w
Subjects without allergy							
20	F/28	no symptoms	no	<0.35	<0.35	<2.00	0
21	F/34	no symptoms	no	<0.35	<0.35	2.65	0

a, Animals; A-J, subjects studied by CD203c expression experiments; AD, atopic dermatitis; AH, antihistamines; AS, asthma; BD, bronchodilator; F, female; g, grass; M, male; mi, mites; mo, molds; no, no therapy at the time of analysis; npf, nonplant-derived food; OAS, oral allergy syndrome; pf, plant food; RC, rhinoconjunctivitis; SIT, specific immunotherapy; TCt, topical corticosteroids; UV, UV light therapy; w, weeds; 0, no allergy.

TABLE E2. Inhibition of IgE binding of patients with allergy to rBet v 1 by IgG antibodies raised with Al(OH)₃-adsorbed proteins

Patient	OD values		% Inhibition	OD values		% Inhibition	OD values		% Inhibition	OD values		% Inhibition
	nrs	αBet v 1		nrs	αBet v 1-rs1		nrs	αBet v 1-rs2		nrs	αBet v 1-mosaic	
1	0.471	0.268	43.1	0.536	0.241	55	0.529	0.359	32.1	0.553	0.07	87.3
2	0.153	0.132	13.7	0.142	0.128	9.8	0.141	0.126	10.6	0.86	0.808	6
3	1.674	0.535	68	1.874	0.415	77.8	1.885	1.043	44.7	2.128	0.102	95.2
4	0.821	0.218	73.5	0.926	0.189	79.6	0.924	0.497	46.2	0.95	0.073	92.3
5	0.769	0.269	65	0.933	0.211	77.4	0.932	0.798	14.4	0.953	0.087	90.9
6	3.448	1.426	58.6	3.447	2.393	30.6	0.091	0.065	28.6	3.42	0.143	95.9
7	0.739	0.348	53	0.794	0.259	67.4	0.813	0.477	41.3	0.098	0.054	44.9
8	0.274	0.191	30.1	0.291	0.118	59.4	0.298	0.199	33.2	0.317	0.052	83.6
9	0.088	0.063	28.4	1.171	0.667	43	1.12	0.94	16.1	1.153	0.329	71.5
10	2.676	0.774	71.1	2.906	0.903	68.9	3.125	2.088	33.2	3.187	0.115	96.4
11	1.39	0.694	50.1	1.51	0.44	70.9	1.535	0.889	42.1	1.587	0.05	96.8
12	3.629	0.348	90.4	3.619	1.026	71.6	3.63	1.985	45.3	3.618	0.11	96.9
13	2.566	0.675	73.7	2.876	0.7	75.7	2.949	1.582	46.3	2.929	0.141	95.2
14	0.218	0.097	55.5	0.234	0.065	72.2	0.342	0.126	63.1	0.237	0.043	81.8
15	0.302	0.145	52	0.31	0.114	63.2	0.324	0.178	45.1	0.331	0.107	67.7
16	1.397	0.183	87	1.529	0.415	72.8	1.49	0.833	44.1	1.583	0.142	91
17	1.02	0.289	71.6	1.113	0.308	72.3	1.157	0.665	42.5	1.155	0.095	91.8
18	0.306	0.137	55.2	0.329	0.1	69.6	0.318	0.182	42.8	0.332	0.096	71.1
% Mean inhibition			58			59			37			81

nrs, Rabbit preimmune sera; *αBet v 1*, rabbit IgG antibody raised against rBet v 1; *αBet v 1-mosaic*, rabbit IgG antibody raised against rBet v 1-mosaic; *αBet v 1-rs1*, rabbit IgG antibody raised against rBet v 1-rs1; *αBet v 1-rs2*, rabbit IgG antibody raised against rBet v 1-rs2.

1-18, Patients with birch pollen allergy. OD values correspond to the amounts of bound IgE antibodies. Percentages (%) of inhibition of patients' IgE binding to rBet v 1 obtained with each antiserum versus the preimmune sera as well as the mean inhibition values are shown.