

Allergy Vaccine Engineering: Epitope Modulation of Recombinant Bet v 1 Reduces IgE Binding but Retains Protein Folding Pattern for Induction of Protective Blocking-Antibody Responses¹

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Human type 1 immediate allergic response symptoms are caused by mediator release from basophils and mast cells. This event is triggered by allergens aggregating preformed IgE Abs bound to the high-affinity receptor (FcεRI) on these cells. Thus, the allergen/IgE interaction is crucial for the cascade leading to the allergic and anaphylactic response. Two genetically engineered forms of the white birch pollen major allergen Bet v 1 with point mutations directed at molecular surfaces have been characterized. Four and nine point mutations led to a significant reduction of the binding to human serum IgE, suggesting a mutation-induced distortion of IgE-binding B cell epitopes. In addition, the mutated allergens showed a decrease in anaphylactic potential, because histamine release from human basophils was significantly reduced. Retained α -carbon backbone folding pattern of the mutated allergens was indicated by x-ray diffraction analysis and circular dichroism spectroscopy. The rBet v 1 mutants were able to induce proliferation of T cell lines derived from birch pollen allergic patients. The stimulation indices were similar to the indices of nonmutated rBet v 1 and natural Bet v 1 purified from birch pollen. The ability of anti-rBet v 1 mutant specific mouse IgG serum to block binding of human serum IgE to rBet v 1 demonstrates that the engineered rBet v 1 mutants are able to induce Abs reactive with nonmodified Bet v 1. rBet v 1 mutants may constitute vaccine candidates with improved efficacy/safety profiles for safer allergy vaccination. *The Journal of Immunology*, 2004, 173: 5258–5267.

Specific allergy vaccination (SAV),⁵ i.e., allergen-specific immunotherapy, is an effective and well-tolerated treatment for allergic disease in selected patients. Controlled studies have shown efficacy in patients with allergic rhinitis/conjunctivitis, allergic asthma, and allergic reactions from stinging insects (1, 2). Effective treatment is correlated with the use of standardized vaccines in maintenance doses containing 5–20 μ g of major allergen for 3–5 years (3). The effect of allergy vaccination lasts at least several years after discontinuing treatment (4–7) and prevents the development of hay fever into asthma in children (8), and thus, allergy vaccination is currently the only treatment to alter the natural course of allergic disease.

Several studies addressing the mechanisms of allergy vaccination have shown effects on both innate and acquired immunological parameters (9, 10). The most pronounced serological marker of successful vaccination is a 30- to 100-fold increase in allergen-specific IgG (11, 12), whereas there is no substantial change in IgE comparing pre- and posttreatment (6). Furthermore, as shown for pollen allergy, the treatment gradually attenuates the seasonal rise in IgE (11) and leads to a seasonal rise in IgG, particularly IgG₄ (13).

There is general agreement (10, 14) that CD4⁺ T cells are involved in the regulation of allergic immune responses and the changes induced by SAV, but some controversy as to the particular effects of allergy vaccination on T cells. Studies based on T cells derived from peripheral blood show ambiguous results; some studies find a reduction in the production of Th2 cytokines combined with a reduction in allergen-specific stimulation (15), i.e., tolerance induction, whereas other studies find a reduction of the Th2 phenotype, but not a reduction in stimulation (16). Other studies demonstrate a reduction in Th2 cytokine production accompanied by an increase in Th1 cytokine production (17, 18), i.e., immune deviation, whereas yet other studies fail to demonstrate a reduction of the Th2 phenotype in PBMC (13, 19). Recently, a mechanism based on the induction of allergen specific CD4⁺CD25⁺ regulatory T cells has been proposed, a mechanism resulting in the production of IL-10 and TGF- β and a down-regulation of Th1 as well as Th2 allergen-specific T cell responses (20). The ambiguity of these observations may be due to the fact that the active cells of the allergic immune response are physically located in the airway mucosa, and that changes in T cell phenotypes are not properly reflected in the circulation (19).

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⁵ Abbreviations used in this paper: SAV, specific allergy vaccination; RLU, relative light unit; n, natural; 3-D, three-dimensional; R.S.E., relative solvent exposure; r.m.s.d., root mean square deviation; CD, circular dichroism.

The substantial rise in IgG indicates that the stimulation of a new allergen-specific immune response is an important aspect of the mechanism, because the sequential nature of Ig gene recombination prevents cells that have already recombined and become IgE producing to switch back to IgG. Histological studies of nasal biopsies lend support to the idea of a new immune response by demonstrating an increase in IFN- γ -producing cells, but no change in IL-5-producing cells, following SAV (19, 21).

A new allergen-specific immune response could exert beneficial effects at several levels. First, introduction of IFN- γ -producing cells into the mucosal tissue infested with a Th2-driven inflammation would counterbalance the allergic immune response leading to a reduction in the persistent inflammation (19). Second, allergen-specific IgG Abs would interfere with allergen-IgE interactions. A direct reduction in allergen-IgE interaction by competitive inhibition has been demonstrated (22). Furthermore, Ag presentation facilitated by the interaction of allergen-IgE complexes with the low-affinity IgE receptor (CD23) on B cells is reduced (23). Third, IgG may reduce histamine release from basophils by negative regulation of the Fc ϵ RI receptor signal transduction through ligation of the Fc γ RII receptor (24). Based on these observations, it may be suggested that the induction of a new allergen-specific immune response coexisting in parallel with the allergic immune response account for many of the immunological effects observed as a consequence of SAV.

Many approaches for the improvement of vaccines for SAV based on genetic engineering have been proposed (for review, see Ref. 25). Most of these approaches aim at a disruption of the tertiary structure of the allergen, leading to a reduction or elimination of IgE binding for the purpose of generating a nonanaphylactic vaccine that can alter the phenotype of allergen-specific T cells. However, such vaccines may not have the capacity to induce IgG Abs reactive with the natural allergen. According to the concept presented in this study, amino acid point mutations are introduced on the molecular surface of the allergen while not disturbing the overall folding pattern of the molecule (26). The idea is to manipulate surface topography in selected areas, thereby reducing IgE binding, while other surface areas are maintained for the capacity of the vaccine to stimulate an allergen-specific non-IgE Ab response. In contrast to the other approaches mentioned above, this concept enables well-defined molecules, i.e., potential pharmaceutical medicaments, to be generated, displaying reduced IgE binding and maintained immunogenicity. This paper describes the production and preclinical testing of vaccine candidates based upon this concept.

Materials and Methods

Site-directed mutagenesis

Bet v 1.2801 (27) inserted into the pMAL-c vector (New England Biolabs, Hertfordshire, U.K.), was modified by site-directed mutagenesis by PCR-based overlap extension (28). Briefly, mutagenesis was performed using two generally applicable oligonucleotide primers and mutation-specific oligonucleotide primers accommodating each mutation. The generally applicable upstream primer containing a *SacI* restriction site also coded for a factor X_a cleavage site inserted directly upstream to the *Bet v 1* gene. The generally applicable downstream primer containing an *EcoRI* restriction site corresponded in sequence to the 3' end of the *Bet v 1* gene. The mutated final PCR product from a third PCR using only the generally applicable primers were directionally ligated into the pMAL-c vector and transformed into *Escherichia coli* DH5 α cells (29) by electroporation (Bio-Rad, Hercules, CA). Full nucleotide sequence was obtained on both DNA strands of inserts of all constructs using the Ready reaction dye terminator cycle sequencing kit and a Fluorescence Sequencer AB PRISM 377, both from PerkinElmer (Hvidovre, Denmark), following the recommendations of the supplier.

Expression and purification of rBet v 1

Plasmids containing DNA encoding rBet v 1 (Table I) and the mutated molecules were overexpressed in *E. coli* K-12, strain DH5 α , yielding fusion proteins with N-terminal maltose-binding protein. Purified rBet v 1 and mutants with the authentic N terminus were obtained in yields of 1–5 mg/L after factor X_a cleavage, as described in Ref. 27. Protein concentrations were determined by UV absorption at 280 nm in a Shimadzu (Columbia, MD) UV-1601PC spectrophotometer and by amino acid analysis.

Crystallization and x-ray diffraction analysis

Crystals of purified rBet v 1 with four point mutations grew after microseeding (30) with ammonium sulfate as the precipitating agent. Crystallization was performed as described elsewhere (31) by sitting-drops vapor diffusion with 10- μ l drops containing 5 μ l of reservoir solution containing 2.2 M ammonium sulfate, 100 mM sodium citrate (pH 6.3), 1% dioxane, and 0.01% sodium azide, and 5 μ l of rBet v 1 mutant, dialyzed against 10 mM Tris-HCl (pH 7.5) before concentration to an OD₂₈₀ of 5.0. Using horsehairs, drops were seeded with crunched crystals of nonmutated rBet v 1 obtained as in Ref. 31 after overnight equilibration at 25°C. Diffraction data were collected at room temperature at the local (University of Copenhagen) Rigaku (Tokyo, Japan) R-axis IIC image plate system with a Rigaku RU200 rotating anode supplying the CuK α x-ray beam at 50 kV and 180 mA. The crystal-to-detector distance was 120 mm, the oscillation range was 2.0°, and the exposure time was 30 min. A complete model of rBet v 1 (Brookhaven Protein Data Bank (PDB) entry 1BV1) (32) was used for molecular replacement with the program AmoRe (33). The structure was refined including 30- to 2.15-Å resolution data with the program CNS (34) and with a test set data size of 5%. Individual but restrained *B* factors were refined.

Circular dichroism (CD) spectroscopy

CD spectra were obtained using an OLIS DSM 10 CD spectrophotometer (On Line Instrument Systems, Bogart, GA), equipped with cylindrical (31-Q-1/CD) or square (21-Q-1/CD) 0.1-cm light path quartz cuvettes (Starna,

Table I. *Bet v 1 and mutated allergens*

Bet v 1
rBet v 1.2801, ^a GenBank accession number z80104.
Amino acid sequence of Bet v 1:
GVFNYETTTSPVPAARLFKAFILDGDNLFQKVPQAISSEVENIEGNGGPGTIKKISFPEGLPFKYVKDRVDE
VDHTNFKYNYSVIEGGPIGDTLEKISNEIKIVATPDGGSILKISNKYHTKGDHEVKAQVKASKEMGETLLR
AVESYLLAHS ^{DAYN}
Amino acid residues in the sequence of Bet v 1 substituted in the mutated allergens are underlined.
Bet v 1 mutant with four point mutations
rBet v 1.2801 (T28, Q32, S45, G108) ^a
Amino acid substitutions: (Asn ²⁸ Thr, Lys ³² Gln), (Glu ⁴⁵ Ser) and (Pro ¹⁰⁸ Gly).
Bet v 1 mutant with nine point mutations
rBet v 1.2801 (V5, S42, S45, K78, V103, I123, E134, H156, +N160) ^a
Amino acid substitutions/(+)insertion: (Tyr ⁵ Val, Lys ¹³⁴ Glu), (Glu ⁴² Ser, Glu ⁴⁵ Ser), (Asn ⁷⁸ Lys, Lys ¹⁰³ Val), (Lys ¹²³ Ile), (Asp ¹⁵⁶ His, + ¹⁶⁰ Asn).

^a In accordance with International Union of Immunological Societies nomenclature (46).

Hainault, U.K.). The spectra were recorded from 260–184 nm, collecting data at every second nanometer, 38 data points per spectrum. The temperature of the cuvette was maintained constant using a Julabo Model F30-C bath/circulator temperature control module (Julabo Labortechnik, Seelbach, Germany). Spectra were obtained in 0.01 M sodium phosphate buffer (pH 7.2) at a concentration of 0.2 mg of protein per milliliter. Each spectrum represents the arithmetic mean of four determinations corrected for buffer absorption and normalized to $\Delta\epsilon = 0$ at 260 nm. The mDeg recorded was transformed to $\Delta\epsilon$ using the following: $\Delta\epsilon = \text{mDeg}/(32,980 \cdot c \cdot l)$ $\text{Abs}_{\text{units}}/(\text{M} \cdot \text{cm})$, where mDeg is the CD signal, c is the concentration in moles per liter as measured by amino acid analysis, and l is the length of the light path in centimeters.

Specific human serum IgE inhibition assay

A pool of equal volumes of sera from seven birch pollen allergic patients was used for specific serum IgE inhibition assays. All patients had a clinical history of birch pollinosis and were radioallergosorbent test class 2 or more. rBet v 1 was biotinylated at a molar ratio of 1:5 (rBet v 1:biotin). The inhibition assay was performed on ADVIA Centaur System (Bayer, Kgs. Lyngby, Denmark) as follows: A serum sample (25 μL) was incubated with paramagnetic beads (solid phase) coated with a monoclonal mouse anti-human IgE Ab (ALK-Abelló, Hørsholm, Denmark), washed, resuspended, and incubated with a mixture of biotinylated rBet v 1 and inhibitor (non-biotinylated rBet v 1 or mutated allergens) in dilution series. The amount of biotinylated rBet v 1 bound to the serum IgE on the solid phase was estimated from the measured relative light units (RLUs) after incubation with acridinium-ester labeled streptavidin. The degree of inhibition was calculated as the ratio between the RLUs obtained using buffer and mutant as inhibitor.

Histamine release in human basophiles

Histamine release experiments were performed as in Ref. 35. In brief, heparinized blood (20 ml) was drawn from eight birch pollen-allergic patients with a history of seasonal hay fever, stored at room temperature, and used within 24 h. A volume of 25 μL of heparinized whole blood was applied to glass fiber-coated microtiter wells (Reference Laboratory, Copenhagen, Denmark) and incubated with 25 μL of 10, 100, 1,000, 10,000, 100,000, or 1,000,000 ng/L Ag or anti-IgE (DakoCytomation, Glostrup, Denmark) for 1 h at 37°C. The plates were washed, and histamine bound to the microfibers was measured spectrophotofluorometrically. All histamine release experiments were performed in triplicate.

T cell reactivity

PBMC isolated from freshly drawn heparinized blood were stimulated with serial dilutions of purified natural (n) Bet v 1 or rBet v 1 (10, 3, 1, or 0 $\mu\text{g}/\text{ml}$). PBMC stimulated with 25 $\mu\text{g}/\text{ml}$ purified protein derivative (State Serum Institute, Copenhagen, Denmark) were used as positive control. At day 6, culture supernatants were harvested for cytokine determination followed by an 18- to 20-h [^3H]thymidine pulse and harvest for scintillation counting. Stimulation index was calculated as the ratio of counts per minute at the optimal Ag concentration (10 $\mu\text{g}/\text{ml}$ for all Bet v 1 preparations) in the stimulated cultures relative to the counts per minute in the unstimulated cultures. All results represent the mean of four replicas.

Generation of Bet v 1-specific T cell lines

T cell lines specific to Bet v 1 were established from PBMC of birch-allergic patients as described elsewhere (36). In brief, isolated PBMC were stimulated with purified Bet v 1 (10 $\mu\text{g}/\text{ml}$) for 14 days in bulk cultures in medium containing 5% human AB-negative serum (AH Diagnostics, Aarhus, Denmark), with the addition of rIL-2 at days 5–7. At day 14, T cells were restimulated with autologous irradiated PBMC, Bet v 1, and 0.05% PHA-P (Boehringer Mannheim, Mannheim, Germany). rIL-2 was added at days 5–7. The T cell lines were kept in culture with restimulation every 10th day. The cultures were terminated after a culture period of 8 wk.

T cell stimulation assay

On day 10 after restimulation, T cells were cultured with autologous irradiated PBMC and the Bet v 1 preparations as described for the PBMC assays. After 24 h, culture supernatants were harvested for detection of cytokines (IL-2, IL-4, IL-5, IL-10, IFN- γ , and TNF- α). After 48 h, the cultures were pulsed with [^3H]thymidine for 18–20 h, followed by harvest for scintillation counting to assess cell proliferation. Results are expressed as mean count per minute values of four replicas.

Cytokine measurements

Cytokine concentrations (IL-4, IL-5, IL-10, and IFN- γ) in the culture supernatants were determined by human Th1/Th2 cytokine beads array kit (BD Biosciences, Mountain View, CA) according to the manufacturer's instructions.

Mouse immunization

The ability of rBet v 1 and mutated allergens to induce Bet v 1-specific IgG Abs was assessed in mouse immunization experiments. BALB/cA mice (eight in each group) were immunized by i.p. injections with rBet v 1 or mutated allergens. The mice were immunized four times biweekly using a dose of 10 μg of protein conjugated to 1.25 mg/ml Alhydrogel (aluminum hydroxide gel, 1.3%, pH 8.0–8.4; Brenntag Biosector, Frederikssund, Denmark). Blood samples were drawn by orbital bleed at days 0 and 63. Specific IgG Ab levels were analyzed by direct ELISA using rBet v 1-coated microtiter plates and biotinylated rabbit anti-mouse IgG Abs (Jackson ImmunoResearch Laboratories, West Grove, PA).

Inhibition of specific human serum IgE binding by Bet v 1-specific mouse IgG Abs

To measure inhibition, serum samples from birch pollen-allergic patients were incubated with paramagnetic beads (solid phase) coated with a monoclonal mouse anti-human IgE Ab (ALK-Abelló). After incubation, beads were washed and resuspended in buffer or diluted samples (1/100) of mouse serum from nonimmunized mice (control) or mouse serum from mice immunized with either rBet v 1 or mutated allergens as described above. Biotinylated rBet v 1 was added to the mixture of beads and mouse serum Abs. After incubation, beads were washed, and biotinylated rBet v 1 bound to the solid-phase serum IgE was estimated from the measured RLUs after incubation with acridinium-ester-labeled streptavidin. The inhibition caused by Bet v 1-specific mouse IgG Abs was expressed as the ratio between the RLUs obtained using buffer and mouse serum as inhibitor.

Statistical analyses

All curve-fitting and statistical analyses were performed using GraphPad Prism, version 4.0 (GraphPad Software, San Diego, CA). Inhibition curves were fitted to a sigmoidal dose-response (variable slope) function, and the curves were considered parallel if the slopes did not differ significantly for the compared data sets. Matched data sets were compared by paired t tests, and unmatched data sets were compared by unpaired t tests or, in the case of multiple comparisons, by ANOVA using a Bonferroni correction of the significance level. All stochastic probabilities of <0.05 were considered significant.

Results

Recombinant mutated allergens

Two rBet v 1 mutants were cloned and expressed in *E. coli* in operon with the N-terminal *E. coli* protein fusion partner, maltose binding protein, enabling purification by affinity chromatography using amylose coupled to agarose. The fusion proteins were subsequently cleaved with factor X_a , and rBet v 1 mutants with authentic N termini were purified at 1- to 5-mg yields per liter of culture medium. All purified rBet v 1 preparations appeared as single bands with an apparent molecular mass of 17.5 kDa after silver-stained SDS-polyacrylamide electrophoresis.

The selection of amino acid residues for mutagenesis was based on the crystal structure of rBet v 1.2801 (PDB entry 1BV1) and targeted to the molecular surface aiming at a modulation of the Ab-binding properties. One mutant was designed with four amino acid substitutions, representing three different areas on the molecular surface of Bet v 1 (see Fig. 1). Asn²⁸Thr and Lys³²Gln are located next to each other, affecting one area, and Glu⁴⁵Ser and Pro¹⁰⁸Gly affect two additional nonoverlapping surface areas. Another mutant was designed with nine point mutations, i.e., eight amino acid substitutions and one C-terminal extension (+¹⁶⁰Asn), affecting five different areas on the molecular surface: (Tyr⁵Val, Lys¹³⁴Glu), (Glu⁴²Ser, Glu⁴⁵Ser), (Asn⁷⁸Lys, Lys¹⁰³Val), (Lys¹²³Ile), (Asp¹⁵⁶His, +¹⁶⁰Asn) (see Fig. 1). In

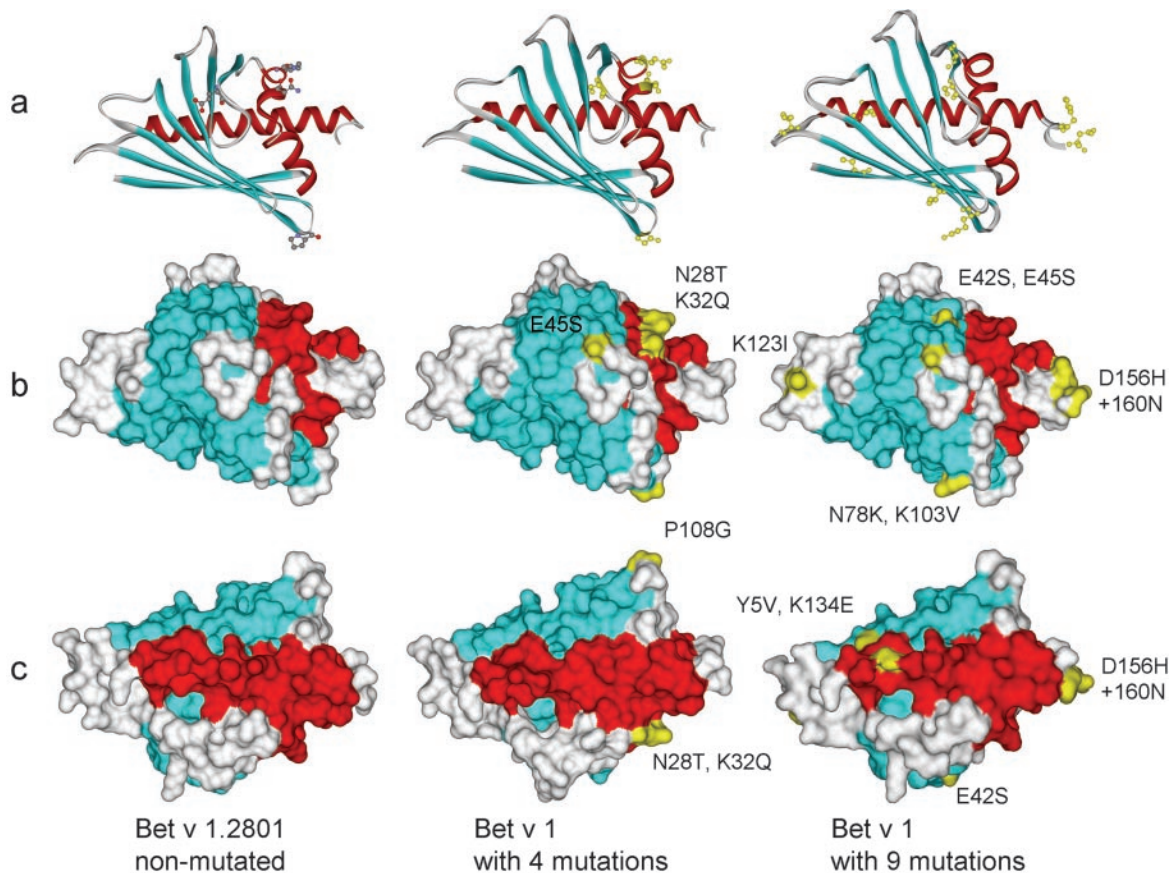


FIGURE 1. View of Bet v 1 and mutated allergens in ribbon style and space-filling models. *Left column*, Bet v 1 (PDB 1BV1). *Middle column*, Mutated Bet v 1 with four point mutations (PDB 1QMR). *Right column*, Mutated Bet v 1 with nine point mutations (SWISS-MODEL) (the model of mutated Bet v 1 with nine point mutations was modeled using PDB 1BV1 and the comparative protein-modeling software SWISS-MODEL at the ExPASy Molecular Biology Internet Server). *a*, Ribbon models of Bet v 1 and derived mutant molecules, color-coded as follows: red, α -chain; green, β -sheet with affected/introduced residues shown as ball and stick; yellow, introduced amino acid residues. *b/c*, *c* turned 180° around horizontal axis. Molecular surfaces of Bet v 1 and derived mutant molecules (Connolly surface), color-coded as follows: red, α -chain; green, β -sheet; yellow, introduced amino acid residues.

the three-dimensional (3-D) structure of rBet v 1.2801, asparagine 28 (47% relative solvent exposure (R.S.E.)) and lysine 32 (86% R.S.E.) are located in the second of the two shorter α -helices present in Bet v 1, whereas lysine 134 (63% R.S.E.) is positioned within the C-terminal α -helix with aspartic acid 156 (69% R.S.E.) in the C terminus. Glutamate 42 (52% R.S.E.) and lysine 103 (45% R.S.E.) are both found in the prominent β -sheet, whereas glutamate 45 (62% R.S.E.), asparagine 78 (54% R.S.E.), proline 108 (93% R.S.E.), and lysine 123 (86% R.S.E.) are positioned in loop regions.

3-D structure of mutants

rBet v 1 with four point mutations was crystallized by microseeding using crystals of rBet v 1.2801 and the same buffer composition as described for the crystallization of rBet v 1.2801 (31). The 3-D structure of rBet v 1 with four point mutations was solved by x-ray diffraction at 30- to 2.15-Å resolution, and the coordinates were published in the PDB database (PDB entry 1QMR). Crystals appeared within 1 wk and were allowed to grow for 2 mo before diffraction data were collected. The crystals were C-centered orthorhombic C222₁ with cell lengths as follows: $a = 32.3$, $b = 74.2$, $c = 119.5$ Å; diffraction data statistics are listed in Table II. The four point mutations Asn²⁸Thr, Lys³²Gln, Glu⁴⁵Ser, and Pro¹⁰⁸Gly, were evident from the $F_o - F_c$ and the $2F_o - F_c$ electron density maps. The final R factor of the model is 0.196, whereas the free R factor is 0.238 including 46 solvent molecules. The average

backbone atom B factor for the structure is 19.7 Å². The Ramachandran plot showed 92.6% of the residues to be in the most favorable regions and only one (Asp⁹³) in disallowed regions. The overall structure of Bet v 1 with four point mutations is comparable with the structure of native nonmutated Bet v 1 with a root mean square deviation (r.m.s.d.) of C α positions of 0.255 Å. Ribbon style and molecular surface models of the crystal structures of rBet v 1 and rBet v 1 with four point mutations are shown in Fig. 1, *left* and *middle columns*, respectively. Statistics for the refinement are found in Table II.

Secondary structure elements were analyzed by CD spectroscopy applying nBet v 1, rBet v 1, and the two mutated recombinant allergens. Fig. 2 shows an overlay of CD spectra obtained at 15°C of purified nBet v 1 obtained from birch pollen and the rBet v 1 derivatives. A CD spectrum of heat-denatured rBet v 1 obtained at 90°C is included for comparison. The spectra of rBet v 1, rBet v 1 with four point mutations, and rBet v 1 with nine point mutations all have negative and positive amplitudes at 212–216 and 192–194 nm, respectively, characteristic for nBet v 1. All four spectra are different from the CD spectrum of heat-denatured rBet v 1, which is characterized by negative and positive amplitudes at 200 and 186 nm, respectively.

Reduced capacity to bind human-specific serum IgE

Introduction of four or nine point mutations decreased the binding of a pool of patients' serum IgE to rBet v 1. Fig. 3, *A* and *B*, shows

Table II. Statistics for crystallographic structure determination of the Bet v 1 mutant with four point mutations: rBet v 1.2801 (T28, Q32, S45, G108)

Data collection	
Resolution range (Å)	37–2.15
Observations	55,800
Unique reflections	7,832
Completeness (%) ^a	96.6 (78.5)
R _{sym} (%) ^{a,b}	4.0 (13.5)
Refinement	
Resolution range (Å)	30–2.15
Unique reflections (<i>I</i> > 0)	7797
R _{cryst} (%) ^c	19.6
R _{free} (%) ^d	23.8
Number of nonhydrogen atoms	
Protein	1,223
Solvent	46
r.m.s.d. from ideal values	
Bond length (Å)	0.006 ^e
Bond angle (°)	1.2 ^e
Estimated coordinate error (Å)	
Luzzati plot	0.30 ^e
Ramachandran plot ^f	
Residues in most favored (disallowed) regions (%)	92.6 (0.7)
Mean B factor (Å ²)	
Protein	22.1
Solvent	34.5

^a Numbers in parentheses are for the highest resolution shell, 2.23–2.15 Å.

^b $R_{\text{sym}} = \sum_{hkl} (\sum_i (I_{hkl,i} - \langle I_{hkl} \rangle)) / \sum_{hkl,i} \langle I_{hkl} \rangle$, where $I_{hkl,i}$ is the intensity of an individual measurement of the reflection with Miller indices *h*, *k*, and *l*, and $\langle I_{hkl} \rangle$ is the mean intensity of that reflection.

^c $R_{\text{cryst}} = \sum_{hkl} (|F_{o,hkl}| - |F_{c,hkl}|) / |F_{o,hkl}|$, where $|F_{o,hkl}|$ and $|F_{c,hkl}|$ are the observed and calculated structure factor amplitudes.

^d R_{free} is equivalent to the *R* factor, but calculated with reflections omitted from the refinement process (5% of reflections omitted).

^e Calculated with the program CNS (34).

^f Calculated with the program PROCHECK (47).

the binding of human serum IgE to biotinylated rBet v 1 inhibited by rBet v 1 or mutants. Compared with the inhibition curves obtained with rBet v 1, both of the mutants' inhibition curves are shifted toward higher Ag concentrations, slopes are reduced, and the mutations induce a reduction in the maximum binding levels compared with rBet v 1. For the fitted inhibition curve of the mutant with four amino acid substitutions (Fig. 3A), the Hill slope is -0.682 ± 0.061 (SEM) compared with -0.991 ± 0.017 (SEM) for rBet v 1, and the asymptote representing the maximum inhibition level is at 0.066 ± 0.018 (SEM) for the mutant compared with 0.001 ± 0.002 (SEM) for rBet v 1. For the mutant with nine amino acid substitutions, the fitted inhibition curve (Fig. 3B) has a Hill slope of -1.111 ± 0.180 (SEM) compared with -1.849 ± 0.197 (SEM) for rBet v 1, and the asymptote representing the maximum inhibition level is at 0.292 ± 0.035 (SEM) compared with 0.039 ± 0.021 (SEM) for rBet v 1. Although all differences are statistically significant (*t* test, *p* < 0.05), deviations are more pronounced for the mutant with nine substitutions compared with the mutant with four amino acid substitutions.

Reduced capacity to trigger human basophile histamine release

rBet v 1 and mutated allergens were tested in standard histamine release assays with human basophils from eight individual birch pollen-allergic patients. Fig. 4 shows the result of nine histamine release experiments. Patient A was tested twice with similar results (A*). Histamine release assays ideally produce bell-shaped curves when plotting released histamine as a function of Ag concentration. At high Ag concentrations, the release is restricted by the number of cells in the assay and eventually reduced as IgE becomes saturated. At low Ag concentrations, potency differences

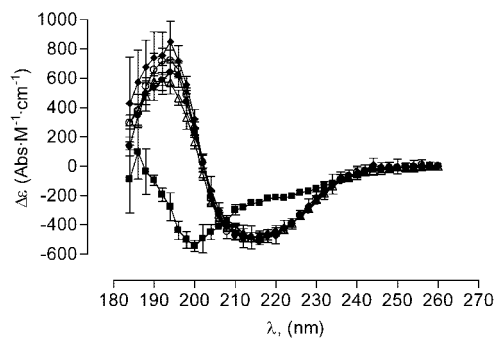


FIGURE 2. CD spectra of nBet v 1 obtained from birch pollen (◆), nonmutated rBet v 1 (●), mutated rBet v 1 with four point mutations (○), and mutated rBet v 1 with nine point mutations (△) obtained at 15°C, and heat denatured rBet v 1 (■) obtained at 90°C. Spectra were recorded from 184 to 260 nm and mean values of four spectra are shown; error bars show 95% confidence limits for each data point.

between Ags can be scored as a displacement along the abscissa. Thus, at low Ag concentration, a reduction in the capacity to release histamine leads to a shift toward higher Ag concentration of the release curve. Due to the low number of data points, statistical analysis based on curve fitting was not possible. Instead, the displacement of the curves was analyzed comparing the level of histamine release in the dynamic range of the assay at 1000 ng/ml (Fig. 5). Both mutants induce significantly less histamine release compared with rBet v 1, but there is no significant difference between the mutants.

T cell reactivity

T cell reactivity in PBMC cultures was determined to compare the T cell reactivity to purified nBet v 1, rBet v 1, and rBet v 1 with four or nine point mutations in a mixed T cell population directly isolated from peripheral blood. There were minor differences in the

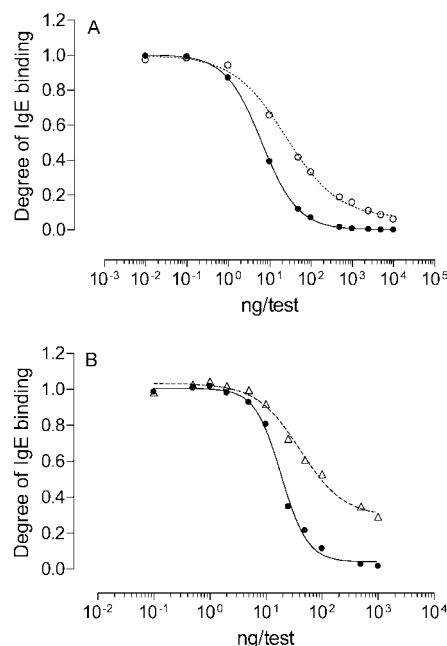


FIGURE 3. IgE inhibition assay. Binding of patients' pooled serum IgE to biotinylated rBet v 1 inhibited by rBet v 1 or mutants. A, rBet v 1 (●), rBet v 1 with 4 mutations (○). B, rBet v 1 (●), rBet v 1 with nine mutations (△). Two different batches of biotinylated rBet v 1 were used in experiments A and B.

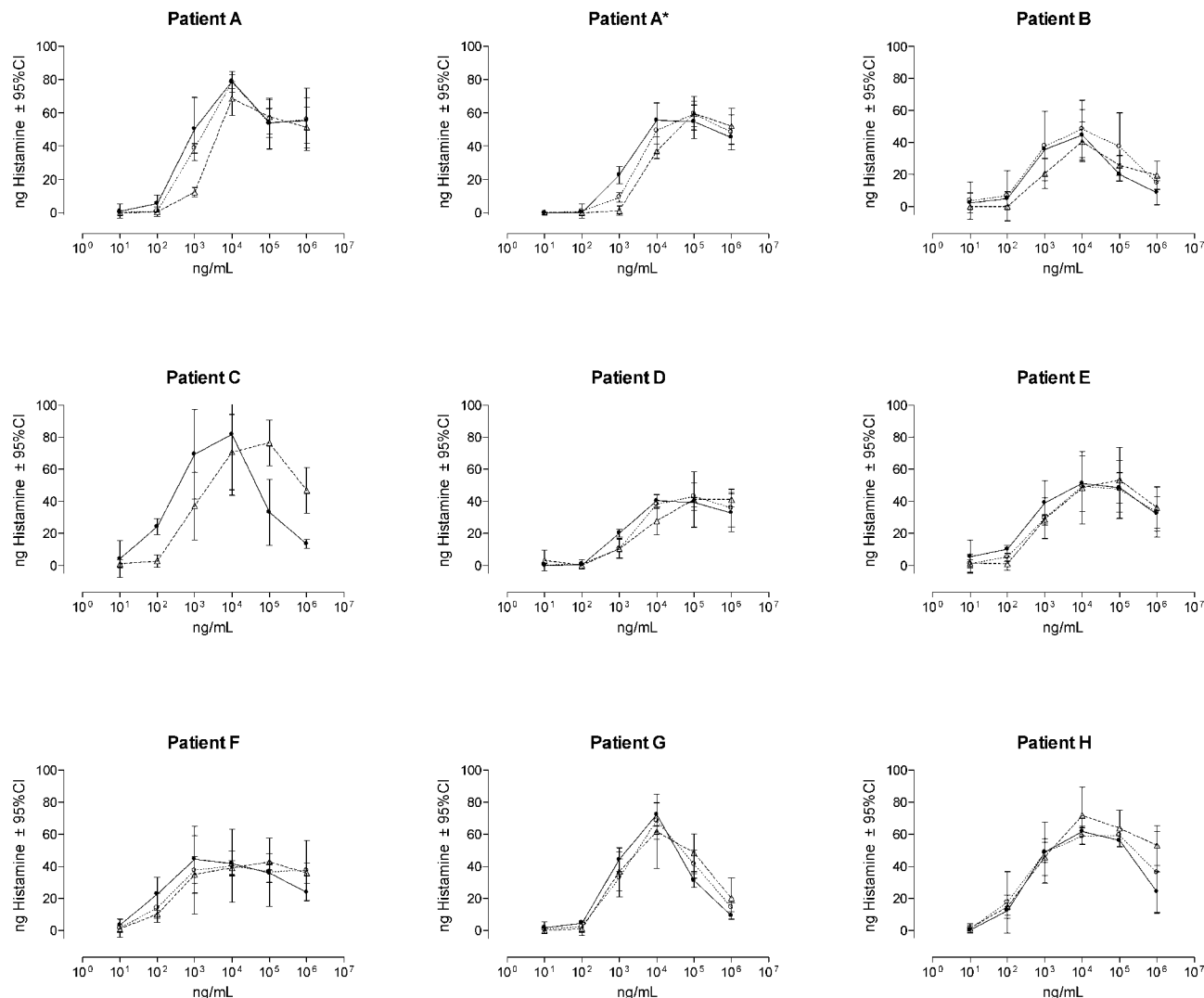


FIGURE 4. Histamine release experiments with nonmutated rBet v 1 (●), mutated rBet v 1 with four point mutations (○), and mutated rBet v 1 with nine point mutations (△) from basophils obtained from eight birch pollen-allergic patients are shown. Patient A was tested twice at 3-wk intervals with similar result (A*). x-axis, Ag in picograms per milliliter; y-axis, histamine release in nanograms. Release is plotted as mean values of triplet measurements with error bars showing 95% confidence limits for each data point.

T cell proliferation toward each Bet v 1 preparation in the individual donors, but no significant differences were observed in this group of 10 patients (Fig. 6). Similarly, the cytokines produced primarily reflected the cytokine profile of the individual patients and were not changed in response to the different Bet v 1 preparations. Cytokine production increased proportionally with PBMC proliferation (data not shown).

Bet v 1-specific T cell lines were established using standard methodology. The T cell lines showed similar responses to natural, recombinant, and mutated rBet v 1 molecules (Fig. 6). Four T cell lines showed a Th0-like cytokine pattern, three showed a Th1 pattern, and one showed a Th2 pattern determined on the basis of the IFN- γ /IL-5 ratio. As observed in the PBMC assays, the cytokine production profile (Th1/Th0/Th2) was a result of the T cell population selected and did not reflect the molecular differences of the Bet v 1 preparations.

The content of LPS was assessed in the Ag preparations. For rBet v 1 and the mutant with four substitutions, contamination was below detection limit; for nBet v 1 and the mutant with nine substitutions, endotoxin content was ~ 200 U/mg. T cell stimulation in PBMC cultures as well as the production of IL-5 and IFN- γ from

established T cell lines within the time frame of the assays were unaffected by this level of contamination.

Ab responses following immunization in mice

Immunization in mice with rBet v 1 or rBet v 1 mutants induced strong Bet v 1-specific IgG responses, demonstrating that the mutated allergens are able to induce Abs cross-reactive with nonmutated rBet v 1. To assess the induction of blocking Abs, mouse antisera were tested in an immunoinhibition assay using a pool of human serum IgE. Serum from nonimmunized mice did not inhibit the binding of human IgE to biotinylated rBet v 1, whereas sera from mice immunized with rBet v 1 or mutated rBet v 1 allergens significantly reduced binding of human IgE to biotinylated rBet v 1. As shown in Fig. 7, at day 63, three or more mouse sera from all immunization groups inhibited the binding of nonmutated rBet v 1 to human IgE by $>80\%$.

Discussion

SAV, i.e., allergen-specific immunotherapy, is an effective and well-tolerated treatment for allergic disease in selected patients, and currently the only treatment with the potential to alter the

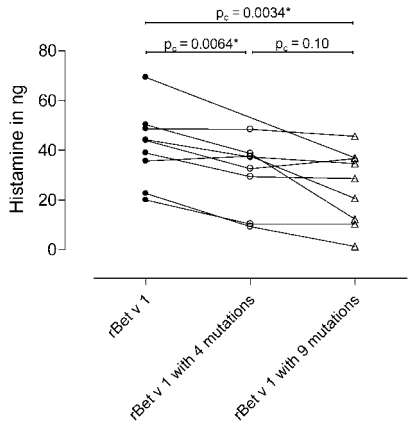


FIGURE 5. Comparison of histamine release at 1000 ng/ml triggered by rBet v 1 or mutants. Both mutants induce significantly less histamine release than rBet v 1, but there is no significant difference between the mutants.

natural course of allergic disease. Despite having been used for almost a century, the immunological mechanisms underlying successful therapy are still only partially understood. The lack of a comprehensive model has resulted in several approaches to the

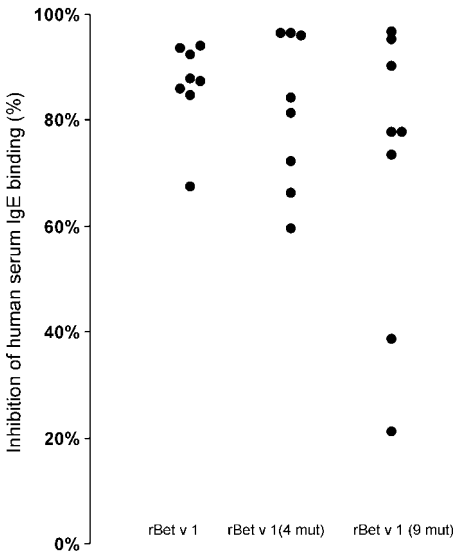


FIGURE 7. Inhibition of binding of specific human serum IgE to biotinylated rBet v 1 by mouse IgG Abs raised against nonmutated rBet v 1 and mutated rBet v 1 allergens.

modification of allergen molecules for the purpose of eliminating a small but significant risk of inducing anaphylactic side reactions.

SAV induces changes in the activities of both innate and acquired immunological parameters. IgE plays a major role in the pathology of allergic asthma and rhinitis; however, the IgE Ab titer against injected allergen is not dramatically affected by the treatment despite clinical improvement of the patient. A more pronounced effect is a marked increase in allergen-specific IgG soon after onset of therapy (11, 12). Allergen-specific IgG remains elevated while therapy continues, and has the capacity to compete with IgE for binding to the allergen, an observation which has led to the term “blocking IgG” (37, 38). Increases in allergen-specific IgA and IgG have also been observed in nasal secretions (39).

Human mast cells and basophils from allergic individuals release histamine in a dose-dependent manner on exposure to allergen in vitro. Allergy vaccination reduces the activity of human basophils, an effect that could be explained by interference from allergen-specific IgG Abs induced by the treatment. However, qualitatively different mechanisms may contribute to this effect. Thus, human basophils, in addition to the FcεRI receptor, express FcγRII receptors on the cell surface, and in vitro experiments have indicated that costimulation of FcεRI and FcγRII leads to reduced basophil activity mediated by alterations in signal transduction (40). Reduced activity of basophil histamine release in these studies was accompanied by a reduction of IL-4 production, implying that allergen-specific IgG induced by allergy vaccination is linked to a reduction in the production of inflammatory cytokines in the allergic immune response.

The idea behind the recombinant mutated allergens described here is to modify the surface topography to reduce IgE binding, while retaining the folding pattern of the α-carbon backbone to preserve surface structures capable of generating an IgG response that will compete with IgE for binding to the natural allergen. We have previously shown that one single amino acid substitution in Bet v 1, i.e., glutamic acid to serine in position 45, covering ~1% of the surface area, inhibits IgE binding between 20 and 50%, when comparing sera of individual tree pollen-allergic individuals (26). The 3-D structure of the Glu⁴⁵Ser mutant was determined by x-ray crystallography, and in comparison with the 3-D structure of the nonmutated rBet v 1.2801, there was an r.m.s.d. of 0.46 Å, in

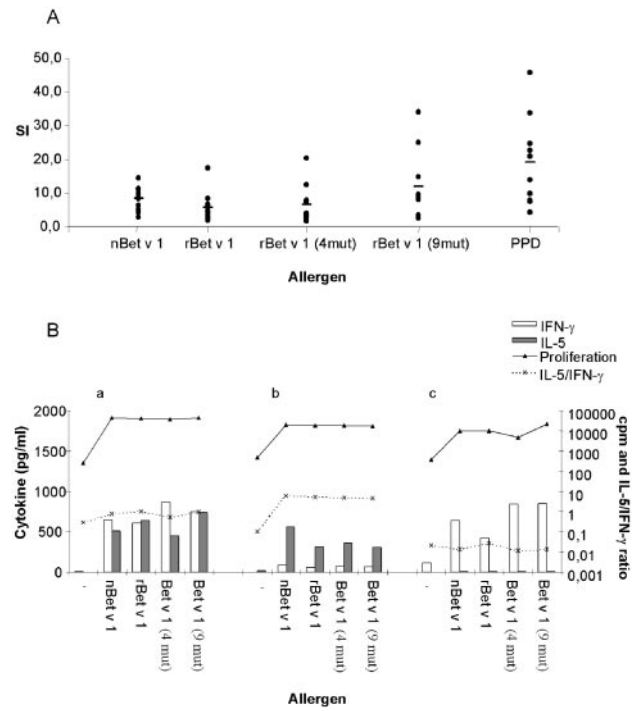


FIGURE 6. nBet v 1 obtained from birch pollen and rBet v 1 with or without point mutations induce comparable T cell responses. **A**, Freshly isolated PBMC from 10 birch pollen-allergic patients were stimulated with the different Bet v 1 preparations (10 μg/ml) and purified protein derivative (PPD) (positive control, 25 μg/ml) to determine T cell proliferation. Results are expressed as stimulation index (SI = counts per minute in allergen-stimulated cultures/counts per minute in cultures without allergen). Similar results were obtained at 3 μg/ml Bet v 1. **B**, nBet v 1-specific T cell lines were stimulated with the different Bet v 1 preparations to compare the proliferation and cytokine production induced. **a**, **b**, and **c** represent examples of three typical T cell phenotypes. Bars indicate cytokine production (IL-5 and IFN-γ; left y-axis), solid lines indicate cell proliferation (cpm; right y-axis), and dashed line indicates IL-5/IFN-γ ratio (Th1 < 0 and Th2 > 0; right y-axis).

accordance with the observation that surface topography deviated only in the vicinity of the mutation.

The crystal structure of a complex between rBet v 1 and a monoclonal mouse IgG Ab, (BV16) Fab recently defined an epitope contact region of 931 Å² (41). This area represents the maximal extension of the epitope and should be compared with the total solvent-accessible surface of rBet v 1 calculated to be 9119 Å² including the interior cavity (41), and 7203 Å² counting only the outward surface accessible for Ab binding (26). For comparison, a single amino acid residue in rBet v 1 constitutes from 0 to 110 Å² of the molecular surface, suggesting that introduction of four or nine point mutations in rBet v 1 affect <10% of the total surface area of rBet v 1, leaving ample space for epitopes with the potential to induce allergen-specific IgG Abs reactive with the natural allergen.

In the present study, amino acid residues selected for mutation all had relative solvent accessibility values >45% as calculated from the structure of rBet v 1.2801 (PDB entry 1BV1) (32), using a probe radius of 1.4 Å (Connolly surface) and the program Insight II, version 97.0 (Accelrys, San Diego, CA). As the majority of birch pollen-allergic patients have serum IgE that cross-react with major allergens of *Fagales* comprising birch, alder, hazel, and hornbeam (42), amino acid residues selected for mutation were all located in surface areas conserved among the *Fagales* major allergens as determined by primary sequence alignments. Selected amino acid residues were substituted to residues not found in the corresponding positions within any known isoallergen sequence of *Fagales* major allergens. Amino acid residues introduced by mutation were present in corresponding positions of the structurally homologous pathogenesis related (PR-10) proteins (43) showing no or limited Ab cross-reactivity to Bet v 1. Preferentially acidic or basic amino acid residues were substituted to neutral residues or vice versa to maximize changes in the surface properties. Comparison of Cα positions in the crystal structure of rBet v 1 carrying four point mutations with the structure of nonmutated rBet v 1.2801 showed an average r.m.s.d. of 0.255 Å, indicating identical Cα folding patterns. The 3-D structure of both mutants was further assessed by CD spectroscopy, resulting in spectra similar to those obtained with nBet v 1 purified from pollen extract and recombinant nonmutated Bet v 1, and distinctly different from the spectrum of heat-denatured rBet v 1.

Crystallization and x-ray diffraction analysis of rBet v 1 with four mutations enabled structural comparison with nonmutated rBet v 1.2801. Introduction of point mutations locally altered both the topography as well as the electrostatic potential of surface areas positioned in the close surroundings of introduced amino acid substitutions (see Fig. 8). Deviations in surface topography outside the areas affected by the mutations (i.e., see Fig. 1) involve only side-chain movement of surface exposed amino acids insignificant for the overall folding pattern of the α-carbon backbone. Whether these deviations are significant for Ab binding is doubtful, because side-chain movement of surface-exposed amino acids has been demonstrated in Ab-Ag complex formation.

Introduction of four or nine mutations in rBet v 1 resulted in reduced binding of patients' specific serum IgE. The changes in the slopes of the mutants' inhibition curves compared with the curves obtained with rBet v 1 (see Fig. 3) were significant. This indicates considerable changes in the epitope structures of the modified allergens. In addition, the asymptote of the fitted curves of the mutants with four and nine mutations, representing the maximum inhibition levels, were estimated to be 6.6 ± 4.1 and $29.2 \pm 8.1\%$, respectively, and were significantly different from the asymptotes of the fitted curve of rBet v 1. This indicates a reduced number of

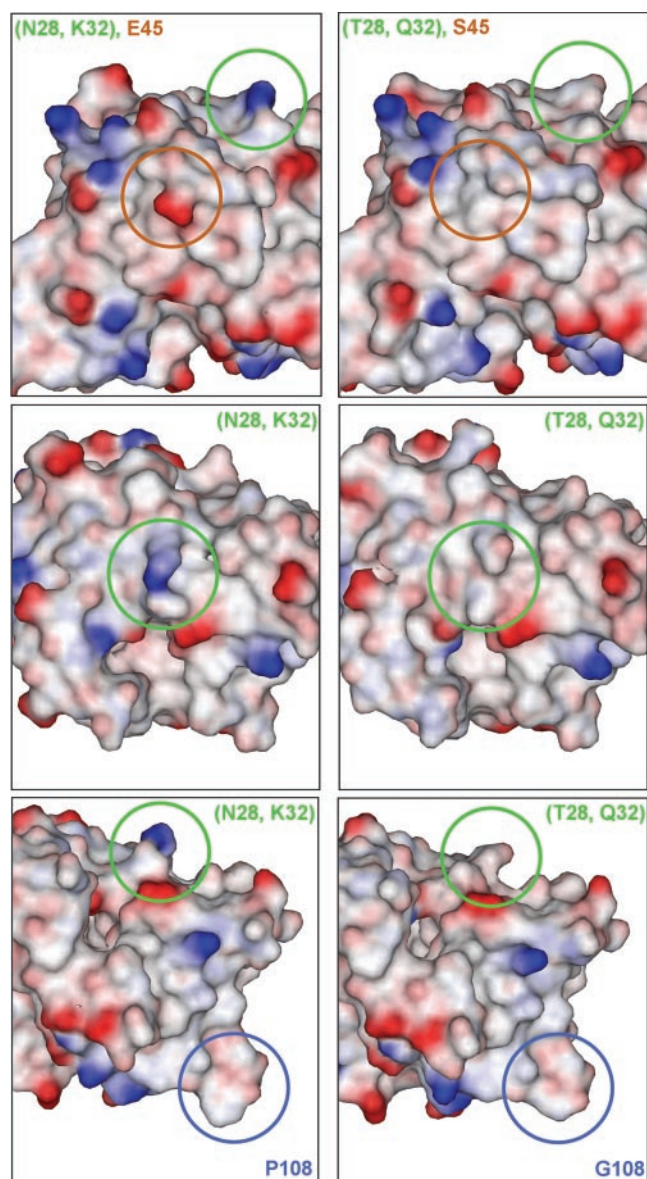


FIGURE 8. Comparison of molecular surfaces colored according to electrostatic potential. Molecular surface models of crystal structures of rBet v 1.2801 (PDB 1BV1) and rBet v 1 mutant with four mutations (PDB 1QMR) are shown. *Left side* shows different views of the molecular surface of nonmutated rBet v 1.2801. *Right side* shows corresponding views of the molecular surface of the mutant. Green circles mark the position of the double-mutation Asn²⁸Thr, Lys³²Gln. The position of the Glu⁴⁵Ser mutation is marked with orange circles. Blue circles mark the position of the Pro¹⁰⁸Gly mutation. Introduction of mutations changed both the topography and the charge distribution locally, whereas many surface structures positioned outside encircled areas are retained.

epitopes recognized by Bet v 1-specific IgE in the serum pool as compared with rBet v 1.

The reduction in IgE binding of the mutants was confirmed in results obtained by standard histamine release assays using basophils from eight birch pollen-allergic patients. When analyzed as a group, release of histamine was significantly reduced for the mutants compared with nonmutated rBet v 1 at Ag concentrations of 1000 ng/ml (see Fig. 5), however, with some patient-to-patient variation. For two patients, the mutations did not reduce histamine release at all. The differences in patient reactivity to the mutants may be explained by variations in individual patients' IgE Ab repertoires recognizing different subsets of epitopes. The spectrum of

epitopes recognized by IgE in any individual patient is thought to be determined by a combination of genetic factors and history of allergen exposure and therefore subject to variation between patients. Variation in dominating IgE-binding epitopes between patients has also been indicated by studies of cross-reactivity, for example, between Bet v 1 and the major allergen from apple, Mal d 1 (44). According to these studies, cross-reactivity may be explained by patients having IgE directed toward molecular surface areas, which are homologous in structure, whereas patients having IgE directed toward structurally different areas do not show cross-reactivity.

The apparent discrepancy between the results obtained by solid-phase immunoinhibition assays and basophil histamine release may be ascribed to fundamental differences in the allergen-IgE interaction in the assays. In the immunoinhibition assays, a reduction in maximal IgE binding of the mutants of 7 and 29%, respectively, was found, whereas the two mutants could not be distinguished in the histamine release experiments. For the solid-phase immunoinhibition assay, interaction of one IgE Ab with Ag is sufficient to produce a signal, and the degree of inhibition observed by the mutated allergen is solely dependent on the difference in affinity for complex formation between IgE and the mutated and the nonmutated allergen, respectively. Due to substantial differences in epitope structure between the mutated and nonmutated allergens, the inhibition curves are nonparallel, and it is therefore not possible to obtain a relative potency measure. Alternatively, the activities of the mutants can be compared by their extrapolated maximal binding levels, which are not directly comparable with the results obtained by histamine release. Furthermore, they cannot be directly compared with earlier results based on the Glu⁴⁵Ser single point mutant, which showed a reduction in relative potency of IgE binding between 20 and 50%.

The interaction of allergen and IgE on the surface of mast cells and basophils necessary for initiation of the signaling cascade leading to release of histamine is much more complicated and not completely understood. An essential difference is that two IgE molecules need to bind to the allergen simultaneously. However, it is not clear whether these two interactions both need to be of high affinity, or if cooperative binding can facilitate the second interaction. It has been suggested (45) that binding of one IgE/FcεRI-receptor complex may facilitate binding of the allergen to a second IgE/FcεRI-receptor complex even if the second Ab binds the allergen with low affinity.

Therefore, two aspects of the present study indicate the importance of neutralizing all dominating IgE-binding epitopes to obtain a vaccine candidate with an improved safety profile in a larger patient population. First, dominating epitopes seem to differ between patients, and second, the apparent cooperative binding associated with the IgE-allergen interaction on the surface of mast cells and basophils.

The ability of rBet v 1 mutants to induce Abs capable of binding to the natural allergen was assessed in mouse immunization experiments showing that rBet v 1 modified with four or nine point mutations induce Bet v 1-specific IgG Ab responses in mice. For both mutants, the mouse Abs inhibited the binding of human IgE to the nonmutated allergen. IgG Abs induced by vaccination based on the mutated allergens may inhibit allergen-IgE interaction by binding to the same epitopes, or they may bind to surface areas not affected by the mutations and cause reduction in IgE binding to the natural allergen by steric hindrance.

rBet v 1 and mutated allergens did equally well stimulate T cell lines established using PBLs from birch pollen-allergic patients and nBet v 1 purified from pollen. These data suggest that rBet v 1 and mutated allergens in most patients can address the pre-ex-

isting nBet v 1-specific T cell population, which may be important for providing T cell help for Ab production.

In conclusion, recombinant mutated allergens with modified surface topography but retained α -carbon backbone folding pattern, can be designed having reduced anaphylactic potential, but retained capacity to induce allergen-specific non-IgE Abs. The optimal number of mutations is likely a compromise between reducing histamine release and maintaining the ability to induce IgG Abs reactive with the natural allergen. A reduction or elimination of the anaphylactic potential of allergy vaccines would greatly facilitate increased dissemination of SAV. Considering the curative potential of the treatment, this would benefit large numbers of allergic patients.

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