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Genetic engineering of trimers of hypoallergenic fragments of the major birch pollen allergen, Bet v 1, for allergy vaccination

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Abstract

An immunotherapy trial performed in allergic patients with hypoallergenic recombinant fragments, comprising aa 1–74 and 75–160 of the major birch pollen allergen, Bet v 1, has indicated that the induction of allergen-specific IgG responses may be an important mechanism of this treatment. To investigate whether the immunogenicity of the rBet v 1 fragments can be increased, recombinant trimers of the fragments were produced. For this purpose, DNA trimers of rBet v 1 aa 1–74 as well as of rBet v 1 aa 75–160 were subcloned into expression plasmid pET 17b, expressed in *Escherichia coli* and purified. The fragments as well as the fragment trimers showed a reduced IgE-binding capacity and allergenic activity compared to rBet v 1 wildtype when tested in allergic patients. Both rBet v 1 aa 75–160 monomer and trimer induced high titers of allergen-specific IgG1 Abs in mice. Interestingly, rBet v 1 aa 1–74 trimer induced a much higher IgG₁ response to rBet v 1 than rBet v 1 aa 1–74 monomer. Consequently, IgG Abs induced with the rBet v 1 aa 1–74 trimer inhibited birch pollen allergic patients' IgE-binding 10-fold more efficiently than IgG Abs induced with the monomer. Our data show that the immunogenicity of allergy vaccines can be increased by oligomerization.

Keywords

Allergy; Immunotherapy; Oligomerization; Immunogenicity

1. Introduction

Birch pollen allergy represents an important health problem in industrialised countries. Specific immunotherapy is the only causative treatment of birch pollen allergy and several

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clinical studies have demonstrated that this treatment is clinically effective [1]. However, the administration of crude allergen extracts may induce severe side effects [2]. Therefore, several strategies have been applied to develop hypoallergenic allergen derivatives with reduced anaphylactic activity [3,4]. For treatment of birch pollen allergy, the major birch pollen allergen, Bet v 1, was selected for the production of hypoallergenic derivatives. Bet v 1 is recognized by more than 90% of birch pollen allergic patients and 60% of the patients show exclusive reactivity to Bet v 1. Two fragments of Bet v 1 were constructed with reduced anaphylactic activity [5] and their suitability for immunotherapy of birch pollen allergic patients has been shown in extensive *in vitro* and *in vivo* studies [6-11]. A multi-centre, placebo-controlled immunotherapy study with the two hypoallergenic Bet v 1 fragments showed that the induction of blocking IgG antibodies, which inhibit allergen-induced basophil degranulation, IgE-facilitated allergen presentation to T cells and the boosting of allergen-induced IgE production, is an important mechanism underlying this treatment [12-16]. In this study, a mix of the two fragments has been used to treat birch pollen allergic patients which induced IgG₁ and IgG₄ responses to both fragments and the Bet v 1 allergen [17]. However, mouse immunization studies with the single components indicated that the N-terminal fragment rBet v 1 aa 1–74 has low immunogenicity [8]. Allergen-specific IgG antibodies were only induced with the N-terminal fragment when CFA was used as adjuvant, whereas the use of AluGel-S did not induce relevant IgG responses in mice [8]. It is well established that immunogenicity of poor immunogens such as haptens can be increased by coupling them to carrier molecules containing unrelated and new T cell epitopes [18-20] and it was shown that the immunogenicity of allergens/allergen peptides can also be increased by coupling them to virus-like particles [21], to immunostimulatory DNA sequences [22], to carrier proteins [20,23-25] or to other allergens and allergen derivatives [26-30]. Interestingly, an increase of immunogenicity was also obtained when proteins were made as oligomers without additional T cell epitope containing sequences. For example, it was shown that the immunogenicity of the major birch pollen allergen Bet v 1 and of IgE domains was increased when these molecules were made as oligomers [31,32].

In this study we have investigated whether it is possible to engineer trimeric forms of hypoallergenic rBet v 1-fragments which retain their low allergenic activity but exhibit increased immunogenicity. Trimers of hypoallergenic Bet v 1 fragments were expressed in *Escherichia coli* and purified to homogeneity. The IgE reactivity and allergenic activity of these molecules was studied in allergic patients. Furthermore, mice were immunized with the monomeric and trimeric forms to study the development of allergen-specific IgG antibodies and whether these IgG antibodies can inhibit allergic patients' IgE binding to the Bet v 1 allergen. We found that the rBet v 1 aa 1–74 trimer induced significantly higher levels of Bet v 1-specific IgG₁ antibodies than the monomer. Consequently, Abs induced with the rBet v 1 aa 1–74 trimer inhibited patients' IgE-binding to rBet v 1 wildtype more than 10-fold more efficiently than Abs induced with rBet v 1 aa 1–74 monomer. Our data thus show that the immunogenicity of small molecules with low immunogenicity can be increased by oligomerization of the same molecule.

2. Materials and methods

2.1. Human sera, antibodies, plasmid vectors, *E. coli* strains, recombinant allergens and derivatives

Forty-four birch pollen allergic patients (#1–44) were investigated. The patients were characterized by case history, skin prick testing with birch pollen extract and a serological analysis was performed as described to establish the diagnosis of birch pollen allergy [33]. A non-allergic individual (*N*) was included for control purposes.

A rabbit antiserum specific for Bet v 1 was obtained by immunization of New Zealand white rabbits with purified rBet v 1 as described [8]. Plasmid pET17b and *E. coli* strain BL21 (DE3) were purchased from Novagen (Madison, WI) [34]. Recombinant Bet v 1 and rBet v 1 derivatives (aa 1–74 and aa 75–160) were expressed in *E. coli* strain BL 21 (DE 3) (Novagen) and purified as described[5,35].

2.2. Expression of a rBet v 1 aa 1–74 and a rBet v 1 aa 75–160 trimer in *E. coli*

cDNAs coding for the three Bet v 1 aa 1–74 segments and the three Bet v 1 aa 75–160 segments of the trimers were obtained by PCR amplification using the Bet v 1 cDNA as template and the following oligonucleotide primers (MWG, Ebersberg, Germany): rBet v 1 aa 1–74 trimer:

rBet v 1 aa 1-74 trimer:

First segment:	Forward 5': GAGGAATTCATATGGGTGTTTCAATTAC 3'
	Reversed 5': CGGGGTACCAACTCATCAACTCTGTCCTT 3'
Second segment:	Forward 5': CGGGGTACCGGTGTTTCAATTACGAA3'
	Reversed 5': CCGAATTCAGTAGTGGCTCATCAACTCTGTCCTT 3'
Third segment:	Forward 5': CCGGWTCGGAAGTAGGTGTTTCAATTAC3'
	Reversed 5': CCGGMTTCCTATTACTCATCAACTCTGTCCTT3'.

rBet v 1 aa 75-160 trimer:

First segment:	Forward 5': GAGGAATTCATATGGTGGACCACACAAACTTC 3'
	Reversed 5': CGGGGTACCAAGTTGTAGGCATCGGAGTG 3'
Second segment:	Forward 5': CGGGGTACCGGTGGACCACACAAACTTC 3'
	Reversed 5': CCGAATTCAGTAGTGGTTGTAGGCATCGGAGTG 3'
Third segment:	Forward 5': CCGGAATTCGGAAGTAGTAGTGGACCACACAAACTTC 3'
	Reversed 5': CCGGAATTCCTATTAGTTGTAGGCATCGGAGTG 3'.

Eco R I (GAATTC), Nde I (CATATG), Kpn I (GGTACC) and Spe I (ACTAGT) sites in the primer sequences have been printed in italics.

The first DNA segments were cloned into the Nde I/Kpn I sites of plasmid pET 17b. These constructs were then digested with Kpn I/Spe I and the Kpn I/Spe I-digested second segments were inserted. The plasmids containing the two segments were digested with Spe I/Eco R I and the Spe I/Eco R I-digested third segment was inserted (Fig. 1A). The DNA sequences of the constructs were confirmed by sequencing both DNA strands on an

automated sequencing system (MWG). Recombinant rBet v 1-fragment trimers were expressed in *E. coli* BL21 (DE3) in liquid culture (luria broth medium containing 100 mg/l ampicillin) by induction with 0.5 mM isopropyl- β -thiogalactopyranoside (IPTG) at an optical density at 600 nm (OD₆₀₀) of 0.8 for 5 h at 37 °C. Cells were harvested by centrifugation at 4000 $\times$ g for 15 min at 4 °C.

2.3. Purification of rBet v 1 aa 1–74 trimer and rBet v 1 aa 75–160 trimer

Bacterial cells from 1 l culture were resuspended in 10 ml 25 mM imidazole pH 7.4, 0.1% (v/v) Triton X-100 and treated with 100 μ g lysozyme for 20 min at room temperature. The lysates of the bacterial cells were frozen three times in liquid nitrogen and defrosted in a 50 °C water bath. DNA was degraded by addition of 1 μ g DNa-seI and incubation for 10 min at room temperature. Cell debris was removed by centrifugation of the cell lysate at 10,000 g for 30 min at 4 °C in an SS34 rotor (RC5C Sorvall, DuPont, Boston, MA). Recombinant rBet v 1 aa 1–74 trimer and rBet v 1 aa 75–160 trimer were expressed in the inclusion body fractions which were isolated as described [36]. Inclusion bodies were solubilized in 8 M urea, 10 mM Tris-Cl, pH 8.0, 1 mM EDTA, 5 mM β -mercaptoethanol. The supernatants, containing the recombinant proteins, were dialyzed to a final concentration of 2 M urea and centrifuged for 15 min at 18,000 rpm to remove insoluble materials. The supernatants were applied to a DEAE Sepharose column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and the proteins were eluted with a combined 0–500 mM NaCl and pH 8–5 gradient. Fractions containing rBet v 1 aa 1–74 trimer or rBet v 1 aa 75–160 trimer were dialyzed against 6 M urea, 10 mM NaH₂PO₄, pH 4.8 and applied to a SP Sepharose Fast Flow column (GE Healthcare Bio-Sciences AB). The recombinant proteins were eluted with a 0–500 mM NaCl gradient and fractions containing more than 90% pure protein were pooled, dialyzed against 10 mM Na₂HPO₄ pH 7.0 and stored at –20 °C. The proteins were analyzed by SDS-PAGE [37] and by Coomassie brilliant blue staining [38]. The protein concentrations of rBet v 1 aa 1–74 trimer and rBet v 1 aa 75–160 trimer were determined with the Micro BCA Protein Assay Kit (Pierce, Rockford, IL) and by UV spectrometry [39].

2.4. IgE-reactivity of rBet v 1 and rBet v 1 derivatives

Purified recombinant Bet v 1, rBet v 1 aa 1–74, rBet v 1 aa 75–160, rBet v 1 aa 1–74-trimer, rBet v 1 aa 75–160-trimer and, for control purposes, BSA, were tested for IgE reactivity by non-denaturing dot blot assays. Two μ l of the proteins (0.25 mg/ml) were dotted onto nitrocellulose membrane strips (Schleicher & Schuell, Dassel, Germany) and incubated with sera from allergic patients with IgE-reactivity to Bet v 1 (dilution 1:10), or, for control purposes, with serum from a non-allergic individual, with a rabbit anti-rBet v 1 antiserum and the rabbit's pre-immune serum (dilution 1:1000) [40]. Bound IgE antibodies were detected with 1:10 diluted ¹²⁵I-labeled anti-human IgE antibodies (Phadia AB, Uppsala, Sweden), bound rabbit IgG antibodies were detected with 1:1000 diluted ¹²⁵I-labeled donkey anti-rabbit IgG antibodies (GE Healthcare Bio-Sciences AB) and visualized by autoradiography (Kodak XOMAT film; Kodak, Heidelberg, Germany).

2.5. Proliferation assays

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinised peripheral blood samples of four birch pollen allergic patients by Ficoll-Paque Plus (Amersham, GE Healthcare, Buckinghamshire, UK) separation. PBMCs (2×10^5) were cultured in triplicates in 200 μ l of Ultra Culture Medium (Lonza Group Ltd., Basel, Switzerland) supplemented with 2 mM L-glutamine (GIBCO, Invitrogen, USA), 50 μ M β -mercaptoethanol (GIBCO, Invitrogen), and 0.1 mg/ml gentamicin (GIBCO, Invitrogen) in the presence of rBet v 1, rBet v 1 aa 1–74 trimer and rBet v 1 aa 75–160 trimer (1 μ g/well). IL-2 (4 U/well) (Roche, Basel, Switzerland) was used as positive control and medium alone as negative control (six replicate wells each). Cells were incubated for 6 days at 37 °C in 96-well plates (Nunc, Roskilde, Denmark) in a humidified atmosphere containing 5% CO₂. At day six, 0.5 μ Ci per well of [³H] thymidine (Amersham, GE Healthcare) was added for the final 16 h of culture. Supernatants were harvested and incorporation of radioactivity was measured in counts per minute (cpm). The mean cpm from the triplicates was calculated and results are expressed as stimulation index which was calculated as the quotient of cpm in stimulated and in unstimulated cultures.

2.6. Allergen-induced upregulation of CD203c expression on allergic patients' basophils

Peripheral blood was collected from allergic patients with IgE-reactivity to Bet v 1 after informed consent was given. Blood samples (100 μ l) were incubated with serial dilutions (0.00005–0.5 nM) of rBet v 1, an equimolar mixture of rBet v 1 aa 1–74 trimer and rBet v 1 aa 75–160 trimer, anti-IgE (1 μ g/ml) (Immunotech, Marseille, France) or PBS for 15 min at 37 °C. After incubation, cells were washed in PBS containing 20 mM EDTA and incubated with 10 μ l of PE-conjugated mAb 97A6 specific for CD203c (Immunotech) for 15 min at room temperature. Thereafter, samples were subjected to erythrocyte lysis with FACS™ Lysing Solution (Becton Dickinson, San Jose, CA). Cells were then washed, resuspended in PBS, and analyzed by flow cytometry on a FACSCalibur (Becton Dickinson), using a Paint-a-Gate Software. Allergen induced upregulation of CD203c was calculated from mean fluorescence intensities (MFI) obtained with stimulated (MFI_{stim}) and unstimulated (MFI_{control}) cells, and expressed as stimulation index (SI = MFI_{stim}:MFI_{control}). An SI of 2.0 (2-fold upregulation) was considered to be indicative of a significant response [41].

2.7. Immunization of mice and measurement of Bet v 1-specific antibody responses

Eight-week-old female BALB/c mice were purchased from Charles River (Sulzfeld, Germany). Animals were maintained in the animal care unit of the Department of Pathophysiology and Allergy Research of the Medical University of Vienna according to the local guidelines for animal care. Groups of five mice each were immunized five times in monthly intervals with 5 μ g of purified rBet v 1 aa 1–74, rBet v 1 aa 75–160, rBet v 1 aa 1–74-trimer or rBet v 1 aa 75–160-trimer adsorbed to 200 μ l of AluGel-S (SERVA Electrophoresis, Heidelberg, Germany) by subcutaneous injections in the neck as described [42]. Blood samples were taken one day before each immunization (Immune sera 0–IV) and three months after the last immunization (Immune serum V) and stored at –20°C until use. IgG₁ responses to rBet v 1 wildtype were measured by ELISA as described [42]. Statistically significant differences between IgG₁ levels induced with rBet v 1 aa 1–74 and

rBet v 1 aa 1–74-trimer were assessed with unpaired Mann–Whitney tests. SPSS statistical software system (SPSS Inc., Chicago, IL) was used for calculations. The reported *p*-values are the results of a two-sided test and a *p*-value <0.05 was considered statistically significant.

2.8. Inhibition of allergic patients' IgE binding to rBet v 1 with rBet v 1 derivative-specific antibodies as determined by ELISA

ELISA plates (Greiner, Kremsmünster, Austria) were coated with purified rBet v 1 (5 µg/ml in PBS) overnight at 4 °C. Plates were washed two times with PBS, 0.05% (v/v) Tween 20 and blocked for 3 h at room temperature with PBS, 1% (w/v) BSA, 0.05% (v/v) Tween 20. Then plates were incubated overnight at 4 °C with mouse anti-rBet v 1 aa 1–74, anti-rBet v 1 aa 75–160, anti-rBet v 1 aa 1–74 trimer, anti-rBet v 1 aa 75–160 trimer, a mixture of anti-rBet v 1 aa 1–74 and anti rBet v 1 aa 75–160, or with a mixture of anti-rBet v 1 aa 1–74 trimer and anti rBet v 1 aa 75–160 trimer antibodies or, for control purposes, with the corresponding mouse pre-immune sera, diluted 1:20 in PBS, 0.5% (w/v) BSA, 0.05% (v/v) Tween 20. Immune sera were obtained shortly before the fourth immunization and pooled from each group for inhibition experiments. After washing for five times with PBS, 0.05% (v/v) Tween 20, plates were incubated overnight at 4 °C with 1:5 in PBS, 0.5% (w/v) BSA, 0.05% (v/v) Tween 20 diluted sera from birch pollen allergic patients. After washing the plates for five times with PBS, 0.05% (v/v) Tween 20, bound human IgE antibodies were detected with HRP-coupled goat anti-human IgE antibodies (KPL, Gaithersburg, MD), diluted 1:2500 in PBS, 0.5% (w/v) BSA, 0.05% (v/v) Tween 20, for 30 min at 37 °C and 30 min at 4 °C. Plates were again washed five times with PBS, 0.05% (v/v) Tween 20 and incubated in the dark with ABTS (60 mM citric acid, 77 mM Na₂HPO₄·2H₂O, 1.7 mM ABTS (Sigma, St. Louis, MO), 3 mM H₂O₂) until a colour reaction was visible. Absorbance was determined with an ELISA reader (Dynatech, Denkendorf, Germany) and the percentage reduction of human IgE binding after pre-incubation with the mouse immune sera was determined according to the formula: % inhibition of IgE binding = 100 – OD_I/OD_P × 100, where OD_I and OD_P represent extinctions after pre-incubation with immune serum and pre-immune serum, respectively.

3. Results

3.1. Expression and purification of rBet v 1 aa 1–74 trimer and rBet v 1 aa 75–160 trimer

Three copies of cDNAs coding for rBet v 1 aa 1–74 or rBet v 1 aa 75–160 containing cleavage sites for different restriction enzymes were ligated with each other. The resulting constructs contained an open reading frame from the Start-Codon of the first cDNA copy to the Stop-Codon of the third cDNA copy and were expressed in *E. coli* as recombinant fragment trimers. Expression resulted in stable recombinant trimers of rBet v 1 aa 1–74 and rBet v 1 aa 75–160 where the three fragments in each of the trimers were separated by spacers of three amino acids (LVP, PLV) as shown in Fig. 1A. Similar as the isolated rBet v 1 fragments, the Bet v 1 fragment-trimers accumulated in the inclusion body fraction of *E. coli* but the level of expression of the trimers was considerably higher (>10-fold) than that of the isolated fragments (data not shown). After solubilization in urea, the recombinant fragment trimers were purified to homogeneity by ion-exchange chromatography. When

urea had been removed by dialysis, the proteins remained soluble at concentrations of 0.2 mg/ml in physiological buffers. Coomassie blue-stained SDS-PAGE indicated that the proteins were more than 90% pure (Fig. 1B). The protein migration observed in SDS-PAGE corresponded to the molecular masses of the trimers (24.7 kDa and 29.2 kDa) calculated from their deduced amino acid sequences (Fig. 1B).

3.2. rBet v 1 fragment-trimers show reduced IgE-reactivity comparable to the isolated rBet v 1 fragments

In order to compare the IgE reactivity of the recombinant fragment trimers with that of the isolated fragments and rBet v 1 wildtype, non-denaturing dot blot assays were performed (Fig. 2). Sera from 26 birch pollen allergic patients (lanes 1–26) showed IgE-reactivity to nitrocellulose-dotted rBet v 1 wildtype, whereas almost no IgE-reactivity was found to the isolated rBet v 1 fragments or the rBet v 1 fragment-trimers. Only one patient showed weak IgE-reactivity to rBet v 1 aa 75–160 (#24) and one to rBet v 1 aa 1–74-trimer (#6) (Fig. 2). A rabbit antiserum raised against rBet v 1 showed reactivity with rBet v 1 wildtype and also reacted with each of the rBet v 1 derivatives, indicating that the proteins had been transferred to the membrane (data not shown). Serum from a non-allergic person (lane N) did not show reactivity with any of the proteins. None of the patients showed IgE reactivity to the control protein, BSA (Fig. 2).

3.3. rBet v 1 fragment-trimers induce lymphoproliferative responses in PBMCs from birch pollen allergic patients

Using Bet v 1-specific T cell clones established from birch pollen allergic patients, it had been shown that the two rBet v 1 fragments aa 1–74 and 75–160 harbor the relevant T cell epitopes of complete Bet v 1 [5]. We used PBMCs from four birch pollen allergic patients (#27–30) to investigate whether the Bet v 1 derivatives induce lymphoproliferative responses. In those patients where rBet v 1 induced T-cell proliferation (i.e., SI > 1: patients 28–30), fragment trimers induced equal or even higher responses (Table 1).

3.4. rBet v 1 fragment-trimers display reduced allergenic activity as determined by CD 203c expression

To investigate the allergenic activity of the rBet v 1 fragment-trimers, basophils from ten birch pollen allergic patients were exposed to the derivatives and rBet v 1 wildtype and the upregulation of CD203c expression was measured by FACS (Fig. 3). Incubation of basophils with different concentrations of rBet v 1 wildtype led to significant (SI > 2) upregulation of CD203c expression between 0.0005 nM (e.g., patient 35) and 0.5 nM (e.g., patient 34) depending on the individual patients' sensitivity. An equimolar mixture of the two fragment trimers showed an at least 10-fold (e.g., patients 36, 37, 40) and up to 1000-fold (e.g., patient 31, 33, 35) reduction of allergenic activity compared to rBet v 1 wildtype. No upregulation was obtained with the buffer alone. Cells from each but one of the donors (i.e., patient 34) responded to crosslinking with 1 µg/ml of anti-IgE antibodies (Fig. 3).

3.5. The rBet v 1 aa 1–74 trimer induces much higher IgG₁ antibodies than the isolated rBet v 1 aa 1–74 protein

We have previously noted that the rBet v 1 aa 1–74 fragment induces only weak IgG₁ responses to the Bet v 1 wildtype allergen whereas rBet v 1 aa 75–160 induced strong Bet v 1-specific IgG₁ antibody responses [8,11]. Again we found that mice immunized with rBet v 1 aa 1–74 developed only very low IgG₁ responses to Bet v 1 wildtype which became detectable only after several courses of immunization (Fig. 4A). When mice were immunized with the rBet v 1 aa 1–74 trimer a much stronger Bet v 1-specific IgG₁ response could be induced which became detectable earlier (i.e., after the second immunization) and the IgG₁ responses after the third immunization were significantly higher than those obtained with the isolated fragment (Fig. 4A). The magnitude of Bet v 1-specific IgG₁ levels induced with the rBet v 1 aa 1–74 trimer was almost comparable with that induced with the highly immunogenic rBet v 1 aa 75–160 fragment and the corresponding trimer (Fig. 4B). The levels of Bet v 1-specific IgG₁ antibodies were similar in mice which had been immunized with the isolated rBet v 1 aa 75–160 fragment and the corresponding trimer indicating that the already high immunogenicity the rBet v 1 aa 75–160 fragment could not be further increased with the trimeric form of the latter fragment (Fig. 4B).

3.6. Mouse antibodies induced with each of the rBet v 1 fragment trimers strongly inhibit human IgE binding to rBet v 1 wildtype

Mouse antibodies induced by immunization with the rBet v 1 fragments or rBet v 1 fragment trimers were compared for their ability to inhibit birch pollen-allergic patients' IgE binding to rBet v 1 wildtype in ELISA inhibition experiments (Table 2). The inhibition obtained with mouse anti-rBet v 1 aa 75–160 antibodies and anti-rBet v 1 aa 75–160 trimer antibodies was between 86 and 98% (mean 93%) and between 85 and 98% (mean 92%), respectively. Only marginal inhibition of birch pollen allergic patients' IgE-binding to rBet v 1 (0–22%, mean 7%) was obtained with sera from mice which had been immunized with the isolated rBet v 1 aa 1–74 fragment. The inhibition of IgE-binding was increased more than 10-fold when sera from mice immunized with the rBet v 1 aa 1–74 trimer (75–95%, mean 84%) were used for inhibition (Table 2). Mixtures of the mouse anti-rBet v 1 fragment antisera or the mouse anti-rBet v 1 fragment trimer antisera inhibited IgE binding to rBet v 1 between 87% and 98% (mean 92%) and between 85% and 98% (mean 92%), respectively (Table 2).

4. Discussion

Allergen-specific immunotherapy represents the only antigen-specific and disease-modifying therapy for allergy but is limited by poor quality of allergen extracts used for vaccine production [1]. Recombinant allergen-derivatives made to reduce the allergenic activity and thus side effects have been produced for several important allergens [43,44], but these derivatives may exhibit reduced immunogenicity [45–47]. It is known that the immunogenicity of low immunogenic peptides can be increased by coupling them to suitable carrier molecules which provide additional T cell epitopes [18,19]. Several recent studies showed that the immunogenicity of allergens/allergen derivatives can be increased by covalent binding to non-allergenic carrier molecules or unrelated allergens [20,23,24,26,27,30]. In this study we have produced covalently bound trimers of

hypoallergenic Bet v 1-fragments by genetic engineering in order to study if it is possible to increase the immunogenicity of one of the weakly immunogenic fragments comprising the N-terminal portion of Bet v 1 (i.e., aa 1–74).

We found that trimeric forms of the hypoallergenic Bet v 1 fragments [5] also exhibited almost no IgE-reactivity in dot blot assays and showed strongly reduced allergenic activity when compared to Bet v 1 wildtype using basophils from birch pollen allergic patients. Thus, oligomerization of the fragments did not increase their IgE-reactivity and allergenic activity making them interesting candidate vaccines for the treatment of birch pollen allergy. rBet v 1 fragment-trimers induced similar or higher lymphoproliferative responses in PBMCs from birch pollen allergic patients indicating that Bet v 1-specific T cell epitopes were preserved.

Several studies indicated that the induction of allergen-specific IgG antibodies, which inhibit allergen-induced mast cell degranulation, IgE-facilitated T cell activation and boosts of secondary IgE responses, is an important mechanism in allergen-specific immunotherapy [12-16]. We were thus interested to investigate whether the trimeric forms of the fragments would induce stronger allergen-specific IgG responses compared to the isolated fragments. We therefore immunized mice with the Bet v 1 fragments and fragment trimers to study the induction of Bet v 1-specific IgG antibodies. Interestingly, rBet v 1 aa 1–74 trimer induced significantly higher Bet v 1-specific IgG₁ antibody levels than the monomeric fragment. As a consequence of the much stronger allergen-specific IgG₁ response, antibodies induced with the fragment trimer inhibited birch pollen allergic patients' IgE-binding more than 10-fold better than antibodies induced with the monomer. This finding was quite unexpected because the immunodominant Bet v 1 T-cell epitope for BALB/c mice is located in the C-terminal part of Bet v 1 whereas the N-terminal part of Bet v 1 seems to contain only minor T cell epitopes [48]. Yet it has been possible to induce Bet v 1-specific IgG responses when the fragment aa 1–74 was administered with a highly potent adjuvant (i.e., CFA) [8].

There are several explanations for the increased immunogenicity of the fragment 1 oligomer. First, it is possible that the few minor T cell epitopes present in the fragment become better presented in the context of the trimer at the level of antigen presentation. Second, it is possible that processing of a trimeric form results in a mixture of peptides which may bind better to the MHC or T cell receptors than those resulting from processing of the monomer. In this context it has been shown that peptide flanking residues which extend from the central MHC class II binding core can enhance T cell activation [49]. Alternatively it is possible that processing of the trimeric form which also contains a few foreign amino acids linking the individual fragments may have resulted in the formation of new T cell epitopes. Moreover, the trimeric form may be more potent than the monomer in activating specific B cells via their antigen receptor. In fact, we noticed that immunization with the trimeric form strongly augmented the secondary immune responses after the second immunization which would be in accordance with the latter assumption (Fig. 4A).

The C-terminal fragment which contains the immunodominant T cell epitope induced already in its monomeric form robust IgG production which is in agreement with our earlier results [8]. These IgG responses were not increased by immunization with the trimeric form.

It thus seems that oligomerization of antigens could be an approach for increasing the immunogenicity of poorly immunogenic structures. This assumption is supported by results from another study which demonstrated that immune responses against weakly immunogenic IgE domains were increased by oligomerization [32]. Another potentially important aspect for vaccine production is our finding that the level of expression of the trimeric forms of the fragments was more than tenfold higher than that of the monomers which may facilitate large scale production of the vaccines.

In summary, it seems that oligomerization of antigens could be a possibility to increase their immunogenicity by a mechanism different from the hapten carrier principle which may be exploited for vaccine production.

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Fig. 1.

(A) Overview of rBet v 1 derivatives. Recombinant Bet v 1 consists of 160 amino acids including the first methionine. Two hypoallergenic fragments comprise aa 1–74 and aa 75–160. A recombinant trimer of fragment aa 1–74 consists of three fragments of which the first two N-terminal fragments are linked by the spacer LVP and the two C-terminal fragments by the spacer PLV. A recombinant trimer of fragment aa 75–160 has been constructed in the same way. (B) Expression and purification of rBet v 1 and rBet v 1-derivatives. rBet v 1 and rBet v 1-derivatives were expressed in *E. coli* and purified to homogeneity by ion-exchange chromatography. Aliquots of 3 µg of each protein were analyzed by SDS-PAGE and Coomassie blue staining. In lane M, a molecular weight marker was loaded. Molecular weights are indicated in kDa on the left margin.

Fig. 2.

IgE-binding capacity of rBet v 1 and rBet v 1-derivatives. Sera from 26 birch pollen allergic patients (lanes 1–26) and from a non-allergic individual (lane N) were tested for IgE-reactivity with dot-blotted rBet v 1, rBet v 1 aa 1–74, rBet v 1 aa 75–160, rBet v 1 aa 1–74 trimer, rBet v 1 aa 75–160 trimer and BSA. Bound IgE-antibodies were detected with ^{125}I -labeled anti-human IgE antibodies.

Fig. 3.

Reduced allergenic activity of rBet v 1 derivatives compared to rBet v 1, as measured by CD203c expression. Blood samples from 10 birch pollen allergic patients (patients 31–40) were exposed to rBet v 1, an equimolar mixture of rBet v 1 aa 1–74 trimer and rBet v 1 aa 75–160 trimer (F1trimer + F2trimer) (0.5–0.00005 nM), anti-IgE (1 µg/ml) or buffer (0) (x-axes). Up-regulation of CD203c expression is expressed as stimulation index (SI) (y-axes).

Fig. 4.

Bet v 1-specific IgG₁ antibodies induced by immunization of mice with hypoallergenic rBet v 1-derivatives. Groups of 5 mice each were immunized monthly with 5 µg of rBet v 1 aa 1–74 or rBet v 1 aa 1–74 trimer (A), rBet v 1 aa 75–160 or rBet v 1 aa 75–160 trimer (B). Blood samples were taken shortly before each immunization and tested for IgG₁-reactivity with rBet v 1-wildtype in an ELISA assay. On the x-axis, the preimmune sera (lanes 0) and the immune sera (lanes I–V) are shown. The optical densities (OD) corresponding to the amount of bound antibodies are displayed on the y-axis. Data are expressed as mean ± SD. Statistically significant differences of antibody reactivity ($p < 0.5$) between mouse-groups are indicated for the different blood samples.

Table 1

Lymphoproliferative responses to rBet v 1 and rBet v 1 fragment trimers PBMCs from birch allergic patients (27–30) were stimulated with rBet v 1, rBet v 1 aa 1–74 trimer and rBet v 1 aa 75–160 trimer. The stimulation indices (Sis) are listed for each patient and antigen.

	<u>Patients</u>			
	27	28	29	30
rBet v 1	1.0	1.8	3.6	1.1
rBet v 1 aa 1–74 trimer	1.1	8.0	13.3	1.5
rBet v 1 aa 75–160 trimer	1.1	4.7	15.6	4.3

Table 2

Inhibition of allergic patients' IgE-binding to rBet v 1 wildtype with mouse-antibodies induced with rBet v 1-derivatives. ELISA-plate bound rBet v 1 was preincubated with mouse immune sera induced with rBet v 1-derivatives or with the corresponding preimmune sera and subsequently incubated with sera from eight birch pollen allergic patients (patients 1, 2,4,5,41–44). Bound IgE antibodies were detected with an HRP-conjugated anti-human IgE antiserum. The percentage inhibition of serum IgE-binding to rBet v 1 is displayed for each patient and as mean inhibition for the group.

	Patients #								Mean
	1	2	4	5	41	42	43	44	
% Inhibition of IgE-binding									
rBet v 1 aa 1–74	13	12	0	0	4	5	22	0	7
rBet v 1 aa 1–74 trimer	82	75	81	88	93	95	82	79	84.38
rBet v1 aa 75–160	93	88	86	98	98	96	94	93	93.25
rBet v1 aa 75–160 trimer	92	88	85	98	97	96	92	89	92.13
rBet v1 aa 1–74 +rBet v 1 aa 75–160	92	90	87	98	98	95	89	92	92.63
rBet v 1 aa 1–74 trimer + rBet v 1 aa 75–160 trimer	92	88	85	98	97	97	93	89	92.38