

Genetic engineering of a hypoallergenic trimer of the major birch pollen allergen Bet v 1¹

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SPECIFIC AIMS

Our aim was to obtain by genetic engineering a recombinant allergen derivative with profoundly reduced allergenic activity but that would preserve structural features, B cell as well as T cell epitopes of the wild-type allergen. A recombinant trimer consisting of three covalently linked copies of one of the most frequent environmental allergens, the major birch pollen allergen, Bet v 1 was expressed in *Escherichia coli* and analyzed for secondary structure content by circular dichroism (CD). The presence of immunoglobulin E (IgE) epitopes was investigated by IgE binding/competition assays; allergenic activity was analyzed by basophil histamine release and skin testing in allergic patients. T cell epitopes and the cytokine release profile were studied using cultured T cells from sensitized patients. We also investigated whether rBet v 1 trimer induces IgG antibodies in vivo that block patient's IgE binding to Bet v 1 and related allergens.

PRINCIPAL FINDINGS

1. Recombinant Bet v 1 trimer contains Bet v 1-specific IgE and IgG epitopes

Stable recombinant dimers and trimers of Bet v 1 were generated by expressing two or three copies of the Bet v 1 cDNA, linked by short oligonucleotide spacers with an open reading frame, in *E. coli*. We found that *E. coli*-expressed rBet v 1 monomer, dimer, and trimer exhibited a comparable ability to bind IgE antibodies from allergic patients and rabbit antisera raised against the recombinant monomer and trimer, respectively (Fig. 1A). When analyzed by ELISA competition, the oligomers inhibited IgE binding to plate-bound monomer, albeit less efficiently than the monomer itself (Fig. 1B).

The Far-UV CD spectra of the recombinant covalently

linked Bet v 1 dimer and trimer (Fig. 1C) exhibited the overall shape typical of folded proteins with mixed α -helical/ β -sheet secondary structure and were similar to those of recombinant monomeric Bet v 1.

2. Recombinant Bet v 1 trimer exhibits profoundly reduced allergenic activity

The in vitro allergenic activity was studied by exposing basophils from birch pollen-allergic patients to various equimolar concentrations of rBet v 1 monomer, dimer, or trimer. rBet v 1 dimer and trimer induced a profoundly reduced release of preformed (histamine) as well as de novo synthesized (leukotriene) mediators (Fig. 2) when compared with the monomer. The trimer, which exhibited in all donors a 100-fold or even greater reduction of mediator release vs. the monomer (Fig. 2a-f). The dimer induced significantly fewer skin reactions (mean wheal diameter: mean \pm SD) than the monomer at 10 μ g/ml (monomer: 7.33 \pm 1.9; dimer: 4.1 \pm 2.4) ($P=0.027$) and at 100 μ g/ml (monomer: 13.08 \pm 4.5; dimer: 7.3 \pm 2.5) ($P=0.0018$). The reduction in allergenic activity of the trimer vs. monomer was even more significant (10 μ g: trimer: 0.7 \pm 1.2; $P=0.0001$; 100 μ g: trimer: 3.6 \pm 2.1; $P=0.0001$).

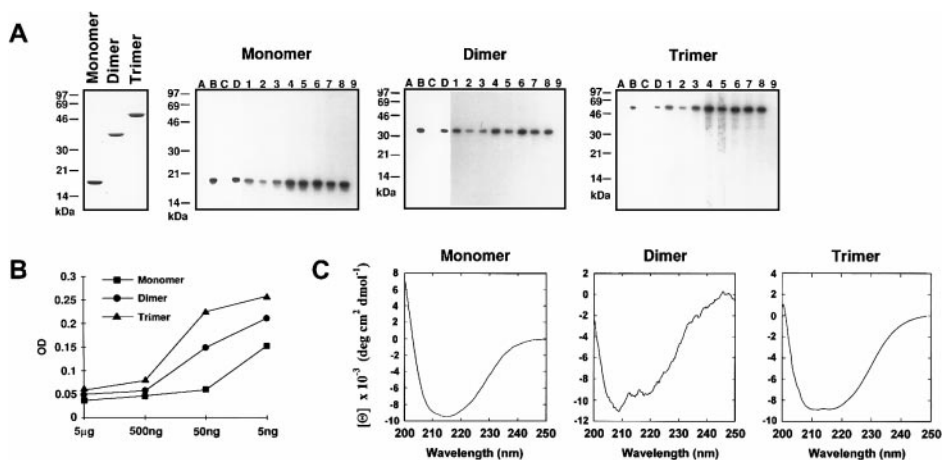
3. rBet v 1 trimer induces proliferation and release of Th1 cytokines in Bet v 1-specific T cells

In five experiments performed with equimolar antigen doses, the trimer induced significantly higher periph-

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Figure 1. Characteristics of purified recombinant Bet v 1 monomer, dimer, and trimer. *A*) SDS-PAGE containing 5 μ g aliquots of purified rBet v 1 monomer, dimer, and trimer. Reactivity of nitrocellulose-blotted monomer, dimer, and trimer with a rabbit anti-rBet v 1 monomer (lanes B), rabbit anti-rBet v 1 trimer antiserum (lanes D), and corresponding preimmune sera (lanes A, C), and with serum IgE from 8 birch pollen-allergic (lanes 1–8) and a non-atopic individual (lanes 9). Molecular mass is displayed in the left margins. *B*) Preincubation of the serum of a birch pollen-allergic patient with various concentrations (*x* axis) of rBet v 1 monomer, dimer, or trimer inhibits IgE binding to ELISA plate-bound rBet v 1 monomer (*y* axis: OD=optical density). *C*) CD spectra of the rBet v 1 monomer, dimer, and trimer. Results are expressed as mean residue ellipticity (*y* axis) at a given wavelength (*x* axis).



eral blood mononuclear cell (PBMC) proliferation than the monomer ($P < 0.05$). The rBet v 1 monomer and rBet v 1 trimer induced dose-dependent release of interleukin 4 (IL-4), IL-5, IL-10, IL-13, and interferon γ (IFN- γ). The rBet v 1 trimer induced significantly higher IFN- γ than the rBet v 1 monomer ($P < 0.05$). In contrast, the overall Th2 response, as demonstrated by IL-4, IL-5, and IL-13 ($P < 0.05$) production, was higher in rBet v 1 monomer-stimulated PBMC (data not shown). This divergence in cytokine profiles between monomer and trimer was also reflected by the significantly higher ratios of IFN- γ /IL-4, IFN- γ /IL-5, and IFN- γ /IL-13 ($P < 0.05$) induced by the rBet v 1 trimer.

The rBet v 1 dimer and trimer induced proliferative responses comparable to those induced by the rBet v 1 monomer in 21 Bet v 1-specific clones derived from different patients regardless their epitope specificities.

4. Recombinant Bet v 1 trimer induces protective antibodies in vivo that block human IgE binding to Bet v 1 and Bet v 1-related plant allergens

The rBet v 1 trimer induced in mice and rabbits IgG antibodies that cross-reacted with natural Bet v 1 and Bet v 1-homologous allergens in tree pollens (e.g., alder, hazel, hornbeam, and oak pollen) and plant food (e.g., apple, hazelnuts, carrots, celery) (data not shown). Trimer-induced rabbit antibodies inhibited human IgE binding to Bet v 1, Bet v 1-related pollen (alder: *Aln g 1*), and plant food allergens (apple: *Mal d 1*). Quantitative competition experiments with sera from 20 birch pollen-allergic patients showed that anti-trimer antibodies inhibited 17–95% (mean inhibition 80%) of IgE binding to Bet v 1 wild-type, which is

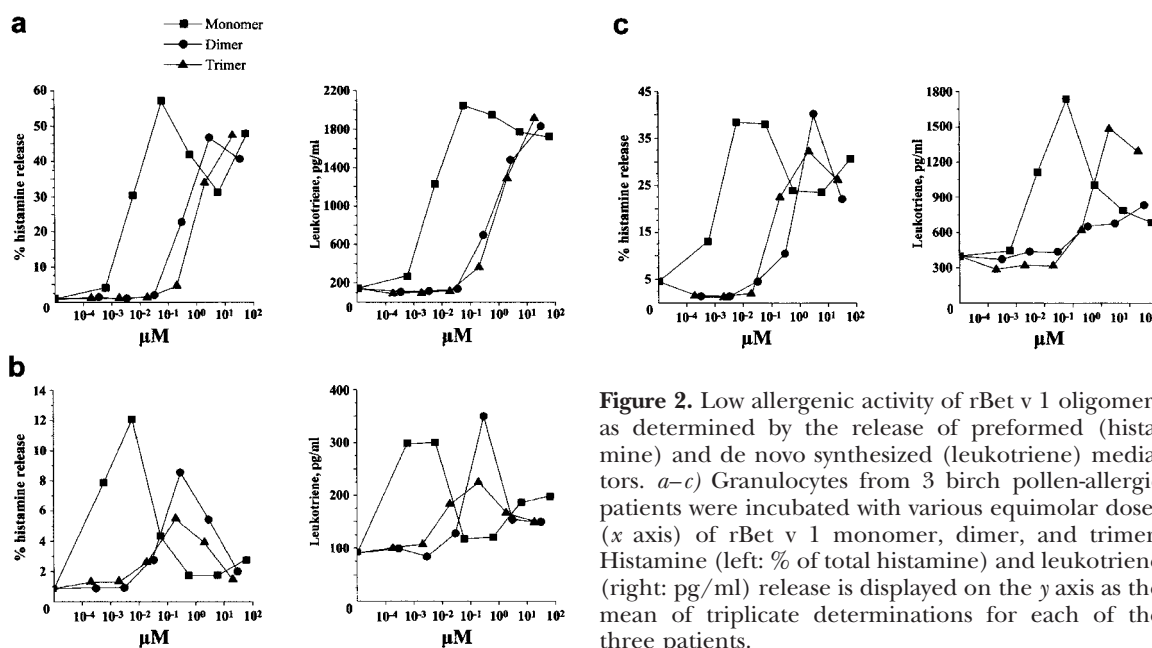


Figure 2. Low allergenic activity of rBet v 1 oligomers as determined by the release of preformed (histamine) and de novo synthesized (leukotriene) mediators. *a–c*) Granulocytes from 3 birch pollen-allergic patients were incubated with various equimolar doses (*x* axis) of rBet v 1 monomer, dimer, and trimer. Histamine (left: % of total histamine) and leukotriene (right: pg/ml) release is displayed on the *y* axis as the mean of triplicate determinations for each of the three patients.

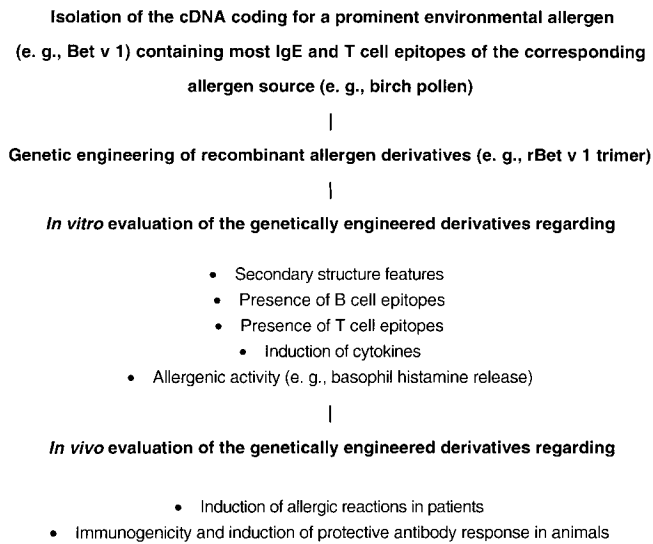


Figure 3. Schematic diagram illustrating the scheme for the construction and evaluation of hypoallergenic allergy vaccines.

in the range of the inhibition obtained with a rabbit anti-rBet v 1 monomer antiserum (data not shown).

CONCLUSIONS

In this study, we show that it is possible to genetically engineer a major allergen so as to profoundly reduce its allergenic activity and to preserve those properties required for the induction of a protective immunity (i.e., blocking antibody responses) of a favorable Th1 type. The diagram in **Fig. 3** shows the scheme for the construction and evaluation of hypoallergenic allergy vaccines applied in this study.

It has been demonstrated that the allergenic activity of Bet v 1 and other allergens can be reduced by destroying IgE epitopes through disruption or mutation of the molecule. However, the Bet v 1 trimer described here represents the first genetically engineered hypoallergenic allergen derivative that simultaneously contained Bet v 1-specific B cell (IgE, IgG) and T cell epitopes as well as a secondary structure similar to the wild-type allergen. There are several, not mutually exclusive explanations for this behavior. 1) It is possible that the covalent tail-to-head linking of monomeric units causes local relative reorientation of epitopes without changing the secondary structure. Although reoriented IgE epitopes would still be able to bind IgE antibodies, they may cross-link FcεRI-bound IgE less efficiently. This assumption would agree with a study showing that the mode of FcεRI cross-linking may have different effects on cell activation and mediator release. 2) The different capacity of rBet v 1 trimer vs. the monomer to activate effector cells may result from IgE recognition of the derivatives with varying affinity/avidity. It has been shown that ligands with different affinities to their receptor can induce antagonistic effects upon binding to the corresponding receptor. 3) The third explana-

tion for the reduced allergenic activity of the trimer would be that microaggregation, steric hindrance, and/or unfavorable charge interactions hide some of the IgE epitopes required for efficient cross-linking. The latter possibility would be consistent with our finding that the trimer inhibited IgE binding to monomer less efficiently when used in ELISA competition studies.

The profoundly reduced allergenic activity of rBet v 1 trimer together with its ability to induce IgG antibodies *in vivo* that block the binding of birch pollen-allergic patients IgE to the wild-type allergen suggests that it can be used as a vaccine to treat birch pollen allergy and perhaps allergies to pollen and plant-derived food containing Bet v 1-related allergens. This assumption is supported by our finding that trimer-induced antibodies inhibited IgE binding to the major allergen of alder pollen Aln g 1 and the major apple allergen Mal d 1.

The decreased *in vitro* allergenic activity demonstrated for the rBet v 1 trimer in this study agrees with results from skin testing (**Fig. 2**). Two independent clinical studies comparing the allergenic activity of rBet v 1 monomer with that of rBet v 1 trimer in a group of 23 Swedish and 36 French birch pollen-allergic patients by skin prick and intradermal testing confirmed that rBet v1 trimer had a >100-fold reduced allergenic activity than the monomeric wild-type molecule.

The possibility of administering high doses of the hypoallergenic rBet v 1 trimer together with its ability to stimulate an altered cytokine expression profile will thus likely favor a Bet v 1-specific Th0-Th1 immune response associated with production of blocking IgG antibodies. Trimer-induced blocking antibodies may contribute to clinical improvement by at least two mechanisms that have been described for conventional immunotherapy. First, they may suppress allergen-induced activation of effector cells, block mediator release and thus reduce immediate symptoms. Second, blocking antibodies may inhibit IgE-mediated presentation of allergens to T cells, suppress T cell activation and release of Th2 cytokines, and thus prevent chronic symptoms and disease progression.

To the best of our knowledge, rBet v 1 trimer represents the first modification of an allergen achieved by genetic engineering in which, despite preservation of secondary structure, T cell and B cell epitopes of the wild-type molecule exhibited a profoundly reduced allergenic activity. It may therefore serve as a paradigmatic model for the development of a novel generation of genetically modified hypoallergenic allergen derivatives that preserve the immunogenic and (perhaps) tolerogenic properties of the corresponding wild-type allergens. Since >95% of birch pollen-allergic patients are sensitized against Bet v 1, we estimate that the hypoallergenic trimer vaccine can be applied to treat almost 100 million allergic patients. **[F]**

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