

## Letter to the Editor

**Nasal application of rBet v 1 or non-IgE-reactive T-cell epitope-containing rBet v 1 fragments has different effects on systemic allergen-specific antibody responses***To the Editor:*

Allergen-specific IgE antibodies are key elements in allergic inflammation and in the immunoregulation of allergic disease.<sup>1</sup> Mucosal allergen contact induces allergic inflammation and also boosts systemic allergen-specific IgE production, which leads to increased sensitivity of mast cells and basophils, to the upregulation of Fcε receptor expression on allergic effector cells and antigen-presenting cells, and thus to increased T-cell activation.<sup>1-3</sup> Increases in allergen-specific IgE levels are also associated with increased clinical sensitivity and aggravation of symptoms.<sup>4,5</sup>

Unwanted boosts of allergen-specific IgE production occur during seasonal allergen contact through the respiratory mucosa, which can be reproduced in clinical studies by controlled nasal allergen provocation.<sup>4</sup> However, strong boosts of IgE production are also observed in the course of allergen-specific immunotherapy, reaching up to 5-fold of pretreatment levels, for example, after sublingual immunotherapy when allergens are applied through the mucosa.<sup>6</sup> In immunotherapy studies performed with recombinant allergen derivatives, which have been engineered to eliminate IgE reactivity and to preserve specific T-cell epitopes, only mild boosts of allergen-specific IgE production were observed that did not cause clinically relevant sensitization.<sup>7,8</sup>

Here we conducted a nasal provocation test study with rBet v 1, the major allergen of birch pollen, and 2 non-IgE-reactive rBet v 1 fragments. These fragments have previously been shown to induce proliferation in 9 different T-cell clones specific for 8 different T-cell epitopes spread over the whole Bet v 1 molecule, indicating that the combination of the 2 fragments harbors all T-cell epitopes of the complete Bet v 1 molecule.<sup>9</sup> The aim of the present study was to investigate the effects of nasal application of these molecules on systemic allergen-specific IgE production, on allergen-specific IgG, IgA, and IgM antibody levels and on allergic symptoms. We hypothesized that in allergic patients nasal contact with T-cell epitopes from a relevant allergen without concurrent contact with B-cell epitopes from the same allergen would not be sufficient to induce a secondary IgE response.

Nineteen patients with birch pollen allergy were enrolled in the study to receive either nasal Bet v 1 (patient B1-B10) or Bet v 1 fragments (patients F1-F9, see Table E1 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). All patients had symptoms of allergic rhinitis during the birch pollen season, and birch pollen allergy was confirmed by means of standard skin prick testing with birch pollen extract (Allergopharma, Reinbek, Germany) and Bet v 1-specific serum IgE levels of greater than 0.7 kU/L at the screening visit. Patients with unstable lung function, persistent allergy (as defined by the Allergic Rhinitis and Its Impact on Asthma working group, [www.whiar.org](http://www.whiar.org)), or airway infection or those who had used antihistamines 2 weeks before or corticosteroids 3 months before the study were excluded. Subjects were randomly allocated to the 2 study groups, and demographic data, allergic history, and sensitization profiles in the 2 groups were comparable at the beginning of the study (see Table E1).

Single-blind nasal provocation was performed on 2 consecutive days (days 1 and 2 of the study). Nasal provocation was followed by anterior rhinomanometry to examine inflammation-induced nasal blockage, and provocation-induced symptoms were evaluated by using a symptom score. Blood samples were obtained on the first day of nasal provocation and 1, 2, 4, 6, and 8 weeks thereafter and were stored at  $-20^{\circ}\text{C}$  until use (study protocol, see Fig E1 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). The study was performed in winter outside the pollen season with the approval of the local ethics committee, and written informed consent was obtained from each patient.

Recombinant Bet v 1 was from BIOMAY (Vienna, Austria), and the 2 recombinant fragments (F1 and F2) were expressed in *Escherichia coli* and purified as previously described.<sup>9</sup> For nasal provocation, rBet v 1, F1, and F2 were reconstituted and diluted in sterile 0.9% sodium chloride solution at 20  $\mu\text{g/mL}$ . F1 and F2 were then mixed 1:1 to obtain nearly equimolar solutions. Recombinant antigens were administered as previously described,<sup>10</sup> yielding a cumulative dose of 1.6  $\mu\text{g}$  of recombinant antigen per study day. Nasal flow was measured at 150 Pa by means of anterior rhinomanometry after measurements had been performed without administration of test substances to determine baseline levels, 10 minutes after application of 0.9% sodium chloride solution to rule out any nonspecific hyperreactivity, and 20 minutes after provocation with the test solutions to determine nasal flow reduction.<sup>10</sup> In addition, patients were asked to score their symptoms of nasal itching, nasal congestion, and nasal discharge by using a 4-point scale (0, no symptoms; 1, mild symptoms; 2, moderate symptoms; and 3, severe symptoms) and to count the number of sneezes (0, no sneeze; 1, 1-5 sneezes; 2, 6-10 sneezes; and 3,  $\geq 11$  sneezes). The scores were added to calculate a total nasal symptom score with a maximum of 12 points.

In the 10 patients who underwent nasal provocation with 1.6  $\mu\text{g}$  of the rBet v 1 allergen (patients B1-B10), we observed a significant decrease of nasal flow 20 minutes after nasal provocation on both study days (day 1:  $-49.3\% \pm 22.0\%$ ,  $P < .001$ , Fig 1, A; day 2:  $-48.7\% \pm 22.3\%$ ,  $P < .001$ , Wilcoxon signed-rank test). This reduction in nasal flow was observed in all patients and on both provocation days. By contrast, nasal provocation with 1.6  $\mu\text{g}$  of the 2 fragments did not significantly affect nasal flow (day 1:  $-7.3\% \pm 16.5\%$ ,  $P = \text{not significant}$ , Fig 1, B; day 2:  $+14.4\% \pm 78.5\%$ ,  $P = \text{not significant}$ , Wilcoxon signed-rank test).

Nasal provocation with Bet v 1 led to a significant increase of symptoms (symptom score on day 1: 7.1, Fig 1, B; day 2: 7.0), whereas nasal exposure to the 2 fragments did not cause any relevant symptoms (average symptom scores on day 1: 0.6, Fig 1, B; day 2: 0.7). None of the patients in either study group had late-phase reactions ( $>4$  hours after nasal provocation). The difference between the effect of Bet v 1 and the 2 fragments was statistically significant regarding both flow reduction ( $P < .001$ , Wilcoxon rank sum test) and induction of allergic symptoms ( $P < .001$ , Wilcoxon rank sum test) for both days of nasal provocation.

The blood samples obtained immediately before and 4 weeks after nasal provocation were analyzed for Bet v 1-specific IgE levels by using the Phadia ImmunoCAP system (Phadia, Uppsala, Sweden). All serum analyses were performed by an investigator who was not aware of whether the patients had

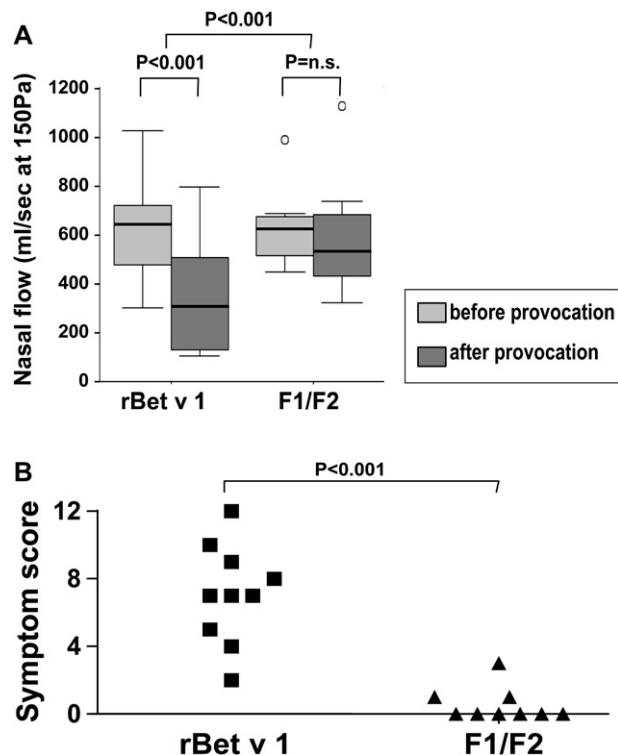


**FIG 1.** rBet v 1 but not the non-IgE-reactive rBet v 1 fragments reduces nasal flow and induces allergic symptoms. **A**, Box plots showing the reduction of nasal flow before or after provocation with rBet v 1 (x-axis) or rBet v 1 fragments (x-axis). Median values and outliers are indicated. **B**, Symptom scores (y-axis) after the first nasal provocation for patients who had received rBet v 1 (x-axis, squares) or fragments (x-axis, triangles). *P* values are shown.

received rBet v 1 or Bet v 1 fragments during nasal provocation (observer blind).

We found that nasal provocation with rBet v 1 was followed by an increase in Bet v 1-specific IgE antibodies 4 weeks after nasal provocation ( $P < .01$ , Wilcoxon signed-rank test, Fig 2; mean IgE levels before provocation, 22.6 kUA/L; mean IgE levels 4 weeks after provocation, 34.1 kUA/L; mean individual increase, +39.3%). The relative difference in IgE levels compared with baseline values between patients who received nasal Bet v 1 and nasal fragments was statistically significant ( $P = .02$ , Wilcoxon rank sum test). By contrast, 4 weeks after nasal provocation with the fragments, IgE levels to Bet v 1 remained unchanged (Fig 2). The samples obtained immediately before and 1, 2, 4, 6, and 8 weeks after nasal provocation were also analyzed for Bet v 1- and fragment-specific IgE, IgA, IgM, and IgG1 through IgG4 levels by means of ELISA. A detailed description of the ELISA experiments is given in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org). The time course of the development of IgE levels as determined by means of ELISA is shown in Fig E2 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org). IgE levels reached their maximum after 4 weeks and began to decrease 6 weeks after nasal provocation.

We observed no relevant changes in Bet v 1-specific and fragment-specific IgA, IgM, and IgG1 through IgG4 levels after nasal provocation with rBet v 1 and after provocation with the 2 fragments (see Fig E2, data for fragment-specific antibody levels and IgG3 levels are not shown).

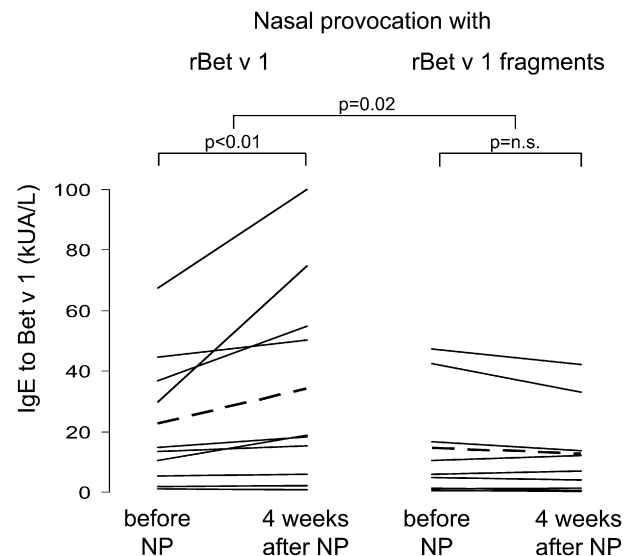


**FIG 2.** Bet v 1-specific IgE levels before and 4 weeks after nasal provocation (NP) with rBet v 1 and the rBet v 1 fragments. Bet v 1-specific IgE levels (kUA/L; y-axis) in sera obtained immediately before and 4 weeks after nasal provocation with rBet v 1 (left panel) and the 2 Bet v 1 fragments (right panel) are shown. Dashed lines represent mean values.

Our results thus demonstrate that the presence of IgE epitopes is required for the induction of allergen-specific IgE production through the nasal mucosa. Non-IgE-reactive allergen derivatives containing only the allergen-specific T-cell epitopes neither boosted allergen-specific IgE production nor induced *de novo* IgE responses. For the boost of IgE by allergens, at least 2 explanations are conceivable. First, allergens can directly activate allergen-specific B cells through surface immunoglobulin receptors to produce more IgE. Second, it is possible that the boosts of IgE production through mucosal allergen contact requires IgE-facilitated antigen presentation, whereas antigen presentation without IgE is not sufficient for the boosting of IgE responses.

Besides these mechanistic considerations, the results of this study might also have implications for the therapy and prevention of allergy. Non-IgE-reactive recombinant allergen derivatives might thus be useful for preventive and therapeutic tolerance strategies based on mucosal routes without inducing IgE-mediated side effects, unwanted IgE sensitizations, or boosts of IgE production.

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## ELISA MEASUREMENTS

ELISA plates (Nunc Maxisorb, Roskilde, Denmark) were coated with 5 µg/mL rBet v 1 (Biomay, Vienna, Austria) diluted in PBS- overnight at 4°C. Plates were blocked with PBS-T+BSA (PBS containing 0.05% vol/vol Tween 20 [PBS-T] and 0.5% wt/vol BSA) for 2 hours at room temperature and 2 hours at 4°C. Sera were diluted 1:5 for the detection of IgE antibodies and 1:50 for the detection of IgG1, IgG2, IgG3, IgG4, IgA, and IgM. Murine mAbs (IgE, clone G7-26; IgG1, clone JDC-1; IgG2, clone G18-21; IgG4, clone JDC-14; IgA, clone G18-1; IgM, clone JDC-15 [all BD PharMingen, San Diego, Calif]; and IgG3, clone HP-6050 [Sigma, St Louis, Mo]) were used for the detection of immunoglobulins. A peroxidase-linked sheep anti-mouse IgG antibody (Amersham Biosciences, Little Chalfont, Buckinghamshire, United Kingdom) diluted 1:1,000 was used as a secondary antibody. The coating step and each incubation step were

followed by 5 washing steps with PBS-T. Each ELISA plate included a buffer control, a negative control (serum from a nonallergic subject), and reference sera from patients with birch pollen allergy (positive control subjects) whose Bet v 1-specific IgE levels had been measured with the CAP system. To allow a comparison of OD values from different ELISA plates and to avoid plate-to-plate variability in OD measurements, results for each immunoglobulin isotype were normalized by using the positive control sera as a reference, as described previously.<sup>E1</sup> The cutoff level for positive IgE measurement was set at 2-fold the OD value measured for a negative control serum.

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**A**

|                |                      |   |    |    |    |    |
|----------------|----------------------|---|----|----|----|----|
|                | Nasal<br>provocation |   |    |    |    |    |
| Day            | ↓ ↓                  | 7 | 14 | 28 | 42 | 56 |
| -----          |                      |   |    |    |    |    |
| Blood sample # | 1                    | 2 | 3  | 4  | 5  | 6  |

**B**

|                              |                           |
|------------------------------|---------------------------|
| Recombinant Bet v 1: aa1-159 |                           |
| Fragment 1 (F1): aa1-73      | Fragment 2 (F2): aa74-159 |

**FIG E1.** **A**, Study design (timeline). **B**, Graphic depiction of Bet v 1 and the 2 rBet v 1 fragments. *aa*, Amino acid.

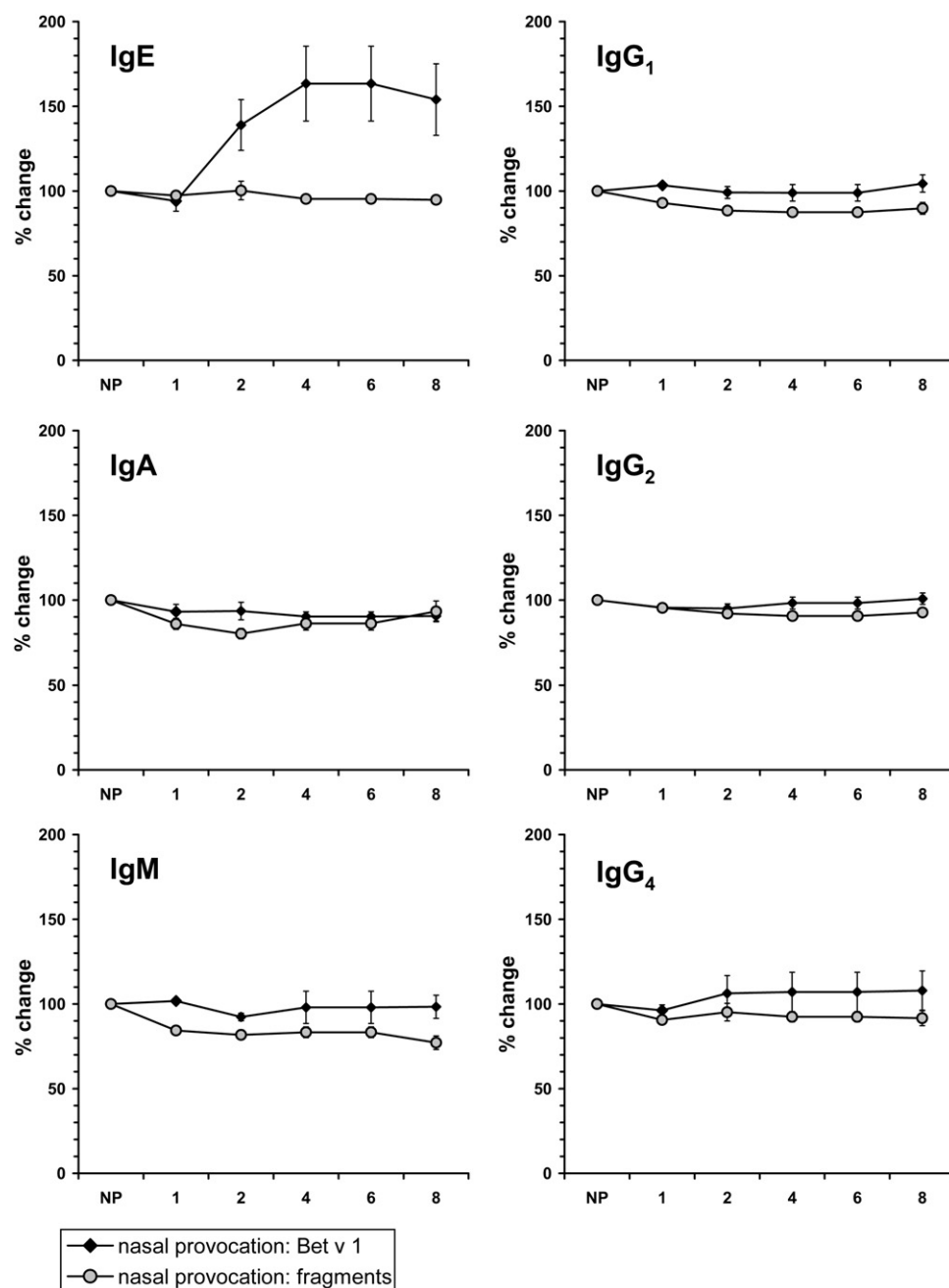


**FIG E2.** Time course of Bet v 1-specific IgE, IgG<sub>1</sub>, IgG<sub>2</sub>, IgG<sub>4</sub>, IgA, and IgM antibody responses in patients after nasal administration of rBet v 1 or fragments. Percentage changes in Bet v 1-specific IgE, IgG<sub>1</sub>, IgG<sub>2</sub>, IgG<sub>4</sub>, IgA, and IgM antibody levels measured by means of ELISA (*y*-axes) in patients who had received nasal provocation (NP) with Bet v 1 (black diamonds) or fragments (gray circles) are displayed at the time of nasal provocation and at 1, 2, 4, 6, and 8 weeks thereafter (*x*-axes). SEMs are shown as error bars.

TABLE E1. Demographic and clinical data

| Patient no. | Age (y) | Sex | Sensitization     |           |                 | Nasal provocation |                                        |
|-------------|---------|-----|-------------------|-----------|-----------------|-------------------|----------------------------------------|
|             |         |     | Allergen*         | Symptoms† | Bet v 1 (kUA/L) | Provoking agent‡  | Duration of symptoms after provocation |
| F1          | 28.5    | F   | b, g, h           | r, c, a   | 1.87            | F1 + F2           | —                                      |
| F2          | 33.9    | F   | b, g, a, h, c     | r, c, a   | 0.84            | F1 + F2           | —                                      |
| F3          | 20.5    | F   | b, g, h           | r, c      | 14.3            | F1 + F2           | —                                      |
| F4          | 21.3    | M   | b, g, h, c        | r, c      | 36.6            | F1 + F2           | —                                      |
| F5          | 22.5    | F   | b, g, h, c, d     | r, c      | 17.0            | F1 + F2           | —                                      |
| F6          | 27.6    | F   | b, g, c           | r, c      | 16.3            | F1 + F2           | —                                      |
| F7          | 33.6    | M   | b, g, h           | r, c      | 0.94            | F1 + F2           | —                                      |
| F8          | 24.0    | F   | b, g, a, c        | r, c      | 1.09            | F1 + F2           | —                                      |
| F9          | 23.9    | F   | b, g, h, c        | r, c      | 9.13            | F1 + F2           | —                                      |
| B1          | 22.6    | F   | b, g, h           | r, c      | 36.2            | Bet v 1           | 60 min                                 |
| B2          | 23.9    | F   | b, g, h           | r, c, a   | 5.37            | Bet v 1           | 3-4 h                                  |
| B3          | 26.0    | M   | b, g, cl, a, h, c | r, c      | 12.9            | Bet v 1           | 2-3 h                                  |
| B4          | 25.9    | M   | b, g, h           | r, c, a   | 48.0            | Bet v 1           | 3-4 h                                  |
| B5          | 20.9    | F   | b, g              | r, c      | 1.91            | Bet v 1           | 3 h                                    |
| B6          | 30.7    | F   | b, g, h           | r, c      | 49.9            | Bet v 1           | 30-60 min                              |
| B7          | 25.6    | F   | b, g, h           | r, c      | 13.8            | Bet v 1           | NK                                     |
| B8          | 23.6    | M   | b, g, h           | r, c, a   | 1.18            | Bet v 1           | 30 min                                 |
| B9          | 24.4    | M   | b, g, h           | r, c      | 30.5            | Bet v 1           | 3-4 h                                  |
| B10         | 20.7    | F   | b, g, h, c        | r, c      | 16.6            | Bet v 1           | 1-2 h                                  |

F, Female; M, male; NK, not known.

\*a, *Alternaria* species; b, birch; c, cat; cl, *Cladosporium* species; d, dog; g, grasses; h, house dust mites.

†a, Asthma; c, conjunctivitis; r, rhinitis.

‡F1, Fragment 1; F2, fragment 2.