# Naturally occurring hypoallergenic Bet v 1 isoforms fail to induce IgE responses in individuals with birch pollen allergy

**Stefan Wagner, PhD,[a] Christian Radauer, PhD,[a] Merima Bublin, PhD,[a] Karin Hoffmann-Sommergruber, PhD,[a] Tamara Kopp, MD,[b] Elli K. Greisenegger, MD,[b] Lothar Vogel, PhD,[c] Stefan Vieths, PhD,[c] Otto Scheiner, PhD,[a] and Heimo Breiteneder, PhD[a]** *Vienna, Austria, and Langen, Germany*

**Background: Engineered hypoallergens are currently being investigated for specific immunotherapy of allergic diseases in preclinical and clinical studies. Naturally occurring hypoallergens have by and large not been considered as a source of vaccine candidates.**
**Objective: Evaluation of the antibody response in atopic individuals induced by birch pollen containing isoforms of the major birch pollen allergen Bet v 1.**
**Methods: Isoform-specific antibody isotype responses for Bet v 1.0101, Bet v 1.0401, and Bet v 1.1001 were determined for 35 sera of individuals with birch pollen allergy. Isoform structures were compared and related to IgE-binding inhibitory capacities and induction of mediator release in human Fcε receptor transformed rat basophilic leukemia cells.**
**Results: Bet v 1.0101 induced a predominant IgE response, whereas the significant highest levels of $IgG_4$ antibodies were directed against Bet v 1.0401. Bet v 1.1001 induced only a minimal antibody response. Structural comparisons revealed that most of the amino acid differences between the isoforms were located on the protein surfaces. IgE induced by Bet v 1.0101 only partly cross-reacted with the 2 other isoforms and bound to them with notably lower affinity. Bet v 1.0401 and Bet v 1.1001 also were poor inducers of mediator release.**
**Conclusion: Bet v 1 isoforms possess highly variant immunogenic and allergenic properties. Bet v 1.0101 acts as the sensitizing agent, whereas Bet v 1.0401 and Bet v 1.1001 can induce only a minimal IgE response.**
**(J Allergy Clin Immunol 2008;121:246-52.)**

***Key words:*** *Bet v 1, Bet v 1.0101, Bet v 1.0401, Bet v 1.1001, isoforms, birch pollen allergy, hypoallergenic variant*

*Abbreviations used*
AP: Alkaline phosphatase
r: Recombinant
SIT: Specific immunotherapy

Allergen-specific immunotherapy (SIT) is an effective treatment for allergic diseases with a long-lasting relief of symptoms. The success of allergen-SIT is associated with the induction of IgG antibodies that intercept allergens before they are able to cross-link mast cell–associated IgE.[1] This is accompanied by a shift from $T_H2$ cytokines like IL-4 and IL-5 produced from $CD4^+$ T cells to $T_H1$ cytokines like IFN-γ,[2] and the induction of IL-10 and TGF-β producing $CD4^+CD25$ regulatory T cells inducing an anergic or tolerant state in peripheral T cells.[3]

Problems associated with allergen extract–based immunotherapy include the risk of inducing local and systemic side effects and the induction of new sensitizations.[4] Allergens with reduced IgE-binding capacity have been proposed to improve the safety of allergen-SIT. Numerous new candidates for safer immunotherapy were generated through great efforts of recent years in identifying allergens and in the production of recombinant proteins.[5,6]

For birch pollen allergy, the new vaccine strategies are focused on hypoallergenic variants of Bet v 1 because more than 62% of all patients with pollinosis exhibit IgE antibodies to this allergen.[7] Interestingly, the birch genome contains at least 7 pollen-expressed genes that encode distinct Bet v 1 isoforms with varying IgE reactivity.[8] Using such isoforms as recombinant proteins, IgE reactivity has been tested identifying high, moderate, or low IgE-binding proteins whereby the isoform Bet v 1.0101 showed the highest IgE-reactivity.[9] In contrast, the isoforms Bet v 1.0401 and Bet v 1.1001 showed very low IgE reactivity and were considered promising candidate molecules for SIT.[9,10] Bet v 1.0401 and Bet v 1.1001 showed higher stimulatory capacity for T cells than Bet v 1.0101.[9] High T-cell reactivity is another prerequisite for a candidate reagent for SIT. Bet v 1.0401 applied as a DNA vaccine in mice showed a different cellular response than Bet v 1.0101.[11] This difference was explained with a minimal sequence alteration of Bet v 1.0401 that could lead to different processing products with a reduced T-cell activation potency.[11] In contrast, Bet v 1.0401 induced a similar humoral response to Bet v 1.0101 in this model.

The humoral response directed against allergens is not restricted to allergen-specific IgE antibodies. Data on the humoral response of atopics and nonatopics to different allergens show that antibody titers change during immunotherapy. Subclasses of

From [a]the Department of Pathophysiology, Center for Physiology and Pathophysiology; and [b]the Division of Immunology, Allergy and Infectious Diseases, Department of Dermatology, Medical University of Vienna, Austria; and [c]the Paul Ehrlich Institute, Department of Allergology, Langen, Germany.

Supported by the Austrian Programme for Advanced Research and Technology of the Austrian Academy of Sciences (to S.W.) and grant SFB F01802 of the Austrian Science Fund (to M.B. and H.B.).

Disclosure of potential conflict of interest: K. Hoffmann-Sommergruber has consulting arrangements with Biomay. S. Vieths is an associate of the Institute for Product Quality and has received grant support from the European Union, the German Research Society, the Research Fund of the German Food Industry, and Monsanto Co. The rest of the authors have declared that they have no conflict of interest.

Received for publication May 9, 2007; revised August 1, 2007; accepted for Publication August 6, 2007.

Available online September 24, 2007.

Reprint requests: Heimo Breiteneder, PhD, Department of Pathophysiology, Center for Physiology and Pathophysiology, Medical University of Vienna, Austria EBO 3Q, Waehringer Guertel 18-20, 1090 Vienna, Austria. E-mail: heimo.breiteneder@meduniwien.ac.at.

0091-6749/$34.00



doi:10.1016/j.jaci.2007.08.006

IgG antibodies, especially $IgG_4$, are thought to capture the allergen before reaching the effector cell–bound IgE and thus prevent the activation of mast cells and basophils.[12] There are no data of individuals with allergy on the humoral response induced by hypoallergenic isoforms of allergens. We assessed the coexpression of IgE and IgG subclasses to the 3 isoforms Bet v 1.0101, Bet v 1.0401, and Bet v 1.1001 in individuals with allergy naturally exposed to birch pollen and demonstrated that small structural differences drastically influence immunogenicity. The isoforms Bet v 1.0401 and Bet v 1.1001 were not able to induce measurable IgE levels in individuals with birch pollen allergy, characterizing them as ideal candidates for allergy immunotherapy.

## METHODS

### Study group

Sera of 35 patients with documented clinical reactivity to birch pollen and specific IgE values to recombinant (r) Bet v 1.0101 (Immuno CAP System FEIA; Phadia, Uppsala, Sweden) were recruited from the Allergy Unit at the Department of Dermatology at the Medical University of Vienna. None of the patients had received an immunotherapy except patient 27 (from 1993 to 1996). Control sera were taken from healthy volunteers with no history of reactions to birch pollen. Blood sampling was authorized by the Ethical Committee of the Medical University of Vienna.

### Allergens

Recombinant Bet v 1.0101, Bet v 1.0401, and Bet v 1.1001 were purchased from Biomay (Vienna, Austria).

### Measurement of antibodies specific for rBet v 1.0101, rBet v 1.0401, and rBet v 1.1001 in ELISA

Maxisorp plates (Nalge Nunc, Rochester, NY) were coated with Bet v 1 isoforms (5 μg/mL in 50 mmol/L carbonate buffer, pH 9.6) overnight at 4°C. Plates were blocked with TRIS-buffered saline/0.5% vol/vol Tween-20 containing 3% milk powder. Patients' sera were diluted in TRIS-buffered saline/0.5% vol/vol Tween-20 1:5 for IgE and 1:50 for $IgG_1$, $IgG_2$, $IgG_3$, and $IgG_4$, added to duplicate antigen-coated wells, and incubated overnight at 4°C. After washing, bound $IgG_{1\text{-}4}$ antibodies were detected with monoclonal antihuman $IgG_1$, $IgG_2$, $IgG_3$, and $IgG_4$ antibodies (BD Pharmingen, San Diego, Calif), respectively, and an alkaline phosphatase–conjugated anti-mouse IgM+IgG antibody (Jackson ImmunoResearch, West Grove, Pa). Bound human IgE was detected using an AP-conjugated mouse monoclonal antihuman IgE antibody (BD Pharmingen). Color development was performed by addition of p-nitrophenyl phosphate (Sigma, St Louis, Mo). The absorbance was measured after 1 hour at 405 nm. To avoid plate-to-plate variabilities, all sera were tested on the same plate for each allergen and antibody subclass. Five control sera were included on each plate to establish a cutoff base line that was calculated by the mean value of the control sera plus 3 times SD.

For inhibition experiments, rBet v 1.0101 or rBet v 1.0401 was coated, and sera of patients were preincubated overnight with increasing concentrations (0, 0.01, 0.1, 1, and 10 μg/mL) of the 3 isoforms before they were applied to the plates. Color development was performed as described.

### Circular dichroism spectroscopy

Structural integrity of the allergens was determined using circular dichroism) spectroscopy. The samples were dialyzed against 10 mmol/L potassium phosphate buffer, pH 7.5, and measured at concentration of 0.2 mg/mL in a 0.1-cm quartz cuvette using a J-810 spectropolarimeter (Jasco, Easton, Md). Far UV spectra were recorded in the range between 190 and 260 nm with a resolution of 0.5 nm and a scanning speed of 50 nm/min. Data of 3 measurements were averaged.

### Homology modeling of Bet v 1.0401

The structure of Bet v 1.0401 was modeled by using the Swiss-Model server[13] with the structure of Bet v 1.1001 (PDB:1fm4) as the template.

### Mediator release assay

Degranulation assays using the humanized rat basophilic leukemia cell line RBL 730/21 were performed as previously described.[14] Cells were plated in 96-well flat-bottomed tissue culture plates ($10^5$/well) and passively sensitized overnight with human sera diluted 1:20. After washing the cells in Tyrode buffer (137 mmol/L NaCl, 2.7 mmol/L KCl, 0.5 mmol/L $MgCl_2$, 1.8 mmol/L $NaHCO_3$, 10 mmol/L HEPES and 0.1% BSA, pH 7.2), cross-linking of the FcεR-bound IgE was induced by adding 0.1 ng/mL to 100 ng/mL proteins in Tyrode buffer for 60 minutes at 37°C. Spontaneous release and nonspecific effects were measured by incubation of naive RBL cells with Tyrode buffer, cell culture medium, and patients' sera, respectively. β-Hexosaminidase release into the supernatant was measured by enzymatic cleavage of p-nitro-N-acetyl-β-D-glucosaminide (Sigma) in citrate buffer (0.4 mol/L, pH 4.5) at 405 nm (reference at 620 nm). Results are reported as percentage of total β-hexosaminidase release after addition of 1% Triton X-100 (Sigma).

### Statistical analysis

Statistical significance was assessed by the *t* test for paired samples. *P* values of less than .05 were considered significant.

## RESULTS

### Patients

Table I summarizes the data of subjects with birch pollen allergy. Twenty-three women and 12 men with a mean age of 49 years were analyzed. All patients had a history of moderate to severe seasonal allergic rhinoconjunctivitis and/or asthma and atopic dermatitis attributable to birch pollen. Sensitization to Bet v 1.0101 was confirmed for all patients by serologic measurement of IgE antibodies in the CAP system.

### Antibody responses to rBet v 1.0101, rBet v 1.0401, and rBet v 1.1001

Antibodies of several isotypes directed against the Bet v 1 isoforms were determined in ELISA. Patients' sera were numbered after their kU/L levels in the Bet v 1.0101 CAP starting with the lowest titers (Table I). All 35 sera displayed IgE to rBet v 1.0101 with antibody levels corresponding to the IgE titers obtained by the CAP assays, whereas 21 sera showed IgE-reactivity to rBet v 1.0401 and 22 sera to rBet v 1.1001 (Fig 1, *upper left panel*). For rBet v 1.0401 and rBet v 1.1001, IgE values were significantly lower ($P < .01$) than for Bet v 1.0101, and many of the IgE values were close to the baseline values.

Low levels of specific $IgG_1$ antibodies reactive with Bet v 1.0101 were detectable in sera of 10 individuals, and with rBet v 1.0401 or rBet v 1.1001 in 6 and 11 sera, respectively (Fig 1, *upper right panel*). Specific $IgG_2$ antibodies were displayed by 27 sera against rBet v 1.0101, by 21 sera against rBet v 1.0401, and by 15 sera against rBet v 1.1001 (Fig 1, *lower left panel*). $IgG_2$ antibody levels were moderate without significant difference for the 3 isoforms. $IgG_4$ antibodies reactive with rBet v 1.0101 were detectable in 25 sera, for rBet v 1.0401 in 14 sera, and for rBet v 1.1001 in 18 sera. The values were significantly higher for rBet v 1.0401 than for either rBet v 1.0101 ($P = .015$) or rBet v11 ($P = .043$). No specific antibodies were detectable for $IgG_3$.