Reshaping the Bet v 1 fold modulates T_H polarization

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Background: Several alternative mechanisms have been proposed to explain why some proteins are able to induce a T_H2-biased and IgE-mediated immune response. These include specific interactions with receptors of the innate immune system, proteolytic activities, allergen-associated carbohydrate structures, and intrinsic structural determinants.

Objectives: Available data suggest that a fold-dependent allergy-promoting mechanism could be a driving force for the T_H2-polarization activity of Bet v 1, the major birch pollen allergen.

Methods: Computer-aided sequence and fold analysis of the Bet v 1 family identified a short stretch susceptible for mutations inducing an altered fold of the entire molecule. With this knowledge, 7 consecutive amino acids of Bet v 1 were replaced with the homologous Mal d 1 sequence, creating the derivative BM4.

Results: The minimal changes of the sequence led to a loss of the Bet v 1–like fold and influenced the immunologic behavior. Compared to wild-type Bet v 1, BM4 induced elevated T-cell proliferation of human PBMCs. In the mouse model, immunization with Bet v 1 absorbed to aluminum hydroxide triggered strong T_H2 polarization, whereas BM4 immunization additionally recruited T_H1 cells. Furthermore, the fold variant BM4 showed enhanced uptake by dendritic cells and a decreased susceptibility to endo-/lysosomal proteolysis.

Conclusion: Modifications in the 3-dimensional structure of Bet v 1.0101 resulted in a change of its immunologic properties. We observed that the fold alteration led to a modified crosstalk with dendritic cells and a shift of the immune response polarization toward a mixed T_H1/T_H2 cytokine production. (J Allergy Clin Immunol 2011;127:1571-8.)

Key words: *Protein remodeling, tree pollen allergens, Bet v 1 family, T_H-cell polarization, IgE reactivity, allergenicity, antigen uptake, lysosomal degradation*

Recently the search of inherent properties of allergens causing a T_H2 immune polarization and the respective cytokine milieu led to the proposal of several alternative mechanisms of allergenicity. Interactions of allergens with receptors of the innate immune system, proteolytic activities, allergen-associated carbohydrate structures, and yet undefined intrinsic structural properties of allergens have been proposed as driving forces of allergenicity.[1] In this context, the intrinsic structural properties of Bet v 1, the major birch pollen allergen, have been intensively investigated. The protein has been shown to be the main cause of spring pollinosis in the temperate climate zone of the northern hemisphere. In addition, a large panel of Bet v 1–homologous allergens has been identified and described in many *Fagales* pollens.[2] Remarkably, only selected Bet v 1 isoforms seem to be accountable as main sensitizers and triggers of *Fagales* pollen allergies. Thus, recent studies focused on the comparison of immunogenicity as well as antigen uptake and processing of the high IgE-binding Bet v 1 isoform 0101 with the low IgE-binding isoform 0401. Despite an amino acid sequence identity of 95% and an almost identical tertiary structure, isoform Bet v 1.0401 was shown to induce a strong IgG but a moderate IgE immune response in a murine model, whereas Bet v 1.0101 induced high serum IgE titers and low IgG levels, a serologic status also reflected in patients with birch pollen allergy.[3] With murine bone marrow–derived dendritic cells (BMDCs), Bet v 1.0401 was shown to be more efficiently taken up than isoform 0101, stimulated an increased activation of costimulatory molecules, and caused an increased activation of IFN-γ–producing T cells compared with Bet v 1.0101.[4] Interestingly, the Bet v 1 homolog from celery, Api g 1, also showed this T_H1 skewing of T cells cultured with antigen-loaded human monocyte-derived dendritic cells (DCs) isolated from patients with birch pollen allergy as well as healthy donors.[5] Structural analysis of the Bet v 1 isoforms revealed that Bet v 1.0401 forms dimers via its single free cysteine, which is not present in isoform 0101. Therefore, the differences related to the decreased ability of Bet v 1.0401 to elicit an allergic immune response are probably a result of aggregate formation.[4] Likewise, although not investigated in detail, a similar process of dimerization could be accountable for the T_H1-polarizing mechanism of Api g 1,

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Abbreviations used
ALUM: Aluminum hydroxide
ANS: 8-Anilino-1-naphtalene sulfonic acid
APC: Allophycocyanin
BMDC: Bone marrow–derived dendritic cell
DC: Dendritic cell
DLS: Dynamic light scattering
FITC: Fluorescein isothiocyanate
FTIR: Fourier-transformed infrared
LAL: Limulus amebocyte lysate
RBL: Rat basophilic leukemia
SI: Stimulation index
WT: Wild-type

because the molecule also has a single free cysteine in its amino acid sequence. However, these observations do not explain the allergenic properties of Bet v 1.0101. Thus, we speculate that the fold of Bet v 1.0101 *per se* plays a role in the T_H2 polarization. To test this hypothesis, we identified a sequence on the backbone of Bet v 1.0101 that is highly sensitive to mutations because amino acid exchanges lead to a change in fold stability of the entire protein. With this knowledge, a destabilizing sequence stretch from apple Mal d 1 was grafted onto the Bet v 1 backbone. The resulting mutant allergen, BM4, was unable to adopt the typical Bet v 1–like fold, as was demonstrated by the characterization of the molecule in terms of physicochemical properties, aggregation state, folding, and antibody binding. Furthermore, T-cell activation, interaction with antigen-presenting cells, and resistance to digestion by endo-/lysosomal proteases isolated from DCs revealed functional differences between the derivative and the wild-type (WT) molecule.

METHODS

Patients and sera

Patients with *Fagales* pollen allergy were selected on the basis of case history, positive *in vivo* skin prick test, and *in vitro* IgE detection (CAP System; Phadia AB, Uppsala, Sweden). Patients with the IgE values greater than class 3 were selected. Experiments with blood samples from patients with pollen allergy were approved by the ethics committee of the Medical University and General Hospital of Vienna (no. EK028/2006). Informed written consent was obtained from all subjects included in the study.

Recombinant allergens

Recombinant Bet v 1.0101, termed *Bet v 1* hereafter, was purchased from Biomay AG (Vienna, Austria). Endotoxin content was 1.09 ng/mg protein as determined by limulus amebocyte lysate (LAL) assay (Associates of Cape Cod, Inc, East Falmouth, Mass).

Cloning and expression of BM4

The Bet v 1 variant BM4 was generated by PCR amplification of mutated fragments of *bet v 1.0101* (X15877) by using internal mismatch primers (see this article's Table E1 in the Online Repository at www.jacionline.org) as described in the subsection on cloning of BM4 in this article's Online Repository at www.jacionline.org. BM4 was cloned into a pET28b vector (Novagen; Merck KGaA, Darmstadt, Germany) by using NcoI and EcoRI restriction sites. Protein expression and purification was performed as described in the subsection on expression and purification of BM4 in this article's Online Repository at www.jacionline.org. Endotoxin content was 1.18 ng/mg protein as determined by LAL assay (Associates of Cape Cod, Inc).

Physicochemical analysis of recombinant proteins

Purity of recombinant proteins was analyzed by SDS-PAGE, identity by amino acid analysis and mass spectrometry,[6] secondary structure by circular dichroism, and homogeneity and aggregation behavior by dynamic light scattering (DLS) and online high-performance size exclusion chromatography light scattering.[7] A brief description of these analyses is given in the subsection on physicochemical analysis of recombinant proteins in the Online Repository at www.jacionline.org. Shelf life and storage stability of recombinant allergens were tested in 5 mmol/L sodium phosphate pH 7.4 at 4°C and –20°C, respectively, for 30 days and analyzed by dynamic light scattering.

Fourier-transformed infrared spectroscopy

Infrared spectra of BM4 and Bet v 1 were recorded at 0.5 and 1.0 mg/mL, respectively, in 5 mmol/L sodium phosphate by using the Confocheck Fourier-transformed infrared (FTIR) system (Bruker Optics, Ettlingen, Germany) equipped with a mercury cadmium telluride detector.[8] A description of the analysis is given in the subsection FTIR in the Online Repository at www.jacionline.org.

ELISA experiments

ELISA experiments using human serum samples in a 1:10 dilution were performed as described in the subsection ELISA experiments in this article's Online Repository at www.jacionline.org.

Activation of basophils

The allergenic potential of BM4 was measured by rat basophilic leukemia (RBL) assay as previously described.[9] Antigen-dependent β-hexosaminidase release into the supernatant was measured by enzymatic cleavage of the fluorogenic substrate 4-methyl umbelliferyl-N-acetyl-β-glucosaminide and expressed as a percentage of total enzyme content of Triton X-100–treated cells. Direct activation of human basophils from donors with birch pollen allergy was determined by upregulation of CD63 surface expression. Basophils from donors with allergy were stimulated with titrated concentrations of the proteins. Samples were stained with BD FastImmune CD63/CD123/anti–HLA-DR reagent (BD Biosciences, Franklin Lakes, NJ). Basophils were selected as $CD123^{high}$, side $scatter^{low}$, and $HLA\text{-}DR^{low}$ leukocytes, and CD63 upregulation was assessed in a minimum of 500 basophils per assay.[10]

T-cell proliferation assays

PBMCs obtained from donors with birch pollen allergy were stimulated with increasing concentrations of antigens as previously described.[11] The stimulation index (SI) was calculated as a ratio between counts per minute obtained in cultures with T cells plus autologous antigen-presenting cells plus allergen and counts per minute obtained in cultures containing T cells and antigen-presenting cells.

Animal experiments

Female BALB/c mice (Charles River Laboratories, Wilmington, Mass) were purchased at 8 to 10 weeks of age and used for experiments 4 days after arrival. All experiments including immunizations, ELISAs, and ELISPOT assays were performed at the same time. Animals were injected subcutaneously with 5 μg antigen adsorbed to Alugel-S (Serva, Heidelberg, Germany) given as two 50-μL subcutaneous injections administered bilaterally in the lumbar region and boosted on days 14, 21, and 42. Sera were collected on days 0, 21, 49, and 180. Per group, 8 animals were tested. Serum IgG_1, IgG_{2a}, and IgE was analyzed by ELISA as described.[4] Antigen-specific IL-2, IL-4, IL-5, IL-10, or IFN-γ–secreting splenic lymphocytes were analyzed by ELISPOT assay using matched-pair mAbs for IL-2, IL-4, IL-5, IL-10, or IFN-γ detection, respectively.[12] All animal experiments were conducted according