Correlation of sensitizing capacity and T-cell recognition within the Bet v 1 family

Claudia Kitzmüller, PhD,[a,b] Nora Zulehner, MSc,[b] Anargyros Roulias, MSc,[c] Peter Briza, PhD,[c] Fatima Ferreira, PhD,[c,d] Ingrid Faé, MSc,[e] Gottfried F. Fischer, MD,[e] and Barbara Bohle, PhD[a,b] *Vienna and Salzburg, Austria*

Background: Bet v 1 is the main sensitizing allergen in birch pollen. Like many other major allergens, it contains an immunodominant T cell–activating region (Bet v $1_{142-156}$). Api g 1, the Bet v 1 homolog in celery, lacks the ability to sensitize and is devoid of major T-cell epitopes.

Objective: We analyzed the T-cell epitopes of Mal d 1, the nonsensitizing Bet v 1 homolog in apple, and assessed possible differences in uptake and antigen processing of Bet v 1, Api g 1, and Mal d 1.

Methods: For epitope mapping, Mal d 1–specific T-cell lines were stimulated with overlapping synthetic 12-mer peptides. The surface binding, internalization, and intracellular degradation of Bet v 1, Api g 1, and Mal d 1 by antigen-presenting cells were compared by using flow cytometry. All proteins were digested with endolysosomal extracts, and the resulting peptides were identified by means of mass spectrometry. The binding of Bet v $1_{142-156}$ and the homologous region in Mal d 1 by HLA class II molecules was analyzed *in silico*.

Results: Like Api g 1, Mal d 1 lacked dominant T-cell epitopes. The degree of surface binding and the kinetics of uptake and endolysosomal degradation of Bet v 1, Api g 1, and Mal d 1 were comparable. Endolysosomal degradation of Bet v 1 and Mal d 1 resulted in very similar fragments. The Bet v $1_{142-156}$ and Mal d $1_{141-155}$ regions showed no striking difference in their binding affinities to the most frequent HLA-DR alleles.

Conclusion: The sensitizing activity of different Bet v 1 homologs correlates with the presence of immunodominant T-cell epitopes. However, the presence of Bet v $1_{142-156}$ is not conferred by differential antigen processing. (J Allergy Clin Immunol 2015;136:151-8.)

Key words: *Allergic sensitization, Bet v 1, birch pollen–associated food allergy, immunodominant T-cell epitope, molecular allergology*

In patients with IgE-mediated allergy, the full manifestation of symptoms is preceded by a sensitization phase in which antigen-presenting cells (APCs) take up allergens and degrade them in endosomes/lysosomes. The generated peptides are then transferred into exocytic vesicles, where they bind to HLA class II molecules. HLA/peptide complexes are transported to the plasma membrane, where they can be recognized by T cells through their T-cell receptor.[1] In atopic subjects the cytokine milieu during presentation favors the development of naive T cells into T_H2 cells,[2-4] which in turn produce cytokines that lead to immunoglobulin class-switching in B cells and the production of allergen-specific IgE.[5] The latter is bound to high-affinity receptors on effector cells, so that on each subsequent contact, sensitized subjects can have allergic symptoms to the respective allergens.

One of the most common causes of IgE-mediated allergy in Northern and Central Europe and North America is birch pollen. Its main sensitizing allergen is Bet v 1, to which 93% of patients with birch pollen allergy have specific IgE.[6] Homologs of Bet v 1 have been identified in a wide range of foods[7]; they show sequence similarities of 50% to 80% and share a tertiary structure called the Bet v 1 fold.[8] With the exception of Dau c 1 from carrot[9,10] and Cor a 1 from hazelnut,[11,12] Bet v 1–related food allergens are considered to be unable to initiate sensitization in atopic subjects. However, because of IgE cross-reactivity, they cause immediate allergic symptoms in more than 70% of Bet v 1–sensitized patients.[6]

It is still not known which properties make a protein allergenic. For Bet v 1[13] and other major allergens, such as Der p 1 and Der p 2 from house dust mite,[14,15] Ves v 5 from wasp venom,[16] Art v 1 from mugwort,[17] Amb a 1 from ragweed,[18] Hev b 6.01 from latex,[19] Cry j 1 and Cry j 2 from Japanese cedar,[20] Pru p 3 from peach,[21-23] Fel d 1 from cat,[24] or Equ c 1 from horse,[25] it has been shown that they contain 1 or more immunodominant T-cell epitopes recognized by more than 50% of allergic patients. However, the nonsensitizing Bet v 1 homolog from celery, Api g 1, is devoid of frequently recognized T-cell epitopes.[26] Hence the presence of immunodominant T cell–activating regions might be an intrinsic feature of sensitizing allergens.

To further elucidate this hypothesis, we analyzed the T-cell epitopes of Mal d 1, another highly homologous but nonsensitizing Bet v 1–related protein in apple.[27] To assess whether the presence or absence of an immunodominant T cell–activating region depends on antigen processing and presentation, we used recombinant Bet

From [a]the Christian Doppler Laboratory for Immunomodulation and [b]the Department of Pathophysiology and Allergy Research, Medical University of Vienna; [c]the Department of Molecular Biology and [d]the Christian Doppler Laboratory for Allergy Diagnosis and Therapy, University of Salzburg; and [e]the Department of Blood Group Serology and Transfusion Medicine, Medical University of Vienna.

Supported by the Christian Doppler Research Association, Biomay AG, Austria, and the Austrian Science Fund, projects SFB F4610 and W1212 and W1213.

Disclosure of potential conflict of interest: P. Briza is employed by the University of Salzburg. F. Ferreira is a board member for HAL Allergy (NL), Indoor Biotechnologies, and AllergenOnline; is employed by the University of Salzburg; and has been supported by Austrian Science Funds (FWF). B. Bohle has been supported by Austrian Science Funds and the Christian Doppler Laboratory for Immunomodulation. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication September 5, 2014; revised November 28, 2014; accepted for publication December 29, 2014.

Available online February 7, 2015.

Corresponding author: Barbara Bohle, PhD, Department of Pathophysiology and Allergy Research, Division of Experimental Allergology, Medical University of Vienna, Waehringer Guertel 18-20, A-1090 Vienna, Austria. E-mail: barbara.bohle@meduniwien.ac.at.

0091-6749

http://dx.doi.org/10.1016/j.jaci.2014.12.1928

Abbreviations used
aa: Amino acid
APC: Antigen-presenting cell
DC: Dendritic cell
DOL: Degree of labeling
FITC: Fluorescein isothiocyanate
mDC: Myeloid dendritic cell
mdDC: Monocyte-derived dendritic cell
nsLTP: Nonspecific lipid transfer protein
pDC: Plasmacytoid dendritic cell
TCL: T-cell line

v 1, Api g 1, and Mal d 1 labeled with different fluorescent dyes. We studied their internalization by different APCs from human blood. Furthermore, we followed these proteins through the endocytic pathway in monocyte-derived dendritic cells (mdDCs) and analyzed their degradation both in mdDCs and by endolysosomal extracts derived from mdDCs. Finally, we compared the binding affinities of T-cell epitopes derived from Bet v 1 and Mal d 1 to MHC class II molecules *in silico*.

METHODS

Patients and allergens

Twelve patients with birch pollen allergy had typical case histories, positive skin prick test responses to birch pollen extract (ALK-Abelló, Hørsholm, Denmark), and birch pollen–specific IgE levels of greater than 3.5 kU_A/L (ImmunoCAP; Thermo Fisher Scientific, Uppsala, Sweden). Patients with birch pollen allergy with associated apple allergy additionally reported oral allergy syndrome to apple. Bet v 1– and Mal d 1–specific IgE levels were determined by using ImmunoCAP. HLA-DRB and HLA-DQB1 typing was performed with a commercial sequence-specific oligonucleotide typing kit (Histo Spot SSO HLA-DRB and HLA-DQB1 typing kit; BAG Healthcare, Lich Germany). Samples with only a single detectable HLA-DRB1 or HLA-DQB1 allele were also typed by using sequence-specific primers (All Set SSP DRB and DQB1 low resolution; Olerup, Vienna, Austria). High-resolution typing was performed by means of nucleotide sequencing (SeCore Invitrogen, Life Technology, Brown Deer, Wis).

Three included nonallergic donors had no case history of early spring pollinosis and no Bet v 1–specific IgE. The study was approved by the local ethics committee. Donors provided written informed consent.

Recombinant Bet v 1.0101 (Bet v 1), Api g 1.0101 (Api g 1), and Mal d 1.0108 (Mal d 1) were purchased from Biomay (Vienna, Austria). These proteins were produced in *Escherichia coli* and contained less than 20 EU LPS/mg of protein. Their IgE binding was demonstrated in ELISAs and immunoblots (data not shown). Proteins were conjugated to pHrodo succinimidyl ester, fluorescein isothiocyanate (FITC)–succinimidyl ester, and Alexa Fluor 488–succinimidyl ester (all from Invitrogen, Carlsbad, Calif), according to the manufacturer's instructions.

The degree of labeling (DOL; moles of dye per mole of protein) was determined by using a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, Del) and the following equation:

$$\mathrm{DOL} = \frac{Abs_{\lambda\ \max\ Dye}}{\varepsilon_{Dye} \times protein\ concentration\ (\mathrm{M})}$$

(λ_{max} pHrodo = 560 nm, λ_{max} Alexa Fluor 488 = 494 nm, ε_{pHrodo} = 65,000 $cm^{-1}M^{-1}$, $\varepsilon_{Alexa488}$ = 71,000 $cm^{-1}Mol^{-1}$).

The DOLs for pHrodo and for Alexa Fluor 488 of Api g 1 were only about a third of the DOLs of Bet v 1 and Mal d 1, probably because of the lower number of lysine residues in its primary structure.

Cell preparation

PBMCs were isolated from peripheral blood by using Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. Monocytes were isolated from PBMCs by using immunomagnetic cell sorting (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany), resulting in greater than 95% $CD14^+$ cells. mdDCs and microsomes thereof were generated, as described previously.[28-30]

Epitope mapping of Mal d 1–specific T-cell lines

Mal d 1–specific T-cell lines (TCLs) were generated from PBMCs (1.5×10^6 per well) by using 10 μg/mL Mal d 1, as previously described.[31] Cultures without Mal d 1 served as controls. After 5 days, suboptimal doses of human rIL-2 (10 U/mL; Boehringer, Mannheim, Germany) were added. At day 7, T-cell blasts were harvested by using density gradient centrifugation and expanded with irradiated PBMCs and IL-2. Ten days after the last feeding, TCLs were stimulated in duplicates with 1×10^5 irradiated (60 Gy) autologous PBMCs plus either Bet v 1, Mal d 1 (5 μg/mL), or 49 overlapping synthetic 12-mer peptides (each 5 μg/mL; Intavis, Köln, Germany), representing the complete amino acid (aa) sequence of Mal d 1. The latex allergen Hev b 3 served as a negative control. Incorporation of tritiated thymidine was measured after 48 hours. Stimulation indices were calculated as the ratio between counts per minute obtained in cultures with T cells plus autologous PBMCs plus peptide and counts per minute obtained in cultures containing T cells and PBMCs alone. A stimulation index of 2.5 was defined as positive T-cell proliferation.

Flow cytometry

The following mAbs were used: CD14–peridinin-chlorophyll-protein, CD19-allophycocyanin (BD Biosciences, San Jose, Calif), CD1c-allophycocyanin, CD141-allophycocyanin, and CD303-phycoerythrin (Miltenyi Biotec). Isotype controls were used to detect nonspecific binding. Flow cytometry was performed with a FACSCanto II (BD Biosciences) and analyzed with FACSDiva software (BD Biosciences) and FlowJo software (TreeStar, Ashland, Ore).

Surface binding, endocytosis, and degradation of labeled proteins

Alexa Fluor 488–labeled proteins (400 ng/mL) were incubated with freshly isolated PBMCs (2×10^6) for 1 hour at 4°C, washed, transferred to 37°C for another 20 hours, and labeled with cell type–specific surface markers to assess protein uptake. mdDCs (1×10^6) were incubated with Alexa Fluor 488–labeled proteins (400 ng/mL) for 3 hours at 4°C, washed, and fixed in 2% paraformaldehyde to assess surface binding. mdDCs (2×10^5) were incubated with pHrodo-labeled proteins (3 μg/mL) for 1 hour at 4°C, washed, and further incubated at 37°C in fresh medium and analyzed at the indicated time points to determine endocytosis. mdDCs (1×10^6) were incubated with FITC-labeled proteins (15 μg/mL) and incubated for 3 hours at 37°C, washed, and further incubated at 37°C for the indicated time periods to assess intracellular degradation. The percentage of antigen degradation is represented as the ratio of $FITC^+$ cells at the indicated time to $FITC^+$ cells at 0 hours.

Degradome assays

Allergens (5 μg each) were digested with microsomal enzymes (7 μg) isolated from mdDCs, as described previously.[28] Reactions were stopped at indicated time points by using heat denaturation and analyzed by means of mass spectrometry with an ESI-QTOF mass spectrometer fitted with a capillary rpHPLC (Waters, Milford, Mass).

HLA-DR–binding predictions

MHC class II–binding predictions were performed with the homologous 15-mer peptides Bet v $1_{142\text{-}156}$ (TLLRAVESYLLAHSD) and Mal d $1_{141\text{-}155}$ (GLFKLIESYLKDHPD) on August 20, 2014, by using the Immune Epitope Database analysis resource Consensus tool (http://tools.immuneepitope.org/mhcii/).[32,33] Percentile ranks are generated by comparing the peptide's score with 15 million random 15-mers from SWISSPROT database; a small percentile rank indicates high affinity.