Naturally processed T cell–activating peptides of the major birch pollen allergen

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Background: Although antigen processing and presentation of allergens to $CD4^+$T lymphocytes are key events in the pathophysiology of allergic disorders, they still remain poorly understood.
Objective: To investigate allergen processing and presentation by dendritic cells using the major birch pollen allergen Bet v 1 as a model.
Methods: Endolysosomal extracts of dendritic cells derived from patients with birch pollen allergy were used to digest Bet v 1. Dendritic cells were pulsed with Bet v 1, and peptides were eluted from MHC class II molecules. Peptides obtained by either approach were sequenced by tandem mass spectrometry. Bet v 1–specific T-cell cultures were stimulated with HLA-DR–eluted Bet v 1–derived peptides. Bet v 1–specific T-cell lines were generated from each patient and analyzed for epitope recognition.
Results: A high proportion of Bet v 1 remained intact for a long period of endolysosomal degradation. The peptides that appeared early in the degradation process contained frequently recognized T-cell epitopes. Bet v 1–derived peptides eluted from MHC class II molecules corresponded to those generated by endolysosomal degradation, matched known T-cell epitopes, and showed T cell–activating capacity. The Bet v 1–specific T-cell line of each individual harbored T cells reactive with peptides located within the MHC class II–eluted Bet v 1–derived sequences demonstrating their occurrence *in vivo*.
Conclusion: We report for the first time how epitopes of allergens are generated and selected for presentation to T lymphocytes. The limited susceptibility of Bet v 1 to endolysosomal processing might contribute to its high allergenic potential. (J Allergy Clin Immunol 2010;125:711-8.)

Key words: *Birch pollen allergy, Bet v 1, antigen processing, antigen presentation, dendritic cells, T cells, T-cell epitopes*

IgE-mediated disorders such as allergic rhinitis and asthma have tremendously increased during the recent decades. Understanding the immune mechanisms underlying allergic disorders is important to develop new strategies for their prevention and treatment. $CD4^+$ T_H lymphocytes play a pivotal role in the sensitization and maintenance of type I allergy. Allergic diseases result from an aberrant T-cell response to allergens dominated by long-lived T_H2 cells.[1,2] Among other cytokines, allergen-specific T_H2 cells secrete high amounts of IL-4 and IL-13, which induce the production of allergen-specific IgE antibodies that mediate immediate allergic symptoms.[3] In addition to this indirect involvement in immediate reactions, allergen-specific T_H2 cells have been demonstrated to be directly involved in clinical late-phase reactions in target organs such as the lung and skin.[4,5]

Allergen-specific $CD4^+$ T lymphocytes are activated by peptides generated by proteolytic degradation of allergens in endolysosomal compartments of antigen-presenting cells (APCs). The T-cell receptor recognizes the peptides in the context of MHC class II molecules exposed on the cell surface of APCs.[6] The most specialized APCs are dendritic cells (DCs).[7] They are capable of priming naive T lymphocytes because they efficiently take up allergens by phagocytosis or pinocytosis or via receptors expressed on their surface (eg, FcεRI) and express a large number of MHC class II molecules and different costimulatory molecules.[8-11] Until now, T cell–activating regions in allergens have been identified on the basis of proliferative and cytokine responses to short overlapping peptides spanning the entire amino acid (aa) sequence of the molecule.[12,13] Although this approach identifies the core epitopes recognized by the T-cell receptor, the actual sequences of naturally processed MHC class II–bound peptides cannot be predicted. Such peptides have been demonstrated to display a high degree of heterogeneity in both length and site of their terminal truncations.[14,15] Furthermore, the aa residues flanking the core epitope recognized by the T-cell receptor have been reported to enhance or hinder T-cell activation, respectively.[16-19]

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Abbreviations used
aa: Amino acid
APC: Antigen-presenting cell
cpm: Counts per minute
DC: Dendritic cell
SI: Stimulation index
TCC: T-cell clone
TCL: T-cell line

Because naturally processed MHC class II–bound peptides derived from allergens have not been identified so far, we investigated such peptides presented by DCs from patients with allergy. We used Bet v 1, the clinically highly relevant major birch pollen allergen, which has been extensively characterized by our group. In addition to dissecting its IgE-binding properties,[20,21] we have identified Bet v 1–T cell epitopes by stimulation of allergen-specific T cells isolated from the peripheral blood of a large number of patients with birch pollen allergy with synthetic overlapping 12mer peptides.[22,23] HLA-DR molecules were shown to be highly relevant restriction elements for Bet v 1–specific T lymphocytes.[24-26]

We isolated HLA-DR–bound peptides from Bet v 1–pulsed DCs derived from individuals with birch pollen allergy. Moreover, Bet v 1 was incubated with microsomal proteases isolated from these DCs. Bet v 1–derived fragments obtained by either approach were sequenced by tandem mass spectrometry. The T cell–activating properties of HLA-DR–eluted peptide sequences were confirmed by using Bet v 1–specific T-cell clones (TCCs). To verify the *in vivo* relevance of naturally processed Bet v 1 peptides, we analyzed the peripheral blood of each patient with birch pollen allergy for the presence of T cells reactive with epitopes matching the eluted peptide sequences.

METHODS

Patients with birch pollen allergy and allergen

All patients with birch pollen allergy included in this study were sensitized to Bet v 1, had rhinoconjunctivitis in spring, and showed specific IgE CAP/RAST class >3 (Pharmacia Diagnostics, Uppsala, Sweden) and positive skin prick reactions (wheal diameter >5 mm) to birch pollen. All patients gave written consent before enrolment in the study, which was approved by the local Medical Ethical Committee of Vienna. Molecular HLA typing for HLA-DRB1 alleles of the patients was performed according to methods previously referenced.[27] Recombinant Bet v 1 was purchased from Biomay (Vienna, Austria).

Endolysosomal degradation assays

Monocyte-derived (md) DCs were generated from $CD14^+$ cells purified from PBMCs as described.[28] DCs were homogenized in 10 mmol/L TRIS acetate pH7 containing sucrose (250 mmol/L), and microsomes were isolated by ultracentrifugation.[29] Bet v 1 (5 μg) was digested with microsomal enzymes (7 μg) in 100 mmol/L citrate buffer pH 4.8 containing dithiothreitol (2 mmol/L) at 37°C. Reactions were stopped by heat denaturation and analyzed by SDS-PAGE and mass spectrometry using an ESI-QTOF mass spectrometer fitted with a capillary reversed phase HPLC (Waters, Milford, Mass).

Allergen-specific T-cell cultures

Allergen-specific T-cell lines (TCLs) were generated by stimulating PBMCs (1.5×10^6) with Bet v 1 (10 μg/mL) as described.[23] TCLs were stimulated with varying concentrations of Bet v 1 (2.5-10 μg/mL) or peptide (5 μg/mL) in the presence of 5×10^4 irradiated (60 Gy) autologous PBMCs for 48 hours in duplicate. Proliferation was assessed by $[^3H]$ thymidine incorporation. The stimulation index (SI) was calculated as ratio between counts per minute (cpm) of TCL plus PBMC plus allergen and cpm of TCL plus PBMC only. Cryopreserved TCCs specific for different Bet v 1 epitopes were thawed, expanded, and stimulated with Bet v 1 (5 μg/mL) or the indicated peptides (0.6-2.5 μmol/L) in the presence of irradiated autologous PBMCs. Supernatants were harvested after 24 hours. Cytokine levels were assessed by ELISA as described.[23] Cytokine levels in controls were subtracted from those determined in unstimulated cultures.

Isolation of peptides from HLA-DR molecules

The monoclonal anti–HLA-DR antibody L243 (ATCC HB-55) was purified by ammonium sulfate precipitation and protein G affinity chromatography. Antibody specificity was checked by flow cytometry (data not shown).

Dendritic cells ($65\text{-}100 \times 10^6$) were stimulated with Bet v 1 (200 μg/mL) plus LPS (1 μg/mL) (Sigma, St Louis, Mo) on day 5 and lysed in 10 mmol/L TRIS/HCl pH 7.6 containing 0.6% CHAPS and Complete Protease Inhibitor (Roche Diagnostics, Mannheim, Germany) for 30 minutes on ice. After ultracentrifugation, protein G beads (GE Healthcare, Vienna, Austria; 200 μL in 200 μL PBS) were added to the supernatant for 2 hours on ice. After centrifugation, L243 antibody (4 mg) was added to the supernatant and incubated for 4 hours at 4°C. Protein G beads (200 μL in 200 μL PBS) were added for 16 hours. After centrifugation, the pellet was washed with PBS, resuspended in 300 μL 10% trifluoroacetic acid, incubated at room temperature for 5 minutes, and ultrafiltrated through a Microcon-10 filter unit (Millipore, Molsheim, France). The eluate was immediately analyzed by tandem mass spectrometry.

Mass spectrometry analysis of HLA-DR–eluted peptides

Peptides from patient 4 were eluted and identified by mass spectrometry as described.[30] For patients 1 to 3, peptides (20 μL) were separated by capillary rpHPLC (Waters) directly coupled to a Q-Tof Global Ultima mass spectrometer (Waters) by using a Nanoflow spray head (precolumn Nanoease Symmetry300 trap column, separating column Nanoease Atlantis dC18; Waters). Peptides were eluted with an acetonitrile gradient (solvent A 0.1% vol/vol formic acid/5% vol/vol acetonitrile; solvent B 0.1% vol/vol formic acid/95% vol/vol acetonitrile; 5% to 45% B in 90 minutes). Data acquisition and instrument control were done with MassLynx software V4.1 (Waters). Singly, doubly, and triply charged ions were selected for fragmentation by collision with argon (collision energy settings, 25-50 kV). Survey and fragment spectra were analyzed by using the software PLGS version 2.2.5 (Waters) with automatic and manual validation with an in-house Bet v 1 database. As control for this analytical system, mdDCs pulsed with the major mugwort pollen allergen were used. No Bet v 1–derived peptides were detected (data not shown).

RESULTS

Endolysosomal processing of the major birch pollen allergen

Microsomal proteases were isolated from DCs of 2 donors with birch pollen allergy and incubated with Bet v 1. At defined intervals, allergen degradation was monitored by SDS-PAGE (see this article's Fig E1 in the Online Repository at www.jacionline.org), and proteolytic fragments were sequenced by tandem mass spectrometry (Fig 1). Intact Bet v 1 was detectable for 24 hours. Taking all time points, 113 different fragments were detected in a total of 368 sequenced peptides. After 0.5 hours, first peptide clusters derived from the N-terminus Bet v $1_{1\text{-}20}$, the central region Bet v $1_{84\text{-}97}$, and the C-terminus Bet v $1_{146\text{-}157}$ were identified. After 1 hour, peptides originating from the region Bet v $1_{104\text{-}115}$ were detected. Some of these initially identified

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