Dissection of the IgE and T-cell recognition of the major group 5 grass pollen allergen Phl p 5

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Background: The major timothy grass pollen allergen Phl p 5 belongs to the most potent allergens involved in hay fever and asthma.

Objective: This study characterized immune-dominant IgE- and T-cell–recognition sites of Phl p 5.

Methods: Seven peptides, P1 to P7 with a length of 31 to 38 amino acids that spanned the Phl p 5 sequence, were synthesized, characterized by circular dichroism spectroscopy, and tested for IgE reactivity, basophil activation, and T-cell reactivity. Carrier-bound peptides were studied for their ability to induce IgG antibodies in rabbits which recognize Phl p 5 or cross-reactive allergens from different grass species. Peptide-specific antibodies were tested for the capability to inhibit IgE reactivity to Phl p 5 and allergen-induced basophil activation of patients with allergy.

Results: The peptides exhibited no secondary structure and showed no IgE reactivity or relevant allergenic activity, indicating that Phl p 5 IgE epitopes are conformational. Except for P3, peptide-specific IgG antibodies blocked IgE binding to Phl p 5 of patients with allergy and cross-reacted with temperate grasses. IgE inhibition experiments and molecular modeling identified several clustered conformational IgE epitopes on the N- as well as C-terminal domain of Phl p 5. P4, which stimulated the strongest T-cell and cytokine responses in patients, was not part of the major IgE-reactive regions.

Conclusion: Our study shows an interesting dissociation of the major IgE- and T-cell–reactive domains in Phl p 5 which provides a basis for the development of novel forms of immunotherapy that selectively target IgE or T-cell responses. (J Allergy Clin Immunol 2014;133:836-45.)

Key words: *Allergy, grass pollen allergen, peptides, epitopes*

Approximately 50% of patients with allergy experience grass pollen-induced allergic symptoms that range from rhinoconjunctivitis (ie, hay fever) to severe asthma attacks.[1-4] Grass pollen has, therefore, been recognized since 1880 as a major allergen source.[5] In addition, the first allergen-specific immunotherapy was performed with grass pollen preparations in 1911.[6] Today, allergen-specific immunotherapy is well established as a clinically effective treatment for allergy.[7-9] New approaches are based on genetically modified recombinant allergens with reduced allergenic activity,[10-13] T-cell epitope-containing peptides for selective targeting of allergen-specific T cells,[14] and carrier-bound allergen peptides for selective induction of protective allergen-specific IgG responses.[15,16]

Group 5 allergens belong to the most frequent and potent grass pollen allergens which, because of their release in the form of submicronic respirable pollen particles, are implicated in asthma attacks.[3,17-20] The complete 3-dimensional structure of group 5 allergens has not yet been solved, but the structures of the N- and C-terminal domains of 2 isoforms, Phl p 5a and Phl p 5b, from timothy grass pollen showed that these domains consist each of anti-parallel 4 helix bundles.[21,22] Both the N-[23] and C-terminal domains of group 5 allergens[20,24,25] are described as containing IgE-binding sites that still need to be characterized in detail.

Different earlier T-cell epitope mapping studies for group 5 allergens performed with T-cell clones and lines report highly discrepant results for the location of major T-cell epitopes.[26-29] Therefore, little is known about the immune dominance of certain T-cell epitopes.

Here, we used Phl p 5 peptide-specific IgG antibodies for IgE competition experiments as an approach for the mapping of conformational IgE epitopes of group 5 allergens.[30,31] For this purpose we synthesized 7 peptides of 31 to 38 amino acids that cover large parts of the Phl p 5 sequence to raise peptide-specific antibodies. With the synthetic peptides the presence of sequential (ie, continuous) IgE epitopes of group 5 allergens was investigated, and peptide-specific antibodies were used for inhibiting the IgE binding to Phl p 5 from a large number of patients with grass pollen allergy and to search for immune-dominant conformational/discontinuous IgE epitopes. Furthermore, we studied the ability of the individual peptide-specific IgG antibodies and mixes thereof to inhibit Phl p 5-induced basophil activation to learn about the clonal distribution of patients' IgE responses. In parallel, we used the synthetic peptides to study lymphocyte proliferation and cytokine responses in peripheral blood mononuclear cells (PBMCs) from patients with grass pollen allergy to identify immune-dominant T-cell epitopes.

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Abbreviations used
7-AAD: 7-Amino-actinomycin D
CD: Circular dichroism
CFSE: Carboxyfluorescein diacetate succinimidyl ester
KLH: Keyhole-limpet hemocyanin
P: Peptide
PBMC: Peripheral blood mononuclear cell

METHODS

Immunization of rabbits with KLH-coupled peptides, reactivity of rabbit antipeptide antibodies with Phl p 5, and natural allergens from different grass species

New Zealand white rabbits were immunized with each of the keyhole-limpet hemocyanin (KLH)-conjugated peptides (200 μg/injection) or with complete rPhl p 5 with the use of Freunds complete and incomplete adjuvans (Charles River, Kisslegg, Germany). IgG reactivity was tested by direct ELISA.[32]

Inhibition of allergic patient's IgE binding to Phl p 5 by peptide-specific IgG

ELISA inhibition experiments were done as described, using 1 μg/mL rPhl p 5 for coating, a 1:250 dilution of each of the rabbit antisera, and 1:10 diluted sera from patients with grass pollen allergy. Detection of bound IgE antibodies and calculation of inhibition of IgE binding was done as described previously.[32]

Allergen-induced upregulation of CD203c and CD63 expression on patient's basophils and inhibition of allergen-induced CD203c upregulation by allergen-specific antibodies

Heparinized blood samples (100 μL) from patients with grass pollen allergy were incubated with serial dilutions of rPhl p 5 (10^{-4} to 10 μg/mL), an equimolar mix of Phl p 5-derived peptides, an equimolar mix of KLH-conjugated Phl p 5 peptides, a monoclonal anti-IgE antibody (Immunotech, Marseille, France; 1 μg/mL), or buffer alone (PBS) for 15 minutes at 37°C.

In CD203c inhibition experiments serial dilutions of rPhl p 5 or an unrelated allergen, that is, birch pollen allergen, Bet v 1 (negative control) (10^{-1} to 10^{-4} μg/mL), were preincubated with a 1:100 diluted mix of rabbit antipeptide antisera or a mix of the corresponding preimmune sera before incubation with the blood samples.[30]

Measurement of CD203c upregulation was done as described.[33]

Lymphocyte proliferation assays, CFSE staining, and multiple cytokine measurements

PBMCs were isolated from patients with grass pollen allergy and were cultured as described previously.[34] Cells were stimulated with different concentrations of each of the synthetic peptides, mixes of the 7 peptides (each peptide 1.25, 0.6, 0.3, and 0.15 μg per well), and equimolar concentrations of rPhl p 5 for comparison. Endotoxin concentrations were determined in each of the tested preparations with the use of the Limulus Amebocyte Lysate QCL-1000 kit (BioWhittaker, Walkersville, Md). The endotoxin concentrations of the peptides and of Phl p 5 (measured at a concentration of 10 μg/mL) were below 1 EU/mL (data not shown). For control purposes 4 U of IL-2 per well (Boehringer, Mannheim, Germany) or medium alone were tested. Cultures were done in triplicates, and results are shown as mean cpm values ± SD.[34]

For carboxyfluorescein diacetate succinimidyl ester (CFSE) staining experiments, cells were labeled with CFSE (Invitrogen, Oslo, Norway) by incubating 5×10^6 cells (triplicates) in a solution of 2.5 μmol/L CFSE in PBS for 10 minutes at 37°C, washed, and then cultured with rPhl p 5 (5 μg/well), the individual peptides or a peptide mix (0.6 μg/well per peptide), medium alone (negative control), or Dynabeads that contained anti-CD3 and anti-CD28 (positive control) (3 μL/well; Invitrogen) for 7 days at 37°C in Ultra Culture serum-free medium (Lonza, Verviers, Belgium). Then cells were centrifuged and resuspended in 50 μL of blocking buffer (PBS, 0.01% wt/vol NaN_3, 1% wt/vol BSA, 10% mouse serum) for 20 minutes on ice, centrifuged, and finally stained with phycoerythrin/cyanine 7-labeled mouse monoclonal IgG_1 anti-human CD3, an IgG_1 isotype control, 7-amino-actinomycin D (7-AAD), phycoerythrin-labeled mouse monoclonal IgG_2 anti-human CD4, and the corresponding isotype control (Biolegend, San Diego, Calif) diluted in fluorescence-activated cell sorting buffer (PBS, 0.01% wt/vol NaN_3, 1% wt/vol BSA) in dark on ice for 20 minutes and analyzed on a Cytomics FC 500 (Beckman Coulter, Fullerton, Calif). Cells were gated according to forward and sideward scatter, dead cells were excluded by 7-AAD staining, and gating was on CD4 cells. After subtraction of results for medium, mean percentages ± SDs of CD4 cells that proliferate in response to each of the agonists are shown.

Levels of IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IFN-γ, TNF-α, and GM-CSF were measured in supernatant fluids from PBMC cultures which were identically prepared as for the proliferation experiments with the use of the xMAP Luminex fluorescent bead-based technology (Luminex Corp, Austin, Tex) and a human MultiAnalyte Profiling Kit A and B (R&D Systems, Abingdon, United Kingdom). Fluorescent signals were read on a Luminex 100 system (Luminex Corp).

Statistical evaluation

Unpaired Kruskal-Wallis tests were used to assess statistically significant differences for T-cell proliferation and cytokines. A P value ≤ .05 was considered statistically significant.

RESULTS

Characterization of synthetic peptides derived from solvent accessible areas of Phl p 5a

It was shown that allergen-derived peptides with a length of >25 amino acids coupled to a carrier gave robust peptide- and allergen-specific antibody responses on immunization.[32,35] Solvent accessibility prediction indicated that the regions of Phl p 5a with predicted high hydrophilicity and surface accessibility can be covered with 7 peptides (P1-P7), ranging from 31 (P1, 3068 Dalton) to 38 (P5, 3853 Dalton) amino acids in length. Table E1 (in the Online Repository at www.jacionline.org) summarizes the position, length, and biochemical properties of the Phl p 5a-derived synthetic peptides. None of the peptides showed any residual secondary structure as determined by circular dichroism (CD) experiments (see Fig E1 in this article's Online Repository at www.jacionline.org), whereas the CD spectrum of rPhl p 5a is characterized by 2 broad minima at 207 and 221 nm, typical for folded α helical proteins. P2, P3, and P4 represent surface-exposed areas on the N-terminal domain, whereas P5, P6, and P7 are part of the C-terminal domain (Fig 1). P1 represents a flexible region at the N-terminus of the first domain. In the given view of the model, P1, P2, P3, P5, and P6 appear on the front side, whereas P4 and P7 are located rather on the backside of the modeled structure (Fig 1).

Fig E2 (in the Online Repository at www.jacionline.org) shows a multiple sequence alignment of group 5 allergens from different grasses in which the peptides were highlighted to visualize their sequence homology with the corresponding regions in related grass pollen allergens from other species. The sequences for Phl p 5a, Phl p 5b, Lol p 5b, Poa p 5, Dac g 5, Lol p 5a, Hor v 5, and Sec c 5 are complete sequences, whereas for Tri a 5 only a