Spatial clustering of the IgE epitopes on the major timothy grass pollen allergen Phl p 1: Importance for allergenic activity

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Background: The major timothy grass pollen allergen Phl p 1 is one of the most potent and frequently recognized environmental allergens.
Objective: We sought to study at a molecular and structural level the IgE recognition of Phl p 1 and its relation to allergenic activity.
Methods: Monoclonal human IgE antibody fragments specific for Phl p 1 and group 1 allergens from various grasses were isolated from a combinatorial library made of lymphocytes from patients with grass pollen allergy. Recombinant Phl p 1 fragments and the 3-dimensional structure of Phl p 1 were used to localize the major binding site for the IgE antibodies. A rPhl p 1 fragment containing this binding site was expressed in *Escherichia coli*, purified, and tested for IgE reactivity and allergenic activity with sera and basophils from patients with grass pollen allergy.
Results: Monoclonal antibodies, as well as polyclonal serum IgE, from patients with grass pollen allergy defined a C-terminal fragment of Phl p 1 that represents a sterically oriented portion on the Phl p 1 structure. This Phl p 1 portion bound most of the allergen-specific IgE antibodies and contained the majority of the allergenic activity of Phl p 1.
Conclusion: IgE recognition of spatially clustered epitopes on allergens might be a general factor determining their allergenic activity.
Clinical implications: Geographic distribution of IgE epitopes on an allergen might influence its allergenic activity and hence explain discrepancies between diagnostic test results based on IgE serology and provocation testing. It might also form a basis for the development of low allergenic vaccines. (J Allergy Clin Immunol 2006;117:1336-43.)

Key words: *Grass pollen allergy, allergen, Phl p 1, 3-dimensional structure, recombinant allergen fragment, epitope, monoclonal human IgE antibodies*

The cross-linking of mast cell– and basophil–bound IgE antibodies by allergens induces the release of inflammatory mediators and thus is a key mechanism in acute allergic inflammation.[1] During the last years, the molecular nature of many important allergens has been revealed by means of cDNA cloning, and the 3-dimensional structures of several allergens have been determined.[2,3] *In vitro* and *in vivo* studies have demonstrated that individual allergen molecules can exhibit greatly varying potencies to induce mast cell and basophil activation and thus acute allergic inflammation.[4] One possible explanation for varying allergenic activities of allergens might be differences regarding the number of IgE-binding sites that are present on a given allergen molecule. In fact, early *in vitro* studies have shown that the intensity of mast cell and basophil degranulation increases with the number of IgE molecules that are cross-linked during this process.[5-7] Evidence for the possibility that also orientational constraints might determine secretory signals induced by aggregation of IgE receptors on mast cells comes from experiments performed with different antibodies specific for the high-affinity receptor for IgE (FcεRI).[8]

In this study we have analyzed the IgE recognition of the major timothy grass pollen allergen Phl p 1, which is one of the most potent and frequently recognized respiratory allergens.[9] We isolated human allergen-specific IgE antibody fragments (Fabs) from lymphocytes of an individual with grass pollen allergy using the combinatorial library technology.[10] Monoclonal human IgE antibodies defined a C-terminal Phl p 1 domain that also contained the majority of binding sites for the patient's serum IgE. Inhibition experiments with the mAbs demonstrate that the C-terminus contains binding sites for several different IgE antibodies. This was confirmed by using basophil histamine release experiments showing that the allergenic activity of the C-terminus mimics that of the complete Phl p 1 molecule. Using the crystal structure of Phl p 1, we were able to show that the highly allergenic C-terminal portion of Phl p 1 represents a sterically oriented area on the Phl p 1 surface. Our results suggest that spatial clustering of IgE-binding sites on an allergen might be an important factor involved in mast cell and basophil activation and allergenic activity.

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Abbreviations used
CDR: Complementarity-determining region
Fab: Antibody fragment
FR: Framework region
GST: Glutathione-S-transferase

METHODS

Plasmids, patient sera, peptides, and recombinant allergens

The pComb3H plasmid representing a modification of plasmid pComb3[11] was kindly provided by Carlos F. Barbas, The Scripps Research Institute (La Jolla, Calif). The pLNOH2 and the pLNOK expression plasmids used for the expression of complete IgG1 antibodies and synthetic rPhl p 1 peptides have been previously described.[12,13] Sera were collected from patients with grass pollen allergy who were characterized on the basis of clinical history, skin prick test responses, RAST results, and test results with recombinant grass pollen allergens, as previously described.[14] Purified rPhl p 1 was obtained from Biomay (Vienna, Austria).

Isolation, characterization, and expression of rPhl p 1–specific IgE Fabs in *Escherichia coli*

Three phage clones expressing Fabs with specificity for rPhl p 1 were isolated from an IgE combinatorial library by panning to rPhl p 1, as previously described.[10] All 3 clones (clones 25, 10, and 43) were sequenced with ^{35}S-dCTP (DuPont NEN, Stevenage, United Kingdom) and a T7 polymerase sequencing kit (Pharmacia-Amersham, Buckinghamshire, United Kingdom), with primers from MWG (MWG AG- Biotech, Ebersberg, Germany).[15] The Phl p 1–specific DNA sequences of the variable regions of the heavy-chain fragments and the light chains were compared with germline sequences deposited in the V Base Sequence Directory (Tomlinson, et al; MRC Centre for Protein Engineering, Cambridge, United Kingdom) and were submitted to the EMBL database under the following accession numbers: AJ512645-AJ512650. Recombinant human Phl p 1–specific IgE Fabs were expressed as previously described.[12]

Preparation of natural pollen extracts: Identification of group 1 allergens with recombinant human IgE Fabs by immunoblotting

Pollen extracts were prepared and blotted as previously described.[12] Nitrocellulose membranes containing blotted pollen extracts were blocked twice for 5 minutes and once for 30 minutes with TBST (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.5% wt/vol BSA, and 0.05% vol/vol Tween 20) at room temperature and then incubated overnight with bacterial extracts containing Phl p 1–specific IgE Fabs at 4°C. Bound Fabs were detected with an alkaline phospatase–coupled goat anti-human IgG F(ab)2 (Pierce, Rockford, Ill) diluted 1:5000 in TBST and 0.5% wt/vol BSA.

Construction, expression, and purification of a complete IgE Fab-derived human IgG1 antibody

Complete human recombinant IgG1 antibodies containing the variable regions of IgE Fab clone 25 were obtained by expression in mammalian cells. In detail, cDNAs coding for the heavy and light-chain variable regions of the IgE Fab were amplified from the IgE Fab-expressing pComb3H plasmid by using the VH (5′VH primer: CGG AAT TCG TGC ATT CCG AGG TGC AGC TGC TCG AG; 3′VH primer: CGG AAT TCG *ACG TAC G*AC TCA CCT GAG GAG ACG GTG ACC CG) and VK (5′VK primer: CGG AAT TCG TGC ATT CCG ACA TCG AGA TGA CTC AGT CTC CAT CTT CC; 3′VK primer: CGG AAT TCA *CGT ACG* TTC TAC TCA CCT TTA ATC TCC AGT GC) primer pairs, respectively. *Bsm*I (underlined) and *Bsi*WI (italics) are indicated. The cDNAs of the VH and VK products were subcloned into plasmid pLNOH2 and pLNOK, respectively, and expressed in COS-7 cells as complete Phl p 1–specific IgG1 antibodies, as previously described.[12]

Expression of rPhl p 1–derived allergen fragments and epitope mapping

Nitrocellulose filters containing rPhl p 1 fragments were generated as previously described[16] and were incubated with culture supernatants containing Phl p 1–specific IgE Fab-derived IgG1 or 1:10 diluted sera from patients with grass pollen allergy. Bound patients' IgE antibodies were detected as previously described,[17] whereas bound rPhl p 1–specific IgG1 antibodies were detected with a mouse monoclonal anti-human IgG1 (PharMingen, Becton Dickinson, San Diego, Calif) antibody, followed by iodine 125–labeled sheep anti-mouse antibodies (Amersham, Buckinghamshire, United Kingdom).

Expression, purification, and IgE reactivity of a recombinant C-terminal fragment comprising amino acids 146 through 240 of Phl p 1

The PCR-amplified cDNA coding for the C-terminal rPhl p 1 fragment T147 (amino acids 146-240) was ligated into the *Eco*RI and *Bam*HI sites of plasmid pGEX-6P-1 (Pharmacia Biotech, Uppsala, Sweden), sequenced for its correct insertion and expressed as glutathione-S-transferase (GST) fusion protein. rT147 was purified with glutathione sepharose 4B (Amersham). For control purposes, the original plasmid pGEX-6P-1 without any insert was used to express and purify GST alone. The IgE reactivity of rT147 and GST was tested by IgE immunoblotting, as previously described.[17]

Quantitative IgE inhibition experiments and human basophil histamine release

Serum aliquots from patients with grass pollen allergy were diluted 1:5 in buffer A (50 mM sodium phosphate buffer [pH 7.4], containing 0.5% wt/vol BSA, 0.5% vol/vol Tween 20, and 0.05% wt/vol NaN3) and incubated either with rT147, GST, or rPhl p 1 overnight at 4°C. In pilot experiments it was determined that 2.5 μg of protein per milliliter of diluted patient's serum ensured conditions of antigen excess. The preincubated serum samples (duplicates) were exposed to rPhl p 1 (2 μg), which had been immobilized to nitrocellulose by dot blotting. Bound Phl p 1–specific IgE antibodies were detected as previously described[17] and quantified in a gamma counter (1277 Gammamaster, LKB; Wallac, Gaithersburg, Md). Results represent means of duplicate determinations with variations of less than 10%. The IgE reactivity of sera preincubated with the control protein GST was assumed as 100% binding to rPhl p 1. The percentage inhibition (100-cpm competitor × 100/cpm control) was calculated for rT147 and rPhl p 1 as competitors.

Granulocytes from patients with grass pollen allergy were isolated by dextran sedimentation and exposed to different concentrations (10^{-4} to 1 μg/mL) of purified rPhl p 1 allergen, rT147, or GST for 30 minutes at 37°C. Cells were then centrifuged at 4°C, and the cell-free supernatants were recovered. Histamine released into the cell-free supernatants was determined in triplicate determinations by radioimmunoassay (Immunotech, Marseille, France) and was expressed as a percentage of total histamine determined after cell lysis.[18]