Genetic Engineering of the Major Timothy Grass Pollen Allergen, Phl p 6, to Reduce Allergenic Activity and Preserve Immunogenicity

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Type I allergy is a genetically determined hypersensitivity disease that affects >25% of the population in industrialized countries (1). It is characterized by the formation of IgE Abs to otherwise harmless Ags from pollen, mites, molds, and animal dander (2), which can activate a variety of immune cells via the high- and low-affinity receptors for IgE (3). Allergen-induced cross-linking of IgE Abs bound to effector cells (i.e., mast cell and basophil) via the high-affinity receptor, FceRI, leads to release of inflammatory mediators (histamine, leukotrienes) and thus to the immediate symptoms of type I allergy, such as allergic rhinitis, conjunctivitis, asthma, and anaphylactic shock (4).

Specific immunotherapy, the only allergen-specific approach for the treatment of type I allergy and for preventing its progression to severe disease manifestations (5–7) involves the administration of increasing doses of allergen extracts to patients. Although several controlled clinical studies have demonstrated that this treatment is clinically effective (8), one major disadvantage is that the administration of crude allergen extracts may induce severe and life-threatening anaphylactic side effects. Several approaches are currently under development to overcome the problem of therapy-induced IgE-mediated anaphylactic side effects. They include the adsorption of allergen extracts to novel adjuvants to delay systemic release of allergens, the coupling of allergens to immunomodulatory DNA sequences, and the design of allergen-derived peptides or recombinant allergen derivatives with reduced allergenic activity (9–17).

Several clinical studies have been performed in patients with allergen-derived T cell epitope-containing peptides and CpG-conjugated allergens demonstrating immunomodulatory activity in allergic patients (10, 18–22). Furthermore, immunotherapy trials with recombinant allergens and recombinant hypoallergenic allergen derivatives were performed indicating that beneficial immunomodulatory effects, reduction of clinical symptoms, and inhibition of IgE memory responses are associated with the induction of IgG Abs that compete with patients IgE binding to the allergens (23–25).

Grass pollen belongs to the most important respiratory allergen sources against which >40% of allergic individuals are sensitized (26). In vitro experiments, studies in experimental animal models, and a recent clinical trial performed with recombinant grass pollen allergens indicate that four major grass pollen allergens (i.e., Phl p 1, Phl p 2, Phl p 5, and Phl p 6 from timothy grass pollen) comprise most of the relevant epitopes needed for the diagnosis and treatment of grass pollen allergy (24). Hypoallergenic derivatives for

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On the basis of IgE epitope mapping data, we have produced three allergen fragments comprising aa 1–33, 1–57, and 31–110 of the major timothy grass pollen allergen Phl p 6 aa 1–110 by expression in *Escherichia coli* and chemical synthesis. Circular dichroism analysis showed that the purified fragments lack the typical α-helical fold of the complete allergen. Superposition of the sequences of the fragments onto the three-dimensional allergen structure indicated that the removal of only one of the four helices had led to the destabilization of the α-helical structure of Phl p 6. The lack of structural fold was accompanied by a strong reduction of IgE reactivity and allergenic activity of the three fragments as determined by basophil histamine release in allergic patients. Each of the three Phl p 6 fragments adsorbed to CFA induced Phl p 6-specific IgG Abs in rabbits. However, immunization of mice with fragments adsorbed to an adjuvant allowed for human use (AluGel-S) showed that only the Phl p 6 aa 31–110 induced Phl p 6-specific IgG Abs. Anti-Phl p 6 IgG Abs induced by vaccination with Phl p 6 aa 31–110 inhibited IgE reactivity to the wild-type allergen as well as Phl p 6-induced basophil degranulation. Our results are of importance for the design of hypoallergenic allergy vaccines. They show that it has to be demonstrated that the hypoallergenic derivative induces a robust IgG response in a formulation that can be used in allergic patients. The *Journal of Immunology*, 2007, 179: 1730–1739.

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Phl p 1 (B cell peptides) (27) and Phl p 5 (deletion variants) (28) have been characterized but are not yet available for Phl p 6. Phl p 6 represents an 11.8-kDa, α helical protein located on the polysaccharide-rich wall precursor bodies (P-particles) of timothy grass pollen (29). It is recognized by serum IgE from 75% of grass pollen allergic patients (29, 30) but despite high sequence homology with group 5 grass pollen allergens (29, 31) shows almost no cross-reactivity with Phl p 5 (29). In this study, we report the construction and characterization of hypoallergenic Phl p 6 derivatives for immunotherapy of grass pollen allergy.

Materials and Methods

Sera from allergic patients, Abs, plasmid vectors, and
Escherichia coli strains

Grass pollen allergic patients were characterized by history, skin prick testing, and serology as described (29). Rabbit sera were obtained by immunizing rabbits three times with purified rPhl p 6, rPhl p 6 aa 31–110, and keyhole limpet hemocyanin (KLH)3 (Pierce)-coupled polypeptides (Phl p 6 aa 1–33, rPhl p 6 aa 6 aa 1–57, rPhl p 6 aa 31–110) (Charles River Breeding Laboratories). Plasmid pET17b and E. coli strain BL21 (DE3) were purchased from Novagen.

Expression of rPhl p 6 fragments in E. coli

cDNAs coding for rPhl p 6 aa 1–57 and aa 31–110 were obtained by PCR amplification using the following oligonucleotide primers (MWG, Ebersberg, Germany) and the Phl p 6 cDNA (29) (accession no. Y16956) as template: rPhl p 6 aa 1–57, forward, 5′-GGGATCCCATATGGGGAAGGCTTGGGGGCTTGAC-3′; rPhl p 6 aa 31–110, forward, 5′-GGGATCCATATGGGGAAGGCACAGACGGCATATAGAAGGGGTGGTGGGGCGCCTTTGAAAC-3′; and reverse, 5′-GGCGGATCTTGTTGGTGGTGAGGTGTGACGCGGCTTTGAC-3′. E. coli BL21 (DE3) were transformed with the PCR products. Successful transformation was confirmed by PCR with the corresponding primers. Recombinant colonies were grown in 10 ml of LB containing 100 μg/ml of ampicillin. The protein expression was induced by shaking at 37°C to an OD600 of 0.8, followed by induction with 0.5 mM isopropyl-β-D-thiogalactopyranoside at an OD600 of 3.0. After a further 1 h, the cells were harvested by centrifugation at 10,000 rpm and frozen at −80°C. Protein concentration was determined using the BCA assay (Pierce).

Purification of rPhl p 6 fragments

Recombinant Phl p 6 aa 1–57 and rPhl p 6 aa 31–110 were expressed in the inclusion body fraction of E. coli. The inclusion bodies were solubilized in 8 M urea, 100 mM NaH2PO4, 10 mM Tris-HCl (pH 8) (5 ml per gram cells) for 60 min at room temperature. After centrifugation for 30 min at 10,000 g, supernatants were loaded onto Ni-NTA matrix columns (Qiagen) and washed with binding buffer. Bound proteins were eluted according to the manufacturer’s guidelines (Qiagen). Fractions containing rPhl p 6 aa 1–57 or rPhl p 6 aa 31–110 with >95% purity were dialyzed against double-distilled H2O and stored at 4°C until use.

Synthesis and purification of the Phl p 6 peptide aa 1–33

Peptide Phl p 6 aa 1–33 was synthesized using a Fmoc (9-fluorenyl-methoxy-carbonyl)-strategy with 2-(H-benzoyl-1-yl)-1,3,4,5-tetrahydronaphthalen-1-ol hexafluorophosphate activation (0.1 mmol small-scale cycles) on the Applied Biosystems peptide synthesizer model 433A. Preloaded polyethylene glycol-polyesterene resin (0.15–0.2 mmol/g loading; PerSeptive Biosystems) were used as solid phase to build up the peptide. Chemicals were purchased from Applied Biosystems. Coupling of amino acids was confirmed by conductivity monitoring with feedback control. The peptide was cleaved from the resin with the following mixture: 250 μl of distilled water, 250 μl of triisopropylsilan (Fluka), and 9.5 ml of trifluoroactic acid for 2 h and precipitated in tert-butyl methyl ether.

IgE and IgG reactivity of rPhl p 6 and Phl p 6 fragments

Ab reactivity of purified rPhl p 6 and Phl p 6 fragments was studied by immunoblotting and ELISA.

For IgE immunoblotting, the purified proteins were separated by SDS-PAGE (1 μg protein/cm gel) (32) and blotted onto nitrocellulose (33). Nitrocellulose strips were incubated with 1/10 diluted sera from grass pollen allergic patients, serum from a nonallergic individual, and for control purposes, with a 1/1000 diluted rabbit anti-rPhl p 6 polyclonal serum and the rabbit preimmune serum. Bound IgE Abs were detected with 125I-labeled anti-human IgE Abs (Phadia), bound rabbit Abs with a 125I-labeled donkey anti-rabbit Ig antiserum (Amersham Biosciences), and visualized by autoradiography using Kodak XOMAT films and intensifying screens (Kodak) at −70°C.

For ELISA experiments, ELISA plates (Greiner) were coated with 5 μg/ml purified proteins, incubated with 1/10 diluted sera from grass pollen allergic patients, and bound IgE detected with alkaline phosphatase-coupled anti-human IgE Abs. For the detection of IgG reactivity, coated ELISA plates were incubated with 1/50 diluted sera from grass pollen allergic patients, and bound IgG detected with HRP-coupled anti-human IgG Abs (BD Pharmingen) as described (34).

Basophil histamine release assays, skin prick testing

Granulocytes were isolated from heparinized blood samples of grass pollen allergic individuals by dextran sedimentation (35). Cells were incubated with different concentrations of purified rPhl p 6, rPhl p 6 aa 1–57, rPhl p 6 aa 31–110, and Phl p 6 aa 1–33. Histamine release into the supernatant was measured by RIA (Immunotech) and is expressed as percentage of total histamine.

After informed consent was obtained, skin prick tests were performed on the forearms of four grass pollen allergic patients with 20-μl aliquots containing different concentrations (100, 10, and 1 μg/ml) of purified rPhl p 6 or rPhl p 6 aa 31–110 as described (36).

Immunization of mice and measurement of Phl p 6-specific IgG1 Ab levels

Eight-week-old female BALB/c mice were obtained from Charles River. Animals were maintained in the animal care unit of Department of Pathophysiology of the Medical University of Vienna according to the local guidelines for animal care. Four microliters of purified rPhl p 6 or the Phl p 6 derivatives were mixed with 200 μl of AluGel-S (Serva). To determine the binding of the polypeptides to AluGel-S, dot blot assays were performed. Samples of the protein-adjuvant mixtures were centrifuged (5 min; 14,000 rpm; room temperature) and 2 μl of the supernatants were dotted onto nitrocellulose. As reference, 5 μg of the purified proteins were diluted in 200 μl of double-distilled H2O without AluGel-S and centrifuged, and 2 μl of the solutions was dotted onto nitrocellulose. The dotted proteins were detected with rabbit anti-rPhl p 6 Abs and a 125I-labeled donkey anti-rabbit Ig antiserum (Amersham Biosciences).

Groups of five mice each, were immunized monthly with 5 μg of purified rPhl p 6, rPhl p 6 aa 1–57, rPhl p 6 aa 31–110, or Phl p 6 aa 1–33, adsorbed to 200 μl of AluGel-S (Serva) by s.c. injections as described (37). Blood samples were taken before each immunization and stored at −20°C until use. IgE and IgG1 responses to complete rPhl p 6 were measured by ELISA as described (37).

MALDI-TOF mass spectrometry and circular dichroism (CD) analysis

Laser desorption mass spectra were acquired in a linear mode with a TOF Confocal MALDI II instrument (Kratos; pHEM). CD measurements of Phl p 6 (aa 1–110), Phl p 6 aa 1–33, and rPhl p 6 aa 31–110 were performed in double-distilled water with protein concentrations of 90.9, 42.8, and 6.56 μM, respectively. Phl p 6 aa 1–57 was measured in 10 mM phosphate buffer (pH 7.0) at a concentration of 28.1 μM. The CD measurements were conducted on a Jasco J-715 spectropolarimeter using a 0.1-cm path length cell with cooling jacket connected to a water thermostating device. Far-UV CD spectra of all samples were taken at 20°C.

1 Abbreviations used in this paper: KLH, keyhole limpet hemocyanin; CD, circular dichroism.
Reactivity of rabbit anti-Phl p 6 Abs with rPhlp6 and Phlp6 derivatives as demonstrated by ELISA

ELISA plates (Greiner) were coated with rPhlp6, rPhlp6 aa 1–57, rPhlp6 aa 31–110 and Phlp6 aa 1–33 (5 μg/ml in PBS) and incubated with serial dilutions (1/1,000, 1/10,000, 1/100,000, and 1/1,000,000) of rabbit anti-rPhlp6 or other rabbit anti-Phl p 6 derivative antisera. Bound rabbit IgG were detected with a 1/2,000 diluted HRP-labeled donkey anti-rabbit IgG antiserum (Amersham Biosciences) (27).

Inhibition of allergic patients’ IgE binding to rPhlp6 with rPhlp6 aa 31–110-specific IgG Abs as determined by ELISA

ELISA plates (Greiner) were coated with purified rPhlp6, rPhlp6 aa 1–57, rPhlp6 aa 31–110 and Phlp6 aa 1–33 (5 μg/ml in PBS) and incubated with serial dilutions (1/1,000, 1/10,000, 1/100,000, and 1/1,000,000) of rabbit anti-rPhlp6 or the rabbit anti-rPhlp6 6 derivative antiserum. Bound rabbit IgG were detected with a 1/2,000 diluted HRP-labeled donkey anti-rabbit IgG antiserum (Amersham Biosciences) (27).

Rat basophil leukemia (RBL) cell degranulation experiments

rPhlp6 (0.1 μg/ml) was preincubated with different dilutions (0, 2, 5, and 10%) of rabbit anti-rPhlp6, rabbit anti-rPhlp6 aa 31–110, rabbit anti-rPhlp6 aa 31–110 KLH, or, for control purposes, of a normal rabbit serum, in Tyrode’s buffer for 2 h at 37°C. The mixtures were exposed to RBL cells, which had been passively sensitized with Phlp6-specific mouse IgE. Supernatants were analyzed for β-hexosaminidase activity as described (39). Data were expressed as mean ± SEM. Statistical significance was assessed using an unpaired Student’s t test. Statistically significant differences (p < 0.05) between preimmune serum values and the corresponding data values were indicated.

Results

Production and purification of Phlp6 fragments

We have recently isolated cDNAs coding for the complete Phlp6 allergen (accession no. Y16956) and for N-terminally truncated Phlp6 fragments. rPhlp6 lacking the first 4 aa had shown almost comparable IgE reactivity as the complete rPhlp6 molecule. However, deletion variants lacking 30 aa (accession no. Y16958), 53 aa (accession no. Y16959), and 57 aa (accession no. Y16960) exhibited considerably reduced IgE reactivity in first pilot experiments (29). Based on these observations, we expressed two recombinant Phlp6 fragments of the mature Phlp6 protein comprising aa 1–57 and 31–110 in E. coli. In addition, a synthetic peptide comprising the first 33 aa of Phlp6 was synthesized. Fig. 1A shows a graphic representation of the three Phlp6 fragments. In Fig. 1B, the fragments were colored in a ribbon representation of the crystal structure of Phlp6, which has been recently solved by...
x-ray crystallography (A. A. Fedorov, unpublished data; Protein Data Base (PDB) ID 1NLX).

The two recombinant fragments (aa 1–57, 31–110) were expressed using plasmid pET 17b in *E. coli* BL 21 (DE3) as C-terminally hexa-histidine-tagged proteins. Both recombinant proteins accumulated in the inclusion body fraction of *E. coli* and after solubilization in urea were purified to homogeneity by nickel affinity chromatography. The synthetic peptide comprised the N-terminal 33 aa of Phl p 6. The Coomassie blue-stained SDS-PAGE gel in Fig. 1C showed that recombinant and synthetic Phl p 6 fragments of >90% purity were obtained.

**FIGURE 2.** MALDI-TOF MS analysis and far-UV CD analysis of purified recombinant rPhl p 6 and Phl p 6 derivatives. A, MALDI-TOF MS analysis was performed with purified Phl p 6 (a), Phl p 6 aa 1–33 (b), rPhl p 6 aa 1–57 (c), and rPhl p 6 aa 31–110 (d). The x-axes show the mass/charge ratio, and signal intensities are displayed on the y-axes as percentage of the most intensive signal obtained in the investigated mass range. B, Results of the far-UV CD analysis are expressed as mean residue ellipticity (y-axis) at a given wavelength (x-axis).
MALDI-TOF analysis of purified rPhl p 6 and the purified rPhl p 6 fragments confirmed their calculated molecular masses deduced from the sequences of the molecules (Fig. 2A).

Recombinant and synthetic Phl p 6 fragments have lost the typical α helical fold of rPhl p 6

As previously reported, the far-UV CD spectrum of purified rPhl p 6 indicates that the protein contains a considerable amount of α helical secondary structure with minima at 208 and 220 nm and a pronounced maximum at 192 nm (29). Secondary structure analysis of rPhl p 6 using the program CDSSTR (40, 41) yielded 68% α helix, 7% β strands, 9% turns, and 16% random coil structures (Fig. 2B).

The CD analysis is in good agreement with the results obtained by crystallographic study of rPhl p 6, which showed that the Phl p 6 monomer forms a four-helical up-and-down bundle (Fig. 1B) (A. A. Fedorov, unpublished data; PDB ID 1NLX), a common structural motif in globular proteins (42). This motif can also be found in Phl p 5 (1L3P) and cytochrome b562 (1QPU) by performing a search with the SSM server (43). The four helices 1, 2, 3, and 4 are composed of residues 3–27, 32–53, 59–77, and 81–98 with the residue numbering for mature sequence of Phl p 6 (accession no. Y16956). The hydrophobic core of the Phl p 6 monomer is formed by Ile11, Val14, Phe18 from the helix α1; by Phe37, Phe41, Ala52 from the helix α2; by Leu59, Leu63, Ala70, Ala74 from the helix α3; and by Phe86, Val87, Phe90, Leu94 from the helix α4. All hydrophilic residues are exposed to solvent.

When the Phl p 6 fragments were designed, the three-dimensional structure of Phl p 6 was not yet available. It was therefore interesting to note that the purified Phl p 6 derivatives represented more or less complete isolated helices. The synthetic N-terminal peptide comprised the first helix 1 (aa 1–33), fragment aa 1–57 included helices 1 and 2, and Phl p 6 aa 31–110 represented helices 2 to 4. Although each of the fragments contained at least one complete helix, we found that they all had lost their α helical structure as shown by the far-UV CD spectra (Fig. 2B). The spectra of the fragments are dominated by patterns of typical random coil secondary structures with a strong negative band at 200 nm, a shoulder at 220 nm, and a rise at 212 nm (Fig. 2B).

The recombinant and synthetic Phl p 6 fragments exhibit reduced IgE and IgG reactivity

Detailed IgE reactivity studies were conducted with the three Phl p 6 fragments in 54 grass pollen allergic patients. In a first series of studies, the IgE reactivity of the Phl p 6 fragments (aa 1–57, 31–110, 1–33) was compared with that of complete rPhlp6 by Western blotting with sera from 37 grass pollen allergic patients. The OD corresponding to the amount of bound Abs are displayed on the y-axis. The results are shown as box-and-whisker plots where 50% of the values are within the boxes and nonoutliers are between the bars. Lines within the boxes indicate the median values. The open circles and stars indicate outliers and extremes.
pollen allergic patients used in the immunoblotting experiment showed IgE reactivity to Phl p 6, whereas no IgE reactivity could be detected to rPhl p 6 aa 1–57 and Phl p 6 aa 1–33. Only three sera exhibited weak IgE reactivity to rPhlp6a a1–57 and Phlp6a a1–33. Only three sera exhibited weak IgE reactivity to rPhlp6a a31–110 (Fig. 3A). A rabbit anti-rPhl p 6 antiserum showed reactivity with rPhl p 6 as well as with each of the Phl p 6 fragments (lanes I) indicating that the proteins had been transferred to the membranes (Fig. 3A). Serum from a nonallergic person (lanes N) and the rabbit’s preimmune serum (lanes P) did not show any binding (Fig. 3A).

In addition, ELISA experiments were performed with 37 sera from grass pollen allergic patients (Fig. 3B). Also in the ELISA we found that rPhl p 6 aa 1–57, rPhl p 6 aa 31–110, and Phl p 6 aa 1–33 showed a strong reduction of IgE reactivity compared with complete Phl p 6 in the range of 68, 66, and 81%, respectively.

Similar results were obtained when we compared the IgG reactivity of the Phl p 6 fragments with that of complete rPhl p 6 in ELISA using sera from additional 37 grass pollen allergic patients with IgG reactivity to Phl p 6 (Fig. 3C). rPhl p 6 aa 1–57, rPhl p 6 aa 31–110, and Phl p 6 aa 1–33 showed also a strong reduction of IgG reactivity compared with complete Phl p 6 (66, 60, and 62%, respectively).

Reduction of allergenic activity of Phl p 6 fragments

To compare the allergenic activity of complete rPhl p 6 with that of the Phl p 6 fragments, granulocytes from four grass pollen allergic patients were incubated with different concentrations of recombiant Phl p 6, rPhl p 6 aa 1–57, rPhl p 6 aa 31–110, and Phl p 6 aa 1–33 (Fig. 4). In each of the four patients, complete rPhl p 6 induced strong basophil degranulation already at a concentra-
of immunogenicity could not be overcome when mice were immunized with the KLH-coupled Phl p 6 peptide (data not shown). Likewise, immunization with rPhl p 6 aa 1–57 failed to induce IgG1 Abs against the Phl p 6 wild-type allergen (data not shown).

Comparison of Phl p 6-specific IgG titers in rabbit antisera obtained by immunization with complete rPhlp6 and Phlp6 derivatives

The lack of immunogenicity of the AluGel-S bound N-terminal Phl p 6 fragments in mice prompted us to perform further immunization experiments in rabbits using KLH-coupled proteins and a strong adjuvant, i.e., CFA. The titers of IgG reactivity to rPhl p 6 and to Phl p 6 fragments were determined in the final bleedings obtained from rabbits that had been immunized with rPhlp6, Phlp6 aa 1–33 KLH, rPhlp6 aa 1–57 KLH, or rPhlp6 aa 31–110 KLH by ELISA titration experiments. Each of the three KLH-coupled Phlp6-derivatives induced Phl p 6-specific IgG responses in rabbits (Fig. 5). According to the serum dilution experiment, immunization with rPhlp6 induced a higher titer of Phl p 6-specific IgG Abs than immunization with rPhlp6 aa 1–57 KLH > rPhlp6 aa 1–33 KLH > rPhlp6 aa 31–110 KLH (Fig. 5). The anti-rPhlp6 antisera reacted also stronger with rPhlp6 aa 31–110 than the anti-Phlp6 aa 31–110 antiserum. The anti-rPhlp6 aa 1–33 antisera, but not the anti-Phlp6 aa 1–33 antisera showed IgG reactivity with the C-terminal fragment that can be attributed to the overlapping sequence of aa 31–57. Phlp6 aa 1–33 was recognized by the anti-rPhlp6, anti-Phlp6 aa 1–33, and anti-rPhlp6 aa 1–57 antisera but not by the anti-Phlp6 aa 31–110 antisera. Each of the antisera showed IgG reactivity with rPhlp6 aa 1–57 (Fig. 5).

Anti-rPhlp6 aa 31–110 Abs inhibit human IgE binding to complete rPhlp6

Only rPhlp6 aa 31–110 had induced Phl p 6-specific IgG Abs in both mice and rabbits. Next, we investigated whether IgG Abs induced with the hypoallergenic rPhlp6 aa 31–110 can inhibit grass pollen allergic patients’ (n = 11) IgE binding to rPhlp6 wild type by ELISA inhibition experiments. Preincubation of Phlp6 p 6 with rabbit IgG raised against rPhlp6 aa 31–110 or KLH-coupled rPhlp6 aa 31–110 inhibited 21–67% (average, 41%) and 50–84% (average, 67%) of human IgE binding to complete Phlp6 p 6, respectively. Rabbit anti-rPhlp6 aa 31–110 Abs inhibited 78–96% (average, 90%) of IgE binding to rPhlp6 (Table II). Similar results were obtained with mouse anti-Phlp6 aa 31–110 and mouse anti-Phlp6 aa 6 Abs (data not shown).

Abs induced by immunization with hypoallergenic rPhlp6 aa 31–110 inhibit basophil degranulation

The protective activity of IgG Abs induced with rPhlp6 derivatives was further investigated using RBL cell mediator release

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**Table I. Induction of Phlp6-specific IgG1 Abs in mice**

<table>
<thead>
<tr>
<th>Group</th>
<th>OD (Preimmune sera)</th>
<th>OD (4 wk)</th>
<th>OD (8 wk)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse anti-rPhlp6 IgG1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.060</td>
<td>0.445</td>
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</tr>
<tr>
<td>2</td>
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<td>1.528</td>
<td>&gt;2.5</td>
</tr>
<tr>
<td>3</td>
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<td>0.253</td>
<td>&gt;2.5</td>
</tr>
<tr>
<td>4</td>
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<td>0.508</td>
<td>&gt;2.5</td>
</tr>
<tr>
<td>5</td>
<td>0.062</td>
<td>0.864</td>
<td>&gt;2.5</td>
</tr>
<tr>
<td>Mean</td>
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<td>0.720</td>
<td>&gt;2.5</td>
</tr>
<tr>
<td>Mouse anti-rPhlp6 aa 31–110 IgG1</td>
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<tr>
<td>Mice</td>
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<tr>
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<td>1.218</td>
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<td>Mean</td>
<td>0.057</td>
<td>1.394</td>
<td>&gt;2.5</td>
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*Groups of five mice were immunized either with rPhlp6 or with rPhlp6 aa 31–110. Serum samples obtained before immunization (preimmune sera) and after 4 and 8 wk of immunization were tested for IgG1 reactivity with rPhlp6. The OD values corresponding to the amount of bound Abs are displayed.*

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**FIGURE 5.** Reactivity of rabbit anti-rPhlp6 and Phlp6 fragment antisera with rPhlp6 and Phlp6 fragments. ELISA plate-bound rPhlp6, rPhlp6 aa 31–110, rPhlp6 aa 1–57, and Phlp6 aa 1–33 were tested for reactivity with different dilutions (x-axes) of rabbit antisera raised against rPhlp6, Phlp6 aa 1–33 KLH, rPhlp6 aa 1–57 KLH, or rPhlp6 aa 31–110 KLH. The OD (y-axes) correspond to the amount of bound Abs.
inhibition experiments. rPhl p 6 was preincubated with increasing concentrations of rabbit anti-rPhl p 6, anti-rPhl p 6 aa 31–110, or anti-rPhl p 6 aa 31–110 KLH Abs and a normal rabbit serum, respectively. The immune complexes were then exposed to RBL cells that had been preloaded with Phl p 6-specific IgE. Fig. 6 shows that anti-rPhl p 6 as well as anti-rPhl p 6 aa 31–110 Abs led to a statistically significant inhibition of Phl p 6-induced mediator release from RBL cells. In agreement with the results obtained in vitro (Table II), antisera induced with rPhl p 6 inhibited degranulation and β-hexosaminidase release from RBL cells more efficiently than antisera induced with rPhl p 6 aa 31–110 alone (Fig. 6). However, when the concentration of Abs raised against KLH-coupled rPhl p 6 aa 31–110 was increased (i.e., addition of 10% v/v of the antiserum), the inhibition of degranulation was almost as good as that obtained with the rabbit anti-Phl p 6 antisera and no statistically significant difference was observed between the inhibition obtained with Abs raised against rPhl p 6 or with anti-rPhl p 6 aa 31–110 Abs at this concentration (Fig. 6). No inhibition of mediator release was noted when the allergen was preincubated with a rabbit serum obtained before immunization (Fig. 6).

Discussion

Grass pollen belongs to the most important allergen sources worldwide (44). The molecular and immunological characterization of the allergenic components in grass pollen, IgE reactivity studies conducted in several populations, and a recent immunotherapy trial performed with recombinant timothy grass pollen allergens have identified a panel of four timothy grass pollen allergens for vaccination against grass pollen allergy (24, 45–52). In this study, we constructed and characterized hypoallergenic derivatives of one of these four major allergens, Phl p 6, from timothy grass pollen (29, 31). The hypoallergenic derivatives represented recombinant fragments that were based on previous IgE epitope mapping data (29). The three Phl p 6 fragments included a synthetic peptide comprising aa 1–33 of Phl p 6 and two recombinant fragments representing aa 1–57 and aa 31–110 of Phl p 6. Each of these fragments exhibited strongly reduced IgE and IgG binding capacity as well as reduced allergenic activity compared with the wild-type allergen. Similar as found for other important respiratory allergens (53) and in particular for hypoallergenic fragments of the major birch pollen allergen, Bet v 1 (36), the loss of allergenic activity was associated with a loss of their native-like structure as shown by CD analysis, indicating that Phl p 6 contains predominantly conformational IgE epitopes. The recently solved three-dimensional structure of Phl p 6 (A. A. Fedorov, unpublished data; PDB ID 1NLX) shows that the allergen forms a four-helical up-and-down bundle. Interestingly, we found that the aa 31–110 Phl p 6 derivative lacks almost parably to the complete Phl p 6 molecule (29). Also, Phl p 6 aa 1–57, representing the sequence of helices α1 and α2, exhibited random coil structure suggesting that helices α3 and/or α4 are also

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**Table II. Inhibition of allergic patients’ IgE binding to rPhl p 6 with rPhlp6a 31–110-specific IgG Abs**

<table>
<thead>
<tr>
<th>Patients</th>
<th>OD</th>
<th>OD</th>
<th>% Inhibition of IgE binding</th>
<th>OD</th>
<th>OD</th>
<th>% Inhibition of IgE binding</th>
<th>OD</th>
<th>OD</th>
<th>% Inhibition of IgE binding</th>
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<td>1</td>
<td>0.303</td>
<td>0.099</td>
<td>67</td>
<td>0.048</td>
<td>0.84</td>
<td>83</td>
<td>0.050</td>
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<tr>
<td>2</td>
<td>0.980</td>
<td>0.778</td>
<td>21</td>
<td>0.423</td>
<td>0.57</td>
<td>90</td>
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<tr>
<td>3</td>
<td>0.512</td>
<td>0.360</td>
<td>30</td>
<td>0.187</td>
<td>0.63</td>
<td>94</td>
<td>0.044</td>
<td>0.91</td>
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<tr>
<td>4</td>
<td>0.345</td>
<td>0.251</td>
<td>27</td>
<td>0.171</td>
<td>0.50</td>
<td>82</td>
<td>0.061</td>
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<tr>
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<td>0.698</td>
<td>56</td>
<td>0.295</td>
<td>0.81</td>
<td>95</td>
<td>0.082</td>
<td>95</td>
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<tr>
<td>6</td>
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<td>0.528</td>
<td>52</td>
<td>0.274</td>
<td>0.75</td>
<td>89</td>
<td>0.054</td>
<td>95</td>
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<tr>
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<td>23</td>
<td>0.439</td>
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<tr>
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<td>51</td>
<td>0.212</td>
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<tr>
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<tr>
<td>Mean</td>
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<td>41</td>
<td>0.275</td>
<td>0.67</td>
<td>90</td>
<td>0.069</td>
<td>90</td>
<td></td>
</tr>
</tbody>
</table>

*ELISA plate-bound rPhl p 6 was preincubated with a normal rabbit serum, rabbit anti-rPhlp6a 31–110 Ig, rabbit anti-rPhlp6a 31–110-KLH Ig, or rabbit anti-rPhlp6 6 Ig, and thereafter incubated with sera from 11 grass pollen allergic patients. The amounts of bound IgE Abs are expressed as OD, and the percentage inhibitions of serum IgE-binding to Phlp6b were calculated. The immune complexes were then exposed to RBL cells that had been preloaded with Phl p 6-specific IgE. Fig. 6 shows that anti-rPhlp6 as well as anti-rPhlp6 aa 31–110 Abs led to a statistically significant inhibition of Phlp6aa 6-induced mediator release from RBL cells. In agreement with the results obtained in vitro (Table II), antisera induced with rPhlp6 inhibited degranulation and β-hexosaminidase release from RBL cells more efficiently than antisera induced with rPhlp6 aa 31–110 alone (Fig. 6). However, when the concentration of Abs raised against KLH-coupled rPhlp6 aa 31–110 was increased (i.e., addition of 10% v/v of the antiserum), the inhibition of degranulation was almost as good as that obtained with the rabbit anti-Phlp6 antisera and no statistically significant difference was observed between the inhibition obtained with Abs raised against rPhlp6 or with anti-rPhlp6 aa 31–110 Abs at this concentration (Fig. 6). No inhibition of mediator release was noted when the allergen was preincubated with a rabbit serum obtained before immunization (Fig. 6).
needed for stabilization of the structure. The peptide comprising aa 1–33 was unfolded.

Immunotherapy trials performed with hypoallergenic rBet v 1 derivatives (36, 54) as well as with recombinant wild-type-like allergens (24) indicated that, besides other mechanisms, the induction of blocking IgG Abs that inhibit a patient’s IgE recognition of the allergens is important for a successful outcome (23–25, 55). The rBet v 1 fragments used in this clinical trial induced Bet v 1-specific IgG Abs, although they were not recognized by IgG from patients before the treatment (23). This finding could have been almost predicted on the basis of immunization experiments conducted in BALB/c mice showing that the rBet v 1 fragments formulated with aluminum hydroxide as in the human trial induced robust Bet v 1-specific IgG responses (56).

We were therefore interested to study the three hypoallergenic Phl p 6 derivatives for their potential to induce blocking IgG Abs. When we immunized mice with Phl p 6 fragments bound to an adjuvant allowed for human use (i.e., aluminum hydroxide), only the C-terminal fragment rPhl p 6 aa 31–110 induced Phl p 6-specific IgG Abs that inhibited grass pollen allergic patients’ IgE binding to the natural allergen. The fragment comprising aa 1–33 failed to induce any detectable IgG response to Phl p 6 in mice using aluminum hydroxide as adjuvant, regardless of whether it was used as isolated peptide or whether it was coupled to a carrier protein. Using CFA, which is a much stronger adjuvant than aluminum hydroxide and a large amount of protein (200 μg/injection), it was possible to induce Phl p 6-specific IgG responses in rabbits with both N-terminal fragments Phl p 6 aa 1–33 and rPhl p 6 aa 1–57. Similar results were obtained for an N-terminal fragment of Bet v 1, the major birch pollen allergen, which induced weaker IgG responses in mice with aluminum hydroxide than with CFA (57). There are several explanations for these results. The possibility that poor adsorption of the N-terminal fragments to aluminum hydroxide was responsible for the lack of immunogenicity in mice can be excluded, because we found that the polypeptides were indeed bound. A more likely explanation is that CFA is a stronger adjuvant than aluminum hydroxide and that different animals show varying immune responses to the polypeptides.

Our data therefore emphasize that it is important to test candidate molecules in a formulation that can be used in allergic patients before they are considered as suitable allergy vaccines. Because the N-terminal fragments were not immunogenic under conditions comparable to those used for humans and because CFA cannot be used in humans, it seems that rPhl p 6 aa 31–110 represents the most suitable molecule for vaccination against allergy to Phl p 6. This was further demonstrated by the finding that rPhl p 6 aa 31–110 coupled to KLH was almost as immunogenic as Phl p 6 and induced almost as high titers of IgG Abs competing with patients’ IgE reactivity as those induced with the Phl p 6 wild-type allergen. Increases of immunogenicity have recently been reported for allergens that were expressed as hybrid molecules together with other allergens, and it may therefore be considered to fuse the rPhl p 6 aa 31–110 derivative or even the N-terminal fragments with other hypoallergenic grass pollen allergen derivatives to increase their immunogenicity and facilitate the production of a composite grass pollen vaccine (58–60).

Disclosures

The authors have no financial conflict of interest.

References


42. Rossi, R., E. G. Monasterolo, and S. Monasterolo. 2001. Measurement of IgE antibodies against purified grass-pollen allergens (Phl p 1, 2, 3, 4, 5, 6, 7, 11, and 12) in sera of patients allergic to grass pollen. Allergy 56: 1180–1185.

