

Different allergenic activity of grass pollen allergens revealed by skin testing

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ABSTRACT

Background Grass pollen is one of the most important allergen sources. The aim of this study was to compare the *in vivo* allergenic activity of two recently characterized major grass pollen allergens, Phl p 4 and Phl p 13, with three established major grass pollen allergens, Phl p 1, Phl p 2 and Phl p 5 as a basis for the formulation of a grass pollen allergy vaccine based on purified allergens.

Material and methods Eighty-two grass pollen allergic patients were skin prick tested with serial dilutions of approximately equimolar concentrations of the purified allergens in a double-blind study.

Results Phl p 4 and Phl p 13 were identified as major grass pollen allergens according to IgE binding frequency (Phl p 4: 85%; Phl p 13: 56%), but exhibited a five to nine-fold lower allergenic skin reactivity compared to Phl p 1, Phl p 2 or Phl p 5.

Conclusion Our results indicate that Phl p 4 and Phl p 13 are not essential components for a therapeutic grass pollen vaccine and underpin the importance of evaluating the *in vivo* allergenic activity of individual allergens for the formulation of therapeutic vaccines based on purified allergens.

Keywords Allergenic activity, grass pollen allergy, human, immunotherapy, recombinant allergen, skin prick test.

Eur J Clin Invest 2008; 38 (4): 260–267

Introduction

More than 25% of the population suffers from IgE-mediated allergies [1]. As a result of extensive research carried out in the field of allergen characterization during the last 30 years, the disease-eliciting allergen molecules have been purified from natural allergen sources or have been produced as recombinant (r) allergens for diagnosis and the first immunotherapy studies based on recombinant allergen molecules have been conducted [2–4]. Detailed molecular, structural and immunochemical data are available for most of the characterized allergens, but relatively few studies have been conducted to analyse the *in vivo* allergenic activity of individual allergen molecules [5–7]. A project within the European GA²LEN (*Global Allergy and Asthma European Network*) research network is dedicated to study factors responsible for differences between asymptomatic persons and patients with a clinically relevant sensitization to allergens [8]. One factor for this phenomenon may be the existence of IgE-reactive ‘allergens’ with low allergenic activity. If this is the case, the evaluation of the *in vivo* allergenic activity of individual allergens in a given allergen source will become an important issue for the design of diagnostic tests and vaccines based on purified natural (n) and recombinant

(r) molecules, particularly for allergen sources containing several different allergen molecules.

Grass pollen represents one of the most frequent and potent elicitors of respiratory allergies (e.g. rhinoconjunctivitis, bronchial asthma) [9]. So far, 11 different groups of grass pollen allergens have been characterized regarding their structural, biochemical and immunochemical properties [9,10]. IgE binding studies performed in different populations of grass pollen allergic patients, as well as studies evaluating the *in vitro* and *in vivo* allergenic activity, indicate that four recombinant major timothy grass pollen allergens (Phl p 1, Phl p 2, Phl p 5 and Phl p 6) include a major part of the allergenic activity of grass pollen (Table 1) [11–19]. This conclusion is supported by the demonstration of clinical improvement in an immunotherapy trial using a cocktail of recombinant Phl p 1, Phl p 2, Phl p 5 and Phl p 6 in subjects with hay fever [20].

More recently, two additional timothy grass pollen allergens, Phl p 4 and Phl p 13, representing glycoproteins from the high molecular weight fraction of grass pollen, were described as major allergens according to IgE binding frequencies (Table 1)

Table 1 Characteristics of major grass pollen allergens. Major timothy grass pollen allergens are described according to molecular weight, biological function, distribution in various allergen sources, IgE-recognition frequency and allergenic activity as evaluated by *in vitro* cell activation or *in vivo* provocation testing

Allergen	Molecular weight (kDa)	Biol. Function/ Biochem. property	Distribution	Reported IgE recognition frequency	Allergenic activity confirmed by provocation testing	Ref.
Phl p 1	31–35	similar to expansins/glycoprotein	Grass pollen (<i>Pooideae</i> + <i>Chloridoideae</i>)	90%	+	10–15
Phl p 2	10–12	unknown/protein	Grass pollen (<i>Pooideae</i>)	40–60%	+	10–16
Phl p 5	32–38	ribonuclease/protein	Grass pollen (<i>Pooideae</i>)	65–85%	+	10–15
Phl p 6	13	P-particle associated/protein	Grass pollen (<i>Pooideae</i>)	60–70%	+	18,19
Phl p 4	55	Similar to beberine bridge (BBE)-like oxido-reductases/glycoprotein	Grass (<i>Pooideae</i> + <i>Chloridoideae</i>) tree and weed pollen, plant-derived food	80%	–	21–24
Phl p 13	55–60	polygalacturonase/glycoprotein	Grass pollen (<i>Pooideae</i> + <i>Chloridoideae</i>)	50%	–	22,25,26

[21–26]. The allergenic activity of these two molecules has not yet been evaluated and thus the question remains whether they are essential components of a therapeutic grass pollen vaccine.

In order to answer this question we conducted a skin test study to compare the IgE reactivity and allergenic potency of Phl p 4 and Phl p 13 with the established major grass pollen allergens, Phl p 1, Phl p 2 and Phl p 5.

Patients and methods

Study population

Eighty-two grass pollen allergic subjects (age 21–37, mean age 27; 31 males, 51 females) were included in the study. Patients were selected according to their history of seasonal grass pollen allergy, a positive skin prick test (SPT) reaction > 3 mm mean wheal diameter to timothy grass pollen extract and grass pollen-specific serum IgE levels of at least 0.7 kUA L⁻¹ (≥ RAST class 2) (CAP, Phadia, Uppsala, Sweden). All individuals had allergic rhinitis and conjunctivitis and/or mild to moderate asthma, attributable to grass pollen. Patients with clinically active atopic dermatitis were excluded. At the time of the study all subjects had a stable lung function (on the basis of spirometry measurement) and there was no evidence of current airway infection. None of the subjects had ever received immunotherapy against grass pollen. Furthermore, subjects had not been treated with anti-inflammatory drugs (corticosteroids, antihistamines, immunosuppressive drugs) in the six week period before the study. Pregnant or lactating females were excluded.

Study design

The study was conducted in 82 Austrian grass pollen allergic subjects. Half of the patients were tested before (May 2004) and

the other half after (October 2004) the grass pollen season when patients exhibit higher grass pollen-specific IgE levels and increased sensitivity, [27] in order to study whether the seasonal boosting effect would affect test results. From each subject serum samples were collected shortly before skin testing. Skin prick tests were performed with recombinant (Phl p 1, Phl p 2, Phl p 5a) and natural (Phl p 4, Phl p 13) timothy grass pollen allergens, commercially available timothy grass pollen extract, histamine solution and sterile glycerol saline as a negative control (Allergopharma, Reinbek, Germany). The purified grass pollen allergen molecules were applied in three dilutions and in approximately equimolar concentrations as follows: rPhl p 1 and rPhl p 5a: 9 µg mL⁻¹, 3 µg mL⁻¹, 1 µg mL⁻¹; rPhl p 2: 4.5 µg mL⁻¹, 1.5 µg mL⁻¹, 0.5 µg mL⁻¹; nPhl p 4 and nPhl p 13: 18 µg mL⁻¹, 6 µg mL⁻¹, 2 µg mL⁻¹. Skin prick tests were performed in a double-blinded manner and in duplicates.

The study was approved by the Ethical Committee of the Austrian Community for Clinical Pharmacology and Therapy, Vienna, Austria. Each patient gave written informed consent.

Purified recombinant (r) and natural (n) timothy grass pollen allergens

Recombinant timothy grass pollen allergens rPhl p 1 [28], rPhl p 2 [16], and rPhl p 5a (accession number AJ555152) were expressed in *Escherichia coli* and purified as described previously [29]. The recombinant allergens have previously been shown to be immunologically equivalent to the corresponding natural allergens. Natural Phl p 4 and Phl p 13 were purified from timothy grass pollen by chromatography as previously described [22]. The allergens were provided in a coded manner as 10-fold concentrated sterile stock solutions in phosphate-buffered saline and were stored at –20 °C. Prior to being used the allergens were

diluted in sterile phosphate-buffered saline containing 50% glycerol added to the final concentrations. Allergen solutions for skin testing were freshly prepared for each test series and used within 24 h.

Skin prick testing

For SPT, drops of approximately 15 µL of the test solutions were placed on the volunteers forearms at a distance of more than 2 cm between individual application points. Each drop was pricked with a fresh sterile lancet (Allergopharma) and results were recorded after 20 min with a ballpoint pen by transferring the wheal area with a tape to paper. Wheal areas were measured by computerized digital planimetry using the software program AnalySIS Version 3.2 (Soft Imaging System GmbH, Münster, Germany). All skin tests were performed in duplicate and a positive reaction was defined as a mean wheal area of at least 2 mm².

Measurement of allergen-specific IgE antibodies

Detection of allergen-specific IgE antibodies was performed by ELISA (*Enzyme-Linked Immunoabsorbent Assay*) as described [30]. IgE levels measured by ELISA were found to correlate well with quantitative CAP (Phadia) measurements and hence were chosen for semi-quantitative IgE measurements [13,31]. ELISA plates (Nunc, Roskilde, Denmark) were coated with 5 µg mL⁻¹ purified timothy grass pollen allergens or 100 µg mL⁻¹ of timothy grass pollen extract. After blocking with PBS (*phosphate-buffered saline*) containing 1% w/v BSA (*bovine serum albumin*), plates were incubated with patients' sera diluted 1 : 5 in PBS/0.5% w/v BSA overnight at 4 °C. Detection of bound IgE antibodies was performed using a mouse monoclonal antihuman IgE antibody (Pharmingen, San Diego, CA, USA) followed by a peroxidase-labelled sheep anti-mouse antiserum (Amersham, Buckinghamshire, UK) and visualized using ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)) as a colour substrate. Serum from a non-allergic individual was included as a negative control and results were normalized between plates by including reference sera and allergens on each of the plates. Optical density (OD) values above the mean value +2 × SD of the negative controls were defined as positive.

Statistical analysis of the data

For correlations between wheal areas (mm²) and specific IgE (OD) the Spearman rank correlation coefficient was used. SPTC₁₀₀ (log concentration of an allergen that would induce a wheal size of 100 mm²) was calculated by the following procedure: Linear regression of wheal size on log concentration of the allergen was computed and if the coefficient of determination was 90% or greater and the slope was positive SPTC₁₀₀ was determined as the abscissa for a wheal size of 100 mm². Frequencies of IgE reactivity were compared across allergens by Cochran's Q-test and IgE levels

Table 2 IgE reactivity of individual grass pollen allergen molecules as determined by ELISA. The numbers and percentages of grass pollen allergic patients (*n* = 82) with IgE reactivity to the allergen molecules as well as the mean OD levels corresponding to the amount of bound IgE antibodies are shown

Allergen	Number of IgE-reactive sera (%) <i>n</i> = 82	Mean IgE level (OD)
rPhl p 1	69 (84.1%)	0.658
rPhl p 2	68 (82.9%)	0.290
rPhl p 5	61 (73.1%)	1.001
nPhl p 4	70 (85.3%)	0.259
nPhl p 13	46 (56.1%)	0.290

by Friedman's test and Wilcoxon-Wilcox post hoc tests. For each of the tests *P* < 0.05 was considered to be significant.

Results

Phl p 1, Phl p 2, Phl p 4, Phl p 5a and Phl p 13 are major allergens according to frequencies of IgE recognition but allergen-specific IgE levels vary

The frequency of IgE reactivity to rPhl p 1, rPhl p 2, rPhl p 5a, nPhl p 4 and nPhl p 13 was determined by ELISA in 82 grass pollen allergic individuals (Table 2). We found that 84% of the sera showed IgE reactivity with rPhl p 1, 83% with rPhl p 2, 73% with rPhl p 5a, 85% with nPhl p 4 and 56% with nPhl p 13 (Table 2). According to the Allergen Nomenclature Guidelines the proteins under investigation can be classified as major allergens, because each of them was recognized by 50% or more of the tested subjects.

However, we observed considerable variability of allergen-specific serum IgE antibody levels to the individual major grass pollen allergens (Table 2). The mean IgE levels were highest for rPhl p 5 (mean OD: 1.001), followed by rPhl p 1 (mean OD: 0.658), rPhl p 2 (mean OD: 0.290), nPhl p 13 (mean OD: 0.290) and nPhl p 4 (mean OD: 0.259). OD values were significantly different with distinctly lower values for rPhl p 2 and nPhl p 13 (*P* < 0.001).

Discrimination of highly allergenic from weakly allergenic allergen molecules according to skin reactivity

Skin prick testing was performed with three serial dilutions of approximately equimolar concentrations of the grass pollen allergens rPhl p 1, rPhl p 2, rPhl p 5, nPhl p 4 and nPhl p 13.

The mean wheal area induced with the highest applied concentration (*c* = 4.5 µg mL⁻¹) of rPhl p 2 was 22.59 mm² with a

Table 3 Immediate type skin reactions to purified grass pollen allergens. The numbers and percentages of grass pollen allergic patients exhibiting a positive skin reaction to a given allergen and allergen concentration as well as the corresponding mean, median and maximum wheal areas are shown

Allergen	Number of positive skin reactions (%) (<i>n</i> = 82)	Mean wheal area (mm ²)	Median wheal area (mm ²)	Maximum wheal area (mm ²)
rPhl p 1 (9 µg mL ⁻¹)	76 (93%)	16.07	13.03	78.95
rPhl p 1 (3 µg mL ⁻¹)	65 (79%)	10.09	9.09	46.3
rPhl p 1 (1 µg mL ⁻¹)	36 (44%)	6.40	5.61	18.49
rPhl p 2 (4.5 µg mL ⁻¹)	49 (60%)	22.59	17.53	109.76
rPhl p 2 (1.5 µg mL ⁻¹)	43 (52%)	16.63	13.50	59.18
rPhl p 2 (0.5 µg mL ⁻¹)	35 (43%)	10.29	8.50	34.24
rPhl p 5 (9 µg mL ⁻¹)	59 (72%)	22.82	21.23	80.35
rPhl p 5 (3 µg mL ⁻¹)	55 (67%)	12.20	10.18	45.57
rPhl p 5 (1 µg mL ⁻¹)	30 (37%)	7.57	7.98	18.56
nPhl p 4 (18 µg mL ⁻¹)	55 (67%)	10.92	10.16	24.27
nPhl p 4 (6 µg mL ⁻¹)	46 (56%)	7.97	7.74	16.75
nPhl p 4 (2 µg mL ⁻¹)	30 (36.7%)	5.05	5.11	11.22
nPhl p 13 (18 µg mL ⁻¹)	24 (29%)	6.05	5.80	13.68
nPhl p 13 (6 µg mL ⁻¹)	11 (13%)	4.2	4.04	10.04
nPhl p 13 (2 µg mL ⁻¹)	6 (7%)	4.43	3.93	6.9

maximum wheal area of 109.76 mm² (Table 3). The highest concentration of rPhl p 5 (*c* = 9 µg mL⁻¹) induced a mean wheal area of 22.82 mm² with a maximum value of 80.3 mm² (Table 3). Fifty percent of the patients corresponding to the 50th percentile exhibited a wheal area greater than 21.23 mm² to rPhl p 5 and of more than 17.53 mm² to rPhl p 2 at equimolar concentrations (Table 3, Fig. 1b,c). rPhl p 1 elicited a maximum wheal area of 78.95 mm² with a mean wheal area of 16.07 mm² at the highest applied concentration (*c* = 9 µg mL⁻¹). The maximum wheal areas induced with the highest concentration of Phl p 4 and Phl p 13 (*c* = 18 µg mL⁻¹) were 24.7 and 13.6 mm², respectively (mean values: 10.9 and 6.0 mm²). nPhl p 13 appeared as the least allergenic molecule of the 5 allergens tested because 50% of the patients exhibited wheal areas smaller than 5.8 mm² (median value; mean wheal area: 6.05 mm²) to the highest concentration tested (Table 3, Fig. 1e). The low allergenic activity of Phl p 4 and Phl p 13 was found before, as well as after, the grass pollen season.

SPTC₁₀₀ values of the grass pollen allergens were calculated to allow a comparison of the allergenic potencies of the individual allergen molecules. The SPTC₁₀₀ describes the calculated allergen concentration which would induce a wheal area of 100 mm².

According to SPTC₁₀₀, rPhl p 2 and rPhl p 5 are the most potent allergens (median concentration inducing a 100 mm² wheal area: 29.7 µg mL⁻¹ and 38.6 µg mL⁻¹, respectively) and thus approximately twice as potent as rPhl p 1 (median SPTC₁₀₀

Table 4 Calculated allergenic activity of grass pollen allergen molecules. The median SPTC₁₀₀ predicts the allergen concentration leading to a wheal area of 100 mm² in 50% or less of patients, whereas 25th and 75th percentile values show the calculated concentrations which would yield such a reaction in up to 25% and up to 75% of patients

Allergen	SPTC ₁₀₀ median	SPTC ₁₀₀ 25–75th percentile
rPhl p 1	73.7 µg mL ⁻¹	34.8–160.8 µg mL ⁻¹
rPhl p 2	29.7 µg mL ⁻¹	13.2–67.9 µg mL ⁻¹
rPhl p 5	38.6 µg mL ⁻¹	21.9–62.3 µg mL ⁻¹
nPhl p 4	191.1 µg mL ⁻¹	122.2–721.0 µg mL ⁻¹
nPhl p 13	237.4 µg mL ⁻¹	148.8–340.9 µg mL ⁻¹

SPTC₁₀₀, estimated allergen concentration inducing a wheal area of 100 mm².

73.7 µg mL⁻¹) (Table 4). Compared to rPhl p 2 and rPhl p 5, the allergenic potency of nPhl p 4 is five to six times lower (median SPTC₁₀₀: 191.1 µg mL⁻¹) and that of nPhl p 13 is approximately six to nine times lower (median SPTC₁₀₀: 237.4 µg mL⁻¹) (Table 4).

Poor correlations of allergen-specific IgE antibody levels and cutaneous reactivities

First of all we noted that the presence of allergen-specific IgE antibodies was not always associated with positive skin test

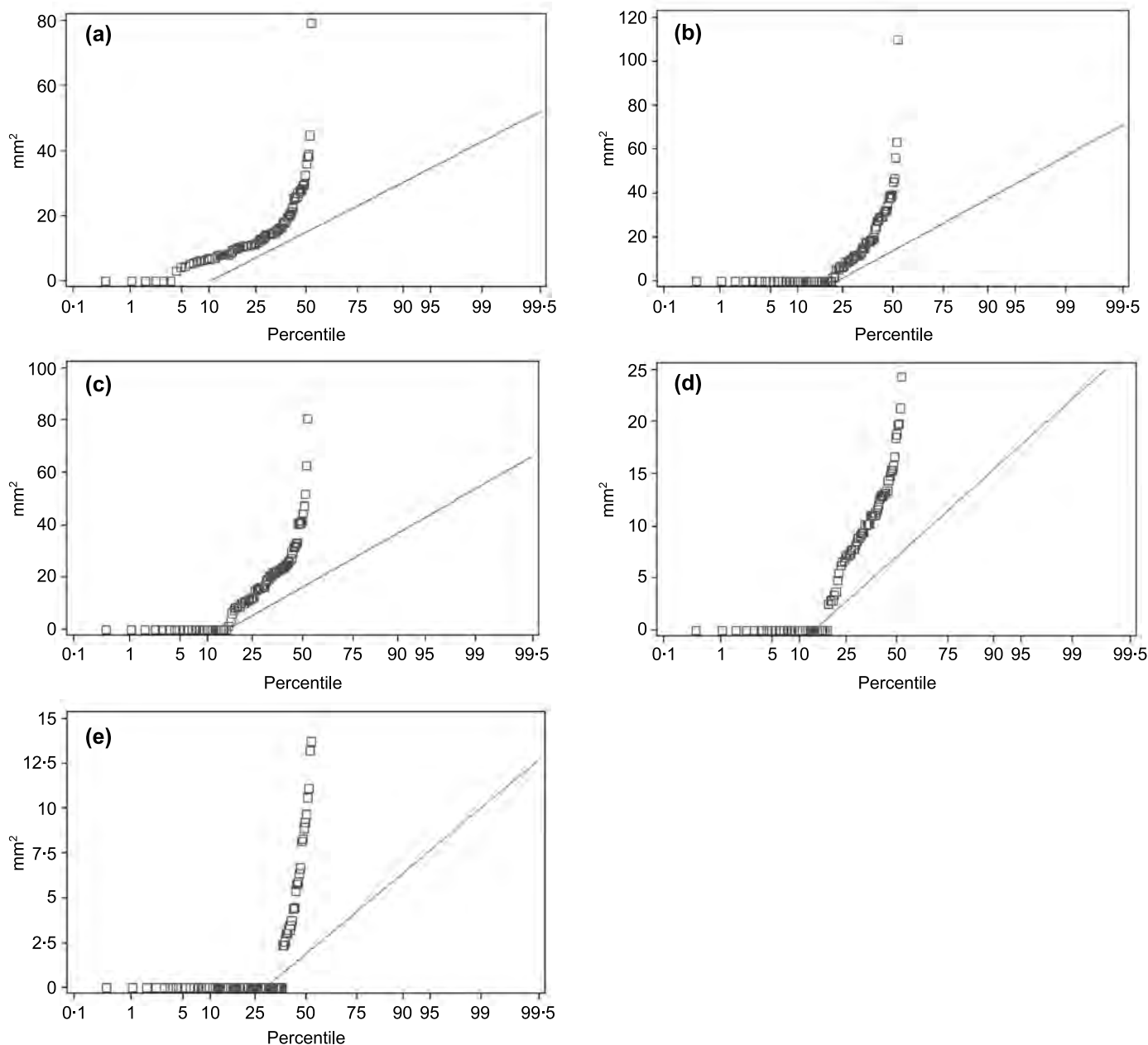


Figure 1 Distribution analysis of wheal areas induced with timothy grass pollen allergens applied in the highest approximate equimolar concentrations. The sizes of the wheal areas (mm^2) are shown on the y-axes. The percentile scales (x-axes) are cut-off values which allow determination of the percentages of reactive patients falling within a given wheal area size. (a) rPhl p 1 ($9 \mu\text{g mL}^{-1}$) (b) rPhl p 2 ($4.5 \mu\text{g mL}^{-1}$) (c) rPhl p 5 ($9 \mu\text{g mL}^{-1}$) (d) nPhl p 4 ($18 \mu\text{g mL}^{-1}$) (e) nPhl p 13 ($18 \mu\text{g mL}^{-1}$).

reactions. The percentages of reactive patients defined by IgE serology and skin testing differed from each other for each of the allergens tested. For example, we found that Phl p 1 induced positive skin reaction in 93% of the patients, but specific IgE antibodies were only detected in 84% of the

patients by ELISA (Tables 2 and 3). On the other hand, IgE reactivity with nPhl p 4 (85%) was higher than skin reactivity (67%) and 56% of the grass pollen allergic patients had IgE specific for nPhl p 13, but only 29% had positive skin reactions (Tables 2 and 3).

Table 5 Grass pollen related symptoms in patients with skin reactivity to certain grass pollen allergen molecules. Displayed are the numbers of patients with positive skin test results, grass pollen-related rhinoconjunctivitis (RC) or rhinoconjunctivitis plus asthma for *exclusively one allergen as displayed, †at least two out of Phl p 1, 2 and 5 or ‡at least one of Phl p 1, 2, 5 plus at least one of Phl p 4, 13

	Phl p 1*	Phl p 2*	Phl p 5*	Phl p 1, 2, 5†	Phl p 1, 2, 5 + Phl p 4, 13‡	Phl p 4*	Phl p 13*
Skin reactivity	2	1	1	12	58	1	0
RC	1	0	1	9	50	0	0
RC + Asthma	1	1	0	3	8	0	0

RC, rhinoconjunctivitis.

*exclusive reactivity.

†at least two of Phl 1, 2, 5.

‡at least one of Phl p 1, 2, 5 plus at least one of Phl p 4, 13.

Accordingly, the correlation of allergen-specific IgE levels with an allergen's SPTC₁₀₀ was low, being significant ($P < 0.05$) only for Phl p 2, Phl p 5 and Phl p 13 (correlation coefficients: Phl p 1-IgE/Phl p 1 SPTC₁₀₀: -0.092; Phl p 2-IgE/Phl p 2 SPTC₁₀₀: -0.289; Phl p 5-IgE/Phl p 5 SPTC₁₀₀: -0.303; Phl p 4-IgE/Phl p 4 SPTC₁₀₀: -0.102; Phl p 13-IgE/Phl p 13 SPTC₁₀₀: 0.272).

Association of grass pollen-related symptoms with skin reactivity to certain allergens

Table 5 displays the occurrence of grass pollen-related symptoms in patients with skin reactivity to certain grass pollen allergen molecules. In 74 out of 82 patients symptoms of rhinoconjunctivitis (RC) or rhinoconjunctivitis plus asthma could be unambiguously attributed to grass pollen exposure. No patient with skin reactivity to Phl p 13 alone was found and the only patient with skin reactivity to Phl p 4 did not show grass pollen related symptoms in the grass pollen season (Table 5). In contrast, four patients who had shown skin reactivity to either Phl p 1, Phl p 2 or Phl p 5 suffered from grass pollen-related symptoms. The majority of patients reacted simultaneously with several grass pollen allergens (Table 5).

Discussion

Advances made in the field of allergen characterization have made it possible to conduct several promising immunotherapy studies with purified allergen molecules [20,32–36]. For those allergen sources (e.g. cat, birch pollen) where the allergenic activity can be attributed primarily to one molecule, therapeutic vaccines can be composed of a single major allergen or derivatives thereof [33]. However, for allergen sources containing several major allergens it has to be decided which molecules need to be included in a therapeutic vaccine cocktail.

In principle, it would be possible to formulate allergy vaccines individually according to the sensitization profile of each patient. In this scenario, the IgE reactivity and clinical sensitization profile

would be determined and the vaccine freshly formulated by mixing the individual allergen molecules. Major problems regarding this approach are the design of clinical studies to evaluate the efficacy of such patient-tailored vaccines and the complex manufacturing process for such products.

Another more feasible approach is to prepare vaccines consisting of a defined mix of allergens, which are suitable for the majority of patients. Optionally, such vaccines could be spiked with additional allergens which are recognized by certain subgroups of patients. The definition of the essential components of therapeutic mixes which are suitable for the majority of patients may take several factors into consideration. The vaccine could be formulated on the basis of the frequencies of IgE recognition and co-recognition of certain allergens. An even more rationale approach would be to limit the components by evaluating the allergenic activity of the individual allergen molecules. In this paradigmatic study we have compared the allergenic activity of two recently described grass pollen allergens, Phl p 4 and Phl p 13 with already established major timothy grass pollen allergens by quantitative skin prick testing in a double-blind study. Other provocation testing methods (e.g. nasal or bronchial provocation) might have been used to obtain information about the allergenic activities in the target organs of respiratory allergy, but it has been shown that immediate skin inflammation is a useful surrogate marker for nasal symptoms [15]. Our results show that Phl p 4 and Phl p 13 represent major allergens according to their IgE recognition frequency, but exhibit a five to nine fold lower *in vivo* allergenic activity compared to the already established major timothy grass pollen allergens Phl p 1, Phl p 2 and Phl p 5. This suggests that Phl p 4 and Phl p 13 may make a proportionally weaker contribution to grass pollen-induced allergic symptoms and hence may be considered as non-essential components of a therapeutic grass pollen allergy vaccine. This assumption is also supported by the fact that we could not identify any patient in our population who showed exclusive reactivity to Phl p 4 or Phl p 13 and exhibited

symptoms of grass pollen allergy. Additional studies analysing the allergenic activity of Phl p 4 and Phl p 13 in other target organs (e.g. nose, lung) may be considered to exclude the clinical relevance of these molecules.

We do not have precise data regarding the amounts of Phl p 4 and Phl p 13 to which patients are naturally exposed, but we found that extremely high concentrations of these allergens would be necessary to induce clinically relevant reactions. The evaluation of the allergenic activity of Phl p 4 and Phl p 13 was made using purified natural allergens so as to exclude the possibility of reduced allergenic activity due to inappropriate folding of the recombinant derivatives or lack of post-translational modification, particularly glycosylation. However, the higher incidence of detection of IgE by comparison with positive skin prick test responses for both Phl p 4 and Phl p 13 may point to IgE-reactivity with cross-reactive carbohydrate determinants on these allergens. On the other hand the reverse was seen with Phl p 1, which occurs naturally in a glycosylated form, but which was produced and tested as a non-glycosylated protein. This indicates that protein structure of Phl p 1 rather than carbohydrate determinants play a dominant role in determining immunological reactivity.

In summary, the results of this study may contribute to the design of a grass pollen allergen mix consisting of the established major grass pollen allergens, Phl p 1, Phl p 2, Phl p 5 and Phl p 6 and which may be suitable for the treatment of the majority of grass pollen allergic patients. This conclusion is supported by the results of a recent clinical study documenting clinical efficacy of such a vaccine consisting of the recombinant wild type allergens [20]. In this study no de novo sensitization was observed even when patients who had not been sensitized against each of the components were treated. A potential risk of inducing new sensitizations with a vaccine cocktail that is not exactly tailored to the patients sensitization profile may be further reduced by using a mix of hypoallergenic derivatives for treatment [37].

Generally speaking, we suggest that similar studies analysing the in vitro [38] and in vivo allergenic activity of allergen molecules should be performed for the selection of allergen molecules for allergy vaccines for other complex and important allergen sources such as house dust mites and weed and tree pollens.

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Received 23 September 2007; accepted 25 January 2008

Acknowledgements

This study was performed with the help of the Allergy Center Wien West, Vienna, Austria and was supported by grant Nos F01804 and F01815 from the Austrian Research Fund (FWF), by a research grant from Biomay, Vienna, Austria and by the Christian Doppler Association.

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