

Human monoclonal antibody-based quantification of group 2 grass pollen allergens

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Background: Grasses belong to the most potent allergen sources worldwide. Group 2 grass pollen allergens are recognized by more than 100 million allergic patients.
Objective: The aim was to develop an assay for the specific detection and quantification of group 2 grass pollen allergens.
Methods: We have isolated a monoclonal human IgE Fab specific for group 2 grass pollen allergens by combinatorial cloning from lymphocytes of a grass pollen-allergic patient. This Fab was converted into a complete human IgG₁ antibody and used together with rPhl p 2 to develop a competitive ELISA for the specific measurement of group 2 allergens.
ELISA plate-bound purified recombinant human Phl p 2-specific IgG₁ is incubated with a constant amount of biotinylated rPhl p 2 competing with increasing concentrations of group 2 allergens to be determined. Defined concentrations of purified rPhl p 2 are used to establish a standard curve. The concentration of unlabeled group 2 allergens can thus be deduced from the displacement of biotinylated rPhl p 2, which can be detected with peroxidase-labeled streptavidin.
Results: The competition-ELISA measured rPhl p 2 concentrations ranging from 10 ng/mL to 500 ng/mL and allowed to quantify group 2 allergens from 9 different grass families. The results were in good agreement with immunoblot data.
Conclusions: The described assay can be used for standardization of diagnostic and therapeutic vaccines as well as for the quantification of group 2 allergens in environmental samples. (*J Allergy Clin Immunol* 2004;113:470-4.)

Key words: Grass pollen allergy, group 2 allergens, competitive ELISA, allergen measurement, recombinant human antibody, recombinant allergen

Grass pollens represent one of the most potent allergen sources worldwide.^{1,2} The grass pollen vaccines used for allergy diagnosis and immunotherapy are raw extracts that contain widely varying amounts of allergens, depending on the grass species and degree of pollen mat-

Abbreviation used
r: Recombinant

uration.³⁻⁵ Furthermore, it is known that the contents of certain allergens may differ, depending on the applied extraction procedure and extract stability.⁶

The most important grass pollen allergen groups comprise three distinct protein families (groups 1, 2, and 5), each of them recognized by more than 70% of grass pollen-allergic patients.^{1,7} Group 1 allergens represent proteins of mainly β -sheet, 2-domain structure, with a molecular weight of approximately 31 to 35 kd.⁸⁻¹² Group 2 allergens are proteins of approximately 10 to 12 kd, with significant sequence homology to the C-terminal domain of group 1 allergens.¹³⁻¹⁵ Group 5 allergens, the third major allergen group, are α -helical proteins with a molecular weight of approximately 27 to 33 kd.¹⁶⁻¹⁸

Currently, immunologic assays based on antibody-based quantification of major allergens represent the only technology available for the standardization of allergen extracts used for diagnosis and therapy.^{19,20} At present, it is only possible to measure the contents of group 1 and group 5 allergens in diagnostic and therapeutic grass pollen extracts by sandwich ELISA technology.²¹⁻²³ However, no assay is available for the specific detection and quantification of group 2 grass pollen allergens.

Group 2 allergens are recognized by more than 100 million grass pollen-allergic individuals.^{9,24} These proteins are expressed in certain grass species (eg, subfamily *Pooideae*), whereas others (eg, *Panicoideae*, *Chloridoideae*) lack immunologically active group 2 allergens.³ rPhl p 2 from timothy grass pollen containing the majority of group 2-specific IgE epitopes has been produced by expression in *Escherichia coli*.^{14,24} Nuclear magnetic resonance analysis of Phl p 2 shows that its 3-dimensional structure mimics an immunoglobulin-like fold.¹⁵ Using rPhl p 2, we have isolated a group 2-specific monoclonal human IgE Fab by combinatorial cloning from lymphocytes of a grass pollen-allergic patient and converted it into a complete human IgG₁ monoclonal antibody.²⁵

In this study, we aimed to use this human monoclonal antibody to develop a sensitive assay for the specific detection and measurement of group 2 grass pollen allergens in diagnostic and therapeutic allergen vaccines and for the quantification of group 2 allergens in environmental samples.

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METHODS

Construction and purification of a monoclonal human antibody specific for group 2 grass pollen allergens

A group 2 grass pollen allergen-specific human IgE Fab was isolated from an IgE combinatorial library, which had been constructed from lymphocytes of a grass pollen-allergic patient.^{25,26} By grafting of the IgE variable regions onto a human IgG1 backbone and transfection into Chinese hamster ovary cells–K1, a complete human IgG₁ with specificity for group 2 grass pollen allergens was expressed. The group 2–specific human IgG₁ (huMab2) was expressed as soluble protein in Chinese hamster ovary cells–K1 supernatants²⁵ and purified with an Immuno Pure IgG Purification Kit from Pierce (Rockford, Ill), according to the manufacturer's instructions. The antibody concentration in the eluted fractions was determined by photometry at 280 nm, and its activity was determined by direct binding to purified rPhl p 2 by immunoblotting and ELISA.

Preparation of grass pollen extracts and specific detection of group 2 allergens by immunoblotting

Pollen of *Phleum pratense*, *Lolium perenne*, *Dactylis glomerata*, *Poa pratensis*, *Secale cereale*, *Hordeum vulgare*, *Avena sativa*, *Triticum aestivum*, *Cynodon dactylon*, and *Anthoxanthum odoratum* were purchased from Allergon Pharmacia (Välinge, Sweden). Aqueous extracts were prepared by suspending the pollen in distilled water and extraction by continuous shaking for 3 hours at room temperature.⁶ Insoluble particles were removed by centrifugation for 30 minutes at 12,000g. The supernatants were dialyzed against distilled water at 4°C overnight and stored at –20°C. The total protein content of the pollen extracts was determined by the method of Bradford.²⁷ Equal protein amounts of each grass pollen extract (22.5 µg/mL) were separated by SDS-PAGE (15% separation gel)²⁸ and blotted onto nitrocellulose membranes (Schleicher and Schüll, Dassel, Germany).²⁹ Membranes were blocked (blocking buffer is 50 mmol sodium phosphate buffer, pH 7.5, 0.5% Tween 20, 0.5% wt/vol BSA, Fraction V, >98% pure, Roth GmbH, Karlsruhe, Germany, 0.05% wt/vol sodium acid) and incubated with purified huMab2 in a dilution of 1:1000 (c = 0.1 µg/mL) overnight at 4°C.²⁵ Membranes were washed and incubated with a mouse monoclonal anti-human IgG₁ (Pharmingen, San Diego, Calif) in a concentration of 1:1000 overnight at 4°C followed by an alkaline phosphatase–coupled rat anti-mouse monoclonal IgG₁ antibody (Pharmingen, 1:1000). Bound antibodies were visualized with 5-bromo-4-chloro-3-indolyl-phosphate/4-nitro blue tetrazolium chloride substrate (Sigma-Aldrich, Vienna, Austria).

Biotinylation of recombinant Phl p 2

Purified rPhl p 2, which had been expressed in *E coli*, was obtained from BIOMAY (Vienna, Austria). rPhl p 2 was labeled with biotin,³⁰ using N-succinimidyl N-[6-(biotinylamino)caproyl]-6-aminocaproate, (Fluka Biochemica, Buchs, Switzerland). After testing, different amounts of biotin (12.5, 37.5, 50, 60 µg biotin/100 µg rPhl p 2), a ratio of 50 µg biotin/100 µg rPhl p 2, delivered a labeled rPhl p 2 molecule with the best test performance regarding sensitivity and homogeneity of results.

Development of a group 2–specific competition ELISA

MaxiSorp ELISA Plates (Nunc, Roskilde, Denmark) were coated with purified huMab2 in a concentration of 2.3 µg/mL diluted in 0.1 mol/L carbonate buffer, pH 9.6, at 4°C overnight. Plates were

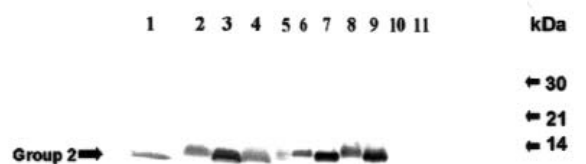


FIG 1. Cross-reactivity of human monoclonal anti-Phl p 2 antibody with group 2 pollen allergens from different grass species. Nitrocellulose membranes containing pollen extracts from 9 grasses with (2: *Phleum pratense*; 3: *Poa pratensis*; 4: *Dactylis glomerata*; 5: *Triticum aestivum*; 6: *Avena sativa*; 7: *Anthoxanthum odoratum*; 8: *Hordeum vulgare*; 9: *Lolium perenne*), one (10: *Cynodon dactylon*) without group 2 allergens, rPhl p 2 (lane 1) and birch pollen extract (lane 11: negative control) were incubated with the anti-Phl p 2 antibody. Molecular weights are indicated (in kd) on the right margin; position of group 2 allergens is indicated.

washed with PBS containing 0.05% vol/vol Tween 20 (PBST) 3 times. After blocking for 2 hours with 2% BSA-PBST, wells were filled with 50 µL of biotinylated Phl p 2 (c = 75 ng/mL) and 50 µL of unlabeled rPhl p 2 in a concentration range between 1 ng/mL and 1000 ng/mL in 1:2 dilution steps (dilution buffer: 0.5% wt/vol BSA-PBST) to establish a standard curve. After an incubation period of 2 hours at 37°C, plates were washed 5 times and incubated with 100 µL horseradish peroxidase–conjugated streptavidin (Endogen, Woburn, Mass, c = 1.25 mg/mL), which was diluted 1:10,000 in dilution buffer. The color reaction was developed by adding 100 µL peroxidase substrate (2,2'-azino-bis [3-ethylbenzthiazoline-6-sulfonic acid]) and analyzed in an ELISA reader (Dynatech, Denkendorf, Germany).

Statistical methods

Interassay and intra-assay variabilities were assessed by descriptive methods. Geometric mean coefficient of variation across concentrations was determined. Number of replicates was chosen such that the difference between the upper 95% confidence interval of the pooled coefficient of variation and the estimate (projected to be within the range of 10% to 20%) would be <5%.

No curve-fitting was conducted for the standard curve. Concentrations were obtained by linear interpolation between contiguous points of the standard curve.

RESULTS

HuMab2 cross-reacts with group 2 allergens of the most common grass species

We have previously converted a recombinant human IgE Fab specific for group 2 grass pollen allergens into a complete human IgG1 antibody (huMab2).²⁵ This antibody, designated huMab2, reacts specifically with rPhl p 2 and natural group 2 allergens in pollen of the most common grass species (*Poa pratensis*, *Phleum pratense*, *Lolium perenne*, *Dactylis glomerata*, *Anthoxanthum odoratum*, *Hordeum vulgare*, *Avena sativa*, and *Triticum aestivum*) (Fig 1). No reactivity was observed with pollen from *Cynodon dactylon*, a grass belonging to the family of *Panicoideae* lacking group 2 allergens³ and birch pollen extract (negative control). HuMab2 did not bind to group 1 allergens at approximately 30 kd, although group 1 allergens contain a C-terminal domain with sequence homology to group 2 allergens.^{13,14}

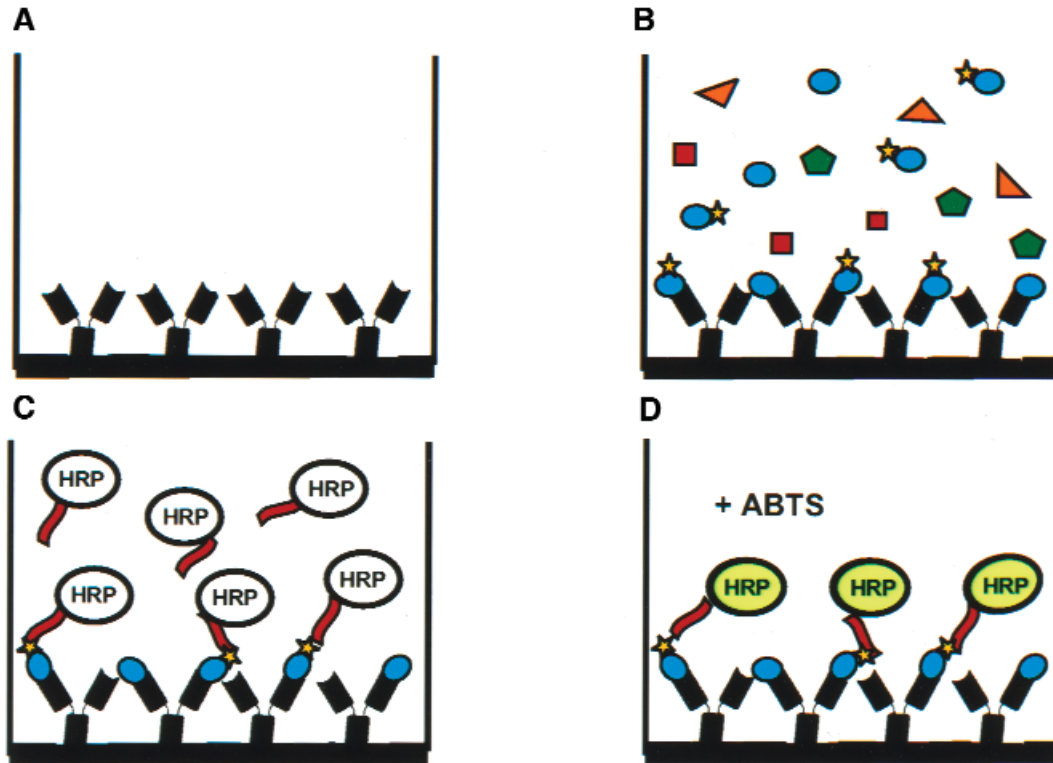


FIG 2. Description of the ELISA for quantification of group 2 grass pollen allergens. **A**, ELISA plates are coated with the huMab2 and **B**, incubated with a mixture containing defined amounts of biotinylated rPhl p 2 (competitor: blue dots with yellow asterisks) and unknown contents of group 2 allergens (blue dots). **C**, Labeled rPhl p 2 is detected with horseradish peroxidase-conjugated streptavidin (red) and **D**, visualized by color reaction (green) by using 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid). The intensity of the color reaction is inversely related to the concentration of group 2 allergens. According to a standard curve established with defined amounts of unlabeled rPhl p 2, the concentration of group 2 allergens can be determined.

Principle of the competition ELISA for quantification of group 2 allergens

Since no other group 2 allergen-specific antibodies besides huMab2 were available to establish a sandwich ELISA, we developed a competitive ELISA for quantification of group 2 allergens by using purified rPhl p 2, from timothy grass, which contains most of the group 2-specific IgE epitopes.^{3,14} The principle of this assay is described in Fig 2. ELISA plates are coated with purified huMab2 (Fig 2, A). Defined amounts of purified rPhl p 2 are used to compete with biotinylated rPhl p 2 to establish a standard curve for group 2 allergens. According to this standard curve, it is possible to determine the amounts of group 2 allergens in allergen extracts (Fig 2, B). The optical density measured by ELISA corresponds to the amount of bound biotinylated rPhl p 2 and is inversely related to the amounts of group 2 allergens (Fig 2, C and D).

Sensitivity, precision, and reproducibility of the group 2 allergen-specific ELISA

Fig 3, A, shows the interassay reproducibility of the group 2 allergen-specific ELISA, displaying the means and standard deviations for 7 concentrations (500, 250, 125, 62.5, 31.25, 15.6, 7.8 ng/mL) of rPhl p 2 determined

in 6 independent measurements performed at different days by the same investigator. The mean interassay standard deviation was 20%.

The intra-assay precision of the ELISA was validated by performing 5 determinations for 7 allergen concentrations, on the same plate, at the same time (Fig 3, B). A low intra-assay mean standard deviation of 11% was found.

Pollen extracts prepared from 10 of the most common grass species contain widely varying amounts of group 2 allergens

The contents of group 2 allergens were measured in pollen extracts (prepared by the same method) from 10 of the most common allergenic grasses (*Phleum pratense*, *Poa pratensis*, *Dactylis glomerata*, *Secale cereale*, *Triticum aestivum*, *Avena sativa*, *Anthoxanthum odoratum*, *Hordeum vulgare*, *Lolium perenne*, and *Cynodon dactylon*). We found that extracts from different grass species contained widely varying amounts of group 2 allergens. *Cynodon dactylon* contained no detectable amounts of group 2 allergens at all (Table I). In the other grasses, group 2 allergen contents ranged between 0.4% (ie, 412 ng/100 µg total protein: *Hordeum vulgare*) and 1.9% (ie, 1874 ng/100 µg total protein: *Poa pratensis*) (Table I). The ELISA results were in good agreement

TABLE I. Contents of group 2 allergens in pollen extracts from different grass species and in commercially available grass pollen preparations

	Group 2 allergens (ng/100 μ g of protein)
Grass pollen extracts	
<i>Phleum pratense</i>	1172
<i>Poa pratensis</i>	1874
<i>Dactylis glomerata</i>	1304
<i>Secale cereale</i>	1425
<i>Triticum aestivum</i>	915
<i>Avena sativa</i>	719
<i>Anthoxanthum odoratum</i>	1524
<i>Hordeum vulgare</i>	412
<i>Lolium perenne</i>	1291
<i>Cynodon dactylon</i>	0
Commercial grass pollen preparations	
<i>Phleum pratense</i> #1	52.5
<i>Phleum pratense</i> #2	373.9
<i>Phleum pratense</i> #3	411.7
<i>Phleum pratense</i> #4	245

with semiquantitative results obtained by immunoblotting when the same extracts and huMab2 for direct detection were used (Fig 1).

We also tested standardized grass pollen allergen vaccines from 4 companies and found that the contents of group 2 allergens ranged from 0.05% to 0.42% wt/total protein (Table I).

For control purposes, we tested recombinant Bet v 1 ($c = 500$ ng/mL) and birch pollen extract ($c = 300$ μ g/mL), which contain no group 2-related allergens, and found no immunoreactivity (data not shown).

DISCUSSION

Although group 2 grass pollen allergens are among the most frequently recognized and important allergens, no assay was available for the identification and quantification of these allergens in biological samples. We have developed a competition ELISA that is highly specific for group 2 allergens and can be applied for detecting these allergens in the most common allergenic grasses. The specificity of our ELISA derives from a human monoclonal IgE antibody from a grass pollen-allergic patient obtained by combinatorial cloning.²⁵ The antibody that is used in the assay was designated huMab2. It recognizes in a highly specific manner a major IgE epitope, which is conserved on group 2 allergens of the most common allergenic grasses.²⁵ Importantly, huMab2 does not show any cross-reactivity with other grass pollen allergens, although group 1 allergens contain a C-terminal domain with close structural similarity to group 2 allergens.¹³⁻¹⁵ Although huMab2 represents a monoclonal antibody, it cross-reacted with group 2 allergens from most grasses, as should be to a polyclonal antiserum. The cross-reactivity with group 2 allergens from the major allergenic grasses may be attributed to the fact that huMab2 recognizes a major IgE epitope-containing portion on group 2 allergens. Consequently, we believe that huMab2 is espe-

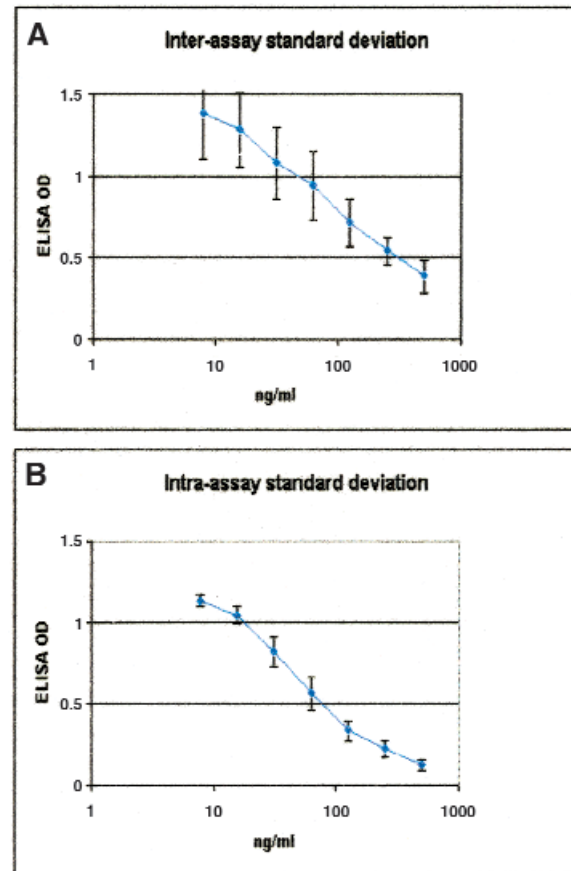


FIG 3. Dose-response curves of rPhl p 2 determined by competition ELISA. **A**, Interassay standard deviations (means of 6 determinations) and **B**, intra-assay standard deviations (means of 5 determinations) shown for different concentrations of rPhl p 2 (x-axis: logarithmic values).

cially suited for the quantification of group 2 allergens in grass pollen vaccines made from different grass species. This is important, considering that natural allergen extracts may also contain allergen variants with reduced IgE-binding capacity and low allergenic activity.³¹⁻³³

The second reagent that contributes to the sensitivity and specificity of the described assay is purified rPhl p 2, the group 2 allergen from timothy grass. It has been isolated with serum IgE from grass pollen-allergic patients from a cDNA expression library constructed from mature timothy grass pollen. By IgE competition assays using sera from grass pollen-allergic patients from different populations, rPhl p 2 has been shown to contain most of the IgE epitopes present in natural group 2 allergens of the most common grass species.³ Therefore, rPhl p 2 can be used as competitor in the described ELISA system to quantify natural group 2 allergens in the most common allergenic grasses.

The described assay uses biotinylated rPhl p 2 as a standardized reagent; it is robust and easy to perform. It showed good reproducibility both in terms of interassay and intra-assay variation. We have demonstrated the suitability of the assay for the measurement of group 2 aller-

gens in extracts prepared from various grass species as well as for the quantification of group 2 allergens in commercially available grass pollen vaccines. Our results showed a broad variability of group 2 allergen contents in these vaccines. This fact needs considerable attention because it is known that grass pollen-specific immunotherapy performed with natural allergen vaccines induces little or no protective immunity against group 2 allergens.³⁴ We therefore suggest using this assay to measure group 2 allergens in natural grass pollen vaccines used for diagnostic and therapeutic purposes for better standardization. Furthermore, our assay can be used to measure the loads of group 2 allergens in the environment to allow prophylactic measures for those patients with high sensitivity to group 2 allergens.

In summary, the described assay will contribute to the improvement of diagnosis, treatment, and prevention of grass pollen allergy.

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