

Antigenic Similarity among Group 1 Allergens from Grasses and Quantitation ELISA Using Monoclonal Antibodies to Phl p 1

Oscar Duffort^a Juan Quintana^a Henrik Ipsen^b Domingo Barber^a
Florentino Polo^a

ALK-Abelló, Research Department, ^aMadrid, Spain, and ^bHørsholm, Denmark

Key Words

Allergen · Grass group 1 allergen · Grass pollen allergy · Immunoassay · Monoclonal antibody · *Phleum pratense* · Pollinosis · Standardization

Abstract

Background: Group 1 allergens elicit a specific IgE response in about 90% of grass pollen-allergic patients. The aim of this work was to study the antigenic similarity among group 1 allergens from different grasses and to develop a monoclonal antibody (MAb)-based quantitation ELISA. **Methods:** Twenty specific MAbs were produced from BALB/c mice immunized with natural Phl p 1. These MAbs were tested for specificity with thirteen different grass pollen extracts from the Poaceae family and in cross-inhibition experiments for the binding of Phl p 1. Purified group 1 allergens from Poaceae grasses (*Dactylis glomerata*, *Lolium perenne*, *Festuca pratensis* and *Poa pratensis*) were tested for parallelism in quantitation ELISA. **Results:** Eighteen to nineteen anti-Phl p 1 MAbs recognized the homologous allergen in pollen extracts from grasses of the Poaceae tribe. In contrast, only four MAbs recognized group 1 from *Cynodon dactylon* and *Phragmites communis*. Four groups of MAbs with different epitope specificity were identified. A grass group 1 quantitation ELISA was developed using a mix of three MAbs on the solid phase and a polyclonal rabbit antibody as the second antibody. The group 1 content could be measured in different batches of

Phleum pratense as well as in pollen extracts from Poaceae grasses, since they showed parallel dose-response curves.

Conclusions: MAbs produced in this work enabled us to show the high antigenic similarity between group 1 allergens from temperate grasses. The results prove the usefulness of the ELISA method developed for standardization of grass allergen products.

Copyright © 2007 S. Karger AG, Basel

Introduction

Major allergen quantitation is an increasing demand for products used for specific immunotherapy. Monoclonal antibody-based ELISAs are the preferred tool to achieve this goal [1]. In addition, the simplification of immunotherapy formulations to single species or even to combinations of recombinant allergens makes it necessary to investigate the immunological cross-reactivity of similar allergens from different species. Grass pollen allergens are one of the leading causes of seasonal allergic diseases worldwide, with an incidence of sensitization estimated to be about 20% of the general population and 40% of atopic individuals [2]. Although the Poaceae family comprises about 9,000 species and its members are widespread nearly everywhere throughout the world, only a limited number of species have been identified as relevant sources of allergy and are used nowadays in clin-

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

© 2007 S. Karger AG, Basel
1018–2438/08/1454–0283\$24.50/0

Accessible online at:
www.karger.com/iaa

Correspondence to: Dr. Domingo Barber
Departamento de Investigación, ALK-Abelló
C/Miguel Fleta, 19
ES-28037 Madrid (Spain)
Tel. +34 91 327 6100, Fax +34 91 327 6117, E-Mail domingo.barber@alk-abello.com

ical practice for diagnosis and treatment. Most of these species belong to the Pooideae subfamily (including *Phleum pratense*, *Lolium perenne*, *Poa pratensis*, *Festuca pratensis*, *Dactylis glomerata* and *Secale cereale*) and grow in temperate regions. A few other important species, growing in temperate, subtropical and tropical habitats, are members of different subfamilies, such as *Cynodon dactylon* (Chloridoideae) and *Phragmites communis* (Arun-dinoideae). The allergenic similarity among grasses has been investigated in a number of studies using different approaches, showing a high degree of cross-reactivity among grasses from the Pooideae subfamily and a lower degree with grasses from other subfamilies [2–8]. To date, eleven different groups of allergens have been identified in grass pollen extracts, *P. pratense*, *L. perenne* and *C. dactylon* being the best-studied species. Groups 1 and 5 are the predominant allergens with an IgE prevalence >80% [8–10]. Group 5 is exclusive of the Pooideae subfamily [11–13], whereas group 1 has also been identified in members from other subfamilies [14–17]. Group 1 allergens are 30- to 37-kDa glycoproteins [14–24] which make up a subgroup of the β -expansin family of cell wall-loosening proteins in plants and may play a role in pollen germination on the stigma [25–27], through a biochemical mechanism that has not yet been fully elucidated [27–30]. The objective of the present work was to develop a method for quantifying Pooideae group 1 pollen allergens and to study the antigenic similarity among grass group 1 from different grasses using a broad panel of monoclonal antibodies (MAbs) directed towards Phl p 1.

Materials and Methods

Source Material, Pollen Extracts and Purification of Grass Group 1 Allergens

All pollen source materials were purchased from Biopol Laboratory (Spokane, Wash., USA). *Dermatophagoides pteronyssinus*, *Gadus morhua*, *Vespula vulgaris* and *Felis domesticus* were internally produced at ALK-Abello. Purified Phl p 3 and Phl p 6 were kindly provided by Mercedes Ferreras (ALK-Abello A/S, Hørsholm, Denmark). Purification of grass group 1 allergens was carried out essentially as previously described [24]. Briefly, a pollen extract was prepared in 0.125 M NH_4HCO_3 (pH 8.3) at a 1:10 (w/v) ratio. The soluble fraction was separated by centrifugation, filtered and dialyzed against 5 mM NH_4HCO_3 . The pollen extract was then subjected to salting out with 3 M ammonium sulfate. The supernatant was dialyzed against 0.100 M buffer acetate (pH 5.0) and separated by hydrophobic interaction (HCl-octyl) chromatography sequentially eluting with three different buffers: buffer 1: 50 mM CH_3COONa (pH: 5.0) + 1 M $(\text{NH}_4)_2\text{SO}_4$; buffer 2: PBS (pH: 7.2) and buffer 3: 50 mM sodium carbonate buffer (pH 10) +

50% ethylene glycol. The final flow through was dialyzed, concentrated and applied on gel filtration (Superdex 75 16/60) columns eluted with 150 mM NH_4HCO_3 . The fractions containing group 1 [24] were pooled, dialyzed against 5 mM NH_4HCO_3 , freeze-dried and stored at -20°C until use. Protein purity was assessed by SDS-PAGE applying 10 μg of the purified proteins. The protein concentration was measured by quantitative amino acid composition analysis using reagents and equipment from Biochrom (Cambridge, UK). The protein concentration of pollen extracts was determined by the method of Lowry et al. [31].

Production of Antibodies

Female BALB/c mice (Criffa, Barcelona, Spain) were injected intraperitoneally with 50 μg of Phl p 1 in Freund's complete adjuvant. On days 15 and 30 after the injection, mice were boosted with an identical amount of antigen in Freund's incomplete adjuvant. On day 52, mice were boosted intravenously with the same dose of antigen in PBS and, 3 days later, spleen cells from immunized mice were fused with Sp2/0-Ag 14 myeloma cells according to the method of Galfré and Milstein [32]. Ten days after the fusion, cell culture supernatants were screened for anti-Phl p 1-specific antibodies using an antibody capture ELISA with Phl p 1 adsorbed to the solid phase. The positive hybridomas were cloned by limiting dilution. Two successive cycles were used to assess monoclonality.

Polyclonal antibodies against *P. pratense* pollen extract were produced in New Zealand rabbits, basically following procedures described elsewhere [33].

Both monoclonal and polyclonal antibody production was carried out in accordance with the Principles of Good Laboratory Practice of the Organization for European Economic Cooperation (revised in 1997) and European Council Directive No. 609 (1986).

Purification and Characterization of Antibodies

Selected MAbs were purified from hybridoma culture supernatants using a protein G Sepharose affinity column (GE Healthcare, Uppsala, Sweden), following the manufacturer's instructions. The isotype of the antibodies was determined by ELISA with anti-mouse subclass antisera (Nordic, Tilburg, The Netherlands).

Specificity of the anti-Phl p 1 MAbs to homologous allergens in pollen extracts from other grasses was investigated by means of an antibody capture ELISA with the pollen extracts adsorbed to the solid phase.

Cross-competition experiments between MAbs for the binding of Phl p 1 were carried out by a RIA with the purified allergen, which had been radiolabelled with ^{125}I according to the method of Hunter and Greenwood [34]. Briefly, removable polystyrene Nunc-Immuno BreakApart wells (Nunc, Roskilde, Denmark) were coated overnight at 4°C with polyclonal rabbit antibodies against mouse Ig (RAM, DAKO, Glostrup, Denmark) at a 1:250 dilution in PBS. Following saturation with PBS containing 1% BSA and 0.1% Tween 20 (PBS-BSA-T), the wells were incubated with 100 μl of anti-Phl p 1 secreting hybridoma culture supernatant for 2 h at room temperature and, after washing with PBS containing 0.1% Tween 20 (PBS-T), unoccupied RAM binding sites were blocked by incubation for an additional hour with a 1:20 dilution of a mouse ascitic fluid containing irrelevant antibodies at high concentration. Subsequent to washing with PBS-T,

the adsorbed anti-Phl p 1 MAb was left to react overnight at room temperature with a mixture of ^{125}I -labelled Phl p 1 (1.5×10^5 cpm/well) and serial dilutions of the competitor MAb in PBS-BSA-T. Finally, the wells were washed and the radioactivity bound was measured in a γ -counter. The homologous MAb and PBS-BSA-T were used as controls for 100 and 0% inhibition, respectively. Control with the cell growth media was carried out along the experiments to rule out a possible interference of IgG antibodies from the fetal calf serum used in the hybridoma culture medium.

Grass Group 1 ELISA

Microtiter 96-well plates (EIA/RIA plates, Costar, Cambridge, Mass., USA) were coated overnight at 4°C with $100\ \mu\text{l}$ of a mixture of anti-Phl p 1 mAbs 1.1, 1.8 and 1.10 at $5\ \mu\text{g}/\text{ml}$ in PBS each. After blocking with PBS-BSA-T, wells were sequentially incubated with samples and references, anti-*P. pratense* rabbit serum (1:4,000 dilution) and horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin antibodies (Calbiochem, San Diego, Calif., USA). Samples, controls and reagents were conveniently diluted in PBS-BSA-T, and all incubations were carried out for 2 h at room temperature with intermediate washes between successive steps. Detection was accomplished with *o*-phenylenediamine (DAKO). After the addition of 2 M HCl, absorbance was measured at 490 nm with a reference filter at 650 nm. The concentration of group 1 in samples was calculated by interpolating from a standard curve constructed with serial twofold dilutions of purified Phl p 1, starting from $2.5\ \mu\text{g}/\text{ml}$.

Results

Characterization of MAbs – Antigenic Similarity among Grasses

Grass group 1 allergens (Phl p 1, Dac g 1, Fes p 1, Lol p 1 and Poa p 1) were purified from pollen extracts by hydrophobic interaction chromatography and gel filtration. These allergens were chosen as they are the most widely used grass species in diagnosis and immunotherapy. Purity of the preparations was above 95%, as determined by SDS-PAGE (fig. 1). Amino acid analysis was performed for quantification of the purified proteins. The composition of the different group 1 allergens was found to be highly similar, and also similar to that deduced from the published Phl p 1 sequence. Purified Phl p 1 was used to immunize BALB/c mice for MAb production. Twenty hybridomas secreting MAbs of IgG class were obtained from the fusion of spleen cells from immunized mice and Sp2/0-Ag 14 myeloma cells. The selection of specific monoclonal antibodies was carried out by means of an antibody capture ELISA with nPhl p 1 on the solid phase. Since the final purpose of the work was to develop an MAb-based ELISA method to quantify group 1 in pollen extracts from different grasses, a battery of

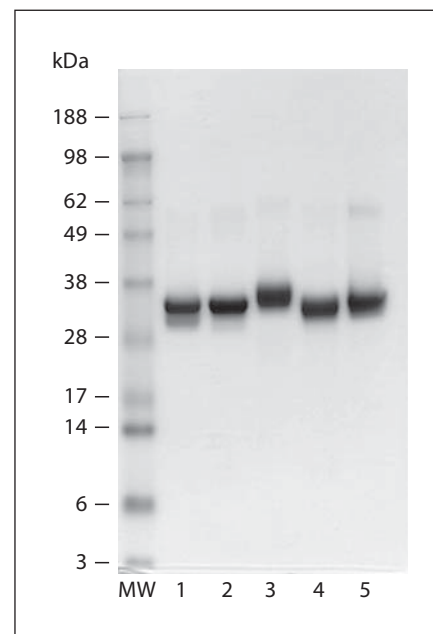


Fig. 1. SDS-PAGE analysis of purified grass group 1 allergens: Dac g 1 (lane 1), Fes p 1 (lane 2), Lol p 1 (lane 3), Phl p 1 (lane 4) and Poa p 1 (lane 5). MW = Molecular weight markers. Electrophoresis was carried out on NuPage Bis-Tris gel (Invitrogen, Carlsbad, Calif., USA), under non-reducing conditions, and protein bands stained with Coomassie blue.

thirteen grass pollens was screened in order to select those MAbs with the broadest specificity. Apart from *P. pratense*, pollen extracts from three other grasses from the Aveneae tribe (*Avena sativa*, *Holcus lanatus* and *Anthoxanthum odoratum*), three from the Triticeae tribe (*S. cereale*, *Triticum aestivum* and *Hordeum vulgare*) and four from the Poeae tribe (*L. perenne*, *P. pratensis*, *F. pratensis* and *D. glomerata*), all of them belonging to the Pooideae subfamily, were included in this experiment, as well as representatives of the Chloridoideae (*C. dactylon*) and Arundinoideae (*P. communis*) subfamilies. Two out of the twenty MAbs bound to the thirteen pollen extracts tested, three of them to twelve, nine to eleven, three to ten, and the other three MAbs to six, four and two extracts, respectively. As shown in table 1, pollen extracts from Pooideae grasses were recognized by at least 75% of the anti-Phl p 1 MAbs, with grasses from the Poeae tribe, *S. cereale* and *A. sativa*, binding to 18–19 of the 20 MAbs. In contrast, a significantly smaller number of MAbs bound to grasses from the other subfamilies. We have defined an immunogenic similarity index by comparing the strength of recognition to that of Phl p 1. As can be

Table 1. Immunological similarity of group 1 allergens

Subfamily/tribe/species	anti-Phl p 1 MAb																				ISI
	1.1	1.2	1.3	1.4	1.5	1.6	1.7	1.8	1.9	1.10	1.11	1.15	1.16	1.17	1.18	1.19	1.20	1.21	1.23	1.24	
Pooideae																					
Poeae																					
<i>Dactylis glomerata</i>	4	4	4	4	0	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	1.07
<i>Lolium perenne</i>	4	4	4	3	0	4	3	4	4	4	0	4	4	4	4	4	4	4	4	4	0.99
<i>Poa pratensis</i>	4	4	4	4	1	3	3	4	4	4	0	4	4	4	2	2	4	4	4	4	0.94
<i>Festuca pratensis</i>	4	4	4	4	0	4	1	4	4	4	0	4	4	4	4	4	4	4	4	4	0.97
Aveneae																					
<i>Phleum pratense</i>	4	4	4	4	1	4	1	4	4	4	3	4	3	4	4	4	4	4	4	3	1
<i>Avena sativa</i>	4	4	4	3	0	4	3	3	4	4	0	3	4	4	1	4	4	4	4	4	0.92
<i>Holcus lanatus</i>	4	4	4	1	0	2	0	2	2	1	0	1	3	0	1	1	1	3	1	1	0.45
<i>Anthoxanthum odoratum</i>	3	2	2	1	0	1	0	3	3	1	0	3	3	1	1	1	3	4	1	1	0.48
Triticeae																					
<i>Triticum aestivum</i>	4	4	4	1	0	4	0	1	4	4	1	1	4	4	1	4	2	1	0	0	0.62
<i>Secale cereale</i>	4	4	4	3	0	4	0	2	4	4	2	2	4	4	1	4	3	4	1	4	0.82
<i>Hordeum vulgare</i>	4	4	4	1	0	4	0	4	1	0	0	4	4	0	1	4	4	4	3	4	0.70
Chlorideae																					
<i>Cynodon dactylon</i>	0	0	0	0	0	1	0	2	0	4	0	0	0	4	0	0	0	1	0	0	0.17
Arundineae																					
<i>Phragmites communis</i>	0	0	0	0	0	0	0	4	0	1	0	0	0	0	0	0	1	4	0	0	0.14
Negative control	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Cell culture supernatant from anti-nPhl p 1-secreting hybridomas were tested in an antibody-capture ELISA with different grass pollen extracts adsorbed onto the solid phase. According to the signal strength, MAb recognition was classified as very strong (4; optical density, OD > 2.0), strong (3; OD 1.5–2.0), moderate (2; OD 1.0–1.5), low (1; OD 0.1–1.0) and negative (0; OD < 0.1). For each species, the sum of individual recognition of individual MABs divided by that of Phleum was defined as an immunological similarity index (ISI) for the corresponding group 1 allergen.

Table 2. Cross-inhibition among MABs for the binding of Phl p 1 (results of a competition RIA with ¹²⁵I-nPhl p 1 as described in the Materials and Methods)

Inhibitor	1.1	1.2	1.3	1.16	1.24	1.8	1.15	1.20	1.10	1.21
1.1	++	++	++	++	++	–	–	–	–	–
1.2	++	++	++	++	++	–	–	–	–	–
1.3	++	++	++	++	++	–	–	–	–	–
1.16	++	++	++	++	++	–	–	–	–	–
1.24	+	+	+	+	++	–	–	–	–	–
1.8	–	–	–	–	–	++	++	++	–	–
1.15	–	–	–	–	–	++	++	++	–	–
1.20	–	–	–	–	–	++	++	++	–	–
1.10	–	–	–	–	–	–	–	–	++	–
1.21	–	–	–	–	–	–	–	–	–	++

++ = >80% inhibition; + = 10–80% inhibition; – = <10% inhibition.

seen in table 1, most of the group 1 allergens of the Poeae tribe have an almost identical immunogenic similarity index to that of Phl p 1, supporting an almost complete immunological cross-reactivity. These results suggest that the antigenic similarity among group 1 from Pooideae grasses is very high, and is lower with *C. dactylon* and *P. communis*.

The ten MABs with the highest affinity among those recognizing a broad spectrum of Pooideae grasses were selected for cross-inhibition experiments aimed at identifying those reacting to non-overlapping epitopes on the Phl p 1 molecule. The results of these experiments are compiled in table 2. Binding of radiolabelled Phl p 1 to MABs 1.10 and 1.21 was not inhibited by any other MAB

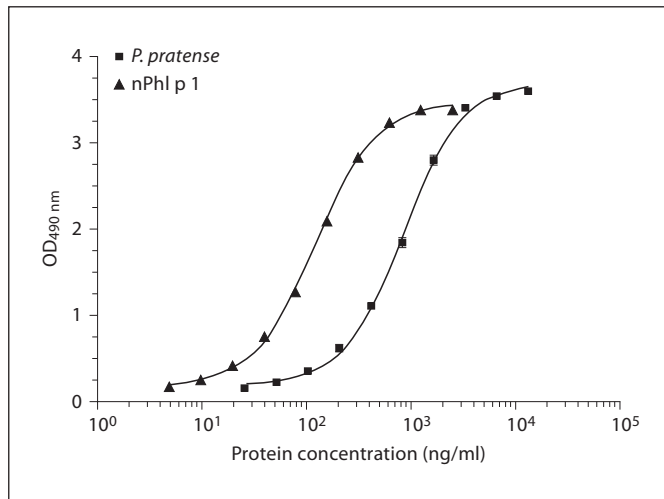


Fig. 2. Dose-response curves obtained in the MAb-based group 1-ELISA for a *P. pratense* pollen extract and purified nPhl p 1 allergen. Determinations were carried out in triplicate. Bars indicate standard error of the mean.

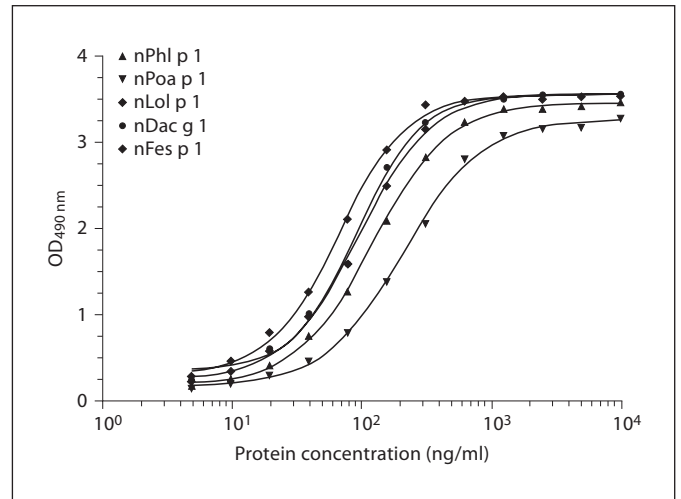


Fig. 3. Purified grass group 1 allergens were tested in duplicate in the group 1 MAb-based ELISA and the linear stretch of the dose-response curves were compared for parallelism.

nor were they able to produce any significant inhibition. In addition, two other groups of MAbs, with five and three members, defining non-overlapping epitopes were identified. Thus, it can be concluded that the MAbs produced delineate four different binding sites on Phl p 1. Looking at the combined data in tables 1 and 2, it can be seen that the epitope defined by MAbs 1.2, 1.2.1.3, 1.16 and 1.24 is only present in the Pooideae subfamily, whereas the other three epitopes are distributed along all grass species tested.

ELISA for Grass Group 1 Quantitation

Different combinations of MAbs from the four different groups identified by the cross-inhibition experiments were tested for their performance in a sandwich ELISA for grass group 1 quantitation. The best combination turned out to be a mixture of three anti-Phl p 1 MAbs (1.1, 1.8 and 1.10) adsorbed to the solid phase and an anti-*P. pratense* pollen rabbit serum as the second antibody. The selection was made taking into account the best signal/noise ratio and to maximize the number of detected epitopes. MAb 1.21, defining a fourth epitope, was not considered since the cell line was unstable. Detection of the rabbit antibodies bound was accomplished with goat anti-rabbit immunoglobulin antibodies conjugated with horseradish peroxidase.

The dose-response curves obtained in the ELISA with nPhl p 1 and *P. pratense* pollen extract were parallel

(fig. 2), suggesting that the allergen had not been altered during the purification process and that the assay was measuring the same molecule in the different preparations. Therefore, the concentration of nPhl p 1 was accurately measured by quantitative amino acid analysis, and this preparation was used as the primary standard in the ELISA method. The assay has a detection limit of 15 ng/ml and a practical working range of 30–500 ng/ml. The lower limit of this range (30 ng/ml) can be considered the limit of quantitation of the method. The specificity of the method was evaluated against a large battery of tree pollen and weed pollen extracts (data not shown). Furthermore, the assay is highly reproducible. The mean intra-assay coefficient of variation was 6.5%, determined as the coefficient of variation of five samples in quintuplicate, and the interassay coefficient of variation was 16.3%, determined over the same samples in five separate assays.

The potential application of the developed ELISA to quantify group 1 in pollen extracts from wild temperate grasses was investigated by comparing the dose-response curves of nPhl p 1 with those obtained with purified group 1 allergens from *L. perenne*, *P. pratensis*, *F. pratensis* and *D. glomerata* (fig. 3). All of them showed comparable slopes (fig. 3), and it can be concluded that the ELISA method may also be appropriate for group 1 determination in these species. Nevertheless, the curves were shifted to some extent along the x-axis, suggesting that the affinity of the anti-Phl p 1 MAbs to the different iso-

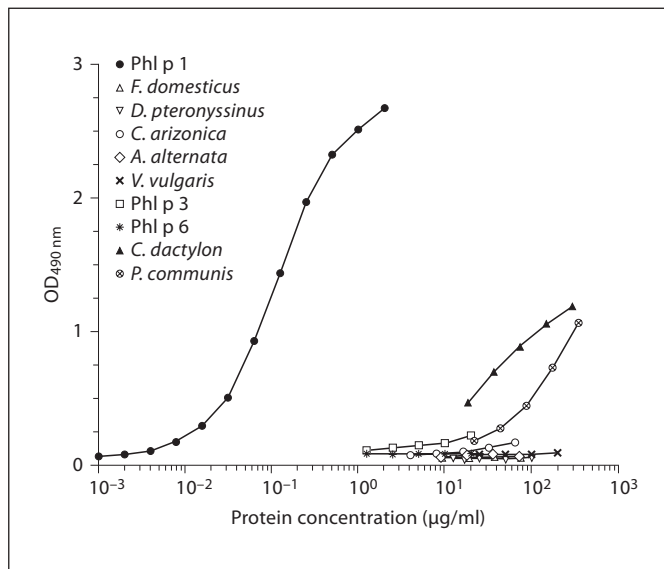


Fig. 4. Specificity of the group 1 MAb-based ELISA. Several allergenic extracts and purified allergens were tested in duplicate in the assay at the protein concentration indicated.

form composition is not the same for all group 1 allergens. This means that individual references from each grass should be used for an accurate determination of the group 1 content. An alternative approach might be to use Phl p 1 as a standard and then multiply the results obtained for the other species by a factor to correct them for the different responses of the MAbs. These correction factors can be deduced from the dose-response curves produced by the purified allergens. This approach was used to determine group 1 allergen concentrations in pollen extracts from Poaceae grasses shown in table 3. Group 5 allergen content was also measured, and the group 1: group 5 ratio was estimated. Four out of the five *P. pratense* pollen extracts showed a ratio close to one. Values found for the other grasses ranged from 0.19 to 0.62. Nevertheless, it should be noted that these are only preliminary results based on the determination in a single pollen batch.

To evaluate the specificity of the method, different extracts and purified allergens were tested. The results are shown in figure 4. Phl p 3, a grass allergen with sequence homology to Phl p 1, was not detected. Phl p 6, another *Phleum* allergen, was equally undetected. Extracts known to contain glycosylated allergens, e.g. *V. vulgaris* (Ves v 1) and *Cupressus arizonica* (Cup a 1), yielded negative responses. Non-Poaceae group 1 allergens were detected with a much lower sensitivity.

Table 3. Ratio group 1/group 5 in grass pollen extracts

Grass	Group 1 µg/ml	Group 5 µg/ml	Group 1/ group 5
<i>Phleum pratense</i>			
Batch 1	708.1	1,035.0	0.68
Batch 2	828.0	1,046.0	0.79
Batch 3	906.0	881.0	1.03
Batch 4	1,122.8	867.0	1.30
Batch 5	356.2	736.3	0.48
<i>Lolium perenne</i>	226.6	1,221.6	0.19
<i>Dactylis glomerata</i>	256.1	757.6	0.34
<i>Poa pratensis</i>	360.0	578.0	0.62
<i>Festuca pratensis</i>	283.8	823.0	0.34

Results of allergen quantitation in different batches of grass pollens. Group 5 was determined as described by Ramírez et al. [13]. The method is also part of the CREATE project and has been extensively evaluated in that context [1]. Group 1 was measured using the ELISA method described in this work. nPhl p 1 was used as a standard and correction factors deduced from the results shown in figure 3 were applied for the estimation of group 1 allergens in other grasses. Figures represent the mean value of two independent determinations. The following correction factors were applied: Poa p 1, 1.97; Lol p 1, 0.49; Dac g 1, 0.66; Fes p 1, 0.72.

Discussion

It has long been recognized that multiple sensitivities to different grass pollens may occur following exposure to and sensitization by a single grass pollen. Allergenic and antigenic cross-reactivities among components of grass pollens have been widely studied. Most of these studies have been based on RAST inhibition with human sera [3–6] or on the ability of antiserum raised against pollen antigens from one or several species to bind to components from the related grasses [35, 36]. The major grass pollens allergens, groups 1 and 5, have been by far the main allergens investigated and, in general, it has been demonstrated that the degree of allergenic cross-reactivity between allergens from different grasses is correlated with the taxonomic relationship and, above all, with the identity of the amino acid sequence of the proteins compared [6, 7, 11]. For this kind of studies, the use of MAbs adds a further dimension, as their monoclonal nature enables investigation of the fine specificity of allergen epitopes [37–39]. In this work, we have obtained MAbs directed to Phl p 1 that define at least four different epitopes on the allergen molecule. The results presented demonstrate that these epitopes are shared with group 1

allergens from other Pooideae grasses. In particular, the most representative wild grasses of the temperate climatic regions, the Poeae tribe, were recognized by these anti-Phl p 1 MAbs with the same immunological strength. As mentioned above, this close antigenic relationship can be explained by the high identity of the Phl p 1 amino acid sequence with the homologous group 1 allergens of the Poeae tribe (about 90%), whereas a more distant relationship was found with Cyn d 1, the sequence of which displays only about 70% identity with Phl p 1. Our findings are in agreement with those described by Esch and Klappper [38], who showed that 91 out of a panel of 123 MAbs directed to the group 1 allergens from five different grasses of the Pooideae subfamily were cross-reactive to epitopes present on all group 1 allergens tested. Moreover, we have shown that purified group 1 allergens from the Poeae tribe produced dose-response curves parallel to Phl p 1 when tested in an ELISA with three MAbs, recognizing non-overlapping epitopes, on the solid phase and an anti-*P. pratense* rabbit serum as the secondary antibody. Altogether, the results presented in this work confirm the close antigenic relationship among group 1 allergens of temperate grasses, and support the idea already suggested by other authors that the number of temperate grasses used for diagnosis and immunotherapy of grass pollen allergy may be reduced to a single grass species [7, 39, 40].

The quality of allergenic extracts intended for clinical use has remarkably improved in the last years with the use of MAb-based methods for measuring the concentration of allergens. The ELISA to quantify grass group 1 allergens described in this work will also be a very useful tool for standardization of grass pollen extracts. As demonstrated by the parallelism of the dose-response curves with the purified grass group 1 allergens, the method may be applied to measure not only the concentration of Phl p 1 in *P. pratense* pollen extracts, but also the concentration of group 1 in other grasses of the Pooideae subfamily. However, correction factors have to be established

to compensate for the observed shift along the x-axis, which is most likely due to the fact that the affinity of the antibodies is not exactly the same for all allergens. This ELISA, along with the previously described ELISA method to quantify group 5 allergens [13], will allow us to standardize allergen products on the basis of the content of the two major grass pollen allergens. The exact ratio between these two major allergens must be defined after careful evaluation of the natural variability. Group 1 and 5 allergens are independent from the point of view of sensitization, thus a biological activity measurement can hardly account for differences in the ratio between the two allergens. Group 1 is more prevalent but group 5 usually induces higher IgE titers than group 1. As a consequence, when a serum pool is used, there is a good correlation between group 5 content and biological activity determined by RAST inhibition [13]. We have not seen this clearly in the case of group 1 (data not shown). This fact stresses the importance of monitoring group 1 content in allergy vaccines and allergy diagnostics in addition to biological activity and group 5 determination. In addition, the ELISA method to quantify grass group 1 allergens can also be used to study their stability in preparations intended for clinical use.

Further development of the method with the production of a reference comprised of a mixture of pure group 1 allergens and a polyclonal antibody directed towards such a mixture might also allow the development of a group 1 assay to monitor group 1 concentrations in mixtures or used to test environmental exposure and will be a subject of further studies.

Acknowledgments

This work was supported by ALK-Abelló. We thank Ms. Lotte Friberg for her skilful technical assistance, Ms. Linda Puebla for language revision of the manuscript and Ms. Carmen Barrio for expert secretarial assistance.

References

- 1 Becker MW, van Ree R, Fiebig H, Cromwell O, Weber B, Monsalve R, Barber D, Raulf-Heimsoth M, Moingeon P, Didierlaurent A, di Felice G, Pini C, Fötisch K, Vieths S: Validation of quantitative, allergen-specific ELISAs. *Arb Paul Ehrlich Inst Bundesamt Sera Impfstoffe* 2006;95:91–97.
- 2 Andersson K, Lidholm J: Characteristics and immunobiology of grass pollen allergens. *Int Arch Allergy Immunol* 2003;130:87–107.
- 3 Leiferman KM, Gleich GJ: The cross-reactivity of IgE antibodies with pollen allergens. I. Analyses of various species of grass pollens. *J Allergy Clin Immunol* 1976;58:129–139.
- 4 Martin BG, Mansfield LE, Nelson HS: Cross-allergenicity among the grasses. *Ann Allergy* 1985;54:99–104.
- 5 González RM, Cortés C, Conde J, Negro JM, Rodríguez J, Tursi A, Wuthrich B, Carreira J: Cross-reactivity among five major pollen allergens. *Ann Allergy* 1987;59:149–154.

- 6 Van Ree R, Driessen MN, Van Leeuwen WA, Stapel SO, Aalberse RC: Variability of cross-reactivity of IgE antibodies to group I and V allergens in eight grass pollen species. *Clin Exp Allergy* 1992;22:611–617.
- 7 Ipsen H, Lowenstein H: Basic features of crossreactivity in tree and grass pollen allergy. *Clin Rev Allergy Immunol* 1997;15:389–396.
- 8 van Ree R, van Leeuwen WA, Aalberse RC: How far can we simplify in vitro diagnostics for grass pollen allergy? A study with 17 whole pollen extracts and purified natural and recombinant major allergens. *J Allergy Clin Immunol* 1998;102:184–190.
- 9 Laffer S, Vrtala S, Duchene M, van Ree R, Kraft D, Scheiner O, Valenta R: IgE-binding capacity of recombinant timothy grass (*Phleum pratense*) pollen allergens. *J Allergy Clin Immunol* 1994;94:88–94.
- 10 Smith PM, Avjioglu A, Ward LR, Simpson RJ, Knox RB, Singh MB: Isolation and characterization of group-I isoallergens from Bermuda grass pollen. *Int Arch Allergy Immunol* 1994;104:57–64.
- 11 Matthiesen F, Lowenstein H: Group V allergens in grass pollens. II. Investigation of group V allergens in pollens from 10 grasses. *Clin Exp Allergy* 1991;21:309–320.
- 12 Smith PM, Ong EK, Knox RB, Singh MB: Immunological relationships among group I and group V allergens from grass pollen. *Mol Immunol* 1994;31:491–498.
- 13 Ramirez J, Obispo TM, Duffort D, Carpizo JA, Chamorro MJ, Barber D, Ipsen H, Carreira J, Lombardero M: Group 5 determination in Pooideae grass pollen extracts by monoclonal antibody-based ELISA. Correlation with biologic activity. *Allergy* 1997;52:806–813.
- 14 Matthiesen F, Schumacher MJ, Lowenstein H: Characterization of the major allergen of *Cynodon dactylon* (Bermuda grass) pollen, Cyn d I. *J Allergy Clin Immunol* 1991;88:763–774.
- 15 Suphioglu C, Singh MB, Simpson RJ, Ward LD, Knox RB: Identification of canary grass (*Phalaris aquatica*) pollen allergens by immunoblotting: IgE and IgG antibody-binding studies. *Allergy* 1993;48:273–281.
- 16 Broadwater AH, Rubinstein AL, Chay CH, Klapper DG, Bedinger PA: Zea mI, the maize homolog of the allergen-encoding Lol pI gene of rye grass. *Gene* 1993;131:227–230.
- 17 Xu H, Theerakulpisut P, Goulding N, Suphioglu C, Singh MB, Bhalla PL: Cloning, expression and immunological characterization of Ory s 1, the major allergen of rice pollen. *Gene* 1995;164:255–259.
- 18 Mecheri S, Peltre G, David B: Purification and characterization of a major allergen from *Dactylis glomerata* pollen: the Ag Dg1. *Int Arch Allergy Appl Immunol* 1985;78:283–289.
- 19 Petersen A, Becker WM, Schlaak M: Characterization of grass group I allergens in timothy grass pollen. *J Allergy Clin Immunol* 1993;92:789–796.
- 20 Laffer S, Valenta R, Vrtala S, Susani M, van Ree R, Kraft D, Scheiner O, Duchene M: Complementary DNA cloning of the major allergen Phl p I from timothy grass (*Phleum pratense*); recombinant Phl p I inhibits IgE binding to group I allergens from eight different grass species. *J Allergy Clin Immunol* 1994;94:689–698.
- 21 Cottam GP, Moran DM, Strandberg R: Physicochemical and immunochemical characterization of allergenic proteins from rye-grass (*Lolium perenne*) pollen prepared by a rapid and efficient purification method. *Biochem J* 1986;234:305–310.
- 22 Perez M, Ishioka GY, Walker LE, Chesnut RW: cDNA cloning and immunological characterization of the rye grass allergen Lol p I. *J Biol Chem* 1990;265:16210–16215.
- 23 Lin ZW, Ekramoddoullah AK, Kisil FT, Herbert J, Mourad W: Isolation and characterization of Poa p I allergens of Kentucky bluegrass pollen with a murine monoclonal anti-Lol p I antibody. *Int Arch Allergy Appl Immunol* 1988;87:294–300.
- 24 Aasmul-Olsen S, Wurtzen PA, Lombardero M, Lowenstein H, Ipsen H: Characterization of group 1 allergens from eleven grass species. *Adv Exp Med Biol* 1996;409:261–265.
- 25 Cosgrove DJ, Bedinger P, Durachko DM: Group I allergens of grass pollen as cell wall-loosening agents. *Proc Natl Acad Sci USA* 1997;94:6559–6564.
- 26 Li LC, Bedinger PA, Volk C, Jones AD, Cosgrove DJ: Purification and characterization of four beta-expansins (Zea m 1 isoforms) from maize pollen. *Plant Physiol* 2003;132:2073–2085.
- 27 Yennawar NH, Li LC, Dudzinski DM, Tabuchi A, Cosgrove DJ: Crystal structure and activities of EXPB1 (Zea m 1), a beta-expansin and group-I pollen allergen from maize. *Proc Natl Acad Sci USA* 2006;103:14664–14671.
- 28 Grobe K, Becker WM, Schlaak M, Petersen A: Grass group I allergens (beta-expansins) are novel, papain-related proteinases. *Eur J Biochem* 1999;263:33–40.
- 29 Li LC, Cosgrove DJ: Grass group I pollen allergens (beta-expansins) lack proteinase activity and do not cause wall loosening via proteolysis. *Eur J Biochem* 2001;268:4217–4226.
- 30 Grobe K, Poppelmann M, Becker WM, Petersen A: Properties of group I allergens from grass pollen and their relation to cathepsin B, a member of the C1 family of cysteine proteinases. *Eur J Biochem* 2002;269:2083–2092.
- 31 Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with the Folin phenol-reagent. *J Biol Chem* 1951;193:265–275.
- 32 Galfré G, Milstein C: Preparation of monoclonal antibodies: strategies and procedures; in van Vunakis H (ed): *Methods in Enzymology*, vol. 73, part B. New York, Academic Press, 1981, pp 1–46.
- 33 Jimeno L, Duffort O, Serrano C, Barber D, Polo F: Monoclonal antibody-based ELISA to quantify the major allergen of *Artemisia vulgaris* pollen, Art v 1. *Allergy* 2004;59:995–1001.
- 34 Hunter WM, Greenwood FC: Preparation of iodine-131 labelled human growth hormone of high specific activity. *Nature* 1962;194:495–496.
- 35 Lowenstein H: Immunological partial identity and in vitro inhibitory effect of two major timothy pollen allergens to whole pollen extract of four grasses. *Int Arch Allergy Appl Immunol* 1978;57:379–383.
- 36 Chakrabarty S, Lowenstein H, Ekramoddoullah AK, Kisil FT, Schon AH: Detection of cross-reactive allergens in Kentucky bluegrass pollen and six other grasses by crossed radioimmuno-electrophoresis. *Int Arch Allergy Appl Immunol* 1981;66:142–157.
- 37 Singh MB, Knox RB: Grass pollen allergens: antigenic relationships detected using monoclonal antibodies and dot blotting immunoassay. *Int Arch Allergy Appl Immunol* 1985;78:300–304.
- 38 Esch RE, Klapper DG: Cross-reactive and unique grass group I antigenic determinants defined by monoclonal antibodies. *J Allergy Clin Immunol* 1987;79:489–495.
- 39 Fahlbusch B, Muller WD, Diener CH, Jager L: Detection of crossreactive determinants in grass pollen extracts using monoclonal antibodies against group IV and group V allergens. *Clin Exp Allergy* 1993;23:51–60.
- 40 Dorward AJ, Waclawski E, Kerr JW: A comparison of the clinical and immunological effects of an alum-precipitated five-grass extract with a conjugated two-grass extract in the desensitization of hay fever. *Clin Allergy* 1984;14:561–570.
- 41 van Ree R, Brewczynski PZ, Tan KY, Mulder-Willems HJ, Widjaja P, Stapel SO, Aalberse RC, Kroon AM: Grass pollen immunotherapy induces highly cross-reactive IgG antibodies to group V allergen from different grass species. *Allergy* 1995;50:281–283.