

Group 5 determination in Pooideae grass pollen extracts by monoclonal antibody-based ELISA. Correlation with biologic activity

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A solid-phase, monoclonal antibody-based ELISA was set up to quantitate group 5 allergens in pollen extracts of wild and cultivated Pooideae grasses. The method was able to evaluate group 5 concentration in mass units with a sensitivity in the ng/ml range and a practical working range of 1–100 ng/ml. The group 5 ELISA was compared with rocket immunoelectrophoresis for determination of allergen levels in several *Phleum pratense* extracts, and a very good quantitative correlation was found ($r=0.98$; $P<0.0001$). A highly significant correlation ($r>0.8$) was also obtained in comparing allergenic potency determined by RAST inhibition to group 5 content in several wild and cultivated grass species. The results proved the usefulness of the method in the standardization of Pooideae pollen extracts employed in diagnosis and treatment.

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Grass pollen allergens constitute an important cause of seasonal allergic diseases worldwide. Groups 1 and 5 are the predominant allergens with an IgE prevalence >80% (1). Group 5 is exclusive of the subfamily Pooideae (2), the most representative and abundant species of which are *Phleum pratense*, *Lolium perenne*, *Dactylis glomerata*, *Festuca pratensis*, and *Poa pratensis* (wild grasses) and *Secale cereale*, *Avena sativa*, *Hordeum vulgare*, and *Triticum sativum* (cultivated grasses). The specific immunologic characteristics of group 5, as compared to group 1 (3–5), explain its differential behavior; thus, the quantitation of this allergen is also becoming important in order to standardize grass pollen extracts for clinical use.

We have developed a monoclonal antibody (mAb)-based ELISA to quantitate the group 5 content in mass units as an alternative method to RAST inhibition. The method is based on the use of two different mAbs recognizing nonoverlapping epitopes, coated on the solid-phase, and purified group 5 allergens as standards. The use of two different mAbs as capturing antibodies is convenient due to the presence of several isoallergens

in many Pooideae group 5 allergens (6–9). The group 5 content in several wild and cultivated Pooideae grasses was compared to the corresponding biologic activity values, determined by RAST inhibition, to establish a practical correlation between both analytic approaches.

Material and methods

Pollen extracts

Pollen of *P. pratense*, *L. perenne*, *D. glomerata*, *F. pratensis*, *P. pratensis*, *S. cereale*, *A. sativa*, *H. vulgare*, and *T. sativum* were purchased from Allergon (Engelholm, Sweden), Biopol (Spokane, WA, USA), and Crystal Laboratories (Luther, OK, USA), or collected by Dr J. A. Jimenez (Madrid, Spain). Aqueous extracts were prepared by suspending the pollen material in 50 mM NH_4HCO_3 buffer pH 7.7 (10% w/v), while continuously shaking, for 30 min at room temperature, and then centrifuging at 4°C, 12 000 g, for 45 min. The supernatant was filtered through 0.4- μm pore size membrane

and dialyzed against water, at 4°C, overnight. The samples were finally filtered through 0.2- μ m pore size membrane and stored in aliquots at -20°C. The total protein content of pollen extracts was determined by the Lowry method (10).

Antibodies

Two anti-group-5-allergen mAbs (Lol 1/7 and Pp v 08) were obtained as previously described (11, 12). Each mAb recognized a different epitope on the group 5 allergen molecules, as was demonstrated by a two-site radioimmunoassay (RIA) (unpublished data), and were purified from ascitic fluid by protein G Sepharose-CL4B affinity chromatography (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions. The purity of the mAbs preparations was assessed by SDS-PAGE, and the protein concentration was measured by a specific mouse IgG ELISA using purified mouse IgG as standard (13).

Anti-wild and cultivated grass pollen rabbit sera used in the ELISA method were raised by immunizing rabbits with respective mixtures of the five wild and four cultivated grass pollen extracts, as previously described (14). The monospecific anti-Phl p 5 rabbit serum employed in rocket immunoelectrophoresis (RIE) experiments was obtained as indicated (3).

Purification of grass group 5 allergens

Phl p 5, Lol p 5, Dac g 5, Fes p 5, and Poa p 5 (wild grasses) and Sec c 5, Ave s 5, Hor v 5, and Tri s 5 (cultivated grasses) were purified by mAb affinity chromatography. The mAbs Lol 1/7 and Pp v 08 were coupled to CNBr-activated Sepharose-CL4B gel (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions (20–50 mg mAb/10 ml gel). The column was equilibrated with phosphate-buffered saline (PBS) before applying the pollen extract samples (20–50 mg protein). Unbound material was eliminated by washing with 0.1% Tween in PBS until optical density (OD) at 280 nm reached the baseline. Bound allergen was eluted with 0.1 M glycine/HCl, pH 2.8. The collected 1-ml fractions containing significant protein amounts were immediately neutralized with 1 M tris-HCl, pH 8.0, pooled, and then dialyzed overnight against 1/10 diluted PBS at 4°C, and stored in aliquots at -20°C. The allergen yield obtained was ~0.5–2% of total protein content. The purity of the preparations was confirmed by SDS-PAGE and crossed immunoelectrophoresis (CIE), and allergenic activity was assessed by RAST inhibition and IgE immunoblotting.

The exact protein concentration of the purified group 5 allergen preparations was determined by amino acid analysis using a Biochrom 20 analyzer (Pharmacia, Uppsala, Sweden). The samples were previously hydrolyzed in a vapor phase technique, as previously described (15).

SDS-PAGE and IgE immunoblotting

Electrophoresis of whole extracts and purified preparations was performed by the method of Laemmli (16) in a Hoeffer vertical slab system with 14% acrylamide separation gel and 5% stacking gel under reducing conditions. The protein bands were detected with Coomassie brilliant blue, and the molecular weight markers were supplied by BioRad Laboratories (Richmond, CA, USA). The electrotransfer onto PVDF membrane strips was performed by the Towbin procedure (17), 50 mM tris-HCl, 0.2 M glycine, and 20% methanol serving as transfer buffer at 250 mA for 2 h. The PVDF strips were saturated with 3% bovine serum albumin (BSA) in PBS for 30 min and then incubated overnight at room temperature with a 1/4 diluted human serum pool from 20 grass-allergic patients (RAST classes 3 or 4). The membranes were washed and then incubated for 3 h with 100 000 cpm/strip of the ¹²⁵I-labeled anti-human IgE mAb HE2 (18). Finally, the membranes were dried and autoradiographed.

Immunoelectrophoretic methods

CIE of the purified group 5 allergens was carried out according to Løwenstein (19). The first dimension was performed in a 1% agarose gel at 10 V/cm for 30 min, at 10°C, and the second dimension in an anti-grass rabbit antibodies-containing gel (40 μ l/cm²) at 4 V/cm, for 18 h, at 10°C. Precipitated protein arcs were visualized by Coomassie brilliant blue staining.

RIE of *P. pratense* extracts using purified Phl p 5 as standard (range 0.6–5 μ g) was performed, according to Laurell (20), in a 1% agarose gel containing anti-Phl p 5 rabbit serum (1 μ l/cm²). Staining was performed with Coomassie brilliant blue, and the Phl p 5 content of extracts was determined by interpolating in the standard curve (rocket peak area vs amount of Phl p 5 for standards).

RAST inhibition

The allergenic activity of the purified grass group 5 allergens and whole extracts was measured by RAST inhibition, as described by Ceska et al. (21). Paper disks were activated with CNBr and

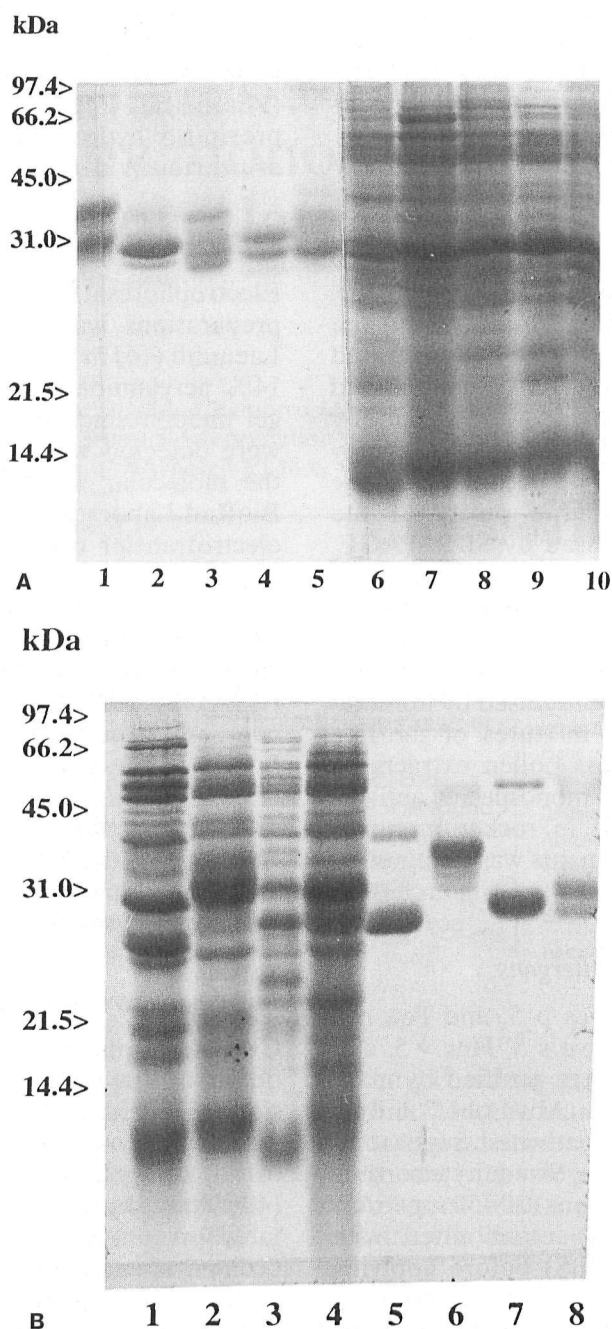


Fig. 1. SDS-PAGE analysis of purified group 5 allergens (10 µg protein applied) and whole-protein extracts (150 µg protein applied). A) Phl p 5 (1), Lol p 5 (2), Dac g 5 (3), Fes p 5 (4), Poa p 5 (5), corresponding whole extracts (6–10); B) Sec c 5 (5), Ave s 5 (6), Hor v 5 (7), Tri s 5 (8), corresponding whole extracts (1–4).

sensitized with an in-house reference of each pollen extract. A volume of 50 µl of a 1/3 dilution of the anti-grass human serum pool was added per well to a 96-well microtiter plate (Costar, Cambridge, MA, USA) and incubated together with the same volume of threefold serial dilutions of samples and in-house reference for 30 min at 37°C. The corresponding allergen disks were then added and incubated for another 3 h at room temperature.

After washing, 100 000 cpm/well of the ^{125}I -labeled anti-human IgE mAb HE2 was added and incubated overnight. Finally, the disks were washed, and the bound radioactivity was measured in a gamma counter. The allergenic activity of grass extracts, in BU/ml, was calculated from the data of three assays by comparison with the corresponding in-house reference previously calibrated by the skin prick test (22).

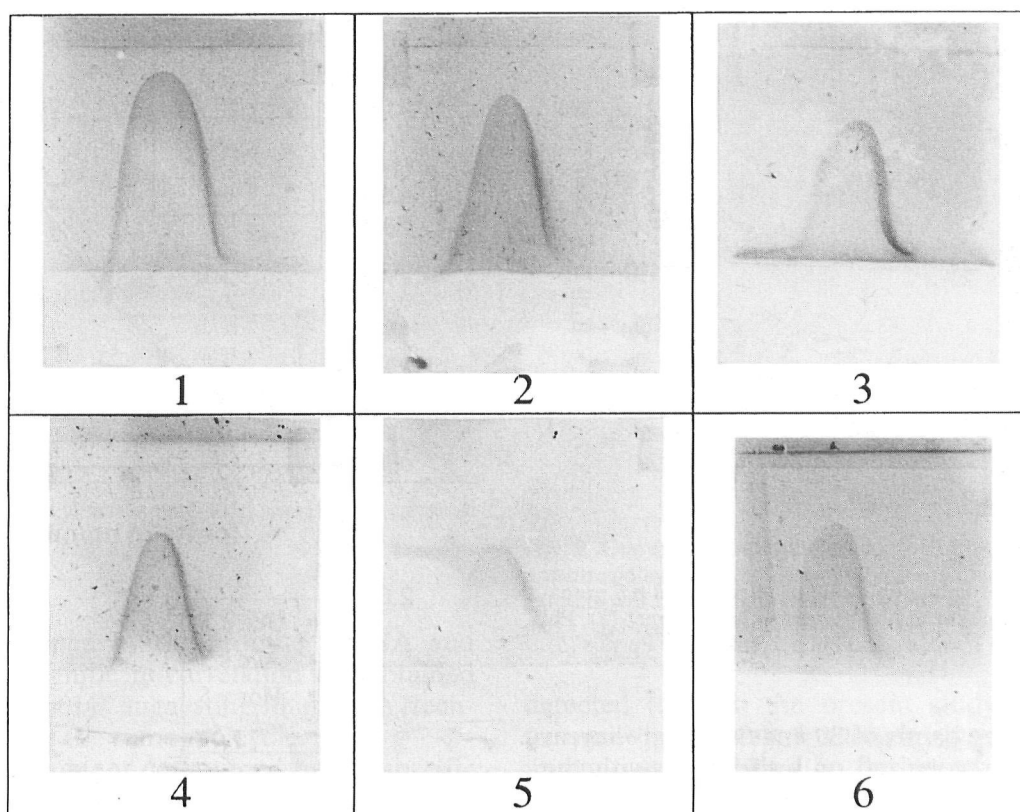


Fig. 2. CIE analysis of purified group 5 allergens (5 μ g protein applied): Phl p 5 (1), Lol p 5 (2), Dac g 5 (3), Fes p 5 (4), Poa p 5 (5), Sec c 5 (6).

Group 5 ELISA

Microtiter 96-well plates (EIA/RIA plate, Costar, Cambridge, MA, USA) were coated overnight at 4°C with 50 μ l of a mixture of the anti-group 5 mAbs Lol 1/7 and Pp v 08 at 2.5 μ g/ml each. This optimum mAb concentration for coating had been previously determined. Plates were then saturated with 1% BSA in PBS (100 μ l/well) for 30 min, at room temperature and incubated for 1 h with 50 μ l/well of samples diluted with 0.1% BSA, 0.05% Tween 20 in PBS. After washing with 0.1% Tween 20 in PBS, the plates were incubated with 1/20 000 (wild grasses) or 1/6000 (cultivated grasses) dilutions of the corresponding rabbit antiserum. After four washes, 50 μ l of a 1/20 000 dilution of peroxidase conjugated goat antirabbit IgG (Tago, Burlingame, CA, USA) was added to each well and incubated for another hour. Finally, the color reaction was developed by incubating with 50 μ l/well of peroxidase substrate buffer (0.3% H_2O_2 , 1 mg/ml *o*-phenylenediamine, in 50 mM citrate-phosphate buffer, pH 6.0). After 30 min, the color reaction was stopped by adding 50 μ l/well of 2 M HCl, and the OD was read at 490 nm with a reference filter at 650 nm. Samples were assayed in duplicate at several threefold dilutions. The group 5 content, in

mass units, of samples was obtained by interpolating in standard curves from different concentrations of the corresponding purified group 5 allergen (range 500–0.2 ng/ml for wild grasses and 200–0.2 ng/ml for cultivated grasses).

Results

Allergen purification

Phl p 5, Lol p 5, Dac g 5, Fes p 5, and Poa p 5 (wild grasses) and Sec c 5, Ave s 5, Hor v 5, and Tri s 5 (cultivated grasses) were purified and characterized from respective whole-pollen extracts by mAb-based affinity chromatography. In SDS-PAGE (Fig. 1), different mol.-wt. isoforms were detected, in agreement with published data (3, 7), corresponding to IgE-reactive components on immunoblotting. The molecular weights found for group 5 allergens ranged from 28 to 38 kDa, Sec c 5 being the smallest (~28 kDa) (Fig. 1B). In CIE (Fig. 2), the purified group 5 allergens showed single anodic arcs, indicating unique antigenic entities regarding the anti-whole grass pollen extract rabbit sera. Affinity chromatography based on a single mAb demonstrated, in the case of *P. pratense* (Fig. 3), that mAb Pp v 08 recognized

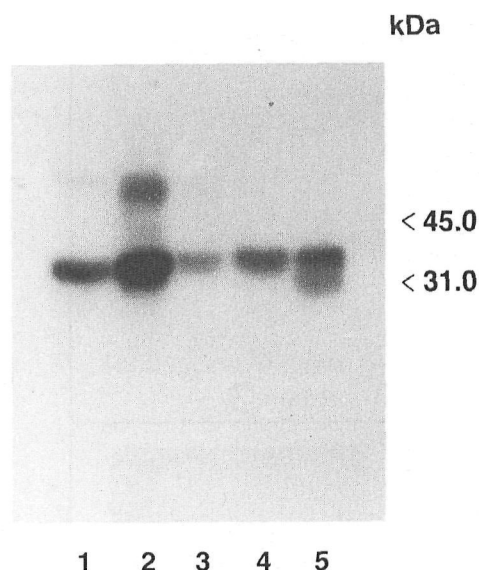


Fig. 3. Specific IgE immunoblotting of purified Phl p 5 preparations: Phl p 1 marker (1), *P. pratense* whole extract (2), Phl p 5 purified by Lol 1/7 (3, 4), and Phl p 5 purified by Pp v 08 (5).

two IgE-binding bands of 30 and 36 kDa, whereas mAb Lol 1/7 recognized only the band with the highest mol.-wt.

The RAST-inhibition assay for the purified group 5 allergens yielded maximum percent inhibition values, ranging between 60–75% (wild grasses) and 55–60% (cultivated grasses). These data confirmed that the purified preparations had retained allergenic activity after the purification process.

Group 5-specific ELISA

For quantitation of group 5 allergens, two specific mAbs, recognizing nonoverlapping epitopes, were bound to the plastic surface of microtiter plates to capture the group 5 molecules of samples, and, as a second antibody, rabbit sera against wild/cultivated grass pollen extracts were used. Fig. 4 shows the binding curves of the affinity-purified allergens in the solid-phase ELISA. The protein content of purified preparations had been previously determined by quantitative amino acid analysis. The dose-response curves showed very similar slopes, and there was no shift on the x-axis, indicating that the group 5 purified allergens are similarly recognized by the mAb. The binding curves of the purified allergen and the corresponding whole extract also showed identical slopes (data not shown), indicating that the immunoassay detected the same molecule in the different preparations, and that no practical alteration had occurred during the purification process. With affinity-purified allergen as standard, it was possible to determine

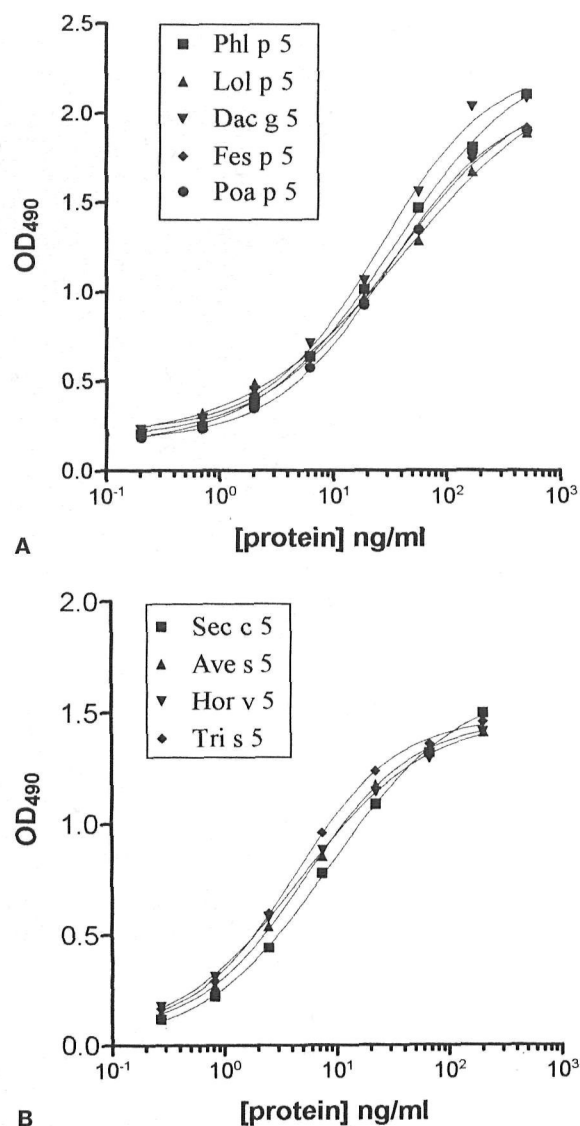


Fig. 4. Dose-response curves in ELISA of purified group 5 allergens from wild grasses (A) and cultivated grasses (B).

the group 5 content in the range of 1–100 ng/ml. The specificity of the group 5 ELISA was ascertained with a set of different allergenic extracts, and the results are presented in Table 1. There was no significant group 5 content in allergenic material known to lack group 5, including non-Pooideae grass pollen extracts (*Cynodon* and *Phragmites*). The content of group 5 allergen in a number of pollen extracts from different Pooideae species, including both wild and cultivated grasses, was determined by ELISA. On average, *P. pratense* had the highest relative group 5 content and *L. perenne* and *S. cereale* the lowest (Table 2). Important differences in group 5 content were observed (up to a factor of 10) within each species.

Fig. 5 shows the comparison by linear regression analysis of the Phl p 5 content of 14 different pollen

Table 1. Specificity of group 5 mAb ELISA

Sample ^a	OD ₄₉₀	Group 5/total protein (%)
<i>Cynodon dactylon</i>	0.378	<0.01
<i>Phragmites communis</i>	0.125	<0.005
<i>Olea europaea</i>	0.138	<0.005
<i>Betula verrucosa</i>	0.174	<0.005
<i>Parietaria judaica</i>	0.226	<0.005
<i>Artemisia vulgaris</i>	0.142	<0.005
<i>Dermatophagoides pteronyssinus</i>	0.092	<0.005
Cat dander	0.105	<0.005
Egg white	0.101	<0.005
Bee venom	0.145	<0.005
<i>Alternaria alternata</i>	0.125	<0.005
<i>Phleum pratense</i>	>2.0	8.2
<i>Secale cereale</i>	>1.5	4.5

^a Group 5 allergen content of extracts listed (10 µg protein/ml) was determined by mAb ELISA.

extracts determined by the group 5 ELISA and RIE. A highly significant correlation was obtained ($r=0.98$; $P<0.0001$), suggesting that both techniques measured the same molecule.

The group 5 content determined by the specific ELISA of several pollen extracts of wild and cultivated grasses was also compared to their allergenic potency, as determined by RAST inhibition, and a good correlation was observed (Table 2). For example, Fig. 6 shows the linear regression analysis for the species *P. pratense* (A) and *S. cereale* (B).

Discussion

The quantitation in mass units of main allergens has become of great importance for the standardization of products for clinical use. Group 5 allergens are major allergens in pollen from grasses belonging to the subfamily Pooideae, and high cross-reactivity, at both the IgE (23) and IgG levels, has been

Table 2. Relative group 5 content and coefficient of correlation between group 5 specific ELISA and RAST inhibition

Species ^a	n	% group 5 (SD)	BU/mg pollen	r
<i>Phleum pratense</i>	15	8.2 (7.8)	538	0.92
<i>Lolium perenne</i>	15	3.4 (1.5)	793	0.90
<i>Dactylis glomerata</i>	7	3.9 (3.0)	388	0.92
<i>Festuca pratensis</i>	14	7.2 (6.0)	991	0.82
<i>Poa pratensis</i>	11	7.1 (2.9)	264	0.95
<i>Secale cereale</i>	14	3.3 (2.4)	528	0.97

^a Different pollen extracts (n =number of samples) of species listed were determined by group 5 specific ELISA and RAST inhibition, and results compared by linear regression (r =correlation coefficient). % group 5=average group 5 content relative to total protein content; SD=standard deviation, BU/mg pollen=average biologic units per mg of total protein.

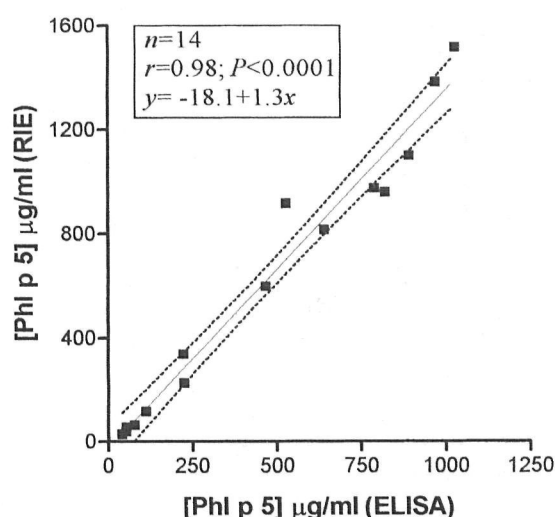


Fig. 5. Correlation between group 5 ELISA and RIE in *P. pratense* pollen extracts. Concentration of Phl p 5 in 14 different extracts was determined by ELISA (abscissa) and RIE (ordinate), and results were analyzed by linear regression.

detected (24). In the present study, an ELISA to quantitate group 5 allergens was set up. The immunoassay is based on the use of two different mAbs as catching antibodies recognizing nonoverlapping epitopes on the allergenic molecule. In Phl p 5, two groups of isoforms (Phl p 5a and Phl p 5b) with molecular weights of 29–32 and 33–38 kDa have been identified (6–9). The epitope recognized by mAb Lol 1/7 is present only in Phl p 5a, whereas the one recognized by mAb Pp v 08 is present in both groups of isoforms (Fig. 3). The existence of different allergen isoforms constitutes an important fact to consider in the design of an mAb-based immunoassay to measure the allergen, and the use of more than one mAb has been recommended (25). The use of two different mAbs in the present immunoassay may explain the high sensitivity of the method (<1 ng/ml) and the uniformity of the response obtained in different species (Fig. 3), as compared to conventional two-site immunoassays (26), thus validating the method for measuring group 5 in mixtures of different Pooideae grasses.

The corresponding group 5 allergen, purified by affinity chromatography, has been used as a primary standard of the assay. The suitability of these preparations was checked by different techniques. By SDS-PAGE, the preparations were shown to have a molecular weight in the range described for group 5 allergens, whereas the CIE technique revealed the characteristic arc corresponding to these proteins. The amino acid composition was very similar to reported data (5), the high content of alanine (24–26%), as well as the absence of tryptophan, being characteristic of group 5 allergens.

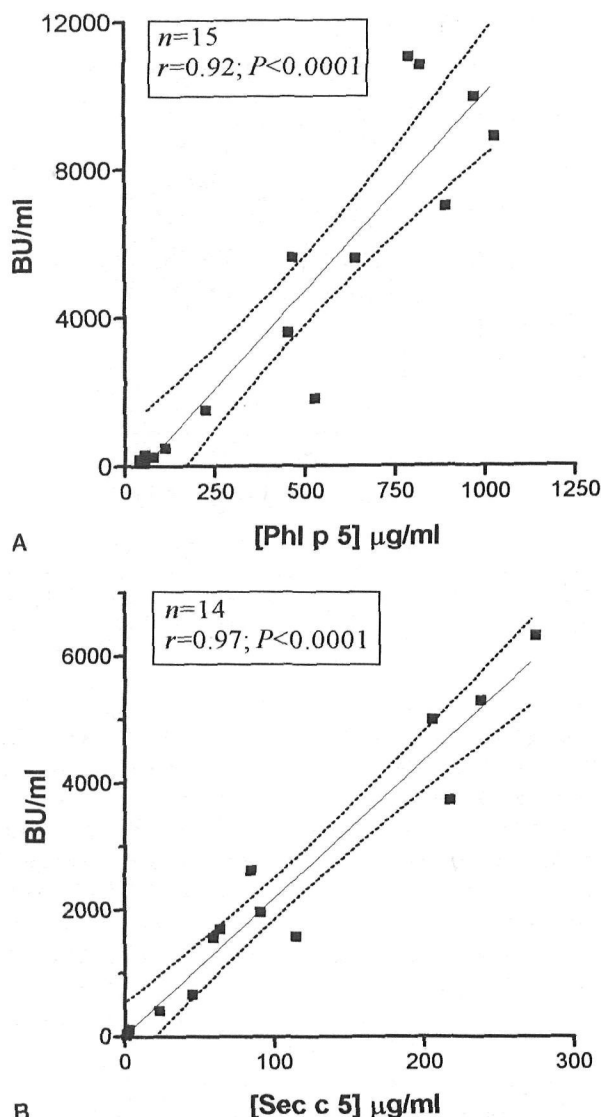


Fig. 6. Correlation between allergenic activity (BU/ml) determined by RAST inhibition and group 5 content determined by ELISA in 15 different *P. pratense* pollen extracts (A) and 14 different *S. cereale* pollen extracts (B).

The allergenic activity of purified group 5 allergens was determined by immunoblotting and RAST inhibition. Compared with the whole-pollen extract, the group 5 allergens could inhibit approximately 55–75% of the grass-specific IgE found in a grass serum pool, demonstrating the importance of these allergens for determining the total activity of Pooideae pollen extracts. The group 5-specific ELISA was compared with two different techniques, RIE and RAST inhibition. A very good quantitative relation was found between the content of Phl p 5 in several *P. pratense* extracts determined by ELISA and RIE (Fig. 5), suggesting that both techniques measure the same component. In addition, a good correlation was obtained

between the group 5 content, as determined by ELISA, and the allergenic activity determined by RAST inhibition, in a number of allergenic extracts prepared from different pollen batches of the Pooideae species studied (Fig. 6). On the average, 1 BU corresponded to about 100 ng of group 5 allergen.

These results confirm that group 5 proteins are major allergens in pollen from Pooideae grasses and that the group 5 ELISA could be very useful for quality control of preparations intended for clinical use, including evaluation of allergenic activity, mass units standardization, and studies on the stability of the allergen in these preparations. Interestingly, due to the uniformity of the response obtained among different group 5 allergens from distinct grass species, the method can be used with mixtures of different Pooideae grass pollen extracts, which are very common in clinical practice. Finally, the group 5 ELISA could be used to monitor the potential contamination of tree and weed pollen raw materials with grass pollens, which, due to their ubiquity, are the main contaminants of other pollen materials.

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