

Monoclonal antibody immunoassay for quantitative analysis of group V allergens in grass pollen extracts

B. FAHLBUSCH, W.-D. MÜLLER, G. SCHLENVOIGT, L. JÄGER, R. WAHL* and B. WEBER*

Department of Clinical Immunology, The University of Jena, Jena, Germany and *Allergopharma, Joachim Ganzer KG, Reinbek, Germany

Summary

A two-site monoclonal antibody (MoAb) ELISA has been developed for the quantification of the *Phleum pratense* major allergen, *Phl p* V. The assay is based on two MoAbs which recognize different non-overlapping epitopes on the *Phl p* V molecule; one antibody (1D11) was immobilized on the solid phase and the other (3B2) was biotinylated. An affinity-purified *Phl p* V preparation (purity of 95%) was used as standard. The assay has a sensitivity of 10 ng/ml of allergen and is suitable for the detection of group V allergen in aqueous grass extracts. The specificity of the assay was investigated with 14 grass pollen and five non-grass pollen extracts. Different levels of group V allergen were detected in extracts of grasses, but not in non-grasses. The assay gives a good correspondence with allergenic activity of extracts as determined by ELISA inhibition using serum pool of allergic patients. The results indicate that the two-site MoAb ELISA could be very useful in the standardization of allergenic extracts from grass pollen.

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Introduction

It is widely agreed that a better standardization of grass pollen extracts is necessary for further improvement of diagnosis and therapy of pollinosis. The standardization of grass pollen allergens is often complicated by the large number of allergenic components to which an individual may be sensitized as well as an incomplete knowledge of allergenic and antigenic relationships among different components of grass pollens. Most important in grass pollinosis are the group I and group V allergens by virtue of their high sensitization index [1,2]. Several *in vitro* methods have been developed for standardization of allergens in crude extracts [3-5]. At present, RAST inhibition is widely used to evaluate extracts quantitatively and compare them with national standards. The main problems with RAST inhibition are that the results depend on the extract used for the solid phase and on the selection of donors for the serum pools, and consequently it is difficult to achieve reproducible results [6].

Recently, group V allergens were identified in eight out of 10 selected temperate grasses using immunoelectrophoretic techniques (CIE) with a monospecific rabbit anti *Phl p* V serum [7]. The patterns were similar to the timothy extract indicating a high degree of common structural features. The crossreactivity of group V allergens has been confirmed with MoAbs [8]. Certain MoAbs have been used to develop specific inhibition immunoassays for quantification of group I allergens in crude extracts [9,10]. To the best of our knowledge a two-site binding assay for the direct measurement of defined components in grass pollen extracts has not been published previously.

The essential factors required to provide a basis for quantification are suitable MoAbs and sufficient amounts of pure and standardized preparations of allergen. Recently, we have raised MoAbs against *Phl p* V that show a broad crossreactivity among different grass pollens [11]. We have now taken advantage of this property to investigate the usefulness of these MoAbs for the quantification of group V allergens in grass pollen extracts in a two-site binding immunoassay. We have isolated the allergen *Phl p* V from whole extracts of *Phl p* by means of affinity chromatography with murine MoAb

Correspondence: Dr B. Fahlbusch, Institute of Clinical Immunology, Friedrich-Schiller-University, Humboldtstr. 3, Jena 0-6900, Germany.

(1D11 or 3B2), and used this preparation as a primary standard. Here we demonstrate that the combination of MoAbs 1D11 and 3B2 or 2D1 provide a basis for a promising sandwich ELISA.

Material and methods

Pollen grains were obtained from Sächsisches Serumwerk GmbH, Dresden. The following species were investigated:

1 Pooid (festucoid) grasses: *Holcus lanatus* (Hol l); *Bromus spec.* (Bro); *Avena elatior* (Ave e); *Alopecurus pratensis* (Alo p); *Dactylis glomerata* (Dac g); *Secale cereale* (Sec c); *Calama grost* (Cal g); *Deschampsia spec.* (Des); *Molina coerulea* (Mol c); *Phleum pratense* (Phl p); *Lolium perenne* (Lol p); *Poa pratensis* (Poa p); *Festuca pratensis* (Fes p).

2 Arundinoid grass: *Phragmites* (Phr).

The following non-grass pollens were used as controls: *Rumex triangulivalvis* (Rum t); *Salix* (Sal); *Solidago canadensis* (Sol c); *Plantago* (Pla); *Carex spec.* (Car).

Pollen from all the species tested were extracted at 4°C for 16 hr with a 0.125 M NH₄HCO₃ buffer, pH 8.0 (10% w/v), centrifuged and dialysed against water. The extracts were stored at -20°C until use. The protein content of extracts was determined between 0.3 and 0.4 mg/ml according to the method of Bradford [12].

Antibodies

Sera were collected and pooled from 60 non-treated patients with hay fever. Diagnosis was based on a detailed clinical questionnaire, prick test and RAST (Phadebas RAST, Pharmacia, Uppsala, Sweden). Sixteen individual sera from allergic patients were used for detection of allergenicity of standard preparation (Phl p V). The production and characterization of MoAbs to group V allergens of Phl p have been described previously [11].

Purification and biotinylation of MoAbs

Ascites of crossreacting MoAbs, 1D11, 3B2 and 2D1, were produced by injecting 3×10^6 cells intraperitoneally into pristan-primed BALB/c mice. The ascitic fluid of the mice was harvested about 14 days later and antibodies purified by high performance anion exchange chromatography with FPLC and Mono Q (HR 5/5, Pharmacia). Each MoAb (1 mg/ml) in 0.1 M sodium bicarbonate, pH 8.4, was mixed with 50 µl D-biotinyl-ε-aminocaproic acid *N*-hydroxysuccinimide ester (Boehringer Mannheim; 1 mg/ml in dimethyl sulphoxide) and left for 30 min at room temperature. Unreacted biotin was removed by

fast desalting column (HR 10/10, Pharmacia). The final concentration of biotinylated MoAbs was 0.5 mg/ml as judged by absorbance at 280 nm.

Purification of Phl p V

An immunosorbent column was prepared by coupling 30 mg of the purified 1D11 to 10 ml of CNBr-activated Sepharose 4B-CL as described earlier [13]. 10 ml extract of Phl p (25 mg protein) was applied to column. After washing with PBS (flow 0.5 ml/min, 20°C) the bound allergen was eluted with 0.1 M glycine/HCl, pH 2.8, and immediately dialysed at 4°C against 0.125 M NH₄HCO₃ followed by 0.0125 M NH₄HCO₃, pH 8.0. The resulting material (≈3.7 mg protein) was aliquoted and freeze dried. The purification of the allergen was confirmed by SDS-PAGE [14]. The allergenic activity of the preparation was assessed by immunoblotting after SDS-PAGE [15] and by binding of specific IgE using direct ELISA (Table 1).

Table 1. IgE-binding reactivity of Phl p (crude extract) and purified Phl p V in patients allergic to grass pollens

Patient number	Phl p (OD)*	Phl p V (OD)*
<i>Allergic</i>		
1	1.28	1.84
2	0.38	0.78
3	0.426	0.64
4	0.85	0.028
5	0.76	0.94
6	0.98	1.28
7	1.26	1.74
8	1.273	1.164
9	0.421	0.253
10	0.511	0.21
11	1.171	1.095
12	1.144	0.962
13	1.342	1.348
14	1.095	1.304
15	1.264	1.39
16	1.332	1.424
<i>Normal</i>		
Serum pool	0.05	0.035

* IgE binding capacity determined by direct ELISA. Phl p or Phl p V coated plates were incubated with serum of patients and the specific IgE binding was detected using biotinylated anti-IgE and streptavidin-HRP.

ELISA inhibition for epitope mapping

Microtitre plates (NUNC, MaxiSorp) were coated with 50 μ l of *Phl p V* (10 μ g/ml) diluted in sodium carbonate buffer (0.05 M, pH 9.6). After washing and blocking with PBS, 0.5% Tween 20 (v/v) and 5% gelatine (w/v) (TPBS/G), each biotinylated MoAb was added alone (no inhibition) or in the presence of homologous or unrelated unlabelled MoAb. The concentration of MoAbs used as inhibitors was adjusted to similar immunoglobulin content [16]. After a 2 hr incubation, wells were washed and the binding of biotinylated MoAb detected with streptavidin-HRP (Boehringer Mannheim; diluted 2×10^{-3}). The substrate used was *o*-phenylenediamine.

Double-binding ELISA

These experiments were designed to establish whether or not the epitopes recognized by the MoAbs were expressed only once or several times on *Phl p V* and to identify a suitable combination for two-site binding assay. Briefly, wells were coated with 50 μ l of each MoAb (5 μ g/ml) in sodium carbonate buffer. Wells were blocked and washed as described above and then incubated for 2 hr with different concentrations of *Phl p* extract (2500–0.025 μ g/ml). After incubation unbound proteins were removed by extensive washing and 50 μ l of biotinylated MoAb were added. Optimal concentrations of labelled MoAbs were established before bound. The ELISA was then continued as described above.

Detecting of group V allergens in pollen extracts using IgE antibodies

Crossreactive determinants in different pollen extracts were detected by EIA-inhibition. Briefly, microtitre plates were coated with 50 μ l of *Phl p V* (1 μ g/ml). In separate tubes mixtures of different extracts (dilution of 10^{-1} , 10^{-2} , 10^{-3}) and serum pool from allergic patients were incubated overnight at 4°C. The mixtures containing IgE-allergen complexes were added into *Phl p V* coated plates for 2 hr. After washing, specific IgE was determined by addition of 50 μ l monoclonal mouse anti-human IgE (BL-IgE, University of Leipzig) followed by incubation of HRP labelled goat anti-mouse globulin.

Two-site binding assay

Quantification of *Phl p V* was achieved by a two-site solid-phase ELISA as follows: microtitre plates were coated with 50 μ l/well of purified 1D11 at 10 μ g/ml in sodium carbonate buffer overnight at 4°C. All subsequent incubations were performed at room temperature for 2 hr. After blocking with TPBS/G and further two washes 50 μ l

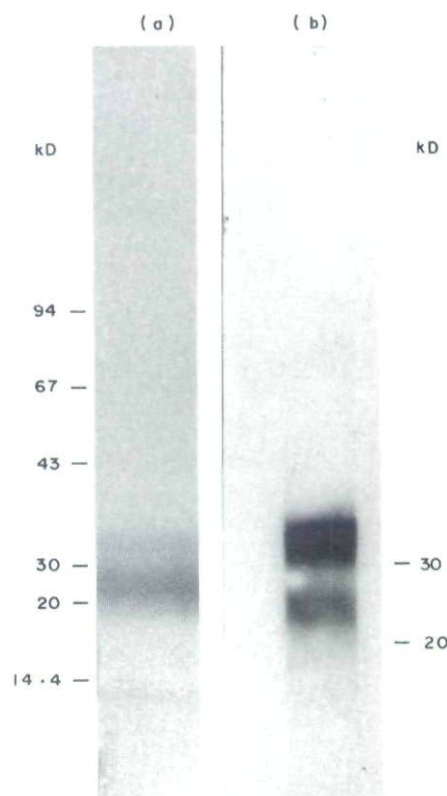


Fig. 1. SDS-PAGE (a) and Western blot (b) of affinity-purified *Phl p V*. *Phl p V* (20 μ g) was separated on an 8–18% gradient polyacrylamide gel (ExcelGel™, Pharmacia) and stained with Coomassie brilliant blue R-250 (run a). In a second run (b) the *Phl p V* was transferred to nitrocellulose and incubated with pooled sera. The bound IgE was detected by using biotinylated anti-IgE and streptavidin-HRP. The blot was developed in ECL substrate solutions [15].

of *Phl p V* standard solution (concentration range 1–1000 ng/ml), or dilutions of grass extracts (in most cases 10^{-2} , 10^{-3} , ...) were added to the wells. The plates were washed three times and incubated with 50 μ l of biotinylated 3B2 (dilution of 10^{-3}). After a further three washes, 50 μ l of streptavidin-peroxidase was added. The ELISA was developed with *o*-phenylenediamine as substrate.

Results

Phl p V was isolated from pollen extract of *Phl p* by means of affinity chromatography using MoAb 1D11 (or 3B2) coupled onto Sepharose. Purity and activity were monitored by SDS-PAGE and immunoblot analysis. As presented in Fig. 1, two major proteins were isolated with molecular weights of 29 kD and 34 kD, consistent with *Phl p Va* and *Vb* [2]. The degree of purity was estimated at 95.2% by scanning with a laser densitometer. The yield of

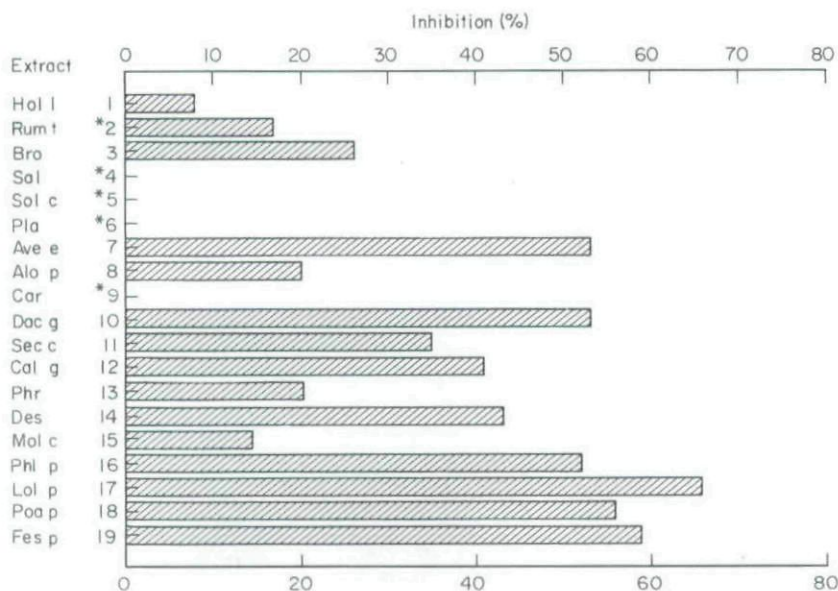


Fig. 2. Inhibition of IgE binding by extracts of several grasses or non-grasses (*). *Phl p V* was adsorbed to microtitre plates and incubated with a mixture of extracts (3–4 µg/ml) and pooled sera diluted 1:2.

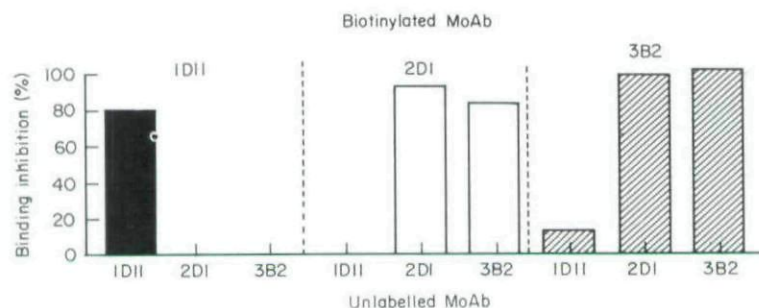


Fig. 3. Binding inhibition of biotinylated MoAb to solid phase *Phl p V* by unlabelled MoAb. Each biotinylated MoAb was added alone (no inhibition) or in presence of homologous or unrelated unlabelled MoAb to *Phl p V* coated immunosorbent plates as described in Materials and methods.

Phl p V allergen was approximately 15% of the protein applied. The allergenicity of isolated fraction was confirmed by means of a panel of individual sera from patients being allergic to grass pollen (Table 1).

Furthermore we investigated the ability of 14 grass pollen extracts and five non-grasses (as controls) to inhibit the binding of IgE antibodies to group V allergen (Fig. 2). Extracts from non-grass pollen did not interfere with binding of IgE, with the exception of *Rumex*. On the other hand significant inhibition was observed with extracts from other grass pollen, but to different degrees. The results in Fig. 2 show that extracts of *Ave e*, *Dac g*, *Phl p*, *Lol p*, *Poa p*, *Fes p* inhibited very effectively IgE binding to *Phl p V*, suggesting that they contain analogues of *Phl p V*. These data confirm similar results obtained with MoAbs [11] and are an essential requirement to quantify group V allergens in different extracts.

As it was our intention to use the MoAbs 1D11, 3B2 and 2D1 for a two-site binding assay, it was necessary to investigate whether these MoAbs recognize different non-

overlapping epitopes. Two different ELISA assays were selected for the epitope mapping of *Phl p V*: the ELISA inhibition and double-binding ELISA. First of all, the MoAbs were compared according to their relative inhibitory capacity in an ELISA using biotinylated and unlabelled MoAbs respectively, and *Phl p V* coated immunosorbent plates (Fig. 3). As expected binding inhibition was observed with homologous MoAb, but also between MoAb 3B2 and 2D1. These data suggest that 3B2 and 2D1 recognize a similar or neighbouring region of *Phl p V*. On the contrary the antigenic epitope detected by MoAb 1D11 seems to be distinct from the binding sites for 3B2 and 2D1. As these results could be influenced by the affinity of MoAbs further investigations were performed using double-binding ELISA (Fig. 4).

Microtitre plates were coated with a MoAb, and then *Phl p* extract diluted in PBS/Tween was added. After washing only *Phl p V* will be bound by the immobilized MoAb and remain in the well. The epitope by which the *Phl p V* molecule is held should be inaccessible to any

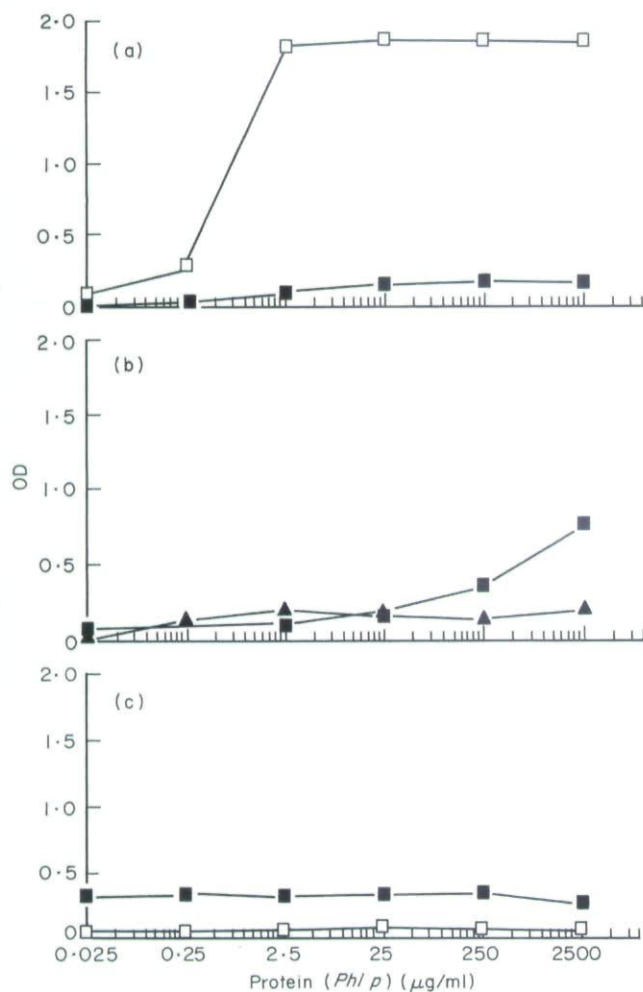


Fig. 4. Double-binding ELISA of labelled MoAb and unlabelled MoAb to *Phl p* V. *Phl p* at concentration of 0.025–2500 µg/ml were allowed to react with capture MoAbs 1D11 (□), 3B2 (■) and 2D1 (▲). As indicator antibodies were used biotinylated 3B2 (a), 2D1 (b) and 1D11 (c).

other MoAb, and consequently biotin-labelled monoclonal anti-*Phl p* V will be found only if it recognizes a different epitope on the *Phl p* V molecule.

Using 3B2 coated onto the microtitre plate as the capture antibody and 1D11 as indicator (Fig. 4(c)) no dose-response curves were detected. The MoAb pair 3B2 and 2D1 showed only at extreme high concentration (≈ 2 mg/ml) a binding capacity (Fig. 4(b)). The best results were obtained with MoAb 1D11 immobilized on the solid phase and 3B2 as second antibody (Fig. 4(a)), and for this reason this combination was used for the following experiments.

The specificity and sensitivity of the two-site MoAb ELISA was studied with a purified *Phl p* V as standard preparation. Figure 5 shows typical titration curves with

biotinylated 3B2. Similar dose-response curves were obtained with labelled 2D1 (results not presented). The assay permitted the detection of nanogram amounts of *Phl p* V per millilitre (linear range between 10 and 120 ng/ml). Simultaneous dilutions of two different batches of *Phl p* extracts provided parallel curves with same slopes. The only difference found in the binding curves is that there was a shift on the x-axis according to the different allergen content. The total *Phl p* V concentrations of both extracts were calculated using the standard curve. The in-house extract (HE, protein content of 2.5 mg/ml) contains 350 µg/ml *Phl p* V and a production extract (PE, protein content of 1.33 mg/ml) of Allergopharma (Reinbek, Germany) 260 µg/ml, respectively.

Specificity was checked furthermore with *Lol p* I (kindly provided from A. Ansari, Baltimore, U.S.A.). *Lol p* I reacted in this assay only using a ≈ 500 times higher concentration (results not shown). In preliminary studies group V levels were measured in a panel of grass extracts (10% w/v) (Fig. 6). Group V allergen was detected in all grass extracts in different quantities. *Alo p*, *Phr c* and *Mol c* contained very low levels of group V. It should be noted that there was no reaction with control extracts, with the exception of *Rumex*.

Discussion

It is evident that a reproducible and exact quantification of defined allergens is the key to successful standardization of grass pollen extracts. MoAbs against the various major allergens have been produced to develop specific immunoassays for quantification of individual allergens in crude extracts [17–21].

It has been demonstrated by Krilis *et al.* [9] with anti-short ragweed Ag E MoAbs, and by Kahn and Marsh [10] with anti-ryegrass Gp I MoAbs, that MoAb-based immunoassays are able to detect nanogram quantities of the specific antigen in whole pollen extracts. In the latter case, a single MoAb recognizing a crossreactive determinant was used to estimate Gp I content of extracts derived from several grass species. In this connection a fundamental question arises, whether a MoAb to any single epitope would be representative for allergenic potency, in terms of allergen standardization. In previous investigations using EIA inhibition we showed that with MoAb 1D11 the determination of allergenic potency of aqueous or depot pollen extracts is possible [22]. Identical results were achieved with different treated extract batches using both human IgE antibodies as well as the group V specific MoAb 1D11.

The availability of group V specific MoAb has the advantage that this major allergen could be easily purified by affinity chromatography. The *Phl p* extract (2.5 mg/ml)

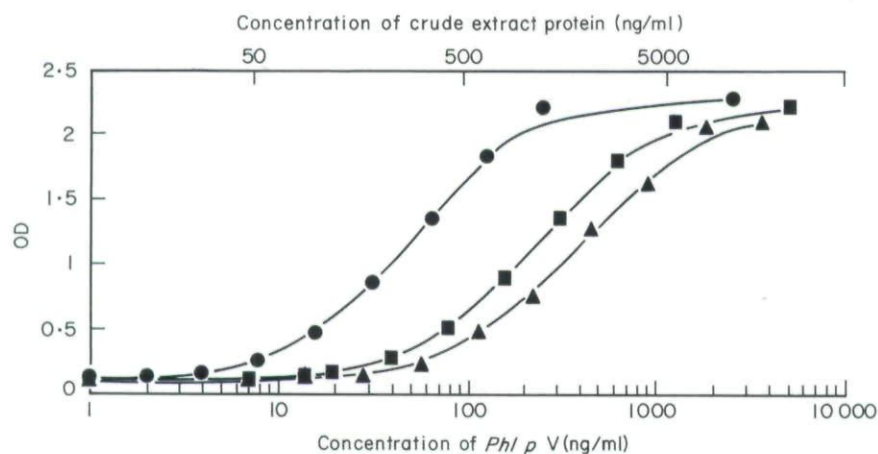


Fig. 5. Dose response curves of purified *Phl p* V and two different *Phl p* extracts. ●, Affinity-purified *Phl p* V. ■, Production extract (PE) obtained from Allergopharma. ▲, In-house extract (HE) prepared as described in Materials and methods.

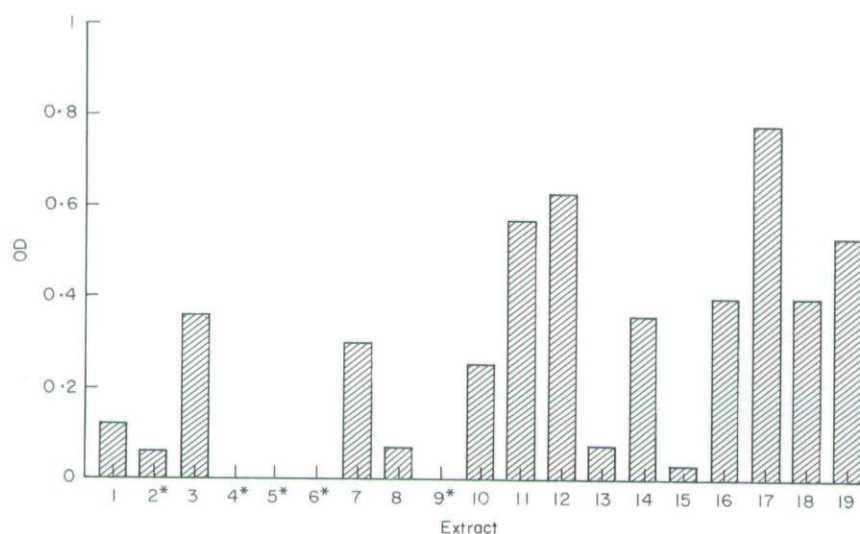


Fig. 6. Two-site binding assay for detection of group V in pollen extracts of different plants. The extracts were adjusted to 300–400 ng/ml protein and incubated in 1D11 (500 ng/well) coated plates. Biotinylated 3B2 (dilution 10^{-3}) was used as indicator antibody. The number of extracts is the same as that shown in Fig. 2. *, Control extracts.

used as raw material contained about 200–300 μ g *Phl p* V. The purity of isolated protein was about 95% and it was suitable as a primary standard for quantification of group V allergen. *Phl p* V was recognized by specific IgE with high frequency (>90% of allergic patients, Table 1), suggesting that it is indeed a major allergen according to the definition of Marsh [23].

The aim of the present studies was to demonstrate that our MoAbs against *Phl p* V are useful for quantitative analysis of group V allergen in grass pollen extracts using a two-site binding ELISA. For this reason epitope mapping was performed with three MoAbs (1D11, 2D1 and 3B2). Using two separate test systems we established that 2D1 and 3B2 recognize a similar site on *Phl p* V, whereas 1D11 clearly binds to an unrelated region. The lack of inhibition in competitive ELISA (Fig. 3) could result theoretically from repeated epitopes on *Phl p* V. To analyse this possibility a second assay was performed (Fig. 4). The results of double-binding ELISA suggest

that all three antibodies bind to antigenic sites that are non-repeated, i.e. present only once on each *Phl p* V molecule. Furthermore, these studies were useful to identify an optimal combination for the two-site binding assay. The highest sensitivity was obtained with MoAb 1D11 immobilized on the solid phase and biotinylated 3B2 or 2D1 as second antibodies. Interestingly, the reversed combination is unsuitable. Obviously MoAbs 3B2 or 2D1 lose their binding ability to allergen after coating to microtitre plate or the affinity to soluble *Phl p* V is too low. Previous studies confirmed that 3B2 and 2D1 have been bound better to solid-phase allergen whereas 1D11 has a higher affinity to soluble molecules [11].

The standard curve (Fig. 5) with purified *Phl p* V showed a high sensitivity in the range from 10 to 120 ng/ml. The same figure shows the binding curve for *Phl p* extracts, an in house (HE) and a production extract (PE) of Allergopharma [4]. It can be seen that the binding curves are parallel to that obtained with the purified *Phl p*

V preparation, suggesting that the allergen had not been antigenically altered during the purification procedure and that the assay detects indeed the same molecule in different extracts. The *Phl p* V content varied between 14% and 20% of the total protein content, as determined according to the method of Bradford [12].

Matthiesen *et al.* [7] have shown that a high degree of common structural features exists between group V allergens of temperate grasses. Recent studies [25] confirmed that both the amino acid compositions and the NH₂-terminal sequences obtained for each group V allergen are almost similar to each other and to the sequence and composition of the *Phl p* V from *Phl p*. The previously reported crossreactivity of MoAbs raised against *Phl p* V and the group V allergens from related species [11, 24] could itself be seen as an indication of similarities at the protein structure level. However, it can not be excluded that the MoAbs used recognize only defined isoallergenic variants. Therefore, we performed preliminary studies for semi-quantification of group V allergens in several pollen extracts. Indeed the level of group V allergen seems to vary and corresponds well to the allergenic potency of extracts (Fig. 2). Group V was not found in control extracts (non-grasses) as expected. The low content in *Rumex* extract could correspond to a contamination of *Rumex* pollen with grass pollen or to a small crossreactivity between the *Phl p* V molecule and a *Rumex* component. Interestingly, *Alo p*, *Phr c* and *Mol c* contain only low quantities of group V. This fact is in agreement with Matthiesen *et al.* [7] and our own studies [11]. The precise levels of group V allergen are currently being investigated in different source materials.

In conclusion we believe that the principles we have described for the measurement of a major allergen in *Phl p* extract can be used to quantify group V allergen in other grass pollen as well as in mixtures.

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