

Detection and quantification of group 4 allergens in grass pollen extracts using monoclonal antibodies

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Summary

Background Grass pollen extracts are complex mixtures consisting of different major allergenic and non-allergenic components. Phl p 4 is an important allergen, because more than 75% of grass pollen allergic patients produce specific IgE antibodies against group 4 allergens.

Objective This study was designed to investigate the specificity of monoclonal antibodies (MoAbs) produced against Phl p 4 and to verify the presence of group 4-like proteins in different grass pollen. Furthermore the usefulness of MoAbs for quantification of group 4 allergens was studied.

Methods Group 4 analogues were investigated by immunoblotting and ELISA inhibition using three MoAbs. The specificity of antibodies was studied using isolated group 1 and group 5 allergens. Quantification of group 4 allergen was achieved by a two-site solid-phase ELISA. Phl p 4 was purified from whole pollen extract by chromatographic or electrophoretic techniques and used as standard.

Results The MoAbs studied bound strongly to proteins from timothy grass pollen extract at a mw of 55 kDa and a pI of 9.0–9.3. Phl p 4 homologues with similar mw were detected in *Dactylis glomerata*, *Festuca pratensis*, *Holcus lanatus*, *Poa pratensis*, *Lolium perenne*. Epitope mapping showed that all three MoAb recognized unrelated regions on Phl p 4. A two-site binding ELISA using MoAbs was developed for determination of Phl p 4 in *Phleum pratense* extracts. The method was able to evaluate group 4 in mass units with a working range between 150 and 2000 ng/mL. The absolute amounts of group 4 in extracts of several grasses varied considerably but was always less than 1% of the total protein.

Conclusion Group 4 homologues are present in the various grass extracts but to different extents. The group 4 ELISA could be very useful as a additional tool for providing information concerning the composition of grass pollen extracts.

Keywords: grass pollen extracts, Phl p 4, purification, monoclonal antibodies, cross-reactivity, two-site binding ELISA

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Introduction

Pollen extracts are commonly used for diagnosis and therapy of grass pollen allergy. These extracts are frequently mixtures of different grass species and contain beside non-allergic components a series of relevant and other less

important allergens. The allergens in grass pollen extracts can generally be divided into six major groups (1–6) [1,2], of which group 1, 4 and 5 allergens are the most dominant by virtue of their high IgE-reactivity [3,4]. Group 4 allergen seems also to be important because it is frequently recognized in IgE-immunoblotting studies [5,6], although the levels of specific-IgE are relatively low compared with groups 1 and 5 [7]. Group 1 and group 5 allergens are present in most grass species and show immunochemical

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similarities between different grasses [8,9]. Matthiesen *et al.* showed however, that there are differences in group 1 and group 5 concentrations between species [8]. Similar results were obtained by immunoblot analysis and quantification of group 5 allergens by a two-site monoclonal antibody ELISA [10]. Since the efficiency of extracts used for diagnostic and therapeutic purposes depends on the one hand on the level of major allergens in the various extracts and on the other on the individual sensitization of the patients the quantity of individual allergens in extracts could be of interest [11].

Little is known about group 4 grass pollen allergens, although more than 75% of grass pollen allergic patients produce specific IgE antibodies against this allergen [5,6]. Group 4 allergens were described as basic glycoproteins of 50–60 kDa molecular weight (mol.wt.) with pI about 9.4 in different grass species [5,12,13]. Recently, Phl p 4 was characterized as a trypsin resistant protein [14].

In a previous paper we described the production of MoAbs against major allergens of *Phleum pratense* [15]. In our continuing attempts to complete the repertoire of MoAbs to grass allergens, three MoAbs produced against Phl p 4 were identified. In the present study we used these antibodies to verify the presence of group 4-like proteins in different grass pollen and to investigate the usefulness of group 4 specific MoAb for the quantification of allergen in grass pollen extracts in a two-site binding immunoassay.

Methods

Extracts and antibodies

The following pollen grains were purchased from Sächsisches Serumwerk GmbH, Dresden, Germany: *Dactylis glomerata*, *Festuca pratensis*, *Holcus lanatus*, *Poa pratensis*, *Lolium perenne* and *Phleum pratense*. For the preparation of crude extracts, proteins were extracted by shaking the pollens in 0.125 M NH_4HCO_3 buffer, pH 8.0, at 10% (w/v) for 16 h at 4 °C. The extracts were then centrifuged at 30 000 *g* for 30 min at 4 °C and the supernatants were filtered, dialysed against water and stored at –20 °C until use. The International Standard 82/520 was obtained from the National Institute for Biological Standards and Control (Potters Bar, UK). For the investigations, the lyophilized extract was dissolved in 1 mL of double-distilled water according to the recommendations.

The protein content of extracts was measured using a modified Bradford method on microtitre plates as described by Vieths [16].

MoAbs were produced after immunization with timothy grass pollen extract [17] or enriched Phl p 4 [15].

Purification of grass pollen allergens

Phl p 5 and Lol p 5 was isolated by affinity chromatography with MoAb 1D11 [18]. Phl p 5 depleted *Phleum pratense* extract (designated as 1D11F₁) was prepared by passing the extract through the 1D11 immunosorbent column. Lol p 1 was purified by reversed-phase high-performance liquid chromatography [19].

Group 4 was prepared either from dialysed *Phleum pratense* extract by a combination of ion exchange chromatography with CM-Sepharose (Pharmacia, Uppsala, Sweden) and gel filtration using Sephadex G 100 (Pharmacia) as described earlier [5,15] or from Phl p 5 depleted extract by means of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

Briefly, a crude extract or group 5 depleted fractions were applied to preparative SDS-PAGE (continuous-elution electrophoresis, Bio-rad Model 491 Prep Cell). We electrophoresed 5 mg of protein at 10 W in cylindrical acrylamide gel (10%, 2.8 × 10 cm) containing 0.1% SDS and eluted it from the bottom of the gel at a flow rate of 1 mL/min. Fractions (1 mL) were collected, and their protein content was determined by absorbance at 280 nm. The samples from every fifth fraction were tested by analytic SDS-PAGE (Phast System, Pharmacia). Analysis of both chromatographically or electrophoretically purified Phl p 4 by SDS-PAGE/immunoblotting showed one band at 55 kDa and IgE-binding. The collected purified allergen was dialysed and lyophilized and used for the analyses.

Finally, the purified allergens were used in competition immunoassays to study the extent of allergenic cross-reactivity among them.

Gel electrophoresis

SDS-PAGE

Analysis of proteins was performed under reducing conditions with ready-to-use Excel polyacrylamide gradient gels (8–18%) according to the instructions of the manufacturer (Pharmacia). Protein/lane (2 µg) were applied for silver staining and 5 µg/lane for blotting.

IEF

Polyacrylamide gel isoelectrofocusing was carried out using Ampholine PAGplate 3.5–9.5 (Pharmacia). The samples (20 µg/cm) were focused at 1500 V for 2.5 h.

2D-gel electrophoresis

In the first dimension, isoelectric focusing was performed as described above and after that the flat gel was slice in strips. After equilibration of the IEF strips with SDS containing sample buffer for 15 min, the separation by SDS-PAGE was

performed with Excel polyacrylamide gradient gels (8–18%) and limited to 600 V and 50 mA.

Immunoblot analysis

Proteins separated by SDS-PAGE were subsequently transferred to nitrocellulose (NC) membrane (Schleicher & Schüll, Dassel, Germany) by semi-dry blotting [20] for 30 min at 0.8 mA/cm². Several samples analysed by IEF or 2D-electrophoresis were transferred to NC membranes by two different methods: electrotransfer [21] and passive transfer (thermoblotting) [Pharmacia Development Technique 1987, File no. 220]. For group 4 allergens the passive transfer was more effective and was used in this investigation.

For subsequent immunological investigations the blots were incubated with various antibodies (human serum or monoclonal antibodies) and visualized with appropriate secondary antibodies (peroxidase-conjugated-anti-IgE or antimouse antibodies) [22].

Competitive inhibition ELISAs

To detect crossreactive determinants in different grass pollen extracts polystyrene microtitre 96-well plates (Nunc, MaxiSorp) were coated with purified allergen Phl p 4 at 10 µg/mL overnight. The plates were then blocked for 30 min with phosphate buffered saline, containing 0.1% v/v Tween-20 (TPBS). Afterwards, 25 µL of the MoAb (diluted 1:10) and 25 µL of serial dilutions of different grass pollen extracts were added to the wells and incubated for 2 h at room temperature. The extract concentrations as inhibitors ranged from 0.1 to 1000 µg/mL.

To study the specificity of MoAbs purified Phl p 5, Lol p 5 and Lol p 1 (0.5–50 µg/mL) were used also as inhibitor. After washing, wells were incubated with peroxidase-labelled anti-mouse IgG (Boehringer, Mannheim, Germany) for 1 h at room temperature. The plates were washed again and developed by incubating with 50 µL/well of a solution of o-phenylenediamine (OPD). The reaction was stopped by adding 50 µL of 1.5 M H₂SO₄ and the absorbance was measured at 492 nm. Assays were performed in duplicate.

Epitope mapping was also performed in competitive inhibition experiments with the MoAbs. Briefly, each biotinylated MoAb was added alone (no inhibition) or in the presence of homologous or unrelated unlabelled MoAb on the solid-phase Phl p 4. The concentration of MoAbs used as inhibitors was adjusted to similar immunoglobulin content. The binding of biotinylated MoAbs was detected with peroxidase-labelled streptavidin. Results were calculated as percentage inhibition.

Two-site binding assay

Quantification of Phl p 4 was achieved by a two-site solid-phase ELISA. The purification and labelling of MoAbs have been described previously [23]. Briefly, microtiter plates were coated with 50 µL/well of purified MoAb 5H1 at 10 µg/mL in 20 mM sodium carbonate buffer pH 9.6 overnight at +4 °C. All subsequent incubations were performed at room temperature for 2 h. After blocking with TPBS followed by a further two washes, 50 µL of Phl p 4 standard solution (concentration range 0.025–25 000 ng/mL), or dilutions of grass extracts (in most cases doubling dilutions) were added to the wells. The plates were washed three times and incubated with 50 µL of biotinylated MoAb 3C4 (dilution 10⁻³) for 2 h. After a further three washes, 50 µL of streptavidin-peroxidase was added. The ELISA was developed with OPD as substrate.

Results

Purification of Phl p 4

Phl p 4 was isolated from a pollen extract of *Phleum pratense* by means of chromatographic or electrophoretic techniques. A single protein with a mol. wt. of 55 kDa was isolated (Fig. 1). Densitometric analysis of purified Phl p 4 revealed a purity of >98%. The allergenicity of the isolated

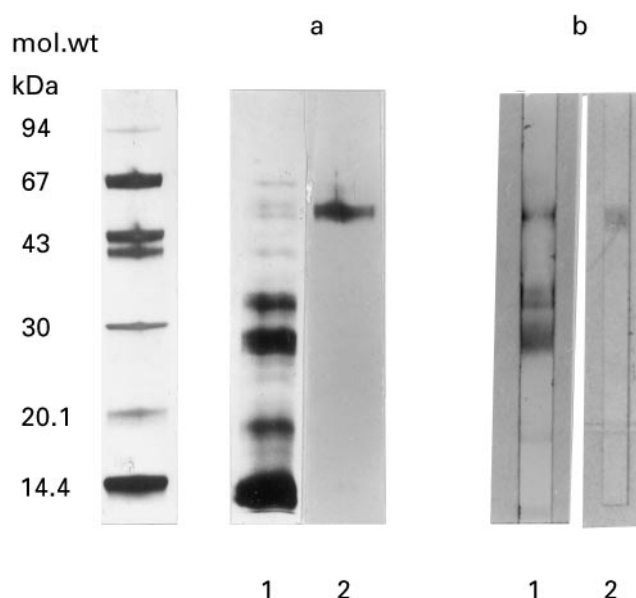


Fig. 1. (a) SDS-PAGE analysis of the whole *Phleum pratense* extract (lane 1) and purified Phl p 4 (lane 2); (b) IgE-reactivity of pooled allergic patients' sera to nitrocellulose blotted timothy grass pollen extract (lane 1) or purified Phl p 4 (lane 2).

protein was confirmed by IgE-binding of pooled sera from grass pollen allergic patients. This purified allergen was used for the studies of specificity of MoAbs and as the primary standard preparation in the sandwich ELISA.

Characterization of MoAbs

As it was our intention to use our murine monoclonal antibodies for a two-site binding assay, it was necessary to investigate whether these MoAbs reacted specifically with Phl p 4 and moreover recognized different non-overlapping epitopes. The specificities of different MoAbs (2D8, 5H1, 3C4) were analysed by immunoblotting after SDS-PAGE and IEF using timothy grass pollen extract (Fig. 2). All three antibodies bound strongly to a protein of about 55–60 kDa. Additional binding of the MoAbs 5H1 and 2D8 was observed to proteins between 30 and 40 kDa and a weak binding at 69 kDa of MoAbs 2D8 and 3C4. This binding pattern was also reported by Fischer *et al.* [14]. The IEF-immunoblots showed binding of MoAbs to proteins in a basic range (pI 7.8–9.5). MoAb 3C4 also recognized structures in a range of pI 6.4 and 7.2, but the binding was weak. After 2D-electrophoresis binding was observed to proteins of 55–60 kDa with pI 9.0–9.5 (Fig. 3). The differences between SDS-PAGE and 2D-electrophoresis suggest that the 30–40 kDa bands observed with the former may result from the cleavage of disulphide bridges which is not achieved under the milder conditions used with the latter method.

On the strength of immunoblotting results after SDS-PAGE it was necessary to determine whether the MoAbs also recognized structures on group 1 or 5 allergens. The specificity of MoAbs was studied by ELISA inhibition with the isolated allergens Phl p 5, Lol p 5 and Lol p 1, and a Phl p 5 depleted fraction (Fig. 4). The binding of MoAbs was inhibited (30–50% inhibition) by Phl p 4 enriched fraction with very low protein content (0.5 ng/mL). By comparison partial inhibition with group 5 allergens was only possible with 10 000-fold (Phl p 5) or 100 000-fold (Lol p 5) higher concentrations. Similar results were obtained with the direct ELISA [15] and confirmed the group 4 specificity of our MoAbs.

Epitope mapping was undertaken using unlabelled MoAb to inhibit the binding of biotinylated MoAb to the allergen (Fig. 5). As expected binding inhibition was observed with homologous MoAb (>70%). In contrast, combination with heterologous MoAb showed less than 20% inhibition. The lowest levels of competitive inhibition were obtained with the MoAbs 5H1 and 3C4, respectively. Therefore, this combination of antibodies was used for the development of a sandwich ELISA.

Detecting crossreactive structures by means of MoAb

The extent of crossreactivity between different grasses was evaluated with an inhibition ELISA, using the MoAb 3C4 (Fig. 6). The different grass pollen extracts inhibited the MoAb binding to the Phl p 4 in a dose-dependent manner,

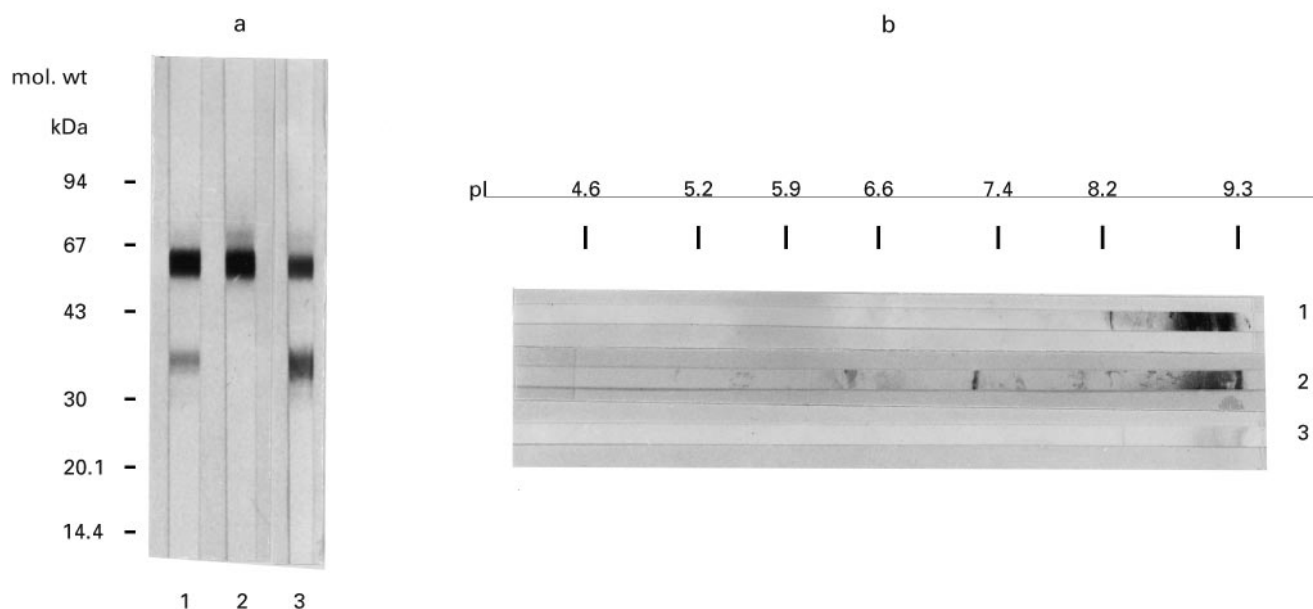


Fig. 2. Immunoblotting of timothy grass pollen proteins using MoAbs after SDS-PAGE (a) and IEF (b). Lane 1: MoAb 5H1; lane 2: MoAb 3C4; lane 3: MoAb 2D8.

reaching complete inhibition. The slopes of the linear range of the inhibition curves were statistically parallel, suggesting the existence of common or related epitopes of group 4 in the extracts studied. In addition, we used the same grass pollens for immunoblotting to ascertain the mw of MoAb-binding components in these species (Fig. 7). Under our experimental conditions, all the extracts studied showed the presence of group 4 homologous proteins, but with differences in the concentrations.

Phl p 4 sandwich-ELISA

We developed a solid-phase ELISA for the determination of Phl p 4 in *Phleum pratense* extracts in which the MoAb 5H1 was used as the capture antibody and the MoAb 3C4 as the detection antibody. Figure 8 shows the binding curve of a purified Phl p 4 preparation in this ELISA. Using this preparation as a standard, it was possible to quantify the Phl p 4 content in the range from 150 to 2000 ng/mL. The same figure shows the binding curves for *Phleum pratense* extracts. As can be seen, the curves are parallel to that obtained with pure Phl p 4, 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.

Furthermore, we investigated purified grass allergens to confirm the specificity of the group 4 assay. The results in Table 1 show that these allergens gave extremely low absorbance measurements at 492 nm by comparison with a similar Phl p 4 concentration.

The Phl p 4 sandwich ELISA was also used to determine the group 4 content in extracts of the other grasses. The absolute amounts varied between 1 µg/mL (*Poa pratensis*) to 20 µg/mL (*Phleum pratense*), but the absolute content of group 4 was in each case <1% relative to the total protein.

Discussion

Groups of grass pollen allergens with extensive immunological crossreactivities have been described in various grass species [24]. Group 4 allergens have been detected in extracts of *Phleum p.* [5], *Lolium p.* [12] and *Dactylis g.* [13] and also in other pollen extracts from grasses [15,25].

MoAbs are valuable tools to identify single proteins in complex extract mixtures or analyse the crossreactivity between them [26]. Therefore, we have evaluated three MoAbs produced against Phl p 4 [15] and used them to study similarity between group 4 allergens from six clinically important grass pollens and to establish a two-site binding assay.

The specificity of MoAbs was proved using immunoblots from timothy grass. All antibodies reacted with a strong basic protein (pI > 9) of about 55–60 kDa. Moreover, weak

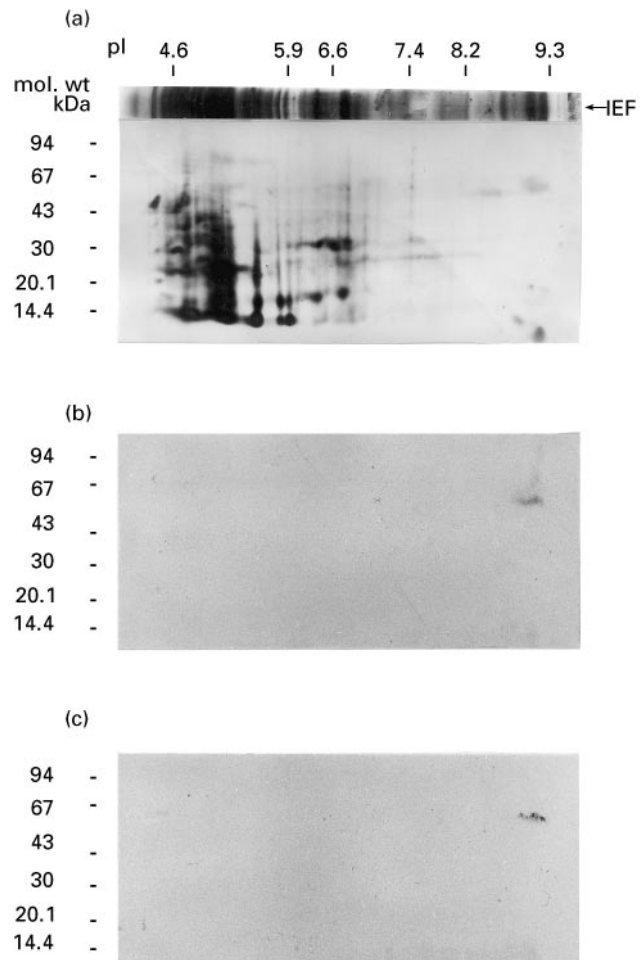


Fig. 3. 2D-electrophoresis and immunoblotting of timothy pollen extract. (a) 2D-gel and IEF strip (on the top) silver stained; after thermoblotting the NC membrane was incubated with supernatants of MoAbs 3C4 (b) or 5H1 (c).

Table 1. Specificity of the two-site ELISA for group 4 quantification

Sample	OD _{sample} – OD _{blank}
Phl p 4 (12 µg/mL)	1.827
Lol p 1 (16 µg/mL)	0
Phl p 1 (30 µg/mL)	0.009
n Phl p 5 (23 µg/mL)	0.131
n Phl p 5a (17 µg/mL) ¹	0.066
n Phl p 5b (20 µg/mL) ¹	0.101
r Phl p 5a (20 µg/mL) ²	0.015
r Phl p 5b (20 µg/mL) ²	0

¹ prepared by HPLC [18]

² kindly provided by Dr A. Bufer, Borstel, Germany.

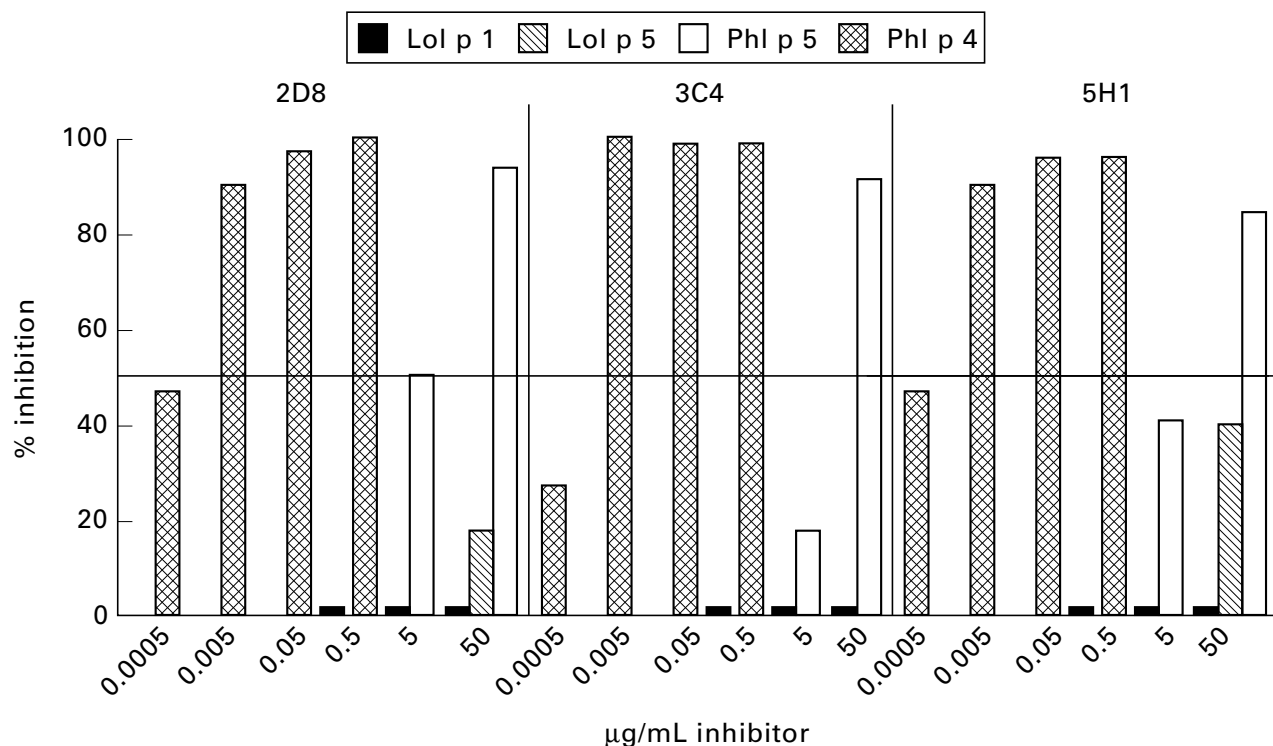


Fig. 4. ELISA-inhibition of the binding of MoAbs to Phl p 4 by major grass pollen allergens. Serial dilutions of purified Lol p 1, Lol p 5, Phl p 5 and Phl p 4 were incubated with MoAbs 2D8, 3C4 and 5H1 and added to wells coated with Phl p 4.

binding of the MoAbs 2D8 and 5H1 was detected between 30 and 40 kDa, in the mw range of two dominant allergens, group 1 and 5. In order to exclude the possibility that similar crossreactive structures exist in Phl p 4 and these major allergens we used for ELISA inhibition. Inhibition experiments indicated that extremely high concentrations of Phl p 5 or Lol p 5 (about 10 000-fold excess in comparison with Phl p 4) are able to inhibit (Fig. 4). This weak reaction could explain the blotting patterns. It is possible that group 5 proteins are denatured during SDS-PAGE in such a way that modified structures are formed which are partially

recognized by group 4 MoAbs. However, the IgE inhibition experiments with a combination of recombinant Phl p 1 [27] and Phl p 5 [28] of Fischer *et al.* [14] indicated that Phl p 4 is indeed immunologically unrelated to any of these allergens. An alternative explanation is that reduction of Phl p 4 with cleavage of disulphide bonds yields a 30–40 kDa component that is recognized weakly by MoAbs 2D8 and 5H1.

The crossreactivity of group 4 allergens in several grass species was studied with two different methods under

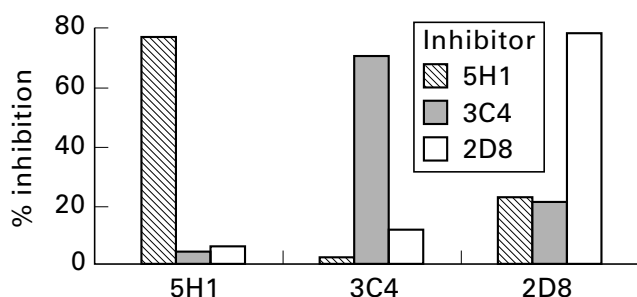


Fig. 5. Competitive inhibition of biotinylated MoAb to solid-phase Phl p 4 by unlabelled MoAb. Each biotinylated MoAb was added alone (no inhibition) or in the presence of homologous or unrelated unlabelled MoAb to Phl p 4 coated immunosorbent plates.

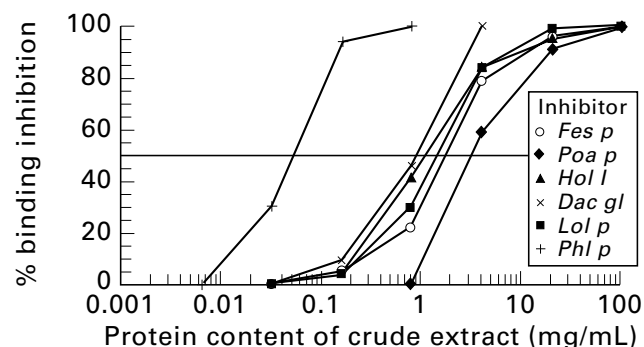


Fig. 6. Dose-dependent inhibition of ELISA activity of MoAb 3C4 to Phl p 4 by several grass pollen extracts. The percentage inhibition was calculated as follows: % inhibition = $(1 - \text{OD with inhibitors} / \text{OD without inhibitors}) \times 100$.

denaturing and native conditions. Using the immunoblotting technique Phl p 4 homologous proteins were detected in the different grasses with all three MoAbs (Fig. 7). In contrast previous reports have indicated that Lol p 4 (pI 9.9, mw 59 kDa) [12] and Dac g 4 (pI 8–9, mol.wt. 67 kDa) [13] for example could be not immunodetected. In this context it is important to remember that during SDS-PAGE the proteins are partially denaturated and therefore effective detection is likely to be more difficult. If such denaturation is commonplace then it limits the applicability of SDS-PAGE in allergen identification.

In order to avoid this possible complicating factor we

analysed the binding of MoAb to Phl p 4 by means of the ELISA inhibition technique with six different grass extracts. Antigenic differences can be detected in terms of differences in the slopes of the inhibition curves. Figure 6 shows that the inhibition curves for Phl p 4 and its analogues in the other species are parallel that indicating antigenic similarities.

Jaggi *et al.* [29] prepared MoAbs against Lol p 4 which recognized different epitopes on the allergen. However, in that study none of the MoAbs could be used successfully to perform a two-site assay. Recently MoAbs were produced against Dac g 4 [25]. One MoAb recognized a 60 kDa

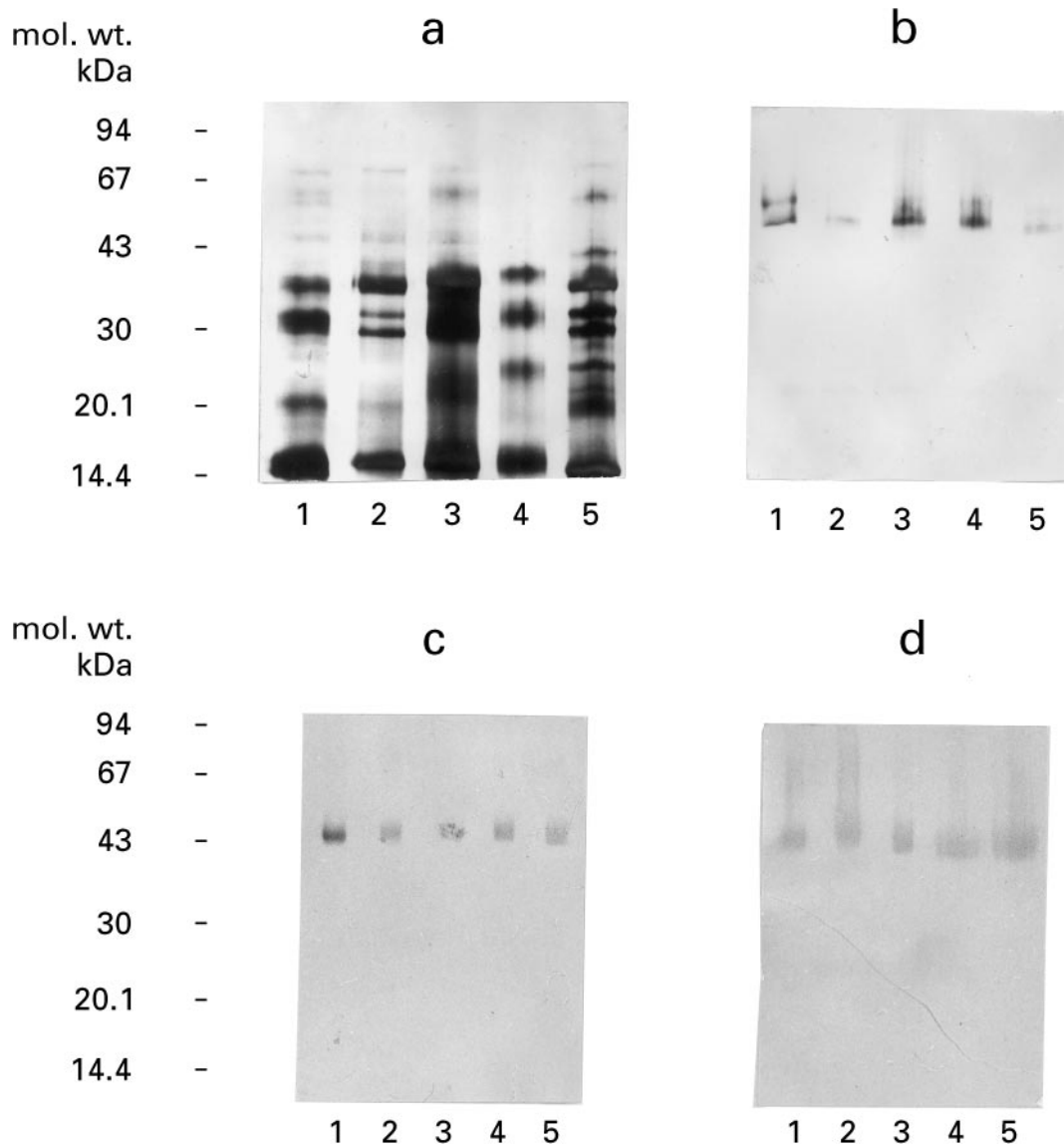


Fig. 7. SDS-PAGE (a) of five different grass pollen extracts and immunoblotting using MoAb 3C4 (b), 5H1 (c) and 2D8 (d) 1 *Dactylis glomerata*, 2. *Festuca pratensis*, 3. *Holcus lanatus*, 4. *Poa pratensis*, 5. *Lolium perenne*.

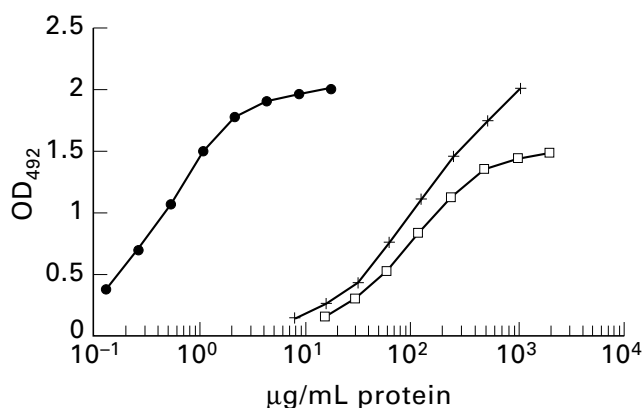


Fig. 8. Dose-response curves in the group 4 ELISA of (●) purified Phl p 4, (+) crude *Phleum pratense* extract and (□) International Standard 82/520.

crossreactive protein in different grass species, detected by immunoblotting. The same antibody was also useful for immunopurification of Dac g 4. Leduc-Brodard *et al* [25], used a pair of MoAbs for a two-site ELISA and found a high specificity for Dac g 4. However, an exact quantification of Dac g 4 analogs was not possible with this approach.

We performed epitope mapping with our MoAbs (Fig. 5) and established that all three MoAb recognized unrelated regions on Phl p 4. These studies were useful to identify an optimal combination for a two-site binding assay. The best results were obtained with MoAb 5H1 immobilized on the solid phase and biotinylated 3C4 as detection antibody. The standard curve with purified Phl p 4 showed a sensitivity in the range from 150 to 2000 ng/mL. The binding curves for two different *Phleum pratense* extracts were parallel to that obtained with the purified Phl p 4 preparation, suggesting that the allergen had not been antigenically altered during the purification procedure. Assaying purified group 1 (Phl p 1 and Lol p 1) or group 5 (r Phl p 5a and 5b, n Phl p 5a and 5b, or n Phl p 5) with the Phl p 4 assay elicited values <0.5% of the true values for Phl p 5 thus testifying to the high degree of Phl p 4 specificity of our two-site binding assay. These results demonstrate that the method is sensitive and specific for Phl p 4, and that it seems to recognize equally well the purified allergen and the same molecule in the complex extract mixture. Likewise parallel binding curves were observed with extracts of the different grass pollens (results not shown), but the percentages of group 4 relative to the total protein content were very different.

In summary, this paper presents evidence that group 4 homologues are present in the various grass extracts. Furthermore, the group 4 ELISA could be very useful together with group 5 [9] and group 1 ELISAs as a tool for providing information concerning the absolute composition of grass pollen extracts.

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