

Subtribe-specific monoclonal antibodies to *Lolium perenne*

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Summary

Background and objectives The ability to measure personal exposure to airborne grass pollen is important in the understanding of allergic diseases. Visual identification is time consuming and it is difficult to distinguish between many grass pollens morphologically. Although grass pollens share common allergenic determinants, we attempted to produce monoclonal antibodies that would distinguish between species, tribes and subfamilies of grasses which would allow immunodetection of pollens.

Methods Monoclonal antibodies raised against *Lolium perenne* were screened for specificity against an extended panel of grass pollen extracts using standard ELISA techniques and a novel particle blotting assay using whole pollen grains.

Results Antibodies showing specificity ranging from subfamily to part-tribe specificity were raised. The most specific monoclonal antibodies (numbers 4, 13 and 17) had reactivity to *Lolium perenne* and *Festuca elatior* but displayed little cross-reactivity to *Phalaris arundinaceae* and the rest of the Poeae tribe when tested by ELISA and no detectable cross-reaction when tested with particle blotting.

Conclusion Monoclonal antibodies that are functionally specific to only two grasses can be produced and used to discriminate between related grass species.

Keywords: grass pollen, immunodetection, monoclonal antibodies, particle blotting

Clinical and Experimental Allergy, Vol. 29, pp. 973–981. Submitted 18 March 1998; revised 13 May 1998; accepted 18 January 1999.

Introduction

Allergy to grass pollen is a common and important worldwide cause of morbidity and economic cost, with an increasing number of grass species being recognized as allergenic. Traditionally pollen exposure has been measured by counting the number of grass pollen grains without any discrimination between grasses. Indeed many grass pollens are morphologically very similar. All grasses, however, are not of equal allergenicity nor produce equally severe symptoms. Different localities may also have a predominance of different grass species. To be able to identify grass pollens more precisely would be of practical and scientific interest. We explored whether this could be achieved using monoclonal antibodies (MoAbs).

Most monoclonal antibodies to pollen allergens reported to date have a degree of cross-reactivity, some react to other grasses [1–5], and others even react to non-grasses [6–12].

The only MoAb without cross-reactivity reported so far inhibits the binding of IgE [13–16], and cannot be used in assays where MoAb and IgE is supposed to bind at the same time.

Similarly patients with grass pollen allergy will often react to pollen from a number of grass species, and IgE from these patients will bind to pollen proteins from the causative agent, as well as to other grasses. Taken together these results suggest a number of shared pollen epitopes. We adopted a strategy of screening a panel of monoclonal antibodies, to see if specific MoAbs could be produced. This report describes the development and testing of these MoAbs to a partially purified *Lolium perenne* pollen extract. In testing the specificity of the MoAbs, four different ELISA reactions and a functional particle blotting assay using whole pollen grains were used. We were able to identify MoAbs specific at a subtribe level.

Materials and methods

Monoclonal antibodies were raised to partially purified extract of *Lolium perenne* pollen and screened against a

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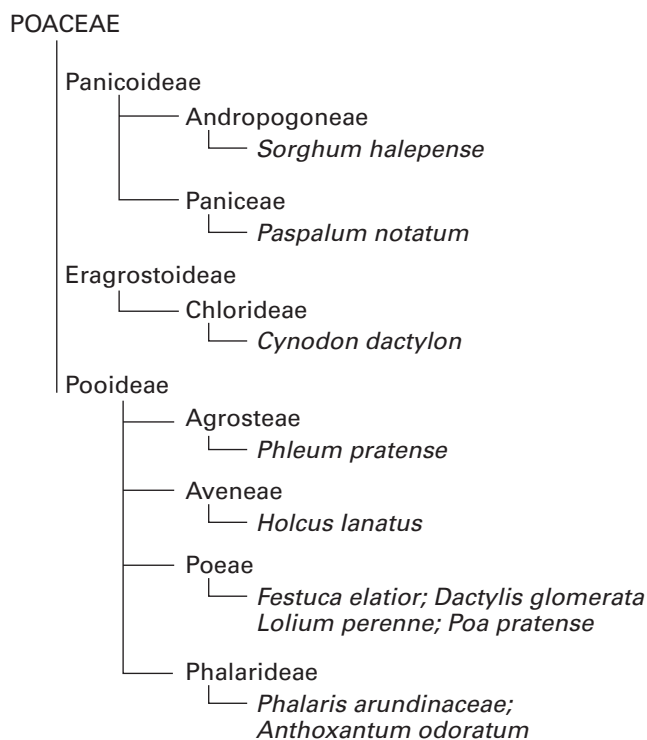


Fig. 1. Grasses in this study and their family relations [20]. Phylogenetic hierarchy is: Family; Sub-family; tribe; *species* (Genus has been omitted for simplicity). All MoAbs in this study react to grasses of the Poideae subfamily, the most specific only to part of the Poeae tribe.

number of related grass pollens. The pollens tested in this study were: *Sorghum halepense* (Johnson grass), *Paspalum notatum* (Bahia grass), *Cynodon dactylon* (Bermuda grass), *Phleum pratense* (Timothy grass), *Holcus lanatus* (velvet grass), *Festuca elatior* (fescue grass), *Dactylis glomerata* (orchard grass), *Lolium perenne* (rye grass), *Poa pratensis* (Kentucky bluegrass), *Phalaris arundinaceae* (canary grass, reed), *Anthoxanthum odouratum* (wweet vernal grass), the control was *Betula verrucosa* (also known as *Betula pendula* or Birch, a tree). The species' interrelations are shown in Fig. 1. All pollens were purchased from Greer Laboratories Inc. (Lenoir, NC, USA). Grass pollens were extracted by adding 1 g of pollen to 10 mL of 0.1 mol/L borate buffer (pH 8.2), with phenyl methyl sulphonyl fluoride (P 7626, Sigma Aldrich Chemical Co., Sydney, Australia), and incubating for 3 h at room temperature on an end-over-end mill. The mixtures were then centrifuged at 2000 *g* for 10 min and the supernatants containing the extracted proteins isolated for further work. The protein concentrations were measured using a protein assay (Bio-Rad 500–0006, BioRad, Sydney, Australia) with bovine serum albumin as standard. The *Lolium perenne* extract was partially purified

on a BioRad BioLogic chromatography system equipped with a 10-mL Bioscale column packed with High Q Anion exchange support (macro prep grade, BioRad). In the elution gradient buffer A was 10 mmol/L TRIS pH 8.8 and buffer B was 150 mmol/L TRIS pH 5.0. Fractions were tested with a pool of human sera in Immunoblot reaction. This was used to identify the molecular weight (M_w) of allergen bands and which of the fractions from the BioLogic purification contained allergens. The human sera pool was from patients with a RAST class 3 or 4 to grass pollen.

Monoclonal antibody production

Four mice strain BALB/c (Culas, Little Bay, Australia) were injected intraperitoneal with 30 μ g of the partially purified material per animal per injection. The first injection was in Freund's Complete Adjuvant, the second in Freund's Incomplete Adjuvant and the booster in phosphate-buffered saline (PBS). MoAbs were produced in cell culture, essentially using the method of Köhler and Milstein [17]. Briefly, NS 1 cells were used as fusion partners for spleen cells of immunized BALB/c mice. The developed cell clones were stabilized in three clonings by limiting dilution.

MoAbs were purified using BioRad BioLogic chromatography system equipped with a 5-mL Bioscale column packed with Prosep-A High Capacity (Protein A) gel from BioProcessing (Durham, UK). The pH of the cell culture supernatants was adjusted to 8.2, the rinse buffer was 0.1 mol/L K-Phosphate pH 8.2 and the elution buffer was 0.2 mol/L Citrate pH 3.0. MoAb activity was tested in supernatants or Protein A purified material with or without biotinylation. Biotinylation of MoAbs was performed essentially as described in Harlow and Lane [18]. Briefly, Biotin N-hydroxy succinimide reagent (Sigma H 1759) was dissolved in di-methyl sulphoxide (Sigma D 8779) and added to the MoAb solution in molecular ratio of between 50 : and 100 : 1. Isotyping of all developed MoAb supernatants were in ELISA using a mouse monoclonal antibody isotyping kit (Sigma ISO-2).

Monoclonal specificity

Testing of the specificity of the developed MoAbs was performed by four different ELISA's methodologies (antigen coating, double MoAb sandwich, MoAb allergen IgE sandwich and IgE inhibition) and by a novel whole-pollen particle-blotting technique. Finally, immunoblotting was used to determine the molecular weights of antigens recognized by selected MoAbs. The assay conditions were similar for all four ELISA methods used. This involved coating in PBS pH 7.2; blocking in PBS with 5% skim milk protein and washing three times in PBS with 0.05% Tween 20 between incubations. All incubations were conducted at

room temperature on an agitator. The dilution buffer used after blocking was PBS with 2% skim milk protein and 0.05% Tween 20. The indicator substrate for the peroxidase-labelled secondary antibodies was OPD/H₂O₂ (Sigma P1526 and H1009) which was stopped using 2.5 mol/L H₂SO₄. All reactions were read in a BioRad Microplate reader model 550 connected to a PC. Data was processed in Excel version 7 or Lotus 1-2-3 version 4.01 both for Windows 95.

Antigen coating ELISA was conducted to determine the binding activity of the MoAbs against pollen antigens coated onto ELISA wells. Standardized concentrations (15 µg/mL) of the 11 different grass pollen extracts were used to coat 11 columns in each microtitre test plate (NUNC, via Medos, Sydney, Australia). As a control, column 12 was coated with the same concentration of *Betula verrucosa* extract. Each MoAb was tested at seven dilutions, starting at 1:100, in fivefold dilution series, and incubated for 2 h. Finally, peroxidase-conjugated rabbit antimouse antibodies (Sigma A3673), diluted 1:500, was added and incubated for 1 h.

Double MoAb Sandwich analysis was conducted to determine the best possible sandwich combination of MoAbs. Wells in microtitre test plate columns were coated separately with each type of MoAb at 10 µg/mL. After blocking, all wells were incubated with *Lolium perenne* extract at 2 µg/mL. After washing, the wells were incubated in a checkerboard pattern with one biotinylated MoAb tested per row of the test plate at 10 µg/mL. Finally, peroxidase-conjugated streptavidin (SAH, Silenus, Vic., Australia), diluted 1:500, was added and the colour of the OPD substrate reaction measured. All combinations of MoAbs were used as capture and detection antibodies. In parallel, the background levels were measured in identical combinations, but with the dilution buffer as replacement for the *Lolium perenne*. In the calculation of results, the individual background levels were subtracted from the corresponding reaction responses. All data were normalized for each biotinylated MoAb, i.e. the maximal response for any combination was set at 100 and the responses from all other combinations in that series are calculated as percentage of this highest response. As reference the maximal responses are listed.

MoAb allergen IgE sandwich assay was conducted to determine whether MoAb and IgE bind to the same or different molecules. This used human IgE from an atopic human sera pool (RAST classes of 3 or 4 to grasses). Test plate wells were coated with MoAbs at 10 µg/mL; blocked and incubated with *Lolium perenne* extract at 2 µg/mL; followed by human IgE. Finally peroxidase-conjugated goat antihuman IgE (Kierkegaard-Perry Labs 074-1004, Bio-Medix, Doncaster, Vic. Australia) was added.

IgE inhibition assay was conducted to determine whether selected MoAbs and human IgE bind to identical or

overlapping epitopes. Test plate wells were coated with *Lolium perenne* extract at 10 µg/mL, followed by an incubation with human atopic sera diluted at 1:5. This binds all recognized epitopes in the *Lolium perenne* extract. The control incubation consisted of dilution buffer only. MoAbs starting at 1:100 and in twofold dilutions were added to bind to any unbound epitopes in the *Lolium perenne* coating. Finally, peroxidase-conjugated rabbit antimouse (Sigma, A3673) diluted 1:500 was bound to the MoAbs. In the calculations, control values were compared with inhibited values at the same dilution of MoAb.

The latter two assays were performed to identify MoAbs that would enable simultaneous binding of MoAb and human IgE with little or no inhibition. This was important as later use of these MoAbs in particle blotting will be for dual detection of (a) patients' allergic responsiveness, and (b) origin of the particle.

Particle blotting. Pollen particles from the 11 different grasses and the *Betula verrucosa* control pollen were trapped on a transparent adhesive tape and overlaid with PVDF membrane (Millipore, Sydney, Australia) [19]. This 'sandwich' was wetted in 50% ethanol for a few seconds and rinsed with three changes of MilliQ water and finally transferred to PBS where it was incubated for 3 h at room temperature on an agitation platform. Proteins eluted from the pollen bound to the membrane in the vicinity of the pollen. Remaining binding spots on the membrane were blocked in PBS with 5% skim milk proteins. The binding of selected MoAbs (4, 9, 13, 16 and 17) to grass pollens and the *Betula verrucosa* control pollen was tested, followed by alkaline phosphatase-conjugated antimouse antibodies (Sigma A3438), and BCIP/NBT (Sigma B8503 and N6876) as an indicator substrate. Purple spots developed around the pollen in a ring or halo if the MoAb had bound to eluted proteins and the results were graded (0-XXXX) depending on the intensity of the halo.

Immunoblotting was performed using three selected MoAbs in individual tests to an electrophoresed and blotted crude extract of *Lolium perenne*. The crude extract was electrophoretically separated in SDS-PAGE 10% precast gels using a Xcell II tank (Novex, Amrad, Sydney, Australia). Proteins were transferred to 0.45 µm nitrocellulose sheets using a blotting cassette fitted into the Xcell tank. Transferred bands were reacted with one out of three selected MoAbs for identification of the molecular weights of recognized bands.

Results

Partial purification of Lolium perenne

Ion exchange purification of the *Lolium perenne* extract produced fractions containing four protein bands identified

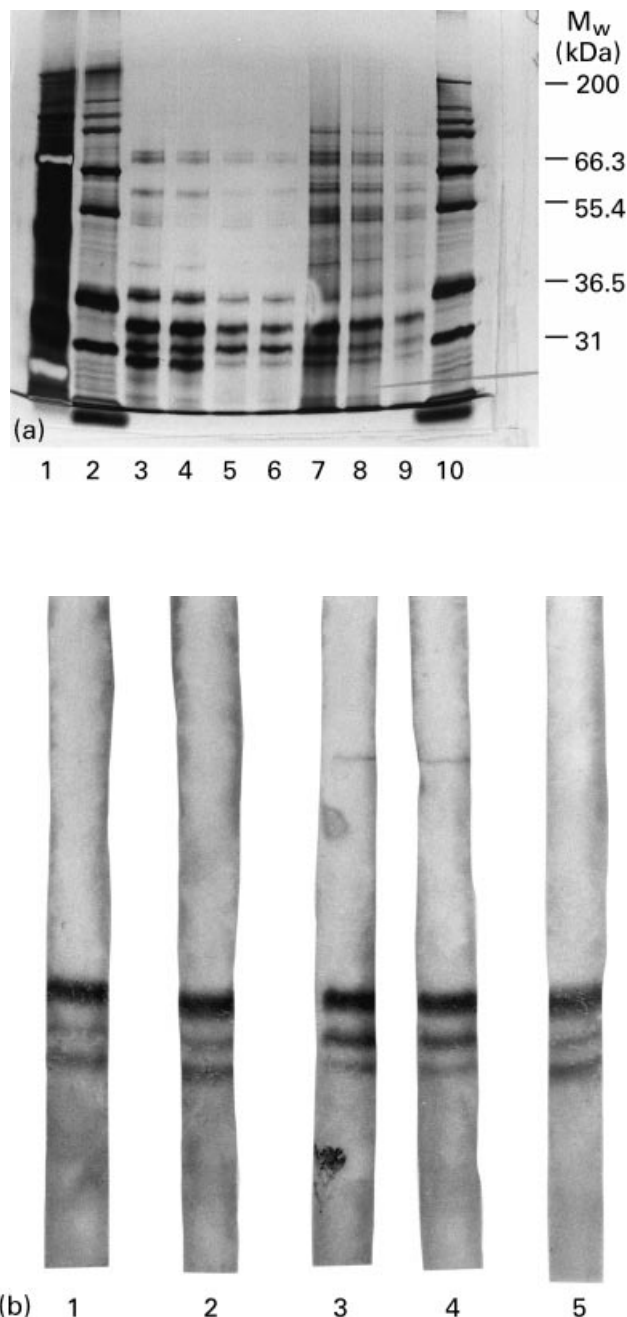


Fig. 2. (a) Purification of *Lolium perenne* allergen. Silver-stained 10% SDS-gel of *Lolium perenne* purifications. Lane 1: BioRad broad range markers (overloaded); lanes 2 & 10: Novex Mark 12 wide range protein standards; lanes 3–6: fractions from ion exchange purification; lanes 7–9: crude *Lolium perenne* extract (different load volumes). M_w of selected marker molecules indicated to the right. (b) Western blotting of isolated fractions from ion exchange purification of *Lolium perenne* extract tested with atopic human serum. Three of the four bands identified in silver staining are recognized by atopic serum where human IgE recognize identical bands in all selected fractions. These fractions were later used for immunization of mice.

by silver staining of SDS gels. These selected fractions eluted at 14% buffer B, corresponding with pH 7.6–7.7. Three of the four bands were recognized by human atopic sera in immunoblotting. These had molecular weights of ≈ 33 , 30 and 28 kDa (see Fig. 2a,b). The non-recognized fourth band at 37 kDa was not as intensely stained and was later found not to be recognized by developed MoAbs.

Monoclonal antibody production

The antibody titre in mouse sera were high after only two immunizations. It was decided to use the spleen from the strongest responding mouse after a subsequent final booster injection. The low number of immunizations was assumed to give the best chance of a high diversity of the antibody repertoire. From the fusion of spleen cells and NS1 myeloma cells, we were able to identify 19 MoAbs, 13 of which were stabilized and grown to 0.5 L scale. Protein A purification of the MoAbs gave consistent single peaks. Each peak eluted when the pH was 4–5, which is typical for mouse IgG1, and was confirmed by isotype analysis.

Monoclonal antibody specificity

Antigen coating ELISA. Each MoAb was tested in serial dilution, the first dilution which did not give a maximal response was selected as having the best discrimination. Responses at this dilution towards all test extracts are reported in Table 1. In general reactivity was only seen to grasses from the Pooideae, with no cross-reactivity to Panicoideae nor Eragostoideae subfamilies [20] (Fig. 1). There was no reaction to the *Betula verrucosa* control pollen. The MoAbs could be divided into three groups: MoAbs 4, 13 and 17 were the most specific to *Lolium perenne* and *Festuca elatior* both from the Poeae tribe with minor reactions to the Phalarideae tribe; MoAbs 3 and 6 reacted to Poeae and Agrostae and a little to Aveneae; and the remaining MoAbs reacted with broad specificity to more or all Pooideae grasses tested.

Double MoAb sandwich ELISA. In nine out of the 13 best combinations the coating MoAb was MoAb 9. Only combinations of MoAb 9 with biotinylated MoAb 6 or 9 (itself) gave low responses. MoAbs 3 and 17 did not coat very well (Table 2). The combinations which gave the most selective assays were either MoAb 4 combined with the biotinylated products of MoAb 13 or 17, or MoAb 13 combined with the biotinylated products of MoAb 4 or 17. The best combination for a general sandwich ELISA assay recognizing the Pooideae subfamily used MoAb 9 as capture antibody combined with biotinylated MoAbs 15 or 16 as detector antibody. The best combinations for a selective sandwich ELISA used the pan-specific MoAb 9 as capture antibody

Table 1. Antigen coating ELISA. Reactions listed are in milli absorbance units (mAbs) after subtraction of background. Each line gives the read-out value of the reactions of one MoAb to all tested allergens. A high absorbance indicates a strong reaction between the MoAb and this particular allergen, whereas a low or missing absorbance indicates that this MoAb is binding weakly or not at all to this allergen. In general we only find reactions to the Pooideae subfamily, with some MoAbs reacting only to part of these

Subfamily	Panicoideae		Eragrostoideae	Pooideae								Control:
Tribe	Andropo- goneae	Paniceae	Chlorideae	Agrosteae	Aveneae	Poeae				Phalarideae		Fagales
Species	Sor h	Pas n	Cyn d	Phl p	Hol l	Fes e	Dac g	Lol p	Poa p	Pha a	Ant o	Bet v
MoAb 2 a Lol p	0	0	0	725	443	807	1583	1511	906	1221	320	6
MoAb 3 a Lol p	4	21	11	388	288	727	476	1392	490	401	194	67
MoAb 4 a Lol p	0	3	0	2	14	344	9	1423	4	23	0	4
MoAb 5 a Lol p	0	5	0	1551	459	1714	2295	2115	1821	2162	22	3
MoAb 6 a Lol p	5	0	15	553	5	172	373	1242	53	21	186	0
MoAb 8 a Lol p	3	6	0	171	307	677	111	898	261	79	9	3
MoAb 9 a Lol p	0	0	0	1796	1690	1732	1699	1933	1656	1920	1162	0
MoAb 10 a Lol p	3	6	2	1405	462	1357	350	1592	697	431	108	5
MoAb 13 a Lol p	5	33	7	25	29	436	66	1260	14	146	10	10
MoAb 15 a Lol p	0	3	0	1635	975	1754	1599	1854	1760	1843	358	8
MoAb 16 a Lol p	7	10	2	1852	873	1912	1364	2080	1980	1932	139	10
MoAb 17 a Lol p	0	23	19	98	84	593	327	1500	176	268	117	10
MoAb 19 a Lol p	0	6	0	951	539	1101	1521	1134	1173	1539	261	5

Table 2. Double MoAb sandwich analysis. All data calculated as percentage of maximal response with this particular biotinylated MoAb. Data can be compared to find the best combination of coating MoAb and biotinylated MoAb. We found that MoAb 3 and MoAb 17 are not efficient for coating, and that certain combinations will not work

Coating Ab no.	Biotinylated Ab no.													@ Max response
	2	3	4	5	6	8	9	10	13	15	16	17	19	
2	72	100	76	97	20	79	14	89	99	100	97	78	96	3.247
3	0	16	0	0	1	2	2	1	1	1	0	1	1	0.227
4	84	43	14	55	79	29	89	26	37	91	62	49	69	2.942
5	65	1	20	3	41	1	93	1	16	13	3	7	20	3.038
6	60	76	78	93	3	69	1	80	94	98	89	76	84	3.137
8	84	46	21	49	65	26	71	8	36	69	42	89	47	2.323
9	77	83	100	100	3	100	5	100	100	100	100	100	100	3.341
10	98	58	65	71	93	54	92	4	93	90	68	92	80	2.998
13	100	45	16	67	100	34	96	40	52	94	70	64	80	3.139
15	80	0	32	2	47	0	100	12	26	9	2	0	19	3.267
16	85	0	23	2	52	0	97	2	16	11	2	1	25	3.182
17	9	5	1	2	6	1	8	0	3	3	2	4	2	0.259
19	81	4	34	4	43	3	97	2	28	14	4	0	14	3.162

With very high responses the maximal response was found by measuring half of the converted substrate and multiplying this result by ≈ 2 .

and one of the three most specific biotinylated MoAbs 4, 13 or 17 as detector antibody.

MoAb allergen IgE sandwich ELISA. All combinations gave medium to strong signals (Table 3).

IgE inhibition ELISA. The binding of MoAbs 4, 9, 13, 15, 16, and 17 were tested for inhibition by human IgE. There seemed to be some degree of inhibition, although in no case above 17%. Inhibition was most pronounced for MoAb 16, followed by 15 and 9, whereas MoAb 4 was not inhibited at all. MoAbs 13 and 17 had little or no inhibition.

Particle blotting. Data from the particle blotting were consistent with those obtained in antigen coating ELISA. Reactions were tested with MoAbs 4, 9, 13, 16 and 17 (Table 4). MoAb 9 gave strong responses towards the same grasses in this and ELISA assays. MoAbs 4, 13, and 17 all gave strong particle blotting reactions corresponding with strong ELISA reactions whereas there was no particle blotting response with *Phalaris arundinaceae* even though there was a weak response in ELISA. MoAb 16 also showed strong particle blotting reactions corresponding with the previously found strong ELISA response, whereas weak particle blotting reactions were seen to *Phleum pratense*, *Holcus lanatus*, and *Dactylis glomerata*, even though there was a medium ELISA response. Particle blotting using MoAb 13 gave halo formation around *Lolium perenne* pollen grains (Fig. 3).

Immunoblotting. MoAbs 4, 13, and 17 were tested in reactions towards the crude *Lolium perenne* extract. All

were found to react to two bands with molecular weights of 28 and 30 kDa. This corresponded with two of three bands seen in Western blotting previously using purified material and human atopic sera pool. All three MoAbs also have a

Table 3. MoAb allergen IgE sandwich assay. Coated MoAb binds allergen that is then tested with human IgE, in this case extract from *Lolium perenne* was tested as the allergen. All MoAbs' responses are well above background, indicating that all MoAbs recognize an allergen molecule that is also recognized by human IgE

MoAb number	Response – background
2	2733
3	649
4	2230
5	1987
6	2767
8	1288
9	2729
10	1888
13	2242
15	2301
16	2026
17	416
19	1964

Table 4. Whole particle blotting. '0' indicates no reaction; 'X' indicates a weak reaction and 'XXXX' indicates a strong reaction, with all or nearly all particles having strong halo formation. These data are comparable with ELISA data in Table 1, where we find correlation with strong reactions. Medium to weak ELISA reactions are weak to non-recognizable in particle blotting.

Subfamily	Panicoideae			Eragrostoideae			Pooideae						Control:	
	Andropogoneae		Panicaceae	Chloridoideae	Agrostaceae	Aveneae	Poeae			Phalarideae			Fagales	
	Sor h	Pas n	Cyn d	Phl p	Hol l	Fes e	Dac g	Lol p	Poa p	Pha a	Ant o	Bet v		
MoAb 4 a Lol p	0	0	0	0	0	XX	0	XXXX	0	0	0	0		
MoAb 9 a Lol p	0	0	0	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	XXXX	0		
MoAb 13 a Lol p	0	0	0	0	0	XX	0	XXXX	0	0	0	0		
MoAb 16 a Lol p	0	0	0	X	X	XXX	X	XXXX	XXX	XXX	0	0		
MoAb 17 a Lol p	0	0	0	0	0	XXX	0	XXXX	0	0	0	0		

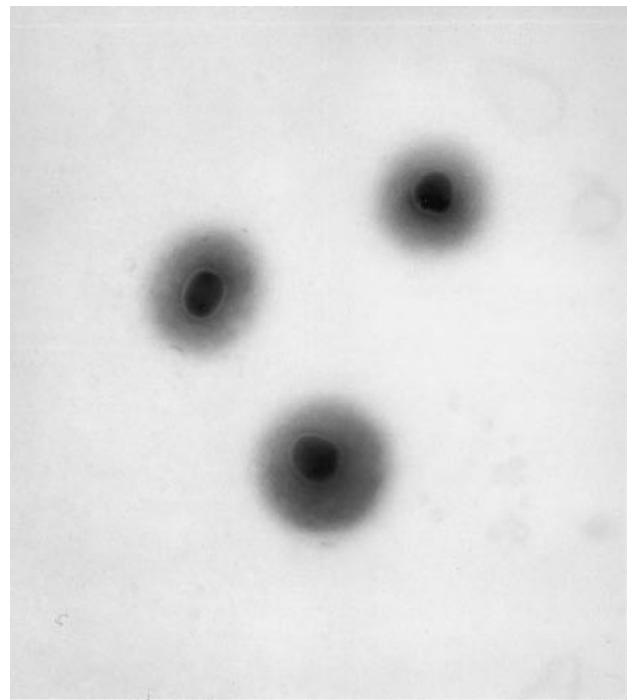


Fig. 3. Particle blotting of *Lolium perenne* showing precipitation of enzyme-converted substrate around the pollen particles. The precipitate forms a ring or a halo around the pollen particles making identification of the recognized pollen grains very easy in the microscope. The reactions may even be seen as small dots by direct observation by eye. The specificity of MoAb will determine which pollens are recognized, in this case MoAb 13 was used to recognize pollen from *Lolium perenne* and/or *Festuca elatior*.

weaker reaction to a band with molecular weight 60 kDa, and none to the 37KDa band (Fig. 4).

Discussion

We have been able to produce monoclonal antibodies which, while not species specific, differentiate between pollens at the subtribe level. In addition we have developed a functional whole-pollen blotting technique which can use these antibodies to differentiate pollen grains from different species.

The extraction of pollen from different grasses, using the same technique, gave widely different concentrations of protein. This observation may help explain the different allergenicity of grasses. Three out of four proteins from purified fractions of *Lolium perenne* extracts were recognized by allergic sera, and presumed to contain allergens. As discussed by Han *et al.* [13,14] and Chang *et al.* [15,16], these could be isotypes of an allergen or degradation products from this allergen. The molecular weights match those of Lol p 1 (27–34) [21–23] and Lol p 5 (28–32)

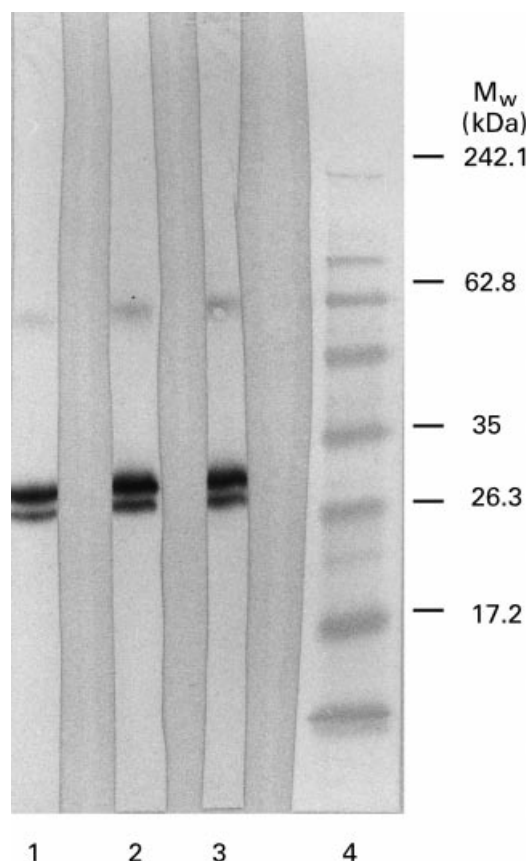


Fig. 4. Immunoblotting of crude Lol p extract probed with anti Lol p MoAbs. Lane 1: MoAb 17; lane 2: MoAb 13; lane 3: MoAb 4; lane 4: Novex SeeBlue prestained standards. All MoAbs recognize two identical bands strongly (30 kDa and 28 kDa) with a weak response to a heavier band ($\approx$ 60 kDa).

[6,8,9]. The pH of the elution buffer (7.6–7.7) match the pI of Lol p 5 (pH 6–8) [6,8,9], whereas that of Lol p 1 is lower (pH 5.5–7) [2,4,5], suggesting that the selected fraction from purification contains Lol p 5. Since most of the protein fraction contained antigenic material it was decided to use this for mouse immunization. The murine antibody response after only two injections was adequate for fusion, which was desirable since this early response was assumed to give MoAbs to the highest number of epitopes. This included potential non-shared epitopes that may only be represented in one or a few species of grasses and to which the antibody response may disappear during prolonged immunization. We stabilized 13 cell lines, all secreting MoAbs to grass pollen allergen. The MoAbs of the 13 cell lines could be divided into three groups according to their specificity, which ranged from subfamily specific to less than tribe specific. We did not find any that reacted to all tested grasses, indicating the family distance between Pooideae and non-Pooideae grasses. The non-Pooideae species listed

may be considered as being of more tropical nature than those belonging to Pooideae (except for the cosmopolitan *Poa pratensis*). The two groups of most interest were the subfamily specific group (MoAbs 9, 15 and 16), with broad reactivity to Pooideae grasses in general, and the most specific group (MoAbs 4, 13 and 17), which only react to two of the four Poeae tribe grasses tested, which would selectively identify *Lolium perenne* and *Festuca elatior*. Potentially, a general Pooideae- (subfamily) detecting ELISA sandwich assay would utilize MoAb 9 and biotinylated MoAb 16 as capture and detection antibodies, respectively. A more specific *Lolium perenne*- and *Festuca elatior*-detecting ELISA would utilize MoAb 9 and biotinylated MoAbs 4, 13 or 17 as capture and detection antibodies, respectively.

Particle blotting of whole pollens gave essentially the same responses as those found in antigen coating ELISA reactions. This was not unexpected as both methods use antigen coating, although to different surfaces. In general, a medium to strong ELISA reaction gave a similar signal in particle blotting experiments, whereas the weakest ELISA reactions did not show any response in particle blotting. This difference in sensitivities probably originates in the different measuring techniques. The ELISA response is measured photometrically as an absorbance reading in optical density units, with linear readings up to 2–2.5 units and with good discrimination between subtle differences of colour intensities. Conversely, particle blotting relies on microscopic inspection by an observer, and as a method is unable to discriminate between subtle differences in intensity, nor is it as sensitive. Despite this, the method of elution of a whole pollen grain on a wet membrane is more akin to what actually occurs on the mucous membrane of an allergic individual. MoAbs and human IgE can bind to the same molecule with little or no interference and are assumed to bind to different epitopes on the same molecule. This means dual recognition of an allergen is possible. It should therefore be possible to use the particle blotting first for detection of allergenicity using human sera reactions and then use MoAbs to determine which species the allergenic particle originated from.

The aim of this study was to produce MoAbs that may be used in a functional assay for discriminating between grass pollen grains of different origin. Immunoblotting of crude *Lolium perenne* extract with the three most selective MoAbs, identified bands with M_w corresponding with those previously found in immunoblotting of selected fractions from ion exchange purification of *Lolium perenne* extract, the identity of bands was supported by positive reactions seen in the MoAb allergen IgE sandwich ELISA reactions. The M_w determined combined with the pH of the fractions suggest that this molecule is Lol p 5. In addition to these bands, there was a weaker reaction to a band with M_w

of ≈ 60 kDa, the nature of which will be investigated further.

In conclusion we found that it was possible to purify *Lolium perenne* allergen and produce MoAbs that were specific to a few closely related grasses. In a functional particle blotting assay these reacted only to *Lolium perenne* and *Festuca elatior*, giving us specific immunoprobes to identify pollen particles.

Acknowledgements

The interest and support of Dr Richard Baker is gratefully acknowledged. Dr Baker has supplied us with a large and good selection of human sera, without which this work could not have been completed. Valentina Razmovski developed the adhesive tape — PVDF sandwich technique. Dr Richard Sporik is thanked for critical review of the manuscript. This project was funded by the Allergen Diagnostic Project Syndicate.

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