

Grass Pollen Allergens: Antigenic Relationships Detected Using Monoclonal Antibodies and Dot Blotting Immunoassay

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Abstract. Monoclonal antibodies against major rye-grass pollen allergens have been used to detect cross-reactive determinants in other grass pollen extracts. Antibody binding was detected by the dot blotting immunoassay in which alkaline phosphatase-conjugated anti-mouse IgG was used as secondary antibody. Taxonomically ordered variations were found between pollen of 22 grass species representing all major natural groups. One of the antibodies, that bound to the 28 to 30Kd allergen, showed high species specificity; immunoblotting showed binding to similar polypeptides in *Festuca elatior* but to none of the other grasses tested. This simple assay has applications in standardizing grass pollen extracts.

Introduction

Monoclonal antibodies provide a powerful tool to analyse molecular relationships between plant proteins and glycoproteins [12, 13]. Such antibodies have now been prepared to the major source of spring and early summer hayfever and asthma in temperate climates, the airborne pollen of grasses [10, 18]. The antigenic proteins and glycoproteins of pollen of several grass species have been partially characterized [6, 8, 9, 16]. Four groups of allergenic proteins and glycoproteins have been isolated, differing in molecular weight, but each comprising 2 or more isoallergens of similar molecular weight. The pollen grains of related species consequently may contain unique as well as common antigens [11, 15]. Since allergic individuals may produce IgE antibodies to either one or both types of allergens, a patient may show a clearcut sensitivity to one single species or to several closely related species, due to the immunological cross-reactivity of the structurally related allergens [21].

Earlier studies with polyclonal antisera raised in rabbits suffered from problems of identity of allergenic and non-allergenic antigens [20] and of specificity [6, 7]. Also quantitation of individual antigens requires crossed rocket immunoelectrophoresis [14, 17], whereas with monoclonal antibodies the individual antigens may be quantified in crude extracts by

methods involving enzyme-linked immunoassays and dot blotting of pollen extracts on nitrocellulose paper [5].

In view of the clinical importance of rye-grass pollen allergens [16], monoclonal antibodies to these were used to assay-related antigens among grass pollens, to provide an alternative approach to use of the radioallergosorbent test (RAST) [1] and RAST inhibition studies [4]. The monoclonal antibodies used were developed and characterized by Smart et al. [18] in terms of immunoglobulin classes, molecular weight classes of antigens, and allergenicity of antigens as assayed with IgE-binding ELISA (table I). The aim of the present work has been to show that hybridoma-derived, allergen-specific monoclonal antibodies have the potential to serve as tools for the characterization of taxonomic relationships among grass pollen that should provide useful information for standardization of grass pollen extracts.

Materials and Methods

Cellulose nitrate membrane (BA 85, pore size 0.45 µm) was obtained from Schleicher and Shull, Inc. Monoclonal antibodies to rye-grass (*Lolium perenne*) pollen allergens were produced at the Flinders Medical Centre, Adelaide, Australia. The detailed protocol and antibody specificities have been published earlier by Smart et al. [18]. Alkaline phosphatase-conjugated anti-mouse IgG, bovine serum albumin (BSA), 5-bromo-4-chloro-3-indoxyl phos-

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3-indoxyl phosphate, and Nitro blue tetrazolium were from Sigma Chemical Company (St. Louis, Mo., USA). Polyoxyethylene sorbitan monolaurate (Tween 20) was obtained from Bio-Rad Laboratories, Richmond, USA.

Pollen grains of all the species used were obtained from Greer Laboratories (Lenoir, N.C., USA). The following were used:

Pooid (festucoid) grasses. Group 1: Triticinae: *Bromus inermis*, smooth brome; *Agropyron repens*, English couch; *A. cristatum*; *Secale cereale*, rye; group 2: Poanae: *Dactylis glomerata*, orchard grass or cocksfoot; *F. elatior*, meadow fescue; *L. perenne*, perennial rye-grass; *L. multiflorum*, Italian rye-grass; *Poa pratensis*, Kentucky blue-grass; *P. compressa*, flattened meadow grass; *Avena sativa*, oat; *Holcus lanatus*, velvet grass or Yorkshire fog; *Anthoxanthum odoratum*, sweet vernal grass; *Arrhenatherum elatius*, oat grass; *Agrostis alba*, red top; *Phleum pratense*, timothy; *Phalaris arundinacea*, reed canary grass.

Chloridoid grass: *Cynodon dactylon*, Bermuda grass, couch. *Panicoid* Grass, *Paspalum notatum*, Bahia grass. Andropogonoid grasses: *Sorghum halepensis*, Johnson grass; *Zea mays*, maize; *Typha latifolia*, bulrush.

As controls of antibody specificity, a number of non-grass pollens were tested: *Ambrosia elatior*, short ragweed; *Cosmos bipinnatus*; *Gladiolus gandavensis*, *Helianthus annuus*, sunflower; and *Populus alba*, white poplar.

50-mg pollen samples from all the species tested were extracted in 1 ml of 0.15 M NaCl in 0.05 M phosphate buffer, pH 7.5. For extraction the pollen was shaken for 3 h in above buffer. The pollen grains were then removed by centrifugation. The protein contents of the extracts were determined by Bio-Rad dye binding protein assay [3]. The volumes of the extracts were adjusted with PBS such that final protein concentration was 1.0 mg/ml⁻¹.

Dot Blotting of Extracts

Long strips, 270 mm long and 10 mm wide, were cut from nitrocellulose membrane. With a pencil 10 × 8 mm rectangular grids were drawn on strips. The membrane was then washed with water for 5 min by gentle agitation in distilled water and left at room temperature to dry. The membrane was handled throughout with forceps. Different pollen antigen extracts (1 µl each) were applied in the centre of rectangular grids. Twenty-one such long strips with antigen spots were prepared. After letting the spot dry for 1 h, the strips were soaked for 1 h in a solution of 3% BSA in phosphate-buffered saline (PBS, 0.05 M sodium phosphate, pH 7.2, and 0.9% sodium chloride) to block the remaining protein-binding sites. The strips were then washed with TAPS buffer (0.5% Tween 20 and 0.05% BSA in PBS), and then dried.

Detection of Monoclonal Antibody Binding

Step I. The strips were incubated with monoclonal antibody hybridoma supernatants (three strips each in individual supernatant) for 3 h at room temperature with gentle rocking. The incubation was followed by five washings with TAPS buffer. All the strips were then incubated with affinity-purified goat anti-mouse IgG-alkaline phosphatase conjugate (Sigma) diluted 1:1000 with 0.05% BSA in PBS, for 2 h.

Step II. After two washes with TAPS buffer and three washes with PBS, alkaline phosphatase positive spots were detected as described by Blake et al. [2]. For enzyme detection, the strips were once washed with 50 mM barbital buffer, pH 9.0 for 5 min. The reaction mixture comprised 200 µl of MgCl₂ (2 M solution), 10 ml

of Nitro BT (1 mg/ml in barbital buffer, pH 9.0), 1 ml of indoxyl phosphate (5 mg/ml 5-bromo-4-chloro-3-indoxyl phosphate in dimethylformamide) and 90 ml of barbital buffer (50 mM, pH 9.0). The mixture was equilibrated at 37°C and then added to nitrocellulose strips in a trough. After 5 min, the strips were rinsed with cold water and air-dried. After drying, the strips were stored in the dark.

For an alternative quantitative assay following step 1, the 10 × 8 mm pieces of nitrocellulose corresponding to individual antigens were cut and put into individual wells of a 96-well ELISA microtiter tray (Dynatech). The alkaline phosphatase activity was quantified by measurement of hydrolysis of *p*-nitrophenyl phosphate (Sigma). For reaction, 150 µl aliquots of *p*-nitrophenyl phosphate (1 mg/ml in 0.1 M carbonate buffer, pH 9.6) were added to each well. After 10 min, the nitrocellulose squares were removed and absorbance at 405 nm of each well was determined using a Titertek Multiskan ELISA plate reader (Flow Labs, McLean, Va. USA). The validity of the assay was checked by spotting multiple dilutions of rye-grass extracts, carrying through the whole procedure and plotting the absorbance (at 405 nm) values against amounts of protein spotted onto nitrocellulose membranes.

Immunoblotting of Antigens in Grass Pollen Extracts

The same grass extracts which were used for dot immunobinding were concentrated by freeze drying and were subjected to SDS-PAGE on 10–15% polyacrylamide gradient gels. Approximately 75 µg protein was loaded into each sample well. Transfer on nitrocellulose paper (S & S BA 85) was carried out at 25 mA for 20 h in 25 mM Tris-HCl/182 mM glycine/20% methanol, pH 8.3 at 15°C. After transfer half of the strip was washed in PBS for 30 min, incubated for 2 h with 3% BSA in PBS. The strips were then incubated in hybridoma supernatant (FMC A7) for 5 h at 25°C. Strips were then washed carefully with TAPS buffer for 30 min with changes, and incubated for 2 h at 25°C with goat anti-mouse IgG-peroxidase (Sigma) diluted 1:1,000 in PBS which also contained 0.5% BSA. After washing with PBS three times and then with TAPS buffer several times, the peroxidase substrate 4-Chloro-1-naphthol was added. The substrate composition was essentially similar as that described by Walsh et al. [19]. After incubation at room temperature in dark conditions for 10 min, the nitrocellulose paper was washed thoroughly in distilled water and allowed to dry; the blue-black colour remained stable for several days.

Results and Discussion

The dot immunobinding assay has been employed to test the cross-reactivity of pollen extracts from 22 species of grasses to 7 different monoclonal antibody preparations directed against allergenic components from rye-grass pollen (fig. 1).

FMC A5 antibodies recognized antigenic determinants in all festucoid genera that were tested (fig. 1, 2). Weak reactions were given with genera from other subfamilies, and no reaction with control pollens. Within the Poideae, results were heterogeneous, and weaker reactions were given by pollen of *Agropyron*, *Agrostis*, *Anthoxanthum*, *Avena* and *Phleum*. This al-

315+3

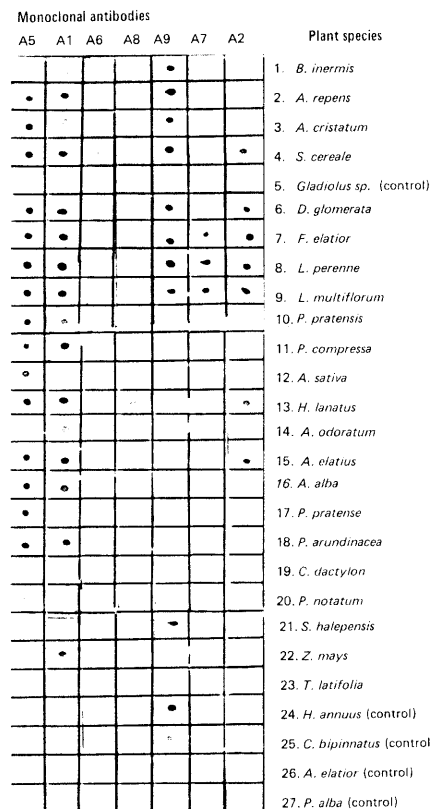


Fig. 1. Detection of antigenic relationships among grass pollen extracts by the dot immunobinding assay with specific monoclonal antibodies. The experimental protocol is described under Materials and Methods.

lergen has a high affinity for IgE, and may possibly be an isoallergen of group 1 allergen [18]. Monoclonal antibody FMC A1 gave a similar pattern of binding to FMC A5 (fig. 1, 2). Binding of this antibody to *Anthoxanthum* and *Zea* is greater than with FMC A5, while the reverse is observed with *Agropyron*. FMC A1 has apparent specificity for one or more isoallergens of group 1 allergen [18]. The molecular weights of the antigens are different (table I), and on this basis, Smart et al. [18] suggested that the 16Kd antigen

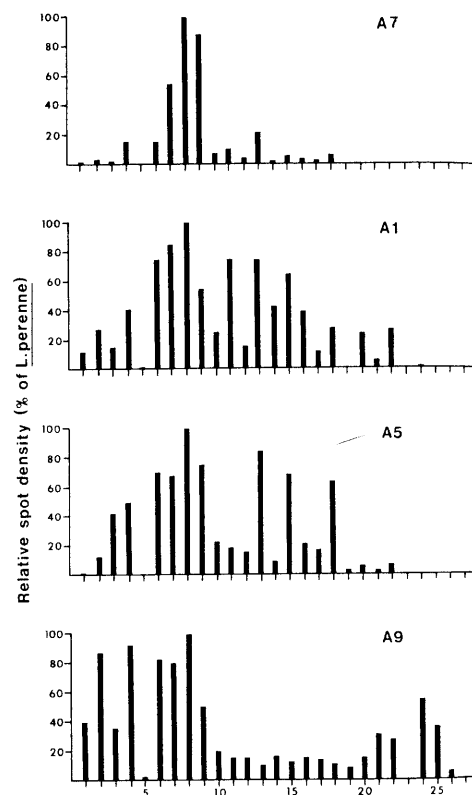


Fig. 2. Quantitative analysis of the binding of the specific monoclonal antibodies to the pollen extracts dot-blotted onto a nitrocellulose membrane. The serial numbers of plant species correspond to those in figure 1.

binding to A5 is not a determinant of group 1 allergen, but a different component. Recently Kahn and Marsh [10] reported results with group 1 allergen content in different grass genera, using specific monoclonal antibodies. No group 1 allergen was detected in pollen of *Cynodon*, results that are similar to those with FMC A1 in the present study. FMC A5 antibodies bound strongly to all festucoid grasses except for *Anthoxanthum* and *Phleum*. Lower levels of binding are present in the chloridoid, panicoid and andropo-

gonoid grasses tested. Monoclonal antibody FMC A2 binds to a 35Kd polypeptide which is an isoallergen of group 1 allergens and shows strong affinity for IgE. The distribution pattern of FMC A2-binding antigen in different grass genera is similar to that recognized by FMC A1.

FMC A6 and FMC A8 antibodies also bound to a range of festucoid genera (fig. 1, 2). On the basis of MW data, it has been suggested that these three antibodies may bind to one or more isoallergens of group 1 allergen (table I and Smart et al. [18]). The distribution of binding patterns certainly supports this interpretation.

FMC A9 antibodies are reported to bind to a number of different pollen components (table I). In our dot assay, this antibody bound to all the pollen extracts tested, with high levels in *Agropyron*, *Agrostis*, *Bromus*, *Festuca*, *Lolium*, *Poa*, *Paspalum* and *Sorghum*, and in the controls, *Ambrosia*, *Cosmos* and *Helianthus*. Low levels were found in *Typha*, and in the unrelated monocot, *Gladiolus*. A possible explanation for these findings is that the antibody is directed against a common carbohydrate sequence in these macromolecules.

FMC A7 bound to an allergen of MW 28 to 30Kd, which has a strong affinity for IgE. The binding pattern of A7 to the range of test antigens is quite distinct and specific in comparison with the other monoclonal antibodies. High levels of binding occur only to closely related genera in the Poanae, especially *L. perenne*, *L. multiflorum* and *F. elatior* (fig. 1, 2). The pollen extracts of other genera, including *Dactylis glomerata*, showed only very weak binding to this antibody. This appears to be a case where the antibody recognizes highly specific sequences common to only these two genera among those tested. Interestingly, those two genera are unusual among grasses in the closeness of their genetic relationships, which has permitted the creation of intergeneric hybrids. The ryegrass pollen component binding to monoclonal antibody FMC A7 was considered distinct from group 1 allergen [18]. This interpretation is now supported by the present findings with the dot blotting immunoassay patterns of 22 grass species. The FMC A7-binding antigen has a lower MW than group 1 allergen (table I), and its strong binding to IgE suggests that this component is a potent allergen. Its presence may explain why pollen of *Lolium* and *Festuca* are such major sources of allergens, in addition to their aerobiological frequency.

Table I. Nomenclature and specificity of the monoclonal antibodies used in this study¹

Preparation	MW (Kd)	IgE binding	Specificity
FMC A1	35	+	group 1 isoallergen
FMC A2	35	++	group 1 isoallergen
FMC A5	16	++	unrelated to group 1
FMC A6	32	+	group 1 allergen
FMC A7	28/30	++	distinct from group 1?
FMC A8	32	+	group 1 isoallergen
FMC A9	18, 47, 49, 71, 87	-?	cross-reacts with several glycoproteins

¹ Adapted from Smart et al. [18].

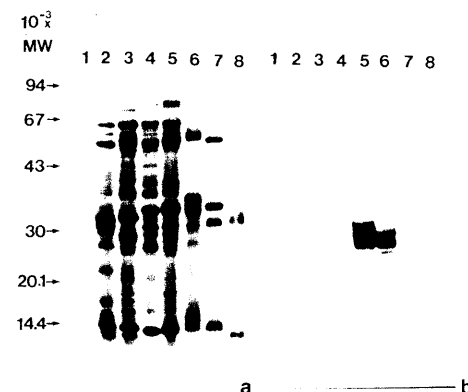


Fig. 3 a SDS-polyacrylamide gel electrophoresis of polypeptides in some grass pollen extracts. Lane 1, molecular weight reference markers 2-10; pollen extracts: 2, *B. inermis*; 3, *S. cereale*; 4, *D. glomerata*; 5, *F. elatior*; 6, *L. perenne*; 7, *P. compressa*; 8, *C. dactylon*. b Immunoblotting of grass pollen extracts with monoclonal antibody FMC A7. The duplicate gel corresponding to that in figure 3 a was blotted onto nitrocellulose and the paper was treated with hybridoma supernatant FMC A7 and stained by ELISA, as detailed in Materials and Methods.

Our immunoblotting experiments confirmed the results of Smart et al. [18] showing that FMC-A7 antibodies bind to antigenic determinants associated with 28 and 30Kd polypeptides of *L. perenne* pollen. In addition to ryegrass, we used other grass pollens for immunoblotting to look for molecular weights of

FMC A7 antibody-binding components in these species (fig. 3). The grasses tested included: *B. inermis*, *S. cereale*, *D. glomerata*, *F. elatior*, *P. compressa*, *Z. mays*, *T. latifolia*, and as a control, sunflower *H. annuus* pollen. Under our experimental conditions, only *L. perenne* and *F. elatior* showed the presence of polypeptides bound by monoclonal antibody FMC A7 (fig. 3). Only trace amounts were recorded from pollens of *D. glomerata* and *P. compressa*. Thus our immunoblotting results exactly correspond to those of the dot immunobinding assay. It is interesting to note that related taxonomic species not only show similarities in FMC A7-binding epitopes (antigenic determinants) but in the molecular weights of the polypeptides as well (fig. 3).

Our studies show that the dot blotting immunoassay can be used as a simple highly reliable assay for detection and quantitation of cross-reacting antigens and allergens from taxonomically related species. The assay can also be used for detection of IgE-binding to various allergenic molecules [19]. Given the availability of monoclonal antibodies precisely characterized for the major rye-grass pollen allergens, this assay provides a ready means for standardization of allergens in both pollen samples and extracts.

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