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Monoclonal Antibodies to Proteins from Cocksfoot Grass (*Dactylis glomerata*) Pollen: Isolation and N-Terminal Sequence of a Major Allergen

David J. Walsh^a, Jayne A. Matthews^a, Russell Denmeade^a, Paula Maxwell^a, Matthew Davidson^b, Matthew R. Walker^a

^a Department of Clinical Chemistry, University of Birmingham, Wolfson Research Laboratories, Queen Elizabeth Medical Centre, Birmingham; ^b Department of Biochemistry, University of Leicester, UK

Abstract. Monoclonal antibodies (Mabs) raised to an aqueous extract of cocksfoot grass (*Dactylis glomerata*) pollen have been characterised. Mab 1B9 was demonstrated by SDS-PAGE and Western blotting to recognise a major allergen of an approximate molecular weight of 28 kD in this extract, termed DG3, and a component with a molecular weight of between 35 and 40 kD in an extract of *Secale cereale* (cultivated rye) pollen. The 28 kD component of cocksfoot grass pollen isolated by affinity chromatography using Mab 1B9 was recognised by IgE antibodies in 80% (8 of 10) atopic sera, but only weakly by 25% (1 of 4) non-atopic sera tested. N-terminal sequencing of DG3 purified by affinity chromatography, 2 D electrophoresis and electroblotting to polyvinylidenedifluoride revealed significant homology with a group-V allergen (Phl p V) from timothy grass (*Phleum pratense*).

Introduction

Plant pollens are the most common cause of seasonal allergic rhinitis. The purification of allergic components from pollen extracts has been achieved by standard physicochemical separation techniques [1-5] and the use of immobilised monoclonal antibodies (Mabs) as immunosorbents [6-8]. The availability of allergens in pure form has facilitated the determination of several partial N-terminal sequences [9-11] and a number of complete protein sequences [12-14] by Edman degradation. In an alternative approach, the complete amino acid sequence of the major birch pollen allergen (Bet v I) has been inferred from the nucleotide sequence of a cDNA clone [15].

Comparison of amino acid sequences has revealed significant N-terminal homology between allergens from closely related species [16] and has shown that cross-reactive epitopes on Group-I grass pollen allergens are highly conserved [17]. Identification of sequence homology with other diverse groups of proteins has allowed speculation regarding the possible biological functions of some allergenic proteins [15]. In addition, sequence data have permitted theoretical

analysis of the epitopes recognised by T and B lymphocytes [18, 19] and the definitive localisation of these sites by epitope-mapping studies with synthetic peptides [20].

The pollen of *Dactylis glomerata* (cocksfoot grass) is a major causative agent of hayfever in Europe [21]. A number of allergenic components have been isolated from the pollen of this species [22, 23] although no sequence information has currently been reported. We have previously reported the purification of a major allergenic component, termed DG3, from cocksfoot pollen by chromatofocusing [24]. The present report describes the use of a Mab for the bulk isolation of DG3 and subsequent N-terminal amino acid sequencing of the affinity purified DG3.

Methods and Materials

Preparation of Pollen Extract

Cocksfoot grass pollen was obtained from Sigma Chemical Co. (Poole, Dorset, UK) and extracted with 0.01 M Tris-HCl, 0.15 M NaCl (pH 8.0) as previously described [24]. This extract was dialysed against 0.01 M sodium phosphate buffer (pH 6.4) and stored at -20°C.

Serum Samples

Serum samples were obtained from grass pollen allergic and non-allergic volunteers. Sera were assayed for IgE reactivity with crude aqueous extract of cocksfoot pollen by chemiluminescent ELISA according to the method of Stiles et al. [25].

Production and Purification of Mabs

Balb/c mice received 100 µg of crude pollen extract in Freund's complete adjuvant at 3-weekly intervals. Response titres were monitored by chemiluminescent ELISA. The highest titre animal was boosted intraperitoneally and intravenously with 50 µg of crude extract in phosphate-buffered saline (PBS) 3 days prior to fusion. Splenocytes were fused with NSO myeloma cells according to the method described by Lowe et al. [26]. Supernates from primary cultures were screened for reactivity with crude extract and DG3 by chemiluminescent ELISA on days 10, 13, and 20 after fusion. Reactive clones were expanded in vitro and ascitic fluid generated by growth in pristane-primed peritoneal cavities of Balb/c mice. Ascitic fluid was dialysed against 0.01 M sodium phosphate buffer (pH 7.0), centrifuged to remove debris, and fractionated by ion exchange chromatography on DEAE cellulose (Pharmacia) using stepwise increases in ionic strength according to the method of Goding [27]. Pollen-specific Mab-containing fractions were determined by ELISA and zone electrophoresis, pooled and dialysed against PBS before storage at -20°C.

Enzyme-Linked Immunosorbent Assay

ELISA was performed on black polystyrene Microfluor™ microtitre plates (Dynatech Laboratories, Billingham, UK). Plates were coated with 1 µg/well crude pollen extract in 0.1 M sodium carbonate buffer (pH 9.6) at 4°C overnight and washed with PBS containing 0.05% Tween 20 (PBST). Remaining binding sites were blocked for 30 min with PBS containing 1% bovine serum albumin (BSA). For hybridoma screening, 100 µl of culture supernatant was incubated per well for 90 min at room temperature, plates washed in PBST and incubated for 60 min at room temperature with 100 µl/well rabbit anti-mouse immunoglobulin conjugated to horseradish peroxidase (Dakopatts, Denmark) diluted 5,000 fold in PBST. Following washing in PBST, 150 µl of chemiluminescent substrate (1 mM sodium luminol, 3 mM hydrogen peroxide, 0.14 mM *p*-iodophenol in 0.1 M Tris-HCl, pH 8.6) was added to each well and light emission monitored in a plate luminometer (ML 100, Dynatech Laboratories). For assaying pollen-specific IgE antibodies in human sera, antigen-coated plates were incubated with 1:10 dilutions of serum in PBST for 90 min at room temperature and revealed using a 1,000 fold dilution in PBST of rabbit anti-human IgE-horseradish peroxidase conjugate followed by chemiluminescent substrate addition.

Preparation of Reverse Immunosorbent

DEAE-cellulose-purified Mab was coupled to Cyanogen bromide-activated sepharose 4 B (Pharmacia) using 10 mg protein/g of dry gel according to the manufacturers recommendations. The resultant gel was poured into a 30 × 1 cm glass column (Pharmacia) and taken through a 3 M potassium thiocyanate (CKNS) elution cycle followed by re-equilibration in double strength PBS (2 × PBS).

Affinity Purification of DG3

10 ml of crude pollen extract in 2 × PBS was applied to the column, cycled for 30 min and unbound material eluted with greater

than 2.5 column volumes of 2 × PBS. Weakly adsorbed material was eluted with 2.5 column volumes of 1 M CKNS and strongly bound material eluted with 2.5 bed volumes of 3 M CKNS. Protein-containing fractions (determined by UV monitoring at 280 nm) were pooled and extensively dialysed against 2 × PBS containing 0.02% sodium azide at 4°C prior to concentration using Centricon concentrators (Amicon) and assessment by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

Polyacrylamide Gel Electrophoresis

One-dimensional separation of proteins was performed in 15% cross-linked polyacrylamide gels in the presence of SDS according to the method of Weber and Osborn [28]. Samples were boiled in sample buffer (2% SDS, 3% sucrose, 5% 2-mercaptoethanol, 0.05% bromophenol blue in 50 mM Tris-HCl, pH 7.0) for 2 min, loaded onto the gel at 1–10 µg protein/lane and electrophoresed at 100 V in the buffer system of Laemmli [29]. Low molecular weight standards (Bio-Rad Laboratories) were used to determine molecular weights of sample components. Two-dimensional PAGE was performed as follows. Affinity purified DG3 was lyophilised and resuspended in 9 M urea containing 4% Nonidet P-40, 5% ampholine (pH 3.5–10; Pharmacia), 5% 2-mercaptoethanol and 2% 3-(cyclohexylamino)-1-propanesulphonic acid. Samples were electrophoresed in polyacrylamide isoelectric focussing gels (pH 3.5–10) for 1–2 h, removed and equilibrated against 0.125 M Tris-HCl (pH 6.8) containing 10% glycerol, 4% SDS, 5% 2-mercaptoethanol and 0.05% bromophenol blue. The tube gel was loaded onto the SDS-PAGE gel and electrophoresed as described above.

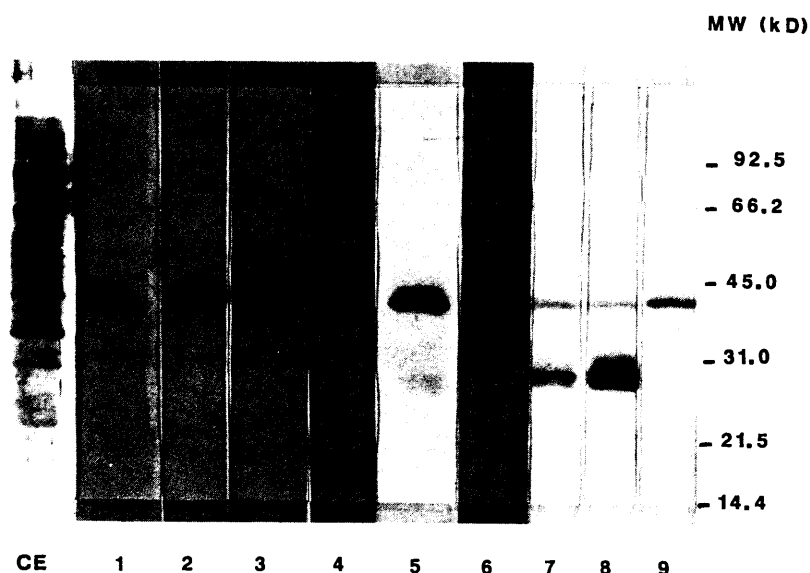
Western Blotting

Proteins separated in one dimension by SDS-PAGE were transferred to nitrocellulose using a method based on Towbin et al. [30]. Transferred proteins were visualised by staining with PBS containing 0.3% Tween 20 and 1 µl/ml Indian ink, according to the method described by Hangcock and Tsang [31]. Unreacted protein binding sites were blocked by overnight incubation in Tris-buffered saline (TBS: 20 mM Tris, 0.5 M NaCl, pH 7.5) containing 2% BSA to facilitate immunoprobings with enzyme-antibody conjugate or TBS containing 5% bovine haemoglobin for immunoprobings by radio-labelled second antibody. Proteins separated by two-dimensional electrophoresis were transferred to polyvinylidenedifluoride (PVDF; Millipore) and stained with Coomassie Blue according to the method of Matsudaira [32]. Electrophoretic transfer was performed for 3 h at 250 mA in transfer buffer in a Bio-Rad Trans-blot cell.

Immunoprobings of Western Blots

After blocking, nitrocellulose blots were incubated overnight at 4°C with Mab ascitic fluid diluted 1:500 with TBS containing 1% bovine haemoglobin. After extensive washing in TBS, the blots were incubated with 125 I-labelled sheep anti-mouse Ig [33] at 0.2 µCi/ml specific activity for 1–2 h at room temperature, washed in TBS extensively and air dried. Dried blots were exposed to X-ray film (Amersham) at -70°C for 24–72 h and developed according to manufacturers instructions. Recognition of transferred bands by human IgE antibodies was assessed as follows: BSA-blocked blots were incubated with atopic or non-atopic sera diluted 1:10 in TBS, 1% BSA overnight at 4°C. Following washing in TBS, blots were incubated for 1–2 h at room temperature with sheep anti-human IgE conjugated to alkaline phosphatase diluted 1:5000 in TBS/

Fig. 1. Western blot analysis of the specificity of cocksfoot pollen-specific Mabs. An aqueous extract of cocksfoot pollen was separated by SDS-PAGE, electroblotted onto nitrocellulose and probed with Mabs 4C10 (lane 1), 6D9 (lane 2), 5D5 (lane 3), 6G7 (lane 4), 2B5 (lane 5), 3D9 (lane 6), 2D8 (lane 7), 1B9 (lane 8), and ZG4 (lane 9). Bound antibody was revealed using ^{125} I-labelled SAM. CE = Pollen extract stained with amido black.



BSA. After washing in TBS, bound antibody was detected using 5-bromo-4-chloro-indolylphosphate/nitroblue tetrazolium substrate [34] and the reactions terminated by replacing substrate with stop solution (20 mM Tris-HCl, pH 8, 5 mM EDTA).

Amino Acid Sequencing

Regions of Coomassie-stained PVDF membrane containing protein bands or spots were excised with a scalpel and sequenced on an Applied Biosystems 470 A gas-phase sequencer.

Results

Specificity of Mabs

From a single fusion, 15 pollen-specific antibody-secreting clone wells were identified by chemiluminiscent ELISA on the basis of signals ≥ 50 times above NSO culture supernatant controls with crude pollen extract. Ouchterlony analysis of the ascitic fluid of the eight clones giving the highest signals in ELISA (data not shown) demonstrated that six clones (1B9, 2B5, 2D8, 3D9, 4C10, 6G7) secreted IgG₁ antibody whilst two (5D5, 6D9) secreted IgM antibody. Immunoprob- ing of nitrocellulose blots of SDS-PAGE-separated crude cocksfoot pollen extract demonstrated that all eight clones recognised a component of an approximate molecular weight of 40 kD (fig. 1). This component was also apparently recognised by Mab ZG4 [26] specific for human IgG₃ (fig. 1, lane 9) suggesting that it may be an 'immunoglobulin binding' protein or lectin. Mabs 2B5, 3D9, 2D8 and 1B9 also revealed a protein of approximately 28 kD with varying intensities. Optimisation of separation conditions

and reagent concentrations demonstrated that this apparent single band was composed of three protein bands of similar molecular weights, the highest intensity band being of intermediate mobility. This was consistent with the physicochemical characteristics of a highly allergenic component (termed DG3) previously isolated by chromatofocusing [24], i.e. 28 kD and pI 6.1. None of the Mabs were reactive with fusion proteins generated from cDNA clones expressed in *Escherichia coli* which have been demonstrated to encode allergenic proteins from cocksfoot pollen [35]. Immunoprob- ing of Western blots of pollen from a number of other plant species with Mab 1B9 revealed a component of 35–40 kD molecular weight extract of *Secale cereale* (cultivated rye) pollen (data not shown).

Physicochemical and Immuno Characterisation of Purified Allergens

SDS-PAGE analysis of specifically bound material eluted from the Mab 1B9 immunosorbent demonstrated it to consist primarily of the 28 kD triplet component (fig. 2, lane 2). The reactivity of human IgE antibodies in atopic and non-atopic sera with this material is shown in figure 3. Eighty percent (8 of 10) of sera from grass pollen-sensitive individuals contained IgE antibodies specific for the 28 kD protein whilst only one of four non-allergic sera showed reactivity with this component and at a much lower level.

N-Terminal amino acid sequencing of the major staining component of the 28 kD triplet following

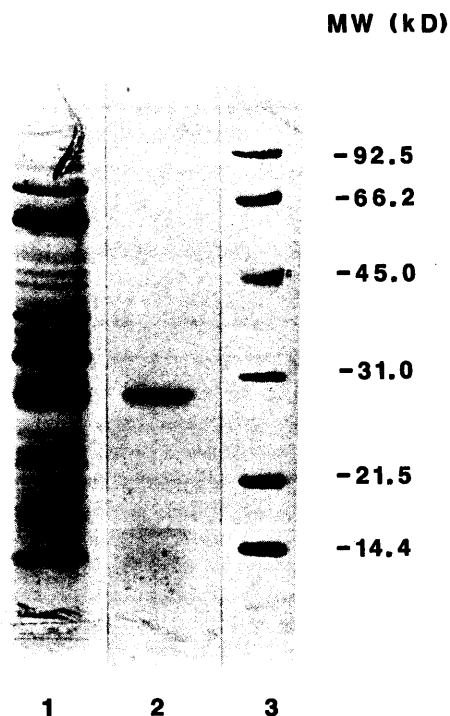


Fig. 2. SDS-PAGE analysis (12% cross-linked gel) of material eluted from Mab 1B9 affinity column. Lane 1 = Cocksfoot pollen aqueous extract; lane 2 = 3 M CKNS fraction; lane 3 = molecular weight markers (Bio-Rad).

one-dimensional SDS-PAGE and electroblotting to PVDF gave two or three different amino acid residues in the same cycle numerous times, indicative of the presence of two or three different protein species. Two-dimensional electrophoresis resolved the affinity product into five proteins of 28 kD with the two major staining components focusing at pI 6.1 (fig. 4). N-Terminal sequencing of components A and B following electroblotting to PVDF showed that their sequences overlapped identically (the first 13 residues of B with residues 9–21 of A) but with A having an additional eight residues at its N-terminus (tables 1, 2). Comparison with the N-terminal sequences of a number of other pollen allergens revealed significant homology between component A of DG3 and the *Phleum pratense* (Timothy grass) allergen Phl p V (table 1, bold face).

Discussion

In this study we demonstrate the use of a Mab, produced using an aqueous extract of cocksfoot grass pollen as immunogen, for the immunopurification of allergenic components directly from pollen extract.

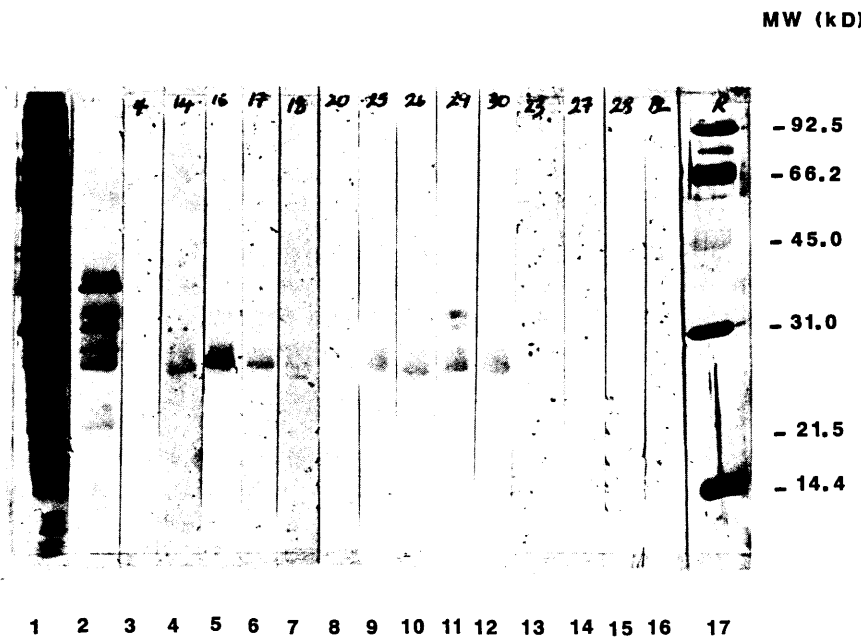


Fig. 3. Immunoblot analysis of reactivity of IgE in serum from grass-allergic (lanes 3–12) and non-allergic individuals (lanes 13–16) with Mab 1B9-purified DG3. Lane 1 = Pollen extract stained with amido black; lane 2 = pollen extract probed with atopic serum pool; lane 17 = molecular weight markers.

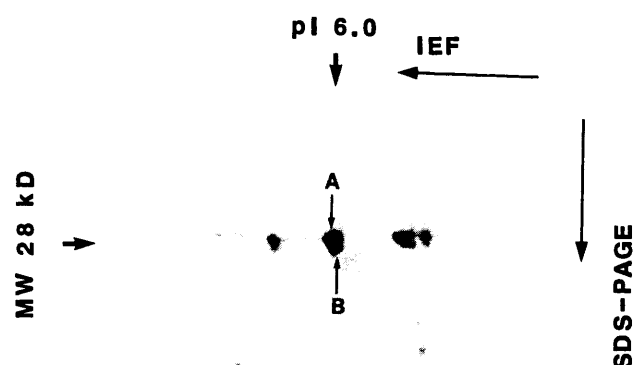


Fig. 4. Two-dimensional electrophoretic separation of Mab 1B9-purified DG3 transferred to PVDF membrane. Principal components (A and B) subjected to sequencing are indicated.

Table 1. N-Terminal amino acid sequences of a number of plant pollen allergens

Allergen	Amino acid residues																						
	1					10										20							
DG3 component A	G	Y	T	H	A	T	H	A	A	A	G	G	K	A	T	(T)	E	(E)	Q	K	L		
DG3 component B	A	A	G	G	(K)	A	T	(T)	E	(E)	Q	(K)	L	M	E	D	I	N	?	(A)			
Phl p V	A	N	L	G	Y	A	H	A	T	H	A	A	H	G	A	G	Y	T	H	A	T	H	
Lol p I	I	A	K	V	?	P	G	?	?	I	T	A	E	Y	G	D	K	W	L	D	A	A	
Cyn d I	A	M	G	D	K	P	G	P	?	I	T	A	T	Y	G	D	K	W	L	D	A		
Lol p II	A	A	P	V	E	F	T	V	E	K	G	S	D	E	K	N	L	A	L	S	I		
Lol p III	-	T	K	V	D	L	T	V	E	K	G	S	D	A	K	T	L	V	L	N	I		
Cry j I	D	N	P	I	D	S	?	W	R	G	D	S	N	W	A	Q	N	?	M	K			
Bet v I	M	G	V	F	N	Y	E	T	E	T	T	S	V	I	P	A	A	R	L	F	K		

- = Introduced for maximum homology; ? = no residue assigned; () = tentative assignment.

Table 2. Amino acid sequence homology of DG3 components A and B with Phl pV

	1					10					20					28									
DG3 component B						A	A	G	G	(K)	A	T	(T)	E	(E)	Q	(K)	L	M	E	D	I	N	?	A
DG3 component A	G	Y	T	H	A	T	H	A	A	A	G	G	K	A	T	(T)	E	(E)	Q	K	L				
Phl pV	G	Y	A	H	A	T	H	A	A	H	G	A	G	Y	T	<u>H</u>	<u>A</u>	<u>T</u>	<u>H</u>	<u>A</u>	<u>A</u>	<u>H</u>			

Repeated homology region in Phl pV underlined.

Of the eight Mabs selected on the basis of reactivity with cocksfoot pollen extract in ELISA, four were found to recognise a 28 kD component by immunoblot analysis. Mab 1B9 also recognised cross-reactive epitopes on a protein of 35–40 kD from the pollen of *S. cereale*. Utilising Mab 1B9 as an immunosorbent

to purify DG3 from pollen extract, the specifically bound material was shown by SDS-PAGE to be composed predominantly of the 28 kD component. This contrasts with the immunoaffinity purification of Dac g I by Mecheri et al. [22] which resulted in recovery of a much smaller product (6–8 kD) that retained some

allergenic activity. The 28 kD protein was strongly recognised by IgE antibodies in eight of ten grass pollen-allergic sera tested by immunoblot analysis, thus allowing it to be considered as a major allergen. This protein has the same physicochemical and immunochemical properties as the DG3 protein previously extracted by chromatofocusing [24].

The physical characteristics of DG 3 suggest that it may be distinct from the two major allergens previously isolated from cocksfoot grass pollen: Dac g I (33 kD) [22] and 4B1 (24–25 kD) [23]. Since no sequence data are yet available for these allergens, and we have been unable to obtain Mabs specific for these proteins, the data presented herein represent the first published amino acid sequence of a cocksfoot pollen allergen and will eventually enable the identity of DG3 and its relationship to Dac g I and 4B1 to be established.

Two-dimensional PAGE resolved the Mab 1B9-purified DG3 into a cluster of 5 components of approximately 28 kD but with differing pI (fig. 4). The two major components showed sequence identity over 13 of their 21 N-terminal amino acids with A having an additional 8 residues at its N-terminus (table 1). Components A and B of DG3 may represent isoallergens, although it is most likely that component B represents a cleavage product of component A generated during sample preparation.

Comparison of our sequence data with other known allergen sequences reveals a significant N-terminal homology between DG3 component A and Phl p V (table 2). Ten of the first 15 amino acids of component A are identical to residues of Phl p V, with an area of repeated sequence in Phl p V also present in component A. In view of this sequence homology and the similarity in physicochemical properties between DG3 and those reported for Phl p V (29–31 kD, pI 5.0–7.0) [36], it may be appropriate to classify DG3 as a group-V allergen. However, according to recent data from Dr. F. Matthiesen and Dr. H. Lowenstein, the molecular weight of Dac g V is 26–27 kD. Clearly, further studies are required to elucidate the nature and the extent of the relationship between these two components.

Determination of the amino acid sequences of allergens is a prerequisite for answering questions regarding the pathogenesis of type-I allergies, including the characterisation of epitopes recognised by T and B lymphocytes. The identification of these epitopes has potential clinical application whereby it fa-

cilitates the production of IgE-reactive monovalent peptides which inhibit allergic sequelae by preventing cross-linking of IgE receptors [37]. Epitope mapping studies may also allow the construction of modified allergen fragments (tolerogens) capable of blocking T-cell responses to native allergens. Epitope mapping can be performed in a number of ways but preferably by testing the reactivity of short recombinant or synthetic peptides. We have previously reported the construction of a cocksfoot grass cDNA library from which clones encoding a pollen allergen distinct from DG3 have been identified [35]. Using the data generated in this study, we now propose to re-screen the library with a synthetic (degenerate) oligonucleotide probe. This may allow the isolation of a full length cDNA clone for DG3 and the subsequent localisation of the allergenic epitopes.

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Correspondence to: M.R. Walker, PhD
Department of Clinical Chemistry
University of Birmingham
Wolfson Research Laboratories
Queen Elizabeth Medical Centre
Edgbaston
Birmingham B15 2TH (UK)