

A Re-examination of Ryegrass (*Lolium perenne*) Pollen AllergensS.A. Ford<sup>a</sup>, B.A. Baldo<sup>a, b</sup><sup>a</sup> Kolling Institute of Medical Research, Royal North Shore Hospital of Sydney, St Leonards, and<sup>b</sup> Department of Medicine, University of Sydney, Sydney, Australia

**Abstract.** Electrophoretic separation of crude ryegrass pollen extracts and purified group I, II and III allergens identified 14 IgE-binding components, 8 of which were previously unrecognized. In addition to allergen groups I, II and III, which are already regarded as clinically important, and on the basis of the frequencies and intensities of IgE binding with sera from 42 ryegrass pollen-allergic patients, proteins with molecular weights (MWs) of 60, 52, 30 and 28 kD were identified as allergens of possible major clinical importance. Six other pollen components with MWs ranging from 23 to 80 kD and which reacted with IgE antibodies in the sera of 33-50% of patients, should also be viewed as proteins with potential clinical relevance for at least a proportion of the patients. The electrophoretic separation patterns of ryegrass pollen extracts in both alkaline and acid gels and IgE-probed membrane transfers produced in this study should serve as useful reference patterns for standardization purposes. In addition, the identification of the complete allergen recognition pattern by individual patients will permit safer and more effective diagnosis and therapy of ryegrass pollen sensitivities.

## Introduction

The pollen of perennial ryegrass is a widespread source of allergens and a major cause of allergic diseases [Marsh, 1975]. The first described allergens from this species were designated groups I, II, III and IV [Johnson and Marsh, 1965a, b, 1966a, b; Marsh, 1975]. Using the recently proposed, but not yet official, International Union of Immunological Societies (IUIS) nomenclature for allergens, the above allergen groups would be designated Lol pI, Lol pII, Lol pIII and Lol pIV, respectively. Group I, the major allergen, is an acidic glycoprotein with a molecular weight (MW) of approximately 32 kD and consists of 4 isoallergenic variants [Marsh, 1975]. Although a number of allergenic components have been identified and/or isolated from ryegrass pollen, most fit the description of one of groups I, II, III or IV [Lynch and Turner, 1974; Smart and Knox, 1980; Howlett and Clarke, 1981]. Smart et al. [1983] produced monoclonal antibodies to what were probably group I and group II allergens. Other monoclonal antibodies produced in the same study reacted with allergenic components with MWs of 28 and 30 kD. Lowenstein

[1980] identified 19 IgE-binding components in crossed radioimmuno-electrophoresis (CRIE) studies on ryegrass pollen. Seven of these corresponded to group I, II or III allergens. Cytochrome c (Lol pX) [Ekramoddoullah et al., 1982] and a basic glycoprotein with a MW of 56.8 kD termed high molecular weight basic allergen (HMBA) [Ekramoddoullah et al., 1983] have recently been shown to be allergenic components of ryegrass pollen. The latter allergen may be the same as allergen group IV.

Because of this uncertainty about the identity or otherwise of the various allergenic components identified in, or isolated from, ryegrass pollen, and in view of the recent success of applying electrophoretic separation to the identification of allergens in complex mixtures [Tovey and Baldo, 1984, 1985; Ford et al., 1985, 1986], we have applied the method to critically re-examine the allergenic spectrum of pollen from this species. Central to this study were the aims of defining a standard reference pattern that can readily be reproduced and interpreted in different laboratories and to apply the method to defining individual patient allergen recognition patterns. Both of these aims must be achieved if standardization of ryegrass pollen ex-

tracts is to be satisfactorily achieved and if such extracts are to be successfully used for diagnosis and therapy. In the study reported here, eight previously unrecognised IgE-binding components are identified, and our current knowledge of the spectrum of ryegrass pollen allergens is reviewed.

## Materials and Methods

### Pollen Extracts and Purified Allergens

Dry *L. perenne* pollen was purchased from Greer Laboratories, Inc., Lenoir, N.C., USA (Lot 51V24-6). The pollen (1.0 g) was extracted in 0.001 M  $\text{NH}_4\text{HCO}_3$  (10 ml) at 4°C overnight, centrifuged (17,300 g, 40 min) and the supernatant freeze-dried. A sample of the freeze-dried extract (52 mg) was then resuspended in 70% saturated  $(\text{NH}_4)_2\text{SO}_4$  (2.8 ml) for 60 min at 4°C, and the precipitated proteins were recovered by centrifugation (12,000 g, 15 min). The freeze-dried material accounted for 34% of the weight of dry pollen and consisted of 4.9% protein [Bradford, 1976]. A lyophilized reference extract of ryegrass pollen (Lot E8-rye) and preparations of ryegrass groups I (cat. No. A611-901-185), II (cat. No. A612-901-185) and III (cat. No. A613-901-185) allergens were purchased from the National Institutes of Allergy and Infectious Diseases (NIAID), NIH, Bethesda, Md., USA. Crude ryegrass pollen extracts from 3 commercial manufacturers (designated CCE1, CCE2, and CCE3) were obtained from Dr. G.A. Stewart (Clinical Immunology Unit, Princess Margaret Hospital, Perth, Western Australia).

### Sera

Sera were collected at the Allergy Clinic, Royal North Shore Hospital (Sydney, Australia) from patients with a history of allergy to ryegrass pollen and a positive skin test to an extract of the pollen. Sera were also obtained from other allergic patients (not allergic to ryegrass pollen) with a negative skin test to a ryegrass pollen extract. All sera were tested for ryegrass pollen-specific IgE in a RAST assay using Pharmacia *L. perenne* discs and  $^{125}\text{I}$ -anti-human IgE (Pharmacia). Fetal cord serum was obtained from the Department of Obstetrics, Royal North Shore Hospital, and used as an IgE-free control.

### Gel Electrophoresis

Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) was carried out as previously described [Tovey and Baldo, 1984; Ford et al., 1985] except that both homogeneous (18%) and gradient (9–27%) acrylamide gels were employed in this study. For the electroblotting experiments, ryegrass pollen extract (800  $\mu\text{g}$  protein) treated with 2-mercaptoethanol was subjected to electrophoresis at 180 V for 16 h.

Alkaline gradient PAGE was carried out in 3–40% highly cross-linked (GG-HX) acrylamide gels (Gradient Pty. Ltd., Pyrmont, Australia) as described by Tovey and Baldo [1984]. Acid gradient PAGE was carried out with glycine/glacial acetic acid buffer (pH 3.5).

With both SDS and gradient PAGE, high- and low-MW markers purchased either from Bio-Rad or Pharmacia were included in each electrophoretic separation.

### Electrophoretic Transfer of Proteins to Nitrocellulose (NC)

Materials and methods for transfer were as previously described [Tovey and Baldo, 1984; Ford et al., 1985], except for the time of transfer which was extended to 120 min.

### Probing of NC Membrane Strips and Autoradiography

NC strips (5 mm in width) obtained after SDS-PAGE were incubated with sera (diluted 1:8 with 0.1% Tween-20, 0.1% sodium azide in PBS) and then with  $^{125}\text{I}$ -anti-IgE (50,000 cpm/strip) overnight. After extensive washing (30 min) with 0.05% Tween-20 in PBS, the NC strips were air-dried at room temperature and loaded into a Kodak X-Omatic cassette (KP68375) together with photographic film (Fuji Rx-safety). The cassette was then stored at  $-70^\circ\text{C}$  for periods of 24–72 h. Fetal cord serum and sera from non-ryegrass pollen-sensitive individuals were used as controls. NC strips obtained after gradient PAGE were assayed as above except that sera were diluted 1:10 and incubation with  $^{125}\text{I}$ -anti-human-IgE (34,000 cpm) was for 7 h.

### Protein Staining

PAGs were stained with Coomassie brilliant blue G250 (CBB; Sigma Chemical Co.) using the enhancing method described by Neuhoof and Stamm [1984]. NC transfers were stained with amido black.

## Results

### SDS-PAGE of Ryegrass Pollen Extracts

Electrophoresis of a crude *L. perenne* pollen extract under dissociating conditions followed by protein staining with CBB resolved 17 bands (fig. 1A c). A standard whole ryegrass pollen extract prepared by the NIH gave 19 bands, most of which were faintly stained (fig. 1A d). Ryegrass pollen extracts CCE1, CCE2 and CCE3 from 3 different manufacturers yielded 24, 21 and 22 bands, respectively (fig. 1A e, f, g). The MW distribution of stained bands when all extracts were taken into account was 2–120 kD. NIAID preparations of purified ryegrass pollen antigens I, II and III were also electrophoresed under the same conditions with the major staining components showing MWs of 35 and 14 kD, respectively (fig. 1A h, i, j). The 3 commercial extracts and the purified antigen preparations were also separated on small 18% homogeneous gels and, in each case, the number of bands resolved was greater than on the large gels. For the commercial extracts CCE1, CCE2 and CCE3, 26, 23 and 32 bands, respectively, were resolved (fig. 1B b, c, d). This increase was due to the improved resolution of components with a MW larger than 43 kD. In addition, several bands with MW <35 kD were observed in the group I allergen sample (fig. 1B e).

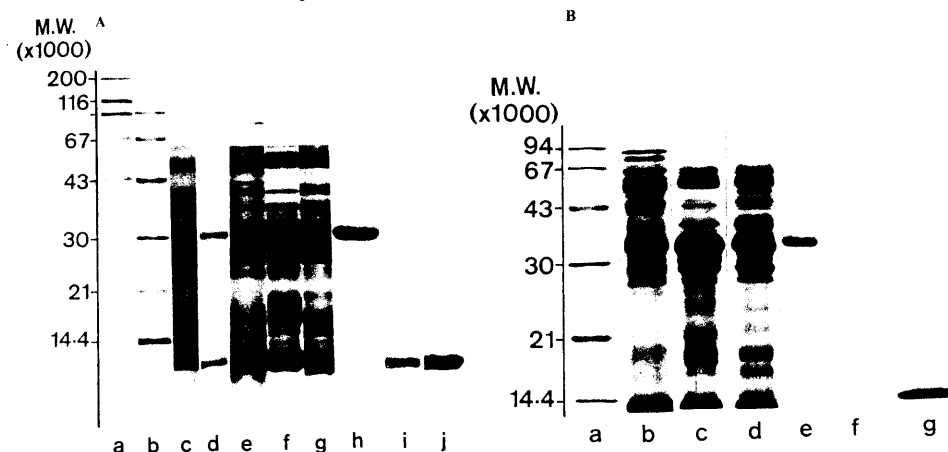


Fig. 1. Separation by SDS-PAGE of different ryegrass pollen extracts on either large ( $14 \times 13$  cm) 9–27% gradient gels (A) or small ( $9 \times 6$  cm) 18% homogeneous gels (B). A a, b: High- and low-MW markers (Bio-Rad); myosin (200 kD),  $\beta$ -galactosidase (116 kD), phosphorylase b (94 kD), bovine serum albumin (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), soybean trypsin inhibitor (21 kD) and lysozyme (14.4 kD). c: Crude ryegrass pollen extract prepared in our laboratory (30  $\mu\text{g}$  protein). d: Whole ryegrass pollen reference extract (NIAID; 30  $\mu\text{g}$  protein). e–g: Crude commercial ryegrass pollen extracts CCE1, CCE2 and CCE3, respectively, from 3 different manufacturers (100  $\mu\text{g}$  protein of each). h–j: Ryegrass groups I, II and III allergens (NIAID), respectively (30  $\mu\text{g}$  protein of each). B a: Low-MW markers (Bio-Rad). b–d: Crude commercial ryegrass pollen extracts CCE1, CCE2 and CCE3, respectively, from 3 different manufacturers (20  $\mu\text{g}$  protein of each). e–g: Ryegrass groups I, II and III allergens (NIAID; 20  $\mu\text{g}$  protein of each), respectively.

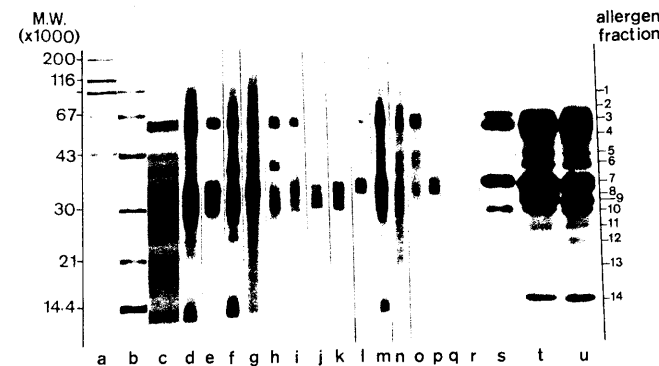


Fig. 2. Identification by autoradiography of the IgE-binding ryegrass pollen components transferred to NC after SDS-PAGE. a, b: PAG-CBB stain of high- and low-MW markers (Bio-Rad). c: Electrophoretically separated crude ryegrass pollen extract stained for protein with CBB. d–p: NC strips with adsorbed transferred pollen proteins incubated with human sera from ryegrass pollen-allergic patients. q, r: NC strips incubated with human sera from a subject not allergic to ryegrass pollen and fetal cord serum, respectively. s–u: IgE-binding patterns for commercial ryegrass pollen extracts (CCE1, CCE2 and CCE3, respectively) from 3 different manufacturers.

### Probing of Proteins Transferred to NC after SDS-PAGE for IgE-Binding Activity

Sera from 42 different patients allergic to ryegrass pollen were examined for IgE-binding to ryegrass pollen proteins separated by SDS-PAGE and then electroblotted onto NC. Of the transferred components, a total of 14 in the MW range 14–80 kD bound IgE antibodies from the patients' sera (fig. 2 a–p). Only one serum demonstrated IgE-binding to all 14 fractions (strip g). Other strips show results with a selection of sera chosen to illustrate individual serum binding patterns (strips d–p). In addition, the corresponding IgE-binding patterns for the 3 commercial extracts have been included (strips s, t, u), clearly demonstrating the allergenic variability between currently used extracts. Sera from subjects not allergic to ryegrass pollen and from fetal cord serum did not bind to any of the transferred proteins as shown by negative autoradiographs (strips q, r).

Band 7 (MW 35 kD) was the pollen component that exhibited the highest frequency of IgE-binding, being recognised by 95% of the sera tested (fig. 3). Only 4 other components bound IgE from more than 50% of the patients' sera tested. These were bands 9 (30 kD), 3 (60 kD), 8 (32 kD) and 10 (28 kD) which bound IgE from individual sera with frequencies of 82, 74, 69 and 55%, respectively (fig. 3).

The IgE-binding patterns of sera from individual patients are shown in table I. While some patients clearly differed in their IgE-binding patterns, for example patient G' (uptake of  $^{125}$ I-anti-IgE 22% in the RAST) and patient H' (RAST uptake 21.9%), there was otherwise a marked similarity in patient reactivity, with the majority of patients recognising over half of the identified IgE-binding components. Sixty per cent of all sera tested demonstrated binding to 7 out of the 14 allergenic components and 38% showed binding to 10 or more.

Table II sets out all of the IgE-binding components detected in this study after subjecting crude ryegrass pollen extract to electrophoresis under dissociating conditions and probing with sera from 42 ryegrass pollen-allergic subjects.

### Gradient PAGE and Probing of Transferred Proteins for IgE-Binding Activity

Figure 4 shows the results of the separation of 2 ryegrass pollen extracts and the NIH-purified group I, II and III allergens on 3–40% acrylamide gradient gels run under either alkaline (fig. 4A) or acid (fig. 4B)

buffer conditions. Gradient PAGE under alkaline conditions (pH 8.6) of both crude pollen extract and the NIH ryegrass pollen extract revealed the presence of 16 protein bands in the MW range 5–140 kD (fig. 4A a–d). This was easily the least number of bands and narrowest MW distribution seen in any of the different pollen extracts so far studied by electroblotting [Ford and Baldo, 1985, 1986, unpublished results]. Electrophoresis of the NIH-purified allergens in this system resulted in multiple bands for group I (7 components in the MW range 30–67 kD with the most heavily stained bands being of MWs 45 and 55 kD) (fig. 4A e) and II allergens (10 components in the MW range 5–45 kD with the most heavily stained band being of MW 14 kD) (fig. 4A f). This degree of heterogeneity was not seen on the dissociating gels. The group III preparation did not resolve well, showing heavy staining at the origin and a densely stained, diffuse band in the 100–140 kD region (fig. 4A g).

Gradient PAGE of a crude extract under acid pH conditions gave 10 bands after protein staining (fig. 4B b) but only one major band with each of the purified allergen samples. Specifically, group I showed a heavily stained band at MW 35 kD (fig. 4B c) and some very lightly stained components down to a MW of approximately 14 kD, while groups II and III each migrated as proteins with a MW of approximately 14 kD (fig. 4B d, e).

Although acid pH gel conditions reduced the molecular aggregation observed under alkaline buffer conditions, the resolution of a complex ryegrass pollen extract separated on acid gels was inferior to the alkaline gel system (c.f. fig. 4A c, d and 4B b). Acid gels were therefore not chosen for electroblotting experiments. After transfer of separated crude pollen extract from alkaline gradient gels and probing with sera from ryegrass pollen-allergic subjects, only 5 distinct IgE-binding bands were visualised by autoradiography (fig. 5 i–n). The MWs of these were approximately 55, 45, 40, 14 and 5 kD. One patient's serum showed binding to all 5 components (strip k) compared with another serum which only showed binding to the 55-kD component (strip j). Strips i, j and n each showed extensive and diffuse shadowing around, and above, MW 55 kD, possibly due to molecular aggregation in the absence of dissociating conditions. Sera from non-allergic subjects and cord sera showed no IgE-binding (strip o).

Allergen groups I and II were also run on alkaline gradient gels, transferred to NC and probed with al-

**Table I.** Patients' sera (n = 42) showing reactions of each with the identified allergenic components obtained by transfer of SDS-PAGE-separated *L. perenne* pollen proteins

| Patient sera | <i>L. perenne</i> allergen fraction and estimated MW (× 1,000) |   |   |   |   |   |   |   |   |    |    |    |    |    |
|--------------|----------------------------------------------------------------|---|---|---|---|---|---|---|---|----|----|----|----|----|
|              | 1                                                              | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
| A            | •                                                              | • | • |   |   |   |   |   |   |    |    |    |    |    |
| B            | •                                                              | • | • |   |   |   |   |   |   |    |    |    |    |    |
| C            | •                                                              | • | • |   |   |   |   |   |   |    |    |    |    |    |
| D            | •                                                              | • | • |   |   |   |   |   |   |    |    |    |    |    |
| E            | •                                                              | • | • |   |   |   |   |   |   |    |    |    |    |    |
| F            | •                                                              | • | • |   |   |   |   |   |   |    |    |    |    |    |
| G            | •                                                              | • | • |   |   |   |   |   |   |    |    |    |    |    |
| H            | •                                                              | • | • |   |   |   |   |   |   |    |    |    |    |    |
| I            | •                                                              | • | • |   |   |   |   |   |   |    |    |    |    |    |
| J            | •                                                              | • | • |   |   |   |   |   |   |    |    |    |    |    |
| K            | •                                                              | • | • |   |   |   |   |   |   |    |    |    |    |    |
| L            | •                                                              | • | • |   |   |   |   |   |   |    |    |    |    |    |
| M            | •                                                              | • | • |   |   |   |   |   |   |    |    |    |    |    |
| N            | •                                                              | • | • |   |   |   |   |   |   |    |    |    |    |    |
| O            | •                                                              | • | • |   |   |   |   |   |   |    |    |    |    |    |
| P            | •                                                              | • | • |   |   |   |   |   |   |    |    |    |    |    |
| Q            | •                                                              | • | • |   |   |   |   |   |   |    |    |    |    |    |
| R            | •                                                              | • | • |   |   |   |   |   |   |    |    |    |    |    |
| S            | •                                                              | • | • |   |   |   |   |   |   |    |    |    |    |    |
| T            | •                                                              | • | • |   |   |   |   |   |   |    |    |    |    |    |
| U            | •                                                              | • | • |   |   |   |   |   |   |    |    |    |    |    |
| V            | •                                                              | • | • |   |   |   |   |   |   |    |    |    |    |    |
| W            | •                                                              | • | • |   |   |   |   |   |   |    |    |    |    |    |
| X            | •                                                              | • | • |   |   |   |   |   |   |    |    |    |    |    |
| Y            | •                                                              | • | • |   |   |   |   |   |   |    |    |    |    |    |
| Z            | •                                                              | • | • |   |   |   |   |   |   |    |    |    |    |    |
| A'           | •                                                              | • | • |   |   |   |   |   |   |    |    |    |    |    |
| B'           | •                                                              | • | • |   |   |   |   |   |   |    |    |    |    |    |
| C'           | •                                                              | • | • |   |   |   |   |   |   |    |    |    |    |    |
| D'           | •                                                              | • | • |   |   |   |   |   |   |    |    |    |    |    |
| E'           | •                                                              | • | • |   |   |   |   |   |   |    |    |    |    |    |
| F'           | •                                                              | • | • |   |   |   |   |   |   |    |    |    |    |    |
| G'           | •                                                              | • | • |   |   |   |   |   |   |    |    |    |    |    |
| H'           | •                                                              | • | • |   |   |   |   |   |   |    |    |    |    |    |
| I'           | •                                                              | • | • |   |   |   |   |   |   |    |    |    |    |    |
| J'           | •                                                              | • | • |   |   |   |   |   |   |    |    |    |    |    |
| K'           | •                                                              | • | • |   |   |   |   |   |   |    |    |    |    |    |
| L'           | •                                                              | • | • |   |   |   |   |   |   |    |    |    |    |    |
| M'           | •                                                              | • | • |   |   |   |   |   |   |    |    |    |    |    |
| N'           | •                                                              | • | • |   |   |   |   |   |   |    |    |    |    |    |
| O'           | •                                                              | • | • |   |   |   |   |   |   |    |    |    |    |    |
| P'           | •                                                              | • | • |   |   |   |   |   |   |    |    |    |    |    |

**Table II.** IgE binding ryegrass pollen components identified by SDS-PAGE and electroblotting

| IgE-binding component <sup>1</sup> | MW kD <sup>2</sup> | Frequency of IgE-binding, % <sup>3</sup> | Intensity of binding <sup>4</sup> |
|------------------------------------|--------------------|------------------------------------------|-----------------------------------|
| 1                                  | 80                 | 45                                       | +                                 |
| 2                                  | 67                 | 48                                       | +                                 |
| 3                                  | 60                 | 74                                       | +++                               |
| 4                                  | 50                 | 17                                       | +                                 |
| 5                                  | 42                 | 50                                       | ++                                |
| 6                                  | 40                 | 33                                       | ++                                |
| 7                                  | 35                 | 95                                       | +++                               |
| 8                                  | 32                 | 69                                       | +++                               |
| 9                                  | 30                 | 82                                       | +++                               |
| 10                                 | 28                 | 55                                       | +++                               |
| 11                                 | 25                 | 36                                       | ++                                |
| 12                                 | 23                 | 43                                       | +                                 |
| 13                                 | 17                 | 19                                       | +                                 |
| 14                                 | 14                 | 43                                       | +++                               |

IgE binding was assessed with sera from 42 patients allergic to ryegrass pollen. SDS-PAGE was performed using dissociating conditions (SDS, heat and mercaptoethanol).

<sup>1</sup> See figure 2.

<sup>2</sup> Determined by SDS-PAGE. See figure 2.

<sup>3</sup> % sera (n = 42) containing IgE antibodies to the ryegrass pollen components.

<sup>4</sup> Assessed from autoradiographs: + = weak, ++ = moderate, +++ = strong.

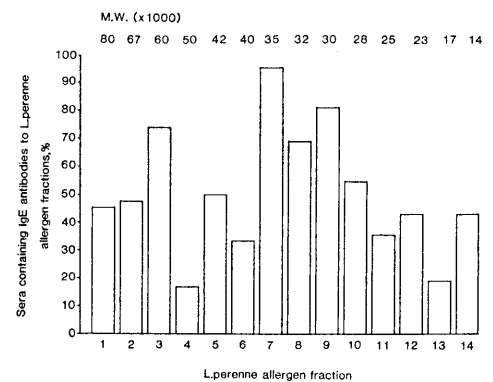


**Fig. 3.** Frequency histogram of patients' sera containing specific IgE antibodies to each of the SDS-PAGE-separated allergenic components of a ryegrass pollen extract.

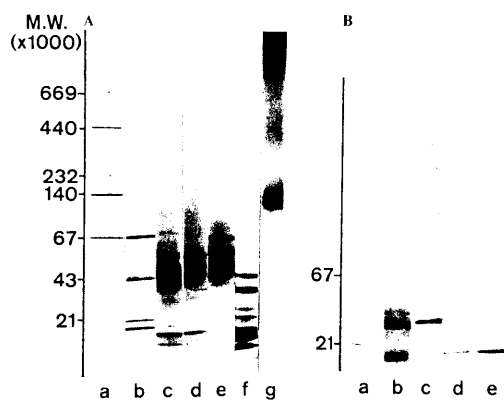


Fig. 4. Separation of ryegrass pollen extracts and purified ryegrass group I, II and III allergens (NIAID) on 3-40% acrylamide gradient gels under either alkaline (A) or acid (B) buffer conditions. 90  $\mu$ g protein of each sample was added to the gels. A a, b: High- and low-MW markers (Pharmacia): thyroglobulin (669 kD), ferritin (440 kD), catalase (232 kD), lactate dehydrogenase (140 kD), bovine serum albumin (67 kD), ovalbumin (43 kD), soybean trypsin inhibitor (21 kD) and  $\alpha$ -lactalbumin (14.4 kD). c: Crude ryegrass pollen extract prepared in our laboratory. d: Ryegrass pollen reference extract (NIAID). e-g: Ryegrass pollen group I, II and III allergens (NIAID), respectively. B. a: Low-MW markers. b: Crude ryegrass pollen extract. c-e: Group I, II and III allergens (NIAID), respectively.

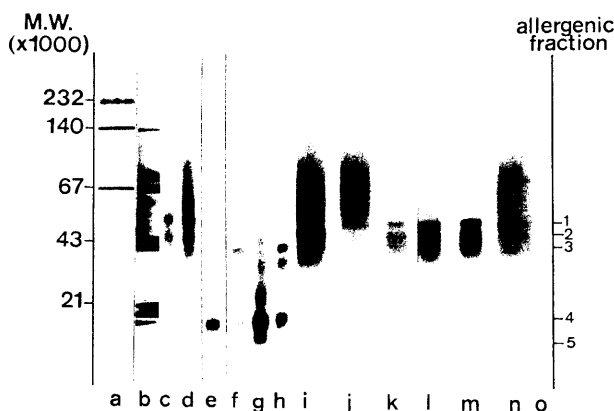


Fig. 5. Identification by autoradiography of the IgE-binding components transferred to NC after gradient PAGE of a crude ryegrass pollen extract and purified ryegrass pollen allergens (NIAID). a, b: High- and low-MW markers (Pharmacia). c, d: Autoradiographs of NC strips of transferred group I allergen incubated with human sera from ryegrass pollen-allergic patients. e-h: Autoradiographs of NC strips of transferred group II allergen incubated with human sera from ryegrass pollen-allergic patients. i-n: NC strips of transferred crude ryegrass pollen extract incubated with human sera from subjects allergic to ryegrass pollen. The positions of 5 IgE-binding components detected in the crude extract are indicated on the vertical axis on the right hand side of the figure.

lergic sera (fig. 5, strips c-h). In the case of group I, distinct IgE-binding occurred to 4 of the 7 protein bands with the most intense binding being to the 55 and 45 kD components (strips c, d). These two bands were also the most intensely stained on the gels and were recognised when a crude extract was probed with atopic sera. With the NC-bound group II allergen, there were differences in the IgE-binding patterns of individual sera. The component binding the greatest quantity of IgE was one with a MW of 14 kD (strips e, g, h). This component corresponded to the most heavily stained band of the group II separation pattern on alkaline gels (fig. 4A f). Over half of the

protein bands resolved in the group I and II allergen samples bound IgE antibodies in the allergic patients' sera.

#### Comparison of NC-Bound IgE-Binding Pollen Components Detected after Transfer from SDS or Gradient PAGs

Comparison of the pollen protein electrophoretic patterns separated on the gradient gels with patterns obtained on SDS-PAGs revealed the greater resolving power and complexity of the separations achieved under dissociating conditions (compare figures 1 and 4). Almost all of the proteins in a crude extract resolved

on gradient gels corresponded to the protein bands obtained when allergen groups I and II were separated under non-dissociating conditions (fig. 4, 5). All 5 allergenic components identified in the crude pollen extract were also found in the 2 purified allergenic preparations. For example, components 1 and 2 were identified in the group I pattern (fig. 5c, d), component 3 was seen in both the group I and the group II patterns (strips d, f-h) and components 4 and 5 corresponded to IgE-binding bands in the group II pattern (strip g). Binding to a 30 kD (approximately) component present in the group II allergen (strips e and g) was not observed when the crude pollen extract was used. This may have been because the group II preparation is greatly enriched with this component.

One serum (RAST uptake with ryegrass pollen extract 38.4%) was found to bind only to components with a MW >45 kD following transfer of crude pollen extract and purified group I proteins from gradient gels to NC (fig. 5j). No binding was observed with group II proteins. By contrast, IgE in this serum bound to 12 SDS-PAG-separated pollen proteins transferred to NC. Again, however, no IgE-binding occurred with group II proteins transferred to NC from SDS-PAGs.

#### Discussion

The allergenicity of ryegrass pollen has been the subject of more investigations than the pollen of any other monocotyledon. However, since the pioneering work initiated in the late 1950s by Marsh and his associates, which led to the findings of allergen groups I, II, III and IV, it was many years before our knowledge of the full allergenic spectrum of *L. perenne* pollen was increased. Recently, allergens that are apparently unrelated to the group I-IV allergens have been identified. Prior to that, many of the published studies on ryegrass pollen allergens described pollen components that could be classified as group I, II, III or IV allergens. Table III summarizes the ryegrass pollen allergens or allergen fractions identified so far and attempts to classify each of the described components as either belonging to, or distinct from, one of the above groups. Table III, incorporating known physicochemical properties, tests for allergenic activity and references, serves as a timely summary of our knowledge so far of the number and nature of allergens in ryegrass pollen.

In the present study, allergens in a ryegrass pollen extract were identified by electroblotting. After transfer from SDS-PAGs, 14 IgE-binding proteins ranging in MW from 14 to 80 kD were found. While different patients often showed different IgE antibody responses to these allergens, the majority of patients exhibited binding to over half of the identified allergens. Of potential clinical significance was the finding that more than one patient's serum recognized the same complement of allergens. For example 5 patients (E, F, G, H and K) of the 42 examined (table I) (that is 12%), had IgE antibodies to pollen components 1, 2, 3, 5, 7, 8, 9, 10, 11, 12, 13 and 14; sera from 4 patients (R, I', K' and O') (9.5%) bound component 7 only; sera from 3 other patients (W, X and Z) (7.1%) reacted with components 7 and 9 and sera from 2 others (D' and J') (4.8%) bound to component 7, 8, 9 and 10. This finding of identical recognition patterns was not observed with patient IgE responses to cocksfoot (*Dactylis glomerata*) pollen or house dust mite allergens where no two sera exhibited identical IgE-binding patterns [Tovey and Baldo 1985; Ford et al., 1985]. Patients with identical IgE responses to groups of allergens were seen, however, with *Parietaria judaica* allergens when these were studied by electroblotting [Ford et al., 1986].

Electroblotting for allergen detection has been of great value for rapidly defining the spectrum of allergens recognized by individual allergic patients. The finding that different patients often demonstrate their own unique allergen spectrum has important implications for the diagnosis and treatment of allergic diseases. For example, a patient may be sensitive to one or more allergens which are found to be lacking in extracts used for diagnosis or therapy. Such information can readily be obtained by electroblotting. In addition, the technique can permit the formulation of 'cocktails' of previously identified allergens. Such cocktails may range from mixtures appropriate for use with a large proportion of the allergic subjects to formulations specifically designed for the needs of only a few, or even a single, patient.

Although ryegrass cytochrome c was not one of the allergens positively identified in our electroblotting study, all of the other previously identified allergens listed in table III appear to have been detected. However, in the absence of authentic reference samples, some uncertainty exists in the identification of the group IV and the HMBA. Not unexpectedly [Marsh, 1975], the group I allergen showed the highest fre-

Table III. Summary of ryegrass (*L. perenne*) pollen allergens identified so far

| Allergen or allergen group <sup>1</sup>                                                     | Physicochemical properties                                                                                                                                      | Test for allergenic activity                             | References                                                                                                  |
|---------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------|-------------------------------------------------------------------------------------------------------------|
| Group I                                                                                     | 4 isoallergens<br>MW 27–34 kD <sup>2</sup><br>pI 5.15–5.25<br>5% carbohydrate<br>S <sub>20</sub> <sup>3</sup> = 2.89 S<br>E <sub>1 cm</sub> <sup>4</sup> = 15.1 | 95% patients<br>skin test-positive                       | Johnson and Marsh [1965a, b, 1966a, b]; Marsh et al. [1966, 1970]; Lichtenstein et al. [1969]; Marsh [1975] |
| <i>Other established or probable preparations and/or descriptions of group I allergens</i>  |                                                                                                                                                                 |                                                          |                                                                                                             |
| Fraction G50.2                                                                              | MW 33 kD <sup>3</sup><br>6.2–7.5% carbohydrate<br>E <sub>1 cm</sub> <sup>4</sup> = 2.1<br>Also exists as dimer                                                  | PCA in monkey and RAST inhibition                        | Lynch and Turner [1974]                                                                                     |
| Lp1                                                                                         | 4 isoallergens<br>MW 32 kD <sup>4</sup>                                                                                                                         | IgE-binding (strong) in radioimmuno-electrophoresis      | Smart and Knox [1980]                                                                                       |
| Ags 21, 22, 23                                                                              | MW 28 and 14 kD<br>pI 5.1–5.25                                                                                                                                  | 40–45% patients' sera show IgE-binding in CRIE           | Lowenstein [1980]                                                                                           |
| Glycoprotein I                                                                              | 3 isoallergens<br>MW 29–33 kD <sup>2</sup><br>pI 5.1–5.5<br>5% carbohydrate                                                                                     | RAST inhibition                                          | Howlett and Clarke [1981]                                                                                   |
| Group I allergens detected with monoclonal antibodies                                       | MW 30–35 kD <sup>4</sup>                                                                                                                                        | IgE-binding ELISA                                        | Smart et al. [1983]                                                                                         |
| Component 7                                                                                 | MW 35 kD <sup>4</sup>                                                                                                                                           | 95% patients' sera IgE-binding following electroblotting | Ford and Baldo (this paper)                                                                                 |
| Group II                                                                                    | 2 isoallergens<br>MW 10–11 kD <sup>3</sup><br>pI acidic<br>E <sub>1 cm</sub> <sup>4</sup> = 10.3<br>S <sub>20</sub> <sup>5</sup> = 1.36 S                       | 60% patients skin test-positive                          | as for group I                                                                                              |
| <i>Other established or probable preparations and/or descriptions of group II allergens</i> |                                                                                                                                                                 |                                                          |                                                                                                             |
| Lp2                                                                                         | MW 10–12 kD <sup>4</sup>                                                                                                                                        | IgE-binding (weak) in radioimmuno-electrophoresis        | Smart and Knox [1980]                                                                                       |
| Ag1                                                                                         | pI 5.4                                                                                                                                                          | 35% patients' sera IgE-binding in CRIE                   | Lowenstein [1980]                                                                                           |
| Group II (?) allergens detected with monoclonal antibodies <sup>6</sup>                     | MW 16 kD <sup>4</sup>                                                                                                                                           | IgE-binding ELISA                                        | Smart et al. [1983]                                                                                         |
| Component 14 <sup>6</sup>                                                                   | MW 14 kD <sup>4</sup>                                                                                                                                           | 43% patients' sera IgE-binding following electroblotting | Ford and Baldo (this paper)                                                                                 |
| Group III                                                                                   | 2 isoallergens<br>MW 10–11 kD <sup>3</sup><br>pI basic<br>E <sub>1 cm</sub> <sup>4</sup> = 10.6                                                                 | 70% patients skin test-positive                          | Marsh et al. [1970]; Marsh [1975]                                                                           |

Table III. (continued)

| Allergen or allergen group <sup>1</sup>                                                                                                                    | Physicochemical properties                                                   | Test for allergenic activities                           | References                   |
|------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------|----------------------------------------------------------|------------------------------|
| <i>Other established or probable preparations and/or descriptions of group III allergens</i>                                                               |                                                                              |                                                          |                              |
| Ags 26, 27, 28                                                                                                                                             | MW 18 kD<br>pI 9.4                                                           | 11–35% patients' sera IgE-binding in CRIE                | Lowenstein [1980]            |
| Component 14 <sup>6</sup><br>(see group II)                                                                                                                |                                                                              |                                                          |                              |
| Group IV                                                                                                                                                   | MW 50 kD <sup>3</sup>                                                        | 20% patients' skin test-positive                         | Marsh [1975]                 |
| <i>Other established or probable preparations and/or descriptions of group IV allergens</i>                                                                |                                                                              |                                                          |                              |
| Antigen A                                                                                                                                                  | pI > 8.6                                                                     | IgE-binding (strong) in radioimmuno-electrophoresis      | Smart and Knox [1980]        |
| Glycoprotein 2                                                                                                                                             | MW 58 kD <sup>3</sup> and 67 kD <sup>3</sup><br>pI > 9.0<br>12% carbohydrate | RAST inhibition                                          | Howlett and Clarke [1981]    |
| Component 2                                                                                                                                                | MW 67 kD <sup>4</sup>                                                        | 48% patients' sera IgE-binding following electroblotting | Ford and Baldo (this paper)  |
| Cytochrome c                                                                                                                                               | MW 12 kD <sup>4</sup>                                                        | Skin tests and RAST                                      | Ekramoddoullah et al. [1982] |
| HMBA                                                                                                                                                       | MW 56.8 kD <sup>4</sup><br>pI 9.9<br>17% carbohydrate                        | Skin tests and direct RAST                               | Ekramoddoullah et al. [1983] |
| <i>Other probable description of HMBA allergen</i>                                                                                                         |                                                                              |                                                          |                              |
| Component 3                                                                                                                                                | MW 60 kD <sup>4</sup>                                                        | 74% patients' sera IgE-binding following electroblotting | Ford and Baldo (this paper)  |
| Ag 8                                                                                                                                                       | MW 22.5 kD                                                                   | 25% patients' sera IgE-binding in CRIE                   | Lowenstein [1980]            |
| Ag 6                                                                                                                                                       | MW < 10 kD                                                                   | 20% patients' sera IgE-binding in CRIE                   | Lowenstein [1980]            |
| Allergenic components detected with monoclonal antibodies                                                                                                  | MW 28 and 30 kD                                                              | IgE-binding ELISA                                        | Smart et al. [1983]          |
| Component 9                                                                                                                                                | 30 kD <sup>4,7</sup>                                                         | 82% patients' sera IgE-binding following electroblotting | Ford and Baldo (this paper)  |
| Component 10                                                                                                                                               | 28 kD <sup>4,8</sup>                                                         | 55% patient's sera IgE-binding following electroblotting | Ford and Baldo (this paper)  |
| <i>Other allergens identified in this study and apparently not belonging to any previously described allergens</i><br>Components 1, 4, 5, 6, 8, 11, 12, 13 |                                                                              |                                                          |                              |

<sup>1</sup> IUIS nomenclature: group I = Lol pI, group II = Lol pII, group III = Lol pIII, group IV = Lol pIV, cytochrome c = Lol pX.<sup>2</sup> MW depending on method used.<sup>3</sup> MW by calibrated column chromatography.<sup>4</sup> MW by SDS-PAGE.<sup>5</sup> Specificity of antibody not fully characterized – may be specific for group II or group III allergens.<sup>6</sup> May be group II or group III allergens since both groups run together in SDS-PAGE.<sup>7</sup> The 30 kD component detected by the monoclonal antibody and fraction 9 may be the sample component.<sup>8</sup> The 28 kD component detected by the monoclonal antibody and fraction 10 may be the same component.

quency of IgE binding (95%) with the 42 different sera from ryegrass pollen-allergic patients. Under dissociating conditions it was not possible to separate the group II and III allergens because of their similar MWs but it was apparent that the frequencies of IgE binding to these and the group IV allergens were all less than 50%. If, as we suspect, component 3 in our study is the same as the HMBA described by Ekramoddoullah et al. [1983], then the 74% frequency of IgE binding that we obtained supports the claim that this protein is a major ryegrass pollen allergen. Two other components, numbers 9 and 10 with MWs of 30 and 28 kD, bound IgE from 82 and 55%, respectively, of the patients' sera. On a frequency basis, this made component 9 the second most important IgE-binding protein identified (table II). Components 9 and 10 appear to be the same IgE-binding proteins previously detected with monoclonal antibodies by Smart et al. [1983]. Eight IgE-binding proteins designated here as components 1, 4, 5, 6, 8, 11, 12 and 13 do not appear to have been previously identified as allergens. Six of these, components 1, 5, 6, 8, 11 and 12, reacted with IgE antibodies in sera from at least one third of the 42 allergic patients tested and 2, components 5 and 8, reacted with antibodies from at least half the patients (table II).

A comparison of results obtained with dissociating and non-dissociating gel conditions suggests that protein denaturation in vivo may be important in the allergic response. Almost all of the resolvable proteins and IgE-binding components observed after gradient-PAGE could be attributed to the group II and III allergens. By contrast, under dissociating conditions a range of allergenic proteins not detected under non-dissociating conditions were revealed. The in vivo implication of this is that after inhalation or ingestion, proteins may be denatured giving rise to a number of IgE-binding peptides and thus a greater range of allergens than can be detected in the intact 'native' pollen proteins. The results also suggest that in the detection of allergens by electroblotting, electrophoresis employing dissociating, and not non-dissociating, gel conditions may, in most cases, be the method of choice to detect the full spectrum of allergens in a crude extract.

In summary, electroblotting of ryegrass pollen proteins has demonstrated 8 previously undescribed IgE-binding pollen components in addition to confirming the existence of those allergens already known (with the exception of cytochrome c). From

the results of this study it is clear that the allergenic complexity of ryegrass pollen extends beyond a definition that includes groups I, II, III and IV, HMBA and cytochrome c. In the absence of clinical assessments of most of the IgE-binding pollen components and ignoring the intensity of IgE-binding detected on electroblots, components 1, 2, (probably group IV allergen), 3 (probably HMBA), 5, 6, 7, 8, 9, 10, 11, 12 and 14 should be considered as potentially important allergens. By taking into account binding intensities as well, this list reduces to components 3, 7, 8, 9, 10 and 14, that is proteins with MWs of 60, 35, 32, 30, 28 and 14 kD, respectively. Of these components, HMBA, group I and groups II and III have already been shown to be important allergens. Side-by-side clinical studies of the 14 ryegrass pollen components identified here are now needed to assess their clinical relevance and rank order of importance for diagnosis and therapy.

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