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IgE-Binding Proteins from Pine (*Pinus radiata* D. Don) Pollen: Evidence for Cross-Reactivity with Ryegrass (*Lolium perenne*)

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Abstract. A complex mixture of pine (*Pinus radiata* D. Don) pollen proteins are rapidly released into aqueous solutions. IgE-binding proteins have been identified in these extracts using combined SDS-PAGE immunoblotting techniques. These IgE-binding proteins were detected using atopic patient and commercial pooled human sera known to be high in ryegrass-specific IgE. Enzyme-immunoassay inhibition studies revealed that leached *P. radiata* pollen proteins could partially inhibit serum IgE binding to ryegrass RAST discs thus providing preliminary evidence for allergen cross-reactivity between these two unrelated species.

Introduction

Pinus radiata D. Don is the most important timber tree in New Zealand. It is the major component of the exotic forests which cover approximately 1,212,000 ha [1] and is also widely planted for wind-break on farms. The atmospheric pollen season in New Zealand commences with the flowering of *Pinus* together with *Acacia* species [2]. *Pinus* species, however, are not generally considered an important cause of allergies. This is despite being wind pollinated and producing enormous quantities of pollen. Suggested reasons for the low allergenicity of pine pollen include the large size and hydrophobicity of the grain, the latter making the release of proteins into aqueous solutions unlikely [3, 4].

Several reports of allergy to pine pollen have, however, been published [5-9]. In a study of pine pollen reactivity in an atopic population in the San Francisco Bay area of the USA [5], a small proportion (3.2%) of those pollen-sensitive individuals who exhibited allergy symptoms during the pine pollen season demonstrated a positive skin test reaction to *P. radiata* extracts. More recently, Kalliel and Settupane [6] demonstrated that 6 patients of 100 tested had a positive skin test reaction to *Pinus strobus* pollen extract. Four of these were tested further by inhalation challenge to which 2 responded positive, suggesting that these patients were hyperreactive to inhaled pine allergen. There are also other reports of pine pollen allergy which

have appeared in the older literature [7-9]. In addition a recent survey of undergraduate students in New Zealand revealed that 5.9% of the hayfever sufferers among them attribute pine as a cause of their allergy [10].

Although pine may not be a major cause of allergy, there does appear to be a proportion of the population who are adversely affected by it. In order to establish whether pine pollen extracts contain allergenic components, we examined aqueous extracts of *P. radiata* pollen for IgE-binding proteins using a combination of SDS-PAGE and immunoblotting procedures.

Some of the characteristics of protein release by pine pollen were compared with those from ryegrass, a well-studied allergen source [11].

Materials and Methods

Pollen

P. radiata pollen was collected from mature male cones of trees growing on the Massey University campus. Pollen was readily obtained by agitating the cones and collecting the pollen released. Ryegrass pollen (*Lolium perenne* cv Grasslands Nui) pollen was obtained by harvesting flowering grass heads from field grown material provided by Grasslands Division of DSIR (Palmerston North, New Zealand). Privet (*Ligustrum lucidum*) pollen was collected from freshly harvested flowers. Pollen was allowed to fall from the flower heads and was collected on polished brown paper. For each species the harvested pollen was separated from extraneous plant debris by sequential sieving and stored dry at -20°C until required. The purity of the pollen samples was checked using a light microscope.

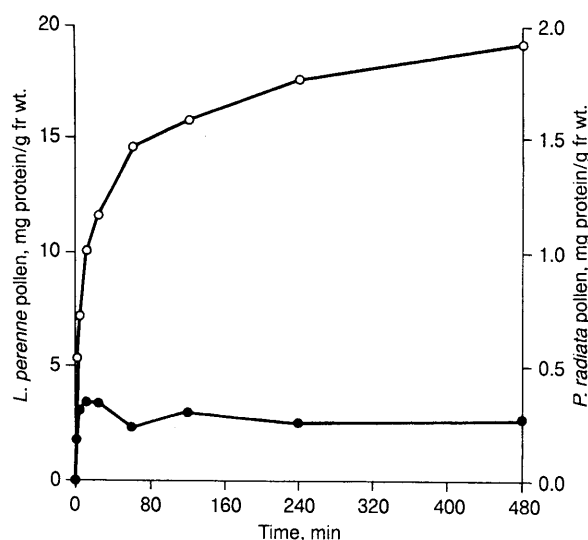


Fig. 1. Time-course of protein release from *P. radiata* (●) and *L. perenne* (○) pollen.

Extracts

Pollen was extracted with a 10-mM ammonium bicarbonate solution (pH 7.8) [12] at a ratio of 1.0 g pollen to 3 ml extraction medium for ryegrass and 5.0 g to 3 ml for pine. Standard extractions were done at 4°C under continuous agitation for 1 h.

For time-course experiments, samples were taken at the intervals indicated (fig. 1). Sampled pollen, collected by centrifugation (5,000 g for 5 min) was returned to the extraction suspension, and leaching under agitation allowed to continue.

Extracts for electrophoretic analysis and immunoblotting were clarified by centrifugation at 5,000 g for 10 min and the supernatant filtered through a 0.45-μm membrane filter (Millipore). The resultant extract was then concentrated either by using Amicon centri-con/centriprep centrifuge concentrators (10,000 molecular weight cut-off) or by lyophilisation. If samples were to be lyophilised they were first dialysed against distilled water for 16 h at 4°C.

The protein concentration of extracts was assayed according to the method of Bradford [13].

Sera

Commercial ryegrass-positive and negative pooled human sera were obtained from Kallestad Diagnostics (USA; Allercoat Positive Control batch numbers 89000433 and 90002583, and Allercoat Negative Control batch number 89000435). We found them to contain 340–390 and 13 kU/l IgE, respectively, using the Phadebas PRIST assay for total IgE and RAST group 3 and 0, respectively, for ryegrass-specific IgE using a Phadezyme RAST test (Pharmacia Diagnostics). Sera was also collected at the Asthma Clinic, Green Lane Hospital, Auckland, from patients with a positive skin test to ryegrass and others who had no history of pollen allergy. Patient sera was stored at –20°C until required.

SDS-PAGE and Electrophoretic Transfer of Proteins to Nitrocellulose

SDS-PAGE was performed using a vertical slab mini-gel system (Bio-Rad Laboratories) using homogeneous 12.5% gels according to the method of Laemmli [14]. Separated proteins were visualized by silver staining (Bio-Rad silver staining kit).

Electrophoretic transfer to nitrocellulose (0.45 μm) was performed using a Bio-Rad Mini-transblot cell. Transfer buffer consisted of 25 mM Tris, 192 mM glycine and 20% (v/v) methanol at pH 8.3. Transfer was for 2 h at 150 mA (constant) at 4°C. Nitrocellulose transfers were stained for protein using the Bio-Rad colloidal gold total protein stain with gold enhancement when appropriate.

Immunodetection of IgE-Binding Proteins

Nitrocellulose filters were blocked overnight at 4°C with 1% (w/v) bovine serum albumin (BSA) in Tris-buffered saline (25 mM Tris-HCl, 0.15 M NaCl, pH 7.5) containing 0.05% Tween 20 (TBS-Tw). Nitrocellulose strips (about 5 × 60 mm) were incubated with serum (undiluted unless otherwise specified) overnight at 25°C; washed with four changes of TBS-Tw over 30 min and then incubated with alkaline phosphatase-conjugated equine anti-human IgE antibodies (Kallestad EAST conjugate) for at least 1 h (up to a maximum of 16 h) at 25°C.

After further washing with four changes of TBS-Tw containing 0.1 mM MgCl₂ and 4 × 10⁻⁷ M ZnCl₂ over 30 min, the nitrocellulose strips were placed in the reaction mixture consisting of 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium with phenazine hydrazide as an enhancer [15] for 0.5–1 h at 37°C, until maximum colour development had occurred.

Negative control sera and sera from non-allergic individuals were used as controls. An additional control was the omission of any serum from the first incubation step.

Dot Immunoblots

These were performed in order to test the response of a number of individual patient sera to pollen extracts. Aliquots (5 μl) of pollen extracts containing either 45 pg (*L. perenne* and *L. lucidum* sp) or 1 pg (*P. radiata*) protein were applied directly to nitrocellulose paper and allowed to dry. Blots were then processed for the immunodetection of IgE-binding proteins as described earlier, except that the first incubation step was for 1 h. The commercially available pooled human serum was diluted 1:5 while patient sera was used undiluted.

Inhibition Studies

Lyophilised pollen extracts were reconstituted in water, and adjusted to contain either 0.5, 0.25 and 0.1 mg/ml protein using TBS as diluent. BSA solutions were similarly prepared.

Aliquots (50 μl) of each dilution were separately incubated with 200 μl of pooled human serum (commercially available high IgE, Kallestad No. 90002583) for 2 h at 37°C with continuous agitation. Following incubation, ryegrass-specific IgE levels were determined by enzyme immunoassay (Phadezym RAST, Pharmacia Diagnostics) using RAST discs coupled with ryegrass pollen extract (provided by the manufacturer).

Results are expressed as percent inhibition of control serum (no pollen extract or BSA) binding. There were two or three replicates per treatment and the experiment repeated at least once for each extract.

Results

Protein Release from Pollen

Protein was released rapidly from *P. radiata* pollen with detectable amounts being observed after only 2 min of incubation (fig. 1). Little further leaching of protein was evident after 5–10 min. The amount of protein released by pine, however, was substantially lower than that detected in ryegrass extracts. For example, after 12 min of incubation protein released from pine was 0.34 mg/g pollen, in contrast with 10.0 mg/g pollen for ryegrass. Pine therefore, released only 3.4% of the level of protein released from ryegrass pollen over this short period.

Electrophoresis of leached proteins from both *L. perenne* and *P. radiata* pollen using denaturing and reducing conditions revealed a complex banding pattern after silver staining (fig. 2). For pine, the most intensely staining band was of an estimated molecular weight of 46 kD (arrowed in fig. 2). There were, however, many other proteins evident covering a wide molecular weight range.

The banding pattern revealed for the ryegrass extract was also complex but quite distinct from that of *P. radiata*. Note that a major protein of approximately 31 kD (arrowed) has been negatively stained. This band, however, stained heavily if Coomassie brilliant blue was used instead of silver staining (data not shown) or if the silver staining was prolonged. Furthermore, when separated proteins were transferred to nitrocellulose this band was readily detected using colloidal gold (fig. 3, lane 6).

IgE Immunoblots

The results of immunoblotting pollen extracts, following SDS-PAGE, using commercially available pooled patient serum and detecting bound IgE with an alkaline phosphatase-conjugated anti-human IgE antibody are shown in figure 3a (*P. radiata*) and figure 3b (*L. perenne*). Additional nitrocellulose strips were stained for total protein in order to demonstrate that transfer of protein from the gel was complete. These results are also presented in figure 3. Note that the protein-banding pattern revealed by the colloidal gold stain differs slightly from that detected in polyacrylamide gels after silver staining.

At least five protein species from *P. radiata* pollen bound IgE when the commercial serum known to contain high levels of ryegrass IgE was used (fig. 3a, lane 2). The molecular weights of these bands range

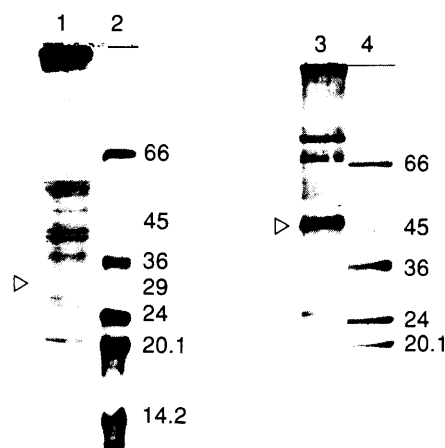


Fig. 2. Banding pattern of leached pollen proteins following SDS-PAGE and silver staining. Lane 1, *L. perenne*; lane 3, *P. radiata*; lanes 2 and 4, molecular weight markers. Molecular weights are expressed in kilodaltons.

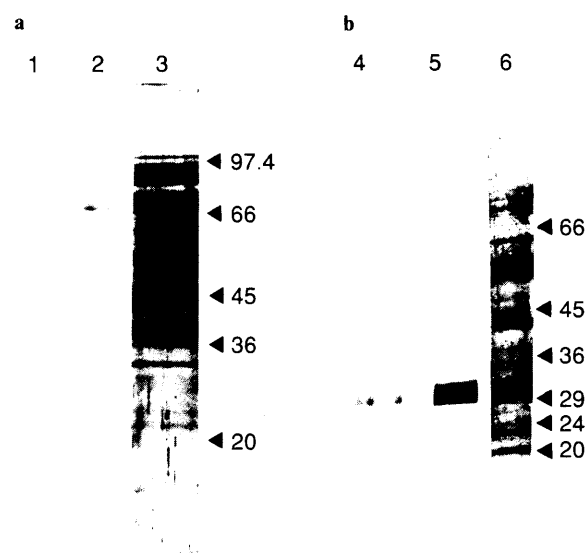


Fig. 3. IgE-binding patterns of *P. radiata* (a) and *L. perenne* (b) immunoblots. Immunodetection of IgE-binding proteins in pollen extracts was performed following their separation by SDS-PAGE and electro-transfer to nitrocellulose. Bound IgE was detected using alkaline phosphatase-conjugated antihuman IgE antibodies (Kallestad). Lanes 1 and 4, commercial low IgE pooled human serum; lanes 2 and 5, commercial high IgE pooled human serum; lanes 3 and 6, total protein stain. Arrows correspond to the position of molecular weight marker proteins and molecular weights are expressed in kilodaltons.

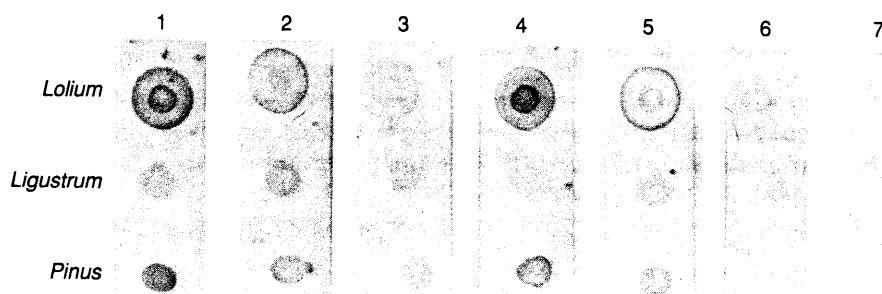


Fig. 4. Dot-immunoblots of IgE-binding proteins in extracts of *P. radiata*, *L. lucidum* and *L. perenne*. Lane 1, commercial high IgE serum; lanes 2-6, individual patient sera; lane 7, commercial low IgE serum (note low level of background staining).

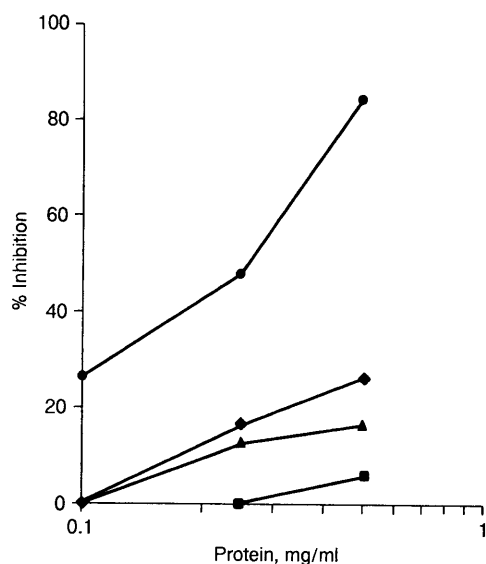


Fig. 5. Inhibition of serum IgE binding to ryegrass RAST disks by varying amounts of pollen extracts. ● = *L. perenne*; ◆ = *L. radiata*, extract 1; ▲ = *P. radiata*, extract 2; ■ = *L. lucidum*. Equivalent amounts of BSA had no inhibitory effect (data not shown).

from about 80 to 38 kD. Of these, the protein species at 67 kD, which may in fact be a duplex, was the most intensely staining band. The band at 46 kD corresponds to the major silver-stained protein arrowed in figure 2 (lane 3). No IgE binding was observed using low IgE commercially available pooled human serum (fig. 3a, lane 1).

Figure 3b shows the results for *L. perenne*. The commercial high IgE sera bound strongly to a mixture of ryegrass proteins (fig. 3b, lane 5). The most intense binding was to a protein with an estimated molecular weight of 31 kD. In contrast, when the negative control serum (low in IgE) was used, only slight binding to the 31-kD protein was observed and no other components of the ryegrass mixture were stained. Sim-

ilarly, no staining was observed when serum was omitted from the first incubation step indicating that non-specific binding of the second antibody had not occurred. The ryegrass IgE-binding pattern was quite distinct from that demonstrated for pine although the same pooled human serum was used for both species.

Dot immunoblots were used to test the response of a number of individual patient sera to the pine and ryegrass extracts (fig. 4). An additional pollen extract (*L. lucidum*, privet) was included in this trial for comparative purposes. Figure 4, lane 1, shows that the commercial high IgE serum was bound by extracts from pine and ryegrass, while only weak binding to privet was observed. It is important to note that the amount of protein applied to the filters for ryegrass and privet was the same (45 µg), whereas only 1 µg pollen protein was applied in the case of pine. Despite this there is strong IgE binding to the pine extract.

Results for individual patients are shown in figure 4, lanes 2-6. Serum from 2 individuals reacted strongly to the pine and ryegrass extracts. Two others exhibited weaker responses and 1 showed no reaction at all. This latter individual had no clinical history of pollen allergy. A feature of these results was the appearance of matching of pine and ryegrass responses. By contrast, only in one instance (fig. 4, lane 2) was there any detectable reaction to privet pollen protein. No IgE binding was seen when the commercial low IgE serum was used (fig. 4, lane 7). The matching of pine and ryegrass responses supports the notion of allergen cross-reactivity between these two species.

Inhibition Studies

The possibility that components of the pine pollen extract bind ryegrass IgE was explored further using enzyme immunoassay inhibition studies. Figure 5 shows the percent inhibition of serum IgE binding to ryegrass RAST discs by varying concentrations of pollen extract. At the highest concentration tested

(0.5 mg protein/ml extract), a locally produced ryegrass extract inhibited IgE binding by 84.3%. In contrast, the equivalent amount of BSA had no effect (data not shown). Two separate extracts of *P. radiata* pollen were found to inhibit IgE binding to ryegrass discs by 16.4 and 26.1%, respectively, when tested at a concentration of 0.5 mg/ml. Only slight inhibition (5.9%) was detected using an extract of privet pollen. Dilution of extracts in each case resulted in a reduction in their inhibitory effect (fig. 5).

Discussion

Whilst it is clear from the literature that pine, although a major contributor of airborne pollen in many parts of the world, is not considered a serious allergenic hazard, there are enough reports to suggest that some people are adversely affected by it [5–9]. Our study provides evidence that leached proteins from *P. radiata* are capable of binding to IgE in atopic serum and, therefore, presumably capable of eliciting an allergic reaction.

Other conifers, especially of the genera *Juniperus* and *Cryptomeria* are widely recognised as serious allergens [16]. One suggestion as to this difference within these major groups of gymnosperms concerns the size of the grains since pine pollen grains, with their distinctive air bladders, fall within the range 60–85 μm , compared with 30 μm for cedar. The smaller grain size of the latter presumably allowing greater penetration into the respiratory tract.

A second possibility for the low allergenicity of pine pollen is its low protein content. Certainly, in comparison with ryegrass and other pollens tested (data not given), the amount of protein released by pine is much smaller. Nevertheless, it is rapidly leached from the grain and of complex composition as revealed by SDS-PAGE. The complexity of pine pollen leachates is comparable with that of other pollens including those known to be strongly allergenic, such as the grasses. Thus the claim that the gymnosperms exhibit reduced antigenic complexity [3] can be questioned. It is perhaps pertinent to note that there is no change in the spectrum of protein species eluting from pine during the time course experiment. This suggests that all the proteins originate from the same pollen storage compartment.

We have shown by immunoblotting techniques that a number of proteins released from *P. radiata*

pollen are capable of binding IgE in human sera. These results were surprising since the sera had not been selected specifically for its reactivity to pine allergen and pine is not considered a major source of allergen. Indeed, the commercially available serum is provided by the manufacturer for ryegrass allergy testing. This serum is reported by the manufacturer to be high in ryegrass-specific IgE which was verified by our own analysis.

The spectrum of proteins released from pine are quite distinct from those leached from ryegrass, as revealed by SDS-PAGE. Moreover, the IgE-binding patterns of these two species are also distinct. The most notable feature of the ryegrass IgE-binding pattern is a strongly staining band at about 31 kD. In contrast the pine IgE-binding proteins were of much higher molecular weight. In this study five such proteins were identified in the pine leachate but the possibility cannot be discounted that with different serum quite different banding patterns will emerge.

An interesting feature of these results is the apparent cross-reactivity of serum for allergens in both ryegrass and pine extract. This cross-reactivity was initially demonstrated by immunoblotting techniques and supported by the inhibition studies. Interestingly, the third pollen used in these studies, privet, and a control protein (BSA) exhibited little or no IgE binding when tested with the same sera which reacted strongly to both ryegrass and pine. This suggests that the cross-reactivity between ryegrass and pine is not a general phenomenon.

Since the commercial serum was pooled human sera, it would be expected to contain IgE against a range of different pollens. Similarly, the patient serum used in this study was obtained from individuals who were known to be atopic particularly to grasses but their reactivity to pine and other agents was unknown. Our studies clearly need now to be extended to a population who exhibit allergy symptoms during both the pine and ryegrass flowering seasons in order to properly characterize the allergenic components in *P. radiata* pollen and their cross-reactivity with ryegrass.

Cross-reactivity between closely related species has been well documented for grasses [12, 17, 18] and trees [17, 19, 20]. Cross-reactivity between unrelated species seems much rarer, but Howlett et al. [21] demonstrated cross-reactivity between *Acacia* and ryegrass pollen allergens using RAST inhibition studies similar to those applied here.

The possibility of cross-reactivity of serum with allergens in both ryegrass and pine is intriguing and merits further study. However, it is perhaps necessary to stress that the RAST inhibition results would suggest that, of the IgE that binds to ryegrass protein, only approximately 20% also binds to pine. Nevertheless, if this cross-reactivity is widespread amongst atopic individuals, and since pine pollen release precedes grass pollen in New Zealand [2] and elsewhere [4], the possibility exists that exposure to pine may play a role in sensitizing individuals to later grass pollen exposure.

Production of *P. radiata* is expanding rapidly in New Zealand and around the world because of its fast growth rate and usefulness for timber. This study would suggest that its pollen should not be overlooked as a contributing factor in allergic disorders.

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