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A comparative biochemical study of conifer pollen allergens

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Abstract

In the order Coniferales, only the family Cupressaceae is regarded as being a significant source of airborne allergens, with *Juniperus ashei* characterized as the most significant aeroallergen. Pollen of the closely related species *J. virginiana* has been shown to cross-react with *J. ashei* pollen, however, *J. virginiana* pollen is not considered an important aeroallergen. Although there have been several reports of allergies to *Pinus* pollen, the pollen of this genus is regarded as hypoallergenic. Our previous studies have shown that pollen extracts of *J. ashei*, *J. virginiana*, *J. pinchotii*, *Cupressus macrocarpa*, *Pinus echinata* and *P. taeda* all contained several proteins with the same molecular weights including the reported allergen of *J. ashei*. The present study compared the biochemistry of *J. ashei*, *J. virginiana* and *P. echinata* pollen. A time course experiment of *J. ashei*, *J. virginiana* and *P. echinata* showed that *J. ashei* released a greater quantity of protein within the first minute of moistening. SDS-PAGE analyses showed that the reported allergen of *J. ashei* pollen extracts was released in large quantities within the first minute of extraction. It was also determined that individual pollen grains of *P. echinata* contained a greater quantity of protein than the pollen of *J. ashei* and *J. virginiana*, but due to the large size of pine pollen there was less protein per gram of pollen. Lipid analysis of these three taxa showed that the pollen of *P. echinata* contained more lipid per grain and per gram of pollen. Results indicate that the rapid release of the reported allergen from *J. ashei* pollen contributes to the allergenicity of this species compared to both *J. virginiana* and *P. echinata*. © 1997 Elsevier Science Ireland Ltd.

Keywords: Conifer pollen allergens; *Juniperus ashei*; *Juniperus virginiana*; *Pinus*; Allergen release

1. Introduction

In 1971, Wodehouse (1971) defined criteria for an allergenic plant. To be allergenic its mode of pollination must be entirely anemophilous and its pollen must be buoyant, abundant and allergenic. Although plants belonging to the order Coniferales meet three of Wodehouse's requirements, only the family Cupressaceae is regarded to be a significant source of airborne allergens, with *Juniperus ashei* (mountain cedar), *J. pinchotii* (Pinchot's juniper), and *Cryptomeria japonica* (Japanese cedar) characterized as the most important aeroallergens (Weber and Nelson, 1985).

J. ashei pollen has been recognized since 1929 as a major winter aeroallergen in central Texas and is considered the most important allergenic species in the genus *Juniperus* (Black, 1929; Wodehouse, 1971; Pence et al., 1976). In addition, Ramirez (1984) reported that the pollen of *J. ashei* was a major aeroallergen in New Mexico and Northern Mexico. This species is distributed throughout central Texas, New Mexico, Northern Mexico, the Arbuckle Mountains of South Central Oklahoma, and the Ozark Mountains of Northern Arkansas and Southwestern Missouri (Ramirez, 1984; Levetin and Buck, 1986; Adams, 1993). Although there are no populations of *J. ashei* in the Tulsa area, Levetin and Buck (1986) reported the presence of this pollen in the Tulsa atmosphere due to transport by southerly winds from populations in the Arbuckle Mountains and possibly Texas. A 40–50 kDa

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glycoprotein has been identified as the major allergen in *J. ashei* pollen extracts (Gross et al., 1978; Budens et al., 1989; Goetz et al., 1989; Schwietz et al., 1989).

It has been known since 1931 that patients allergic to *J. ashei* pollen skin tested (intradermal) positive to the extracts from *J. virginiana* (Eastern redcedar) pollen (Kahn and Grothaus, 1931). Later, Yoo et al. (1975) confirmed this cross-reactivity using immuno-diffusion with rabbit antisera and patient skin testing, scratch and intradermal. In 1975, Lewis and Imber studied a population of atopic individuals to find the rate of allergenicity to tree pollen. These individuals were divided into three groups (PS1, PS4, PS5) and were skin tested with various tree pollen extracts. Only one group (PS1) was tested (intracutaneously) with the extract of *J. virginiana* pollen, the only non-angiosperm. Lewis and Imber (1975) reported that, of the 1307 persons in PS1, 17.3% exhibited strong reactivity. However, Lewis and Imber (1975) did not include these results in their calculations of reactivity level, reaction frequency, or correlation coefficients.

Juniperus virginiana is the most widely distributed conifer in the eastern half of the United States (Adams, 1993). In Oklahoma, this species is abundant throughout the state except the panhandle (Levetin and Buck, 1980). However, despite the geographic abundance and the high concentration of atmospheric pollen of this species and the cross-reactivity with *J. ashei*, *J. virginiana* pollen is not considered an important aeroallergen (Kahn and Grothaus, 1931; Yoo et al., 1975; Levetin and Buck, 1980; Lewis et al., 1983; Adams, 1993). Lewis et al. (1983) suggested that either the previous reports concerning the allergenicity of *J. virginiana* pollen are invalid or there is limited exposure to the pollen. However, more recently, Lewis et al. (1991) suggested that due to the high atmospheric concentrations of Cupressaceae pollen, mainly from *Juniperus*, pollen from this family should not be overlooked as a possible source of airborne allergens.

Pinus, in the family Pinaceae, is one of the most widely distributed genera in the Northern hemisphere (Kral, 1993). Four species of *Pinus* occur in Oklahoma with large populations of *P. taeda* (loblolly pine) and *P. echinata* (yellow pine) (Levetin and Buck, 1980). Although there have been several case studies and population studies showing that pollen extracts from this genus give positive skin test results, *Pinus* pollen is still not considered to commonly cause clinical symptoms (Walker, 1921; Rowe, 1939; Newmark and Itkin, 1967; Kinnas, 1971; Wodehouse, 1971; Lewis et al., 1983; Harris and German, 1985; Kalliel and Settipane, 1988; Armentia et al., 1990; Freeman, 1993). In fact, it has been used as a negative control for nasal and conjunctival provocation tests and for bronchial allergen tests (Frølund et al., 1986; Hosen, 1990).

Because numerous patients complained of allergies due to pine pollen, Farnham and Vaida (1982) investigated the possibility of pine pollen allergenicity. They randomly chose 1000 patients and skin tested with *P. strobus* (Eastern white pine) pollen extracts. Results showed that 16.5% had positive reactions. Farnham and Vaida (1982) later skin tested 100 tree sensitive patients with *P. strobus* pollen extract and 35% exhibited positive reactions. In a later study, they skin tested (prick and intradermal) 1067 new patients with *P. strobus* pollen extract and 27.3% had a positive reaction. These studies suggest that pine pollen allergies need to be investigated further (Farnham and Vaida, 1982; Farnham, 1988).

There have been many theories put forth as to why pine pollen is rarely allergenic. Wodehouse (1971) stated that humans have developed an immunity to pine pollen because pine has been around since the post glacial period; however, this cannot explain the low allergenicity since most allergenic angiosperms have also been around that length of time. Several investigators have hypothesized that the waxy coating on the pollen hinders the processing of the antigen (Howlett et al., 1981; Harris and German, 1985). Others suggest pine pollen is too large to enter the respiratory tract (Armentia et al., 1990). However, the presence of pine pollen in the respiratory tract has been reported in the literature (Michel et al., 1977; Accorsi et al., 1991; Dankaart et al., 1991). Cornford et al. (1990) suggest that low protein content is the reason for *Pinus* pollen being rarely allergenic.

Because of the clinical importance of a few conifers, and the abundance of airborne *Pinus* pollen in some areas, a preliminary aerobiological and biochemical study of conifer pollen was performed (Pettyjohn and Levetin, 1996). Aerobiological results showed that *Pinus* pollen was present in the Tulsa atmosphere approximately 73% of the days from the first week of April to the last week of June during 1989 and 1990 with the highest concentration, 905 grains/m^3, found at Site B on 11 May 1990. However, this concentration was low relative to previously reported *Juniperus* pollen atmospheric concentrations (Chapman and Williams, 1984; Levetin and Buck, 1986; Pettyjohn, 1996; Levetin, 1997).

Comparison of proteins present in the pollen extracts of *J. ashei*, *J. pinchotii*, *J. virginiana*, *C. macrocarpa*, *P. taeda* and *P. echinata* showed that all six extracts shared three proteins with the same molecular weights, one of which was the reported allergen of *J. ashei* (Pettyjohn and Levetin, 1996). Immunoblotting analysis showed binding to the extracts of all six pollen taxa (Pettyjohn, 1996). There was binding to the 42 kDa glycoprotein of *J. ashei*, *J. virginiana*, and *J. pinchotii*, the 45 kDa protein of *J. ashei* and *P. echinata*, the 50 kDa protein of *J. pinchotii*, *C. macrocarpa*, *P. echinata*, and *P. taeda*, and the 66 kDa protein of *P. echinata*.

Many studies have reported that the allergen or allergens of various species are released from the grain rapidly and in large quantities (Knox and Heslop-Harrison, 1971; Marsh et al., 1981; Montero et al., 1992; Vrtala et al., 1993; Taylor et al., 1994). Therefore, to investigate possible reasons for the differences in allergenicity a comparative biochemical study of the highly allergenic pollen of *J. ashei* and the rarely allergenic pollen of *J. virginiana* and *P. echinata* was conducted.

2. Materials and methods

2.1. Extraction methods

To determine the rate and quantity of protein released from nondefatted pollen of *J. ashei*, *J. virginiana*, and *P. echinata* (Greer Laboratories, Lenoir, NC), a timed extraction was performed. Nondefatted *J. ashei* pollen and *J. virginiana* pollen (100 mg) were analytically weighed (Mettler AE163 analytical balance) into nine separate microfuge tubes that were separately placed into a balsa wood holder (one tube for each extraction interval, 1, 5, 10, 60, 120, 240, 360, 480, and 1440 min). Then 1 ml of 0.125 M ammonium bicarbonate (NH_4HCO_3, pH 8.1) was added to each tube, and the tubes rotated at 4°C for each time interval. The pollen mixture was centrifuged for 30 s at $14\,000 \times g$ using an Eppendorf microfuge. The supernatants were collected and the pollen pellets discarded. Because *Pinus* pollen floats, a different procedure was used. Nondefatted *P. echinata* pollen (200 mg) was analytically weighed into nine separate 15 ml tubes, one tube for each extraction interval as above. Extraction was performed by rotating the pollen in 2 ml of 0.125 M NH_4HCO_3 (pH 8.1) buffer at 4°C for the specified extraction interval. After rotating, extracts were collected from each tube using a 45 μm, 1000 μl pipetman tip filter (Danville). The amount of protein in the extracts were analyzed using a Lowry's protein assay kit (P5656, Sigma, St. Louis, MO). The experiment was repeated seven times.

To ascertain the quantity of lipid per grain of pollen of *J. ashei*, *J. virginiana* and *P. echinata*, 100 mg of these three taxa were analytically weighed separately into individual glass vials. Ethyl ether (5 ml) was added to each vial and the ether/pollen mixtures were rotated at room temperature for 18 h. The ether was extracted from the pollen of *J. ashei* and *J. virginiana* using a 5 ml glass syringe and a Millex-GS 0.22 μm filter (Sigma, St. Louis, MO) and placed into separate preweighed glass vials. These vials were left overnight at room temperature in a hood to allow all the ether to evaporate. After evaporation, the vials were reweighed. The experiment was repeated four times. The same procedure was used for *P. echinata* pollen, however, the ether was extracted using a 22 μm 1000 μl pipetman tip filter (Danville).

2.2. Protein analysis

To determine when specific proteins were eluted during the timed extraction, SDS-PAGE was used. Proteins of *J. ashei* timed extracts and *J. virginiana* timed extracts were precipitated from 50 μl of extract by adding 250 μl of 100% ethanol which had been previously cooled to −20°C. The ethanol/extract mixture was kept at −20°C overnight. Next, these mixtures were centrifuged at $14\,000 \times g$ for 30 min in an Eppendorf microfuge at 4°C. After centrifugation, the supernatants were discarded and the protein pellets were allowed to air dry to evaporate any remaining ethanol. The pellets were resuspended in 80 μl of sample buffer (0.0625 M Tris, 10% glycerol, 2% SDS, and 5% 2-β-mercaptoethanol) and loaded onto a 12.5% discontinuous gel that was prepared based on Laemmli (1970) method. Electrophoresis was performed using a Bio-Rad Protean II Slab Cell (Hercules, CA). The molecular weight markers were silver stain SDS-PAGE low range standards (Bio-Rad, Hercules, CA). Gels were silver stained as described in Table 1 (Reese and Horner, 1993, personal communication). A similar procedure was used to precipitate the extracts of *P. echinata*, but 200 μl of extract and 1 ml of 100% ethanol was used.

2.3. Hemacytometer analysis

The number of pollen grains in a mg of pollen was established using the hemacytometer method. *J. ashei* pollen (5 mg) were analytically weighed into a microfuge tube, and 1 ml of FAA (formalin, acetic acid, alcohol) was added. Of the pollen/FAA mixture 10 μl was placed onto a hemacytometer and the number of pollen grains in the large and small squares were counted 17 times at 200×. A similar procedure was used to calculate the number of grains in a mg of *J. virginiana* pollen but 3 mg pollen were used for this species. *P. echinata* pollen (10 mg) was analytically weighed in the same way as for *J. ashei* and *J. virginiana*. A 50:50 solution (1 ml) of distilled water to Tepol was added to the pollen and calculation of the amount of pollen per mg was performed in the same manner.

3. Results

3.1. Soluble pollen protein content

The rate of protein elusion from the pollen of these three taxa showed considerable differences. *J. ashei* pollen released a greater quantity of protein within the

Table 1
Silver stain protocol

Steps	Procedures
1.	Place gel in 200 ml fixing solution (300 ml methanol, 50 g sulfosalicylic acid, 200 g trichloroacetic acid, 1 tab Phast Blue, and dH_2O up to 1000 ml) for 30 min on a rocker.
2.	Add 4 ml glutaraldehyde and 1 g sodium thiosulfate to 200 ml incubation solution[a] (300 ml ethanol, 68.04 g sodium acetate, and dH_2O up to 1000 ml). Decant fixing solution and pour incubation solution over gel. Incubate overnight on a rocker.
3.	Decant incubation solution and rinse gel three times (10 min each wash) in dH_2O on a rocker.
4.	Add 54 μl formaldehyde to 100 ml silver nikate solution[b] (1 g silver nitrate and dH_2O up to 1000 ml) and then pour over gel. Cover with aluminum foil and rock for 1 h.
5.	Add 27 μl formaldehyde to 100 ml developer solution (25 g sodium carbonate, adjust pH to 11.8–11.3 with 2.5 N sodium bicarbonate, dH_2O up to 1000 ml). Decant silver nitrate solution and pour developer solution over gel and rock by hand until bands develop.
6.	Decant developer solution and then pour stop solution (18.61 g EDTA and dH_2O up to 1000 ml) over gel. Gel may stay in this solution until ready to dry.

[a] Store at 4°C.
[b] Must be light protected, either store in a brown bottle or wrap clear bottle in aluminum foil. Store at 4°C.

first minute (15.5 mg/g pollen) of moistening than either *J. virginiana* or *P. echinata* pollen (Fig. 1 and Table 2). In fact, *J. ashei* pollen continued to release more protein per gram than the other two pollen types during the first 60 min. During the first minute of extraction, *J. ashei* pollen released 42% of its protein, whereas only 10% was eluted from *J. virginiana* pollen and *P. echinata* pollen released just 17%. Increasing amounts of protein were eluted from the pollen of *J. ashei* and *J. virginiana* during the entire 1440 min extraction procedure, but the extract of *P. echinata* pollen started to lose protein after 480 min. After 120 min, *J. virginiana* pollen released more protein per gram than either *J. ashei* or *P. echinata* pollen and continued to do so through the remainder of the time. However, *J. virginiana* pollen did not release the same percent of protein (57%) as *J. ashei* pollen until 240 min. By contrast, the pollen of *P. echinata* released 57% of its protein at 5 min, but the overall amount of protein/g was less than *J. ashei* pollen at this time. *J. ashei* pollen and *J. virginiana* pollen both had approximately a 30% increase of protein released between the 480 and 1440 min extraction, but *P. echinata* pollen lost 4% of its protein at the 1440 min time (Fig. 1 and Table 2). Overall, *J. virginiana* pollen released more protein (Fig. 1 and Table 2) at 1440 min time interval (41.4 mg/g pollen) than the pollen of *J. ashei* (37.2 mg/g pollen) or *P. echinata* (13.3 mg/g pollen).

3.2. Protein analysis

SDS-PAGE showed that approximately 13 water soluble proteins were present in the extracts of *J. ashei* and *J. virginiana* pollen, and approximately 20 in the extract of *P. echinata* pollen. All proteins were released from the pollen of *J. ashei*, *J. virginiana*, and *P. echinata* within the first minute (Figs. 2–4). The predominant proteins in all timed extracts of *J. ashei* had masses of 45, 42, 31 and 14 kDa (Fig. 2). The 42 kDa protein was found to be a glycoprotein as shown by the Periodic Acid-Schiff test (data not shown). The 31 kDa polypeptide appeared to be two proteins as evident by the separation of the bands in the 1 and 1440 min lanes. As shown in Fig. 3, the dominant proteins released in the first 10 min from *J. virginiana* pollen had masses of 42 and 26 kDa. After 60 min, the predominant proteins of *J. virginiana* extracts had weights of 66, 45, 42, 31, 26, 19, and 12 kDa. Of these, the 42 kDa polypeptide is a glycoprotein as shown by the Periodic Acid-Schiff test (data not shown). The dominant proteins found in the 1 min extract of *P. echinata* had weights of 66 kDa and 45 kDa (Fig. 4). After 5 min, the additional principal proteins had masses of 85, 77, 60, 55, 14, and 13 kDa. The 42 kDa protein was barely visible in all timed extracts. Of the three taxa examined, *P. echinata* contained a greater number of protein bands (Fig. 4).

3.3. Lipid extraction

Ether extraction of these three pollen types showed there was a difference in the amount of extractable lipid. As shown in Table 3, there was 3.0×10^{-3} mg of lipid extracted from 1 g of *J. ashei* pollen, 5.0×10^{-3} mg of lipid extracted from *J. virginiana* pollen, and 1.4×10^{-2} mg of lipid extracted from 1 g of *P. echinata* pollen. Of these three pollen types, 1 g of *P. echinata* pollen contained a much greater quantity of lipid with 1 g of *J. ashei* pollen containing the least (Table 2).

3.4. Amount of protein and lipid per grain

Analysis of the hemacytometer counts showed there was variation in the quantity of grains per mg of pollen of *J. ashei*, *J. virginiana*, and *P. echinata* and therefore in the weight of an individual grain of these three taxa (Table 4). *J. ashei* pollen (1 mg) contained 2.18×10^5

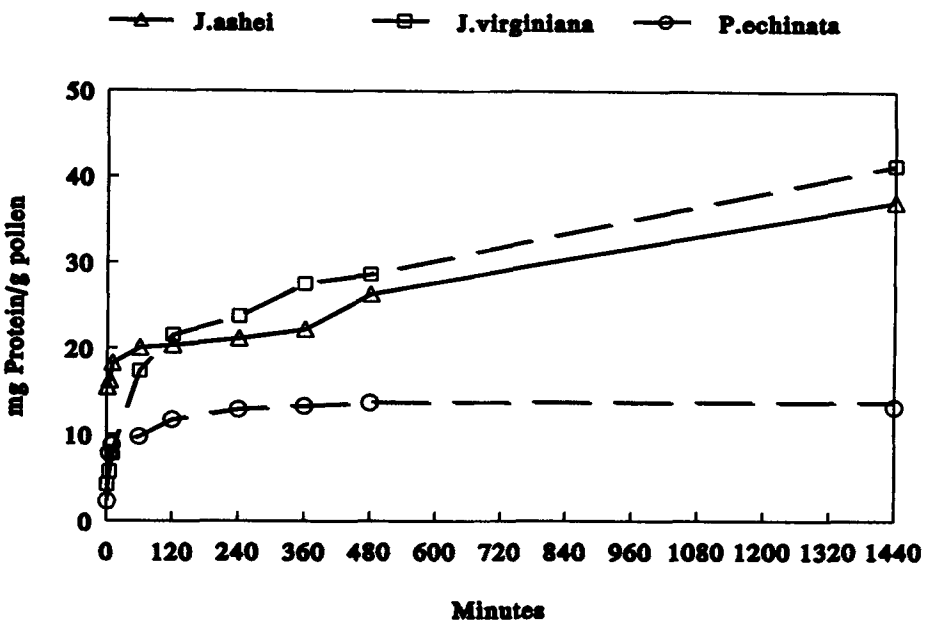

Fig. 1. Timed extraction of nondefatted pollen of *J. ashei*, *J. virginiana*, and *P. echinata*. Results are the mean of seven experiments.

grains. In 1 mg of *J. virginiana* pollen there were 3.63×10^5 grains, while 1 mg of *P. echinata* pollen contained 4.50×10^4 grains. Therefore, of these three species, *J. virginiana* pollen was the lightest at 2.8×10^{-6} mg.

The quantity of extractable protein per grain was different for each pollen type analyzed. As shown in Table 5, the pollen of *J. ashei* contained 1.69×10^{-7} mg of extractable protein per grain. The amount of extractable protein per grain of pollen of *J. virginiana* was found to be 1.14×10^{-7} mg. Extracts of *P. echinata* pollen contained 3.07×10^{-7} mg of protein per grain (Table 5). Therefore, the pollen of *P. echinata* contained the greatest amount of protein and *J. virginiana* pollen had the least. However, the amount of extractable protein as a percentage of mass showed that *P. echinata* pollen had the least with the pollen of *J. virginiana* having the greatest (Table 5).

Although the amount of extractable lipid per grain of pollen of *J. ashei* and *J. virginiana* was 1.38×10^{-11} mg, the percent of extractable lipid per grain of these two pollen types as a percentage of total mass was 0.0003 and 0.0005, respectively (Table 5). The pollen of *P. echinata* had 3.11×10^{-10} mg of exactable lipid per grain and the percentage of total mass was 0.0014. Of these three pollen types, the pollen of *P. echinata* had the greatest quantity and percentage of total mass of extractable lipid.

Table 2
Estimation of protein released from the pollen of *J. ashei*, *J. virginiana*, and *P. echinata*

Extraction time (min)	Mg protein extracted		
	J. ashei	*J. virginiana*	*P. echinata*
1	15.5 (42) ± 0.6	4.2 (10) ± 0.2	2.3 (17) ± 0.3
5	16.3 (44) ± 0.3	5.7 (14) ± 0.4	7.8 (57) ± 0.2
10	18.3 (49) ± 0.2	7.8 (19) ± 0.7	8.6 (62) ± 0.2
60	20.1 (54) ± 0.4	17.4 (42) ± 0.8	9.8 (71) ± 0.4
120	20.4 (55) ± 0.4	21.6 (52) ± 0.4	11.8 (86) ± 0.3
240	21.2 (57) ± 0.2	23.8 (57) ± 0.4	13.1 (95) ± 0.5
360	22.3 (60) ± 0.4	27.6 (67) ± 1.1	13.4 (97) ± 0.3
480	26.4 (71) ± 0.3	28.7 (69) ± 0.8	13.8 (100) ± 0.2
1440	37.2 (100) ± 0.8	41.4 (100) ± 0.6	13.3 (−4) ± 0.2

Values in parenthesis are percent extracted.
Results are the mean ± S.D. from seven experiments.

4. Discussion

Many studies have reported that the allergen or allergens of various species are released from the grain rapidly and in large quantities (Knox and Heslop-Harrison, 1971; Marsh et al., 1981; Montero et al., 1992; Vrtala et al., 1993; Taylor et al., 1994). Particles are cleared from the nasopharynx area by mucociliary flow within 5–10 min and remain in the larynx for approximately 20 min before entering the gastrointestinal tract (Proctor et al., 1973; Marsh et al., 1981; Ong et al., 1995). According to Baranuik et al. (1988), "the nasopharynx is the site of maximum exposure to rapidly released solutes". Therefore, the rapid release of the allergen is an important factor in the allergic reac-

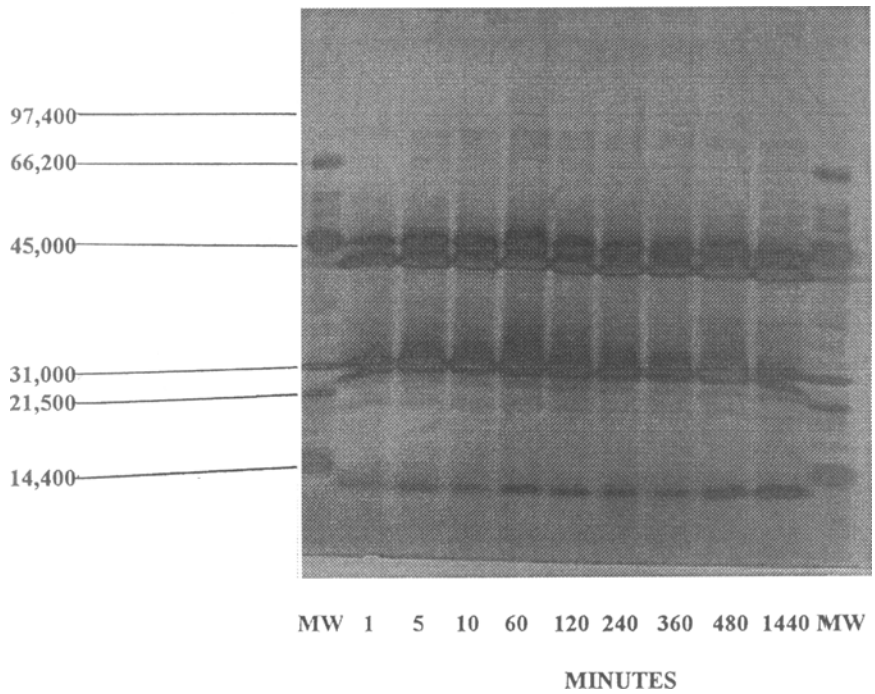

Fig. 2. Proteins present in the timed extracts of *J. ashei* pollen. MW, molecular weight standards.

tion. Data from this study shows that in the first 10 min the pollen of *J. ashei* releases 2–3 times more protein than the pollen of *J. virginiana* and *P. echinata* (Fig. 1 and Table 2). SDS-PAGE analyses showed that *J. ashei* pollen released a significantly greater amount of the 42 kDa glycoprotein than *J. virginiana* pollen within the first 60 min (Figs. 2 and 3). Findings from this study seem to suggest that one reason for the lower allergenicity of the pollen of *J. virginiana* and *P. echinata* might be due to the different rates of allergen release from the grain.

A possible explanation for the slower release of the allergen could be due to differences in the rate of exine rupturing in the pollen of *J. virginiana* compared with *J. ashei* pollen. When pollen from members of the Cupressaceae are placed in an aqueous environment, the intine swells and ruptures the exine. A timed experiment comparing the rupturing kinetics of *J. virginiana* and *J. ashei* and the relationship to the allergen release may validate this explanation. An ultrastructure comparison of the pollen walls of *J. ashei* and *J. virginiana* showed that the sexine of *J. virginiana* was denser than that of *J. ashei* (Pettyjohn, 1996). This too might contribute to the slower release of protein from the pollen of *J. virginiana*.

Immunoblotting analysis (Pettyjohn, 1996) showed binding to the 42 kDa glycoprotein of *J. virginiana* and *J. ashei* pollen extracts suggesting it is the same protein. The slower release of this protein from *J. virginiana* and *P. echinata* may account for the lower allergenicity of this species. Although there is a slower release of extractable protein from *J. virginiana* pollen, aerobiological data showed high concentrations (Levetin and Buck, 1986; Pettyjohn, 1996; Levetin, 1997). These higher concentrations may not compensate for the slower release even though more grains are being inhaled.

Cornford et al. (1990) suggested that the low protein content of *Pinus* pollen might be the reason it is rarely allergenic, however, data from this study shows that the individual pollen grains of *P. echinata* contain a greater quantity of extractable protein than the other two pollen types studied (Table 5). However, it must be stated that in the previous study the pollen was extracted under agitation and in this study extraction proceeded with rotation. Both procedures allowed the majority of individual pollen grains to stay in contact with the extraction buffer and, therefore, may not simulate the natural environment of the nasal passage.

Howlett et al. (1981) and Harris and German (1985) postulated that the waxy coating of *Pinus* pollen might be a reason for its low allergenicity. Results from the ether extraction of pollen of *J. ashei*, *J. virginiana*, and *P. echinata* seem to support this hypothesis. The pollen of *P. echinata* contains a 10-fold greater amount of extractable lipid per grain than the other two pollen

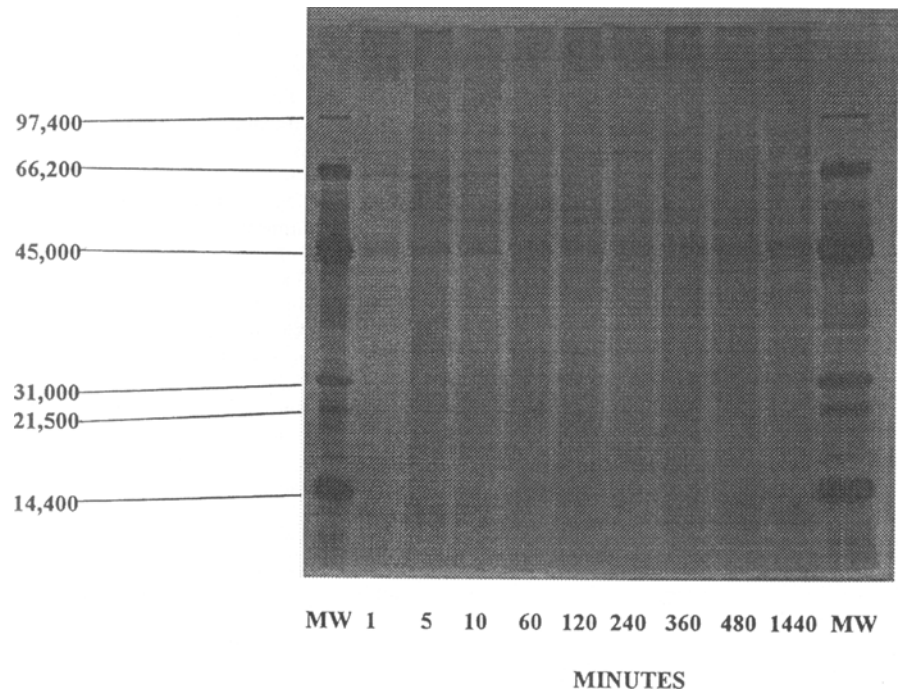

Fig. 3. Proteins present in the timed extracts of *J. virginiana* pollen. MW = Molecular weight standards.

types (Table 5) and may result in a slower release of the allergenic proteins and therefore explain the hypoallergenicity of *Pinus* pollen. Immunoblotting analysis (Pettyjohn, 1996) showed binding to proteins of *P. echinata* and *P. taeda* pollen extracts which suggests there are IgE antibodies specific for or cross-reactive with *Pinus* pollen, thus there needs to be a re-evaluation of *Pinus* pollen being used as a negative control.

Aerobiological data (Pettyjohn and Levetin, 1996) showed that the atmospheric concentration of *Pinus* pollen in Tulsa was low relative to previously reported atmospheric concentrations of *Juniperus*/Cupressaceae

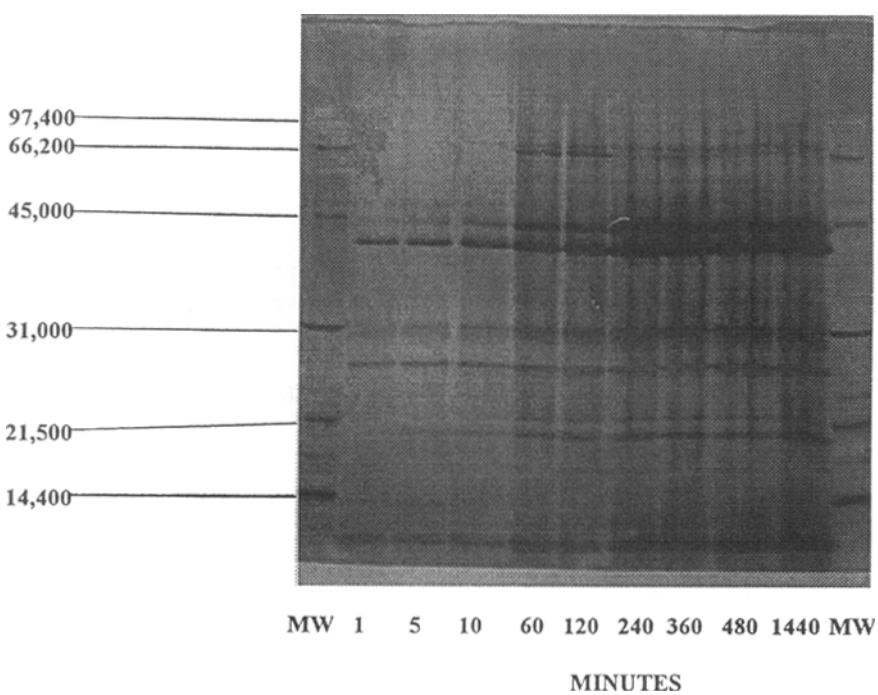

Fig. 4. Proteins present in the timed extracts of *P. echinata* pollen. MW = Molecular weight standards.

Table 3
Estimation of the amount of lipid in 1 g pollen of *J. ashei*, *J. virginiana*, and *P. echinata*

Pollen	Mg lipid extracted
J. ashei	$3.0\times10^{-3}\pm1.0\times10^{-4}$
J. virginiana	$5.0\times10^{-3}\pm1.2\times10^{-3}$
P. echinata	$1.4\times10^{-2}\pm1.0\times10^{-4}$

Results are the mean ± S.D. of four experiments.

pollen (Levetin and Buck, 1986; Pettyjohn, 1996; Levetin, 1997). Others have also reported low atmospheric concentrations of *Pinus* pollen (Fountain and Cornford, 1991; Silvers et al., 1992). Freeman notes that the individuals who tested positive for *Pinus* pollen allergies lived at least 2 years in an area heavily populated with *Pinus*. In fact, Farnham and Vaida (1982) and Farnham (1988) reported a high incidence of positive reactions to *P. strobus* pollen extracts in Massachusetts, a state with large expanses where pine predominates (Little, 1971). The low atmospheric concentration, slower release of possible allergens, and high amount of extractable lipid all might contribute to the low allergenicity of *Pinus* pollen.

5. Conclusion

In conclusion, this is the first study to compare the biochemical differences between a highly allergenic pollen type (*J. ashei*) and related but rarely allergenic pollen types (*J. virginiana* and *Pinus echinata*). The time course extraction showed that *J. ashei* pollen released a greater amount of protein within the first minute of moistening than the other two pollen types. In addition, the quicker release of allergenic protein and the lower amount of lipid may explain the high allergenicity of *J. ashei* pollen. Therefore, results from this study seem to suggest another postulate be added to Wodehouse's definition of an allergenic plant. The pollen must release the allergen or allergens in large quantities within 5–10 min.

Table 4
Estimation of quantity of pollen grains in 1 mg of *J. ashei*, *J. virginiana*, and *P. echinata*

Pollen	Pollen grains/mg	Estimated weight of a single pollen grain (mg)
J. ashei	$2.18\times10^{5}\pm1.0\times10^{4}$	4.6×10^{-6}
J. virginiana	$3.63\times10^{5}\pm1.3\times10^{4}$	2.8×10^{-6}
P. echinata	$4.50\times10^{4}\pm3.0\times10^{3}$	2.2×10^{-5}

Results are the mean ± S.D. from 17 hemacytometer counts.

Table 5
Estimation of extractable protein and lipid per grain of pollen of *J. ashei*, *J. virginiana*, and *P. echinata*

Pollen	Mg protein per pollen grain (% per mass)	Mg lipid per pollen grain (% per mass)
J. ashei	1.69×10^{-7} (3.67)	1.38×10^{-11} (0.0003)
J. virginiana	1.14×10^{-7} (4.07)	1.38×10^{-11} (0.0005)
P. echinata	3.07×10^{-7} (1.40)	3.11×10^{-10} (0.0014)

Values in parenthesis are percent of extractable protein or lipid as a percentage of total mass.

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