

ORIGINAL ARTICLE

Pollen proteolytic enzymes degrade tight junctionsSARAH RUNSWICK,^{1*} THOMAS MITCHELL,^{1†} PAUL DAVIES,^{1,3} CLIVE ROBINSON² AND DAVID R. GARROD¹

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Background and objective: Asthma and allergic rhinitis are significant, increasing causes of morbidity worldwide. Pollen, a major cause of seasonal rhinitis/conjunctivitis, carries proteolytic enzymes on its surface. We showed previously that peptidase allergens from house dust mites compromise epithelial barrier function by degrading the extracellular domains of the tight junction proteins, occludin and claudin, thus facilitating allergen delivery across epithelial layers. In this study, we aimed to determine whether peptidases from allergenic pollens should similarly be considered to have a role in disrupting tight junctions.

Methods: Diffusates from stored pollen of Giant Ragweed, White Birch and Kentucky Blue Grass, and fresh pollen from Easter Lily were applied to confluent monolayers of Madin-Darby canine kidney (MDCK) and Calu-3 cells in serum-free medium. Immunofluorescence was performed for the tight junction proteins, occludin, claudin-1 and ZO-1. The effect of pollen diffusate on occludin was studied by Western blotting, and enzymatic activity in the diffusates was demonstrated by zymography. The ability of protease inhibitors to block the action of the diffusate on tight junctions was investigated.

Results: Diffusates from all four allergenic pollens caused loss of immunofluorescence labelling for tight junction proteins on MDCK and Calu-3 cells. The effect was blocked by inhibitors of serine and cysteine proteases. Degradation of occludin was demonstrated by Western blotting and zymography indicated that diffusates contain proteolytic activity.

Conclusions: Pollen peptidases directly or indirectly disrupt epithelial tight junctions, and this activity should be considered as a possible mechanism for facilitating allergen delivery across epithelia.

Key words: allergy, asthma, pollen, proteolytic enzyme, rhinitis, tight junction.

INTRODUCTION

Tight junctions, the apical element of the epithelial junctional complex, are a key component of epithelial

barriers. Their transmembrane proteins, occludin and claudins, form a selectively permeable seal that regulates the passage of molecules through the paracellular channels.¹ Tight junctions provide a barrier to the entry of allergenic molecules, but this may be compromised by environmental peptidases that degrade tight junction proteins.

Asthma and allergic rhinitis are significant and increasing causes of morbidity worldwide.² Atopic individuals have a heightened predisposition to developing IgE-mediated hypersensitivity responses to specific allergens, most commonly airborne allergens such as house dust mite faecal pellets and pollen.^{3,4} Such responses may be chronic or seasonal in manifestation, depending on whether the sensitizing allergen is continuously or seasonally present in the environment. The mechanism by which such allergens trigger responses is poorly understood.

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Pollen, a major cause of seasonal rhinitis/conjunctivitis, carries on its surface a number of proteolytic enzymes, which are believed to be involved in functional aspects of the fertilization process in plants.⁵ The extent to which these peptidases are allergenic is not well established. Although various enzymes are among the different types of pollen proteins that have been identified as allergens,^{6,7} the majority of this enzymatic activity is directed towards degrading pectin, the major plant cell wall polymer of galacturonic acid. However, it has been shown that peptidases released by a variety of allergenic pollens cause detachment of epithelial cells from the substratum in tissue culture.^{8,9} Thus, pollen peptidases may damage airway epithelium, thereby facilitating a non-specific increase in epithelial permeability and allowing allergens access to the immune system to initiate or exacerbate an allergic response.

We have shown previously that peptidase allergens from house dust mites compromise epithelial barrier function by degrading the extracellular domains of occludin and claudin.^{10–14} It was demonstrated that tight junction degradation facilitated allergen delivery across epithelial cell layers and that delivery was blocked by inhibiting the proteolytic activity of the house dust mite enzymes. Thus, the degradation of tight junctions may be a key initial step in sensitization to airborne allergens, permitting foreign proteins access to the immune system to initiate an allergic response.

In order to determine whether peptidases in pollen have a similar effect on tight junctions, we exposed confluent monolayers of epithelial cells to diffusates of allergenic pollens and determined the effect on the major tight junction proteins occludin, claudin-1 and ZO-1 by immunofluorescence labelling. Striking disruption of tight junctions was observed, suggesting that pollen peptidases may also be key factors facilitating allergic sensitization.

METHODS

Preparation of pollen diffusates

Pollen from Giant Ragweed (GRW; *Ambrosia trifida*), Kentucky Blue Grass (KBG; *Poa pratensis*) and White Birch (WB; *Betula pendula*) were purchased from Sigma (Poole, Dorset, UK). Pollen from the Easter Lily (EL; *Lilium longifolium*) was harvested directly from stamens as it began to detach freely. Samples of pollen were suspended at a concentration of 100 mg/mL in either Dulbecco's modified Eagle's medium (DMEM) or Hanks' Balanced Salt Solution (HBSS) (Invitrogen, Paisley, UK) and incubated at 4°C with regular agitation for 30 min to 2 h. The suspension was then centrifuged at 10 000 g for 10 min, and the supernatant was collected, filtered through a 0.22- or 0.45-µm filter (Millex, Millipore, Watford, UK), and either used immediately or stored in aliquots at –20°C. Once thawed, aliquots were either used immediately or discarded.

Cells and cell culture

Madin-Darby canine kidney (MDCK) cells were used to assay the effects of pollen diffusates on tight junctions, as their tight junctions are well characterized and have been used previously for similar assays, yielding similar results to human airway epithelial cell lines.^{10–14} The cells were maintained in DMEM plus 10% foetal bovine serum (FBS) plus 50 U/mL penicillin and 50 µg/mL streptomycin (Cambridge Bio-Sciences Ltd, Cambridge, UK). For treatment with pollen diffusates and immunofluorescence, cells were seeded at confluent density on 13-mm-diameter glass coverslips in 24-well tissue culture plates (Corning—SelectScience Ltd, Bath, UK) and maintained in culture for 30 h.

Calu-3 (human airway epithelial) cells were propagated in Eagle's minimum essential medium with Earle's salts (EMEM), supplemented with 10% FBS, 2 mmol/L L-glutamine, non-essential amino acids, 10 µmol/L sodium pyruvate, 50 U/mL penicillin and 50 µg/mL streptomycin.

Treatment of cells with pollen diffusates

The medium was removed from confluent cells on coverslips in 24-well plates, and the cells were washed thoroughly with serum-free DMEM (SFM). Pollen diffusates at the appropriate concentration in SFM (see individual experiments) were then applied to the cells in a total volume of 400 µL per well, and the plate was maintained at 37°C for the requisite time.

Examination of tight junctions by immunofluorescence

The culture medium was removed from the cells, which were washed rapidly and thoroughly with HBSS or PBS before fixation in a 1:1 mixture of methanol and acetone at –20°C for 10 min. The coverslips were then washed thoroughly in PBS and either stained immediately or stored at –20°C until required. Before staining, the coverslips were treated with blocking buffer (10% normal goat serum, 2% Triton X-100, 0.5% Tween 20, v/v in PBS containing 0.01 mM Na₃N₃) for 30 min at room temperature. Coverslips were mounted in gelvatol with phenylenediamine as anti-bleaching agent and examined on a Zeiss Axioplan Microscope using a X63 plan-apochromat oil immersion objective (numerical aperture 1.40; Welwyn Garden City, Herts, UK). Images were captured with a MicroMAX digital CCD camera (Princeton Instruments Inc., c/o Roper Scientific, Marlow, Bucks, UK) and MetaMorph software (Universal Imaging Corporation, Downingtown, PA, USA). Images were further processed using Adobe PhotoShop. All experiments were repeated a minimum of three times. In order to avoid inter-observer bias, images were scored blind by six independent observers on a sliding scale of 1–10 for loss of tight junction staining, where 1 = no change from

untreated control and 10 = complete obliteration. Series for which fewer than five observers agreed on rank order were discarded.

Antibodies

Antibodies to occludin, claudin-1 and ZO-1 were purchased from Zymed (San Francisco, CA, USA), antibody to E-cadherin was from BD Biosystems (Oxford, UK), and antibody to desmocollin was JB1.¹⁵ Secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

Western blotting

Cells grown in 25-cm² flasks were lysed in hot Laemmli sample buffer, scraped from the surface of the flask with a cell scraper, boiled for 5 min, centrifuged for 5 min at 13 000 g, and samples of the supernatant were loaded onto 10% polyacrylamide gels for electrophoresis. Separated proteins were transferred to nitrocellulose membranes for blotting, while identical gels were stained with Coomassie blue to confirm equal protein loading. Nitrocellulose membranes were incubated overnight in 5% dried milk powder in Tris-buffered saline, then sequentially exposed to primary and secondary antibodies with appropriate washing steps, and the reactive polypeptides were visualized by enhanced chemiluminescence.

Zymography

Zymograms were run on 7.5% or 10% polyacrylamide gels impregnated with 0.1% gelatin (Sigma). Samples of pollen extract were prepared in non-reducing sample buffer and incubated at 20°C for 10 min prior to loading onto gels. After electrophoresis (approximately 80 min at 100–120 V), the gels were gently agitated in 2.5% Triton X-100 in distilled water for 30 min, then in 50 mM sodium phosphate buffer, pH 6.8 for 12–16 h at room temperature. Gels were stained with Coomassie blue for 60 min and destained for a further 60 min.

RESULTS

Pollen extracts degrade tight junctions

MDCK cells were first exposed to diffusate from KBG pollen for 4, 6 and 8 h, then fixed and stained for the tight junction proteins occludin, ZO-1 and claudin-1, and examined by fluorescence microscopy. After the longest incubation period, the cell monolayers were intact and there was no evidence of cell detachment. After exposure to the extracts for 8 h, the pollen diffusate was removed from sample cultures and replaced with normal tissue culture medium. These cells were then re-incubated for 2 h to determine whether any changes induced by the pollen extracts were revers-

ible. Control cells treated with HBSS alone for 8 h showed continuous peripheral staining for each of the tight junction components, occludin and claudin-1, which are transmembrane proteins, and ZO-1, which is a cytoplasmic protein (Fig. 1, Occ, Cld-1, ZO-1). In the treated cells, the tight junction staining was characterized by breaks in the continuous rings. Such breaks were visible at all time points (Fig. 1, small arrows), and by 8 h, some cell interfaces appeared almost devoid of staining. In the case of claudin-1, loss of peripheral staining was accompanied by the appearance of marked granular or punctate staining within the cytoplasm (Fig. 1, broad arrows) at all time points examined. After a 2-h recovery period, intact rings of occludin and ZO-1 staining were restored, but punctate cytoplasmic staining for claudin-1 persisted. Parallel experiments in which cells were stained for transmembrane adhesion molecules of other junction types, desmocollin of desmosomes and E-cadherin of zonulae adherentes, showed that these were unaffected by the pollen diffusate during the 8-h incubation period (Fig. 1, Dscs and E-cad). These results are strikingly reminiscent of those obtained previously when cells were treated with peptidase allergens from the house dust mite *Dermatophagoides pteronyssinus*.¹² We conclude that KBG pollen diffusate contains components that directly or indirectly lead to the disruption of tight junctions.

To determine whether such an effect on tight junctions is a general feature of allergenic pollens, MDCK cells were similarly treated with diffusates of commercially available GRW and WB pollens, as well as fresh EL pollen. These diffusates produced disruption of tight junctions similar to that observed with the KBG diffusate (Figs 2,3). In each case, no detachment of the cells from the monolayer was observed, the effects were similarly reversible by re-incubation of the cells in normal tissue culture medium, and no effects on desmosomes or zonulae adherentes were observed (results not shown). These results indicate that agents that promote the disruption of tight junctions are readily liberated from several allergenic pollens.

To determine whether these effects are likely to be important in human airway epithelium, the Calu-3 cell line was treated with diffusates of KBG, WB and GRW pollens. The effects of the most concentrated extracts from pollens (100 mg/mL) were quite severe (Fig. 4); both desmocollin and occludin staining were greatly diminished after 8 h of treatment, and some cell detachment from the monolayers was observed. Therefore, the cells were treated with a series of doubling dilutions of the extracts. The effects on the cells were less severe with increasing dilution of the extracts; at concentrations equivalent to 12.5 and 6.25 mg/mL, there was little effect on desmocollin, but gaps in occludin staining were still evident (Fig. 4, arrows). We conclude that the tight junctions of human airway epithelial cells can be degraded by peptidases in pollen diffusates, and that these tight junctions are more sensitive and/or accessible to disruption than intercellular adhesions of other cell types.

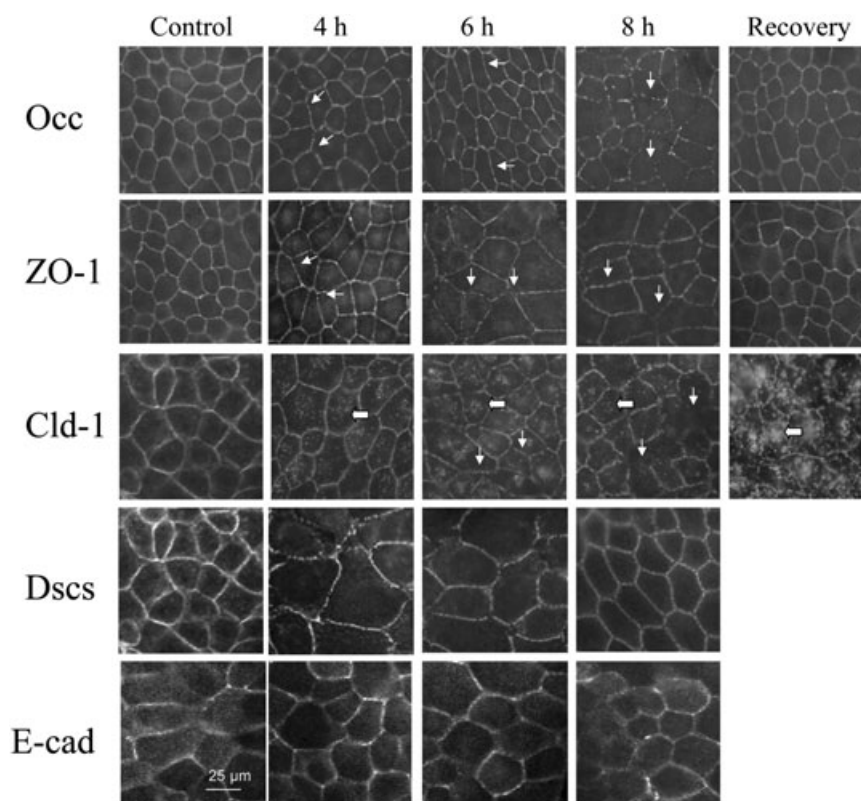


Figure 1 The effect of incubating MDCK monolayers with pollen grain diffusate on the integrity of tight junctions, zonula adherens and desmosomes by indirect immunofluorescence. Monolayers were exposed to diffusate from Kentucky Blue Grass (KBG; suspension concentration of 100 mg/mL) for 4, 6 and 8 h, and then stained for occludin (Occ), claudin-1 (Cld-1) and ZO-1 components of tight junctions (representative images shown). Control incubations were carried out in the absence of diffusate in the medium. In some experiments, the diffusate was incubated with the cells for 6 h, then removed and the cells allowed to recover for a further 2 h (Recovery). Incubation of monolayers with diffusate had no obvious effect on staining for E-cadherin (E-cad; zonula adherens) or desmocollin (Dscs; desmosomes) up to 24 h. Representative images are shown for 4, 6 and 8 h of incubation with KBG diffusate. Small arrows indicate examples of tight junction disruption. Broad arrows indicate punctate staining for claudin-1. All micrographs are shown at the same magnification. MDCK, Madin-Darby canine kidney.

Proteolytic activity in pollen diffusate degrades tight junction protein

In order to confirm that pollen diffusate causes disruption of tight junctions, the effect of KBG extract on occludin of MDCK cells was studied by Western blotting. The antibody used recognizes the carboxy-terminal cytoplasmic domain of the molecule, and thus detects a series of degradation products arising from cleavage of the extracellular domains. The results show degradation products in cells incubated with the diffusate for 4, 6, 8 and 9 h (Fig. 5, bracket). Some degradation was also noted in the untreated control cells (Fig. 5), probably arising from turnover of occludin. Both this and the increased degradation induced by treatment with pollen diffusates were blocked by the cysteine peptidase inhibitor E-64 (Fig. 5). These results indicate that pollen diffusate induces proteolytic degradation of the tight junction protein, occludin, that is consistent with cleavage of the extracellular loops, as the antibody binds to the cytoplasmic region of the protein.

Peptidase inhibitors block the effect of pollen diffusate on tight junctions

To determine whether the effect of pollen diffusate on tight junctions could be blocked by peptidase inhibitors, MDCK cells were treated with KBG diffusate for up to 8 h in the presence of the metallopeptidase inhibitor BB-94, the serine peptidase inhibitor AEBSE, E-64 or soybean trypsin inhibitor. BB-94 did not block the action of KBG diffusate on tight junctions, but each of the other compounds substantially inhibited the disruption of occludin (Fig. 6). These results indicate that inhibition of various classes of proteolytic activity blocks the disruptive effect of pollen diffusate on tight junctions.

Pollen diffusates contain proteolytic enzymes

To demonstrate that the pollen diffusates we used contained proteolytic enzymes, EL and KBG diffusates were analysed by zymography. EL pollen

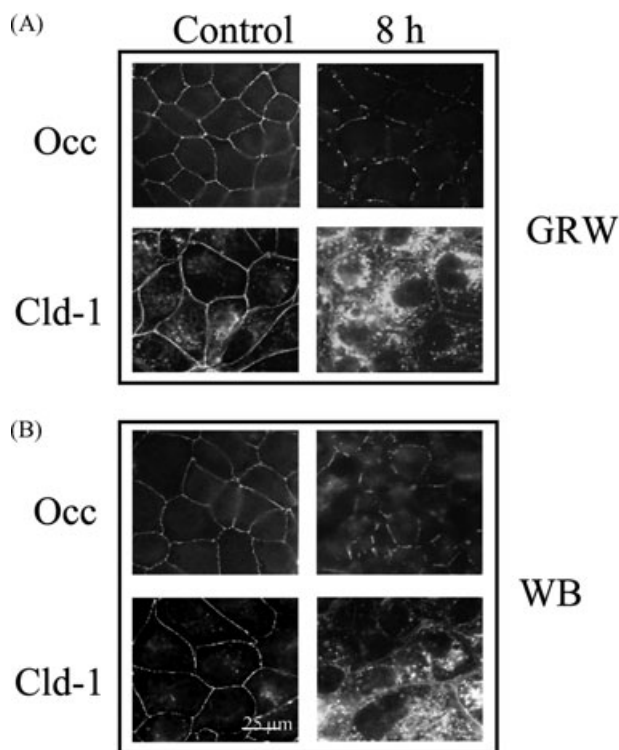


Figure 2 Diffusates from the pollens of White Birch (WB) and Giant Ragweed (GRW) have deleterious effects on the tight junctions of MDCK monolayers. Representative images showing indirect immunofluorescence staining of occludin (Occ) and claudin 1 (Cld-1) after 8 h of incubation with (A) GRW and (B) WB pollen diffusates. Control incubations were carried out in the absence of diffusate. All micrographs are shown at the same magnification. MDCK, Madin-Darby canine kidney.

contained a substantial amount of proteolytic activity with a mobility corresponding to a 45- to 85-kDa protein, and additional activity corresponding to a 20- to 30-kDa protein (Fig. 7A). In KGB diffusate, the principal activity corresponded to 94 kDa, with a substantial amount of weaker activity of lower mobility (Fig. 7B). These results indicate that the pollen diffusates that cause breakdown of tight junctions contain abundant proteolytic activity.

DISCUSSION

Our results show that diffusates from four allergenic pollens disrupt epithelial tight junctions as indicated by loss of immunofluorescence for tight junction proteins. This effect depends upon proteolytic activity within the diffusates as it is blocked by inhibitors of serine and cysteine peptidases. We cannot distinguish between the possibilities that this proteolytic activity directly degrades tight junction proteins or causes their disruption by an indirect mechanism. However, our results with pollen diffusates strongly resemble our previous findings with purified house dust mite peptidase allergens.¹⁰⁻¹⁴ We showed that purified cys-

teine and serine peptidase allergens from *D. pteronyssinus* degrade the tight junctions of MDCK cells and human airway epithelial cells. Loss of tight junctions was demonstrated by decreased immunofluorescence and correlated with increased monolayer permeability to the extracellular marker, mannitol and cleavage of tight junction membrane proteins as shown by Western blotting. Tight junction cleavage and increased permeability were accompanied by enhanced delivery of purified allergen across the epithelial layer. It is likely that these changes resulted from direct cleavage of the extracellular domains of claudin and occludin, because the purified enzymes cleaved peptides corresponding to the extracellular loop sequences of these proteins. The similarity between our current and previous immunofluorescence results suggests the possibility that pollen peptidases similarly damage epithelial tight junctions.

The pollen diffusates had identical effects on tight junction proteins of MDCK cells and Calu-3 human airway epithelial cells. This accords with our previous results with dust mite allergens.¹⁰⁻¹⁴ MDCK cells are the 'gold standard' for tight junction studies because they are easy to grow and have been thoroughly characterized. In addition to the ubiquitous occludin, MDCK cells express claudins 1-4 and 7 as their major tight junction transmembrane proteins.¹⁶ Calu-3 cells clearly express occludin and claudin-1, but their tight junctions have not been fully characterized. We have shown by RT-PCR that they also express claudins 3, 5, 6, 7, 9 and 10, and small amounts of claudins 4 and 14 (S. Runswick and D.R. Garrod, unpubl. obs.). Thus, the junctional composition of these cell types differs, but the common components, occludin and claudin-1, were similarly affected by pollen diffusates. This is to be expected because both are conserved in mammals; occludin shows ~90%,¹⁷ and claudin-1~86% (CLUSTALW alignment of SwissProt database sequences) cross-species amino acid identity. Disruption of these two membrane proteins in these cells is consistent with increased tight junction permeability.¹⁰⁻¹⁴ As Calu-3 cells were affected, it is reasonable to conclude that the results are relevant to human airway epithelium. Indeed, Calu-3 cells were more sensitive to pollen diffusates, showing equivalent disruption at greater diffusate dilution (compare Figs 1,2,4).

Previous work has shown that pollen peptidases can cause substratum detachment of cultured epithelial cells.^{8,9} We found only limited detachment of Calu-3 cells at the highest diffusate concentrations, and it seems unlikely that pollen peptidases reach sufficient concentrations *in vivo* to detach airway epithelial cells. We have achieved widespread effects on tight junctions by bathing cell layers with pollen diffusate, but *in vivo* effects are more likely to be localized. Pollen grains are >10 µm in diameter and probably do not penetrate beyond the upper airways, although pollen fragments may occur as a microaerosol that penetrates the lower airways.¹⁸ A pollen grain or fragment may thus contact the airway epithelium, becoming hydrated and emitting its peptidases onto the apical epithelial surface before being removed by ciliary action. It seems reasonable to propose that

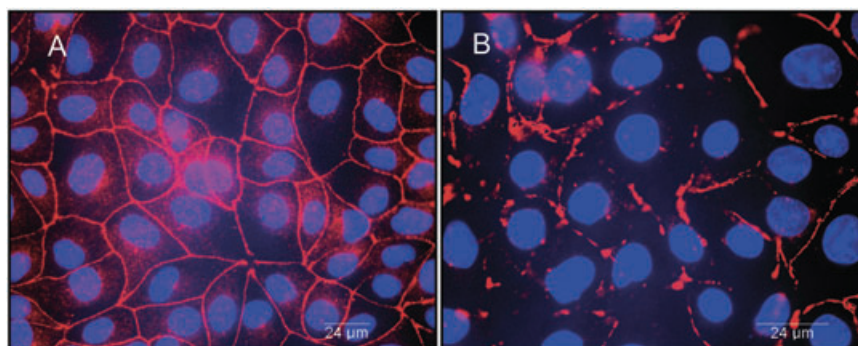


Figure 3 Easter Lily pollen diffusate degrades tight junctions. Immunofluorescence staining for occludin in MDCK cells after exposure to serum-free medium (A) or Easter Lily pollen diffusate (B) for 8 h. Note gaps in staining after exposure to diffusate. Cell nuclei were stained with Hoechst 33258 dye. The micrographs are at slightly different magnifications as indicated by the scale bars. MDCK, Madin-Darby canine kidney.

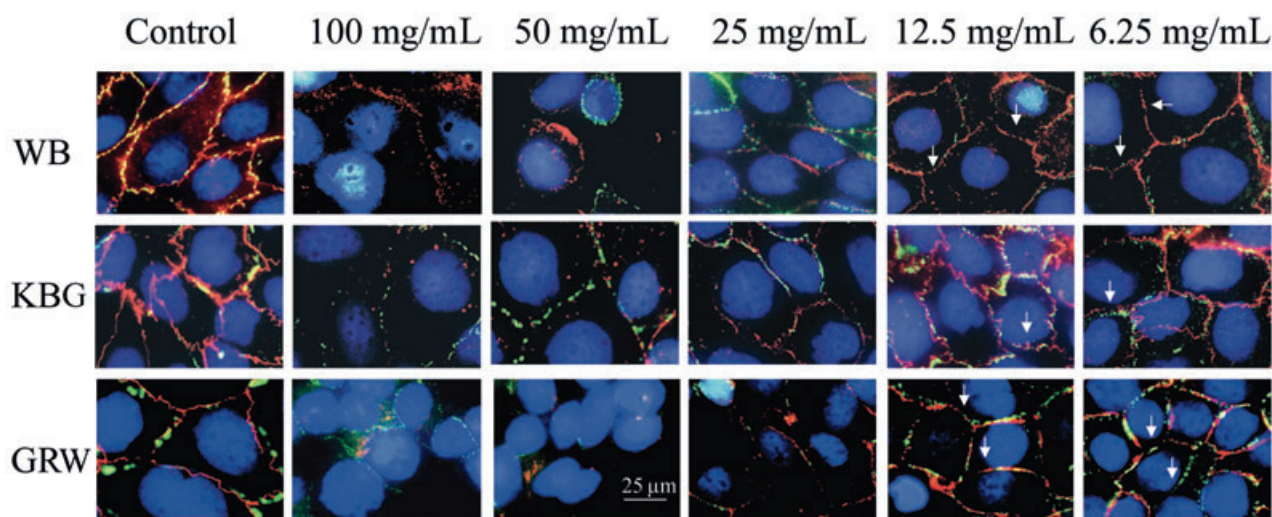


Figure 4 Diffusates from White Birch (WB), Kentucky Blue Grass (KBG) and Giant Ragweed (GRW) degrade tight junctions of a human airway epithelial cell line. Representative images of Calu-3 cells incubated with differing concentrations (shown as initial suspensions of pollen per mL) of diffusate and stained for occludin (red) or desmocollins (green) by indirect immunofluorescence. Incubations were carried out overnight, and control incubations were in serum-free medium. At pollen diffusate, concentrations of 100 and 50 mg/mL staining for both desmocollins and occludin was greatly diminished. At concentrations of 25, 12.5 and 6.25 mg/mL, selective breakdown of occludin occurred as indicated by reduced staining and the appearance of gaps. No difference in staining for desmocollins was observed at these concentrations. Monolayers were counter-stained with Hoechst 33258 dye. All micrographs are shown at the same magnification. Arrows indicate breaks in occludin staining at the two lowest concentrations of diffusate protein.

enzyme concentration would temporarily be sufficient, locally, to damage tight junctions. At present there appears to be insufficient information on the behaviour of pollen grains in the airway and the proteolytic activity associated with individual pollen grains to perform realistic dose calculations. It is also not clear whether extracts of peptidases from commercial sources of stored pollens provide an accurate indication of what occurs with fresh pollen. Therefore, it is interesting that the results obtained with fresh *L. longifolium* pollen were similar to those obtained with the three stored pollens.

Focal degradation of airway tight junctions may facilitate access of allergen to the immune system and

would occur irrespective of whether or not the peptidases themselves are major allergens; the mere presence of active peptidases would be expected to damage tight junctions, facilitating the transepithelial movement of any soluble macromolecules from the lumen. In this context, it has been shown that the proteolytic activity of Der p 1 can drive allergic responses both to Der p 1 itself and to 'bystander allergens' that lack intrinsic peptidase activity.^{19,20}

Promotion of allergen delivery by peptidases may augment the ability of allergens to inactivate antipeptidase defences.²¹ Furthermore, loss of function polymorphisms in endogenous enzyme inhibitor genes (e.g. chromosome 5q32-lymphoepithelial-

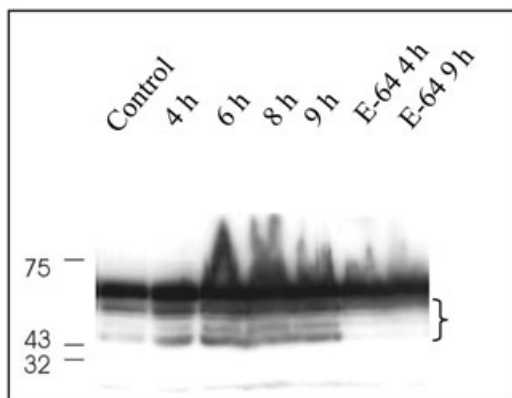


Figure 5 Diffusate from Kentucky Blue Grass (KBG) specifically degrades occludin in tight junctions. Western blotting for occludin in lysates of MDCK monolayers incubated for up to 9 h with 100 mg/mL diffusate. Degradation products of occludin were observed after 6 h of incubation, and these were substantially reduced by the presence of the cysteine protease inhibitor E-64 at 50 μ M (shown at 6 and 9 h time points). Control incubations were carried out in the absence of diffusate. MDCK, Madin-Darby canine kidney.

Kazal-type 5 inhibitor, chromosome 7–plasminogen activator inhibitor-1, chromosome 11–C1 esterase inhibitor, chromosome 14–serpin cluster, chromosome 18q21) predispose to allergic disease.^{22–25} Peptidases from pollens also degrade bioactive peptides that are believed to maintain normal lung function.²⁶

Peptidase allergens contribute to innate immunity and activate cells by IgE-independent mechanisms, including cleavage of tethered ligand receptors. Epithelial cells respond by chronic release of cytokines, including GM-CSF and IL-6, airway levels of which are increased in allergic asthma and rhinitis,^{27–30} and which promote a Th2 allergic bias.^{30,31} Proteolytic activity is therefore associated with both initiation of sensitization and maintenance of the allergic state. Additional consequences of the peptidase activity of these allergens include selective activation of innate immunity and cleavage of cytokine and IgE receptors associated with enhancement of allergic responses.³² Thus, pollen peptidases may contribute to the initiation and development of allergy by other mechanisms in addition to facilitating allergen delivery.

Pollen allergens comprise a diverse collection of proteins with differing functions.^{6,7} It has been suggested that the allergen Phl p 1 from Timothy grass pollen contains cysteine peptidase activity.³³ Other work suggests, however, that apparent peptidase activity in recombinant Phl p 1 arose from contaminants.³⁴ Serine and cysteine peptidase activities were identified in diffusates from several allergenic pollens.³⁵ Diffusates from KBG, rye grass (*Lolium perenne*) and Bermuda grass (*Cynodon dactylon*) contain serine peptidase activity associated with a protein of ~95 kDa.³⁶ An aminopeptidase with specificity for hydrophobic residues and a 92-kDa trypsin-like serine peptidase have been purified from mesquite (*Prosopis velutina*) pollen.³⁷ Similarly, a

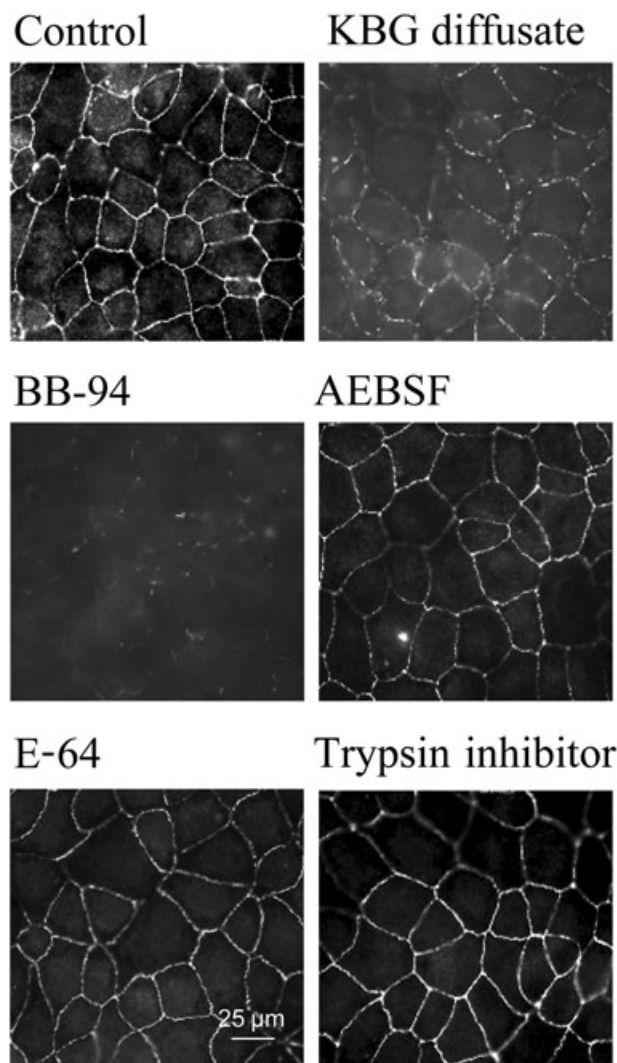
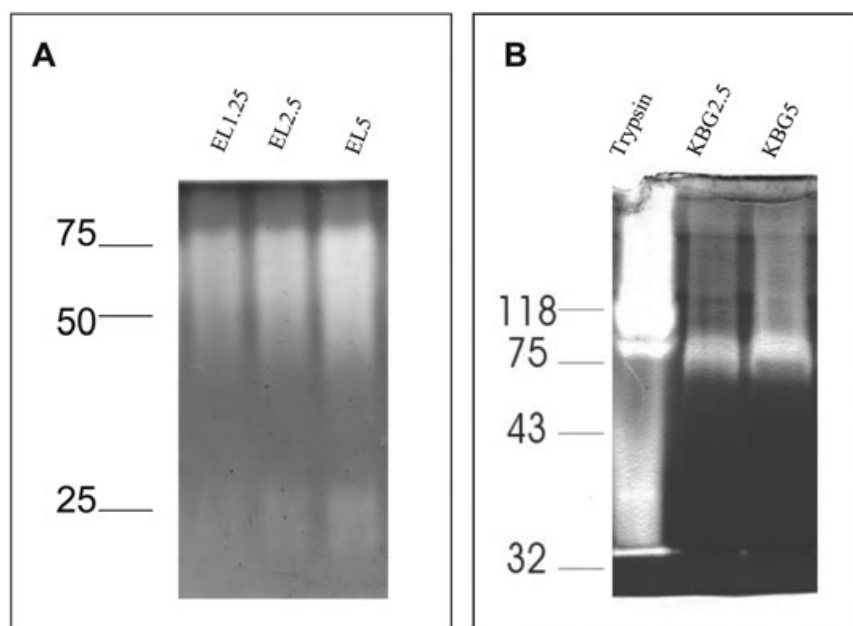


Figure 6 The proteolytic activity of pollen grain diffusates can be inhibited by serine, cysteine and trypsin-like protease inhibitors. Monolayers of MDCK cells were treated with Kentucky Blue Grass (KBG) diffusate (100 mg/mL) for up to 8 h with the addition of protease inhibitors: BB-94 (5 μ M), AEBSF (100 μ M), E-64 (10 μ M) and trypsin inhibitor. The status of tight junctions was observed by staining the cells for occludin by indirect immunofluorescence. All micrographs are shown at the same magnification. MDCK, Madin-Darby canine kidney.

92-kDa chymotrypsin-like aminopeptidase has been isolated from *Parieta judaicia* pollen (Cortes *et al.*⁹). Furthermore, a 35-kDa protein identified as the Bermuda grass allergen Cyn d 1, which shares epitopes in common with Phl p 1, was also shown to contain peptidase activity.³⁶ Serine peptidases of 80 and 82 kDa have been isolated from common ragweed (*Ambrosia artemisiifolia*).^{38,39} The present results from zymography of KBG pollen diffusate are consistent with the presence of peptidase activity in a 95-kDa protein. The results from EL pollen suggest activity in proteins of slightly lower MW and possibly in 20- to 30-kDa

Figure 7 Pollen diffusates contain proteolytically active products. Concentrated Easter Lily (A) and Kentucky Blue Grass (KBG; B) diffusates were subjected to zymography on a gelatin substrate. Strong proteolytic activity was present between 85 and 45 kDa in EL pollen diffusate, with weaker activity between 30 and 20 kDa. Similar activity was found in diffusate concentrated 1.25, 2.5 or 5 times (EL1.25, EL2.5, EL5). A major proteolytic band at 94 kDa was identified in KBG (100 mg/mL) diffusate, which had been concentrated by 2.5-fold (KBG2.5) and 5-fold (KBG5) reductions in volume. Positive control for enzymatic activity was trypsin. MW markers are in kDa.



proteins. Further investigation of the phenomena described here must await isolation and characterization of the peptidases involved. If it transpires that pollen proteases facilitate allergen delivery and the allergic response, inhibition of protease activity may offer an opportunity for therapeutic intervention or prevention of allergic asthma and rhinitis.

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