

Augmentation of Permeability in the Bronchial Epithelium by the House Dust Mite Allergen Der p1

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Sequence analyses have revealed the existence of homology between certain aeroallergens and proteolytic enzymes. This homology can be expressed functionally, but its significance to airway pathophysiology is unknown. Studies with Madin-Darby canine kidney cells and canine tracheal epithelial cells grown on plastic substrata or matrix proteins suggest that Der p1, a major allergen from the house dust mite *Dermatophagoides pteronyssinus* and a cysteine proteinase, or the unfractionated growth medium extract (SGME) from which it was purified, are both capable of causing cell detachment. The ability of both agents to produce functional changes in the barrier function of the epithelium was further demonstrated using isolated bovine airway preparations. Over a 3-h duration, both Der p1 and SGME elicited significant increases in the permeability of isolated sheets of bronchial mucosa to serum albumin. Exposure of isolated bronchial segments to lumenally applied solutions of Der p1 resulted in histologic evidence of epithelial injury. Neither Der p1 nor SGME was active in these experimental systems unless chemically reduced, suggesting that the effect was initiated by cysteine proteinase activity. Similar augmentation of mucosal permeability and tissue injury was produced by bovine trypsin and collagenase from *Clostridium histolyticum*. In both the isolated mucosal sheet model and in cultured cells, the actions of Der p1 or SGME were associated with relatively little cytolysis, suggesting a specific action of the reagents on cell attachment. These results demonstrate a new functional activity of Der p1 that may be germane to the processes of allergen presentation, inflammatory cell activation, and chronic tissue injury.

Asthma is a common, chronic disease that is frequently characterized by the presence of both atopy and bronchial hyperreactivity. These features appear to interact to the extent that continued exposure to a range of aeroallergens contributes not only to the general sequelae of immediate hypersensitivity reactions but also to the development of chronic asthma and bronchial hyperreactivity (1, 2). The allergen sources most often associated with asthma appear to be few in number but include the house dust mite, pollens, animal dander, and fungi. Of these, mites represent one of the more

significant sources (3, 4), and allergy to them represents an independent risk factor for the development of the disease (3–5).

The capacity of an individual to produce IgE antibody is under the influence of a number of genes that control T-cell immunoregulation. However, possession of these genes does not result in obligate expression of IgE, and it has been proposed that environmental exposure to allergens, in concert with other factors, is critical in the induction process (6). The airway mucosa is likely to be a primary site of interaction between putative allergens and the immune system, and it has been suggested that any physicochemical property associated with the allergen, or a situation facilitating contact with immunocompetent cells (e.g., MHC class II–positive dendritic cells) residing within the airway mucosa, is likely to enhance IgE production in genetically susceptible individuals (7, 8). Because the airway epithelium represents a highly regulated and tight barrier (9, 10), it is not self-evident how allergen macromolecules gain ready access to these immunocompetent cells.

Transepithelial solute permeability is governed by a number of factors of which the most important are molecular radius, solute concentration, and the possible presence of

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Abbreviations: [4-(N-hydroxyamino)-2R-isobutyl-3S-(thiophen-2-yl-sulfonylmethyl)succinyl]-L-phenylalanine-N-methylamide, BB-250; bovine serum albumin, BSA; Group I allergen from house dust mite *Dermatophagoides pteronyssinus*, Der p1; dithiothreitol, DTT; L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane, E-64; lactate dehydrogenase, LDH; Madin-Darby canine kidney, MDCK; Eagle's minimum essential medium, MEM; spent growth medium extract, SGME.

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transport processes (11, 12). However, in the case of allergens, it is not obvious how these factors alone can explain the degree of epithelial penetration considered necessary to induce an immune response (13). Data suggest that mucosal inflammation and the accompanying increase in epithelial permeability induced by agents such as irritant gases and respiratory tract viral infections may be necessary adjuvant factors (6). Evidence from *in vitro* models suggests that eosinophil-dependent tissue injury is capable of increasing the permeability of the airway mucosa to macromolecular solutes in a manner that is dependent *inter alia* on the expression and activation of proteinases (14). Taken together with demonstrations that direct addition of assorted proteinases can damage the airway mucosa *in vitro*, these observations further underline the potential importance of this mechanism in augmenting epithelial permeability (15, 16).

Of significance in understanding how allergens might traverse the epithelial barrier is the finding that many of them are enzymatically active. The mite allergens comprising groups 1 through 4 have been shown to correspond to a cysteine proteinase, lysozyme, trypsin, and amylase, respectively (17–20). These data, together with the well-documented observations that occupational exposure to enzymes can result in the development of asthma, raise the possibility that enzymatic activity per se might influence the disposition of allergens in the airway epithelium (21) and, ultimately, their immunogenicity.

The mite proteinases are of particular interest because of their status as major aeroallergens, and preliminary evidence supports the view that proteolytic activity plays a role in a number of potentially significant pathophysiologic processes. Significantly, mite proteinases can alter mucosal permeability (21), activate kallikrein (22), and degranulate mast cells by a mechanism that is independent of IgE crosslinkage (23).

In this paper, we describe studies that confirm and extend our previous preliminary report (21) and show that crude mite proteinase extracts and the highly purified mite cysteine proteinase allergen Der p1 have the capacity to alter the permeability of the bronchial epithelium to albumin. In addition, we show that the mite reagents have the ability to detach epithelial cells from artificial substrata on thin, ungelled coatings of matrix proteins at physiologically relevant concentrations and by mechanisms that are not grossly cytolytic.

Materials and Methods

Materials and Reagents

All reagents were purchased from Sigma Chemical Co. (St. Louis, MO or Poole, Dorset, UK), except where stated. In the present paper, we report experiments performed with the Sigma type IV collagenase (Clostridiopeptidase A [E.C. 3.4.24.3] from *Clostridium histolyticum*), which is derived by ammonium sulfate fractionation of culture supernatants and consequently contains small quantities of other enzyme activities. The Sigma "type" designation does not indicate the type of collagen preferentially degraded by this enzyme, and it is actually an interstitial collagenase. To avoid confusion, we refer to the enzyme using its catalogue number, collagenase C5138. Collagenase C5138 used in these studies had 650 collagen digestion units per milligram of solid and contained clostripain, caseinase, and tryptic activities as im-

purities at specific activities of 0.29, 60, and 0.05 U/mg, respectively.

Collagenase used in the cultured cell detachment experiments was from Boehringer Mannheim (collagenase A from *C. histolyticum*) and had a similar profile of contaminant activities to the Sigma preparation. Elastase from *Pseudomonas aeruginosa* was from Nagase Biochemicals (Fukuchiyama City, Japan). ¹²⁵I-labeled bovine serum albumin (BSA) (specific activity, 2.17 to 3.5 μ Ci/ μ g) was from NEN Du Pont Research Products (Stevenage, Hertfordshire, UK). RPMI 1640 culture medium was obtained from GIBCO BRL (Gaithersburg, MD), and fetal calf serum was from Australian Biosearch (Perth, Western Australia). Matrigel was purchased from Universal Biologicals (London, UK). General laboratory reagents and cell stains were obtained from BDH (Poole, Dorset, UK) and were of the highest grade obtainable. The matrix metalloproteinase inhibitor BB-250 [4-(N-hydroxyamino)-2R-isobutyl-3S-(thiophen-2-yl-sulfonyl methyl)succinyl]-L-phenylalanine-N-methylamide) was kindly provided by British Biotechnology Limited (Cowley, Oxford, UK). Nedocromil sodium was a generous gift of Fisons (Loughborough, Leicestershire, UK). The Madin-Darby canine kidney (MDCK) cell line was kindly provided by Dr. Andrew McWilliam (Institute For Child Health Research, Perth, Western Australia), and enzymatically dispersed canine tracheal epithelial cells were provided by Dr. Peter Sly (Institute For Child Health Research, Perth, Western Australia).

Cell Culture and Detachment Assay

Cell detachment assays were performed by seeding 10^5 MDCK or 2×10^5 enzymatically dispersed canine tracheal epithelial cells (24) into the wells of a culture plate. Cells were incubated with RPMI 1640 medium containing 2 mM glutamine and 10% fetal calf serum at 37°C in a humidified 5% CO₂ atmosphere for 24 h or until confluent. After washing with medium, the cells were then incubated with proteinases in the presence of activators and/or inhibitors for either 2, 5, or 7 h. The proteinases were prepared in serum-free medium and were incubated with activators/inhibitors for 10 min before addition to the cell monolayers.

After the appropriate incubation period, medium was carefully aspirated from each well and the cell monolayers were washed sequentially in serum-containing medium, phosphate-buffered saline, and water before being air-dried. Leishman's stain (50 μ l) was added to each well and incubated for 10 min at room temperature before the addition of 50 μ l of distilled water and incubation for a further 10 min. In those studies performed on plates coated with Matrigel, toluidine blue (0.1% in 1% vol/vol acetic acid) was used in place of Leishman's stain. After removal of the excess stain, monolayers were given three successive washes with distilled water and air-dried before the addition of 200 μ l of 0.5% vol/vol Triton X-100 for 30 min. This solution was then recovered from each well and transferred to a clean 96-well microtiter plate for determination of absorbance at 630 nm. The cell detachment caused by the proteinases was calculated as follows and expressed as a percentage: $1 - (\text{absorbance of test sample} - \text{absorbance of background detachment} / \text{absorbance of positive control} - \text{absorbance of background level of detachment}) \times 100$.

Conditioned medium from MDCK cultures was prepared by growing the cells to confluence and incubating them with serum-free medium for 5 h. The conditioned medium was centrifuged to remove any cellular material, and the resulting supernatant diluted into culture medium for determination of proteinase activity by Azocoll degradation.

Coating of Culture Wells with Matrigel

Thin, ungelled coatings of Matrigel were applied to 96 well culture plates by addition of 50 μ l aliquots to each well of a 1:500 dilution of Matrigel made in serum-free RPMI 1640 medium. After incubation at room temperature for 60 min, the solution was aspirated and each well washed with RPMI 1640 medium before the addition of cells for growth to confluence. Confirmation of the deposition of protein on the bottom of the culture wells was established using Leishman's stain to visualize the protein.

Studies with Tissue Chambers

The methods used in these experiments have been described in detail elsewhere (14). In brief, fresh bovine lungs were obtained at a local abattoir and used within 1 h post mortem. The main bronchi were identified and opened by incision, and the airway mucosa and submucosa carefully removed by dissection. Light and electron microscopy demonstrated that the tissue sheets removed comprised the airway epithelium, basement membrane, and some underlying submucosa (data not shown). A maximum of six pieces were taken from the main bronchus in order to minimize the effects of tissue heterogeneity.

The dissected mucosal tissue was washed extensively with Eagle's minimum essential medium (MEM) and cut into 1- to 2-cm²-sized pieces. These pieces were then mounted between two acrylic plates. Each plate had a central aperture (0.5 cm diameter) to expose a defined area of tissue and to act as a well for the addition of small volumes of solution when the chambers were mounted vertically. The "sandwich" formed by the airway tissue and the acrylic sheets was then interposed between two water-jacketed half chambers as described in full elsewhere (14). This arrangement permits the independent bathing of the apical and basolateral sides of the tissue. Both chambers were initially filled with MEM (2 ml per half chamber) and maintained at 37°C.

After equilibration for 30 min, the chambers were oriented vertically and the apical bathing solution was removed and replaced with 200 μ l medium containing allergen or an appropriate control. In other experiments, we investigated the effects of bacterial collagenase or trypsin. During all of these treatment periods, the chambers were oriented vertically so as to hold the small aliquots of allergen in the central aperture of the acrylic plate. However, for the other periods of the experiment in which the apical chamber was completely filled with solution, the apparatus was mounted horizontally.

Measurement of Albumin Flux

The half chambers on the apical side of the tissues were loaded with 2 ml MEM containing 2 μ Ci of ¹²⁵I-labeled BSA. After addition of tracer, the contents of the chambers were agitated gently throughout the subsequent period of the

experiment to avoid problems caused by incomplete equilibration and formation of diffusion shells. At 0, 15, 30, 60, 90, 120, 150, and 180 min after addition of tracer, 25 μ l samples of medium were withdrawn by microsyringe from the opposite (basolateral) side of the tissue. The amount of radioactivity in each of the samples was quantified, and the data were corrected for the radioactive decay from the reference date of isotope preparation. We have demonstrated elsewhere by radio-high performance liquid chromatography that the radioactivity that traverses the tissue and is measured in this way is associated with intact BSA (14).

Assay of Lactate Dehydrogenase

Lactate dehydrogenase (LDH) activity was measured in chamber fluid pooled from the apical and basolateral sides and expressed as a percentage of total tissue LDH determined after homogenization of matched pieces of untreated airway tissue. The activity of LDH was quantified using a colorimetric assay technique involving the NADH-dependent reduction of pyruvic acid to lactic acid. Unreacted pyruvic acid was treated with 2,4-dinitrophenylhydrazine, with the absorbance of the colored product formed being inversely proportional to LDH activity.

Studies with Isolated Bovine Bronchial Segments

Segments of bovine bronchi (length $\times$ internal diameter approximately 3 $\times$ 0.2 cm) were isolated by dissection, and side branches closed by ligation with surgical silk. Polyethylene cannulae (Portex, Hythe, Kent, UK; size 6FG) were inserted into the ends of the segments and ligated in position, and the preparations placed into a dish containing prewarmed MEM with the ends of the cannulae supported so as to leave them open to the atmosphere. Aliquots of MEM containing experimental additions (dithiothreitol [DTT], Der pl, or collagenase) were introduced into the lumen of each segment by means of a syringe prior to incubation of the preparations at 37°C. At the end of the experiment, the segments were fixed for at least 24 h in neutral-buffered formalin before histologic processing using standard procedures. Transverse sections (5 μ m) were cut serially from the center of each segment and stained with hematoxylin and eosin. Coded slides were examined by an independent observer and scored semiquantitatively for the occurrence of epithelial injury.

Source of Mite Extracts and Allergen Protein

Spent growth medium, in which *D. pteronyssinus* had been cultured, but which was devoid of whole mites, was kindly provided by Commonwealth Serum Laboratories (Parkville, Australia). Spent growth medium was extracted using 0.01 M phosphate buffer, pH 7.2, containing 0.15 M sodium chloride. An ethanolic precipitate of spent growth medium was also used in some experiments. Der pl was isolated from spent growth medium extract (SGME) by monoclonal antibody affinity chromatography as described elsewhere (25), using reagents kindly provided by Dr. Martin Chapman (University of Virginia).

Assay of Proteinase Activity

Nonspecific proteinase activity was determined spectrophotometrically using insoluble diazotized collagen (Azocoll;

Calbiochem, La Jolla, CA) as substrate (26). One unit of proteinase activity was defined as the optical density reading at 520 nm measured after reaction (multiplied by 1,000 for convenience) normalized to unit time of incubation and unit amount of sample protein. In assays designed to measure cysteine proteinase activity, the enzyme was activated with DTT as described (27). Preliminary studies established that Der p1 was activatable for Azocoll degradation either by 0.2 to 3 mM reduced glutathione or by DTT (data not shown), and consequently either of these reducing agents was used in experiments with isolated cells and tissues.

Statistics

Data are presented as the mean value $\pm$ SEM of n separate experiments. Permeability data were analyzed by Kruskal-Wallis ANOVA. Median values were compared between two groups using the two-tailed Mann-Whitney test for unpaired variables. Probability values ≤ 0.05 were considered significant.

Results

Influence of Mite Proteinases on Epithelial Cells

Detachment of MDCK cell line and primary cultures of tracheal epithelial cells from plastic and matrix protein substrata. After 5 h of incubation, SGME (1×10^{-8} to 2×10^{-2} g/ml) promoted a concentration-related detachment of MDCK cells from a plastic substratum in the presence or absence of 0.5 mM glutathione (Figure 1a). Incubation for 2 h also promoted detachment, but higher concentrations were required to elicit similar effects (data not shown). At both 2 and 5 h, there was a slight difference between responses elicited by SGME in the presence and absence of glutathione, although the activity of the unreduced preparation suggested that either the cells had the capacity to activate the latent cysteine proteinase activity or that detachment was induced by other proteinases present in SGME.

Similarly, Der p1 (4.6×10^{-10} to 4.6×10^{-3} g/ml) caused a concentration-related detachment of MDCK cells from a plastic substratum (Figure 1b), with a greater effect being observed after 5 h of treatment than 2 h (data not shown). Unreduced Der p1 was also active in this model (Figure 1b), suggesting activation of the allergen proteinase by the MDCK cells themselves. In this regard, culture medium conditioned for 5 h by MDCK cells was capable of activating Der p1 to degrade Azocoll. In the absence of conditioned medium, proteolytic activity of Der p1 activated by 1 mM glutathione was 160 ± 1.2 U, whereas the activity of that treated with the conditioned medium was 56 ± 4.1 U. Fresh culture medium per se was devoid of Azocoll-degrading activity and incapable of activating Der p1 (data not shown).

Figure 2a illustrates the activity of Der p1 in causing detachment of MDCK cells with respect to Pseudomonas elastase, bovine trypsin, and collagenase determined after 5 h of incubation. The rank order of activity measured from the concentrations required to produce 50% detachment of cells was Pseudomonas elastase > trypsin > glutathione-reduced Der p1 > unreduced Der p1 >> collagenase (Figure 2a). A similar rank order of activities was obtained when the data were compared after standardization of proteinase activity in Azocoll units (Figure 2b). The proteinases also

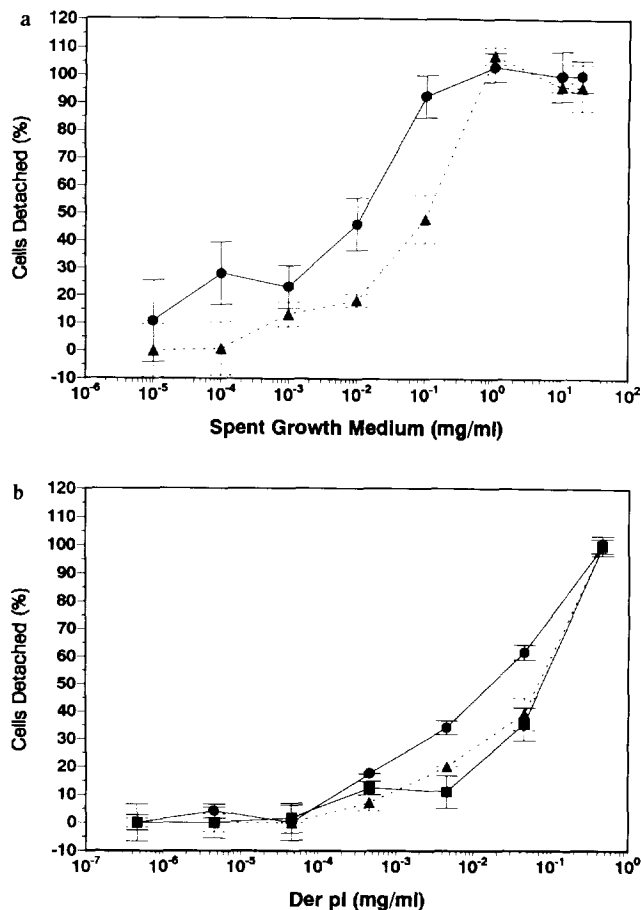


Figure 1. a. Detachment of MDCK cell monolayers from a plastic substratum by treatment for 5 h with SGME in the presence (circles) or absence (triangles) of 0.5 mM glutathione. Data from five replicates in three separate experiments. b. Corresponding study using Der p1 in the absence of exogenous reducing agent (triangles) or the presence of 1 mM DTT (squares) or 0.5 mM glutathione (circles). Data from seven replicates in three separate experiments.

caused detachment of canine tracheal epithelial cells cultured on plastic (Figure 2c), confirming that these actions are not unique to the MDCK cell line and occur in epithelial cells of pulmonary origin.

The proteinases also caused significant detachment of MDCK cells from culture wells treated with a thin, ungelled coating of Matrigel (Figure 2d). As calculated from the relative concentrations of the proteinases to elicit 50% detachment, the rank order of activity was identical to that obtained on untreated plastic (Pseudomonas elastase > trypsin > reduced Der p1 > unreduced Der p1 > collagenase). However, inspection of the concentration-effect curves for individual enzymes under the two conditions clearly shows that cells grown on Matrigel were relatively more resistant to the proteinases than were MDCK cells grown on plastic (Table 1).

That the ability of reduced or unreduced Der p1 to detach MDCK cells was due to cysteine proteinase activity was tested using the cysteine proteinase inhibitor E-64 (L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane). This compound caused a concentration-dependent (2.8×10^{-11} to

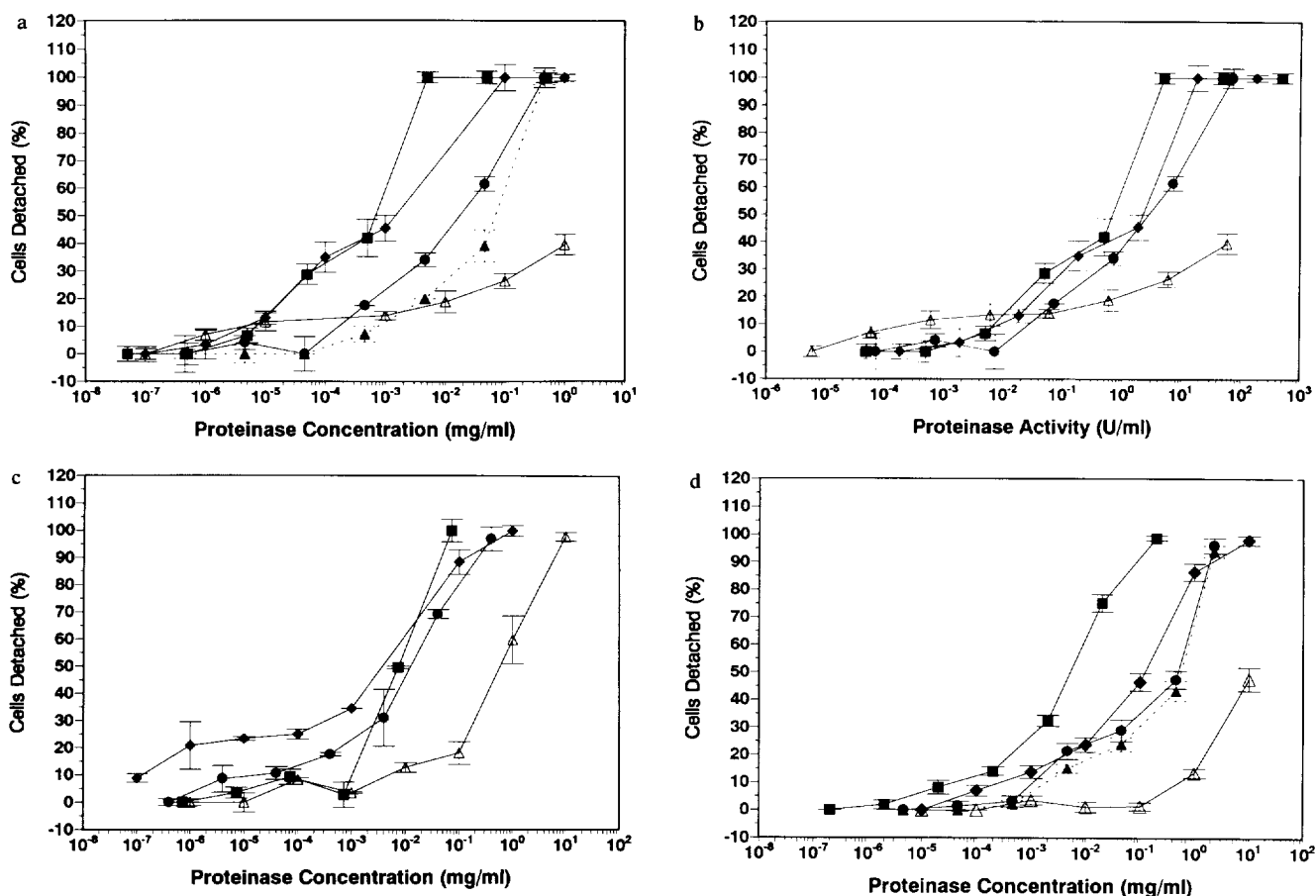


Figure 2. a. Detachment of MDCK cells from monolayer cultures on a plastic substratum by treatment for 5 h with Pseudomonal elastase (squares), trypsin (diamonds), Der p1 (closed triangles), Der p1 and 0.5 mM glutathione (circles), and collagenase (open triangles). Panel b depicts the relative ability to detach cells after normalization of data for the Azocoll-degrading activity of the four enzymes. According to this assay, specific activities of the enzymes were Pseudomonal elastase, 385 U/mg; trypsin, 185 U/mg; activated Der p1, 160 U/mg; and collagenase, 60 U/mg. Unactivated Der p1 is omitted from this set of results because it exhibits zero activity in the Azocoll assay unless a reducing agent is present. Data from four experiments with three replicates. Panel c depicts the relative ability of the enzyme preparations to detach canine tracheal epithelial cells grown in primary culture. Experimental conditions and key are as shown in panel a. Panel d depicts the detachment of MDCK cells from thin, ungelled substrata of Matrigel using the experimental conditions and key depicted in panel a. Data from four experiments performed in triplicate.

2.8×10^{-7} M) inhibition of cell detachment initiated by Der p1 (Figure 3).

Vital dye staining of the MDCK cells detached by 7 h of treatment with a range of concentrations of SGME reduced

by glutathione demonstrated that in all cases the majority of the cells retained their viability and excluded trypan blue (Figure 4), supporting the view that the crude allergen was acting by specific detachment from the culture substratum rather than by cytotoxicity.

Influence of SGME on Epithelial Permeability

The SGME prepared for use in these studies contained a modest amount of proteolytic activity (88 Azocoll U/ml in a 20 mg/ml solution). However, in the presence of 1.5 mM DTT, there was a significant increase in its proteolytic activity, with an estimated activity of 239 Azocoll U/ml. Initial experiments were performed using SGME at this concentration. A total of 200 μ l of this solution, with or without 1.5 mM DTT supplementation, was added to the apical sides of tissues and incubated for 3 h.

In tissues incubated in the presence of medium alone, the net unidirectional flux of BSA was 2.46 ± 0.17 fmol/ cm^2/min ($n = 6$), and this was not significantly different in tissues incubated for 3 h in the presence of medium sup-

TABLE 1

*Calculated concentrations of proteinases causing a 50% detachment (EC_{50} values) of MDCK cells cultured on plastic substrata and surfaces coated with a thin, ungelled layer of Matrigel**

Enzyme	EC_{50} on Plastic (mg/ml)	EC_{50} on Matrigel (mg/ml)
Pseudomonal elastase	0.0007	0.005
Trypsin	0.002	0.14
Der p1 (reduced)	0.02	0.5
Der p1 (unreduced)	0.08	0.6
Collagenase	> 1	> 10

* Contact time with the enzymes was 5 h. Data from three to four experiments performed in triplicate.

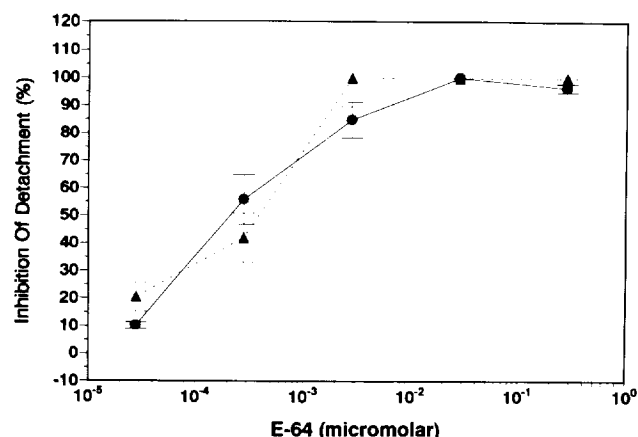


Figure 3. Effect of cysteine proteinase inhibitor E-64 on detachment of MDCK cells mediated by 200 $\mu\text{g}/\text{ml}$ (32 U/ml) Der pI (closed triangles) or Der pI in the presence of 0.5 mM glutathione (circles). Incubation period was 5 h. Data are from triplicate determinations in two separate experiments.

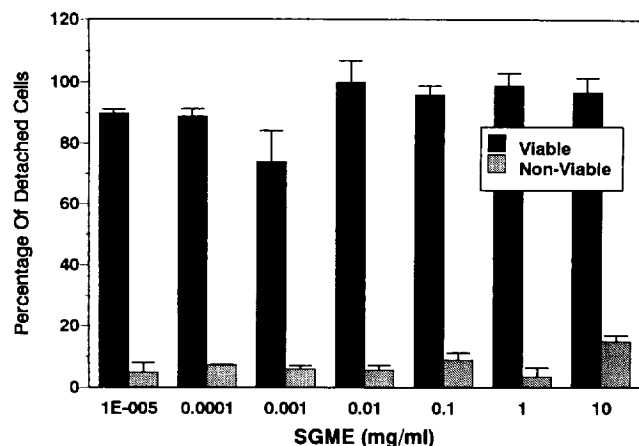


Figure 4. Viability of MDCK cells detached after 7 h of incubation with SGME in the presence of 0.5 mM glutathione. Data are from triplicate determinations in two separate experiments, with solid-filled columns showing the proportion of viable cells and columns with grey shading the proportion of nonviable cells. Viability was determined by trypan blue exclusion.

plemented with 1.5 mM DTT or in medium containing 239 U/ml SGME (Figure 5). In contrast, the net unidirectional flux of BSA was significantly greater in tissues that had been treated with SGME rendered catalytically competent by inclusion of DTT (Figure 5).

The ability of SGME to cause an increase in the net unidirectional flux of albumin across the bronchial mucosa was significantly inhibited by the matrix metalloproteinase inhibitor BB-250 at a concentration of 5 μM but not by the antiasthma drug nedocromil sodium at 100 μM (Figure 6).

Noncytolytic Effect of SGME in Isolated Sheets of Mucosa

The increase in the net unidirectional flux of albumin elicited by treatment of the bronchial mucosa preparations with SGME was not a process involving gross cytolysis. Figure

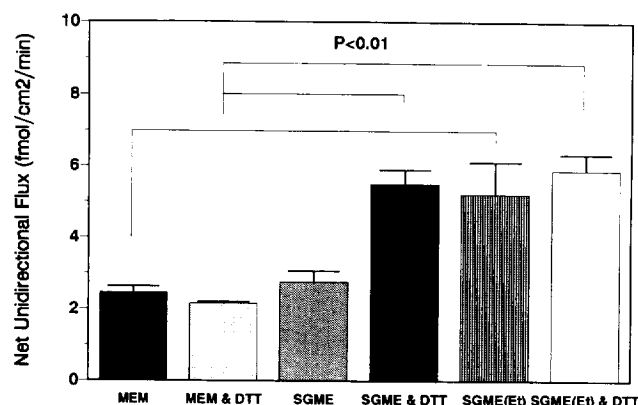


Figure 5. Effect of mite SGME on the net unidirectional flux (apical to basolateral) of BSA in bronchial sheets. Experiments were performed with SGME prepared as described in MATERIALS AND METHODS and compared with an ethanol precipitate (SGME(Et)). SGME (239 Azocoll U/ml when catalytically competent with DTT) was applied to the apical side of the tissue with a contact time of 180 min. Data are mean $\pm$ SEM from six separate experiments.

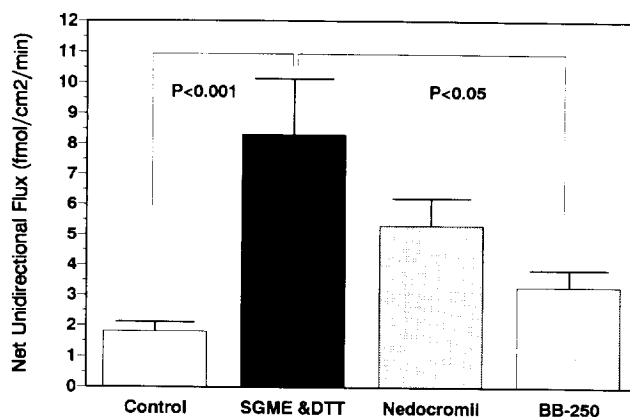


Figure 6. Effect of nedocromil sodium (100 μM) and BB-250 (5 μM) on the increase in albumin flux elicited by SGME activated by DTT. Contact time and Azocoll activity of the SGME are as described in Figure 5. Data are mean $\pm$ SEM from six to eight experiments.

7 indicates the release of LDH from tissues during incubation with activated SGME or after exposure to medium supplemented with DTT. Over the experimental period, there was a net release of $3.99 \pm 2.60\%$ total LDH under control conditions, and, although tissues treated with SGME showed a tendency towards an increased net release of $11.61 \pm 5.13\%$, this difference was not significantly different.

Influence of Der pI on Epithelial Permeability

For comparison, experiments were performed with highly purified Der pI. The allergen was dissolved in medium to give solutions with a proteinase activity of 233 Azocoll U/ml in the presence of DTT (equivalent to between 300 and 3,182 $\mu\text{g}/\text{ml}$ protein depending on the lot of purified allergen used) and 200 μl aliquots of this were incubated with the apical side of the bronchial mucosa for 3 h. The results of these ex-

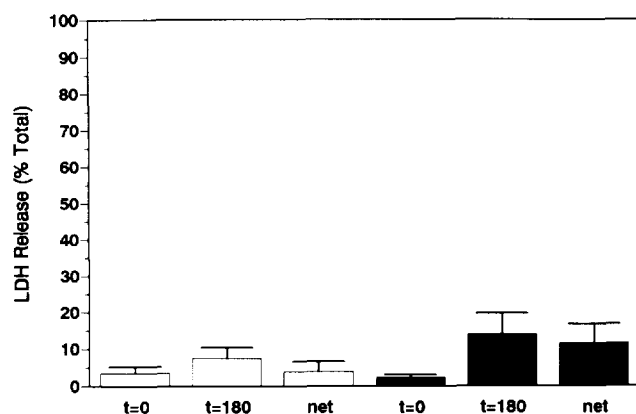


Figure 7. Release of LDH from sheets of bovine bronchial mucosa after exposure for 180 min to medium supplemented with 1.5 mM DTT (open columns) or medium containing both SGME (239 Azocoll U/ml) and 1.5 mM DTT (closed columns). Data are mean $\pm$ SEM from five experiments.

periments are shown in Figure 8. In the absence of reducing agent, Der pl did not significantly affect the baseline net unidirectional flux of BSA. However, in the presence of 1.5 mM DTT, the permeability of the tissues to BSA was doubled ($P < 0.01$; Figure 8).

Table 2 illustrates the effects of collagenase C5138 on epithelial flux of albumin. Concentrations of 100 and 500 $\mu\text{g/ml}$ produced significant increases in the net unidirectional flux of albumin when exposed to the apical side of the tissues. Trypsin (an ethanolic precipitate from bovine pancreas) also produced an increase in the tissue permeability to albumin at a similar range of concentrations to the bacterial collagenase (Table 2).

Histologic Studies Using Isolated Bronchial Segments

Figure 9 illustrates the appearance of bronchial segments after intraluminal exposure to Der pl (233 U/ml) or collagenase C5138 (10 mg/ml) for 5 h. Segments that had been exposed to medium alone or medium with 1.5 mM DTT ap-

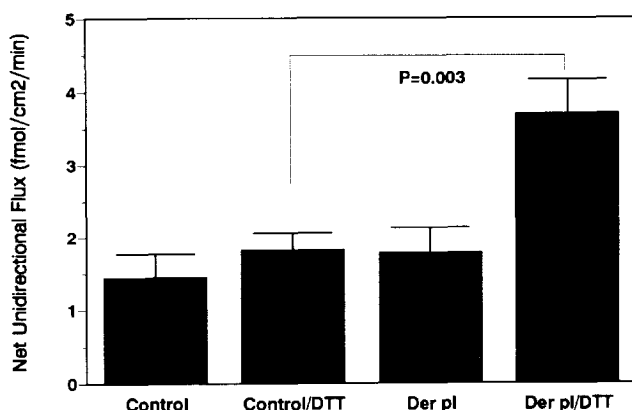


Figure 8. Effect of purified Der pl (233 Azocoll U/ml; 180 min of contact with the apical side) on the net unidirectional flux of albumin in the bronchial mucosa. Data are mean $\pm$ SEM from 10 to 12 experiments.

TABLE 2
Effect of collagenase and trypsin on the transepithelial flux of BSA in the bronchial mucosa

Conditions	Net Unidirectional Flux of BSA (fmol/cm²/min)	
	Trypsin	Collagenase C5138
0	2.42 $\pm$ 0.21	2.34 $\pm$ 0.10
100 $\mu\text{g/ml}$	4.81 $\pm$ 0.18 [†]	5.17 $\pm$ 0.64 [†]
500 $\mu\text{g/ml}$	ND	10.67 $\pm$ 1.58 [†]

* Incubations with the enzymes were performed for 180 min on the basolateral side in the case of trypsin and on the apical side in the case of collagenase. The vectors of albumin flux were basolateral-apical and apical-basolateral for the two enzymes, respectively.

[†] $P < 0.05$ with respect to appropriate enzyme-free control. Data from five experiments.

peared normal, with a well-preserved epithelium and generally a good maintenance of cell-cell and cell-matrix contacts. In contrast, segments exposed to collagenase exhibited a grossly disrupted architecture. Loss of the epithelium was a striking feature, together with evidence of cell shrinkage, ciliary damage, and disruption of intercellular contacts. Although not as extreme as the damage evoked by collagenase, the effects of Der pl were similar in nature (Figure 9), with evidence of columnar cell shrinkage and outright detachment.

Discussion

In this study, we have presented three lines of evidence that suggest that the house dust mite allergen Der pl is capable of causing the bronchial epithelium to become increasingly permeable to macromolecules as a consequence of proteolytic injury. First, we demonstrated the ability of Der pl to elicit detachment of the MDCK cell line and primary cultures of canine tracheal epithelium from artificial and matrix protein substrata. Second, our experiments established that Der pl was capable of increasing the permeability of the bronchial mucosa to albumin. Third, we showed that luminally applied Der pl caused histologically observable damage to the architecture of the epithelium in bovine bronchial segments.

In producing these effects, Der pl behaves in a similar fashion to other proteinases such as elastase, trypsin, and a bacterial collagenase (15, 16, and this study). The SGME from which Der pl was purified was also active, but both of these reagents were dependent on the presence of a reducing agent to unleash this activity. As Der pl exhibits sequence homology with cysteine proteinases, the requirement for a reducing agent is, therefore, an expected component of its catalytic competence. The fact that SGME also required similar activation suggests that although this extracted medium is a source of other putative proteinases (18–20), the most important activity causing the change in tissue permeability is cysteine proteinase dependent.

The precise mechanism of this change in epithelial permeability to albumin is unknown, but the most likely candidates are physical disruption of the epithelial barrier or alterations in mucosal transport of albumin. The similar behavior shown by other proteinases in this study and the characteristics of proteinase-dependent injury of the airway epithelium

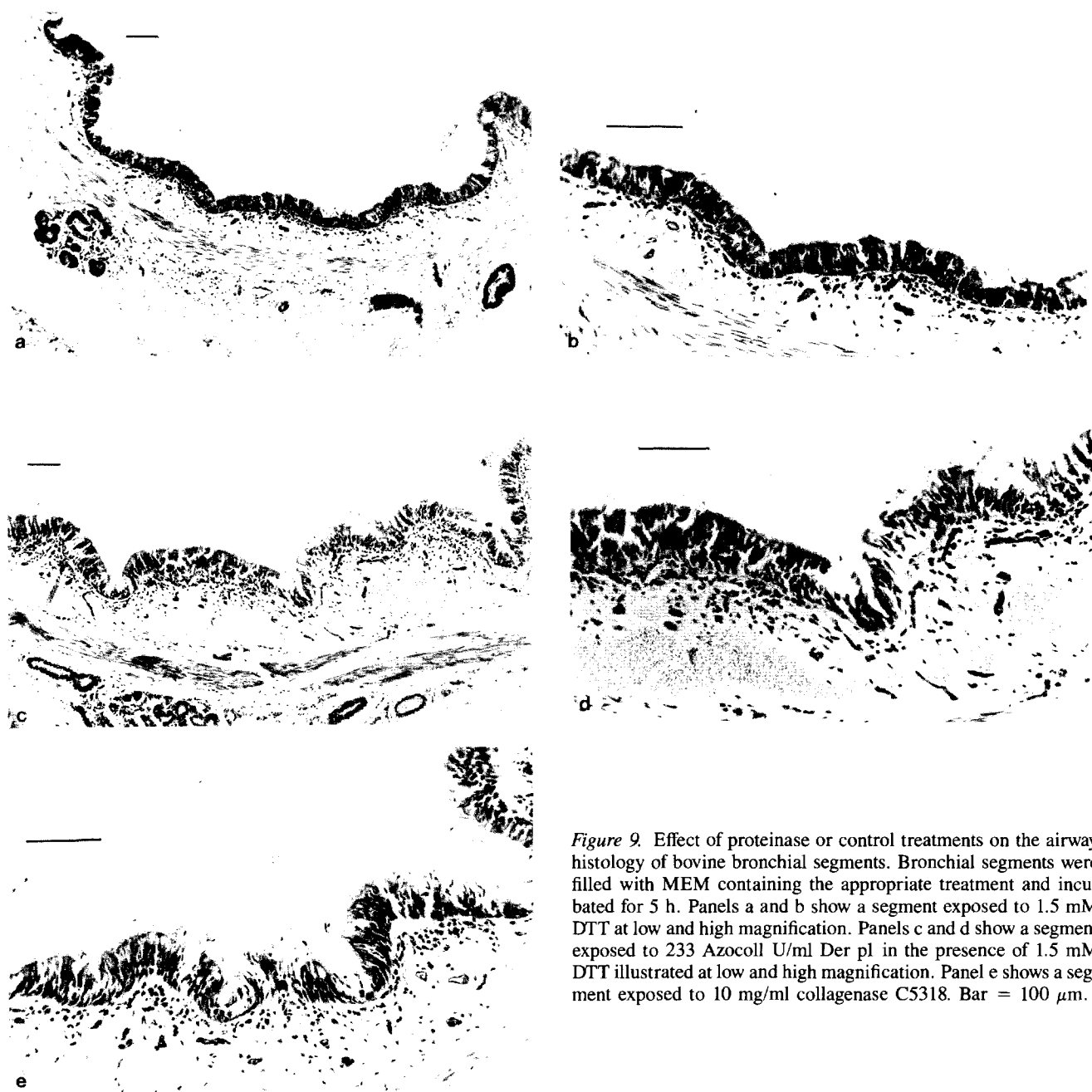


Figure 9. Effect of proteinase or control treatments on the airway histology of bovine bronchial segments. Bronchial segments were filled with MEM containing the appropriate treatment and incubated for 5 h. Panels a and b show a segment exposed to 1.5 mM DTT at low and high magnification. Panels c and d show a segment exposed to 233 Azocoll U/ml Der pl in the presence of 1.5 mM DTT illustrated at low and high magnification. Panel e shows a segment exposed to 10 mg/ml collagenase C5318. Bar = 100 μ m.

by inflammatory cells (14–16) suggest that detachment of cells from each other as well as the biomatrix is the more important component of the response. The view that proteinase-dependent detachment of epithelial cells is a major component of the augmented permeability is further supported by the histologic appearance of bronchial segments exposed to Der pl and also by the greater resilience to detachment of MDCK cells when cultured on Matrigel. Matrigel is a solubilized matrix protein extract from the Engelbreth-Holm-Swarm mouse sarcoma (28). Its principal components are laminin, type IV collagen, heparan sulfate proteoglycans, and entactin. Consequently, the cells cultured on this substratum are likely to make fuller use of

cell-substrate anchorage mechanisms than those cultured on plastic, and this is the likeliest explanation of the different detachability of cells under the two conditions. However, the relative activity of the proteinases is the same in both cases. Although MDCK cells are not of pulmonary origin, we chose to perform our initial studies with these cells because, unlike the pulmonary alternatives, this is an established line in which the mechanisms of cell-cell (tight junctions, intermediate junctions, and desmosomes) and cell-matrix anchorage are extensively characterized (29, 30). This makes them a convenient and useful model with which to undertake future studies of the general mechanisms by which Der pl acts. However, using cells from canine trachea, we con-

firmed that Der p1 was capable of detaching pulmonary epithelial cells in culture. The concordance between the relative activities of the proteinases in MDCK and tracheal cells was generally good, the minor difference between elastase and trypsin possibly being due to antiproteinases or other factors in tracheal cells that are absent in the MDCK line.

Although the cellular site and mechanism of detachment remain to be established, our data suggest that a cascade of proteinases may be involved in the response because the effects of SGME in the bronchial epithelium were inhibited by the matrix metalloenzyme inhibitor BB-250. This possibility could have interesting consequences not only for understanding the pathophysiology of epithelial injury but also for offering a novel pharmacologic potential to interfere with this process. The possible participation of amplification mechanisms was explored further using the antiasthma drug nedocromil sodium, which has modest inhibitory actions on the behavior of a range of inflammatory cells that express matrix metalloproteinases. However, it was without effect on cysteine proteinase-induced injury in our model, suggesting that any amplification that takes place does so in cells or by processes that are insensitive to this drug.

Unlike the sheets of bronchial mucosa, MDCK cells became detached in the presence of Der p1 that had not been activated by glutathione or DTT. Although this might indicate that Der p1 and SGME exhibit actions other than those previously highlighted in the mucosal sheet model, this explanation is unlikely. Our demonstration that medium conditioned by MDCK cells was able to activate Der p1 and that the effects of Der p1 were sensitive to inhibition by E-64 clearly endorses the view that epithelial cell detachment is initiated by cysteine proteinase activity of the allergen.

The significance of these data to allergenic sensitization remains uncertain, but our experiments suggest some exciting possibilities. For example, the ability of a protein to facilitate its own penetration into the airway mucosa could provide greater scope for the three-dimensional dendritic intraepithelial network of antigen-presenting cells (7, 8) to initiate an immunologic response by detecting and shuttling antigen from the airway epithelium to lymph nodes and submucosal T lymphocytes. The expression of enzymatic activity to facilitate antigen presentation might thus represent a fundamental mechanism that distinguishes between the many benign environmental antigens to which the lung is exposed and those that are immunologically significant. Equally, in a sensitized airway, it is conceivable that local changes in airway permeability induced by this enzymatic activity might facilitate access of additional allergen to mast cells in the airway submucosa or, as demonstrated *in vitro*, render airway smooth muscle more susceptible to bronchoconstrictor agents by removal of a diffusion barrier (31, 32). Chronic exposure to enzymatic allergens could be one component of a diverse array of influences that gradually compromise the integrity of the airway epithelium in asthma.

A number of conditions would need to be fulfilled for these actions to be of pathophysiologic relevance. Firstly, the allergen would have to be present in suitable concentration in the airway to overcome endogenous antiproteinases and, secondly, cysteine proteinases such as Der p1 would also need reduction to achieve catalytic activity. Airway surface lining fluid contains a number of potential reducing agents

of which glutathione is probably the most important (33, 34). In the studies presented here, we have shown that the enzymatic activity of Der p1 can be activated by concentrations of glutathione that are encountered in the airway (34).

Whether the amount of inhaled allergen impacting on the epithelium is sufficient to cause physical and functional disruption of this tissue is more difficult to assess. The experiments described herein relate only to acute studies; we have no means of knowing whether longer-term exposure to lower concentrations of Der p1 can reproduce the effects seen or whether they might influence mucosal homeostasis by subtle modification of solute transport processes or by promoting inflammatory events. In this regard, our most recent observations suggest that chronic exposure to lower concentrations of Der p1 is capable of significantly up-regulating cytokine production in human airway epithelial cells (G. A. Stewart *et al.*, unpublished observations). It is also difficult to relate the solution concentrations used in these experiments to the amounts of allergen that reach the airway in typical environmental exposures. Mite fecal pellets are known to contain Der p1 at relatively high concentration (10 mg/ml) (35), and, together with other mite allergens that also have enzymatic activity, this is subject to rapid release to establish what should be high focal concentrations on the apical surface of the epithelium. The extent to which this could then be diluted by mucociliary clearance is unknown, but such a dilution might become less relevant in asthma where this process is impaired (36).

In summary, these studies have demonstrated that the functionally expressed cysteine proteinase activity of the allergen Der p1 causes an increase in the permeability of the bronchial mucosa, a process that may involve detachment of viable epithelial cells. This expression of proteolytic activity may be associated with its allergenicity by promoting antigen presentation and tissue injury.

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