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Class specific inhibition of house dust mite proteinases which cleave cell adhesion, induce cell death and which increase the permeability of lung epithelium

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1 House dust mite (HDM) allergens with cysteine and serine proteinase activity are risk factors for allergic sensitization and asthma. A simple method to fractionate proteinase activity from HDM faecal pellets into cysteine and serine class activity is described.

2 Both proteinase fractions increased the permeability of epithelial cell monolayers. The effects of the serine proteinase fraction were inhibited by 4-(2-aminoethyl)-benzenesulphonyl fluoride hydrochloride (AEBSF) and soybean trypsin inhibitor (SBTI). The effects of the cysteine proteinase fraction could be inhibited by E-64. No reciprocity of action was found.

3 Treatment of epithelial monolayers with either proteinase fraction caused breakdown of tight junctions (TJs). AEBSF inhibited TJ breakdown caused by the serine proteinase fraction, whereas E-64 inhibited the cysteine proteinase fraction.

4 Agarose gel electrophoresis revealed that the proteinases induced DNA cleavage which was inhibited by the matrix metalloproteinase inhibitor BB-250. Compound E-64 inhibited DNA fragmentation caused by the cysteine proteinase fraction, but was without effect on the serine proteinase fraction. Staining of proteinase-treated cells with annexin V (AV) and propidium iodide (PI) revealed a diversity of cellular responses. Some cells stained only with AV indicating early apoptosis, whilst others were dead and stained with both AV and PI.

5 HDM proteinases exert profound effects on epithelial cells which will promote allergic sensitization; namely disruption of intercellular adhesion, increased paracellular permeability and initiation of cell death. Attenuation of these actions by proteinase inhibitors leads to the conclusion that compounds designed to be selective for the HDM enzymes may represent a novel therapy for asthma.

Keywords: Bronchial epithelium; asthma; allergens; apoptosis; proteinase inhibitors; tight junctions; *Dermatophagoides pteronyssinus*

Introduction

Proteins excreted in the faecal pellets of house dust mites (HDM) belonging to the genus *Dermatophagoides* (e.g. *D. pteronyssinus, D. farinae*) are major causes of allergic asthma and are strongly implicated in the expanding prevalence of the disease (Platts-Mills *et al*., 1992; Tovey *et al*., 1981; Sears *et al*., 1989). When inhaled, HDM faecal pellets impact upon the fluid-covered epithelial surface of large diameter airways. The resulting hydration of HDM faecal pellets will trigger a rapid and total discharge of the major allergenic proteins (Tovey *et al*., 1981) thus achieving a high local concentration of HDM proteins on the airway lining. At least nine groups of HDM faecal pellet allergens have been described, four of which are known to express catalytically competent proteinase activity (reviewed by Robinson *et al*., 1997b). The group 1 allergens are cysteine proteinases whilst the group 3, group 6 and group 9 allergens share sequence identity with archetypal serine proteinases and are themselves catalytically competent (Robinson *et al*., 1997b; King *et al*., 1996; Yasueda *et al*., 1993; Stewart, 1994; Stewart *et al*., 1992; Chua *et al*., 1988).

Proteolytic enzymes from diverse sources disturb the normal structure and function of the bronchial epithelium (Omari & Sparrow, 1992; Herbert *et al*., 1993; 1994; 1996; Robinson, 1995). The enzymatic nature of proteins from HDM faecal pellets raises the possibility that their proteolytic activity could explain the enigma of how allergens encounter dendritic antigen presenting cells to promote allergic sensitization (Robinson *et al*., 1997b). Dendritic cells are located beneath intercellular tight junctions (TJs) which are the key components of the paracellular permeability barrier of epithelia (Anderson & Van Itallie, 1995; Robinson *et al*., 1997b). This localization means that they do not directly sample the milieu of the airway lumen for potential antigens (Holt *et al*., 1990; 1991; Holt, 1993; 1995), suggesting that potential allergens must traverse the epithelial barrier for antigen presentation to occur. However, the mechanism responsible for this crossing of the epithelial barrier is obscure (Robinson *et al*., 1997b).

In previous experiments we demonstrated that the cysteine proteinase HDM allergen known as Der p 1 increased the flux of albumin across sheets of airway mucosa and disrupted the normal architecture of epithelia (Herbert *et al*., 1990; 1995). These observations indicated Der p 1 was capable of initiating a breakdown in cell adhesion, thus explaining the increased 'leakiness' of the airway lining to macromolecules (Herbert *et al*., 1995). On the basis of these experiments we suggested that these effects of Der p 1 may be central to the process of allergen sensitization (Herbert *et al*., 1995; Robinson *et al*., 1997b).

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The possibly crucial involvement of a proteolytic event in the process of sensitization to common airborne allergens suggests that inhibition of this step would represent a novel therapeutic approach to the treatment of asthma. Thus, in the present study we sought to examine whether proteinase inhibitors protected bronchial epithelial cells against some of the effects of enzymes released by the HDM *D. pteronyssinus*. We also report a simple fractionation procedure which allows separation of the HDM proteinases class activities. This fractionation procedure will be valuable in screening for potential inhibitors until such times as recombinant catalytically active HDM proteinases become available.

Methods

Cell culture

Calu-3 and MDCK cells were used as paradigms for examining intercellular junctions of epithelia and their susceptibility to HDM proteinases and potential inhibitors. Both cell lines express tight junctions, zonulae adherentes and desmosomes, and are thus acceptable models of cell adhesion mechanisms present in the airway. Calu-3 is an adenocarcinoma cell line derived from a 25-year-old Caucasian male. It has been the subject of relatively few investigations, but is known to express tight barrier properties on the basis of electrophysiological studies (Shen *et al*., 1994; Haws *et al*., 1994) and our own functional and immunocytochemical characterization (Winton *et al*., in press). MDCK cells are widely accepted as a 'gold standard' for studies of intercellular adhesion in epithelia (Stevenson *et al*., 1986; Anderson *et al*., 1988; Howarth *et al*., 1994; Stuart *et al*., 1994; Zhong *et al*., 1994; Stuart & Nigam, 1995). Calu-3 cells were propagated in Eagle's minimum essential medium with Earle's salts (EMEM) supplemented with 10% v/v heat inactivated foetal calf serum (FCS), 2 mM L-glutamine, non-essential amino acids, 10 μM sodium pyruvate and containing 50 U/ml penicillin and 50 μg/ml streptomycin.

Madin-Darby canine kidney (MDCK) epithelial cells were cultured in EMEM containing 50 U ml^{-1} penicillin, 50 μg ml^{-1} streptomycin, 2 mM L-glutamine, non-essential amino acids and 10% v/v heat inactivated FCS. For subculture of both cell types, the cells were rinsed in phosphate-buffered saline (PBS) without calcium and magnesium and then partially digested using a 0.05% (w/v) trypsin and 0.02% (w/v) EDTA solution.

All cultures were propagated at 37°C in a humidified atmosphere of 5% carbon dioxide in air.

Coating of Transwell^{TM} inserts with Matrigel

Measurements of mannitol clearance were performed on confluent cell monolayers that had been propagated on 0.4 μm pore diameter Costar Transwell™ inserts coated with an ungelled ultra-thin undercoat of Matrigel. Coating was achieved by addition of 250 μl aliquots of Matrigel (diluted 1 : 500 v/v in EMEM) to the interior of the insert followed by ambient incubation for 60 min under aseptic conditions. The solution was then aspirated and the inserts gently washed with medium before the addition of a confluent density of cell suspension. Calu-3 monolayers grown for use in these experiments typically developed transepithelial electrical resistances of $359 \pm 47\ \Omega\ cm^2$ whereas MDCK cells developed transepithelial resistances of $50 \pm 8\ \Omega\ cm^2$ ($n = 4$).

Cell treatment protocols and measurement of clearance

Cells ($2-5 \times 10^5$ per cm^2 growth area) were plated onto Matrigel-coated inserts. In this paper we use the term 'insert' as meaning the filter unit containing the cells and the term 'well' as referring to the cavities of the tissue culture plate. To monitor growth and integrity, inserts were taken at random, washed gently in PBS and stained under subdued illumination with acridine orange and ethidium bromide (1 mg ml^{-1} each in PBS). Inserts were examined by fluorescence microscopy and were used only when confluence with high viability was attained.

At confluence, the medium was aspirated from the wells and replaced with serum- and bicarbonate-free EMEM buffered with 20 mM HEPES and containing 2 mM L-glutamine. The medium from the inserts was then gently removed and replaced with 300 μl of serum-free EMEM containing [^{14}C]-mannitol (1 μCi ml^{-1} and 1 mg ml^{-1} unlabelled mannitol in HEPES-buffered medium). The Transwell™ plates were then equilibrated for 30 min at 37°C on a Luckham R100 orbital shaker. Triplicate 100 μl aliquots of the unused labelled mannitol solution were sampled and their radioactivity content determined by liquid scintillation spectrometry (Beckman LS6000IC) following addition of 5 ml Opti-Fluor.

After the equilibration period, the inserts were placed in fresh wells containing 1 ml of serum-free HEPES buffered EMEM and incubated at 37°C with continuous gentle shaking. The medium from the original wells was retained and its radioactivity content determined after the addition of 10 ml Opti-Fluor. These results were used to determine the tracer concentration at time zero. At timed intervals, 20 μl aliquots of the basolateral bathing fluid were removed and the amount of ^{14}C mannitol quantified as described above for the calculation of clearance volume.

Calculation of epithelial permeability

Paracellular permeability of mannitol was calculated from measurements of clearance volume at defined time points. Clearance estimates were made over 3–5 h and were calculated according to the relationship:

$$V_{probe_t} = \sum_{i=1}^{t} \frac{VA_i \Delta[A]_i}{[L]_i} \quad (1)$$

where:

V_{probe_t} is the clearance volume at each time point

VAi is the abluminal volume at each time point

Δ[A]i is the increase in tracer concentration between time points

[L]i is the luminal tracer concentration at each time point.

Under conditions where diffusion is the sole means of transepithelial movement of the solute, dv_{probe}/dt approximates closely to the permeability-surface area product thus allowing estimation of the permeability of mannitol in the composite system of cells, filter, unstirred layers and protein coating (P_t). Epithelial permeability can be calculated from the measured variable by considering the Matrigel-coated filter and unstirred layers as a system of series permeabilities. Thus:

$$\frac{1}{P_t} = \frac{1}{P_1} + \frac{1}{P_2} + \frac{1}{P_3} \quad (2)$$

and

$$\frac{1}{P_4} = \frac{1}{P_2} + \frac{1}{P_3} \quad (3)$$

where

P_t is the composite permeability of the system

P_1 is the component due to the epithelial cells alone

P_2 is the component due to the filter without Matrigel

P_3 is the unstirred layer component

P_4 is the permeability of the filter with the Matrigel coating.

In pure diffusion systems

$$P_3 = \frac{D}{\delta} \quad (4)$$

where

D is the free diffusion coefficient of mannitol

δ is the summed thickness of unstirred layers

Unstirred layer thicknesses are independent of membrane permeability under ideal conditions, thus P_3 in equations (2) and (3) are identical. Subtracting equations (2) and (3) thus permits the calculation of the permeability of the epithelial monolayer (5).

$$\frac{1}{P_1} = \frac{1}{P_t} - \frac{1}{P_4} \quad (5)$$

Analysis of variance was performed after log transformation of the permeability data and probability values for the different treatments assigned using the least significant difference test. Data are presented as the geometric mean values with indicated standard errors of n experimental observations. Probability levels of $P<0.05$ were considered statistically significant.

Preparation of proteinase fractions from HDM culture medium

HDM proteinase allergens have not yet been prepared in catalytically competent form by recombinant cellular expression of the mature enzyme protein. For convenience, and to enable future large scale screening of potential inhibitors in the absence of catalytically active recombinant proteins, we sought to separate the cysteine and serine proteinase activity by simple biochemical fractionation of spent medium in which HDM had been grown. During culture the HDM release allergens into the medium resulting in the accumulation of proteins suitable for purification. Spent medium from cultures of *D. pteronyssinus* (Commonwealth Serum Laboratory, Parkville, Australia) was dissolved in five volumes of phosphate buffered saline and then centrifuged at 48,400 *g* and 4°C for 20 min. Ammonium sulphate was added gradually to the stirred supernatant at 4°C to achieve a 50% saturated solution. After centrifugation (48,400 *g*, 20 min, 4°C) the pellet, found by enzymatic assay to be enriched in cysteine proteinase activity, was redissolved in a minimum volume of distilled water. Ammonium sulphate was added to the supernatant from the first cut to achieve 80% saturation. The pellet resulting from further centrifugation, found to be enriched in serine proteinase activity, was resuspended in a minimum volume of distilled water. The cysteine (50% precipitate) and serine proteinase (50–80% precipitate) fractions were separately dialyzed against distilled water overnight and then lyophilized prior to reconstitution in EMEM. Protein content of the extracts was measured using the Coomassie Blue technique with bovine serum albumin as standard (Smith *et al*., 1985). Proteinase activity was measured using the Azocoll degradation assay as described elsewhere (Herbert *et al*., 1995; Chavira *et al*., 1984). Extracts were also assayed for the presence of endotoxin using the Limulus amebocyte lysis assay (Endotect™, ICN Biomedicals, Thame, Oxfordshire). In all cases the levels of endotoxin were below the limit of assay detection (<0.06 ng ml^{-1}).

Immunoblotting of HDM proteinase fractions

Proteinase fractions were separated by SDS–PAGE and transferred electrophoretically to nitrocellulose membranes. Non-specific protein binding was blocked with 5% w/v non-fat milk and 0.1% v/v Tween-20 in Tris-buffered saline (TBS) followed by incubation with mAb 5H8 (anti-Der p 1) diluted in TBS containing 2% w/v bovine serum albumin and 0.1% v/v Tween-20. Detection was by enhanced chemiluminescence technique (Amersham International, Buckinghamshire).

Assays of cell death

Cells were plated on 60×15 mm petri dishes and grown for 2–4 days in serum-containing EMEM under tissue culture conditions in a 5% CO_2 atmosphere. Cells were then exposed to treatments in serum-free EMEM containing 20 mM HEPES whilst under aerobic incubation at 37°C. At defined time points, cells were harvested with a scraper and pooled with detached cells in the supernatant. Cells were centrifuged at 550 *g* for 5 min and their DNA extracted (Nucleon II, Scotlab, Coatbridge, Strathclyde). The extracted DNA was resuspended in 100 μl TE buffer (10 mM Tris-HCl and 1 mM Na_2EDTA) overnight at room temperature, and its purity determined spectrophotometrically. Equal amounts of DNA were applied in 4:1 ratio with sample buffer (0.25% bromophenol blue and 40% w/v sucrose in water) to each lane of 2% (w/v) agarose gels and electrophoresis performed at 50 V for 2–3 h in TAE buffer (0.04 M Tris-acetate and 0.001 M EDTA). Bands of DNA in the gels (which had ethidium bromide incorporated into them) were visualized by ultra-violet light.

The redistribution of phosphatidylserine into the outer layer of cell membranes which occurs during the initiation of apoptosis was studied using annexin V staining. This was performed in 60×15 mm petri dishes which had been modified by drilling a 1 cm diameter hole into the base of each dish and covering the external face of this with a glass coverslip secured in place by Sylgard (Dow Corning, Midland, MI, U.S.A.). A polyamide ring was mounted by means of cyanoacrylate adhesive onto the inner face of the dish to create a glass-bottomed well which was then coated with an ultrathin layer of Matrigel. Cells (3×10^4) were plated into each well and allowed to grow for 2–3 days, after which they were exposed to the desired experimental treatment. Following this, cells were rinsed in PBS and then in 200 μl binding buffer prior to addition of annexin V-FITC (AV) and propidium iodide (PI) under subdued lighting conditions. After incubation for 15 min the cells were rinsed with binding buffer and examined by fluorescence microscopy using blue and green excitation filter sets (Zeiss Axiovert 10 with oil immersion Fluar objectives). Using this technique, cells in early apoptosis stain green because FITC-conjugated annexin V binds to phosphatidylserine which has become reorientated into the outer leaflet of the cell membrane (Fadok *et al*., 1992; Koopman *et al*., 1994; Vermes *et al*., 1995; Homburg *et al*., 1995). Dead cells also show red staining with PI because leakiness of the nuclear membrane allows it to bind to nucleic acids. Photographic documentation was made using a Contax 167MT camera and Kodak TMAX 400 film for black and white prints or Ektachrome 160T for colour reversal images.

LDH activity was measured using pyruvic acid as substrate and monitoring spectrophotometrically the formation of a

phenylhydrazone derivative from lactic acid. MDCK or Calu-3 cells were seeded onto 12-well plates and grown to confluency. Cell monolayers were exposed for 18 h to either control treatments (serum- and phenol red-free EMEM, with 0.6 mM dithiothreitol in the case of the control for the cysteine proteinase fraction) or the HDM proteinase fractions diluted in the same medium (with 0.6 mM dithiothreitol present in the cysteine proteinase fraction). At the end of the experiment, the incubation medium was harvested and centrifuged at room temperature to sediment any cells which had detached from the wells during treatment. The first supernatant fraction was assayed directly for LDH activity, whereas the pellet formed by any detached cells was subjected to hypotonic lysis with distilled water (5 min at room temperature) prior to brief centrifugation to remove cellular debris. The resulting second supernatant was then assayed for LDH activity. Cells which had remained adherent to the wells during treatment were lysed as described above and LDH activity measured. Because no significant detachment occurred during treatment with control media, total cellular LDH activity was defined as that present in the lysate from adherent cells treated with serum- and phenol red-free EMEM alone.

Immunocytochemical visualization of the effects of inhibitors on proteinase-mediated cleavage of intercellular junctions

To study the effects of proteinases and inhibitors on intercellular junctions, MDCK cells were cultured on coverslips and treated with the appropriate proteinase and/or inhibitor for the desired time period. The cells were fixed in ice-cold methanol before binding of rat anti-ZO-1 (mAb R40.76) (Stevenson *et al*., 1986; Anderson *et al*., 1988) and mouse anti-desmoplakin (mAb 11-5F) (Parrish *et al*., 1987). Indirect fluorescent antibody staining was performed using DTAF- and TRITC-conjugated second antibodies. Microscopy was carried out using a Zeiss Axiovert microscope with $\times 40$ magnification oil immersion Fluar objective. Specimens were illuminated using excitation and emission filter sets for DTAF, FITC and TRITC. Cells were photographed as described above.

Materials

All media and cell culture reagents were purchased from ICN Biomedicals Ltd. (Thame, Oxfordshire), except where stated. HBSS was obtained from Gibco BRL, Life Technologies Ltd. (Paisley). Mannitol and Triton X-100 were obtained from Sigma-Aldrich Ltd. (Poole, Dorset, U.K.) and heat inactivated foetal calf serum was from Labtech International Ltd. (Uckfield, East Sussex, U.K.). Matrigel was obtained from Universal Biologicals, London. Mannitol clearance measurements were made in 12 mm diameter Transwells with 0.4 μm membrane pore size and 10 μm membrane thickness (Costar, U.K. Ltd., High Wycombe, Buckinghamshire). D-[^{14}C]-mannitol was obtained from NEN Du Pont Research Products (Stevenage, Hertfordshire, U.K.), and the Opti-Fluor scintillant and the scintillation vials were from Canberra Packard Ltd. (Pangbourne, Berkshire, U.K.). MDCK cells were grown from stock in our laboratory. Calu-3 cells were originally obtained from the American Type Culture Collection (Rockville, MD, U.S.A.) and expanded by serial passage to create a local bank of cryopreserved cells. Cells were cultured in Falcon 75 cm^2 cell culture flasks (Marathon Laboratory Supplies, London), Costar multiwell tissue culture plates or Transwell inserts according to the nature of the experiment. Agarose (molecular grade) was from Promega (Southampton, Hampshire, U.K.). Assay kits for LDH measurement were purchased from Sigma; Apoalert kits were purchased from Cambridge Bioscience. Acridine orange, ethidium bromide and all other general laboratory reagents were obtained from BDH (Poole, Dorset, U.K.). Compound E-64 (L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane, an inhibitor of cysteine proteinases, was obtained from Sigma. Concentrated aqueous stock solutions were stored frozen until required. The serine proteinase inhibitor 4-(2-aminoethyl)-benzenesulphonyl fluoride hydrochloride (AEBSF) was obtained from Pentapharm, Basle, Switzerland. The matrix metalloproteinase (MMP) inhibitor BB-250 ([4-(N-hydroxyamino)-2***R***-isobutyl-3***S***-(thiophen-2-yl-sulphonylmethyl) succinyl]-L-phenylalanine-N-methylamide) was provided by British Biotech Pharmaceuticals Ltd. All inhibitors were made as concentrated stock solutions in dry Me_2SO and diluted as required with medium for use in experiments. Appropriate controls for the Me_2SO vehicle were incorporated into experiments as required. Monoclonal antibody R40.76 reactive against ZO-1 was generously provided by Dr Bruce Stevenson, University of Alberta, Canada. Monoclonal Der p 1 antibody 5H8 was a kind gift of Dr Martin Chapman, University of Virginia, U.S.A.

Results

Fractionation of spent mite medium

Spent mite medium was separated into two fractions by ammonium sulphate precipitation. The fraction yielded by precipitation with 50% ammonium sulphate consisted of major protein bands at $\sim$22 kDa and 38 kDa (Figure 1a, lane 2). Tests of enzyme degradation using chromogenic substrates showed that the catalytic activity of the 50% precipitate was inhibitable by E-64 (not shown). Immunoblot analysis of the 50% precipitate using mAb 5H8 raised against Der p 1 revealed the presence of a major band with an apparent mass of $\sim$22 kDa and a minor band at 38 kDa (Figure 1b, lane 2). In the SDS–PAGE and immunoblotting analyses the cysteine proteinase fraction behaved identically to Der p 1 purified by a combination of immunoaffinity chromatography, gel filtration and isoelectric focusing (compare lanes 1 and 2 in panels a, b of Figure 1). Comparison of lanes 2 and 3 of the immunoblot shown in Figure 1b demonstrates that the 5H8 mAb also reacted with an additional range of proteins present in the 50–80% ammonium sulphate precipitate. In the absence of reducing agent the 50–80% ammonium sulphate precipitate fraction exhibited high catalytic activity which was then attenuated by inhibitors of archetypal serine proteinases (not shown). For convenience, in this paper the 50% precipitate is subsequently referred to as the cysteine proteinase fraction and the 50–80% precipitate as the serine proteinase fraction.

Effects of HDM proteinase fractions on epithelial permeability

Under the control conditions used in these experiments both MDCK and Calu-3 epithelial cells lines form tight monolayers with mannitol permeabilities in the range $0.7-1.2\times10^{-6}$ cm s^{-1}. Exposure of either MDCK or Calu-3 cell monolayers to the serine proteinase fraction produced a concentration-related change in permeability (Figure 2). The concentration-dependency of the cysteine proteinase fraction was not tested in this particular series of experiments, but the

effects of pure cysteine proteinase allergen Der p 1 have been previously demonstrated by us in similar *in vitro* models (Herbert *et al*., 1990; 1995).

The effects of the cysteine proteinase fraction in MDCK cell monolayers were attenuated by the cysteine proteinase inhibitor E-64 (Figure 3a). E-64 had no observable effect on the intrinsic permeability properties of the cell monolayer (Figure 3a). The serine proteinase inhibitors AEBSF and, less effectively, SBTI both inhibited the action of the serine proteinase fraction in MDCK cells (Figure 3b). Neither inhibitor *per se* exerted any observable effect on epithelial permeability (Figure 3b). The inhibitors were also effective when tested at the same concentration in Calu-3 cell monolayers. Calu-3 cell monolayers treated with the cysteine proteinase fraction had a mannitol permeability of $(8.44 \pm 0.43) \times 10^{-6}$ cm s^{-1} which was reduced to $(4.84 \pm 0.32) \times 10^{-6}$ cm s^{-1} by E-64 ($P < 0.05$, $n = 5$). Calu-3

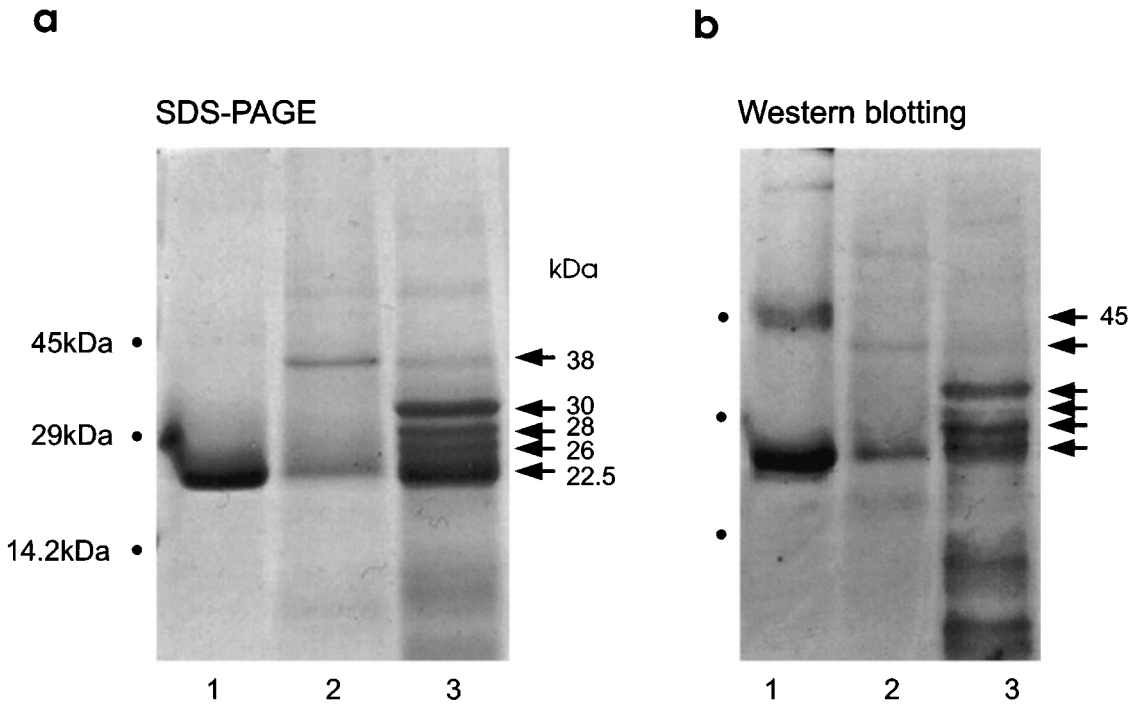

Figure 1 SDS–PAGE (a) and immunoblot analysis (b) of HDM proteinase fractions. Key to lanes: immunoaffinity purified Der p 1 (1); HDM cysteine proteinase fraction (2) and HDM serine proteinase fraction (3). Proteins were visualized by Coomassie blue staining. (b) shows the immunoblot prepared from the gel in (a). Proteins were detected using mAb 5H8 (anti-Der p 1) and visualized by ECL technique. Note the detection of immunoreactive proteins by mAb 5H8 in the serine proteinase fraction in addition to the expected immunoreactivity of the cysteine proteinase fraction. Filled circles indicate mass calibration standards and arrows indicate apparent molecular masses of bands calculated from the mobilities of dye labelled standards.

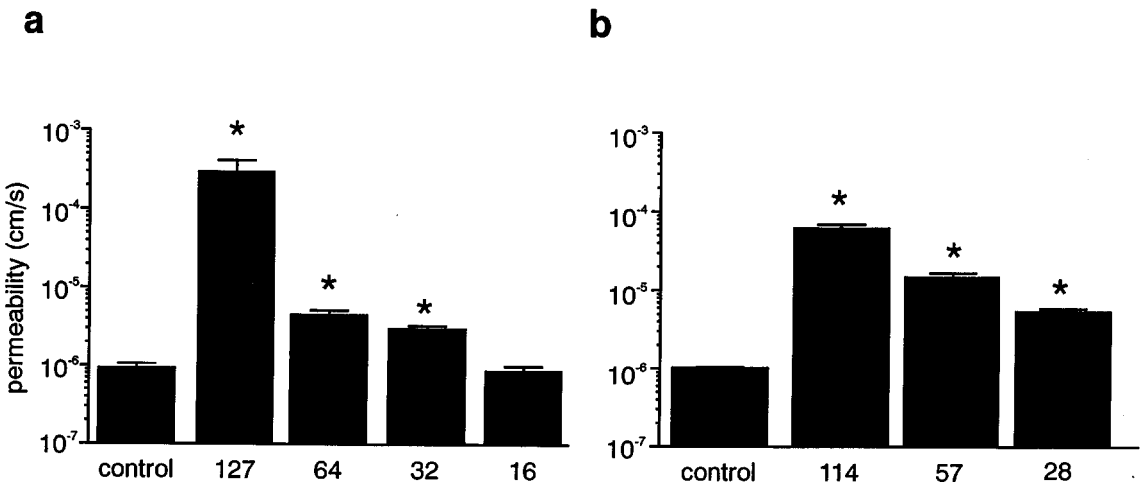

Figure 2 Dilution-response curves showing the effects of 18 h exposure of cell monolayers to the serine proteinase fraction prepared from HDM cultures. (a) shows the mannitol permeability of MDCK epithelial cells under control conditions (serum-free EMEM alone) and following treatment (proteinase fraction diluted in serum-free EMEM). (b) depicts similar studies performed in Calu-3 bronchial epithelial cells. Data are mean ± s.e.mean in four to six experiments. Proteinase allergen activity (expressed in azocoll units ml^{-1}) is indicated by the numbers under the filled bars. Asterisks indicate statistically significant differences with respect to the untreated control response (serum-free EMEM medium) for each cell type.

cell monolayers treated with the serine proteinase fraction had a mannitol permeability of $(18.1 \pm 0.02) \times 10^{-6}$ cm s^{-1} which was reduced to $(10.20 \pm 0.01) \times 10^{-6}$ cm s^{-1} by AEBSF ($P < 0.05$, $n = 5$).

The class-specific proteinase inhibitors were only effective against the cognate enzyme fractions derived from the HDM cultures. Figure 4 shows that AEBSF, at a concentratioan which ablated the effects of the serine proteinase fraction, failed to inhibit the action of the cysteine proteinase fraction on Calu-3 cell monolayers. Conversely, E-64 did not inhibit the permeability change caused by the serine proteinase fraction (Figure 4).

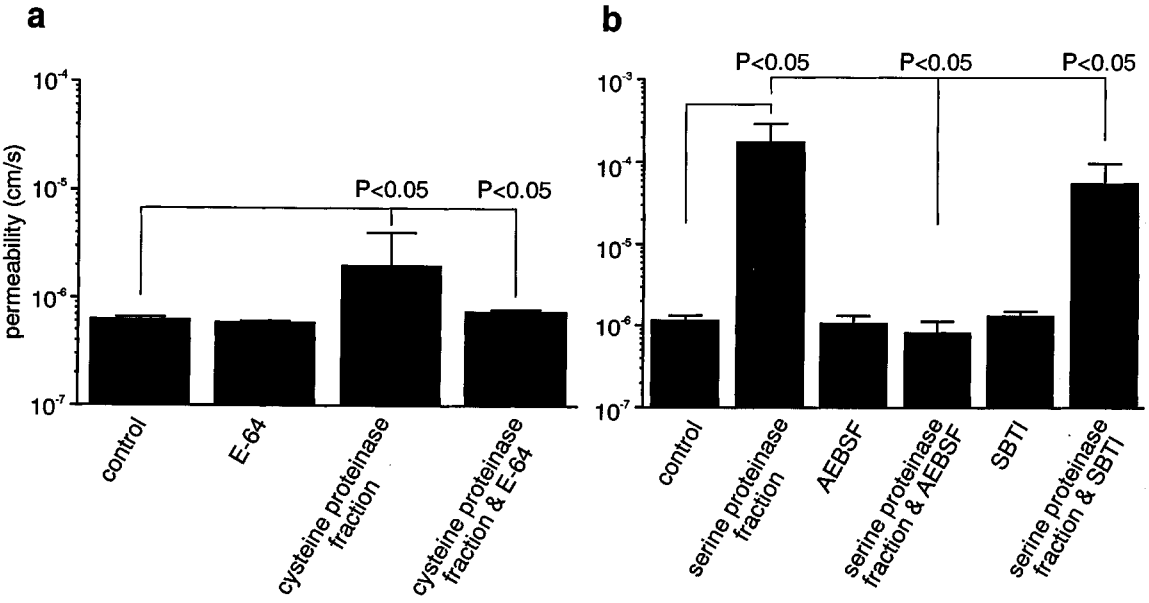

Figure 3 Inhibition of HDM proteinase fractions which increase the permeability of MDCK cell monolayers. (a) shows the effects of E-64 (10 μM) on the effect produced by the cysteine proteinase fraction (80 azocoll units ml^{-1}). Control cells were exposed to serum-free EMEM containing 0.5 mM reduced glutathione. (b) depicts the effects of AEBSF (100 μM) on the effects of the serine proteinase fraction (127 azocoll units ml^{-1}). Data are mean $\pm$ s.e.mean in three to five experiments. Contact time was 18 h. In (a and b) statistically significant differences exist between the permeabilities of control and proteinase treated monolayers and also between the monolayers treated with the proteinases in the presence and absence of inhibitors. Significant comparisons are shown in the Figure by the bracketing lines and indicated probability values.

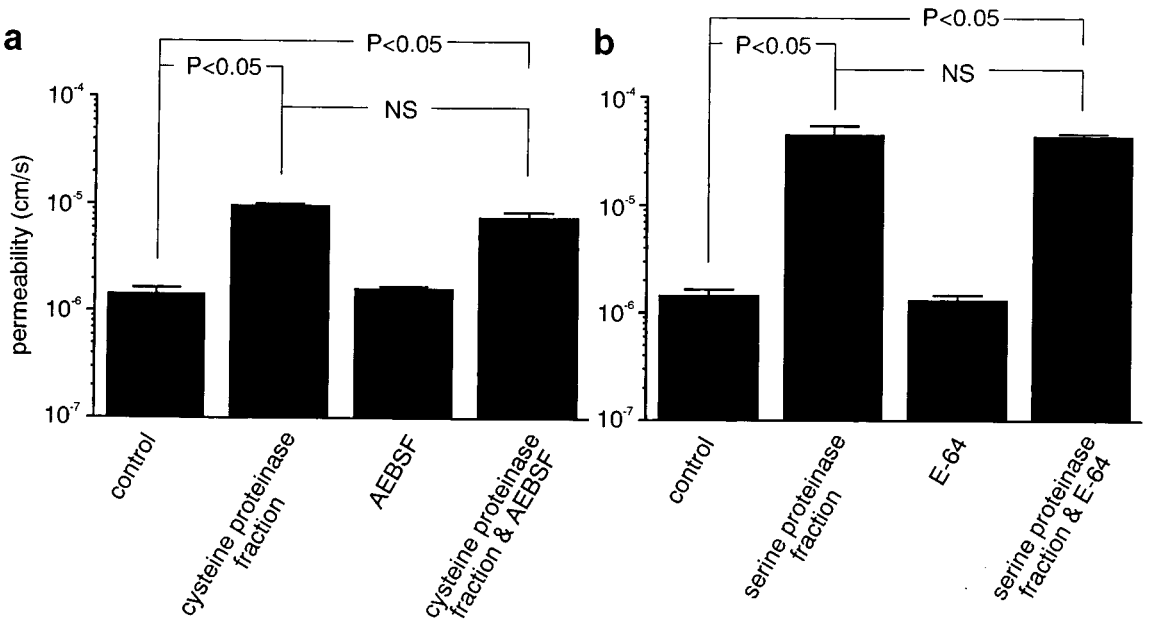

Figure 4 (a) illustrates the effects of 100 μM AEBSF on the changes in mannitol permeability evoked in Calu-3 cell monolayers following 18 h exposure to the cysteine proteinase fraction (80 azocoll units ml^{-1}). Data are mean $\pm$ s.e.mean in three experiments. (b) shows the effect of 10 μM E-64 on Calu-3 cell monolayers treated with the serine proteinase fraction (94 azocoll units ml^{-1}) for 18 h. Data are mean $\pm$ s.e.mean in three experiments. Control cells were treated with serum-free EMEM medium alone (with 0.5 mM reduced glutathione in the case of the cysteine proteinase fraction).

Figure 5 shows that treatment of MDCK cell monolayers with either the cysteine or serine proteinase fractions produced a disruption of the normally contiguous peripheral staining pattern of the TJ protein ZO-1 and of the punctate staining of desmoplakin. Addition of E-64 to the cysteine proteinase fraction or AEBSF to the serine proteinase fraction inhibited the proteinase-dependent changes (Figure 5).

HDM proteinases induce cell death in epithelia

Control incubation of MDCK or Calu-3 cell monolayers for 18 h in serum-free EMEM produced negligible release of LDH until they were subjected to hypotonic lysis at the end of the experiment (Figure 6). Treatment of monolayers of either cell type with the cysteine and serine proteinase fractions also failed to release significant amounts of LDH into the incubation medium (Figure 6), despite the fact that some cells detached from the matrix substratum during the experiment. Lysis of the detached cells and the adherent cells resulted in the recovery of LDH equivalent in amount to that found in untreated cell lysates (Figure 6).

Cell death was also studied by examining DNA fragmentation and studying the staining of cells with AV and PI. Figure 7a,b shows evidence of DNA fragmentation in MDCK and Calu-3 cells following treatment with HDM proteinases under conditions that result in an increase in permeability of epithelial monolayers. The effects of both HDM proteinase fractions were attenuated by the matrix metalloproteinase inhibitor BB-250 (Figure 7b). E-64 inhibited the effects of the cysteine proteinase fraction, but not the serine proteinase fraction (Figure 7b). Figure 7 also shows that treatment of MDCK or Calu-3 cells with the serine proteinase fraction resulted in some cells within monolayers binding annexin V alone (AV^+PI^-), indicative of early apoptosis in the cells, and others which stained with both annexin V and PI (AV^+PI^+) indicating cell death (see Figure 7d,f,h,j). The spatial distribution of AV^+PI^- early apoptotic cells and AV^+PI^+ dead cells was clustered around regions where there was clear disruption of cell adhesion (e.g. see Figure 7d,f).

Discussion

HDM faecal pellet proteins are a major cause of allergic asthma (Tovey *et al*., 1981) and are thought to be key factors which underlie the increasing prevalence of this disease (Dowse *et al*., 1985). In this study we have shown that proteinases from *D. pteronyssinus* faecal pellets exert potent biological effects on epithelial cells. The HDM proteinases were fractionated by ammonium sulphate precipitation into cysteine and serine classes. Both precipitates had similar effects in the experimental systems investigated. They produced an increase in permeability of epithelial monolayers, caused cleavage of lateral cell adhesion, and detached cells from the biomatrix substratum. Cleavage and detachment of cells was not associated with gross release of LDH, but evidence was found of early apoptosis and outright cell death with nuclear rupture. Inspection of cells stained with AV and PI revealed that staining was localized to areas of cell disruption/detachment. We further demonstrated that cell death could be attenuated by proteinase inhibitors.

Ammonium sulphate precipitation proved to be an effective means of separating two classes of proteinase in the spent HDM culture medium. The fraction precipitated by 50% ammonium sulphate had cysteine proteinase activity and its permeability promoting effect on epithelial cell monolayers was inhibited by E-64, a compound known to be an irreversible inhibitor of cysteine proteinases (Barrett *et al*., 1982). However, AEBSF, an inhibitor of serine proteinases (Baker & Cory, 1971; Kagaya *et al*., 1997; Stefanis *et al*., 1997), had no effect on the activity of this fraction. SDS–PAGE and immunoblot analysis with mAb 5H8 raised against the HDM allergen Der p 1 (a cysteine proteinase) revealed the presence of bands with apparent molecular masses of ~22 kDa and ~38 kDa in this fraction. In contrast to the fraction precipitated by 50% ammonium sulphate, that precipitated by 50–80% saturated ammonium sulphate had serine proteinase activity and its effects on epithelial cells were inhibited by AEBSF, and to a lesser degree SBTI, but not at all by E-64. The weak inhibitory effect of SBTI seems to result

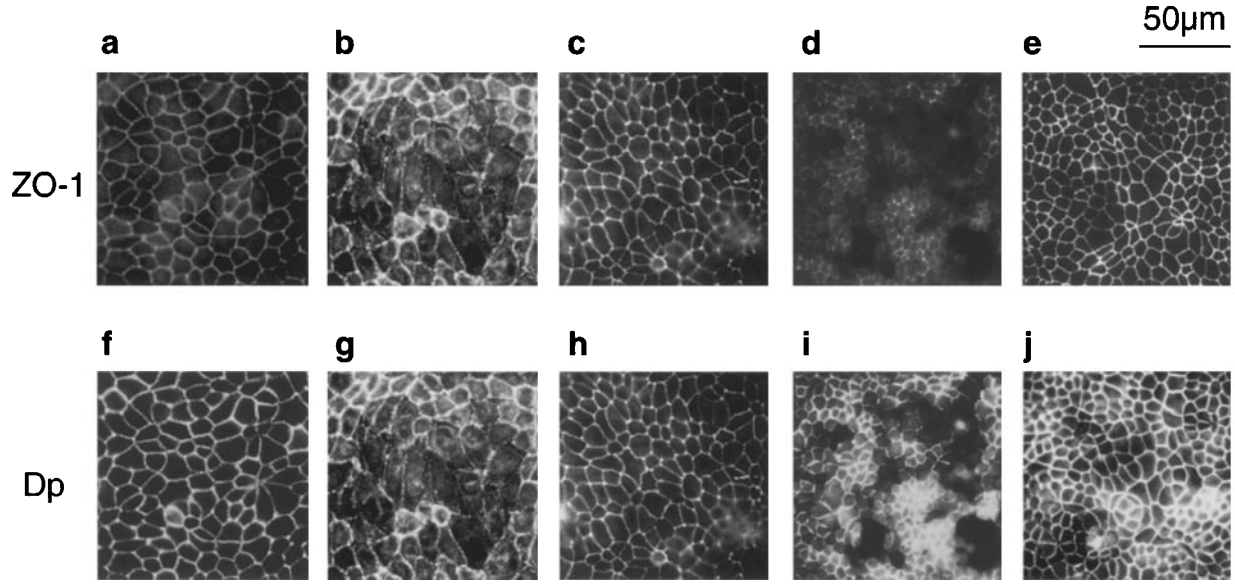

Figure 5 Immunostaining of the TJ protein ZO-1 (a)–(e) and the desmosomal plaque protein desmoplakin (f) to (j) in MDCK cell monolayers. (a) and (f) show immunostaining of cells exposed to serum-free EMEM as control. The pattern of immunostaining after treatment with the cysteine proteinase fraction (80 azocoll units ml^{-1}) is shown in (b) and (g) and the modification of this response by E-64 (10 μM) in (c) and (h). The pattern of immunostaining after treatment with the serine proteinase fraction (94 azocoll units ml^{-1}) is shown in (d) and (i) and the modification of this response by AEBSF (100 μM) in (e) and (j).

from a degradation of this proteinaceous inhibitor by the proteinase allergens themselves (unpublished observations). An analogous degradation of a proteinaceous enzyme inhibitor by a proteinase allergen has been described by others (Kalsheker *et al*., 1996). We conclude from these experiments that the precipitation procedure provides a facile means of separating HDM protineases into functional classes of enzyme activity. The finding that mAb 5H8 detected proteins in the fraction which exhibited cysteine proteinase activity was as expected. The bands detected at ~23 kDa and ~38 kDa in this fraction are consistent with the presence of mature and pro-forms of the cysteine proteinase allergen Der p 1. However, the positive reaction of mAb 5H8 with proteins in the fraction which had functional serine proteinase activity was surprising. The bands with apparent molecular masses in the range 26–29 kDa in the serine proteinase fraction are consistent with the three serine proteinase allergens (Der p 3, 6 and 9) from *D. pteronyssinus*. The existence of this cross reaction of 5H8 with the serine and cysteine proteinase fractions does not affect the interpretation of any results

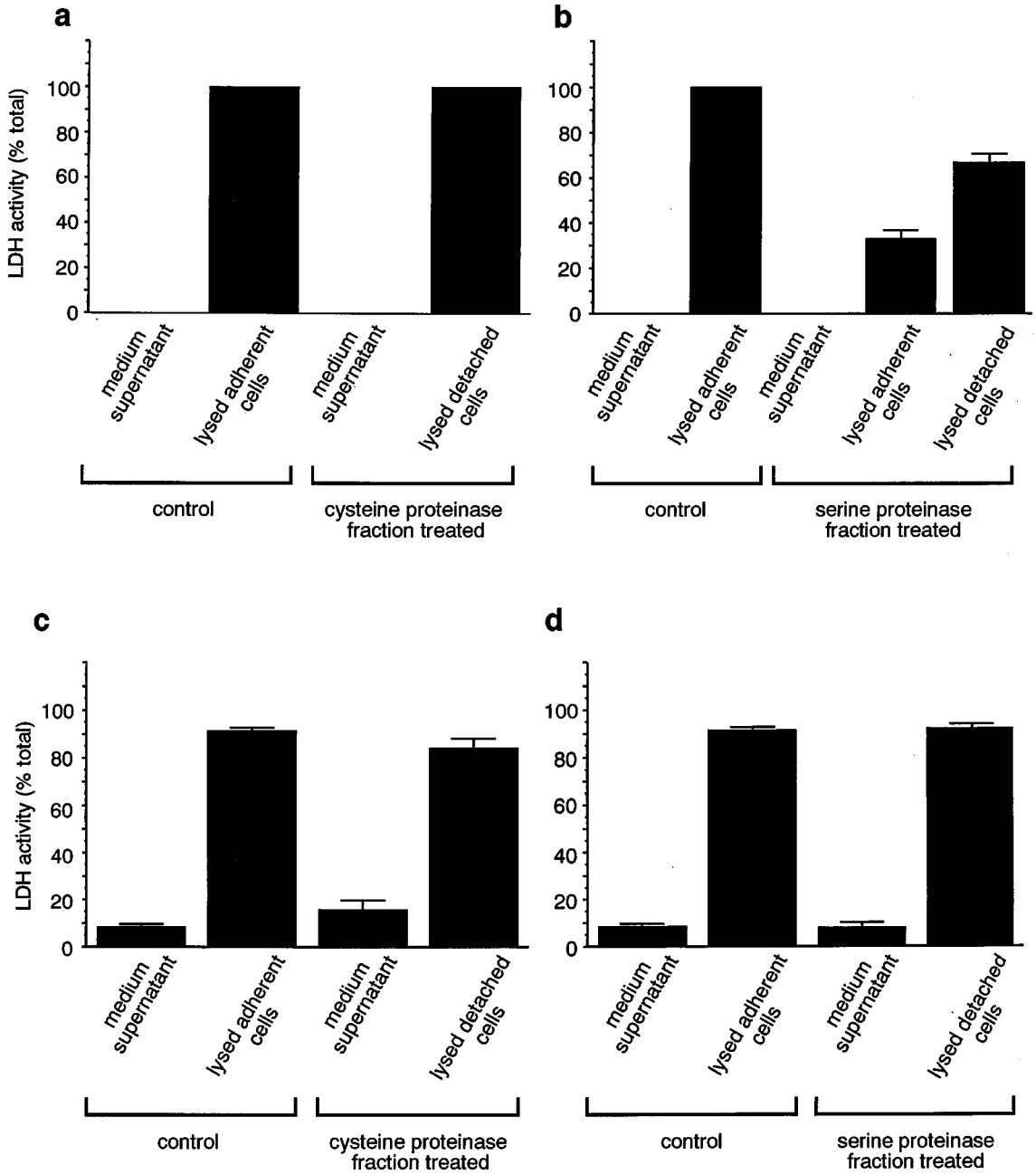

Figure 6 Measurement of LDH release following treatment of MDCK or Calu-3 cells with HDM proteinase fractions for 18 h. (a) shows the lack of release of LDH from cells under control conditions until the monolayer was subjected to hyptotonic lysis. (a) also shows that treatment with the cysteine proteinase fraction (80 azocoll units ml^{-1} after activation with 0.5 mM reduced glutathione) resulted in no release of LDH into the medium during the treatment period. Note that all of the cells were detached from the wells during treatment and that hypotonic lysis of the detached cells resulted in recovery of the same amount of LDH activity measured in the control cells. (b) illustrates a similar experiment using the serine proteinase fraction (114 azocoll units ml^{-1}). Note that not all of the cells were detached during the treatment period, but that the sum of LDH activity in lysed adherent and lysed detached cells correspond to the amount detected in control cells. (c) and (d) show similar experiments performed in Calu-3 cells. Note that there was a small background release of LDH from these cells (<10% total cellular LDH) under control conditions and that this was not significantly altered by proteinase treatment. Data shown are mean ± s.e.mean in four experiments.

printed in this paper. Rather, it provided an unexpected means of studying the proteins in both precipitated fractions. However, the apparent cross reactivity may have implications when this mAb is used to either purify or quantify HDM allergens. The mAb 5H8 is widely used for both of these purposes and it is noteworthy that other independent data exist which question the specificity of this reagent (Cambra & Berrens, 1996). Our data provide further grounds for exercising caution in the use of mAb 5H8 for quantitative or preparative purposes, but further work will be necessary to characterize the extent of the potential problem of cross reaction.

We have previously shown that highly purified Der p 1 allergen induces an increase in the transepithelial flux of serum albumin in the airway mucosa, causes disruption of epithelial architecture and detaches MDCK cells from natural biopolymer substrata (Herbert *et al*., 1990; 1995). We also demonstrated that these effects of Der p 1 were a result of its cysteine proteinase activity because they were sensitive to inhibition by E-64, a relatively specific inhibitor of most cysteine proteinases (Barrett *et al*., 1982; Shaw, 1990; Herbert *et al*., 1995). Although comparisons of amino acid sequence predict that Der p 1 is a putative cysteine proteinase (Chua *et al*., 1988; 1993; Stewart, 1994; Topham *et al*., 1994; Robinson *et al*., 1997b), others have suggested that it might act as a bifunctional cysteine-serine proteinase because its activity has also been reported to be inhibited by APMSF, an inhibitor of serine proteinases (Hewitt *et al*., 1995; 1997). If correct, this proposed bifunctionality would have potentially important implications for the design of specific inhibitors of Der p 1. However, in the present study we argue against the functional significance of the claimed bifunctionality by showing (i) that the cysteine proteinase fraction derived from HDM cultures could not be inhibited by concentrations of AEBSF that significantly inhibited the activity of the serine proteinase fraction, and (ii) the serine proteinase fraction being resistant to inhibition by E-64 at concentrations of this inhibitor which block cysteine proteinase activity. Other evidence against Der p 1 being a mixed cysteine-serine proteinase has also been presented recently (Chambers *et al*., 1997).

The permselectivity of the bronchial epithelium to hydrophilic solutes is governed by TJs which are expressed circumferentially at the apical pole of each cell (Schneeberger & Lynch, 1992). Contiguous expression of the TJ proteins of one cell and their close opposition with TJ proteins on adjacent cells is thought to result in the epithelium being able to develop its tight properties (Anderson & Van Itallie, 1995; Robinson, 1995). Disruption of the interaction of the TJ proteins between

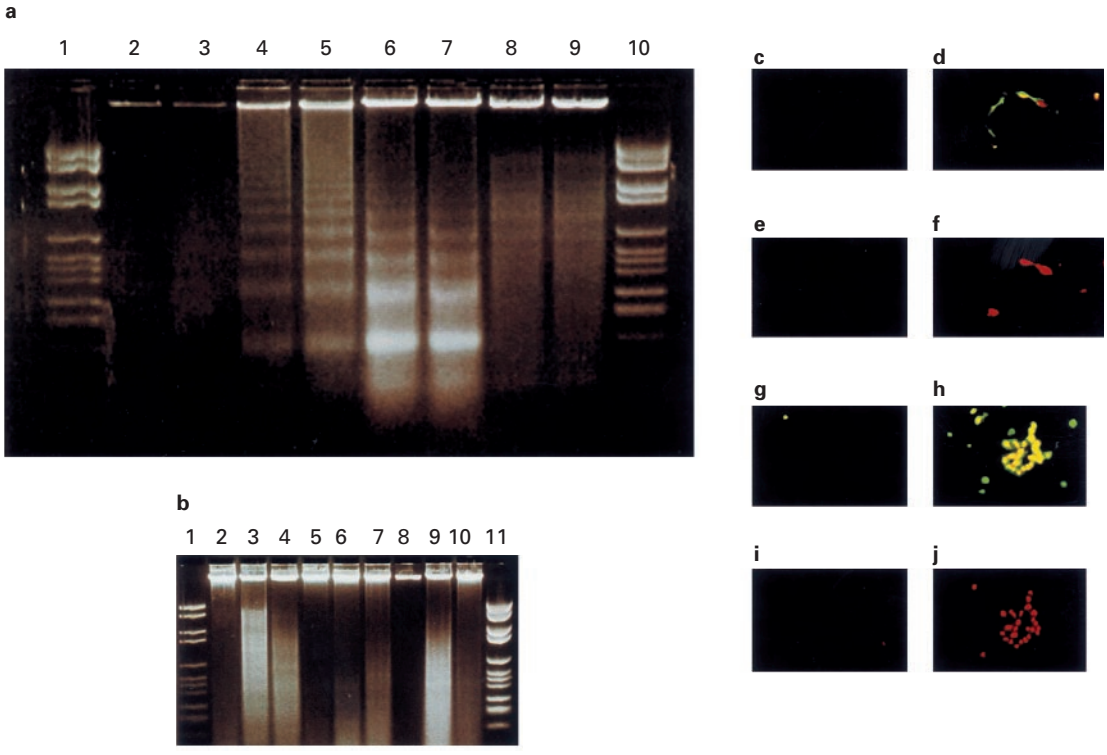

Figure 7 Agarose gel electrophoresis of DNA (a) and (b) and cellular staining with AV and PI (c) to (j) following treatment of MDCK and Calu-3 cells with HDM proteinase fractions. (a) shows proteinase-induced DNA fragmentation in MDCK cells. Key to lanes: DNA markers (1, 10); untreated cells (2, 3); cells treated for 18 h with 10 μM camptothecin (positive control) (4, 5); cells treated for 18 h with serine proteinase fraction (114 azocoll units ml^{-1}) (6, 7) and cells treated with cysteine proteinase fraction (80 azocoll units ml^{-1} after activation) for 18 h (8, 9). (b) shows DNA fragmentation in Calu-3 cells. Key to lanes: DNA markers (1, 11); untreated cells (2); cells treated for 18 h with serine proteinase fraction (94 azocoll units ml^{-1}) (3); cells treated with cysteine proteinase fraction (80 azocoll units ml^{-1} after activation) for 18 h (4); untreated cells in the presence of BB-250 (5 μM) (5); as lane 3, but cells treated in the presence of 5 μM BB-250 (6); as lane 4, but cells treated in the presence of 5 μM BB-250 (7); untreated cells in the presence of 10 μM E-64 (8); as lane 3, but cells treated in the presence of 10 μM E-64 (9); as lane 4, but cells treated in the presence of 10 μM E-64 (10). (c) to (f) show fluorescence microscopy of AV and PI (e) and (f) staining of Calu-3 cell monolayers under control conditions (c) and (e) and following treatment for 18 h with the serine proteinase fraction (135 azocoll units ml^{-1}) (d) and (f). (c) and (d) show the staining pattern under blue light excitation and (e) and (f) show that under green light excitation. (g) to (j) show examples from MDCK cell monolayers with the same layout as (c) to (f). In both cell types, note the virtual absence of AV and PI staining in untreated cells. In (d) and (f) the staining pattern partially circumscribes an area of cell detachment in the monolayer and note that some cells exhibit both AV and PI staining. In (h) and (j) the majority of stained cells are positive for both AV and PI and no significant detachment of cells from the substratum was evident.

cells, for example by the formation of discontinuities in their perijunctional localization, is associated with failure of epithelial barriers (Howarth *et al*., 1994; Zhong *et al*., 1994; Stuart *et al*., 1994; Stuart & Nigam, 1995). Both fractions of HDM proteinase used in this study caused breakdown of TJs as assessed by loss of perijunctional staining of ZO-1. Some disruption of desmosomes was also observed. The breakdown of TJs resulted in an increased permeability of epithelial monolayers, and eventually physical detachment of cells from the substratum occurred. The loss of ZO-1 from TJ and desmoplakin from desmosomes was dependent upon exogenous proteinase activity because the process was attenuated by E-64 (in the case of the cysteine proteinase fraction) and AEBSF (in the case of the serine proteinase fraction). Although ZO-1 and desmoplakin are intracellular proteins, and thus unlikely to be degraded by exogenous proteinases, their breakdown is explicable as a consequence of disruption of other, membrane-exposed, components of TJ and desmosomes. A similar mechanism has been invoked to account for changes in other intracellular proteins following cleavage of intercellular contacts (Volk *et al*., 1990).

The HDM proteinases did not cause significant release of LDH from the cells. However, treatment with either of the proteinase fractions resulted in some cells exhibiting signs of early apoptosis (AV^+PI^-) or outright cell death (AV^+PI^+). Cells may enter apoptosis by multiple mechanisms (reviewed in Hale *et al*., 1996) including changes in homotypic and heterotypic cell adhesion and cell-matrix attachment (Boudreau *et al*., 1995; 1996; Mahida *et al*., 1996; Frisch & Francis, 1994). An early signalling event in programmed cell death is disruption of phospholipid-binding cytoskeletal proteins which leads to the transmembrane redistribution of phosphatidylserine (Martin *et al*., 1995a,b). The framework of cytoskeletal proteins is normally stabilized and restrained by direct interaction with protein components of intercellular junctions (Furuse *et al*., 1994; Anderson & Van Itallie, 1995) which suggests that proteolysis of intercellular adhesions, especially TJ, could be the critical event in orchestrating the cellular response to proteinase allergens. The ability of E-64 to inhibit the action of the cysteine but not serine proteinase fraction suggests that E-64 acted at a proximal step in the process leading to permeability changes and apoptosis, rather than by inhibiting a distal proteolytic step in a convergent transduction pathway. Furthermore, intracellular signalling proteinases of the ICE/ced-3 caspase family that are activated *inter alia* by Fas/APO-1 ligation in apoptosis (Los *et al*., 1995; Mariani *et al*., 1995; Kayagaki *et al*., 1995; Tanaka *et al*., 1996) have an unusual inhibitor profile in being insensitive to E-64.

In contrast to E-64, the matrix metalloproteinase inhibitor BB-250 attenuated the apoptotic response induced by both of the HDM proteinase fractions. We have previously reported (Herbert *et al*., 1995) that this compound inhibited the increase in mucosal permeability produced by a crude extract prepared from HDM cultures. As BB-250 is not an inhibitor of the HDM cysteine or serine proteinases other options must be considered to explain the actions of this compound in our experimental systems. Several possibilities exist. Metalloproteinases are implicated in the release of Fas ligand, an important pathway which can lead to apoptosis (Mariani *et al*., 1995; Kayagaki *et al*., 1995). If the effect of the HDM proteinases were to activate epithelial cells to produce a metalloproteinase capable of cleaving Fas ligand then the inhibitory effect of BB-250 could be simply explained. It is known that epithelial cells derived from lung can produce the matrix metalloproteinases gelatinase A and gelatinase B (Herbert *et al*., 1996; Robinson *et al*., 1997a; Canete-Soler *et al*., 1994; Yao *et al*., 1996) but it is not known whether these enzymes cleave Fas ligand in this situation. A second possibility is that metalloproteinases are embedded in the Matrigel undercoats on which the epithelial cells are cultured and that these become activated by the HDM proteinases. Although biomatrix is known to provide a 'reservoir' for a variety of growth factors and enzymes (Yurchenko & Schnittny, 1990) including matrix metalloproteinases (Mackay *et al*., 1993), we consider this explanation less likely in our experimental setting because the cells were grown on ultra-thin ungelled layers of Matrigel which would provide exposure to significantly lower amounts of these agents than would a thick gelled layer of matrix undercoat.

Lung sensitization to airborne allergens such as those of HDM is central to the pathogenesis of allergic asthma. The lung epithelium forms a barrier that foreign proteins must cross before they can cause allergic sensitization, but the mechanism by which allergens cross the epithelial barrier is poorly understood (Robinson *et al*., 1997b). The enzymatic nature of proteins derived from HDM faecal pellets provides one explanation of the mechanism by which allergens encounter the immune system. By causing focal disruption of tight junctions, and ultimately the loss of moribund cells, HDM proteinases would be able to increase the paracellular permeation of allergens to antigen presenting cells. It is noteworthy that the localized trauma and cell death produced by proteolytic cleavage of intercellular junctions may also fulfil some conditions of the 'danger model' of adaptive immunological response (Matzinger, 1994; Ridge *et al*., 1996) and thus explain why allergens evoke antibody-directed responses. In further support of this view, recent evidence has suggested that when apoptosis (previously considered to be an immunologically 'silent' form of cell death) occurs in the presence of tissue injury, the resulting combination is a potent stimulus for antigen presenting cells (Boockvar *et al*., 1994; Casciola-Rosen *et al*., 1994; Ibrahim *et al*., 1996).

In summary, these results show that HDM proteinases have effects on epithelial cells which are likely to promote allergic sensitization. The ability of specific inhibitors to interfere with epithelial cell responses to proteinase allergens suggests a rationale for the development of novel inhibitors with therapeutic application.

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