

Cell lines of pulmonary and non-pulmonary origin as tools to study the effects of house dust mite proteinases on the regulation of epithelial permeability

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Summary

Background Allergenic and non-allergenic proteinases from house dust mites (HDM) cause loss of adhesion between airway epithelial cells that may result in a loss of functional cohesion between the cells and thus assist in allergen presentation. Improved cellular assay systems are needed to ascertain the mechanisms involved.

Objectives To survey a series of epithelial cell lines (Calu-3, 16HBE14o⁻, NCI-H292 and A549 from human airways, and MDCK from dog kidney) and establish their utility for studies of the effects of HDM proteinases from *D. pteronyssinus* on epithelial permeability. To develop an improved method for measuring changes in epithelial permeability induced by HDM proteinases and other provocants.

Methods The permeability of epithelial monolayer cultures to mannitol was calculated from measurements of clearance using a technique that permits mathematical estimation and reduction of non-cellular diffusional constraints. Permeability was studied under control conditions and after perturbation of monolayers with HDM proteinases (separated into serine- and cysteine-proteinase classes) or chelation of extracellular Ca²⁺. Fluorescent antibody staining was used to investigate whether the cells expressed tight junctions (staining of ZO-1), desmosomes (staining of desmoplakin) and zonulae adherentes (staining of E-cadherin).

Results The Calu-3 line was identified as an airway cell line that expressed functional tight junctions, desmosomes and zonulae adherentes. Calu-3 monolayers exhibited a low clearance and permeability to mannitol, similar to that seen in the extensively characterized MDCK cell line. Clearance and permeability were significantly increased by treatment with either HDM proteinase fraction or by calcium chelation. 16HBE14o⁻ cells also had a low permeability to mannitol under control conditions and expressed a similar repertoire of functional proteins from major intercellular junctions. In contrast, NCI-H292 and A549 cell lines were functionally deficient in tight junctions, although they did express desmosomes and zonulae adherentes to a greater extent. Epithelial permeability was found to be a more appropriate and sensitive index of epithelial perturbation than was tracer clearance.

Conclusion These results suggest that the Calu-3 and 16HBE14o⁻ cell lines are useful tools in studying the mechanism of HDM proteinases on airway epithelial cell function. HDM proteinases of both cysteine and serine mechanistic classes were found to perturb epithelial adhesion and function.

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Introduction

There is an increasing body of evidence that several major allergens from house dust mites exhibit catalytical competence as enzymes [1–3 and reviewed in 4]. This has prompted suggestions that these enzymatic actions might contribute directly to allergic sensitization and to the perpetuation of established allergic inflammatory reactions [1–4].

Most data concerning proteinase activity in mite allergens currently relate to those of groups 1,3,6 and 9 from mites of the genus *Dermatophagoides* [4]. The group 1 allergens are cysteine proteinases and have been subject to the greatest scrutiny, whereas much less information exists concerning the enzymatic effects of the serine proteinases that comprise allergens of groups 3, 6 and 9 [4]. The possession of functional proteolytic activity confers upon allergens the ability *inter alia* to stimulate cytokine expression by epithelial cells and, at least for those of the serine proteinase class, the potential to activate inflammatory cells for degranulation [2–4].

Evidence has recently been presented that the group 1 allergen Der p 1 increases the net unidirectional flux of albumin across isolated sheets of bronchial mucosa [1]. It was also demonstrated that Der p 1 could detach epithelial cell monolayers from both artificial and natural biopolymer substrata with a similar efficacy to trypsin or a metallo-elastase from *Pseudomonas aeruginosa* [1]. Exposure of isolated bronchial segments to Der p 1 also resulted in disruption of lateral and basolateral adhesion between epithelial cells in a manner that resembles the effect of enzymes such as trypsin [1]. It has been suggested that the possession by allergens of functional proteolytic activity might be one factor which explains the insidious nature of these molecules in initiating and subsequently re-stimulating an allergic response in genotypically and phenotypically susceptible individuals [1,4]. By being able to facilitate their own penetration of epithelial barriers through an ability to increase paracellular permeability allergens might gain greater access to antigen-presenting cells in submucosal tissue. In the case of Der p 1, it might also favour IgE synthesis by causing cleavage of the low affinity IgE F_c receptor (CD23) on B lymphocytes [5,6]. In an extension of the concepts outlined above, it is also possible that non-allergenic enzymes from dust mites might serve an accessory role in facilitating the presentation of both catalytically active and inactive allergenic proteins present within mite faeces [4].

Under normal circumstances the healthy tracheo-bronchial epithelium is a barrier that offers significant resistance to the paracellular flow of macromolecules [7]. Tight junctions restrict the paracellular permeability of solutes [8,9], whilst other intercellular junctions regulate epithelial architecture and are thus thought to provide the necessary environment for the normal function of tight junctions [10–12]. Paracellular permeation of hydrophilic solutes is related to the number and density of strands within the tight junction [9,13], although some exceptions to this generalization exist and strand organization rather than absolute abundance may be a significant factor [14]. Disruption of tight junctions and other intercellular junctional complexes by proteinases would therefore be expected to cause an increase in epithelial permeability and alteration of other functions even before epithelial barrier properties become totally compromised by outright cell loss.

Several studies have investigated the influence of a variety of external variables on the permeability of the tracheo-bronchial epithelium. These studies range from clinical investigations in humans [15–19] to examinations at the cellular level [20,21]. Data from whole organism studies are difficult to interpret and are often poorly suited to the elucidation of cellular mechanisms unless they are directed by rationales established in model systems. Isolated tissue studies maintain a quasi-physiological relevance [22,23], but still possess a level of cellular complexity that can confound data interpretation. In contrast, experiments with homogeneous monolayer cultures are potentially the most useful of systems. Mechanisms can be investigated under carefully controlled *in vitro* conditions and the outcomes used to devise strategies for directed investigations *in vivo*. The effects of several perturbants on the barrier properties of epithelial monolayers have been reported, but the interpretation of the data reported is hindered by flaws in the experimental techniques used. Although several such studies report apparent changes in epithelial permeability, this has not usually been measured specifically. For example, studies have involved only single sampling points, the measurement of gross percentage flux of tracers across cellular barriers, or they have neglected the diffusional resistance of the culture substratum and have not quantified the magnitude of unstirred layers in their diffusional systems [22–28]. Experiments have also been performed using a miscellany of airway epithelial cell lines or very variable primary cultures [24–26,28], without consideration of whether these cells exhibit a normal repertoire of functional intercellular adhesion molecules.

The normal function of tight junctions may rely upon the correct expression and function of other intercellular junctions and the cytoskeleton [29], and so when only grossly normal morphology of cultures has been reported, the expression of normal function must be viewed with caution.

As part of a programme of work investigating the relationship between the proteolysis of intercellular adhesion molecules and airway microinjury we have been screening cell lines for their possible use in studies of the effects of dust mite proteinases. We now report a series of studies in which we have compared five cell lines for their utility as permeability models in which the effects of dust mite proteinases can be investigated. We also describe an improved technique for estimating permeability changes in epithelial cell monolayers which should be applicable to the study of a diverse range of epithelial perturbants.

Methods

Cell culture

This study involved a survey of the usefulness of five epithelial cell lines in studying the effects of proteinases on epithelial permeability. Three of the lines investigated were of human pulmonary tumour provenance, the fourth a post-crisis SV40 large T antigen transformed cell line, whilst the fifth was of non-tumour origin from canine kidney.

The A549 line is a human lung adenocarcinoma derived by explant culture from the peripheral airways of a Caucasian male with lung cancer. Stock cultures were grown in Dulbecco's modified Eagle medium (DMEM) containing 50 U/mL penicillin, 50 µg/mL streptomycin, 40 µg/mL gentamicin, 2 mmol/L L-glutamine and 10% v/v heat-inactivated fetal calf serum. Culture medium was changed every 2–3 days. The cells were subcultured by rinsing three times in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free Hanks' balanced salt solution (HBSS) and then by treatment with 0.25% w/v trypsin solution.

The NCI-H292 cell line is a human pulmonary mucopidermoid cell line derived from a carcinoma removed from a 32-year-old black female [30,31]. This cell line has been the subject of relatively little previous investigation, but has been suggested to express tight barrier properties [28]. The cells were propagated in RPMI 1640 medium containing 50 U/mL penicillin, 50 µg/mL streptomycin, 2 mmol/L L-glutamine and 10% v/v heat-inactivated fetal calf serum.

Calu-3 is an adenocarcinoma cell line derived from a 25-year-old Caucasian male. It has been the subject of relatively few investigations [32], but has been suggested to express tight barrier properties on the basis of electrophysiological studies. The cells were propagated in Eagle's minimum essential medium with Earle's salts (EMEM) supplemented with 10% v/v heat inactivated fetal calf serum,

2 mmol/L L-glutamine, non-essential amino acids, 10 µmol/L sodium pyruvate and containing 50 U/mL penicillin and 50 µg/mL streptomycin.

16HBE14o⁻ cells established as previously described [33] were cultured in EMEM containing 50 U/mL penicillin, 50 µg/mL streptomycin, 2 mmol/L L-glutamine, non-essential amino acids and 10% v/v heat-inactivated fetal calf serum. All culture flasks in which these cells were grown were treated with ultra-thin coatings of Matrigel as described later.

Madin-Darby canine kidney (MDCK) epithelial cells were cultured in EMEM containing 50 U/mL penicillin, 50 µg/mL streptomycin, 2 mmol/L L-glutamine, non-essential amino acids and 10% v/v heat-inactivated fetal calf serum. MDCK, Calu-3, 16HBE14o⁻ and NCI-H292 cells were subcultured by rinsing in phosphate-buffered saline (PBS) without calcium and magnesium and then by partial digestion 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 inserts with matrigel

Measurements of mannitol clearance were performed on confluent cell monolayers propagated on 0.4 µm pore diameter Costar Transwell inserts that had been previously coated with a thin, ungelled film of Matrigel. Coating was achieved by addition of 250 µL aliquots of Matrigel (diluted 1:500 v/v in the appropriate medium) 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 near-confluent density of cell suspension.

Cell treatment protocols and measurement of clearance

Cells were plated onto Matrigel-coated inserts at a density of 1×10^5 cells per insert for the A549 cells, $2\text{--}3 \times 10^5$ for NCI-H292 and MDCK cells, and at 5×10^5 for Calu-3 cells and 6×10^5 for 16HBE14o⁻ cells. 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, sample inserts were taken at random, washed gently in PBS and stained under subdued illumination with acridine orange and ethidium bromide. Inserts were used only when confluence had been established in high viability cultures.

Once the cells had become confluent, the medium was aspirated from the wells and replaced with serum- and bicarbonate-free medium buffered with 20 mmol/L HEPES and containing 2 mmol/L L-glutamine. The medium from the inserts was then gently removed and replaced with 300 µL of serum-free medium containing [¹⁴C]-mannitol

(1 μ Ci/mL and 1 mg/mL unlabelled mannitol in HEPES-buffered medium). The Transwell plates were then left to equilibrate for 30 min at 37 °C on a Luckham R100 orbital shaker. Triplicate 100 μ L aliquots of the unused, labelled mannitol solution were sampled and their radioactivity content determined by liquid scintillation spectrometry (Beckman LS6000IC) after the 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 medium 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 μ L 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.

Background to calculation of permeability

The clearance volume of mannitol at each time point in the experiment is given by:

$$V_{\text{mar}_t} = \sum_{i=1}^t \frac{V_{\text{cham}_2} \Delta[\text{mar}]_i}{[\text{L}]_i} \quad (1)$$

where:

V_{mar_t} is the clearance volume of the permeability marker at each time point;

V_{cham_2} is the basal well volume at each time point;

$\Delta[\text{mar}]_i$ is the increase in tracer concentration between time points; and

$[\text{L}]_i$ is the tracer concentration in the insert at each time point.

The volume of medium in the compartments is constant between sampling points, and thus calculation of results according to this approach accounts for changes in the volume of the solution in the well introduced by the sampling process as the experiment progressed. The clearance rate was determined by unweighted least squares linear regression of values from individual time points. Under conditions where diffusion is the sole means of transepithelial movement of the solute, dV_{mar}/dt approximates closely to the permeability–surface area product thus allowing estimation of the apparent permeability of mannitol. However, this is a composite estimate (P_{tot}) that includes the contributions to permeability from the filter itself, its Matrigel coating and the unstirred layers in the system. Estimates of epithelial permeability in which these errors are minimized can be obtained by considering the Matrigel-coated filter and unstirred layers as a system of series permeabilities. Thus:

$$\frac{1}{P_{\text{tot}}} = \frac{1}{P_1} + \frac{1}{P_2} + \frac{1}{P_3} \quad (2)$$

and

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

where:

P_{tot} is the composite permeability of the system;

P_1 is the component due to the epithelial cells alone;

P_2 is the unstirred layer component;

P_3 is the component due to the Matrigel-uncoated filter; and

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

In pure diffusion systems [35,36] it has been shown that

$$P_2 = \frac{D}{\delta} \quad (4)$$

where:

D is the free diffusion coefficient of mannitol; and

δ is the summed thickness of unstirred layers.

Under ideal conditions at planar surfaces, unstirred layer thicknesses are independent of membrane permeability. Thus the term P_2 in equations (2) and (3) is interchangeable allowing estimation of the permeability of the monolayer to be made (5) under error minimization conditions by subtraction of equations (2) and (3).

$$\frac{1}{P_1} = \frac{1}{P_{\text{tot}}} - \frac{1}{P_4} \quad (5)$$

Measurement of transepithelial electrical resistance

Transepithelial electrical resistance (TER) was determined by delivery of a $\pm 20 \mu\text{A}$ square wave alternating current across the monolayers at 12.5 Hz. The resistance of cell-free inserts was $\pm 1 \Omega \cdot \text{cm}^2$.

Preparation of serine proteinase fraction from house dust mites

Spent medium from cultures of *Dermatophagoides pteronyssinus* (Commonwealth Serum Laboratory, Parkville, Australia) was dissolved in five volumes of phosphate-buffered saline and then centrifuged at 48 400 g for 20 min at 4 °C. 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, 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. After centrifugation as above, the pellet containing the serine proteinase fraction was resuspended in a minimum volume of distilled water. The cysteine and serine proteinase fractions were both dialysed against distilled water overnight and then lyophilized prior to reconstitution in minimum essential medium with Earle's

salts, 20 mmol/L HEPES and 2 mmol/L L-glutamine. Protein content of the extracts was measured using the Coomassie Blue technique with serum albumin as standard. Proteinase activity was measured using the Azocoll degradation assay [1] and used in experiments at a final activity of ≈ 90 units/mL. One unit of activity is defined as $1000 \times (\Delta A_{520}/\text{min}/\text{mg protein})$ measured in the Azocoll degradation assay as described previously by us [1]. Extracts were declared free of endotoxin (<0.06 ng/mL) using the Limulus amebocyte lysis assay.

Cellular staining with fluorescent probes and indirect immunocytochemistry

To visualize confluency and viability, the cells grown on Matrigel-coated Transwell inserts were stained with ethidium bromide and acridine orange. The cells were gently rinsed twice with PBS and $100 \mu\text{L}$ of acridine orange and $100 \mu\text{L}$ ethidium bromide (1 mg/mL for each) added under subdued illumination. The cells were immediately visualized by fluorescence microscopy using a Zeiss Axiovert 10 microscope with excitation at 546 nm and emission at >590 nm.

Intercellular junctions were stained and visualized using three monoclonal antibodies (MoAb) for ZO-1, E-cadherin and desmoplakin, respectively. They were rat anti-ZO-1 (R40.86), rat anti-E-cadherin (DECMA-1) and mouse anti-desmoplakin (11-5F) [34]. In addition, a pan-reactive ZO-1 rabbit polyclonal antibody was also used in human cells in which R40.86 proved unsatisfactory. The working dilution of the antibodies was determined by titration assay. Cells were fixed in ice-cold methanol before antibody binding. For ZO-1 and E-cadherin staining in Calu-3, 16HBE14o⁻, A-549 and NCI-H292 cells, modified CSK buffer (composition: NaCl 50 mmol/L, sucrose 3000 mmol/L, PIPES (pH 6.8) 10 mmol/L, MgCl_2 3 mmol/L, 0.5% v/v Triton X-100, 4-(2-aminoethyl)-benzenesulphonyl fluoride hydrochloride 1.2 mmol/L) was applied to remove the 'background' staining before fixation. Indirect immunocytochemistry was performed using FITC-conjugated second antibodies. Specimen excitation was at 450–490 nm and emission at >520 nm with observations made using a Zeiss oil immersion objective.

All photographic documentation of results was made using a Contax 167MT camera and Kodak TMAX 400 film for black and white prints.

Distribution fitting and statistical analyses

Quantile–quantile plots were used to determine the most appropriate population distribution describing clearance and permeability across epithelial monolayers. Observed values

were ranked and plotted against the inverse probability function $F^{-1}(1 - R_a/n + n_a)$, where F^{-1} depends on the distribution and R_a and n_a are adjusted values of rank and number of observations. The maximum likelihood method was used to estimate the shape parameter of non-Gaussian distributions.

Analysis of variance was performed after log transformation of the 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.

Materials

All media and cell culture reagents were purchased from ICN Biomedicals Ltd (Thame, UK), except where stated. RPMI 1640 and HBSS were obtained from GibcoBRL, Life Technologies Ltd (Paisley, UK). Mannitol, gentamicin and Triton X-100 were obtained from Sigma-Aldrich Ltd (Poole, UK) and heat-inactivated fetal calf serum was from Labtech International Ltd (Uckfield, East Sussex, UK). Matrigel was obtained from Universal Biologicals, London, UK. The mannitol clearance measurements were undertaken using 12 mm diameter Transwells with $0.4 \mu\text{m}$ membrane pore size and $10 \mu\text{m}$ membrane thickness, from Costar UK Ltd (High Wycombe, UK). D-[¹⁴C]-mannitol was obtained from NEN Du Pont Research Products (Stevenage, UK), and the Opti-Fluor scintillant and the scintillation vials were from Canberra Packard Ltd (Pangbourne, UK). MDCK and NCI-H292 cell lines were obtained from the European Collection of Animal Cell Cultures (Porton Down, Wiltshire, UK) and the A549 cells were purchased from ICN Biochemicals Ltd. Calu-3 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were cultured in Falcon 75 cm² cell culture flasks (Marathon Laboratory Supplies, London, UK), Costar multiwell tissue culture plates or Transwell inserts according to the nature of the experiment. The monoclonal antibody directed against ZO-1 was a generous gift from Dr Bruce Stevenson (University of Alberta) and the rabbit polyclonal pan ZO-1 antibody was kindly donated by Dr Tom Fleming (University of Southampton). The E-cadherin MoAb (DECMA-1) was obtained from Sigma. Desmoplakin was stained using monoclonal antibody 11-5F as previously described [34]. FITC-conjugated second antibodies for use with MoAbs R40.86 and DECMA-1 were purchased from Vector Laboratories (Peterborough, UK), and those for use with mAb 11-5F and the polyclonal pan ZO-1 antibodies were from Sigma. Acridine orange, ethidium bromide and all other general laboratory reagents were obtained from BDH (Poole, Dorset, UK).

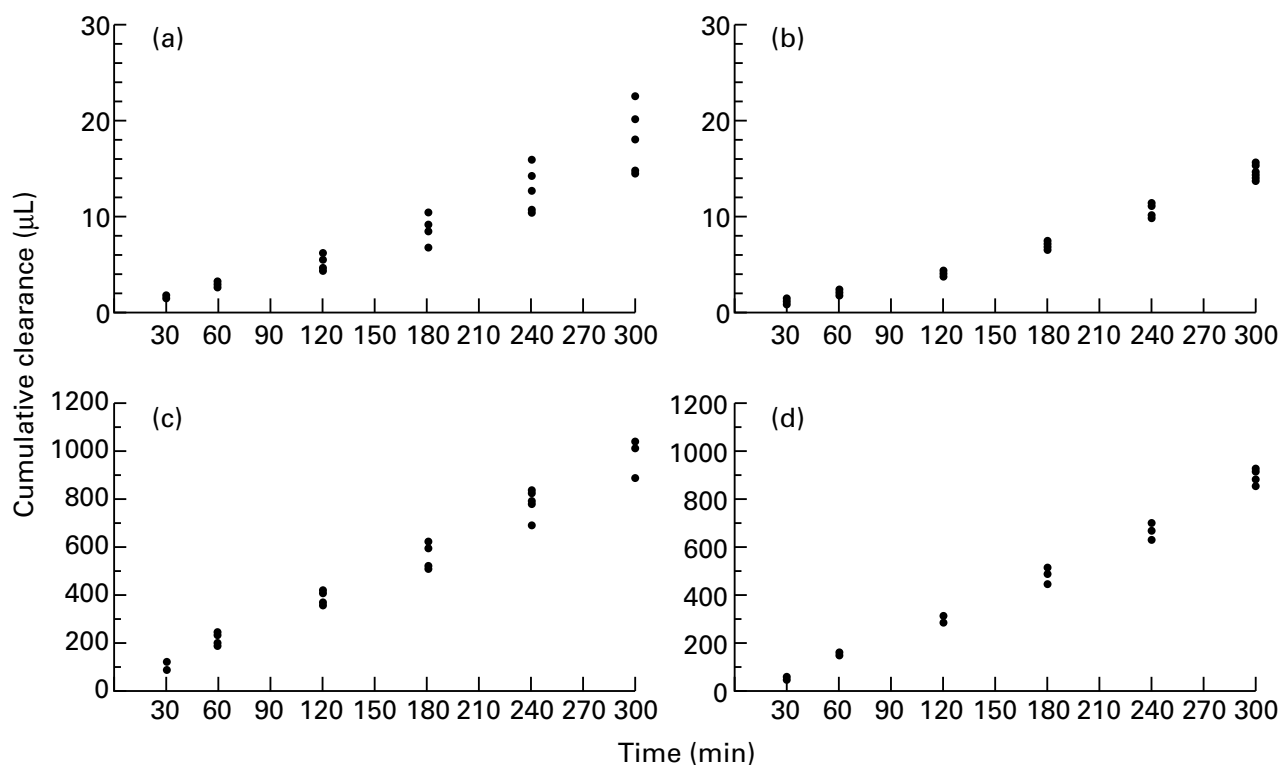


Fig. 1. Time-dependent clearance of mannitol in monolayers of (a) MDCK cells (b) Calu-3 cells (c) A549 cells and (d) NCI-H292 cells. Cells were grown on matrigel-coated Transwell inserts as described. A total of five separate experiments were performed for each of the four cell lines for which data are displayed. At each time point each symbol represents data from one experimental series.

Results

The transepithelial movement of mannitol in confluent cell monolayers was linear over the time in all of the cell lines evaluated in this survey. Four examples of this are shown in Fig. 1(a–d). Data for 16HBE140[−] cells were similar to those displayed for Calu-3 cells (not shown). The rates of clearance and epithelial cell permeabilities in cell-free Transwells coated with Matrigel and on Matrigel-coated Transwells with confluent cell monolayers approximated well to log-normal distributions when analysed by visual inspection of quantile–quantile plots (Fig. 2a–d), and logarithmic transformations were therefore applied to all values for statistical analysis.

Under control conditions, typical clearance rates were $0.0741 \pm 0.0009 \mu\text{L}/\text{min}$ in MDCK cells compared with 0.0520 ± 0.0010 , 0.0208 ± 0.0005 , 2.919 ± 0.089 and $2.787 \pm 0.121 \mu\text{L}/\text{min}$ in Calu-3, 16HBE140[−], A549 and NCI-H292 cell monolayers ($n=5$ experiments for each). Coefficients of variation between experiments were, respectively, 15.5, 22.4, 30.6, 12.4 and 7.5% for each of the cell types based on comparisons in 3–12 separate runs. The clearance values measured in monolayers containing either A549 or NCI-H292 cells were very similar to the clearance

rates of mannitol through cell-free Matrigel-coated Transwells ($3.530 \pm 0.075 \mu\text{L}/\text{min}$, $n=23$). For reference, the clearance in uncoated filters was $4.47 \mu\text{L}/\text{min}$ and the summed thickness of unstirred layers in coated filters was $280 \mu\text{m}$. Epithelial permeability (i.e. values corrected for the permeability of the filter and its Matrigel coating) under control conditions was $(1.18 \pm 0.15) \times 10^{-6} \text{ cm}/\text{s}$ in MDCK cell monolayers, $(0.78 \pm 0.15) \times 10^{-6} \text{ cm}/\text{s}$ in Calu-3 cells, $(0.31 \pm 0.01) \times 10^{-6} \text{ cm}/\text{s}$ in 16HBE140[−] cells, $(2.60 \pm 0.42) \times 10^{-4} \text{ cm}/\text{s}$ in A549 cell monolayers and $(2.10 \pm 0.50) \times 10^{-4} \text{ cm}/\text{s}$ in NCI-H292 cells ($n=5$ for each). The permeability of the Matrigel-coated filters alone was $(5.18 \pm 0.11) \times 10^{-5} \text{ cm}/\text{s}$. The high permeabilities seen in A549 and NCI-H292 monolayers were due to the absence of functional tight junctions as judged by the discontinuous, faint or frankly absent pericellular staining of the tight junction associated protein ZO-1 (Fig. 3). Perijunctional staining of ZO-1 in A549 cells was fragmented (Fig. 3[d]) and never attained the continuous ring-like appearance typically expected of normal tight junctions. NCI-H292 cells expressed little ZO-1 (Fig. 3[c]). E-cadherin staining in NCI-H292 cells was less extensive than in MDCK cells (Fig. 3, compare [k] with [m]) but we noted that the staining of this protein became more pronounced as the NCI-H292

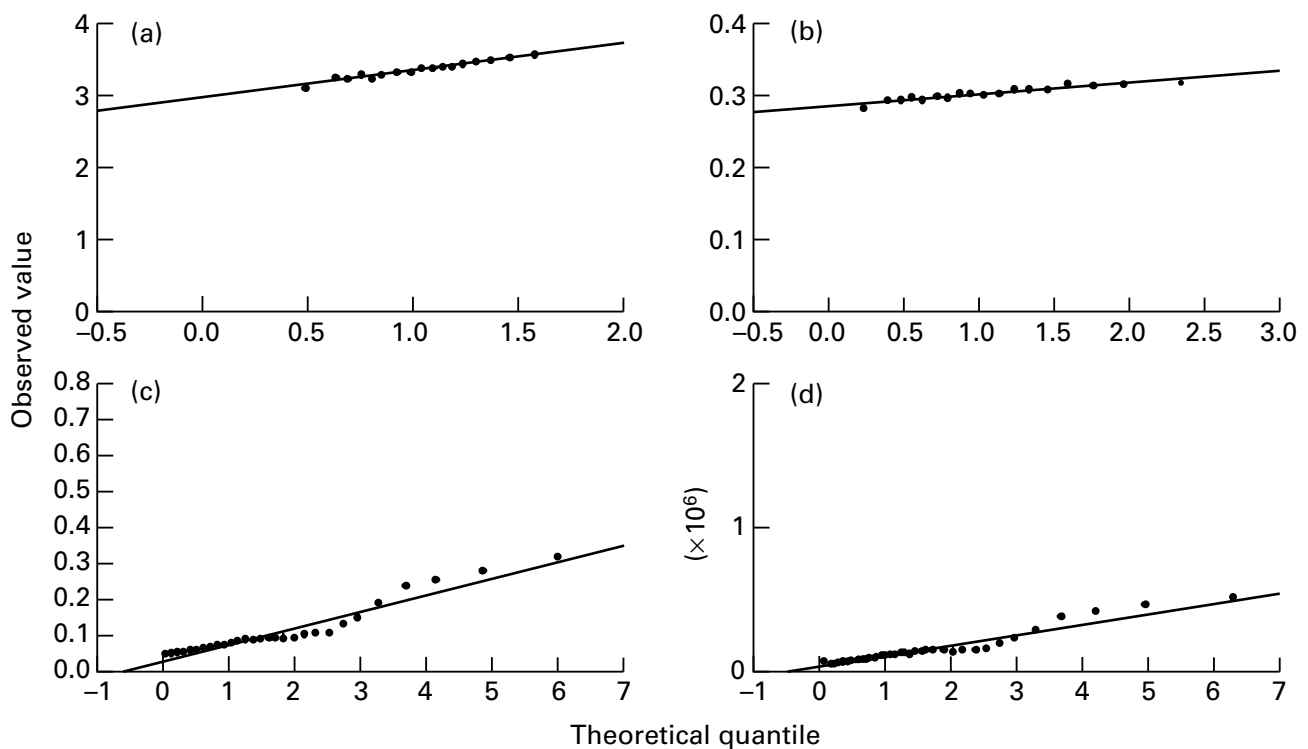


Fig. 2. Quantile–quantile plots of clearance (a), (c) and epithelial permeability (b), (d) in Matrigel-coated Transwells without cells (a), (b) and monolayers of MDCK cells (c), (d). Points depict experimentally observed values and the lines indicate theoretical fits for lognormal distributions.

cells began to stratify (not shown). In contrast, E-cadherin staining of A549 cells was weak (Fig. 3 [n]). NCI-H292 and A549 cells exhibited clearly positive staining of the desmosomal plaque protein desmoplakin, but this was less organized than the staining pattern of this protein in the other three cell lines (Fig. 3). Calu-3 cells and 16HBE14o[−] cells exhibited staining patterns of junctional proteins which were generally similar in appearance to that seen in MDCK cells. Perijunctional staining of ZO-1 was continuous and well-defined (Fig. 3[a], [b], [e]), although the pan ZO-1 antibody also detected some diffuse intracellular staining. Desmoplakin and E-cadherin staining in MDCK, 16HBE14o[−] and Calu-3 cells were characterized by typical punctate appearance of the desmosomes and staining of E-cadherin which extended beyond the continuous zonulae adherens. Qualitatively, the extent and contiguity of ZO-1 immunostaining at the peripheries of the human lung cells is inversely correlated with the permeability characteristics of the individual monolayers. This suggests that contiguous expression of ZO-1 by Calu-3 and 16HBE14o[−] cells contributes to the formation of functional tight junctions between neighbouring cells, a view supported by the TERs developed by these monolayers at confluence (301 ± 16 and $272 \pm 44 \Omega \text{ cm}^2$, respectively, $n = 4$).

Experimental manipulations were performed with proteinases or EGTA to perturb the control rates of mannitol clearance in cell monolayers so as to assess the relationship between clearance and epithelial permeability as indices of impaired epithelial barrier function. There was relatively little scope for augmentation of clearance and permeability in the A549 and NCI-H292 monolayers and consequently these cells proved to be less useful tools than did the MDCK, Calu-3 or 16HBE14o[−] monolayers.

Treatment of MDCK monolayers for 18 h with a cysteine proteinase fraction from house dust mite cultures (including the major allergen and cysteine proteinase Der p 1) produced a 17-fold increase in mannitol clearance across the cell and filter barrier and a 25-fold increase in epithelial monolayer permeability (Fig. 4[a], [b]). A similar extract containing serine proteinases (including the proteinase allergens Der p 3, Der p 6 and Der p 9) when tested under similar conditions produced a 36-fold increase in mannitol clearance across the cell and filter barrier, but a 150-fold increase in epithelial permeability (Fig. 5[a], [b]). The effect of the serine proteinase fraction was similar in magnitude to that of 0.1 mg/mL trypsin (Fig. 5[a], [b]), although it is difficult to make meaningful comparisons of relative activity when there are likely to be differences in the substrates that

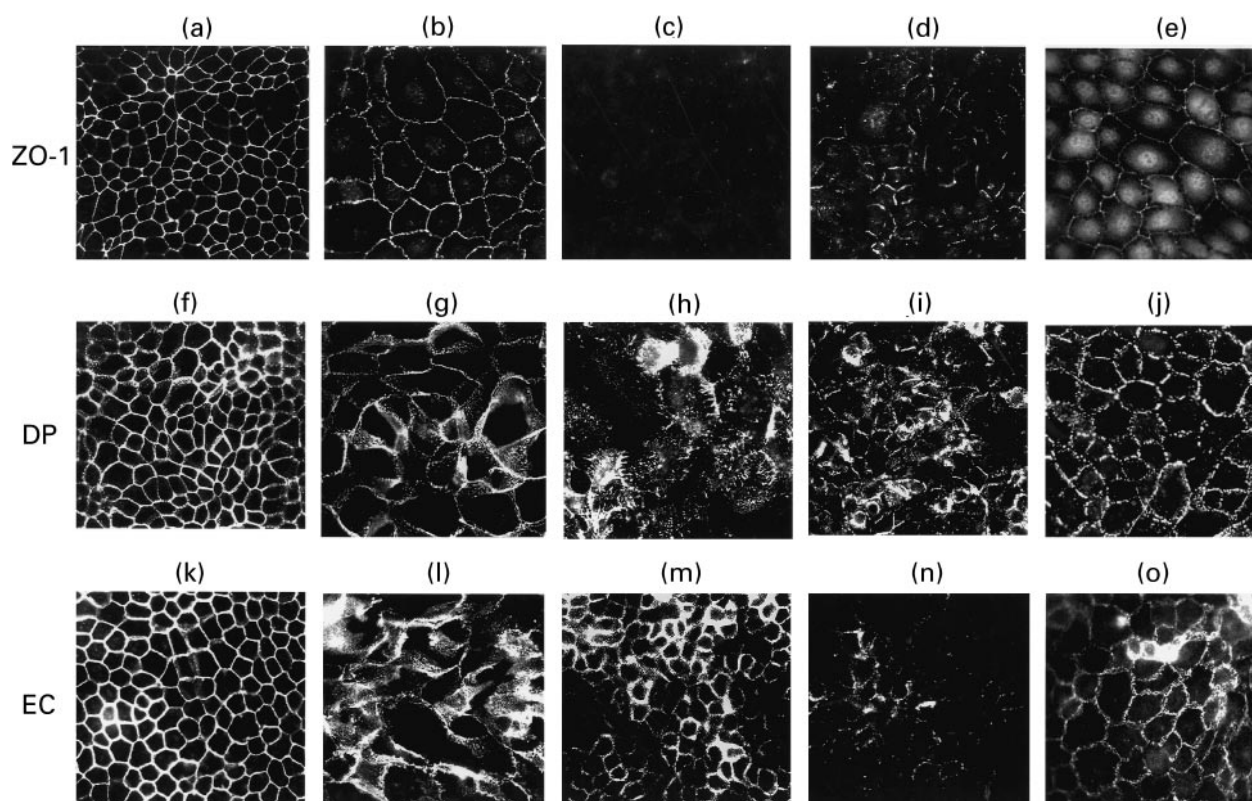


Fig. 3. Immunofluorescent labelling of intercellular junctions in the five cell lines examined in this study. Each row depicts a separate type of intercellular junction. The row labelled 'ZO-1' shows monoclonal or polyclonal antibody staining directed against the tight junction protein ZO-1 ([a]–[e]); row 'DP' shows desmosomes visualized using a monoclonal antibody specific for desmoplakin ([f]–[j]) and row 'EC' shows adherens junctions visualized using an antibody to E-cadherin ([k]–[o]). The different cell lines studied are arranged as separate columns: MDCK cells ([a], [f], [k]); Calu-3 cells ([b], [g], [l]); NCI-H292 cells ([c], [h], [m]); A549 cells ([d], [i], [n]); and 16HBE14o[−] cells ([e], [j], [o]).

the various enzymes attack in the isolated cells and when no means of standardized quantification are defined. In a separate series of experiments, chelation of divalent cations produced a 16-fold increase in mannitol clearance and a 260-fold increase in epithelial permeability (Fig. 5[c], [d]). All of these changes induced in MDCK cells are statistically significant ($P < 0.05$) with respect to the control values. The serine proteinase fraction from the house dust mite culture medium extract produced a 56-fold increase in mannitol clearance in Calu-3 cells and a corresponding 354-fold increase in permeability ($P < 0.001$ for both parameters) (Fig. 5[e], [f]). Treatment of Calu-3 cells for 4 h with 5 mmol/L EGTA increased clearance 22-fold and permeability 54-fold ($P < 0.001$ for both parameters) (Fig. 5[g], [h]). In 16HBE14o[−] cells, treatment of the monolayer with the mite serine proteinase fraction for 3 h under conditions designed to produce less severe disruption of the monolayer produced an increase in permeability from $(0.308 \pm 0.007) \times 10^{-6}$ cm/s to $(0.810 \pm 0.024) \times 10^{-6}$ cm/s

($n = 5$, $P < 0.05$). In contrast, the mannitol clearance volume was increased only 1.2-fold and monolayer permeability by 2.6-fold after treatment of A549 cells with 0.5 mg/mL collagenase for 48 h ($P < 0.05$ for both parameters, data not shown). In NCI-H292 cell monolayers, treatment of the cells with dust mite serine proteinase extract had no significant effect on either permeability or clearance (data not shown). The experiments performed in A549 and NCI-H292 using proteinases which have diverse substrate specificities show that there is little scope to increase the permeability of monolayers comprised of these cells, a finding which is probably related to the apparent absence of contiguous staining of tight junction proteins such as ZO-1. In contrast, monolayers formed from those cells, which exhibit contiguous rings of ZO-1 staining, have lower permeabilities under control conditions and therefore respond more significantly to the effects of proteinase treatment.

Figure 6 shows the relationship between mannitol clearance and epithelial mannitol permeability in the four

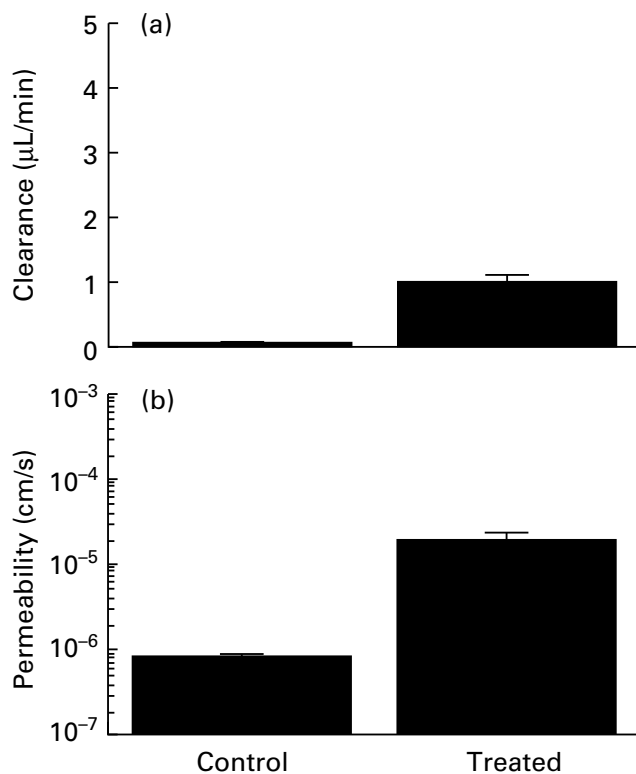


Fig. 4. Effect of 18 h exposure to the cysteine proteinase fraction from house dust mite culture medium on (a) mannitol clearance and (b) epithelial permeability to mannitol in MDCK cell monolayers. For both the clearance and permeability data, the differences between the relevant 'treated' and 'control' groups are statistically significant ($P < 0.05$). Results from three to four experiments.

cell types measured under control conditions and after attempted perturbation of epithelial barrier function. As shown in Fig. 6(a) and (b), the relationship between clearance and permeability in MDCK and also in Calu-3 cells was initially linear over control values but became quasi-exponential as the monolayer was traumatized by treatment with proteinases or EGTA. These results indicate that epithelial permeability is a more suitable and sensitive measure of changes in barrier function than is clearance, especially under conditions where extensive failure of the epithelial barrier is initiated. In contrast, the relationship between clearance and permeability in the intrinsically more permeable A549 and NCI-H292 monolayers (Fig. 6[c], [d]) lacked the initial linear, low permeability portion of the relationship seen with the MDCK cells.

Discussion

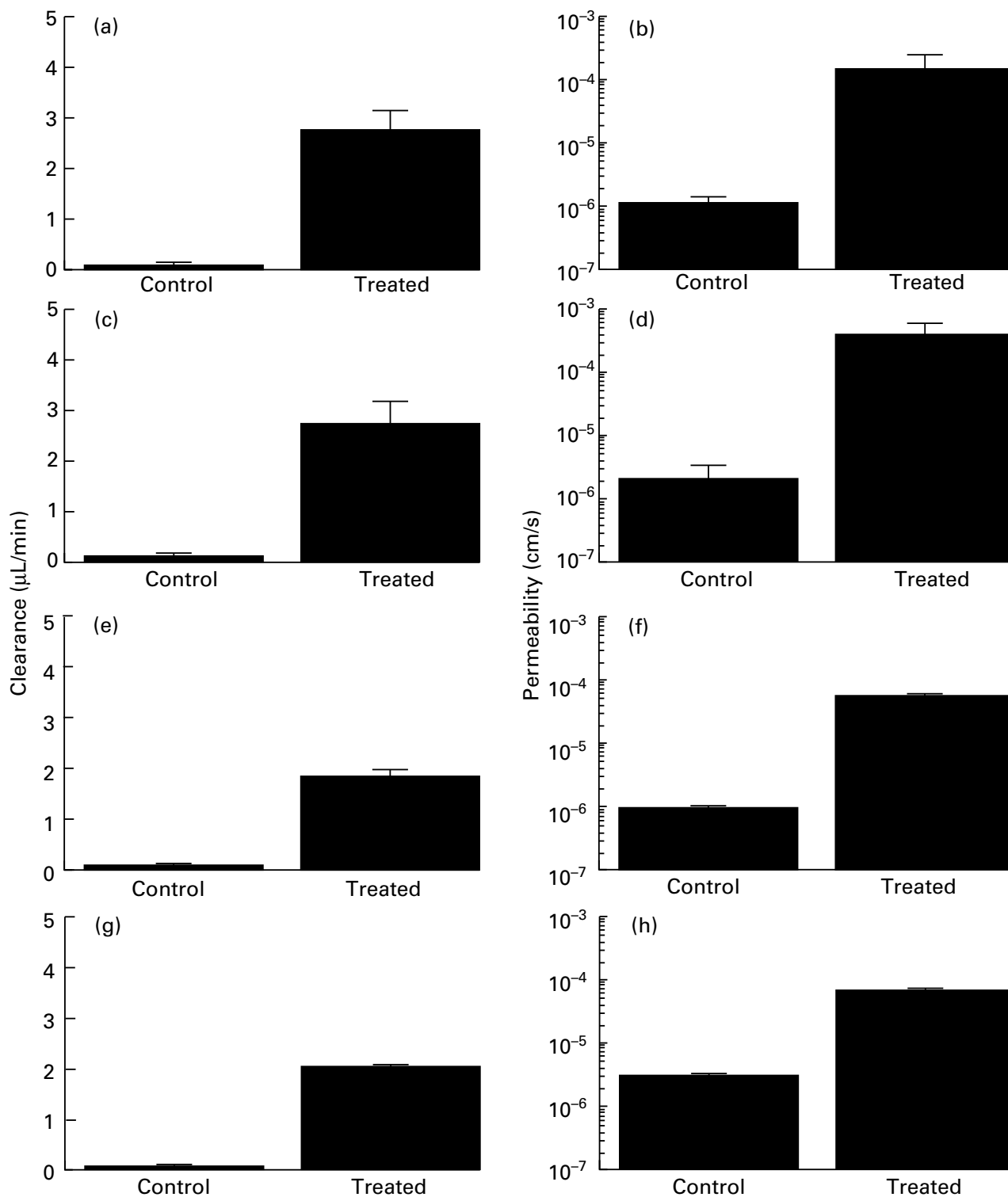
There is growing interest in the possibility that an alteration in the functional properties of the bronchial epithelium could be an important event in the initiation of allergic pulmonary

sensitization [reviewed in 4,7]. It is also suspected that the permeability of the bronchial mucosa is enhanced by the inflammatory events that are triggered in response to an asthma attack [reviewed in 7]. These concepts have prompted several descriptive investigations of changes in epithelial barrier function following exposure to diverse agents such as oxidant gas pollutants, inflammatory mediators and bacteria [24–28]. Most of these experiments have relied upon measurements of electrical resistance or the net percentage leakage of radioactive tracer across control or compromised monolayers of cultured epithelial cells. Whilst the responses demonstrated in these previous studies may reflect real events and are often implied to be 'permeability' changes they may be inaccurate surrogates, potentially misleading and therefore unsuited to the definition of the cellular and molecular mechanisms involved in pathophysiologically significant events. Many previous studies of changes in the epithelial barrier [24–27] have been conducted under conditions where other factors (filters, protein coatings and unstirred/unstirrable layers) have unknown influence on the measures of solute movement. Whilst it may be possible to disregard these factors during control conditions (because of their assumed constancy), perturbation of the epithelial monolayer changes the potential contribution that these variables make to uncorrected measurements. Transepithelial electrical resistance is a useful surrogate which avoids some of the difficulties encountered in diffusional studies, but since it measures the instantaneous open probability of 'pores' within tight junction strands rather than the passage of a solute it can produce results which diverge from measurements of solute permeability. This divergence has been demonstrated in a number of papers [37–39], most elegantly in two studies in which chicken occludin was expressed in MDCK cells and where electrical resistance was found to increase (as expected) in the transfected cells but where solute permeability was found to increase [38,39]. It is also significant that most previous studies investigating permeability responses of the airway epithelium have also failed to provide definitive evidence that mature tight junctional adhesion existed across entire monolayers and that this phenotype is well maintained in culture.

In this paper we describe (i) a method for the measurement of epithelial permeability coefficients in cultured cell monolayers, and (ii) a survey of human airway epithelial cell lines as potential tools to study factors which impair the epithelial barrier. The objective of these investigations was to refine techniques that are necessary to examine the hypothesis that some domestic environmental allergens contribute to airway sensitization and allergic inflammation because they are proteinases which can perturb epithelial cell functions, including the regulation of tissue permeability [1,4]. However, the methods we describe are likely to be of general applicability to study the mechanisms of action of

a variety of inflammatory stimuli on the permeability of the airway lining. Our data highlight the fact that significant discrepancies can occur between crude measurements of 'epithelial leakiness' (uncorrected solute clearance) and

more precise measures of permeability under conditions where changes in physiological function occur. To validate our methods we either chelated calcium ions using EGTA or treated the cell monolayers with collagenase since both of



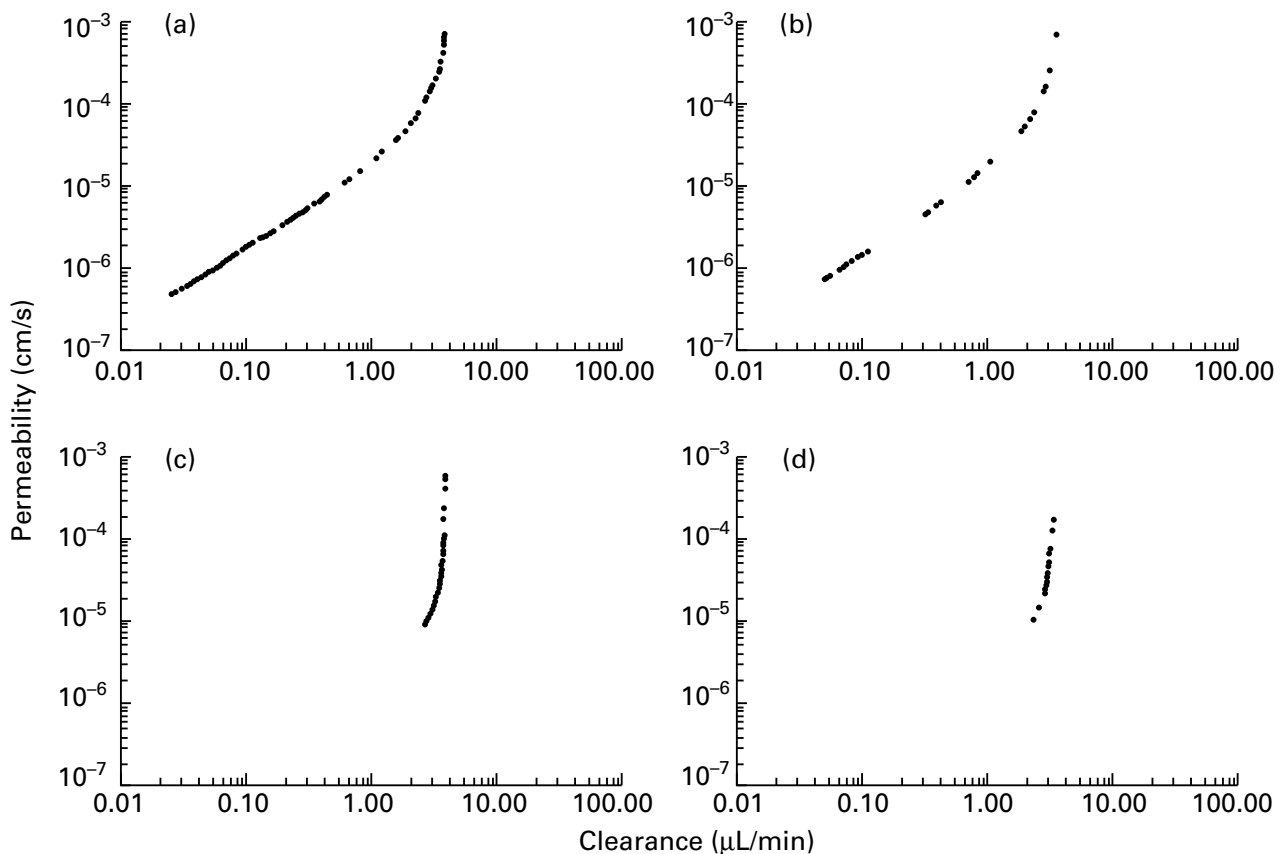


Fig. 6. The relationship between mannitol clearance and epithelial permeability in monolayers of (a) MDCK cells (b) Calu-3 cells (c) A549 cells and (d) NCI-H292 cells. Data are derived from monolayers under control and a wide variety of conditions in which barrier properties had been perturbed by proteinases or by chelation of calcium.

these manoeuvres are known to interfere with epithelial cell adhesion. We also examined the effects of cysteine and serine proteinases from faecal pellets of the house dust mite *D. pteronyssinus*. A more detailed account of the effects of these dust mite proteinases *per se* is beyond the scope of the present manuscript but is given elsewhere [40].

Under control conditions, Calu-3, 16HBE14o⁻ and MDCK cell monolayers exhibited low and similar mannitol clearances and the lowest mannitol permeability coefficients of the cell lines tested. In contrast, the estimates of these parameters in A549 cells and NCI-H292 cells were significantly higher, and approached the characteristics of cell-free filters

alone. The 'leaky' monolayers formed by the latter airway carcinoma cell lines failed to show significant immunostaining of the tight junction protein ZO-1, whereas Calu-3 and 16HBE14o⁻ cells expressed the continuous circumferential staining that is characteristic of cells, such as the MDCK line, which form occluding junctions. The formation of tight junctions in Calu-3 and 16HBE14o⁻ cells is further supported by the development of significant transepithelial electrical resistances by these monolayers whereas A549 and NCI-H292 cell monolayers have nil transepithelial resistance when confluent. This suggests that the formation of peripheral rings of ZO-1 staining is related to the

Fig. 5. Effect of 18 h exposure to the serine proteinase fraction from house dust mite culture medium on (a) mannitol clearance and (b) epithelial permeability to mannitol in MDCK cell monolayers. For comparison, (c) and (d) show the effect of 4 h exposure of the cells to 5 mmol/L EGTA on clearance and permeability. Similar experiments were conducted with Calu-3 cells (e–h). The effect of 18 h exposure of Calu-3 cells to the serine proteinase fraction from house dust mite culture medium on (e) mannitol clearance and (f) permeability is illustrated, whilst (g) and (h) show the responses to EGTA treatment. In each experiment, all of the differences between the 'treated' and control groups are statistically significant ($P < 0.05$) with respect to the appropriate control. Results from four to five data sets in each experiment.

formation of tight junctions and that these junctions are the probable reason why these monolayers have low permeabilities to mannitol. Both A549 and NCI-H292 cells exhibited staining for desmoplakin. Staining of E-cadherin was less intense in NCI-H292 cells than in MDCK cells and was virtually absent in A549 cells. Thus, the presence of tight junctions in Calu-3 and 16HBE14o⁻ cells probably accounts for the low transepithelial movement of mannitol in these cells, since mannitol permeability values were similar to those measured in MDCK cell monolayers in which tight junction formation is known to occur.

Mannitol clearance and permeability in MDCK monolayers were significantly increased by a house dust mite faecal extract containing the cysteine proteinase Der p 1. This result is as expected from our previous studies in other experimental models [1]. For convenience in validating the experimental methodology we did not use Der p 1 that had been purified to homogeneity in the present studies. However, we have previously demonstrated that the cysteine proteinase allergen Der p 1 increases transepithelial solute flux in isolated sheets of bovine airway mucosa and this is associated with changes in lateral and basal epithelial cell adhesion [1]. In previous studies of the effects of Der p 1 on cultured epithelial cells we employed the MDCK cell line because it is known to express intercellular junctional complexes which are involved in normal cell-to-cell adhesion [1]. Furthermore, many of the proteins within the junctions expressed by this cell line have been characterized in detail [reviewed in 8,10,12] and consequently the MDCK cell line is an important experimental paradigm for studying changes in epithelial barrier function. In this study we show that immunostaining of Calu-3 and 16HBE14o⁻ cell lines reveals patterns of protein staining in tight junctions, adherens junctions and desmosomes which are morphologically similar to those of MDCK cells. Both of these human airway cell lines had baseline permeabilities which approximated those of MDCK cell monolayers and both behaved similarly in response to the perturbations investigated. These findings suggest that Calu-3 and 16HBE14o⁻ cell lines could be suitable models in which to study the behaviour of the bronchial epithelium. In contrast, the functional tight junction deficits of the A549 and NCI-H292 cell lines preclude their use in permeability studies because even when cells were stripped from the filters with EGTA or collagenase there was only a small increase above the high baseline permeability. However, these cells with tight junction deficits will nevertheless be useful to examine the effects of epithelial perturbants on intercellular junctions in a cellular setting that is not 'contaminated' by the contribution of functional tight junctions.

Mannitol clearance and permeability in Calu-3 and 16HBE14o⁻ monolayers was also significantly increased by the serine proteinase fraction derived from the house dust

mite cultures. This preparation is enriched in the serine proteinase allergens Der p 3, Der p 6 and Der p 9 [2,4]. Assuming that the proteinase allergens contribute to the effects measured, this is the first evidence that the serine proteinase allergens may augment transepithelial solute flux in epithelial cells. However, even if the allergens themselves are not the causative agents underlying the permeability changes in these preparations, the net effect may provide a mechanism to facilitate the presentation of allergen to dendritic cells in the airway [4].

In conclusion, we have described a method to evaluate the effects of diverse perturbants on the permeability of epithelial monolayers and have characterized a range of epithelial cell lines for their usefulness in such investigations. These techniques are currently being applied to examine the effects of highly purified proteinase allergens and ascertain the mechanisms involved.

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