

Quantitative structural and biochemical analyses of tight junction dynamics following exposure of epithelial cells to house dust mite allergen Der p 1

H. WAN, H. L. WINTON, C. SOELLER, D. C. GRUENERT*, P. J. THOMPSON†, M. B. CANNELL, G. A. STEWART‡, D. R. GARROD§ and C. ROBINSON

Department of Pharmacology & Clinical Pharmacology, St George's Hospital Medical School, London, UK, *Gene Therapy Core Centre, Cardiovascular Research Institute, Department of Laboratory Medicine, University of California, San Francisco, USA, †Department of Medicine, University of Western Australia, Queen Elizabeth II Medical Centre, Nedlands, Australia, ‡Department of Microbiology, University of Western Australia, Queen Elizabeth II Medical Centre, Nedlands, Australia and §School of Biological Sciences, University of Manchester, Manchester, UK

Summary

Background House dust mite allergen Der p 1 is a cysteine peptidase. Previously, we have suggested that the proteolytic activity of this allergen may contribute to asthma by damaging the barrier formed by the airways epithelium.

Objective The present study applied novel techniques to compare changes in permeability with quantitative events in tight junctions (TJs) and desmosomes (DMs) of epithelial cells exposed to Der p 1.

Methods Confluent monolayers of Madin-Darby canine kidney (MDCK) and 16HBE14o-human bronchial epithelial cells were used as experimental models. Permeability was estimated from mannitol clearance. Digital imaging with quantification of TJs and DMs was achieved by fluorescent antibody staining and 2-photon molecular excitation microscopy (2PMEM). Biochemical changes in TJs were studied by immunoblotting, radiolabelling and immunoprecipitation.

Results Der p 1 caused a time-dependent breakage of TJs and reduction in their content of the protein ZO-1. Reduction in ZO-1 immunofluorescence at TJs occurred with a small increase in the amount of diffuse, cytoplasmic immunoreactive ZO-1 staining. Morphological changes in TJs occurred in synchrony with increases in epithelial permeability. DM puncta increased both in size and intensity of staining. Immunoblotting demonstrated that the disruption of TJ morphology was associated with cleavage of ZO-1 and occludin. Cells recovered from allergen exposure by *de novo* synthesis of occludin.

Conclusion Der p 1 could contribute to sensitization and allergic responses by degrading the function of the airway epithelial barrier.

Keywords: house dust mite, allergen, Der p 1, tight junction, epithelium, asthma, cysteine peptidase

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Introduction

The allergen Der p 1 is a cysteine peptidase [1,2] and is one of several house dust mite (HDM) allergens with enzymatic

activity. The enzymatic activity of these allergens is possibly relevant to how they cause allergic sensitization and asthma [2]. Since inhaled allergens are likely to achieve their highest concentrations at the barrier formed by the airways epithelium, this research has focused on the effects of Der p 1 on epithelial function.

A major role of airway epithelium is to prevent the

Correspondence: C. Robinson, Department of Pharmacology & Clinical Pharmacology, St George's Hospital Medical School, Cranmer Terrace, London SW17 0RE, UK.

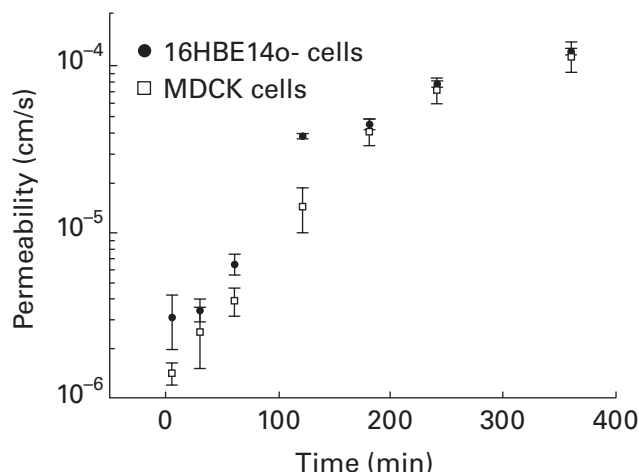


Fig. 1. Effects of Der p 1 on permeability of epithelial monolayers to ¹⁴C mannitol. Closed symbols show responses in MDCK cell monolayers (□) and 16HBE14o-cell monolayers (●) following treatment with proteolytically active allergen. Data are mean ± SE mean in four experiments.

ingress of foreign proteins, micro-organisms and other factors that pose threats to the host. The structural integrity of the epithelium depends, in part, upon various types of cell–cell and cell–matrix adhesions (e.g. tight junctions (TJs), adherens junctions, desmosomes (DMs) and hemidesmosomes) [reviewed in 3]. Intercellular junctions also confer important functional characteristics on the epithelium. This is particularly true for TJs because they regulate access to paracellular shunt pathways and thus exert an important degree of control over the permeability of the epithelial barrier [3–6]. In contrast to TJs, DMs have little influence on paracellular permeability of solutes because of their punctate distribution around cells. Nevertheless, these cell junctions are essential to the maintenance of

epithelial defences because they contribute to epithelial cohesion [7].

Previous studies have shown that Der p 1 can increase the permeability of epithelial barriers [8]. These changes were prevented by inhibitors of cysteine peptidases, indicating that the effects were due to the attack of Der p 1 on protein targets within the airway lining. Until recently, the identities of these targets were unknown, but evidence has now been presented that primary substrates could be the occludin and claudin families of TJ adhesion proteins [9]. Since TJs have a critical role in maintaining epithelial architecture, and are crucial to the regulation of paracellular permeability, cleavage of these junctions could be of major significance to the pathogenesis of asthma. The purpose of the present study was to investigate further the dynamics of TJ cleavage elicited by the allergen Der p 1. The changes which take place in DMs following allergen exposure were also investigated. The objectives of the study were pursued using a novel combination of techniques that allowed 3-dimensional quantitative analysis of TJs and DMs to be performed in relation to studies of epithelial permeability.

Methods

Preparation of Der p 1

Cysteine peptidase activity was fractionated from spent *Dermatophagoides pteronyssinus* culture medium (Commonwealth Serum Laboratory, Parkville, Australia) as previously described [8,9]. Purified Der p 1 was prepared by affinity chromatography and chromatofocusing according to previously published procedures [8–11]. Enzymatic activity was measured spectrophotometrically using N-benzoyl-Phe-Val-Arg-p-nitroanilide. Protein content was measured by Coomassie Blue assay [12]. Preparations were analysed

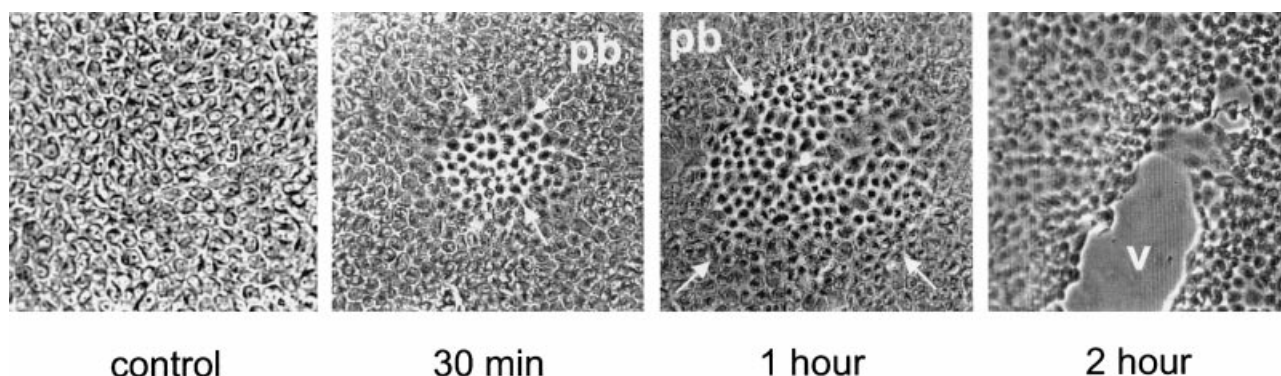


Fig. 2. Phase contrast images of MDCK epithelial monolayers exposed to allergen. Under control conditions, cells form cohesive monolayers. After treatment with allergen, the cohesive appearance of the epithelial monolayer is obviously changed, cells become phase bright (pb, shown in areas demarcated by arrows at 30 min and 1 h) and, in some cases, frank exfoliation leads to the formation of acellular vacancies (v, at 2 h) in the monolayer.

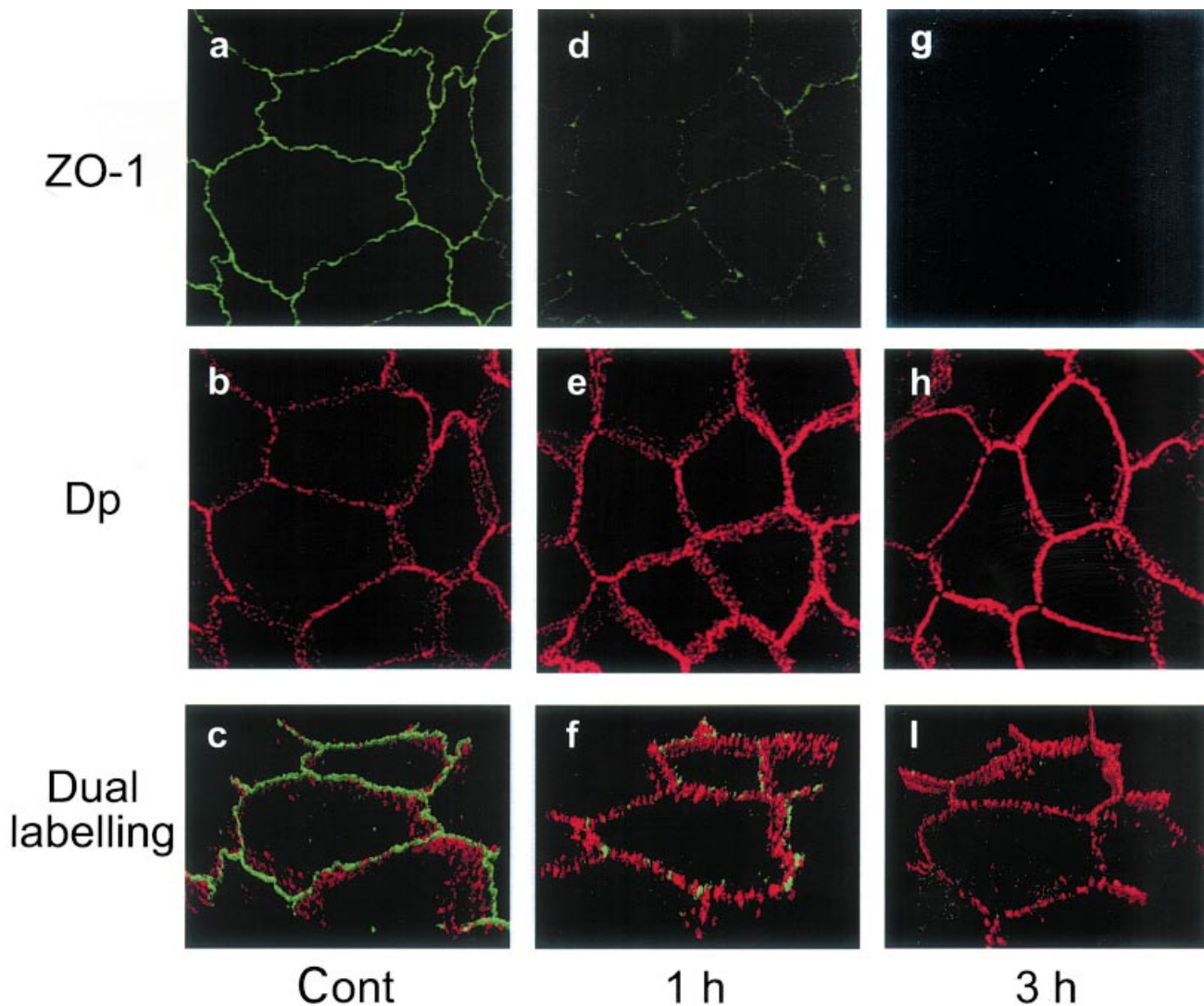


Fig. 3. Effects of Der p 1 on intercellular junctions of MDCK cells. Sample through focus images (8- μ m thick) of (a) ZO-1 and (b) desmoplakin staining in sham-treated monolayers. Panel (c) shows a 3-dimensional isosurface-rendered image of the 3-dimensional distribution of ZO-1 (green) and desmoplakin (red) for the images in (a) and (b). Panels d, e and f show corresponding images in cells treated with Der p 1 for 1 h. Panels g, h and i show responses at 3 h.

for the presence of endotoxin by Limulus amoebocyte lysis assay (ICN Pharmaceuticals Ltd, Basingstoke, UK) and were found to be below the assay limit of detection (0.06 ng/mL). Enzyme activity of the allergen preparations used in these experiments was (nmol/min): 200 (Fig. 1), 20 (Fig. 2) and 2 (Figs 3,4,5,6,7). These amounts are equivalent to the total enzymatic activity of 4–400 HDM faecal pellets, or 0.8–80 ng Der p 1.

Cell culture and permeability measurement

MDCK and 16HBE14o-cells were cultured as described [13]. For immunocytochemistry, cells were grown on glass coverslips coated with an ultra-thin layer of Matrigel

(Universal Biologicals, London, UK) [13]. Permeability of D-[^{14} C]-mannitol (NEN Du Pont, Brussels, Belgium) was measured as previously described [8] in confluent cell monolayers grown on a Matrigel undercoat in 0.1- μ m pore diameter Transwells (Corning-Costar UK Ltd, High Wycombe, UK). Allergen preparations were diluted in minimum essential medium with Earle's salts (EMEM) for addition to cells. Controls comprised EMEM alone.

Immunostaining and 2-photon molecular excitation microscopy

Fluorescent antibody staining was used to study the modulation of TJs and DMs [14]. Secondary antibodies were

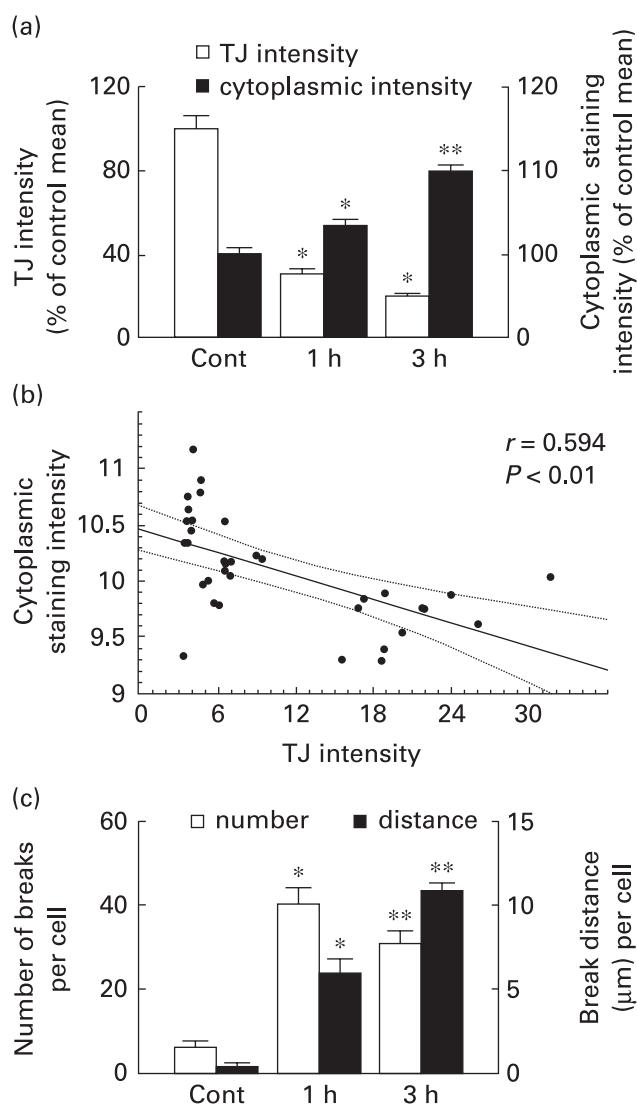


Fig. 4. (a) Graphical analysis of immunoreactive ZO-1 concentration at the cell boundary (TJ intensity) and cytoplasmic fluorescence in MDCK cell monolayers. Cells were sham-treated controls (cont) or treated with Der p 1 for 1 h or 3 h. Bars show mean $\pm$ SE mean of 12 cells. * $P < 0.05$ from control values, ** $P < 0.05$ from control values and the 1 h time point data. (b) Regression analysis showing inverse correlation between intensity of TJ staining and fluorescence intensity of diffuse cytoplasmic staining following treatment of MDCK cells with Der p 1. The solid line shows the fitted regression line with the 95% confidence interval denoted by the envelope formed by the dotted lines. (c) Graphical analysis of the mean number of breaks in TJ (TJ breakage) and the length of the detected breaks per cell. Bars show mean $\pm$ SE mean of 12 cells. Asterisks indicate significant differences as described in (a).

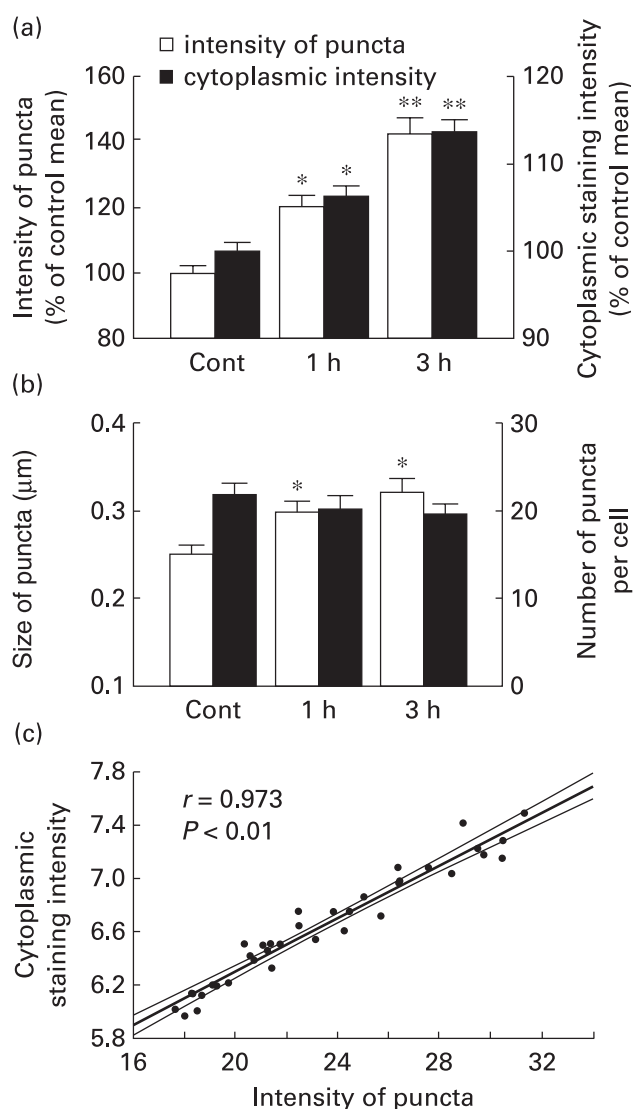


Fig. 5. (a) Graphical analysis showing intensity of staining of desmosomal puncta and changes in cytoplasmic fluorescence in MDCK cell monolayers. Cells were sham-treated controls (cont) or treated with Der p 1 for 1 h or 3 h. Bars show mean $\pm$ SE mean of 12 cells. * $P < 0.05$ from control values, ** $P < 0.05$ from 1 h time point. (b) Graphical analysis of punctate desmosomal staining showing mean size of each punctum and the average number per cell. Bars show mean $\pm$ SE mean of 12 cells. * $P < 0.05$. (c) Regression analysis showing correlation between staining intensity of desmosomal puncta and fluorescence intensity of diffuse cytoplasmic staining following treatment of MDCK cells with Der p 1. The solid line shows the fitted regression line with the 95% confidence interval denoted by the envelope formed by the dotted lines.

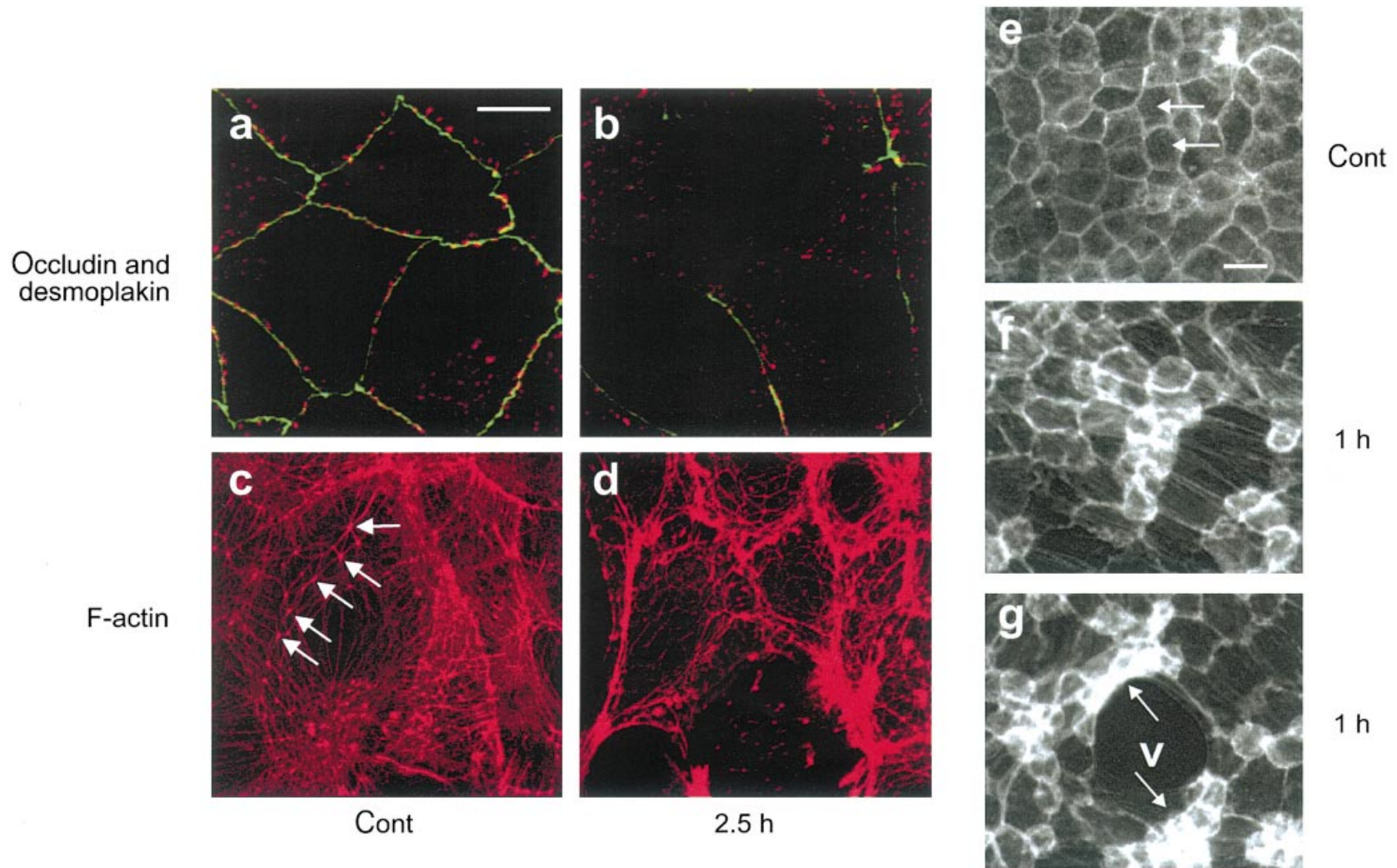


Fig. 6. Effect of Der p 1 on occludin and desmoplakin immunostaining and the actin cytoskeleton of 16HBE14o-cells. (a) Depicts untreated control (cont) cell monolayers showing the contiguous TJs as disclosed by occludin staining (green) and the punctate appearance of desmoplakin (red). (b) 16HBE14o-cells after treatment with Der p 1 for 2.5 h. (c) Actin staining in untreated control (cont) cells visualized by rhodamine phalloidin staining. Note focal points of actin staining (examples indicated by arrows) with radiating actin fibres. (d) Actin staining following treatment with Der p 1 for 2.5 h. Note the reduction in the stress fibre network and the increased clarity of staining at the cell boundaries. Scale bar for a–d is 10 μ m. (e) F-actin staining of MDCK cells under control conditions. Cytoplasm contains areas of punctate staining (examples shown by arrows) and perijunctional rings are clearly visible. (f) Development of actin stress fibres following 2.5 h treatment with Der p 1. (g) MDCK cells treated with Der p 1 showing condensation of F-actin staining (arrows) surrounding areas of allergen induced cell loss (v). Scale bar for e–g is 20 μ m.

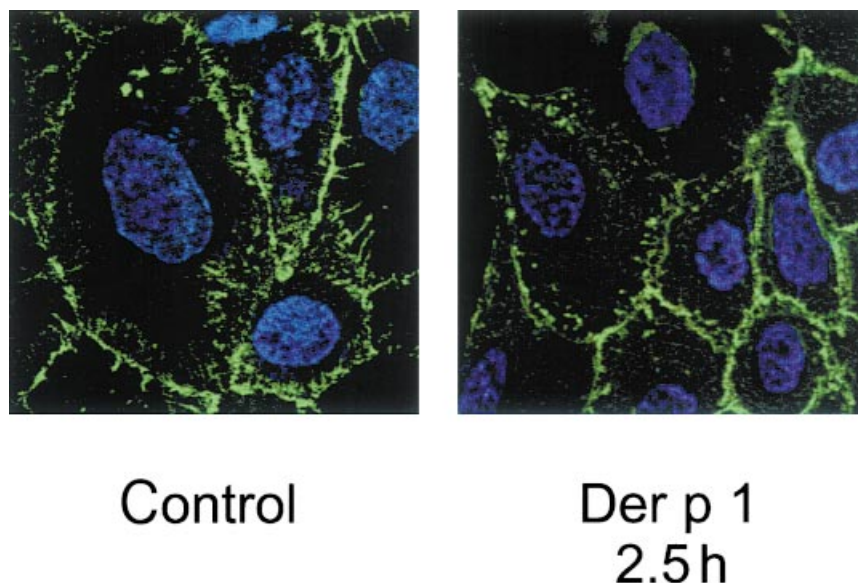


Fig. 7. 2PMEM imaging of E-cadherin immunostaining in 16HBE14o-epithelial cells under control conditions and following 2.5 h exposure to Der p 1. Cell nuclei were stained with 4',6-diaminidino-2-phenylindole (DAPI) in this experiment. Note that the changes in E-cadherin staining are small in comparison with those seen for TJ proteins.

DTAF or TRITC conjugates (Chemicon International Ltd, Harrow, UK). ZO-1 was stained using monoclonal antibody (mAb) R40.76 [15]. Desmoplakin was stained with mAb 11-5F [16]. Cells were fixed in methanol for fluorescent antibody labelling as previously described [14]. Actin was stained using rhodamine phalloidin (Molecular Probes Europe, Leiden, The Netherlands). For actin staining, cells were fixed for 10 min in 3.7% v/v formaldehyde solution in PBS, washed extensively, and permeabilized for 10 min with 0.1% v/v Triton X-100. After washing with PBS, cells were stained with rhodamine phalloidin for 45 min.

Two-photon molecular excitation microscopy (2PMEM) was performed on a modified LSM410 microscope (Carl Zeiss Ltd, Welwyn Garden City, UK) illuminated by an argon ion-pumped Ti:sapphire modelocked laser (Coherent, Santa Clara, CA, USA). Fluorochromes were excited at 810 nm using laser pulses of 80–100 fs duration [9,17].

Quantitative analysis of 2PMEM images

The lack of photobleaching outside the focal plane in 2PMEM permits quantitative analysis of fluorescently-labelled specimens. Quantification was conducted within the IDL software environment (Research Systems Inc., Boulder, CO, USA) using programs written specifically for this purpose [9]. Three-dimensional isosurfaces were reconstructed from image data stacks using IRIS Explorer (Numerical Algorithms Group, Oxford, UK) running on a Silicon Graphics Indigo 2 workstation loaded with SG Extreme Graphics hardware (Silicon Graphics, Reading, UK).

ZO-1 content of TJs was measured as the fluorescence intensity of the label at constant illumination power from the focal volume excited by the laser. Stacks of acquired images

were read into computer memory (Silicon Graphics Indigo2 and Indy workstations) as a 3-dimensional array and the co-ordinates of cell boundaries identified. From a defined start point, the staining pattern throughout the 3-dimensional dataset was automatically traced voxel by voxel. The staining pattern normal to the traced co-ordinates was fitted by a Gaussian function to identify the peak fluorescence as well as its width in each cell. Breaks in TJ ring continuity were quantified as the number of times the staining intensity dropped below twice the average background intensity in adjacent voxels in the image stack along the cell periphery. Data were computed to average fluorescence present in the periphery of each cell, the background fluorescence present, the number of breaks in the peripheral staining pattern and the mean length of the break in the staining pattern.

For DMs, the relevant image stacks were transformed into 3-dimensional arrays. The number of discrete staining sites was counted with a standard region counting algorithm (IDL) from a binary mask. The binary mask was constructed by accepting regions of three or more connected pixels whose individual intensities were more than the mean +4 standard deviations of the surrounding area (10×10 pixels).

Immunoblotting

Immunoblotting was performed as described in full elsewhere [9]. For MDCK cells, mAb MOC37 was used to detect occludin, mAb R40.76 to detect ZO-1 and rat mAb DECMA-1 (Sigma) to detect E-cadherin. For 16HBE14o-cells, rabbit polyclonal anti-ZO-1 and rabbit anti-occludin (Zymed Inc, San Francisco, CA, USA) were used. Further details of these antibodies have been described elsewhere [13].

Detection was by enhanced a chemiluminescence technique (ECL) (Amersham Pharmacia Biotech, Little Chalfont, UK).

³⁵S-labelling and immunoprecipitation of occludin

To study the *in situ* degradation of radiolabelled occludin, confluent monolayers of cells were grown in 60 mm Petri dishes and subjected to two calcium 'switches' prior to labelling. Down-switching of calcium was conducted by addition of medium containing 5 mmol/L EGTA for 1 h, followed by reversal for 30 min in normal medium. A second downward switch in calcium was applied for a further 2 h. After restoration of calcium and re-establishment of TJs, cells were pre-incubated for 30 min with methionine-free medium prior to addition of 3 mL (250 µCi/mL) Tran ³⁵S-label (ICN Pharmaceuticals Ltd) for 18 h. Cells were then extracted immediately, or chased in the presence or absence of allergen. Chase medium was EMEM modified with 150 µg/mL methionine, 5% v/v Ultrosor (GIBCO, Paisley, UK) to replace fetal calf serum and additionally, containing 0.5 mmol/L glutathione as reducing agent for the allergen.

To study the synthesis of occludin, cells were treated for 3 h with allergen or control solution prior to labelling. During the last 30 min of the pre-labelling period, all cells were put into methionine-free medium. Labelling was undertaken for 3 h with 800 µCi/mL Tran ³⁵S-label in methionine-free EMEM containing 5% v/v Ultrosor. Cells were then chased with unlabelled methionine for 30 min prior to immunoprecipitation of occludin. Unchased cells were examined for comparison.

Occludin was immunoprecipitated as follows. Cells were washed twice in ice-cold PBS and extracted for 30 min in ice-cold NET buffer (150 mmol/L NaCl, 50 mmol/L Tris-HCl, pH 7.4, 5 mmol/L EDTA, 1% v/v NP-40) containing peptidase inhibitors (5 mmol/L phenylmethanesulphonyl fluoride, 20 µg/mL aprotinin, 40 µmol/L E-64, 10 µmol/L pepstatin, 10 µg/mL soybean trypsin inhibitor, 100 µmol/L leupeptin). The resultant solution was declared 'NET buffer soluble fraction' and was transferred to an Eppendorf tube. The remaining insoluble material was treated with 0.2 mL boiling buffer A (150 mmol/L NaCl, 25 mmol/L EDTA, 20 mmol/L Na₂HPO₄, 1% w/v SDS) plus the peptidase inhibitor cocktail, and was scraped into an Eppendorf tube. The mixture was triturated 10 times through a 25G needle and boiled for 5 min prior to the addition of 0.8 mL buffer B/peptidase inhibitor cocktail (same composition as buffer A, except 1% v/v NP-40 replaced SDS). The sample was centrifuged at 10 000 g for 5 min and the supernatant fluid declared 'NET buffer insoluble fraction'.

Occludin was immunoprecipitated from the NET buffer soluble and insoluble fractions as follows. Non-immune rabbit serum was added to each fraction and rotated for

2 h at 4 °C. Protein A-Sepharose beads (100 µL of a 10% w/v suspension) were then added and rotation continued for a further 2 h. Beads were removed by centrifugation at 10 000 g for 5 min. Rabbit anti-occludin IgG (4 µL of a 1 mg/mL solution) was added to the supernatant fluid and the sample rotated overnight at 4 °C. Protein A-Sepharose beads (60 µL) were then added and rotation continued for a further 2 h prior to centrifugation at 10 000 g for 5 min. The supernatant fluid was discarded and the beads washed sequentially in: 1 mL RIP buffer (20 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA); RIP buffer with 0.5 mol/L NaCl; twice in RIP buffer plus 0.1% w/v SDS; and finally, RIP buffer with 10 mmol/L Tris-HCl, pH 6.8. Protein was solubilized from the beads by addition of 60 µL boiling SDS-PAGE sample buffer and boiling the mixture for 5 min. The Sepharose beads and other insoluble materials were removed by centrifugation at 10 000 g for 10 min and the supernatant fluid subjected to SDS-PAGE. The gel was fixed with 50% v/v methanol and 10% v/v acetic acid for 1 h and soaked in Amplify solution (Pharmacia Amersham Biotech) for 20 min at room temperature. The gel was dried under vacuum on a slab dryer (Pharmacia) and then exposed to Hyperfilm (Pharmacia Amersham Biotech) at -70 °C.

RT PCR amplification of occludin mRNA

16HBE14o-cells were treated with allergen as in the radiolabelling and immunoprecipitation experiments. Total RNA was extracted conventionally using guanidium isothiocyanate. Occludin cDNA was synthesized from 5 µg RNA using 4 µL reverse transcriptase buffer (5×), 1 µL 0.27 µg/µL p(dT)₁₂₋₁₈ primer (Amersham Pharmacia Biotech), 2 µL 100 mmol/L dithiothreitol, 2 µL dNTP (5 mmol/L each) (Amersham Pharmacia Biotech), 1 µL 40 U/µL RNasin (Promega, Southampton, UK) and 400 U MMLV reverse transcriptase (GIBCO-BRL). PCR amplification was performed using RedTaq DNA polymerase (Sigma), dNTP and the equivalent of 0.2 µg original total RNA. Occludin primers were: 5'-TATGAGACAGACTACACAACCTGGCGGCGAG-3' (forward) and 5'-ATCATAGTCTCCAACCATCTTCTTGATGTG-3' (reverse), generating a 363 bp product. GAPDH was amplified as housekeeping gene using the primer pair 5'-ACCACAGTCCATGCCATCAC-3' (forward) and 5'-TCCACCACCCTGTTGCTGTA-3' (reverse), generating a 451 bp product. Amplification conditions, chosen to avoid template depletion, were: initial denaturation at 94 °C for 3 min, with 25 cycles of 45 s denaturation at 94 °C, 45 s annealing at 60 °C and 90 s extension at 72 °C. PCR products were separated by 1.5% w/v agarose gel electrophoresis and gels digitized for analysis. The identity of the occludin PCR amplicon was confirmed by sequencing using an ABI 377 automated DNA sequencer (Applied Biosystems Inc, Foster City, CA, USA).

Statistics

Statistical significance, calculated using the Statistica program suite (Statsoft, Tulsa, OK, USA), was taken as $P < 0.05$ and is indicated in Figures by asterisks.

Results

Quantification of intercellular adhesions disruption by Der p 1

MDCK and 16HBE14o-cells form monolayers that exhibit low permeability to mannitol (Fig. 1). Following treatment with Der p 1, the permeability of the monolayer increased with time (Fig. 1). Heat inactivation of the peptidase destroyed its enzyme activity and prevented the change in epithelial permeability (not shown). Cells treated with catalytically active allergen showed time-dependent changes in the cohesiveness of the monolayer. Initially, allergen-treated cells became rounded in appearance and bright under phase contrast illumination (Fig. 2). After several hours, there was an exfoliation of cells with the development of small holes within some monolayers (Fig. 2).

As disclosed by immunostaining of ZO-1, TJs appear as contiguous rings around the peripheral margins of epithelial cells (Fig. 3a). In contrast, desmosomes have a punctate appearance when desmoplakin is immunostained (Fig. 3b,c). Visual inspection of acquired images suggested that treatment of MDCK cells with Der p 1 evoked a time-dependent disappearance of TJ immunostaining (Fig. 3d,g,i), but it appeared that desmosomes were less affected (Fig. 3e,h,f,i). One hour after exposure to Der p 1, the immunostaining of TJs was significantly reduced and at 3 h, it was virtually absent (Fig. 3a,d,g), suggesting that the gain in epithelial permeability activated by Der p 1 resulted from a functional disassembly of TJs.

2PMEM imaging enabled the effects of Der p 1 on TJs and desmosomal puncta to be quantified. Figure 4(a) shows an analysis of the amount of ZO-1 localized within TJ rings in cells before and after treatment with Der p 1. Following 1 h exposure to the allergen, there was a marked reduction in fluorescence intensity within junctions. This reduction in junctional immunostaining was synchronous and inversely correlated with changes in spatially diffuse fluorescence (Fig. 4b), suggesting that ZO-1, or fragments derived therefrom, had been translocated from the junctions into the cytoplasmic compartment. Figure 4(c) shows that there are normally few breaks in the continuity of TJ rings in confluent MDCK monolayers, and these breaks constitute only a small proportion of cell circumference. Following treatment with Der p 1, the number and magnitude of TJ breaks increased as ZO-1 disappeared from TJs (Fig. 4c). The small decrease in break number at 4 h compared with 1 h after Der p 1 treatment was caused by short vacancies in

ZO-1 immunostaining coalescing into a smaller number of longer breaks.

Digital image analysis revealed that Der p 1 treatment also resulted in changes in the appearance of desmosomes (Fig. 5a). The fluorescence intensity and diameter of individual puncta was increased in a time-dependent fashion (Fig. 5a,b), and there was a small increase in diffuse cytoplasmic immunostaining (Fig. 5a) which was correlated with the mean intensity of desmosomal puncta (Fig. 5c). However, the number of desmosomes was not altered (Fig. 5b).

Similar changes in TJs and desmosomes were also observed in 16HBE14o-cells. Figure 6 shows the loss of occludin and desmoplakin immunostaining in cells treated with Der p 1. In these experiments, the actin cytoskeleton was also visualized by staining with rhodamine phalloidin. In untreated 16HBE14o-cells, the actin cytoskeleton appears as a dense fibrous meshwork without clearly defined perijunctional rings (Fig. 6c). Occasional focal points of actin staining are present, with actin fibres radiating from them. Upon treatment with Der p 1, there was a partial dissolution of the fibrous network, while ring-like staining patterns at the cell periphery became more visible (Fig. 6d). In contrast, the pattern of F-actin staining in control monolayers of MDCK cells differed from that seen in human bronchial epithelial cells. There was a clearly defined perijunctional ring of actin staining (Fig. 6e), and areas of punctate staining were visible within the cells that probably represent the ends of vertically aligned F-actin fibres. Following treatment with Der p 1, MDCK cell monolayers developed stress fibres (Fig. 6f) and, particularly around areas of cell loss, there was a marked condensation of F-actin staining (Fig. 6g).

Adherens junctions were also examined as potential targets of Der p 1. Figure 7 shows that in contrast to the substantial changes in TJs, E-cadherin staining was less obviously affected 2.5 h after exposure to Der p 1.

Der p 1 activates proteolysis of TJ components

Whole cell extracts of MDCK cells were immunoblotted to ascertain whether the loss of ZO-1 immunostaining within the TJ ring was caused by proteolysis of cell junction proteins. Figure 8(a) shows that ZO-1 from untreated cells migrates with an apparent molecular mass of about 220 kDa. Following 1 h of treatment, the intensity of the 220 kDa band was decreased slightly. After 5 h treatment of cells, the intensity of the 220 kDa band was markedly reduced and a faint band of immunoreactive protein became visible at about 197 kDa.

Changes also occurred in the transmembrane TJ protein occludin. Figure 8(b) shows that Der p 1 treatment of MDCK cells resulted in the cleavage of occludin into two major immunoreactive products. In MDCK cells, occludin normally

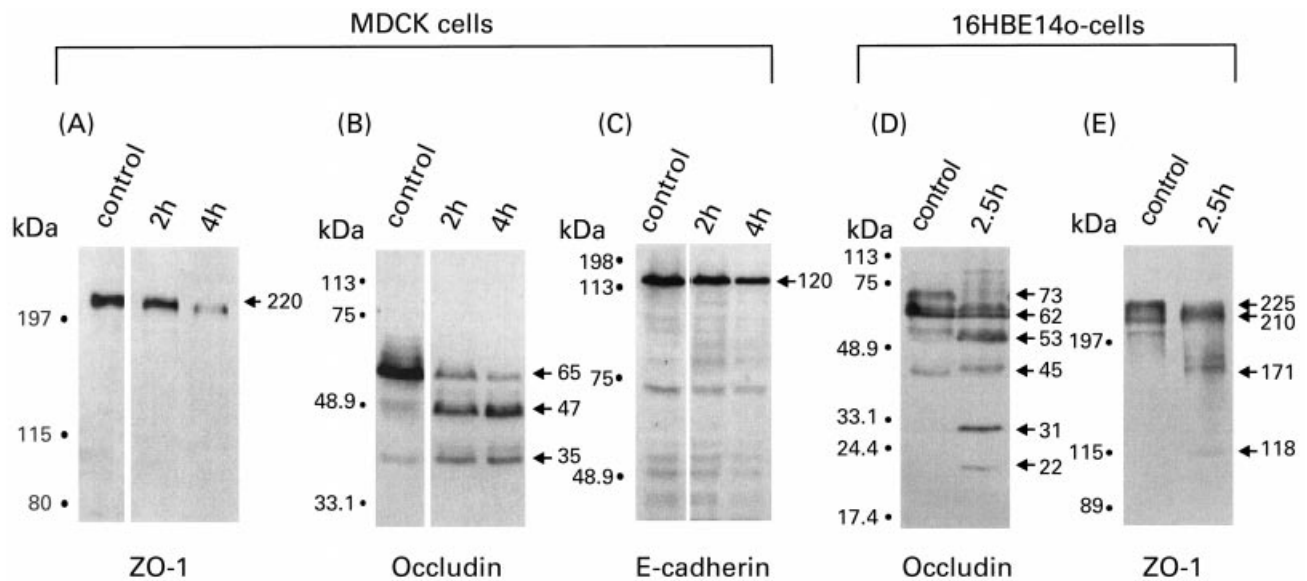


Fig. 8. Immunoblots of junctional proteins from MDCK cell monolayers treated with Der p 1. (a) ZO-1 from control cells or following treatment of cells for 2 h or 5 h. Note the decrease in the intensity of the 220 kDa band on treatment. (b) Occludin. Note the extensive degradation and the detection of two cleavage fragments of 47 kDa and 35 kDa. From the reactivity of the antibody, both fragments must contain the C-terminus of occludin. (c) E-Cadherin. Note the decrease in the intensity of the 120 kDa band on treatment. (d) Immunoblots of occludin from 16HBE14o-cell monolayers under control conditions and after treatment for 2 h with Der p 1. (e) Immunoblots of ZO-1 from 16HBE14o-cell monolayers under control conditions and after treatment for 2 h with Der p 1. Enzyme activity of the allergen was 1600 nmol/min.

migrates with an apparent mass of approximately 65 kDa, but fragments of 47 kDa and 35 kDa were identified after cells had been exposed to Der p 1. As the antibody used for immunoblotting recognizes an epitope within the C-terminal domain of occludin, both of the products detected must be fragments that contain an intact C-terminus. Figure 8(c) illustrates that the effect of the allergen was not restricted to TJ proteins. E-cadherin of zonulae adherentes was degraded, although judging from the small diminution in the staining intensity of the 120 kDa band, it was less susceptible than the TJ protein occludin. No immunoreactive cleavage products were detected by immunoblotting.

TJ proteins of 16HBE14o-cells were also degraded by Der p 1 (Fig. 8d,e). In the case of occludin, the apparent masses of cleavage products differed slightly from those of MDCK cells but were consistent with cleavage of the first extracellular loop (band at approximately 52 kDa) and within the C-terminal domain (band at approximately 34 kDa). Note that the antibodies used to blot canine and human occludin are different. The greater complexity of occludin blots from control 16HBE14o-cells compared with MDCK cells is a feature we have found consistently.

To study the dynamics of occludin processing on Der p 1 treatment, cells were incubated with Tran ³⁵S-label and occludin immunoprecipitated after various labelling and

treatment procedures. Initially, it proved difficult to incorporate ³⁵S amino acids into occludin. This difficulty may have arisen because in unperturbed cell monolayers, either the turnover of occludin is low or, alternatively, the labelled amino acids are taken up into cells at sites that are poorly accessible when TJs are closed. However, two cycles of calcium switching prior to labelling resulted in ³⁵S incorporation mainly into a 66 kDa, NP-40 insoluble form of occludin (Fig. 9a). Some labelling of bands with apparent masses of 76 kDa and 53 kDa was visible also (Fig. 9a), the latter being more distinct in cells chased for 2 h after the labelling period. The intensity of the 53 kDa band was further increased in cells treated with Der p 1 during the chase period, suggesting it to be a cleavage product formed from occludin by the allergen.

On removal of the allergen, re-synthesis of occludin was evident in 16HBE14o-cells. Figure 9(b) shows that following 3 h of treatment with allergen, Tran ³⁵S-label was incorporated into the 66 kDa, NP-40 insoluble fraction of occludin. These cells had not been subjected to an initial calcium switch to break and re-form TJs, indicating that TJ cleavage by the allergen was an effective initiator of TJ re-synthesis. This impression was confirmed by the absence of occludin labelling in cells that were subjected to the labelling procedure without the prior exposure to Der p 1 (Fig. 9b, lane L5). Occludin synthesis was also evident

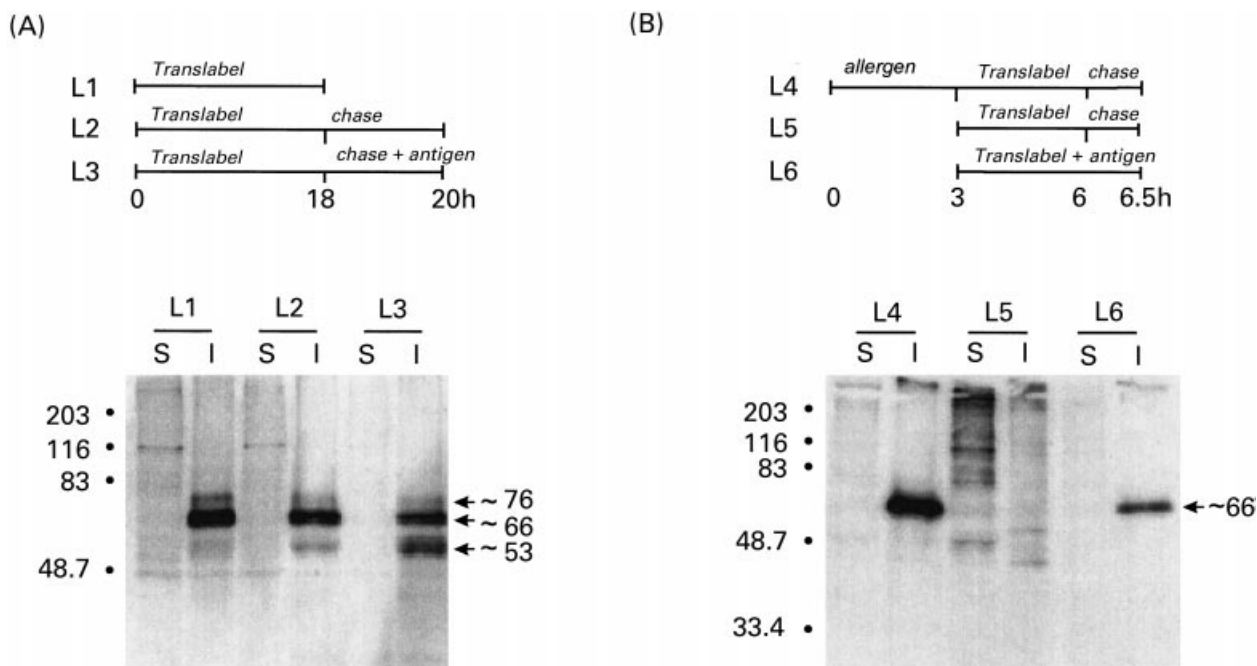


Fig. 9. Immunoprecipitation of occludin from 16HBE14o-cells labelled with Tran ^{35}S -label. (a) Calcium switched cells were subjected to the following treatment protocols which are summarized in the schematic above each fluorogram: incubation with Tran ^{35}S -label for 18 h prior to immunoprecipitation (L1); incubation with Tran ^{35}S -label for 18 h followed by 2 h chase in unlabeled medium prior to immunoprecipitation (L2); incubation with Tran ^{35}S -label for 18 h followed by 2 h chase in unlabeled medium containing allergen prior to immunoprecipitation (L3). Lane headings S and I denote NET buffer soluble and insoluble fractions, respectively. These experiments show degradation of occludin on treatment with allergen. (b) In this series of experiments, cells were treated as follows before immunoprecipitation of occludin: Cells were treated with allergen for 3 h prior to incubation with Tran ^{35}S -label for a further 3 h and then a chase in unlabeled medium for 0.5 h (L4); cells incubated with Tran ^{35}S -label for 3 h with 0.5 h chase in unlabeled medium (L5); cells treated for 6.5 h with Tran ^{35}S -label and allergen (L6). These experiments show activation of occludin synthesis in cells treated with allergen. Note the absence of occludin labelling in cells that were not treated with allergen. Enzyme activity of the allergen was 1600 nmol/min.

during allergen exposure of cells. Lane L6 of Fig. 9(b) shows incorporation of radioactivity into cells co-treated with allergen and Tran ^{35}S -label.

Re-synthesis of occludin was not associated with an obvious alteration in occludin gene transcription, as levels of mRNA appeared qualitatively unchanged (Fig. 10).

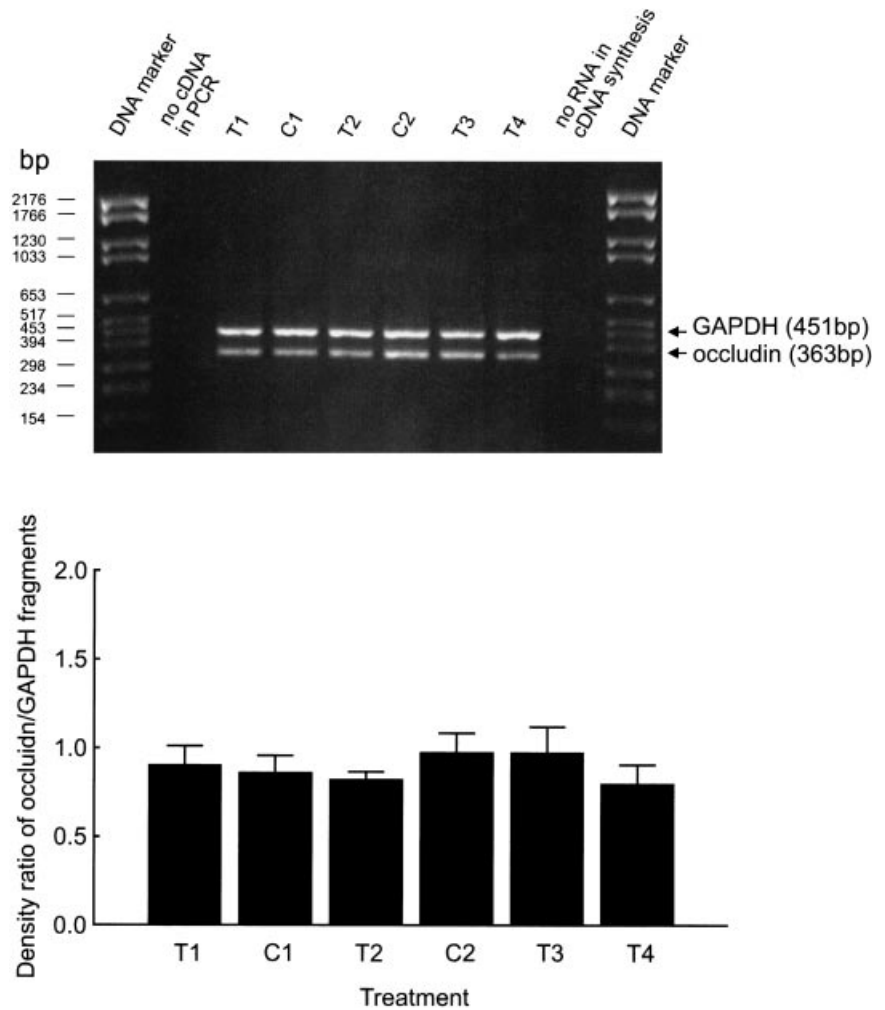
Discussion

This study suggests that the epithelial lining of the airways could be an important biological response target of the HDM allergen Der p 1. Reversible perturbation of inter-cellular adhesion underlies the changes in TJ morphology and loss of control over epithelial permeability that were found to result from exposure to Der p 1. Since the ability to act as a barrier is a major function of the airway lining, these actions of Der p 1 may be of significance to allergic sensitization and asthma. These results support and extend the recent identification of occludin and claudin family

proteins as major substrate targets of house dust mite faecal pellets and purified Der p 1 [9].

The possible importance of enzymatic activity directed against the airways epithelium in allergic sensitization to Der p 1 was first suggested by Herbert and colleagues [18,19]. The unknown mechanism by which Der p 1 increased the leakiness of airways epithelium [18,19] was reliant upon the cysteine peptidase activity of the allergen since the effect was inhibited by E-64, a compound that inactivates a wide range of cysteine peptidases [19]. Progress in characterizing the complex response mechanisms to specific allergens has been impeded by the difficulties involved in obtaining active allergens in high chemical purity. Although the availability of monoclonal antibodies to particular allergens offers immunoaffinity purification as an option, the unknown complexity of the biological matrices in which many allergens are found makes this approach alone unsatisfactory. A number of apparently contradictory claims have been made regarding the enzymatic mechanism of Der p 1 when purified by

Fig. 10. Cellular levels of occludin mRNA are unchanged following treatment of 16HBE14o-cells by Der p 1. (a) RT PCR amplification of occludin mRNA from cells treated as follows: cells treated with allergen for 3 h prior to 3.5 h recovery in normal medium (T1); cells incubated in normal medium for 6.5 h as control (C1); cells treated with allergen for 3 h prior to 0.5 h recovery in normal medium (T2); cells incubated in normal medium for 3.5 h as control (C2); calcium switch with overnight incubation in normal medium and then exposure of cells to allergen for 3 h prior to 0.5 h recovery in normal medium (T3); calcium switch with overnight incubation in normal medium and then exposure of cells to allergen for 3 h prior to 0.5 h recovery in normal medium (T4). DNA markers are shown in the lanes at extreme left and right. Controls lacking cDNA in PCR and no RNA in cDNA synthesis are also shown. Occludin was amplified as a 363 bp RT PCR product. RT PCR of GAPDH is shown for reference as housekeeping gene. (b) Densitometric analysis of occludin RT PCR in the treatments described in (a). Data are from triplicate amplifications and are expressed as the mean ($\pm$ SE mean) ratio of occludin to GAPDH.



immuno-affinity chromatography [20–22], and further purification may be required to remove contaminant serine peptidase activity [23]. We have shown that one Der p 1 monoclonal antibody has cross reactivity with preparations of house dust mite faecal serine peptidases [8]. The purification strategy used for Der p 1 differs from those used by other authors in that several additional steps (ammonium sulphate fractionation, chromatofocusing and affinity chromatography) were used in combination. Although laborious, this approach has the merit of providing a high level of confidence in the molecular and functional identity of the purified protein. Allergen purified in this way has been used in our previous studies of Der p 1–epithelial cell interactions. In these, we demonstrated that E-64 and/or antipain inhibited the Der p 1-induced detachment of MDCK cells from artificial substrata [19], and the cleavage of occludin in 16HBE14o-cells [9]. In contrast, these effects could not be inhibited by inhibitors of serine, aspartic or matrix metallopeptidases [8,9]. We also note *en passant* that the cleavage sites we have identified for Der p 1 in

occludin and claudins [9] differ from those attacked by house dust mite faecal pellet serine peptidases [unpublished observations]. Nevertheless, until catalytically-active peptidase allergens of unambiguous identity and purity are prepared by recombinant expression, it would be prudent to consider the possibility that unknown, low abundance contaminants could contribute to the effects reported for any ‘purified’ allergen.

It now seems that Der p 1 has several discrete actions in epithelial cells that are dependent on its proteolytic activity [8,23], most notably the ability to cause cleavage of the occludin and claudin families of TJ adhesion proteins. In the present study, we have extended these findings to demonstrate the complexity of the overall cellular response, with rapid initiation of measures that may be directed to repair damaged cell–cell adhesions.

Reduction in the protein content of TJs and fragmentation of the TJ rings were early and profound responses in cells exposed to Der p 1. The time course of these changes mirrored the increase in epithelial permeability. This was

expected since TJs comprise the major barrier to paracellular transit pathways in epithelia [4,5]. The increase in diffuse cytoplasmic fluorescence that accompanied these changes in TJs might arise if intact ZO-1 were to be redistributed from TJs into a cytoplasmic pool. Alternatively, it might represent the removal from TJ of ZO-1 cleavage products. While not excluding the former option, our immunoblotting data support the latter possibility, because Der p 1 treatment resulted in cleavage of ZO-1 in both MDCK and 16HBE14o-cell lines. It is not known how ZO-1 is degraded under these circumstances but, as in our earlier study in 16HBE14o-cells [9], it seems reasonable to propose that the initiator of these events is cleavage of TJ adhesion by proteolysis of the transmembrane proteins that seal TJs. Unlike ZO-1, occludin is a transmembrane protein [5] thought to contribute to the adhesive seal of TJs [24–27] and regulate paracellular pathways [26,28]. Immunoblotting confirmed that occludin cleavage occurred in both cell lines after treatment with Der p 1, consistent with the view that TJs undergo comprehensive disassembly following exposure to this allergen [9]. Immunoblots of occludin from 16HBE14o-cells were more complex than those from MDCK cells in which only a major 66 kDa band was detectable. As found previously, blotting of 16HBE14o-cells disclosed higher mass forms of occludin which might be phosphorylated occludin [29] or isoforms of this protein [30].

Recently, a new multigene family of TJ adhesion proteins, the claudins, has been discovered [31,32] that is thought to reconstitute the properties of TJs even more effectively than occludin [33]. In these experiments, we did not study the effects of Der p 1 on the dynamics of claudins, but our previous experiments with synthetic extracellular domains of claudin-1 [9] suggest that claudins contain a conserved Der p 1 cleavage site identified in occludin [9].

TJs are not the only intercellular junctions of epithelia that form contiguous rings at lateral cell boundaries [7,34] and which may be targets for the action of Der p 1. Immunoblotting showed that E-cadherin of zonulae adherentes [35] also underwent proteolysis, but the extent of this seemed much less than that of occludin or ZO-1. Since zonulae adherentes are located beneath TJs, the smaller extent of proteolysis may arise simply because degradation of TJs is an essential prerequisite for diffusional access of Der p 1 to adherens junctions. Alternatively, the extracellular domains of E-cadherin may be less suitable substrates than the extracellular loops of occludin. Further investigations will be necessary to explore these possibilities.

The changes observed in desmosomes were unanticipated from our previous work [9]. Quantitative image analysis clearly showed that MDCK cells responded to Der p 1, with a small but consistent increase in size of puncta which

paralleled an increase in their fluorescence intensity and the background fluorescence of the cytoplasm. A possible implication of these findings is that the MDCK cells responded to TJ breakage by reinforcement of desmosomal adhesion. This may represent a short-term adaptation to the injury produced by the peptidase allergen. However, if the allergen is left in contact with cells, this adaptation is subsumed by other processes, notably apoptosis and frank cellular exfoliation [8]. In connection with the latter event, we observed partial dissolution of the stress fibre network of the actin cytoskeleton in 16HBE14o-cells, a process which is associated with detachment of epithelial monolayers from their substrata [36]. In MDCK cells that remained attached to the substratum, there was apparent reinforcement of the actin stress fibre network and a condensation of F-actin staining, particularly around sites of cell loss. At present, we are unable to determine whether these changes represent cellular responses to the exfoliation of neighbouring cells, or whether they temporally precede cell loss because they are involved in the perturbation of cell adhesion activated by the allergen. Regardless of which of these options is correct, changes in cellular actin organization and cell adhesion are likely to have important consequences for epithelial function, since these events may result in loss of epithelial polarization and disturbance of membrane domain-specific functions.

By radiolabelling of endogenous occludin we were able to show that cells exposed to Der p 1 were attempting to re-synthesize this TJ protein in parallel with its degradation by allergen. In unperturbed cells, we encountered initial difficulties in radiolabelling occludin to a suitable specific activity, either because occludin turnover is normally low, or because the uptake of amino acids is hindered by TJs when they are closed. This problem was circumvented by forcing cells to break and reform TJs by means of two calcium switches [37] prior to incubation with Tran ³⁵S-label. This resulted in the labelling of an insoluble fraction containing high and low mass forms of occludin. Following treatment with Der p 1, there was evidence of occludin cleavage, generally confirming the conclusions of the immunoblotting studies in MDCK cells. The small discrepancy in molecular mass of the cleavage products may represent differences in sequence between human and canine occludin [38] and the low abundance of radioactively-labelled occludin after subjection to further proteolytic processing. In unswitched cells treated with Der p 1, either prior to or simultaneously with labelling, there was incorporation of ³⁵S into a 66 kDa form of occludin. This suggests that occludin re-synthesis is taking place when TJs become cleaved, indicating that either protein re-synthesis is activated or that supply of exogenous amino acids from the apical side of the cell is facilitated. The reformation process seems independent of changes in gene transcription

because levels of occludin mRNA seemed qualitatively unaffected by allergen treatment.

In summary, evidence is provided which supports the view [9] that TJ cleavage and repair occur following exposure of epithelial cells to Der p 1. The changes in TJs are accompanied by alterations in the composition and appearance of desmosomes that may be a defensive adaptation to physical and functional stresses imposed on the epithelial barrier. These observations reinforce the hypothesis [2,8,9,18,19] that disruption of the lung epithelium permeability barrier by Der p 1 may be an important event in allergic sensitization and asthma.

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