

The distribution of adhesive mechanisms in the normal bronchial epithelium

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The distribution of adhesive mechanisms in the normal bronchial epithelium. S. Montefort, J. Baker, W.R. Roche, S.T. Holgate. ©ERS Journals Ltd 1993.

ABSTRACT: The integrity of the bronchial epithelium is dependent on various adhesion mechanisms that serve to hold the composite structure of the epithelium together and anchor it to the underlying basement membrane. Using immunohistochemistry we wanted to map out a number of these junctional and non-junctional adhesion mechanisms in the normal human bronchial epithelium.

The $\beta 1$ -associated integrin subunit $\alpha 2$ was immunolocalized to all of the epithelial intercellular spaces, whilst $\alpha 6$ and $\beta 4$ were strongly evident at the basal cell layer basement membrane junction. The $\alpha 1$ and $\alpha 5$ integrin subunits were not detected anywhere in the epithelium.

Monoclonal antibodies (MoAbs) to tight junction polypeptides and the E-cadherin, liver cell adhesion molecule (LCAM), immunolocalized to the apicolateral portions of the intercellular junctions between all neighbouring columnar cells, with LCAM extending further along the lateral cell membrane. Desmosomal protein (dp) 1 and 2 MoAbs gave a punctate pattern between all of the suprabasal cells, and exhibited the greatest intensity of staining at the junction between the columnar and basal cell layers.

In conclusion, there is an organized distribution of adhesive mechanisms within the normal human bronchial epithelium, which may be targeted by the various insults which lead to epithelial shedding.

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The normal bronchial epithelium is a complex, pseudostratified, ciliated columnar cell structure [1], the best known function of which is to serve as a protective barrier to the underlying submucosa [2]. It fulfils this function by impeding the access of irritant stimuli to the sensory intraepithelial and submucosal nerves, and also by trapping and excluding pollutants/allergens present in the bronchial lumen in its mucociliary escalator [3]. In addition, it forms the supporting matrix for many different cell types involved in immune regulation, detoxification and maintenance of periciliary fluid homeostasis. Apart from these structural functions, the bronchial epithelium is capable of producing proinflammatory mediators, such as leukotrienes [4, 5], endothelin [6], bronchoconstrictor neuropeptides [7], and cytokines, and bronchodilatory substances, such as prostaglandin E_2 and the epithelial cell derived relaxant factor [8, 9]. These varied functions emphasize why epithelial shedding is so important in conditions such as asthma, where focal damage to this barrier and bronchial hyperresponsiveness have been associated [10].

The widely-accepted hypothesis for the cause of epithelial shedding in asthma is that insults, such as ozone [11], viruses [12-14], and eosinophil granule arginine-rich proteins, produce a cytotoxic attack against epithelial cells, leading to disruption and shedding of damaged cells [15, 16]. However, the finding of viable epithelial cells in bronchoalveolar lavage fluid and or "damaged" cells still

attached to the bronchial wall [17] suggests that site-directed cytotoxicity is not the only mechanism leading to epithelial cell shedding in bronchial asthma. Our recent observation of a potential plane of cleavage between the suprabasal and basal cell layers of the epithelium [18] suggests that disturbance to the intraepithelial adhesive mechanisms may be important in epithelial disruption.

The integrity of the bronchial epithelium, like that of other epithelia, is dependent on various adhesive mechanisms, that serve to hold the composite structure together and bind it to the underlying basement membrane. These adhesive mechanisms can be broadly subdivided into junctional and non-junctional types. Some of these adhesive structures have dual roles, such as the tight junction (zona occludens), which, in addition to maintaining the surface seal of the epithelium, also serves to maintain selectivity over epithelial permeability [19], and gap junctions (nexus), which, in addition to binding adjacent epithelial cells, are also involved in intercellular ion exchange [20].

The best characterized epithelial junctional adhesive structures are desmosomes (macula adherens) [21], and hemidesmosomes [22], being important in cell-to-cell and cell-to-basement membrane adhesion, respectively. Although studied extensively in human skin, their distribution has not been extensively investigated in the human bronchial epithelium. Desmosomes exist as discrete structures, visible on electronmicroscopy by the presence of dense plaques on

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each side of a 25–35 nm wide intercellular space, and tonofilaments extending from the plaques into the cellular cytoplasm. Desmosomes are comprised of four desmosomal proteins (dp1–dp4), which are mostly localized within the plaque and around the tonofilaments, and three desmosomal glycoproteins (dg1–dg3), which are mostly found in the extracellular region [23]. Hemidesmosomes are composite structures, which in the skin [24] and cornea [25] have been shown to be largely comprised of $\alpha 6\beta 4$ integrin aggregates, which probably interact closely with basement membrane components, such as laminin. In addition, some of the $\beta 1$ integrin heterodimers, which are known to interact with matrix proteins [26], have also been immunolocalized in the normal human bronchus [27, 28], where they have an extensive distribution. This might be expected, since many of these adhesion molecules are receptors to most of the basement membrane components [29], and form components of junctional adhesive mechanisms in other tissues [24, 25].

Since knowledge concerning adhesive mechanisms which maintain the integrity of the human airway epithelium is rudimentary, we have used immunohistochemical and electronmicroscopic produces to map the distribution of a number of important adhesive mechanisms in normal bronchial epithelium, to provide a database with which to compare diseased states where epithelial damage occurs, such as asthma.

Methods

Bronchial tissue

Four different samples of bronchial tissue were obtained from the segmental bronchi of fresh lobectomy specimens, obtained from two patients undergoing surgery for lung malignancies. One patient had no history of smoking, whilst the other had ceased to smoke 18 months prior to surgery. Neither of the patients suffered from any other illness. The small pieces of bronchial wall were taken

well away from the tumour margin. Each bronchial sample ($n=8$) was divided into three; one for transmission electron-microscopy, one for frozen sectioning, and one for embedding in glycolmethacrylate (GMA) resin, a water soluble resin which has the advantage over other resins of preserving many immunoreactive epitopes [30].

Electronmicroscopy

The tissue sample was immediately transferred into 3% glutaraldehyde fixative for 2 h, and then washed in cacodylate/sucrose buffer overnight. The biopsy was then post-fixed in 2% osmium tetroxide in cacodylate buffer for 2 h, rinsed in millipored double distilled water and incubated in 2% uranyl acetate for 1 h. It was then dehydrated through graded alcohols, cleared in Histosol for 20 min and infiltrated with 50:50 Histosol/ Spurr resin for 30 min. Finally, it was infiltrated in 100% Spurr overnight. The specimen was then embedded in Spurr in a small tube, spun and polymerized at 60°C for 16 h. Ninety nanometre sections were cut on an ultramicrotome (C. Reichart, Austria) and mounted on uncoated copper grids. Sections were examined using a Hitachi H7000 microscope (Hitachi, Tokyo, Japan).

Immunohistochemistry

Table 1 displays the range of monoclonal antibodies (MoAbs) used in this study.

Frozen sections

The tissue samples were snap-frozen in liquid nitrogen. Cryostat sections of 5 μ m were cut, mounted onto a glass slide coated with 0.01% poly-L-lysine as an adhesive, dried for 30 min at room temperature and stored at -20°C until used. For immunostaining the unfixed frozen sections were incubated with the primary monoclonal antibody (MoAb) for 30 min at room temperature. After washing

Table 1. – Monoclonal antibodies (MoAbs) used for immunohistochemistry

Adhesion protein	MoAb	Source	Cryostat	GMA
$\alpha 1$ (CD49a)	TS2-7	D. Garrod	+	+
	mouse IgG1	Manchester, UK		
$\alpha 2$ (CD49b)	CLB-4	A. Sonnenberg	-	+
	mouse IgG1	The Netherlands		
$\alpha 5$ (CD49e)	B1E5	D. Hall	+	+
	mouse IgG1	S. Francisco, US		
$\alpha 6$ (CD49f)	GoH3	A. Sonnenberg	+	+
	rat IgG1	The Netherlands		
$\beta 4$	5B5	J. Aplin	+	+
	mouse IgG1	Manchester, UK		
LCAM	Decma-1	Sigma Chem.	+	+
	rat IgG1	Poole, UK		
Dp1 + 2	11.5F	D. Garrod	+	+
	mouse IgG1	Manchester, UK		
Tight Junction polypeptide	ZO1	T. Fleming	+	-
	mouse IgG1	Southampton, UK		

GMA: glycolmethacrylate resin; IgG: immunoglobulin G; Dp: desmosomal protein; CD: cluster designation; LCAM: liver cell adhesion molecule.

three times in phosphate buffered saline, (PBS) pH 7.4, the appropriate second layer antibody linked to the fluorochrome was added for 30 min. The sections were rinsed and mounted in a mixture of PBS/glycerol in a ratio of 1:9. They were viewed under a Zeiss photomicroscope, fitted with an ultraviolet filtered mercury bulb.

Glycolmethacrylate (GMA) sections

The tissue sample was placed in ice-cooled acetone, containing the protease inhibitors phenylmethyl-sulphonyl fluoride (2 nM) and iodoacetamide (2 nM), and rapidly cooled to -20°C overnight. The following day the sample was transferred into acetone at room temperature for 15 min, and then into methyl benzoyl for a further 15 min. The tissue was immersed in GMA monomer (Polysciences, Northampton, UK) at 4°C for 7 h, during which time the GMA solution was changed three times. On completion, the tissue was embedded in GMA resin (prepared by mixing GMA monomer, *N,N*-dimethyl-laniline in polyethylene glycol (PEG) 400 and benzoyl peroxide) and polymerized overnight at 4°C . The GMA blocks were stored in airtight containers at 20°C until immunostaining.

Sections were cut on an ultramicrotome at $2\ \mu\text{m}$ thickness and floated onto ammonia water (1 in 500), and then picked onto 0.01% poly-L-lysine glass slides, where they were allowed to dry at room temperature for 1–6 h. Endogenous peroxidases were blocked using a solution of 0.1% sodium azide and 0.3% hydrogen peroxide; this was followed by three rinses in Tris buffered saline (TBS) adjusted to pH 7.6. Undiluted culture supernatant, consisting of Dulbecco's minimum essential medium (MEM) + 10% foetal calf serum + 1% bovine serum albumin, was applied for 30 min and, after draining, the primary antibodies were added. The sections were incubated with the primary antibodies overnight at 37°C . After rinsing, biotinylated rabbit anti-mouse immunoglobulin G (IgG) antigen-binding fragment (Fab) (Dako Ltd, High Wycombe, UK) was applied for 2 h and followed by the streptoavidin-biotin-horse-radish-peroxidase complex (Dako Ltd, High Wycombe, UK) for 2 h. After rinsing in TBS, (amino-ethyl carbamazole (AEC) in acetate buffer (pH 5.2) and in hydrogen peroxide, was used as substrate to develop a peroxide-dependent colour reaction. The sections were then counterstained with Mayer's haematoxylin.

Results

No differences in the distribution of any of the adhesive mechanisms studied were noted between the eight different tissue samples.

Electronmicroscopy (EM)

Transmission EM showed intercellular junctions between adjacent columnar cells to be similar to those noted in other epithelia, with tight junctions, intermediate junctions and desmosomes quite easily identified (fig. 1). The

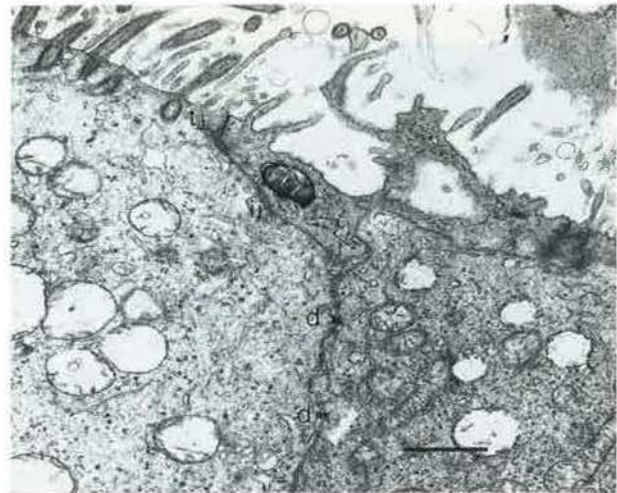


Fig. 1. – Electronmicrograph of intercellular junction between two columnar epithelial cells showing tight junction (tj), intermediate junction (ij), and desmosomes (d). (Original magnification $\times 20,000$. Bar = $1\ \mu\text{m}$).



Fig. 2. – Multiple hemidesmosomes (arrowheads) in basal cell basement membrane interface. (Original magnification $\times 20,000$. Bar = $1\ \mu\text{m}$).



Fig. 3. – Immunostaining for $\alpha 2$ integrin in bronchial epithelium on GMA-embedded biopsy. GMA: glycolmethacrylate resin. (Original magnification $\times 400$. Bar = $10\ \mu\text{m}$).

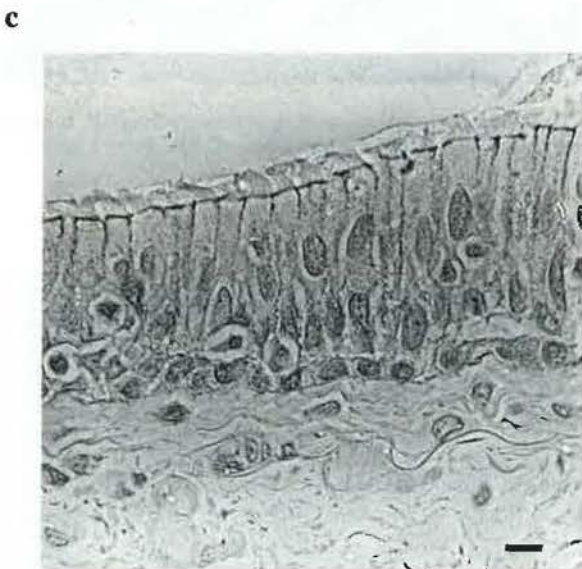
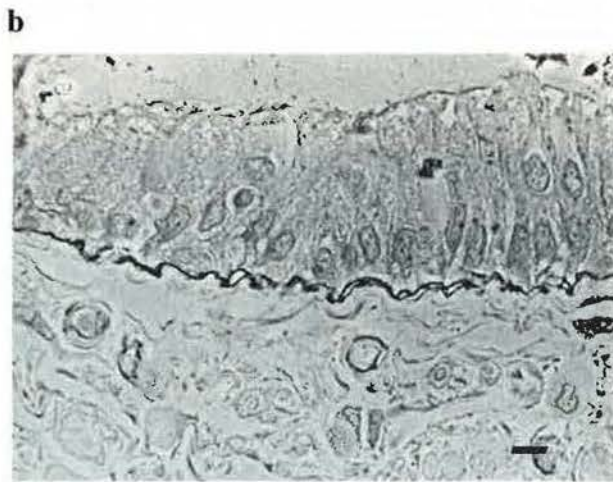
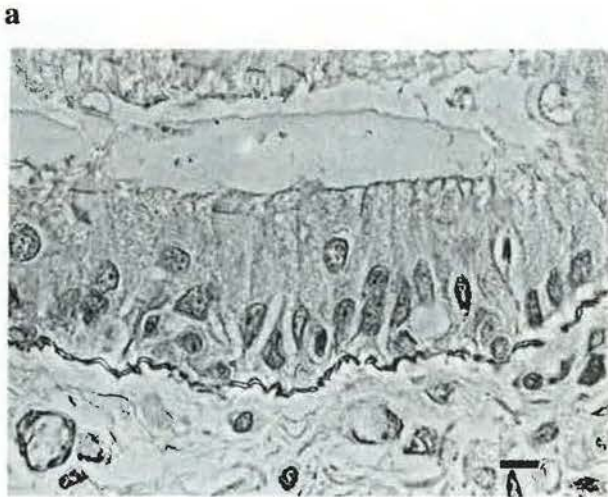


Fig. 4. - Immunostaining in bronchial epithelium on GMA-embedded biopsy for a) $\alpha 6$ integrin b) $\beta 4$ integrin and c) E-cadherin. GMA: glycolmethacrylate resin. (Original magnification for a) and b) $\times 400$, and for c) $\times 450$. Bar = 10 μm).



Fig. 5. - Immunofluorescent punctate staining for desmosomal proteins 1 and 2 on frozen section of bronchial epithelium. (Original magnification $\times 400$. Bar = 10 μm).



Fig. 6. - Immunofluorescent staining for tight junctions in bronchial epithelium on frozen section. Lu: bronchial lumen. (Original magnification $\times 400$. Bar = 10 μm).

desmosomes noted were similar in structure and dimensions to those reported in other epithelia. Hemidesmosomes were observed as multiple electron-dense areas (fig. 2) where the basal cells impinged upon the underlying basement membrane. However, these structures were not as prominent as reported in other human tissues such as skin.

Immunohistochemistry

Glycolmethacrylate. GMA had the advantage of enabling thin sections to be cut, whilst preserving morphology and localization of immunostaining to specific cellular structures. We failed to demonstrate any epithelial staining with the MoAb to the $\alpha 1$ subunit or the $\alpha 5$ subunits. In the case of the $\alpha 2$ subunit, immunostaining was observed around all of the bronchial epithelial cells including the basal cells (fig. 3). The $\alpha 6$ and $\beta 4$ showed similar patterns of distributions, being only expressed in the sub-basal region of the epithelium as a clear linear pattern and less strongly around submucosal vessels (figs 4a and 4b). The MoAb to the E-cadherins immunostained the apical portions of the intercellular junctions found between the columnar epithelial cells (fig. 4c), and to a lesser degree, occasional parts of the sub-basal cell area.

Frozen sections. Cryo-immunohistochemistry was most helpful in the localization of dp1 and dp2 in desmosomes.

A punctate pattern was observed along the lateral border of the suprabasal cells, with a denser distribution at the interface between the suprabasal and basal cell layers (fig. 5). There was a complete absence of staining below the basal cell layer. Immunostaining with the MoAb against

tight junctions, ZO1, was localized to the apical part of the intercellular borders but did not extend down as far as the E-cadherins (fig. 6). The cryo-immunofluorescent technique confirmed the GMA findings for the $\alpha 1$, $\alpha 2$, $\alpha 6$ and $\beta 4$ subunits, with the latter two again being found at the basal cell basement membrane junction (figs 7a and 7b). The E-cadherin was seen in the intercellular borders between neighbouring columnar cells, immunostaining most intensely in the apical region of these junctions (fig. 7c). In cross-section the cadherin immunostaining was seen to surround the individual epithelial cells (fig. 7c).

Discussion

Using a combination of immunohistochemistry and electronmicroscopy it has been possible to build-up a picture of the structures important in maintaining the structural integrity of the bronchial epithelium. Of particular novelty in this study was the clear demonstration of tight junctions and E-cadherin, probably present within intermediate junctions. Whilst these structures may be important in regulating paracellular transport, attachment of the epithelium to its basement membrane appears to rely heavily on the expression of hemidesmosomes, containing the $\alpha 6$ and $\beta 4$ integrin subunits. In contrast to hemidesmosomes, the structures upon which the majority of the epithelial integrity depends are the desmosomes found along the lateral and inferior borders of the suprabasal cells. An increased number of these structures along the apical border of the basal cells indicates an important site of epithelial stress, and corresponds to the site of cleavage of epithelial cells especially in asthma. These observations indicate the complexity of the normal bronchial epithelium and the potential importance of these structures as a target for disruption in inflammatory airway disorders.

The epithelium in the normal human bronchus, is described as pseudostratified, ciliated and columnar [1]. It appears that the basal cells provide a foundation upon which the suprabasal cells attach. In human skin [24], and cornea [25], the basal cells are fixed firmly to the basement proteins *via* hemidesmosomes with their constituents $\alpha 6\beta 4$ integrins. However, while hemidesmosomal structures were visualized in the bronchial epithelium by transmission electronmicroscopy, they seemed less distinct than described by others in the skin. The presence of strong immunostaining for the $\alpha 6$ and $\beta 4$ components of hemidesmosomes along the inferior border of the basal cells adds further evidence to the important role played by these hemidesmosomal integrins in anchoring the epithelium to the basement membrane. Neither of these integrin subunits were evident immunohistochemically above this level, although some staining was observed around submucosal blood vessels. Thus, in addition to forming a laminin receptor with $\beta 1$, $\alpha 6$ is also able to interact with $\beta 4$ to form a further integrin receptor for laminin present in vascular basement membrane [31].

The $\alpha 2$ integrin subunit, which comprises a component of one of the $\beta 1$ collagen/laminin receptors, immunolocalized to the basal cell/basement membrane interface, where it would be expected to interact with basement membrane

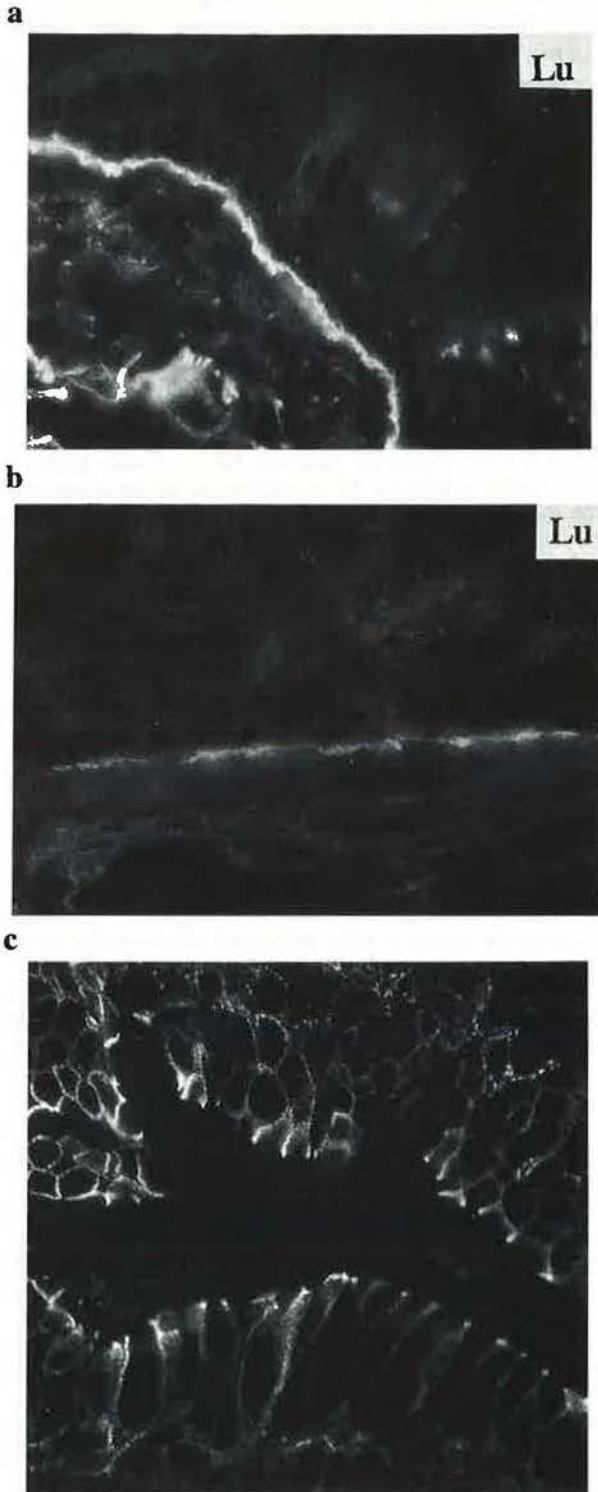


Fig. 7. - Immunostaining on frozen section for a) $\alpha 6$ integrin b) $\beta 4$ integrin and c) E-cadherin. Lu: bronchial lumen. (Original magnification for a) and b) $\times 400$ and for c) $\times 450$. Bar = 10 μm).

components. The immunostaining of this integrin also extended between most of the epithelial cells, confirming the recently published findings of DAMJANOVICH *et al.* [27]. We have failed to demonstrate any immunostaining for either $\alpha 1$ or $\alpha 5$, which forms a component of the classical fibronectin receptor, in the intact normal epithelium, despite the reports of $\alpha 5$ being expressed in cultured human bronchial epithelial cell monolayers [28]. It is possible that these integrin components are only expressed under specific conditions or in the presence of specific growth factors.

It is frequently stated that the integrity of the bronchial epithelium is dependent upon the tight junction, a complex adhesive structure found to surround suprabasal cells. However, this seems unlikely, since immunostaining with the tight junction MoAb, ZO1, was restricted to a discrete structure at the apex of the suprabasal epithelial cells intercellular borders - confirming the findings of GODFREY *et al.* [32], who utilized a freeze-fracture technique for studying tight junctions. The complexity of the tight junction, together with its apical localisation, makes it a strong candidate for controlling paracellular transport, although how this is achieved is not known. A second region of electro-density is observed just below the tight junctions and has been designated the intermediate junction. The distribution of immunostaining with the anti E-cadherin MoAb suggests identity with the intermediate junction. This is in keeping with the finding of BOLLER *et al.* [33], who similarly localized E-cadherin to the intermediate junction of the adult mouse intestinal epithelium. Along with the tight junctions, it is likely that intermediate junctions contribute to the selectivity of paracellular transport, possibly by the epithelial cell regulation of extracellular Ca^{++} concentration. E-cadherin may also contribute towards intercellular adhesion between adjacent suprabasal cells, since immunostaining for this molecule was present in a graded fashion below the site of the intermediate junctions.

The most important adhesion mechanism which operates in epithelial structures is the desmosome. This composite structure was easily identified in the bronchial epithelium, between adjacent epithelial cells and the characteristic plaque and tonofilament structures, as previously described in skin. Using a MoAb directed against shared epitopes expressed on desmosomal proteins 1 and 2, which are situated mainly in the desmosomal plaque, the distribution of desmosomes within the epithelium could clearly be seen as punctate immunofluorescent structures, when viewed by ultraviolet microscopy. Punctate staining was present along the lateral and inferolateral borders of the suprabasal cells. This was also evident between adjacent basal cells, but not along their inferior border. There was a particularly dense population of desmosomes at the junction between suprabasal and basal cell layers, suggesting that a strong intercellular bond is needed in this plane to maintain epithelial integrity. It is possible that a disturbance of desmosomal adhesion could account for the epithelial fragility in asthma, especially since this seems to occur between the suprabasal and basal cells [18]; the site of high desmosomal expression. An alternative explanation is that neutral proteases, either from inflammatory cells *per se*, e.g. trypsin, elastase, or induced by a cognate interaction

between eosinophils and epithelial cells [34], could result in enzymatic degradation of desmosomes, with a subsequent reduction in their capacity to anchor epithelial cells.

Whatever the mechanisms of epithelial damage in asthma and other disorders, the widespread distribution of desmosomes and other adhesive structures within the bronchial epithelium may be the target of inflammatory responses in which the epithelium becomes damaged. Thus, understanding of factors controlling their expression, function and degradation is of clear importance in airway homeostasis and the mechanisms by which it may be disturbed.

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