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In vivo restitution of airway epithelium

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Abstract. Epithelial shedding occurs in health and, extensively, in inflammatory airway diseases. This study describes deepithelialisation, reepithelialisation and associated events in guinea-pig trachea after shedding-like epithelial denudation in vivo. Mechanical deepithelialisation of an 800-μm wide tracheal zone was carried out using an orotracheal steel probe without bleeding or damage to the basement membrane. Reepithelialisation was studied by scanning- and transmission electron microscopy and light microscopy. Nerve fibres were examined by immunostaining. Cell proliferation was analysed by [^{3}H]-thymidine autoradiography. Immediately after epithelial removal secretory and ciliated (and presumably basal) epithelial cells at the wound margin dedifferentiated, flattened and migrated rapidly (2–3 μm/min) over the denuded basement membrane. Within 8–15 h a new, flattened epithelium covered the entire deepithelialised zone. At 30 h a tight epithelial barrier was established and after 5 days the epithelium was fully redifferentiated. After completed migration an increased mitotic activity occurred in the epithelium and in fibroblasts/smooth muscle beneath the restitution zone. Reinnervating intraepithelial calcitonin gene-related peptide-containing nerve fibres appeared within 30 h. We conclude that (1) reproducible shedding-like denudation, without bleeding or damage to the basement membrane, can be produced in vivo; (2) secretory and ciliated cells participate in reepithelialisation by dedifferentiation and migration; (3) the initial migration is very fast in vivo; (4) shedding-like denudation may cause strong secretory and exudative responses as well as proliferation of epithelium, and fibroblasts/smooth muscle. Rapid restitution of airway epithelium may depend on contributions from the microcirculation and innervation.

Key words: Airways – Epithelial repair – Cell migration – Cell proliferation – Reinnervation – Guinea-pig

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Introduction

In inflammatory airway diseases such as asthma, epithelial shedding is considered a common phenomenon, and it has been claimed that denudation of the basement membrane is a characteristic feature of the bronchial mucosa in asthma (Dunnill 1960; Laitinen et al. 1985). In vivo experiments carried out in the 1950s and earlier demonstrated that traumatic removal of tracheal epithelium is followed by a process whereby epithelial cells in the margin of the damage flatten and move medially to eventually cover the wound (Loeb 1919; Wilhelm 1953; O'Corell and Beattie 1956). This migration as well as other aspects of epithelial repair have been further investigated (Hilding 1965; Lane and Gordon 1974; McDowel et al. 1978; Keenan et al. 1982a–c, 1983). The techniques used to denude the airways in vivo have involved neck surgery and damage to the epithelial basement membrane as well as local bleeding. Hence, it is difficult to know to what extent the previous in vivo findings are valid for repair of epithelium that has been sloughed off from an intact basement membrane.

In recent years in vitro cultures of epithelial cells have been employed to study epithelial repair mechanisms (Zahm et al. 1991; Rickard et al. 1992, 1993; Romberger et al. 1992). Rennard et al. (1991) have reviewed several important limitations with in vitro culture techniques including the undefined cultured cell type and the variabilities that occur depending on the source of epithelial cells and culture conditions. Furthermore, it is evident that potentially important contributions from the mucosal microcirculation and innervation are lost in the in vitro situation and that cell culture studies cannot readily reveal the effects that epithelial removal may have on different adjacent tissue elements.

We have now continued the early in vivo approaches in this research area employing novel techniques both for the denudation and for examination of the reepithelialisation. One particular aim has been to produce a well-defined and reproducible denudation without causing damage to the basement membrane. This constitutes a

novel approach because earlier studies have consistently involved both severe mucosal damage and bleeding. A denuded, intact epithelial basement membrane appears to be relevant to conditions of epithelial shedding in health and disease. Epithelial cells may be shed in airway defense reactions, and shedding has been considered a hallmark of asthmatic airways (Houston et al. 1953; Dunill 1960; Naylor 1962; Laitinen et al. 1985). The possibility of studying effects and consequenses of "shedding-like" removal of epithelial cells in vivo may thus provide new information of potential relevance to airway defence mechanisms and to inflammatory airway disease processes.

In the present study we have taken advantage of the technique developed by Erjefält and Persson (1991; Erjefält et al. 1993a) whereby an oral catheter, or a denudation probe, can be introduced into the tracheal lumen without surgery and without causing any disturbance to the tracheal mucosa except for a gentle denudation effect. Well defined zones of denudation could also be demonstrated in preliminary experiments carried out with guinea-pig isolated tracheas (Erjefält et al. 1993b). Employing scanning and transmission electron microscopy as well as selected immunochemistry and histochemistry we have now examined several features of the reepithelialisation process in vivo in the airways of guinea-pigs. Especially the morphology and time-course of reepithelialisation, the mitotic activity, the secretory response, and the intraepithelial nerve fibres are examined.

Materials and methods

Removal of the epithelium

Guinea-pigs, male, DH, from Möllegaard, Denmark weighing 450–500 g, were anaesthetised with a 3:2 mixture of ketamin (50 mg/ml) and xylazin (20 mg/ml) given intramuscularly (1 ml/kg) and placed in a supine position as previously described for orotracheal catheterisation (Erjefält and Persson 1991). A specially designed steel-probe was gently moved into the tracheal lumen under fibre optic illumination and visual inspection down to the carinal region. By applying slight pressure, a longitudinal strip (~800 μm width) of the epithelium was removed along the dorsal side of the trachea, from the carinal region to the larynx (~30 mm length). This technique to remove the epithelium does not cause bleeding or structural damage to the underlying, denuded basement membrane (Erjefält et al. 1994b, c). The absence of extravascular erythrocytes immediately after epithelial removal confirmed that no bleeding had occurred. The presence of extravascular erythrocytes was examined by bright field microscopy and scanning electron microscopy of stretched tracheal preparations (see below). In only a few instances (3 out of a total of 66 animals) the experiments were associated with structural damage to the basement membrane and/or bleeding; these animals were excluded from further evaluation.

Termination of the experiment and tissue processing

In order to monitor the epithelial restitution animals were deeply anaesthetised with an intraperitoneal injection of pentobarbital (60 mg/ml; 5 ml/kg) at different time points after deepithelialisation (see below). The thorax was opened and the animals were exsanguinated by an incision in the still beating heart. The trachea was rapidly excised and placed in a Petri dish, rinsed gently with 0.9% NaCl and cut into segments. Animals (n=3, at each time point) were analysed before (non-deepithelialised controls), and at 0, 15 min, 1, 2, 4, 8, 15, and 30 h and at 5 and 15 days after deepithelialisation. For [^{3}H]-thymidine autoradiographic studies separate animals (n=3; at each time point) were treated as above and analysed before (non-deepithelialised controls) and 2, 8, 16, 30 and 70 h after epithelial removal. Subepithelial proliferating cells were examined by immunohistochemical visualisation of bromodeoxyuridine, BrdU (separate animals, n=6) in non-deepithelialised controls and at 50 h after epithelial removal (see below). For immunohistochemistry and histochemistry, two segments (taken at 2–5 and 23–26 mm from the larynx) were immediately placed in Stefanini's fixative (2% formaldehyde and 0.2% picrid acid in phosphate buffer, pH 7.2) overnight at 4°C. Samples were then rinsed several times with a Tyrode buffer (pH 7.4), containing 10% sucrose, embedded in mounting medium (Tissue-tek®, Miles, USA), frozen, and stored at –70°C until examination. For electron microscopy two segments, taken 5–8 (transmission electron microscopy) and 8–23 (scanning electron microscopy) mm from the larynx were used (see below).

Scanning electron microscopy (SEM)

Tracheal segments, 1.5 cm long, were cut along the ventral side, stretched out on Sylgard-coated Petri dishes using micro needles and gently rinsed with PBS buffer (pH 7.4). Tracheal pieces were immediately fixed in PBS-buffer containing 1% glutaraldehyde and 3% formaldehyde for 2 h and examined in a bright-field microscope for any signs of bleeding. In some of the samples where a gel covered the deepithelialised zone this gel was gently removed with a forceps so that examination of the underlying, denuded surface could be carried out. Specimens were rinsed thoroughly in PBS (3×20 min) and dehydrated through a series of ethanol. Samples were subjected to critical point-drying, using carbon dioxide as the transitional fluid, gold-coated and examined with a Philips 515 SEM. The frequency of ciliated cells (expressed as % ciliated epithelial surface) in the remaining and the new, regenerating epithelium was calculated from scanning electron micrographs using a computerised image analysis system (Image Access, Micro-Macro Image Analysis, Gothenburg, Sweden).

Migratory rate

The migratory rate of the new epithelium was calculated from scanning electron micrographs. At 8 points along the denuded zone (1 mm apart) the distance between the original wound edge and the front of the new migrating epithelium was measured on micrographs and the mean distance calculated. The migration rate (μm/min) for each time point was expressed as mean values ±SEM. (n=3).

Transmission electron microscopy

Tissue samples were immersed in the same fixative as for SEM. After fixation overnight the specimens were post-fixed in 1% osmium tetroxide for 1 h. They were then dehydrated in graded acetone solutions, contrasted in a mixture of 1% phosphotungstic acid and 0.5% uranyl acetate, and embedded in Polarbed 812. Ultrathin sections (60–80 nm) were cut on a LKB MK III Ultrotome and routinely contrasted with uranyl acetate and lead citrate in a LKB 2168 ultra-stainer. The samples were examined using a Jeol 200 CX transmission electron microscope. For high resolution light microscopy, thin sections (1–2 μm) of the embedded tissue were cut and stained with toluidine blue.

Histochemistry

For histochemical staining of secretory cells, cryostat sections (10 μm) from tracheal specimens were stained with periodic acid-Schiff reagent (PAS) and examined for the presence of seromucous cells. This histochemical staining has been widely used for identification of secretory cells in the respiratory tract (Robinson et al. 1986). The proportion (%) of the total epithelial area that displayed PAS-positive staining was quantified (3 sections/animal) using the same computerised image analysis system as above.

Mitotic activity

Cells undergoing mitosis were detected with [^{3}H]-thymidine autoradiography as previously described (Tielemans et al. 1992). For further characterisation of subepithelial proliferating cells immunohistochemical visualisation of the uridine analogue BrdU was used (Jones et al. 1993). Briefly, each animal was given a single pulse of ^{3}H-labelled thymidine (10 mCi/mmol; 0.5 mCi/ml, Amersham) or BrdU (10 mg/kg) intraperitoneally (0.5 ml /animal) 1 h before termination of the experiment (for BrdU-immunostaining, see below). Tracheal specimens were collected at different times after deepithelialisation (see above) and put into a PBS-buffer containing 1% glutaraldehyde and 3% formaldehyde overnight or Stefanini's fixative (2% formaldehyde and 0.2% picric acid in buffer, for BrdU-immunostaining, see below). The specimens were rinsed in Tyrode buffer containing 10% (w/v) sucrose, frozen and sectioned (10 μm) perpendicular to the mucosal surface in a cryostat. Sections were immersed in autoradiographic emulsion (ILFORD K5, diluted 1:1 in distilled water). The slides were then air dried and exposed for 6 weeks at 4°C. Autoradiographs were developed with KODAK D-19 developer, counterstained with hematoxylin, mounted in Kaiser's medium (Merck, Darmstadt, Germany) and quantified in a bright-field microscope. In each section all ^{3}H-labelled nuclei were counted and the mitotic activity in the old, remaining epithelium and new, undifferentiated epithelium was expressed as numbers of ^{3}H-labelled cells/mm of the epithelial lining (3 sections per animal were counted). The mitotic activity in the subepithelial tissue was expressed as the total number of ^{3}H-labelled subepithelial cells/section. Sections immunostained for BrdU were examined in a fluorescence microscope and immunoreactive cell nuclei were recorded by a computerised video system. The same sections were then stained with toluidine blue or haematoxylin to reveal the morphology of the BrdU-labelled cells in the subepithelial tissue.

Immunohistochemistry

Tracheal specimens were sectioned perpendicular to the mucosal surface in a cryostat and the slides were allowed to air-dry. In sections used for BrdU immunostaining the DNA was hydrolysed with 1 M HCl at 56°C for 30 min. All sections were rinsed in phosphate-buffered saline (PBS) and exposed to the primary antibodies in an appropriate dilution overnight at +4°C. After thorough rinsing in phosphate-buffered saline (PBS)+0.25% Triton X-100 the sections were incubated in fluorescein isothiocyanate (FITC)-labelled secondary antibodies for 1 h at room temperature. After rinsing in PBS+0.25% Triton X-100 the sections were mounted in PBS:glycerin (1:1) and examined in a fluorescence microscope. Primary antibodies used were: mouse monoclonal antibodies against BrdU (code M744, Dako, Copenhagen, Denmark, dilution 1:40), rabbit polyclonal antibodies against calcitonin gene-related peptide (code 8427, Euro-Diagnostica, Malmö, Sweden, dilution 1:1280), vasoactive intestinal peptide (code 7852, Euro-Diagnostica, dilution 1:1280), and pituitary adenylate cyclase-activating peptide (PACAP 27, code 88121–3, kindly provided by Dr. A. Arimura, Belle Chasse, La, USA, dilution 1:640). The peptide antibodies had previously been tested for cross-reaction with chemically related or unrelated peptides (Sundler et al. 1985, 1992). As secondary antibody a FITC-labelled goat anti-mouse antibody (Jackson, West Grove, Pa., USA, dilution 1:80) or a FITC-labelled swine anti-rabbit antibody (Dako, Copenhagen, Denmark. dilution 1:80) was used. In control sections, using incubation with only secondary antibodies, no staining was found.

Results

Morphology and time-course of the reepithelialisation

Directly (0 h) after deepithelialisation. The deepithelialisation maneuver produced a sharp edge between the

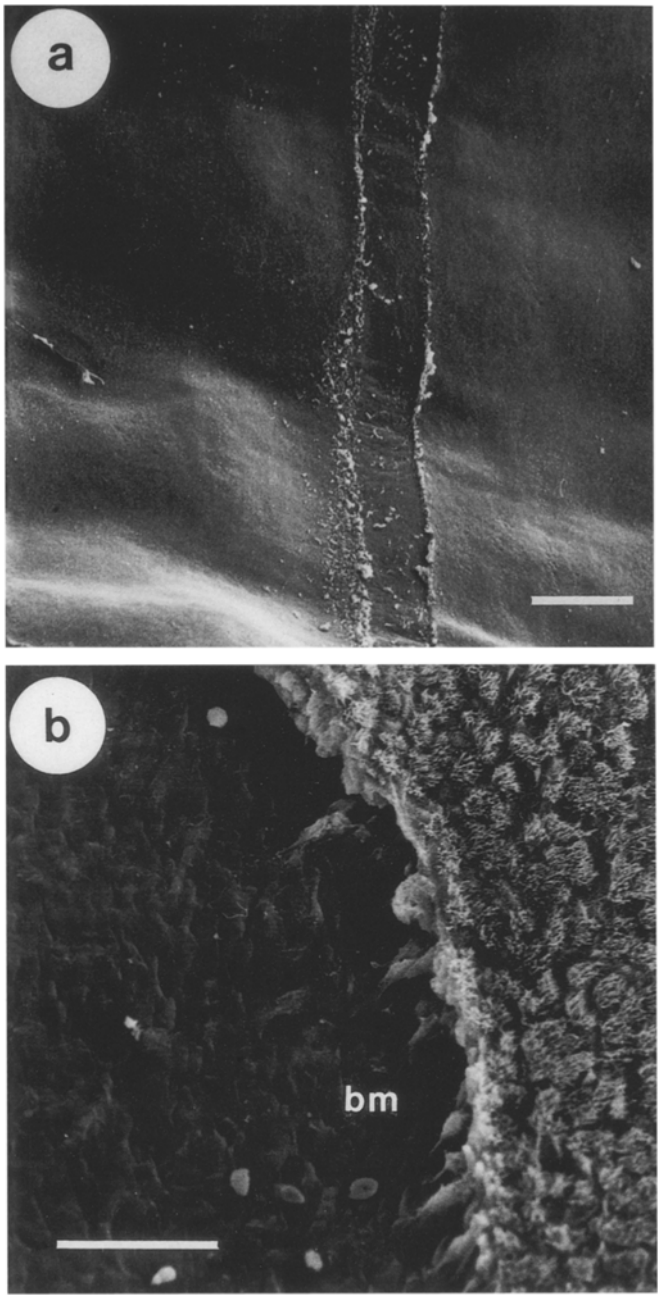

Fig. 1a, b. Scanning electron micrographs of tracheal mucosa immediately after gentle mechanical deepithelialisation. A restricted deepithelialised zone is created along the dorsal side of the trachea (**a**). There is a sharp edge between the remaining epithelium and the denuded but non-injured basement membrane (*bm*) (**b**). Original magnification: **a** ×100; **b** ×800. *Scale bars*: **a** 1 mm, **b** 50 μm

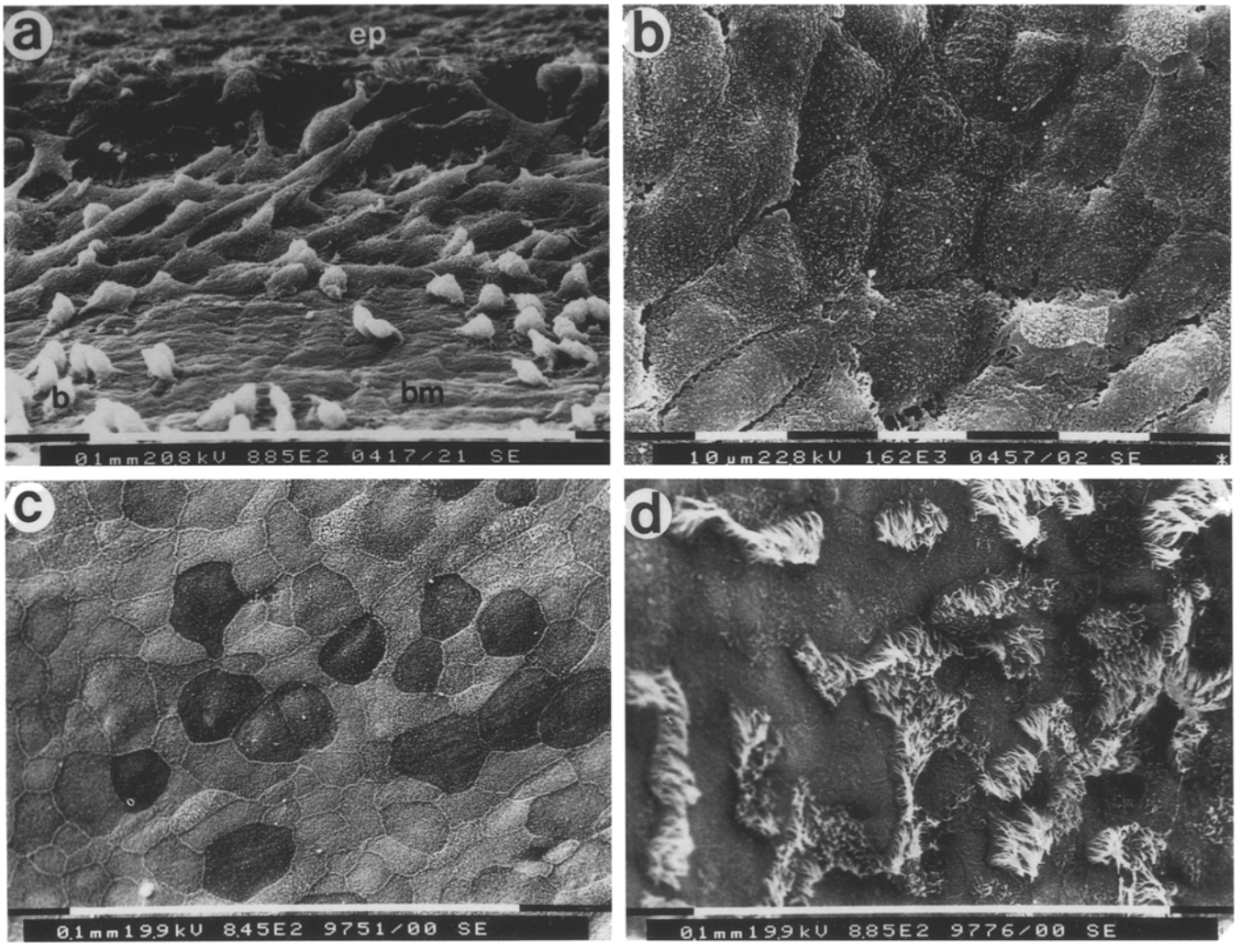

Fig. 2a–d. Scanning electron micrographs of new undifferentiated epithelium during different stages of reepithelialisation. At 15 min (**a**) flattened epithelial cells at the wound margin cover a 40–60 µm wide zone of the denuded basement membrane. At 8 h the denuded area is totally covered by an undifferentiated epithelium (**b**). An apparently tight epithelium with ridgelike seals is observed at 30 h (**c**). After 5 days (**d**) the denuded area is covered by a ciliated, differentiated epithelium. *bm* Basement membrane; *b* basal cell; *ep* normal epithelium. Original magnification: **a, d** ×900; **b** ×1600; **c** 850. *Scale bars*: **a, c–d** 100 µm; **b** 10 µm

intact epithelium and the denuded zone (Fig. 1). The denuded basement membrane was not injured (Fig. 1b). In some areas scattered basal cells remained attached to the basement membrane. Ciliated and secretory cells at the wound edge had a columnar shape. No erythrocytes were found on the deepithelialised surface or in the extravascular space in the subepithelial tissue, as examined by light and transmission electron microscopy.

Migration of epithelial cells (15 min – 8 h). Within 15 min epithelial cells at the wound edge became flattened and migrated over the denuded basement membrane (Fig. 2a). Ciliated cells that lost their cilia and secretory cells that displayed an intense granule discharge were frequently observed at the wound edge (Fig. 3). Migrating cells were attached to each other by patchy cell-cell connections. The speed of migration was several µm/min (Fig. 4), and after 15 min the migratory, flat epithelium covered a 40–60 µm wide zone (Fig. 2a). A common feature for all migratory cells was the sparse number of surface microvilli. Scattered basal cells, remaining on the denuded basement membrane after epithelial removal, did not flatten (Fig. 2a). At 1 h after deepithelialisation the front of the migrating epithelium had moved to cover a 150 µm wide zone. Migrating epithelial cells that displayed signs of intense granule discharge or reduced numbers of cilia were found only within 0–40 µm from the wound edge. At 2–4 h the front of the migrating epithelium had moved 200–300 µm and at 8 h after epithelial removal most of the denuded basement membrane was covered by a flat undifferentiated epithelium (Fig. 2b). The recruitment of epithelial cells did not alter the ratio between ciliated and non-ciliated cells in the remaining epithelium close to the deepithelialised zone. The percent of ciliated epithelial surface area in the remaining epithelium (measured 10–40 µm from the wound edge) was 58±7% at 2 h, and 65±8% at 8 h (compared to 64±9% in controls), n=3 at all

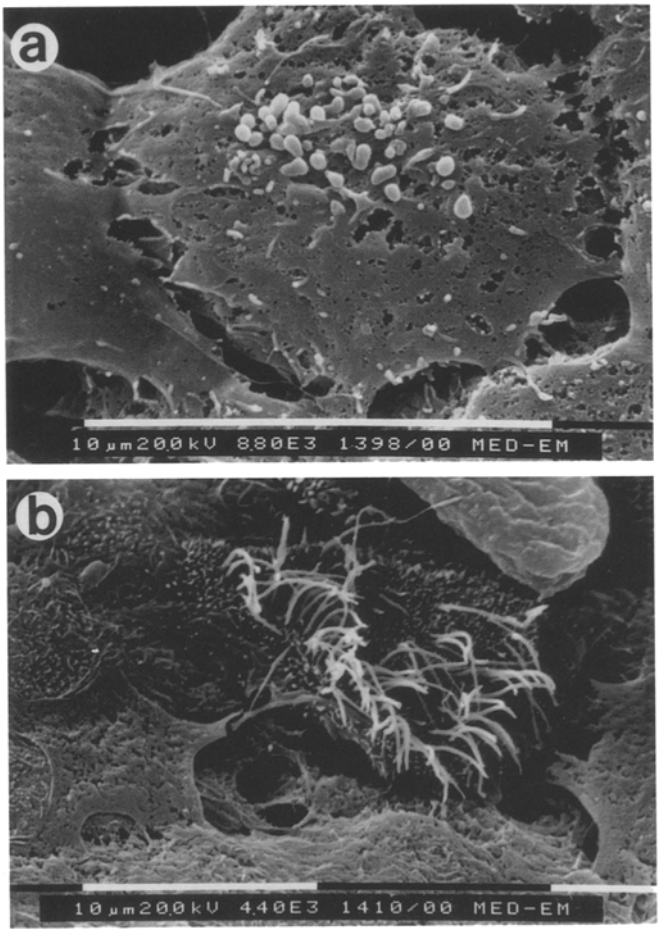

Fig. 3a, b. Scanning electron micrographs of dedifferentiating epithelial cells, located ~30 µm from the original wound edge, 1 h after deepithelialisation. A secretory cell displaying extensive granule discharge (**a**) and a ciliated cell with few scattered cilia (**b**). Note that both cells have a flattened morphology. Original magnification: **a** ×8800; **b** ×4400. *Scale bars*: **a–b** 10 µm

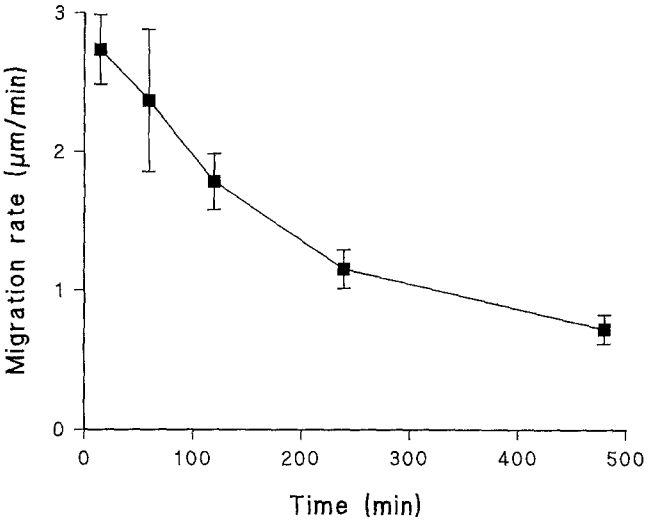

Fig. 4. The rate of epithelial cell migration during 0–8 h after deepithelialisation. The data are given as mean ±SEM

time points. The rate of migration was highest immediately after deepithelialisation and decreased from 2.7±0.3 µm/min to 0.7±0.1 µm/min between 15 min and 8 h after deepithelialisation (Fig. 4).

Differentiation of epithelial cells (15 h-15 days). At 15 h the migrating epithelium covered the entire deepithelialised area. Dedifferentiating epithelial cells were no longer observed at the wound margin. In patchy areas of the new epithelium SEM examination suggested that the cells had tight connections. At 30 h the new epithelium consisted of 2–3 cell layers (Fig. 5) and was characterised by regular tight cell-cell linings, and surface epithelial cells had increased numbers of microvilli (Fig. 2c). Tight junctions and desmosomes were frequently observed (Fig. 6). Many cell nuclei contained well-developed nucleoli or condensed chromatin. A ciliated epithelium was established within 5 days after epithelial denudation (Fig 2d). Light microscopy and histochemistry revealed that a ciliated, pseudostratified, columnar epithelium containing PAS-positive secretory cells was present at the site of deepithelialisation. At 15 days the epithelium had a normal, ciliated surface area (59±10% ciliated surface, compared to 64±9% in controls, n=3) and displayed normal density of PAS-positive cells (9.1±0.6% of the epithelial lining, compared to 8.7±0.7% in controls).

Airway epithelial secretion Two hours after deepithelialisation the remaining tracheal epithelium displayed a dramatic reduction in PAS-positive staining compared to control epithelium (Fig. 7). This decline in epithelial PAS-staining occurred around the entire luminal circumference and was gradually reversed during the course of reepithelialisation, reaching a normal level 5 days after deepithelialisation (Fig. 7).

Mitotic activity during reepithelialisation

Epithelial mitotic activity. In controls only few scattered ^{3}H-thymidine-labelled epithelial cells could be detected (Fig. 8a, b). The new, flat epithelium in tracheas taken 2 and 8 h after deepithelialisation displayed a similar low mitotic activity as the controls (Fig. 8a). A marked increase in mitotic activity was observed in the new epithelium at 15 and 30 h (Figs. 8a, 9b). Seventy hours after deepithelialisation the number of proliferating cells had again decreased compared to the 30-h value (Figs. 8a, 9c). The old, remaining epithelium displayed the same low mitotic activity as studied in controls 2 and 8 h after epithelial removal. A modest increase in mitotic activity was observed in the old epithelium between 15 to 70 h (Fig. 8b).

Subepithelial mitotic activity. Few scattered subepithelial cells undergoing mitosis were observed in control tracheas and at 2 and 8 h after deepithelialisation (Fig. 8c). Between 15 and 70 h an increase in ^{3}H-thymidine-labelled subepithelial cells was observed (Figs. 8c, 9b,c). The increase in mitotic activity was mainly located in

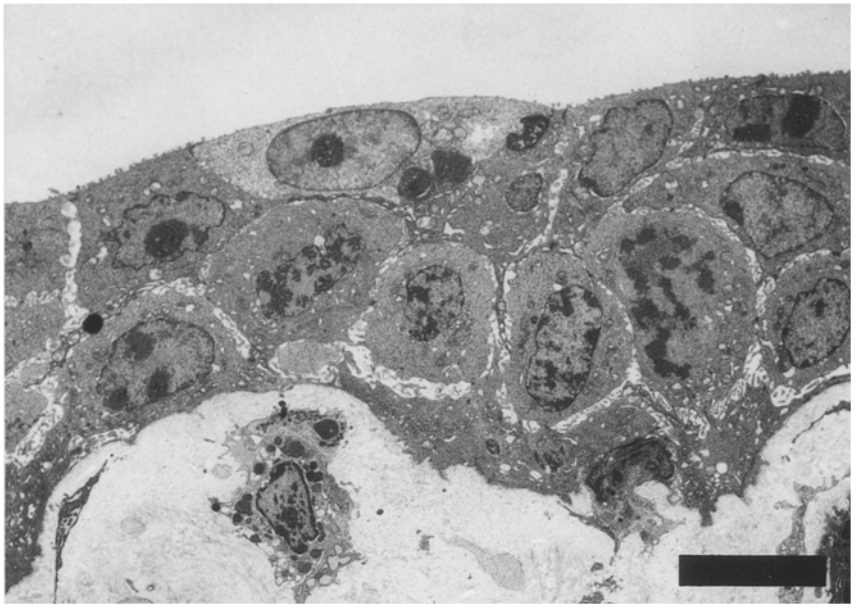

Fig. 5. Transmission electron micrograph of the new epithelium at 30 h after denudation. The epithelium consists of 2–3 layers of flat or cuboidal, undifferentiated cells. Original magnification: ×2000. *Scale bar*: 10 μm

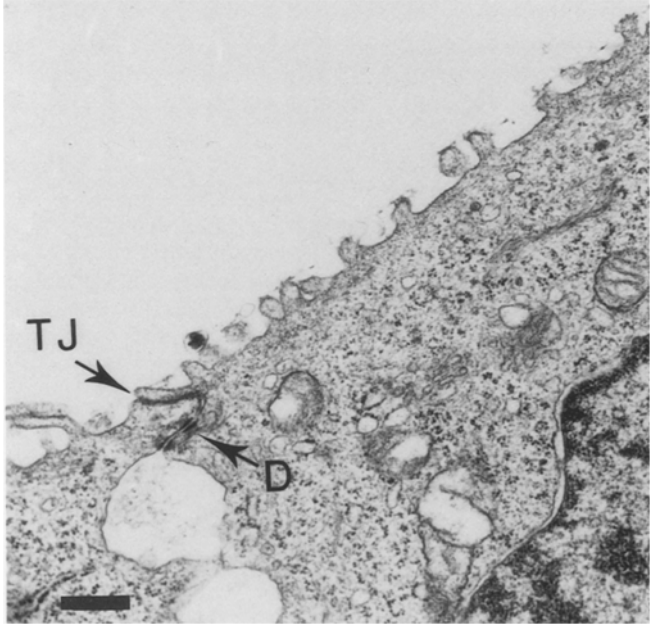

Fig. 6. Transmission electron micrograph of the boundary between two non-differentiated epithelial cells in the new epithelium 30 h after denudation. Note the presence of tight junctions (*TJ*) and desmosomes (*D*). Original magnification: ×10000. *Scale bar*: 0.5 μm

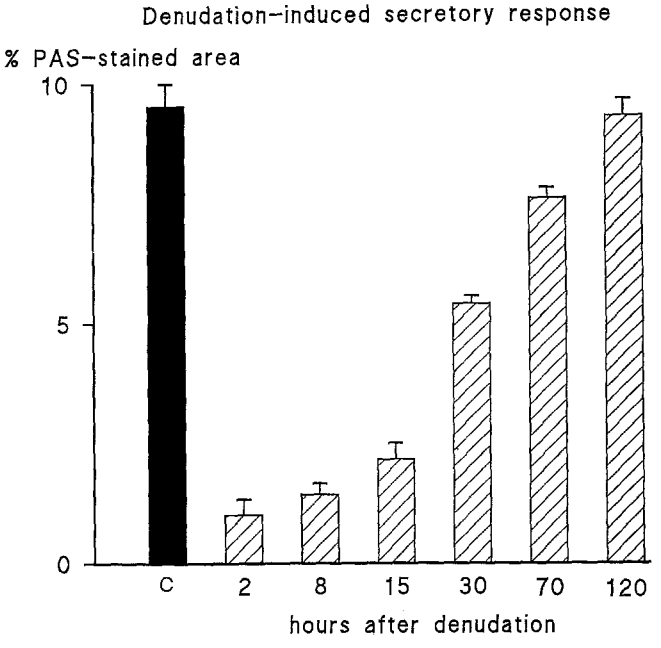

Fig. 7. The proportion (%) of the epithelial area covered by PAS-positive staining in the remaining epithelium during the first 5 days after epithelial removal. The entire tracheal circumference on both sides of the longitudinal denudation zone was analysed. The data are given as means ±SEM (n=9)

the tissue beneath the denuded area. Immunohistochemical visualisation of BrdU combined with toluidine blue staining discerned several elongated BrdU-containing nuclei in cells tentatively identified as fibroblasts and smooth muscle cells.

Reinnervation by intraepithelial nerve fibres

In control tracheal tissue both intraepithelial and subepithelial CGRP-containing nerve fibres were present (Fig. 10a). Epithelial innervation consisted of scattered CGRP-containing fibres, some of which appeared to reach the epithelial surface. Subepithelial CGRP-containing nerve fibres were mainly located in the loose connective tissue. In controls PACAP- and VIP-containing nerve fibres were present in the subepithelial tissue, and were distributed mainly in the smooth muscle region and around subepithelial glands. No intraepithelial, PACAP-containing fibres were observed, and only a few VIP-containing fibres could be detected in the control epithelium.

Fifteen min and 1, 2, 4, 8, and 15 h after deepithelialisation no CGRP-, PACAP- or VIP-containing fibres

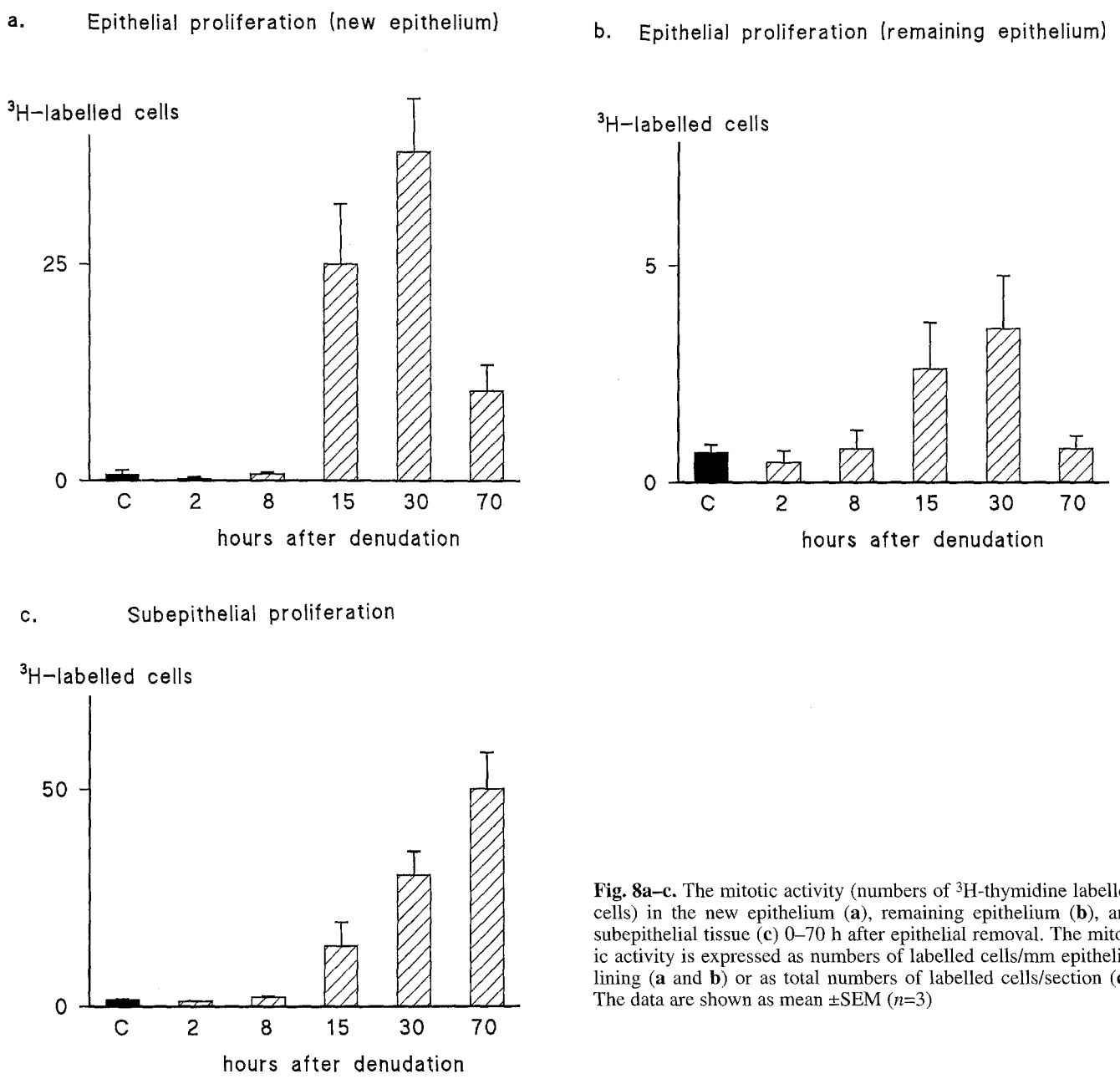

Fig. 8a–c. The mitotic activity (numbers of ^{3}H-thymidine labelled cells) in the new epithelium (**a**), remaining epithelium (**b**), and subepithelial tissue (**c**) 0–70 h after epithelial removal. The mitotic activity is expressed as numbers of labelled cells/mm epithelial lining (**a** and **b**) or as total numbers of labelled cells/section (**c**). The data are shown as mean ±SEM (n=3)

were detected in the new epithelium. However, CGRP-positive nerve fibres were present in the new, undifferentiated epithelium at 30 and 70 h after deepithelialisation (Fig. 10b–c). After 5 days the new, differentiated epithelium displayed a normal distribution of CGRP-containing fibres (Fig. 10d). No PACAP-containing fibres were found in the new epithelium at 30 h or at any later time period. A few scattered, VIP-containing fibres could be detected in the new epithelium at 5 days after deepithelialisation. At all time points examined there was no detectable difference in the distribution pattern of subepithelial CGRP-, PACAP- or VIP-containing fibres.

Discussion

This study describes a novel method whereby the epithelium of a defined zone reproducibly can be removed in guinea-pig trachea in vivo without causing bleeding or damage to the basement membrane. Immediately after epithelial removal epithelial cells at the edge of the denuded zone dedifferentiate, flatten and migrate rapidly to cover the denuded basement membrane. Already after one day the entire new epithelium is tightly sealed and reinnervated, and the mitotic activity is increased. The speed of the reepithelialisation process, particularly the high initial migration rate may be important in airway defence and disease.

Previous studies on in vivo reepithelialisation of tracheal epithelium have used hamsters, rats, rabbits, and dogs (Wilhelm 1953; O'Corell and Beattie 1956; Lane and Gordon 1974; McDowell et al. 1978; Keenan et al. 1982a–c); several important features of the reepithelialisation process have been described, including the observation that the wound is eventually covered by flattened epithelial cells that have migrated from bordering epithe-

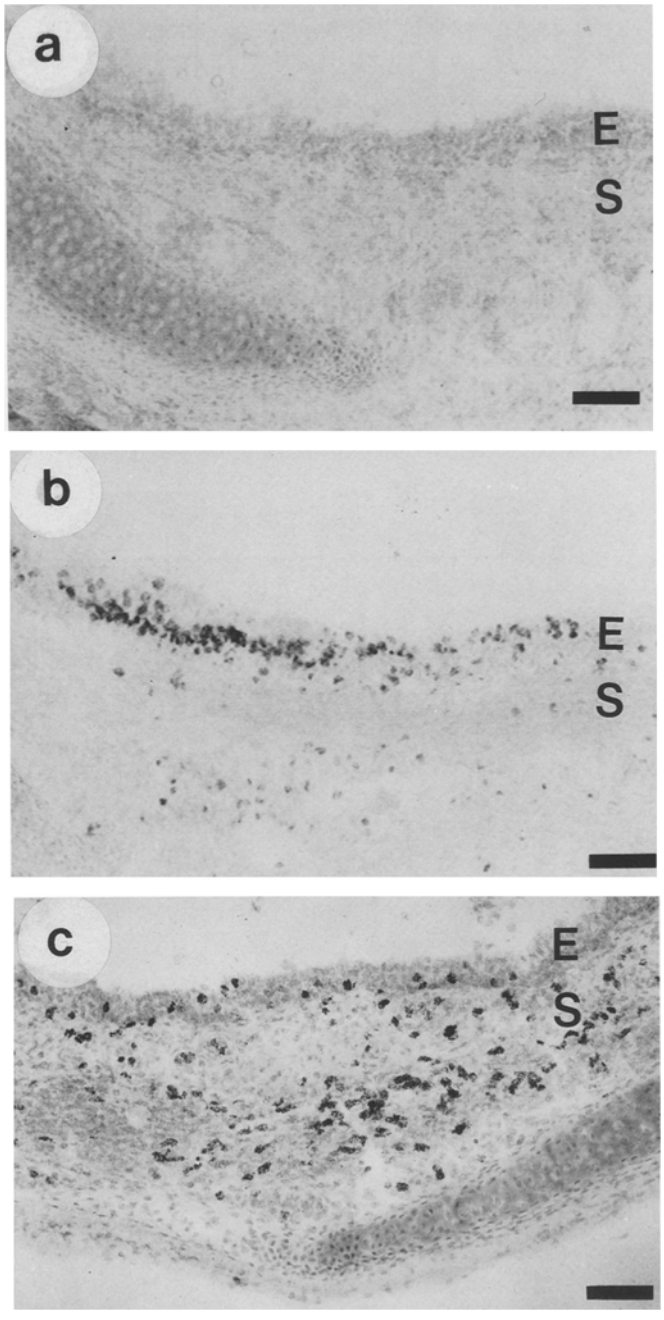

Fig. 9a–c. Autoradiographic visualisation of ^{3}H-thymidine labelled cells in a non-deepithelialised control (**a**) and the denuded area at 30 h (**b**) and 70 h (**c**) after denudation. The mitotic activity is low in the control situation (**a**). At 30 h numerous ^{3}H-thymidine-labelled cells are observed, particularly in the epithelium (**b**). At 70 h most of the mitotic activity is observed in the subepithelial tissue (**c**). *E* Epithelium; *S* subepithelial tissue. Orginal magnification: ×150. *Scale bars*: 50 μm

lium. The previous techniques have produced variable areas of denudation with a non-distinct borderline between denuded and intact tissue. Hence, it has not been possible to study well the early phase of repair. The previous techniques have also caused damage to the basement membrane and local bleeding. Scanning electron and light microscopy indicate that the denuded basement membrane remains intact and that bleeding (bulk extravasation of erythrocytes) does not occur in the present study. The present technique further produces well-defined and sharp edges between intact and denuded zones which is essential for detailed examination of early repair events.

It is evident from the present and previous studies (Wilhelm 1953; Lane and Gordon 1974; Kennan et al. 1982a-b) that the first reepithelialisation of the denuded airway mucosa is via cell migration and not by cell proliferation. Migration of ciliated cells during reepithelialisation in vivo was first reported in the 1950s (O'Corell and Beattie 1956; Wilhelm 1953). Keenan et al. (1982a–c), who more recently examined reepithelialisation in hamster trachea in vivo, argued that secretory cells are the only cell type responsible for migration. The present work gives further evidence that secretory cells (epithelial cells releasing granules) flatten and migrate at the margin of the intact epithelial lining. Moreover, this study demonstrates that ciliated cells also dedifferentiate into the flat poorly differentiated epithelium that migrates. Flattened ciliated cells were thus frequently observed close to the denuded edge. It appears that the cilia are internalised as the cell shape changes from cylindrical to flat. The observation in this study that the proportion of ciliated to secretory cells was not altered at the margin of the old epithelium strengthens our view that secretory and ciliated cells have been equally recruited to the migrating epithelium. Although the isolated basal cells that occasionally were left on the denuded basement membrane did not appear to migrate (this study) basal cells in the remaining epithelium may well have contributed to the present reepithelialisation. The ultrastructural findings in the present study of the new epithelium confirm previous work describing regenerating epithelial cells as "poorly" differentiated, polysome rich, lacking characteristic epithelial organelles such as cilia and secretory granules (Lane and Gordon 1974; Keenan et al. 1983).

The speed of the migrating epithelial cells found in the present study is far greater than that observed in in vitro cell culture models. Zahm et al. (1991), using a wound model of human nasal epithelium in primary culture, have demonstrated epithelial cell migration and closure of small circular wounds (radius=40 μm) within 8 h. Although considered fast, the rate of this in vitro cell migration may be >20 times slower than that observed in the present study. Migration is also exceedingly slow in isolated tracheal preparations where denudation was produced by the same technique as in the present study (Erjefält et al., unpublished observations). Our findings are also in contrast to recent in vivo studies in rats employing traumatic denudation techniques and in which a lag time of at least 1 h before the start of epithelial repair was reported (Shimizu et al. 1994). The high migration speed observed in this study, particularly in the immediate phase, suggests that patchy denuded areas in vivo may be covered by a new migratory epithelium within minutes. When a topical insult in the airways is severe enough to cause epithelial shedding, rapid resealing by migrating cells would thus be an efficient defense mechanism.

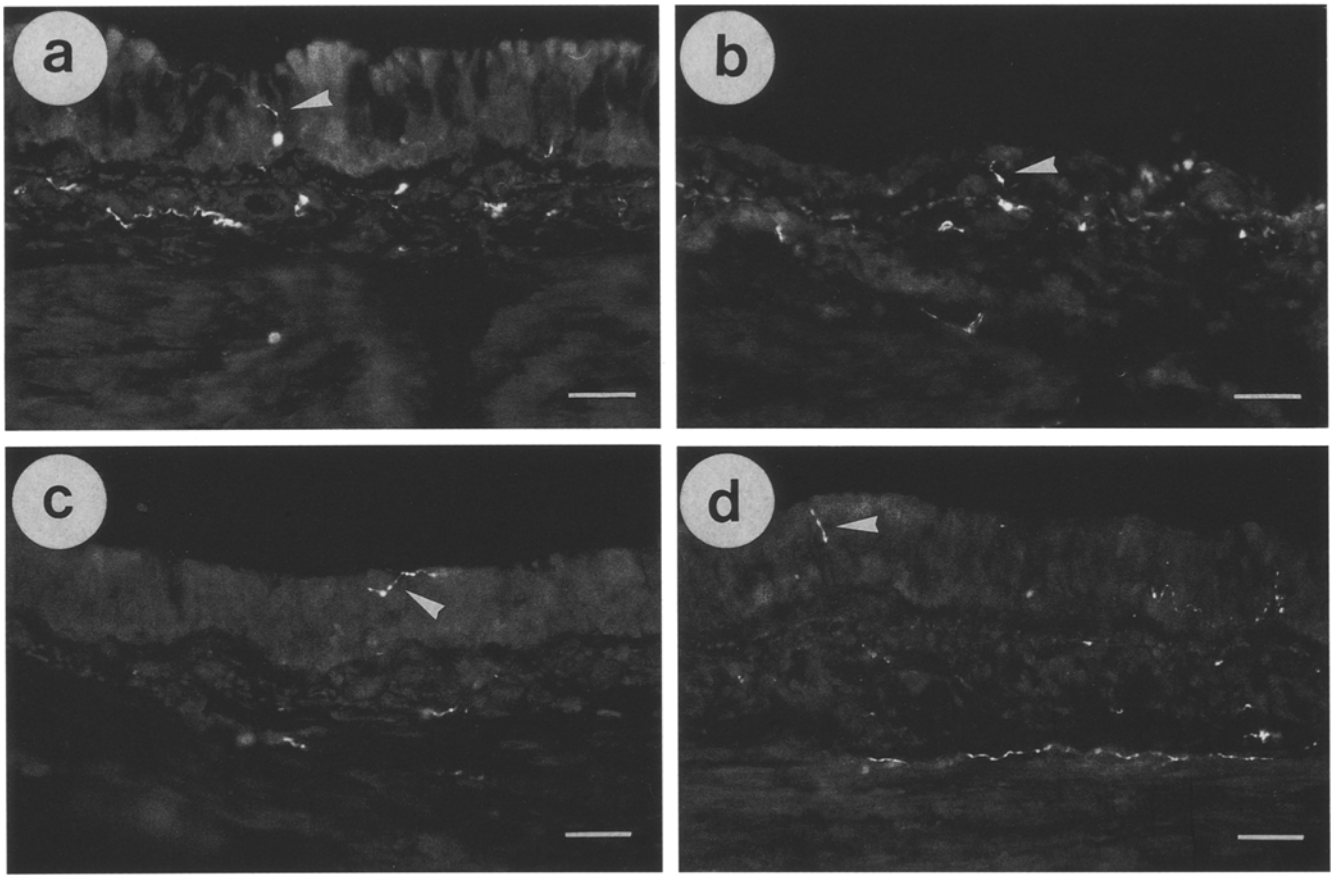

Fig. 10a–d. Fluorescence micrographs demonstrating CGRP-immunoreactive nerve fibres in control trachea (**a**) and 30 h (**b**), 70 h (**c**) and 144 h (**d**) after deepithelialisation. Note that intraepithelial, CGRP-positive fibres (*arrowheads*) are present in the epithelium at all these time points. Original magnification: **a–d** ×300. *Scale bars*: 20 μm

From in vitro studies of migration rates of epithelial cells over different artificial basement membranes it has been inferred that the composition of the normal basement membrane has to be altered, presumably by damage, to yield a matrix suitable for rapid migration (Rickard et al. 1992). However, already in 1953 Wilhelm noted inpassing that a non-disrupted basement membrane was important for a rapid migration of epithelial cells in vivo. We have recently demonstrated that within minutes after epithelial removal bulk plasma is exuded from the underlying microcirculation (Erjefält et al. 1994b), possibly as a consequence of the removal of an epithelial nitric oxide system that tonically suppresses the permeability of the subepithelial microcirculation (Erjefält et al. 1994a). Through a non-bleeding exudative process the denuded and intact basement membrane is rapidly endowed with fibrinogen, fibronectin and other factors emanating from blood plasma (Erjefält et al. 1994b), suggesting that microcirculation-derived factors together with an intact basement membrane may promote a rapid reepithelialisation. Extravasated plasma creates a fibrin-, and fibronectin-rich gel, also rich in infiltrating leukocytes, over the denuded zone (Erjefält et al. 1994b). Thus, the reepithelialisation in this study occurs in close spatial relationship with a protective and active gel.

Increased epithelial secretion is a common response to inhalation of irritants (Dahlgren et al. 1972). Keenan et al. (1982b) reported that mechanical damage to the mucosa, including denudation, produced a secretory response. However, the secretory response to more gentle, shedding-like denudation has not been studied. The marked reduction in PAS-positive mucus staining in the remaining epithelium 2 h after the present deepithelialisation is interpreted to reflect an almost complete mucus discharge from the secretory epithelial cells. Thus, removal of epithelium from a relatively small and restricted area induces an intense secretion in the intact epithelium around the entire luminal circumference (0-0.5 cm from the longitudinal denudation zone). The mechanisms, possibly neural, involved in the spreading of this secretory response remain to be elucidated. This study also demonstrated that PAS-positive mucus cells gradually normalised in numbers during the first 5 days after denudation.

We demonstrate here that epithelial cell proliferation starts at a time (15 h) when the migratory phase is over. Proliferation is particularly high around 30 h after denudation. This is in general agreement with previous reports of mitotic activity during reepithelialisation of mechanically damaged airway surface areas (Lane and Gordon 1974; Keenan et al. 1982a–b). The proliferation

of cells in the regenerating epithelium in vivo is likely to be regulated by a complex mixture of growth factors, derived from different sources. Both fibroblasts and epithelial cells can produce factors that may stimulate growth of airway epithelial cells in vivo (Kelley 1990; Shoji et al. 1990). Leukocytes are present in the deepithelialised area (Erjefält et al. 1994b) and have the capacity to release several growth factors (Bitterman et al. 1983; Takizawa et al. 1990; Kovacs and DiPietro 1994). Furthermore, the plasma exudation response that is induced by this shedding-like denudation (Erjefält et al. 1994b) would continuously supply plasma-derived growth factors at the site of reepithelialisation.

^{3}H-thymidine autoradiography was used during the early phase of this study to quantify proliferating epithelial cells in the denuded zone. When we observed an intense proliferation also in the subepithelial tissue we decided to carry out additional BrdU-labelling permitting counterstaining to more clearly identify these proliferating subepithelial cells. Increased proliferation of smooth muscle cells and fibroblasts has previously been observed in association with airway inflammation in asthma (Roche 1991; Bousquet et al. 1992; Brewster et al. 1993), and increased mitotic activity of the endothelium, smooth muscle and fibroblasts has been reported during the repair of relatively severe wounds in the airways (Keenan et al. 1982a, b). The present observations suggest that also more gentle, shedding-like deepithelialisation can cause proliferation of fibroblasts and smooth muscle cells in the subepithelial tissue. This observation suggests that shedding-restitution processes occurring in airway diseases may cause part of the fibrosis and smooth muscle remodelling that may ensue (Bousquet et al. 1992; Stewart 1993).

The normal guinea-pig tracheal mucosa is innervated by sensory nerve fibres (Luts et al. 1990; Uddman et al. 1991; for review, see also Lundberg et al. 1988). The epithelial innervation is mainly by CGRP-immunoreactive nerve fibres although fibres containing other neuropeptides may contribute (Luts et al. 1990). The present study demonstrates that already 30 h after denudation the new epithelium is reinnervated by CGRP-immunoreactive nerve fibres. Furthermore, the deepithelialisation and the subsequent reepithelialisation may not alter the distribution of subepithelial sensory nerve fibres containing CGRP, VIP and PACAP (this study). The present observations may be compared with previous findings demonstrating reinnervation by vasoactive intestinal peptide (VIP)- and neuropeptide Y (NPY) -immunoreactive fibres within hours after damage to jejunal villi (See et al. 1992) Reinnervation of a damaged pulpa by CGRP-immunoreactive nerve fibres occurred within 48 h after dental extraction and reinsertion (Kvinnsland et al. 1991). In vitro cell culture experiments suggest that CGRP and substance P increase the mitotic activity in guinea-pig airway epithelial cells (Ziche et al. 1990; White et al. 1993). CGRP may also stimulate migration of epithelial cells (Sanghavi et al. 1994). It is not known whether these in vitro mechanisms play a role in vivo. For example, under the present conditions the migration milieu is also endowed with an abundance of other active factors emanating from plasma, leukocytes and structural cells. However, since CGRP appeared in the new epithelium when the mitotic activity was high (this study), it is possible that the present reinnervation has in part contributed to the increased mitotic activity occurring at about 30 h in this study.

There are conflicting reports on the severity of epithelial disruption in inflammatory airway diseases (Laitinen et al. 1993). Increased numbers of columnar epithelial cells have been observed in broncho-alveolar lavage fluids from asthmatic subjects (Beasley et al. 1989, Bousquet et al. 1991), and epithelial denudation in asthmatic bronchial biopsies has been reported (Dunnill 1960; Laitinen et al. 1985). However, other investigators describe no significant differences in epithelial disruption between healthy controls and patients with mild and severe asthma (Lozewics et al. 1990; Caroll et al. 1993). It appears that artefactual loss of epithelium inherent in biopsy techniques has precluded quantitative assessment of the epithelial shedding (Jeffery et al. 1989; Söderberg et al. 1990; Albers and Timens 1993). Indeed, if reepithelialisation in asthmatic bronchi in vivo is as rapid as observed in the present study denudation upon loss of small clusters of epithelial cells may be exceedingly short-lasting.

In conclusion, this study demonstrates that well-defined areas of the airways can be deepithelialised in vivo without damage to the basement membrane. Our findings also demonstrate that epithelial denudation may occur in vivo without bleeding. Airway repithelialisation starts immediately and occurs rapidly through migration of flattened secretory and ciliated cells (and presumably also basal cells). The fast reepithelialisation in the present study might depend on the presence of an intact basement membrane. Furthermore, essential factors from the mucosal microcirculation, the innervation and/or other in vivo specific factors may have contributed. The present shedding-like denudation produces potentially important physiological and cellular effects. In addition to the plasma exudation response there is a marked secretory response, and there is increased mitotic activity not only in the epithelium but also in fibroblasts and smooth muscle cells. The fast and efficient restitution of the epithelium suggests that epithelial shedding may be an important defense mechanism in normal, healthy airways. Our findings further suggest that epithelial shedding by itself may be an inducing stimulus for important facets of the pathophysiology and the remodelling of inflammatory airway diseases.

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