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The cells of the tracheobronchial epithelium of the mouse: a quantitative light and electron microscope study*

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INTRODUCTION

Early observations with the light microscope revealed that the mucous membrane of the mouse lower airway differed from that of other species (Frankenhaeuser, 1879). Frankenhaeuser noted that subepithelial glands were virtually absent and that mucus-containing cells were rare. Whereas this latter observation has been confirmed in several ultrastructural studies of the mouse trachea (Hansell & Moretti, 1969; Hama & Nagata, 1970; Chen & Lin, 1972; Taira & Shibaski, 1978; Pack, Al-Ugaily, Morris & Widdicombe, 1980) and of the bronchioles (Karrer, 1956; Lauweryns, Cokelaere & Boussauw, 1969; Okada, 1969; Petrik & Collet, 1970; Wang, Huang, Sheldon & Thurlbeck, 1970; Etherton, Conning & Corrin, 1973; Stinson & Loosli, 1978), only one electron microscopic study has included the major bronchi (Lauweryns *et al.* 1969); this study was not quantitative. Therefore a systematic comparison of the epithelium at different airway levels has not yet been carried out. In addition, not every study used specific pathogen-free (SPF) animals. The proportions of epithelial cell types are different in SPF and 'stock' rats (Jeffery & Reid, 1975) and mice (Al-Ugaily, Morris, Pack & Widdicombe, 1980), the SPF animals having fewer mucous cells. The scant mucous cells of the mouse occur in increased numbers in the lower trachea, especially at the level of the carina (Korhonen, Halopainen & Paavolainen, 1969; Pack *et al.* 1980), but whether this increase persists in the primary bronchus is unknown.

The present study was carried out to establish the distribution of cell types in the mouse airway and to determine whether cells containing mucus occur in the primary bronchus or smaller airways of the mouse.

METHODS

Seven mice were used, of MFI strain and free of specific pathogens (SPF). Anaesthesia was induced with sodium pentobarbitone (Sagatal, May & Baker; 60 mgm/kg, i.p.). The trachea was then exposed and a shortened 19 gauge syringe needle was tied into the airway immediately below the larynx. The chest was opened by cutting through the abdominal wall and the diaphragm. The lungs were then slowly inflated until they refilled the rib cage (0·6–1·0 ml of fixative). In four mice, neutral buffered formol saline was used and the tissue was taken for light microscopy. In the other

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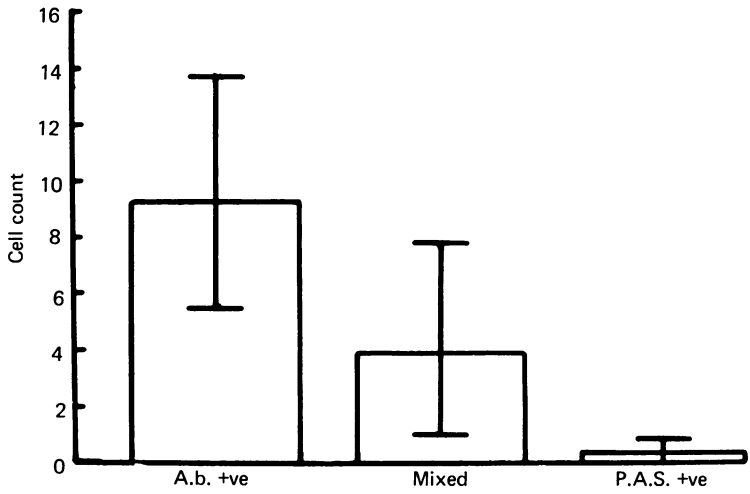


Fig. 1. The relative proportion of cells in the primary bronchus staining with Alcian blue/PAS method. The majority of the cells stained either with Alcian blue or both Alcian blue and PAS indicating acid mucosaccharide. The cell count is the mean number of cells per complete cross section ± S.D.

three mice 3 % glutaraldehyde in Sorensen's phosphate buffer (pH 7·3) was instilled and the tissue was prepared for electron microscopy.

Light microscopy

Following inflation fixation, the lungs and airways were removed and immersed in formol saline for seven days. The tissue was then dissected under fixative and the following four specimens were embedded in paraffin wax: lower trachea, carina and primary (extrapulmonary) bronchus together, main axial bronchus and smaller airways. Transverse, serial, 9 μm thick sections of the entire length of the primary bronchus were cut. Every twelfth section was mounted, stained with Alcian blue (pH 2·6, 30 minutes) and periodic acid–Schiff (PAS) and examined for the presence of positive staining cells or glands. To account for the various lengths of the bronchi, the total length of each primary airway was divided into six equal parts, each part being considered to represent an 'airway level'. Three sections from each level were then selected using random numbers and the cell counts from these were used for further numerical evaluation. Random sections ($n > 6$) of the other tissues were cut, stained in a similar manner, and also examined for glands and mucous (Alcian blue +ve) or serous (PAS +ve) cells.

Electron microscopy

With a separate group of three mice the lungs and airways were removed after inflation with glutaraldehyde and immersed in the fixative at 4 °C for a further 2 hours. The tissue was then dissected under fixative and the same tissues were taken as for light microscopy, except that the carina and main bronchus were separated to reduce the size of the final blocks. The tissues were then post-fixed in 2 % osmium (2 hours, 4 °C), dehydrated in graded ethanols at 4 °C and embedded in Araldite. The location of the distal airway and the orientation of the blocks were achieved by examining 1 μm sections stained with toluidine blue (Mercer, 1963). Thin sections were then cut, mounted on G 200 grids (LKB), stained with alcoholic uranyl acetate

(Stempack & Ward, 1964) and lead citrate (Reynolds, 1963) and viewed in a Philips 301 electron microscope.

The relative numbers of the types of epithelial cell were determined by selecting fields where the epithelium was parallel to a grid bar and counting the numbers of cells between adjacent grid bars, this distance being 85 μm for the grid used. At least twelve such lengths (1·02 mm) were counted for each sample of airway. When, for a given block, it was necessary to count cells in more than one section, 20 μm were removed from the block face between thin sections to avoid biasing the data by recounting the same cells in near adjacent sections.

RESULTS

Light microscopy

Mucous and serous cells were characterized by their being packed with Alcian blue or PAS-positive granules. The present study confirmed previous observations that very few mucus-containing cells occurred in the tracheal epithelium of the mouse, although they were more numerous at the level of the tracheal bifurcation. This increase in the mucous cell count persisted throughout the length (2–3 mm) of the primary bronchus. The count for carina and primary bronchus was $14{\cdot}5 \pm 10{\cdot}6$ (mean and S.D.) mucous cells per complete cross section. However, these cells were absent from the axial bronchus (bronchi less than 0·62 mm diameter) of SPF animals. Examination of both the axial bronchus and trachea of non-SPF animals revealed more mucus-containing cells. Unlike those of the rat (Jeffery & Reid, 1975) the majority of cells in the primary bronchus which stained with Alcian blue/PAS were Alcian blue-positive. This indicates that they contained acid mucosaccharide (Fig.1).

Clara cells were identified at all airway levels by their characteristic apical projection into the lumen (Smith, Heath & Moosavi, 1974). The general reaction of this cell type with the Alcian blue/PAS method was the background 'pale pink' which was also seen in the ciliated cells. As with the ciliated cells, the luminal surface was delineated by an alcianophilic band. Occasionally, PAS-positive granules were seen in the cytoplasm of the Clara cell. In some cells these granules formed a distinct layer immediately below the luminal surface membrane.

Electron microscopy

Three principal cell types were seen in the epithelium of the mouse: basal, ciliated and non-ciliated 'secretory'. Brush cells were also observed, though both these and the basal cells decreased in number in the more distal airways (Table 1). Both the basal cells and the ciliated cells were generally similar to those of other species (Breeze, Wheeldon & Pirie, 1976; Breeze & Wheeldon, 1977; Jeffery & Reid, 1977). The epithelium was reduced in thickness in the more distal airways, the ciliated cells being more cuboidal. The non-ciliated cells were also reduced in height but to a lesser extent than were the ciliated cells, and hence the luminal projection of the Clara cells was more marked in the distal airways (Fig. 2). Both the ciliated and non-ciliated cells were linked near the luminal surface by tight junctions. Throughout the depth of the epithelium there were complex microvilli-like interdigitations between adjacent cells, and desmosomes were occasionally seen.

The non-ciliated cells

The electron microscope observations confirmed that mucus-containing cells occurred in largest numbers at the levels of the tracheal bifurcation and the primary

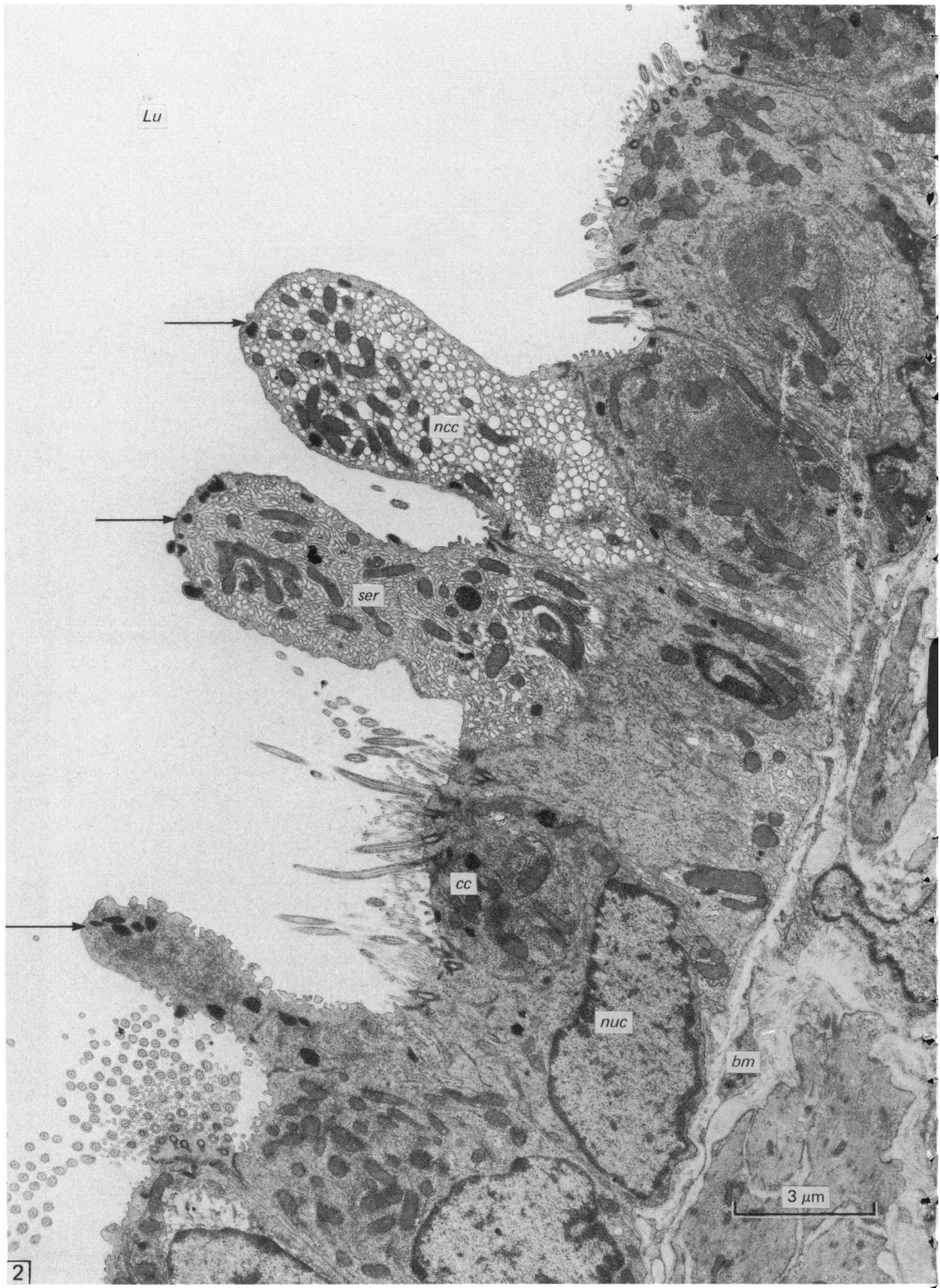


Fig. 2. Clara cells in the mouse primary bronchus. Both ciliated cells (*cc*) and projecting, non-ciliated Clara cells (*ncc*) can be seen. Three different forms of Clara cell (→) occur in a single field of view. *bm*, basement membrane; *Lu*, lumen; *nuc*, nucleus; *ser*, smooth endoplasmic reticulum.

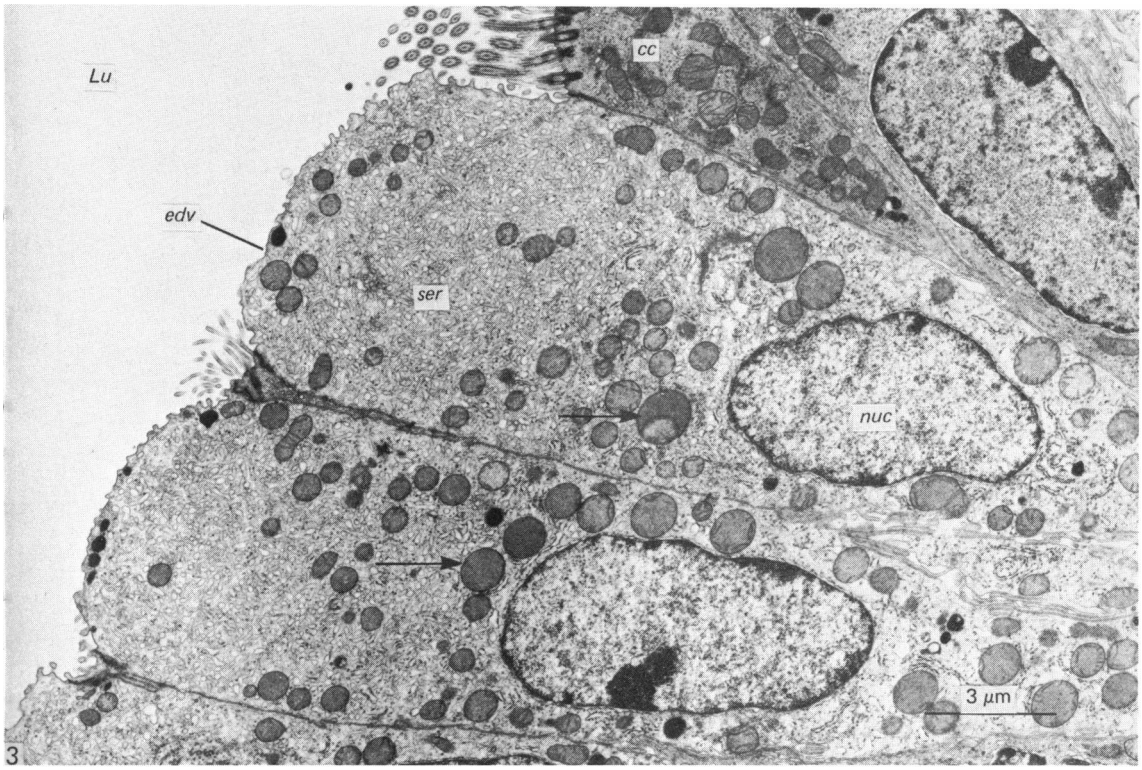


Fig. 3. The most frequent form of Clara cell, The nucleus (*nuc*) is basal and the greater portion of the cell is filled with smooth endoplasmic reticulum (*ser*). Both mitochondria-like bodies (→) and electron-dense vesicles (*edv*) can be seen. *cc*, cilitated cell; *Lu*, lumen.

bronchus. None was found in the intrapulmonary (axial) airways. The majority of non-ciliated cells resembled Clara cells in fine structure (Fig. 2).

On the basis of differences in their cytoplasmic inclusions, three distinct forms of Clara cells were recognized:

(1) The commonest Clara cell (Fig. 3) had a cytoplasm of moderate electron density. The nucleus was basal, had a variable complexity of form and frequently contained a nucleolus. The apical portion of the cell invariably projected into the lumen of the airways and was packed with well organized arrays of smooth endoplasmic reticulum (Fig. 3). In addition to normal mitochondria, mitochondria-like bodies were invariably encountered (Figs. 3, 6, 10). These had few or no cristae and were frequently surrounded by whorls of endoplasmic reticulum. In most cells, electron-dense 'serous type' secretory vesicles were also located, most regularly in the apical portion of the cell. On occasion these occurred as a band immediately below the luminal membrane (Figs. 2, 3, 10). The luminal projection of the cell usually bore numerous short microvilli with glycocalyx-like border. Where this projection was large, however, these microvilli were absent (Fig. 2).

(2) Non-ciliated 'Clara-like' cells (Fig. 4) were also observed which resembled the 'Type B' cells described by Evans, Gabrel-Anderson & Freeman (1978). The cytoplasm was electron-dense and contained many free ribosomes. The nucleus was similar in form to that of the more common Clara cell. The luminal surface of the cell invariably bore microvilli, although the apical projection was not always pronounced.

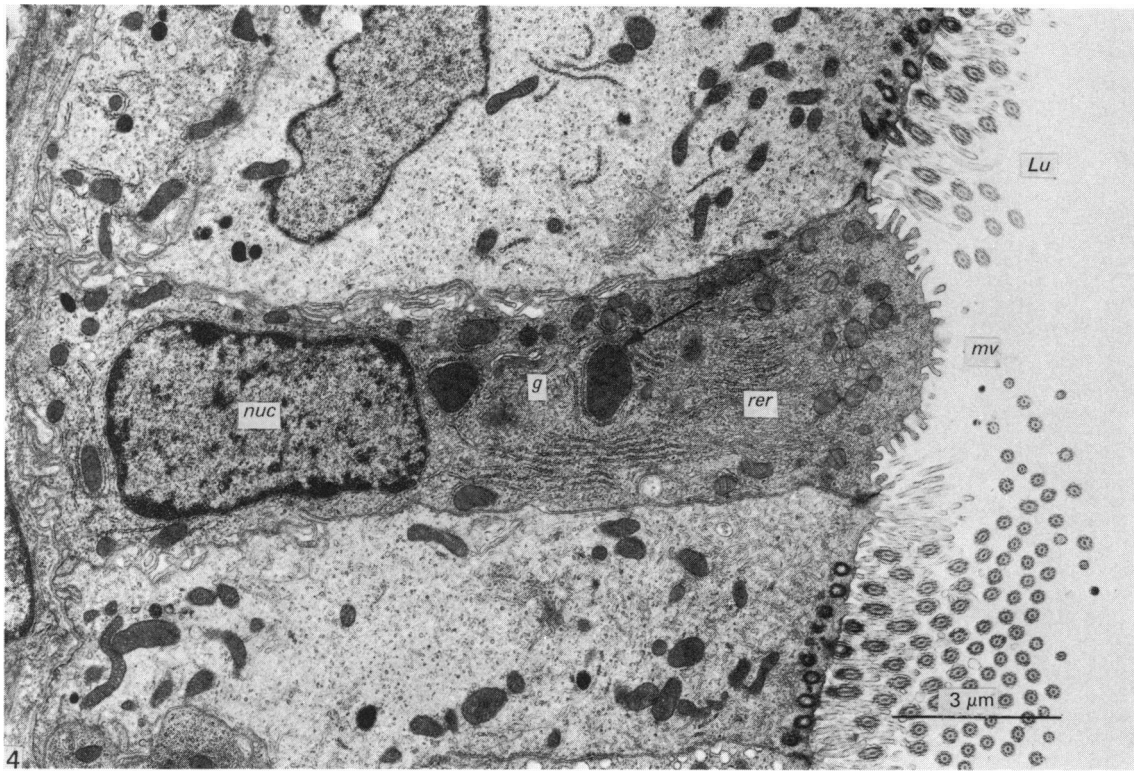


Fig. 4. A Type 2 non-ciliated Clara cell. This cell does not project into the lumen (*Lu*) of the airway. The nucleus (*nuc*) is basal and the apical portion of the cell is filled with rough endoplasmic reticulum (*rer*) which surrounds the mitochondria-like bodies (→), *g*, Golgi body; *mv*, microvilli.

In these cells, smooth endoplasmic reticulum was limited, although a pronounced Golgi body was invariably present. Large areas of lamellar or whorled rough endoplasmic reticulum were seen, usually in the apical portion of the cytoplasm; this frequently surrounded the 'mitochondria-like bodies' of the cell (Fig. 4). In some cells, electron-dense secretory vesicles were located, though not in those which did not project into the airway lumen (Fig. 4).

(3) In the third type of non-ciliated cell the superficial portion of the cell or, on occasion, the whole cell was vesiculated (Fig. 5). A faint matrix was occasionally visible within the vacuoles, possibly representing the vacuoles' content. The cell frequently projected into the airway lumen, and microvilli were usually rare. Both mitochondria-like bodies and normal mitochondria occurred throughout the cell; these normal mitochondria, together with the appearance of adjacent cells (e.g. Fig. 2), seem to eliminate the possibility of this vesiculated appearance being a fixation artifact. Serous-type vesicles were also observed in some cells. The nucleus was complex and frequently polymorphic, and on occasion paired centrioles were present. Although there were many free ribosomes, little rough endoplasmic reticulum was seen. A well organized Golgi body was a regular feature and both lysosome-like structures and multivesicular bodies were also seen. Areas of small vesicles were present in most cells (Fig. 5).

In addition to these three clearly defined types, cells which appeared as transi-

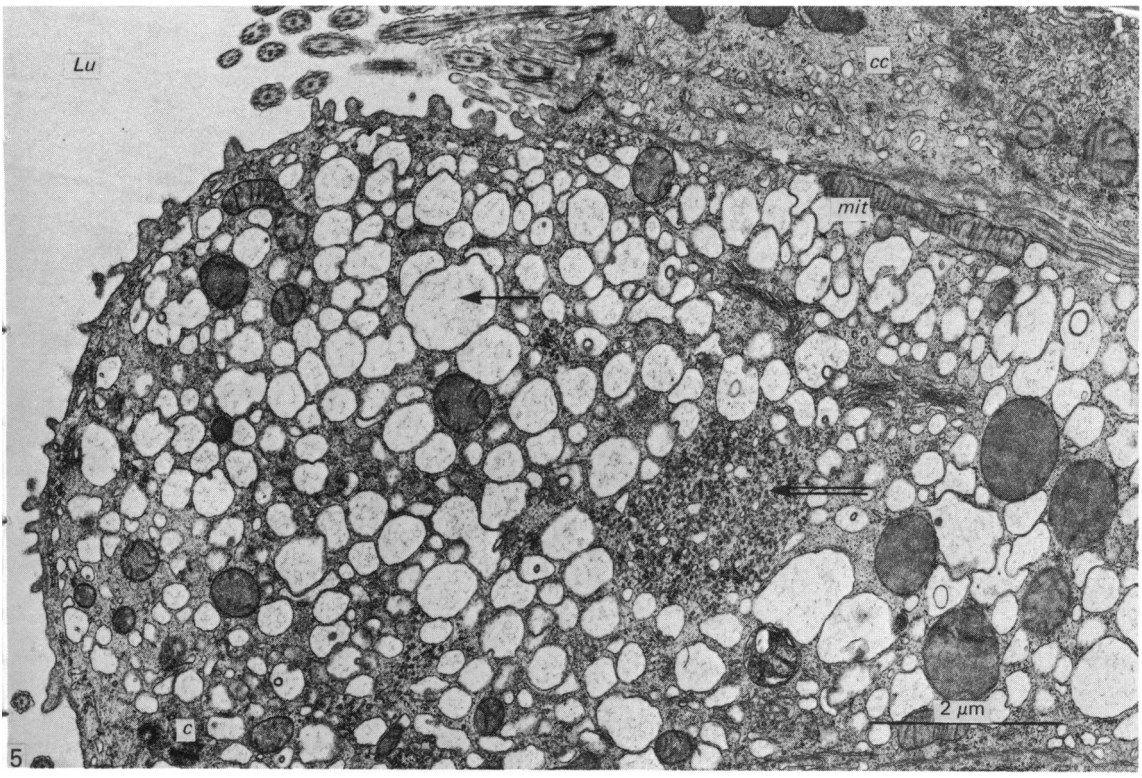


Fig. 5. A vesiculated Clara cell. The cytoplasm of the cell is filled with vacuoles containing a faint matrix (←). The normal mitochondria (*mit*) and normal morphology of an adjacent ciliated cell (*cc*) indicate that this appearance is not a fixation artifact. A dense array of small vesicles (⇐) is a common feature of this cell type. *c*, centrioles; *Lu*, lumen.

tional forms were regularly observed. For example, in some cells the smooth endoplasmic reticulum seemed to be breaking down to form vesiculated areas (Fig. 6). This may represent the process of transition from a Type 1 cell to Type 3. The continuously variable content of smooth and rough endoplasmic reticulum may further indicate a transition from Type 2 to Type 1. Occasionally, cells were observed which, whilst having the characteristic morphology of Clara cells, contained scattered mucous granules or ciliary bases (Fig. 7). These, too, may represent transitional forms. A small proportion of the non-ciliated cells did not fit clearly into any of the above classes, having some features both of Clara cells and of the serous cells of the rat. These are listed separately as non-ciliated in Table 1.

The electron-dense 'serous type' vesicles frequently appeared to be in close apposition to the luminal surface or projecting through the cell membrane (Figs. 8, 9). Such profiles suggested extrusion of the electron-dense vesicles by a merocrine secretory process. In section, apocrine secretion was indicated by extension of the luminal cytoplasmic projection of the Clara cell, loss of microvilli and a narrowing of the region between the luminal projection and the perikaryon of the cell (Fig. 10). Many cytoplasmic organelles were contained within the apical portion of these actively secreting cells.

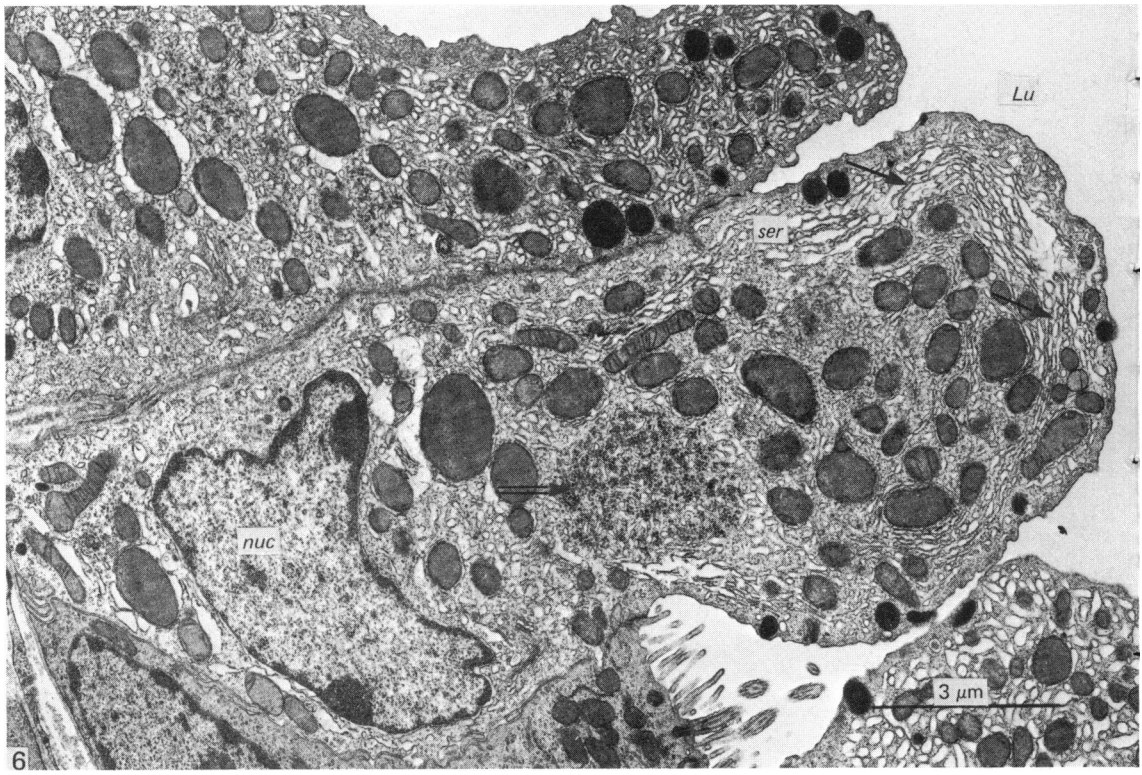


Fig. 6. A transitional Clara cell. In this cell the smooth endoplasmic reticulum (*ser*) appears to be breaking down to form vacuoles (→). A cluster of small vesicles is located in a supranuclear position (⇒), a feature not seen in normal Clara cells. *Lu*, lumen; *nuc*, nucleus.

During the course of this and previous studies we have examined with the electron microscope over 3·3 cm of murine airway epithelium. With the exception of the larynx, we have yet to locate a free nerve fibre within the epithelium, although on occasion nerves were seen immediately below the epithelial basement membrane.

DISCUSSION

The airway levels investigated in the present study were similar to those which have been studied in the rat (Jeffery & Reid, 1975). In the rat the most frequent non-ciliated cell of the larger airways was the serous cell, with its large areas of rough endoplasmic reticulum and 600 nm rounded, electron-dense granules. In the mouse, however, the principal cell type is morphologically identical to the Clara cell of the distal airways (Baskerville, 1970; Basset, Poirer, Le Crom & Turaif, 1971; Kilburn, 1974), with its luminal cytoplasmic projection filled with smooth endoplasmic reticulum, abnormal mitochondria and smaller (90–400 nm) irregular electron-dense granules. On the basis of the apparent absence of mucous and serous cells in the mouse trachea, it has been suggested that this cell is the source of respiratory mucus in the mouse (Hansell & Moretti, 1969).

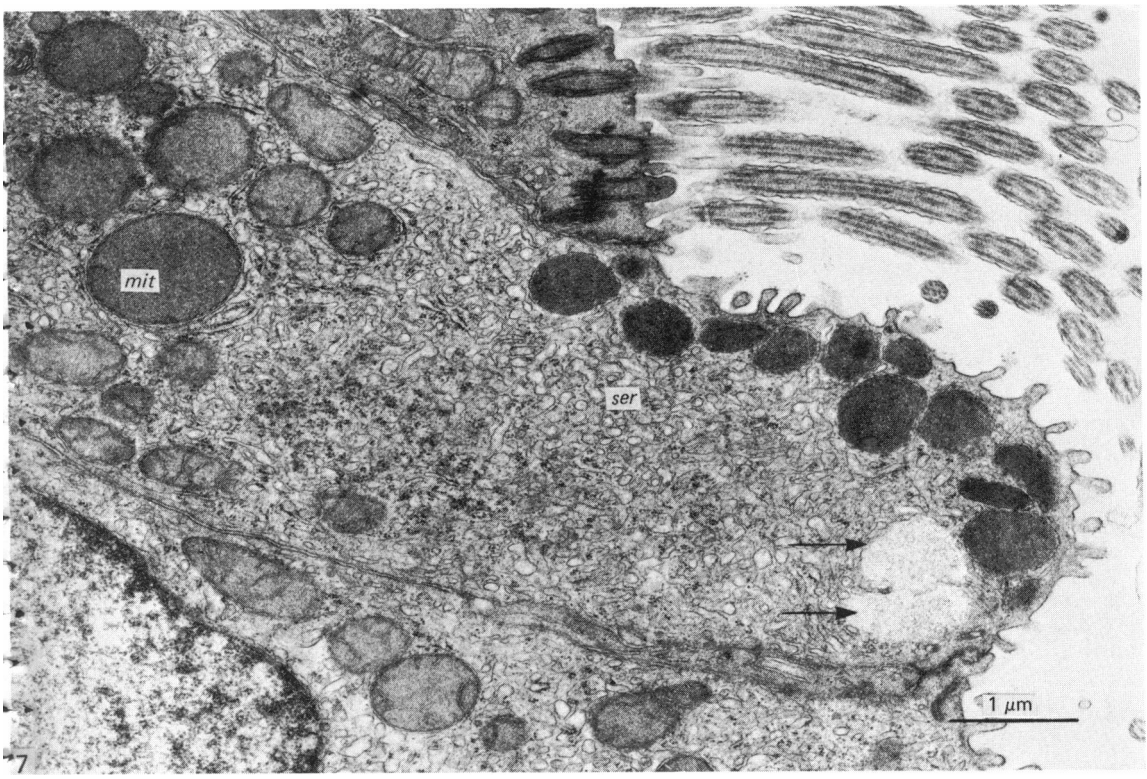


Fig. 7. A Clara cell containing mucous granules. This cell has the characteristic mitochondria-like bodies (*mit*) and smooth endoplasmic reticulum (*ser*) of a Clara cell. Mucous granules (→) are located in the apical portion of the cell.

Sources of mucins in the mouse airways

Respiratory mucus can arise from several distinct anatomical sources. These include the subepithelial glands, the goblet and serous cells of the epithelium itself and the Alcian blue-positive layer lining the luminal surface of the airway (Jeffery, 1978). It has also been suggested that the Clara cells may produce a watery lining layer, the hypophase (Roth, 1973; Petrik & Collet, 1974). In the mouse, submucosal glands are restricted to the larynx and the most rostral trachea, extending to 1·6 mm down from the larynx. The absence of glands and the near absence of mucus-containing cells from the trachea have prompted speculation as to the source of its lining secretion (Hansell & Moretti, 1969; Pack *et al.* 1980). The consistent occurrence of goblet cells at the carina (Korhonen *et al.* 1969; Pack *et al.* 1980; Al-Ugaily *et al.* 1980) and primary bronchus (this study) possibly aids our understanding of the mechanisms of the mouse mucous membrane. Mucociliary transport would presumably move the mucins produced by these cells rostrally, the usual direction for ciliary transport in the airway (Van As & Webster, 1972). It is, therefore, possible that most of the mucins in the mouse trachea could arise in the carina and primary bronchi and 'flush' the trachea from below.

Whilst these goblet cells in the extrapulmonary bronchi enable one to envisage the production of a mucus sheet or plaques in the trachea and primary bronchi, it is unlikely that they protect the more distal airways. However, in such a small animal any inhaled matter may normally be precipitated on the mucus sheet of the small

Table 1. *The number of cells per mm at different levels of airway. Pooled data from three animals, 1·02 mm of epithelium being examined for each animal*

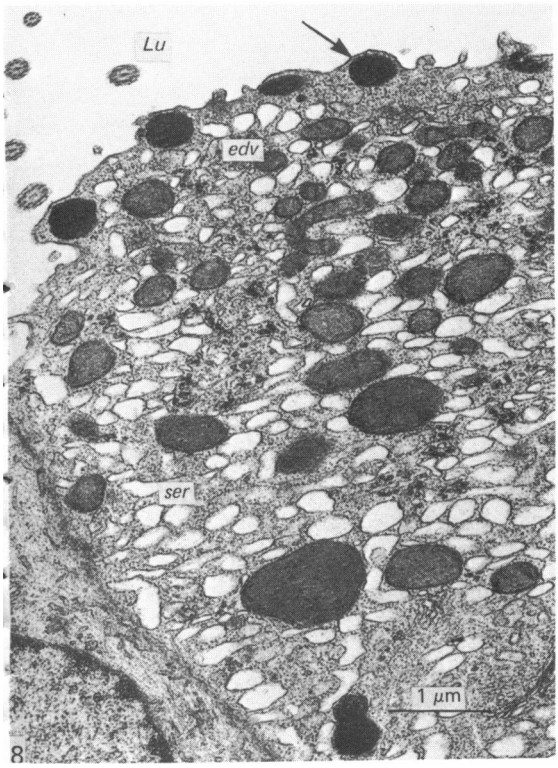
Airway level	Ciliated	Clara	Mucus-containing	Non-ciliated	Brush	Basal
Lower trachea	83·6	121·6	—	5·2	4·9	17·9
Carina	69·3	102·3	0·33	3·3	3·6	11·8
Primary bronchus	103·6	100·3	3·6	4·6	0·33	7·8
Axial bronchus	72·2	123·5	—	4·2	0·33	2·3
Distal airway	50·9	140·5	—	3·9	—	2·3

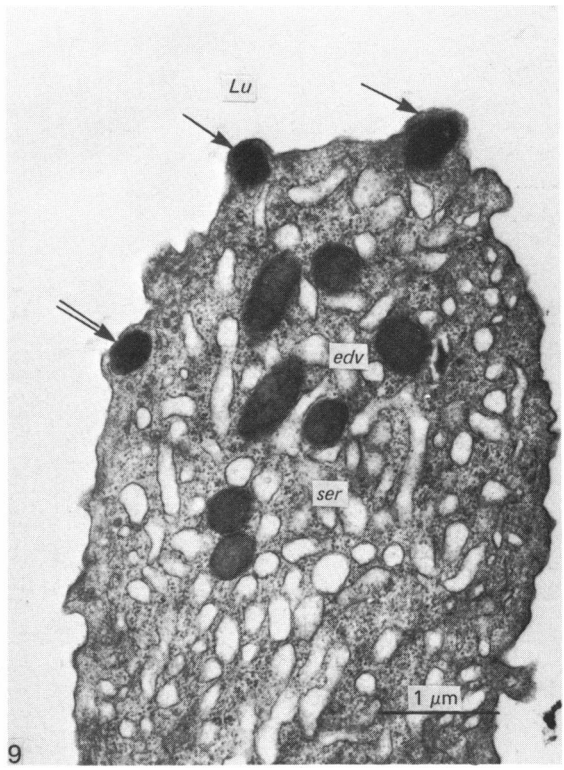
extrapulmonary airways before it reaches the lung lobules. Pertinent to this is the observation that the paucity of mucous cells in the trachea and primary bronchi, and their absence from the axial airways, were observed only in SPF animals. When 'stock' mice were examined some mucus-containing cells were observed at every airway level. This result may suggest that the airway irritation, to which non-barrier maintained animals are normally exposed, causes goblet cell metaplasia in airways usually devoid of these cells. This parallels the situation in many human diseases, where goblet cell metaplasia in small airways is a prime or exacerbating factor (Kilburn, 1974). With this possibility in mind, we suggest that the airway of the mouse is an ideal model with which to study the Clara cell and its role in health and disease.

The Clara cells

Clara cells are located at every airway level in the mouse from the distal airway to the larynx. In addition, the 'secretory (goblet)' cells described by Matulionis & Parks (1972) in the murine nasal septum are morphologically very similar to Clara cells. In the trachea and primary bronchi the 'characteristic luminal projection' (Smith *et al.* 1974) is less pronounced. This, however, is largely due to the more columnar nature of the ciliated cells of the trachea and bronchi. The size and form of the Clara cells themselves change little at different airway levels.

There is much discord in the literature as to the reaction of Clara cells to mucus stains, particularly PAS. It is generally accepted that Clara cells do not stain with Alcian blue, aldehyde fuchsin or mucicarmine and, therefore, do not contain acid mucosaccharide; we have confirmed this observation for the Clara cells of the murine airways. Some authors have demonstrated granules in Clara cells with PAS (Azzopardi & Thurlbeck, 1969; Roth, 1973), or its electron microscopic equivalent PATO (Cutz & Conen, 1971); other workers did not confirm this (Niden, 1967; Kuhn, Callaway & Askin, 1974). We observed PAS-positive granules in a small proportion of Clara cells. These granules were either in a supranuclear position or lying as a distinct layer immediately below the luminal cell membrane. It is interesting to note that the electron-dense vesicles, as seen with the electron microscope, also occurred in a similar location. By analogy with the rat serous cells, which contain electron-dense granules and are also PAS-positive (Jeffery & Reid, 1975), the electron-dense granules of the Clara cell may well be the PAS-positive granules revealed by light microscopy. Morphological studies have indicated that these electron-dense granules may be lost from the cell by a process of merocrine secretion (Stinson & Loosli, 1978; Al-Ugaily *et al.* 1980; Pack *et al.* 1980). Thus, by extrapolation, the mouse Clara cell may normally secrete a PAS-positive material into the airway lumen.





Figs. 8–9. Evidence for merocrine secretion. In some Clara cells the electron-dense vesicles (*edv*) appear to be in the process of extrusion into the lumen of the airway (→). On occasion the luminal membrane appears ruptured above the vesicle (⇒). *Lu*, lumen; *ser*, smooth endoplasmic reticulum.

Several studies (Etherton *et al.* 1973; Etherton, Purchase & Corrin, 1979; Smith *et al.* 1974; Pack *et al.* 1980) have suggested that Clara cells may also undergo apocrine secretion. Profiles were observed in section where the 'apical cap' of the Clara cell appeared to be budding from the body of the cell. It has been proposed that this 'apical cytoplasmic bleb' is a fixation artifact (Jeffery & Reid, 1975). Recent studies, using several preservation techniques, indicate that this is not the case, and that these profiles may genuinely represent apocrine secretion (Etherton *et al.* 1979). In addition to electron-dense vesicles, these cytoplasmic extensions or 'apocrine droplets', also contain mitochondria and large areas of smooth endoplasmic reticulum; the secretory product entering the airway may therefore be complex and derived from several cytoplasmic structures (Etherton *et al.* 1973), which may subsequently undergo dissolution (Smith *et al.* 1974). There is, to date, no direct experimental evidence as to the nature of the secretory product of Clara cells; merocrine and apocrine release may well produce different products.

Evans *et al.* (1978) noted types of non-ciliated cell in the distal airways of rats which, on the basis of autoradiographical studies using nucleic acid precursors, they concluded were developmental forms of Clara cell. We noted similar forms in the various airway levels of the mouse (Type 2). In addition, however, we observed a Clara-like cell which contained many large vacuoles. The normal appearance of the

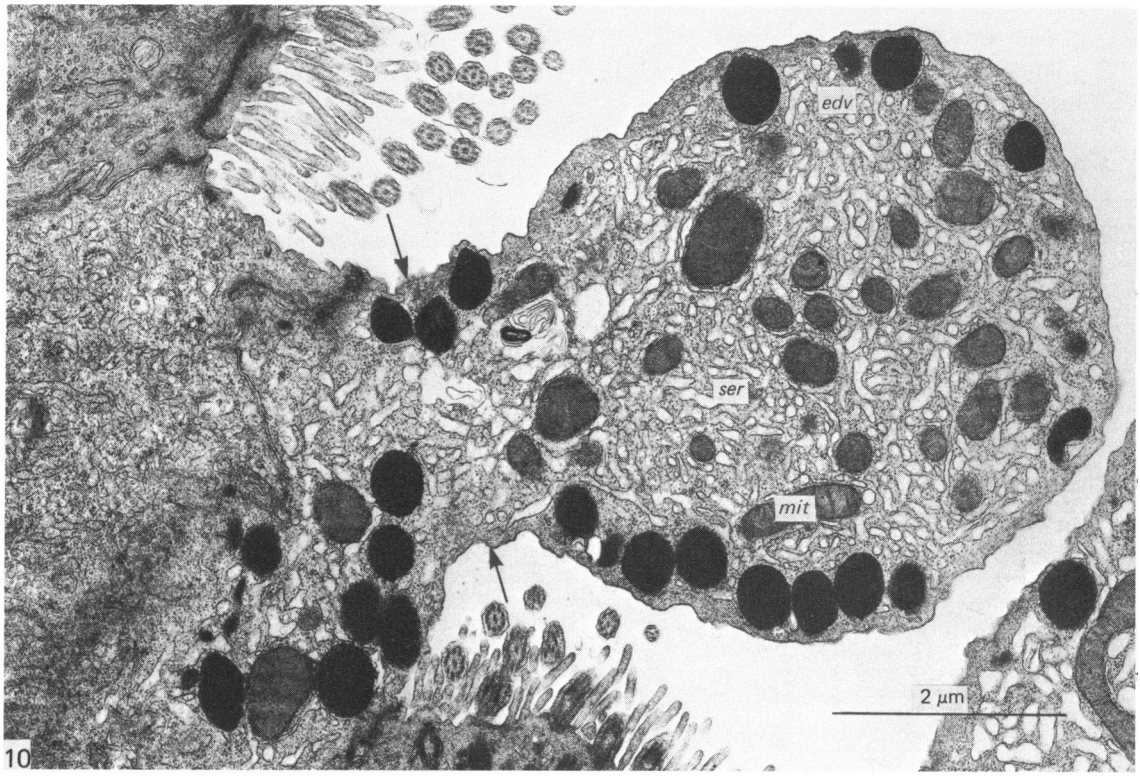


Fig. 10. Evidence for apocrine secretion. In some cases where the luminal projection of the cell was extensive there was a narrowing of the cell at the level of the epithelial surface (→). This suggests that the apical portion of the cell is in the process of being shed. *edv*, electron-dense vesicles; *mit*, mitochondria; *ser*, smooth endoplasmic reticulum.

other cytoplasmic inclusions within these cells and the morphology of adjacent Clara-like cells (Fig. 2) indicate that this vacuolated appearance is not a fixation artifact. Similar vacuoles have been seen in both the mouse (Lauweryns *et al.* 1969; Fig. 4) and the hypoxic rat (Smith *et al.* 1974; Fig. 15). The general appearance of these cells suggests that they represent an involutionary phase in Clara cell turnover.

Clara cells were sometimes observed with a few mucous granules in their apical cytoplasm; cells with Clara cell-like cytoplasmic inclusions bearing cilia were also seen (Pack *et al.* 1980). Thus Clara cells may undergo metamorphosis to become either ciliated (Evans *et al.* 1978) or, especially in the respiratory epithelium of the murine nose (Matulionis & Parks, 1972), mucous cells. The Clara cell, therefore, may have a role to play in the generation of other cell types.

Epithelial nerves

Epithelial nerves have been located in the trachea and primary bronchi of several species of experimental animal (Jeffery & Reid, 1973; Das, Jeffery & Widdicombe, 1978), and it has been suggested that they may be 'irritant' receptors (Widdicombe, 1976) responsible for initiating the cough reflex. We have failed to observe such epithelial nerves in the lower airways of the mouse, except once in the larynx (Pack *et al.* 1980). This result may correlate with the observation that mice do not cough

in response to mechanical stimulation of the airway mucosa, although an 'expiration reflex' may be elicited from the larynx (Korpas & Kalocsayova, 1975).

The profusion of Clara cells, paucity of mucous cells and absence of epithelial nerves and submucosal glands are all features associated with the distal airways of most species. In the mouse, however, this characteristic bronchiolar morphology extends throughout the tracheobronchial tree. This feature may be related to the airway diameter and the unique problems of potential mucus accumulation in small diameter airways to which this species may be subjected.

SUMMARY

The epithelium of the conducting airways of the mouse consists of a single layer of cells. The number, type and form of these cells have been investigated at five airway levels from the trachea to the distal conducting bronchi with both light and electron microscopes. Contrary to what is found in other species, the majority (50–60 %) of cells in the murine airway epithelium are Clara cells. Mucus-producing tissue was infrequent throughout the airways, though epithelial mucous cells occurred in increased numbers at the carina and in the primary bronchus. No mucous or serous cells or submucosal glands were seen in intralobular airways.

On a morphological basis, three distinct forms of Clara cell were recognized. On occasion, cells were observed which were apparently transitional types between these and also between Clara cells and mucous or ciliated cells. It is suggested that the 'transforming' cells may indicate a role for the Clara cell as a developmental cell involved in the epithelial cell turnover. Evidence is also provided that Clara cells may undergo both apocrine and merocrine secretion and, it is argued that the latter may be of a PAS +ve material.

Free nerve endings were not seen in the epithelium. This may be related to the restricted ability of mice to cough. It is suggested that the lack of mucus-producing tissue and of cough reflex may be due to the small diameter of the mouse airways.

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