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Postnatal development of the epithelium of larynx and trachea in the rat: scanning electron microscopy

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INTRODUCTION

The fine structure of the epithelium in the larynx and trachea has been described in detail in a variety of mammals with transmission (Rhodin & Dalhamn, 1956; Rhodin, 1959; Watson & Brinkman, 1964; Rhodin, 1966; Frasca *et al.* 1968; Hansell & Moretti, 1969; Hama & Nagata, 1970; Jeffery & Reid, 1975) or combined transmission and scanning electron microscopy (Dahlgren, Dalen & Dalhamn, 1972; Andrews, 1974; Castleman, Dungworth & Tyler, 1974; Alexander, *et al.* 1975). However, much less is known about the changes that occur in the morphology of this epithelium between birth and maturity. It is therefore the purpose of this paper to describe, with scanning electron microscopy complemented by light microscopy, the changes that occur in the appearance of the epithelium of the larynx and trachea of the rat from birth onwards.

MATERIALS AND METHODS

The extrapulmonary tracts of 28 Porton albino Wistar rats were used in this study. Nineteen were prepared for scanning electron microscopy. These comprised 11 specimens obtained on consecutive days after birth from litter mates, and 8 specimens from another litter, of which 5 were taken at weekly intervals from the second to sixth weeks and the remainder at 5 months. Nine were processed for light microscopy. Four were short term (litter mates with ages of 1, 6, 8, 12 days) and five long term (ages 2, 8, 10, 14, 16 weeks) specimens. A preliminary investigation was performed with scanning electron microscopy on 34 rats with ages ranging from 1 day to 21 weeks, and with light microscopy on 5 rats aged from 5 days to adulthood, to establish areas in need of further investigation and to refine tissue preparation techniques.

Preparation for scanning electron microscopy

Under anaesthesia induced by subcutaneous sodium pentobarbitone, the respiratory tract was exposed and the distance between the larynx and carina was measured. The larynx, trachea and portions of both main bronchi were rapidly excised. The trachea was pinned to a thin rectangular slice of cork, and was flushed through with 0·1 M sodium cacodylate. It was then placed in a modified Karnovsky fixative (Karnovsky, 1965), consisting of 2 % paraformaldehyde and 3 % glutaraldehyde in 0·1 M sodium cacodylate, for 24–48 hours at 4 °C. The tissue, detached from the

cork, was washed in 0·1 M sodium cacodylate and post-fixed in 1 % osmium tetroxide in 0·1 M sodium cacodylate. After this the tissue was again washed in 0·1 M sodium cacodylate and placed overnight in 70 % ethanol at 4 °C. It was then cut into 4–5 mm lengths which were further sliced into rectangular segments 1–2 mm wide. These were placed into individual, numbered glass vials partly filled with 70 % ethanol. The method of Pihl & Bahr (1970) for critical point drying of mitochondria was adapted to these larger specimens from this stage as follows: the tissues were dehydrated in successive changes of 30, 60, 95 and 100 % ethane diol (ethylene glycol), each of 10–12 minutes. A further period of 7–10 minutes in a second change of ethane diol was followed by two changes each of 12–15 minutes in the intermediate fluid, 100 % cellosolve (2 ethoxy-ethanol, ethylene glycol monoethyl ether). The specimens were then transferred into the numbered compartments of a tiered tissue container immersed in cellosolve. The tissue container, rapidly drained of excess cellosolve, was placed in the chamber of the critical point drier. This was sealed and infiltrated with the transitional fluid, liquid carbon dioxide. After critical point drying (Anderson, 1951) the tissues were mounted on to 'Cambridge' specimen studs with silver dag (Muir & Rampley, 1969), coated under vacuum with carbon and gold, and observed at an accelerating voltage of 20 kV in a Siemens Autoscan microscope.

Preparation for light microscopy

All short term, and three of the long term, light microscopic tissues were initially dissected, measured and pinned as for scanning electron microscopy. However, the fixative used was 10 % buffered formol saline and the period of fixation was of 1–2 weeks' duration. The two remaining long term preparations were prepared by alternative methods. In the first, the animal was perfused systemically through the left ventricle of the heart with isotonic saline followed by 10 % buffered formol saline and simultaneously exposed to the same fixative syringed into the tracheal lumen. After perfusion, this material was then treated like the other light microscopic material prepared by aqueous fixation. In the second, the trachea, immediately after its removal, was cut into short loops which were quick-frozen in liquid propane and then further processed by freeze–drying and vapour fixation according to the method of Tock & Pearse (1965). This material was embedded in high melting point paraffin wax (Gurr) and cut serially at 10 μm. Those specimens not processed by the latter method were, after fixation, sliced into convenient segments, dehydrated with ethanol, cleared in chloroform and embedded in paraplast. Serial sections were cut transversely at 4 or 5 μm and longitudinally at 7 μm.

All serial sections were divided into groups of three. The first was stained with haematoxylin and eosin. The second was treated with periodic acid-Schiff (PAS) and alcian blue (Mowry, 1963) to distinguish neutral (PAS) and acidic (alcian blue) mucosubstances. The third was stained with aldehyde fuchsin and alcian blue (Spicer & Meyer, 1960) combined with a counterstain described by Halmi (1952) to differentiate the sulphated (aldehyde fuchsin) and carboxylated (alcian blue) subgroups of the acidic mucosubstances.

RESULTS

Definition of electron response

On critical appraisal of scanning electron micrographs it was apparent that different cells and different regions of the same cell exhibited varying degrees of brightness, or as it will be termed henceforth, 'electron response'. The electron response of a given specimen site is directly related to the number of secondary electrons produced by the impingement of high energy primary electrons on to the central area of that site. This, in turn, is for a given accelerating voltage, determined by the density of the metallic coating and the incident angle of the primary beam. For convenience, electron response was designated as high, medium and low (Fig. 2).

The development of the epithelium

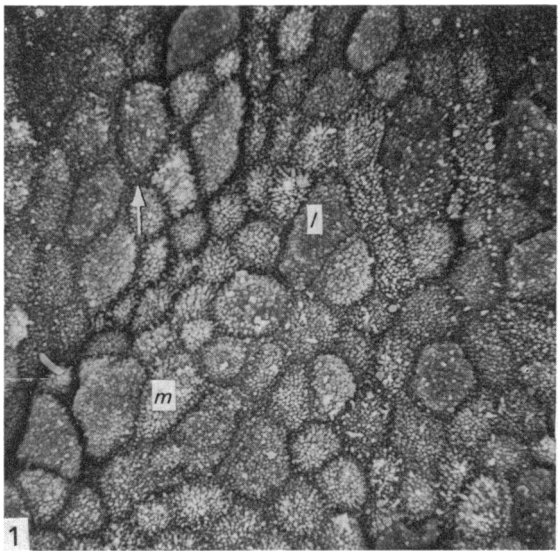
Early postnatal period: scanning electron microscopy

During the early postnatal period, that is in the initial 3 weeks after birth, certain structural features distinguished the epithelium of the various anatomical divisions of the upper respiratory tract.

The *laryngeal* zone, situated above the first tracheal cartilage, showed an uneven and sometimes undulating topography (Fig. 1). The *cartilaginous* and *intercartilaginous* zones of the trachea which respectively overlaid, and alternated between, the cartilage rings had a remarkably flattened appearance (Fig. 2). The *membranous* zone, on the posterior aspect of the trachea, was well demarcated from other areas; it was irregular and marked by occasional proximo-distal depressions (Fig. 3).

Four main cell types were distinguished in the epithelium during this period. The commonest was a medium electron responsive cell with spheroidal or elongated microvilli and dimensions between 3×5 μm and 5×10 μm (Figs. 1–3). In the laryngeal and membranous regions the outline of this cell was polygonal, rounded or spindle-shaped and the apical surface was domed, whilst in the cartilaginous and intercartilaginous areas, the cell perimeter was distinctly polygonal and the upper surface was flattened. In all zones, except the membranous zone, large groups of these cells were sometimes observed with a central, rounded, high electron responsive protrusion 0·5–1·5 μm wide, consisting of an aggregate of microvilli 0·13 μm in diameter and with occasionally a central, elongated projection of similar diameter (Fig. 4). Medium electron responsive cells with a single or 'primary' cilium (Sorokin, 1968) 0·2 μm in diameter and of variable length were usually dispersed within such groups, but also occurred at random throughout the upper respiratory epithelium.

A second cell type, not previously described in the literature, was observed in numbers comparable to those of ciliated cells (Figs. 1–2, 4–7). This cell was larger than other cells of the epithelium (4×5 μm to 10×30 μm) and presented an irregular or polygonal apical surface which had a low electron response. The microvilli were often sparsely distributed and they were variable in diameter (0·05–0·15 μm). This cell occurred in all areas of the respiratory tract examined, but was most easily identified in the anterior and lateral aspects of the trachea either singly, in groups of several cells, or in larger aggregates of up to nine cells which were invariably situated in the intercartilaginous spaces. Regional differences in the cell outline and apical surface occurred, comparable to those seen in the non-ciliated cell. A small proportion of these cells displayed a central high electron responsive microvillous protrusion similar to that seen on medium electron responsive cells, a primary



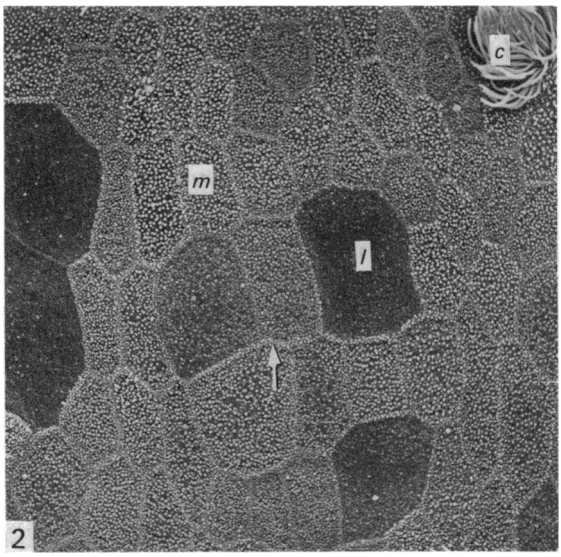
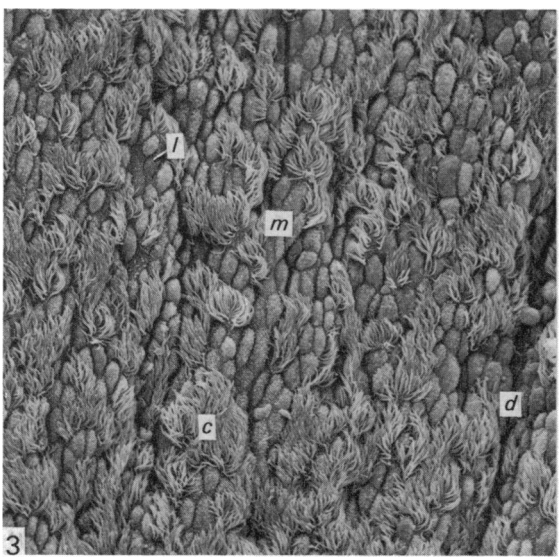


Fig. 1. Scanning electron micrograph of the laryngeal zone during the early postnatal period. Medium electron responsive cells (*m*) outnumber low electron responsive cells (*l*). Cell border (↑). Age: 2 days. ×2000.

Fig. 2. Scanning electron micrograph depicting the typical appearance of the cartilaginous and intercartilaginous regions during the early postnatal period. Medium electron responsive cells (*m*) predominate whilst low electron responsive (*l*) and ciliated cells (*c*) occur with less frequency. The cilia show a high electron response. Cell border (↑). Age: 5 days. ×2000.



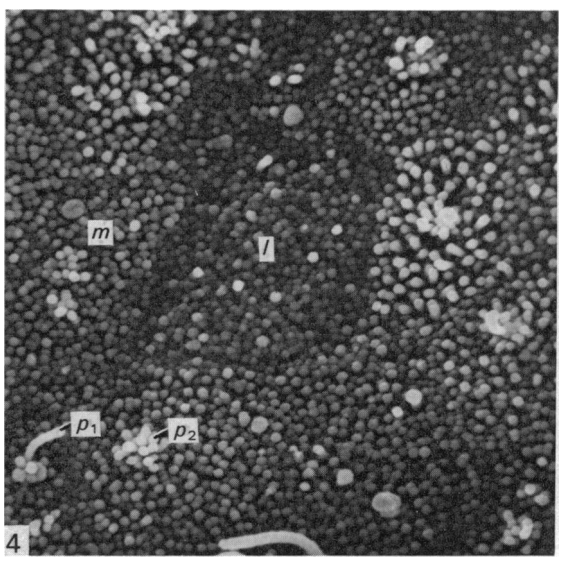
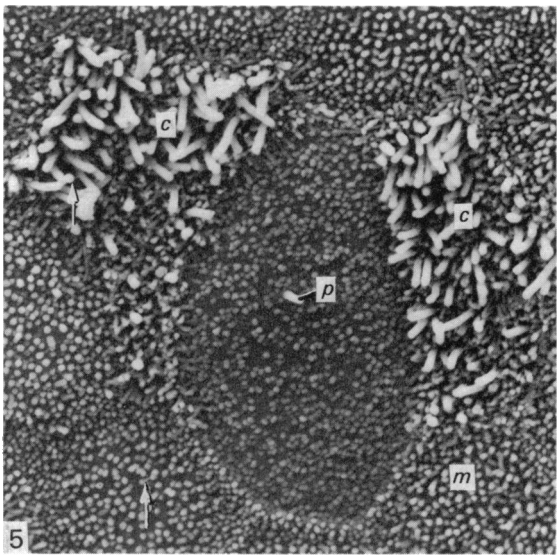


Fig. 3. Scanning electron micrograph of the membranous zone during the early postnatal period. The irregular epithelium consists of similar numbers of ciliated (*c*) and non-ciliated, medium (*m*) and low (*l*) electron responsive cells. Epithelial depression (*d*). Age: 2 days. ×800.

Fig. 4. Scanning electron micrograph of the early postnatal period in which distinct microvillous protrusions (p_2), sometimes associated with a longer, solitary projection (p_1) are evident on the medium electron responsive cells (*m*). Low electron responsive cell (*l*). Age: 2 days. ×6000.



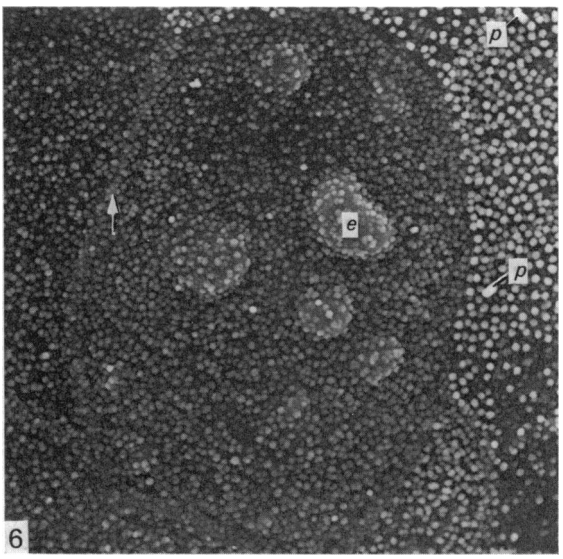
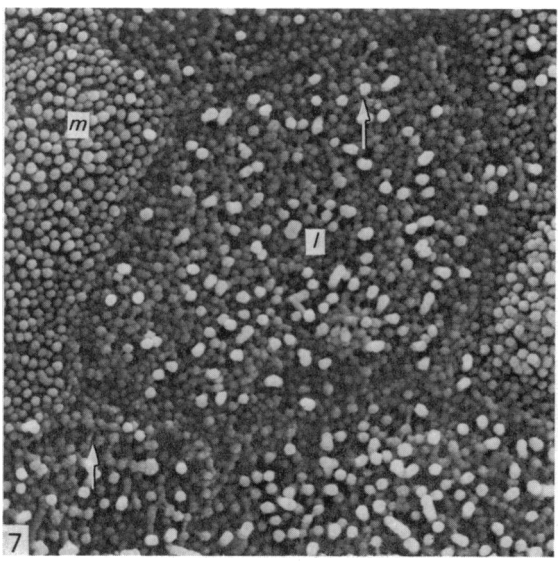


Fig. 5. Scanning electron micrograph of the early postnatal period showing a low electron responsive cell with a central primary cilium (*p*) flanked by two cells undergoing ciliogenesis (*c*). Medium electron responsive cell (*m*). Filamentous strands (↑). Age: 4 days. × 5200.

Fig. 6. Scanning electron micrograph of the circular to oval prominences (*e*) which occasionally occurred on the low electron responsive cells of the early postnatal period. Primary cilium on medium electron responsive cell (*p*). Cell border (↑). Age: 5 days. × 6400.



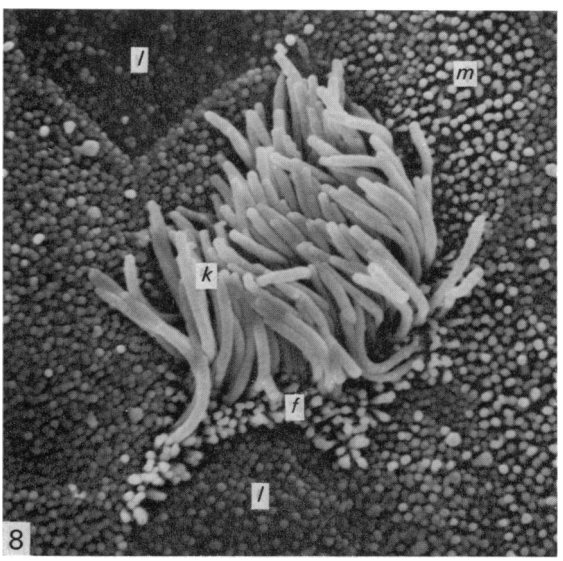
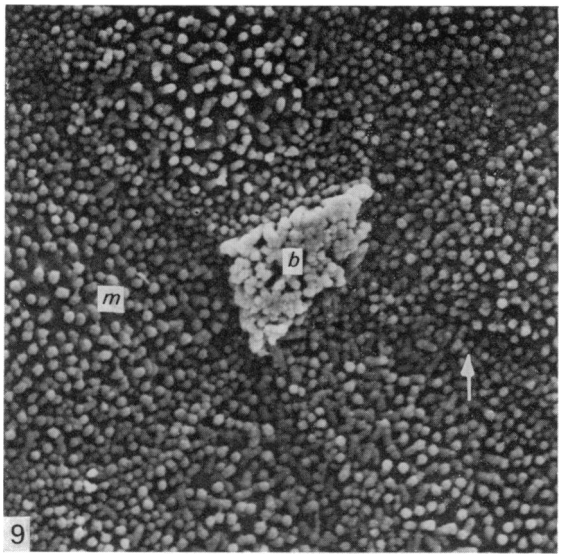


Fig. 7. Scanning electron micrograph of a low electron responsive cell (*l*) with a diffuse covering of high electron responsive microvilli during the early postnatal period. Medium electron responsive cell (*m*). Filamentous strands (↑). Age: 2 days. × 8000.

Fig. 8. Scanning electron micrograph of a ciliated cell of the early postnatal period surrounded by medium (*m*) and low (*l*) responsive cells. Cilia (*k*). Microvilli of ciliated cell (*f*). Age: 9 days. × 6000.



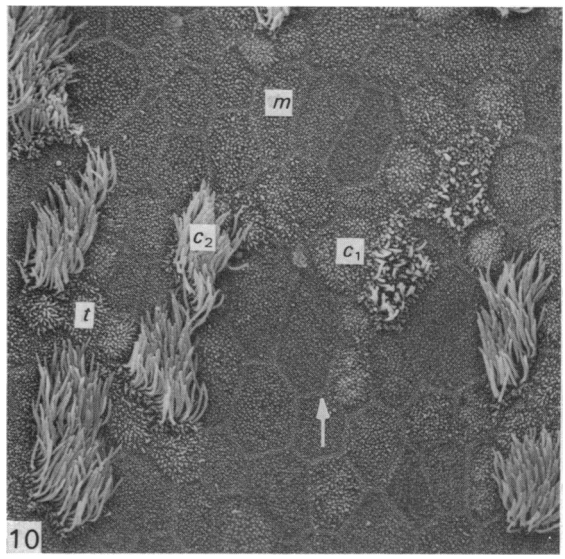
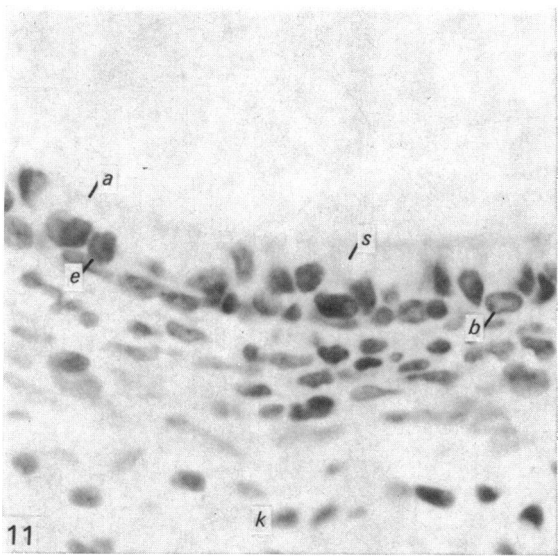


Fig. 9. Scanning electron micrograph of the brush cell (*b*) of the early postnatal period occurring singly among medium electron responsive cells (*m*). Cell border (↑). Age: 4 days. ×8000.

Fig. 10. Scanning electron micrograph of the cartilaginous and inter-cartilaginous zones in the latter part of the early postnatal period. Immature (c_1) and mature (c_2) ciliated cells and the transitional type of non-ciliated cell (*t*) occur less frequently than medium electron responsive cells (*m*). Cell border (↑). Age: 2 weeks. ×2000.



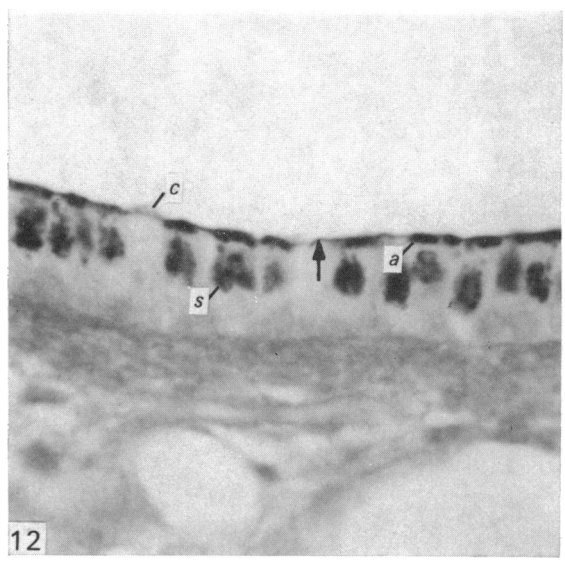


Fig. 11. Light micrograph of the early postnatal epithelium showing basophilic (*b*) and eosinophilic (*e*) nuclei, pale staining supranuclear zones (*s*) and moderate subapical staining (*a*). Cartilage (*k*). Age: 1 day. Stain: haematoxylin and eosin. ×960.

Fig. 12. Light micrograph of the early postnatal epithelium stained for acidic and neutral mucosubstances. Secretory cells react at the supranuclear (*s*) and subapical (*a*) regions. The intracellular contents of the ciliated cell (*c*) are non-reactive. The glycocalyx (↑) is stained with alcian blue. Age: 1 day. Stain: PAS–alcian blue. ×960.

cilium (Fig. 5), one or more spheroidal prominences 1–3 μm in diameter (Fig. 6), or else a diffuse distribution of high electron responsive microvilli (Fig. 7).

The fully developed ciliated cell was polygonal in outline and populated by numerous cilia 0·2 μm in diameter interspersed with characteristic, elongated microvilli consisting of a low electron responsive stem and a high electron responsive, spheroidal termination (Fig. 8). The immature ciliated cell displayed cilia-like projections of a variable length and number. Ciliated cells were variously distributed. In the membranous region these cells occurred with uniform density among equal numbers of non-ciliated cells, whilst in the other zones the proportion of ciliated cells increased from anterior to posterior and from proximal to distal. Thus, in the laryngeal region, ciliated cells were infrequent and occurred singly, but were numerous and arranged in longitudinal linear bands in the cartilaginous and intercartilaginous areas nearer the carina.

The distinctive brush cell was the least common cell during the early postnatal period. Rounded or angular in shape, and small (2×3 μm to 3×4 μm), this cell was singly distributed in the cartilaginous and intercartilaginous zones. Its apical surface consisted of a thick covering of elongated projections, or 'brushes', of uniformly high electron response and 0·15 μm in diameter (Fig. 9). From its initial appearance on the second postnatal day the numbers of this cell increased as development progressed. This cell was not observed in either the laryngeal or membranous regions during the early postnatal period.

During the second week the next phase of development in the epithelium was heralded by the appearance of conspicuous domed cells, 3×4 μm to 4×5 μm in size, with numerous elongated high electron responsive microvilli and they were often situated adjacent to ciliated cells in the cartilaginous and intercartilaginous areas (Fig. 10).

Early postnatal period: light microscopy

The epithelium showed a changing pattern. On the first postnatal day, it was cuboidal to columnar and 10–15 μm in height, and the cells were predominantly non-ciliated (Figs. 11–13). Nuclei occurred in one or two basal layers, and occasionally also near the upper margin of the epithelium. It was found, incidentally, that the stains used distinguished two types of nuclei. Those in the superficial layer were shown by eosin and the orange G of the Halmi counterstain, to belong to secretory cells. Those in the deeper layer were stained by haematoxylin and aldehyde fuchsin. The secretory cells displayed three main intracellular compartments: the apical third was strongly eosinophilic, displayed diffuse and granular reactivity for neutral mucosubstances, and had an upper margin crowned by a fringe of acidic (and sometimes neutral) mucosubstances. The middle third, which often had a concave upper limit, showed a pale, vesicular eosinophilia and contained abundant granules of neutral and acidic (mainly carboxylated) muco-substances. The basal third was occupied by the nucleus.

By the sixth postnatal day, and beyond, the epithelium was reduced to a height of 8–10 μm. The nuclei were more random in distribution and lost their differential staining, except in the membranous region where it lingered for a short period. The secretory cells were similar to those of the first postnatal day, but more cells reacted for neutral mucosubstances. This appearance altered little until the mature period.

Mature period: scanning electron microscopy

Beyond the third postnatal week all zones of the upper respiratory tract differed from the early postnatal period in the proportions of the various cell types and in the morphology of non-ciliated cells. Two broad divisions, the *early* and *late phases* were recognized.

Early phase: The appearance of the laryngeal zone became, in the main, indistinguishable from that of the cartilaginous and intercartilaginous areas (Fig. 14). Preponderant were non-ciliated cells which included, for the first time in epithelial development, the mucin-containing cells usually associated with adult epithelium, and brush cells.

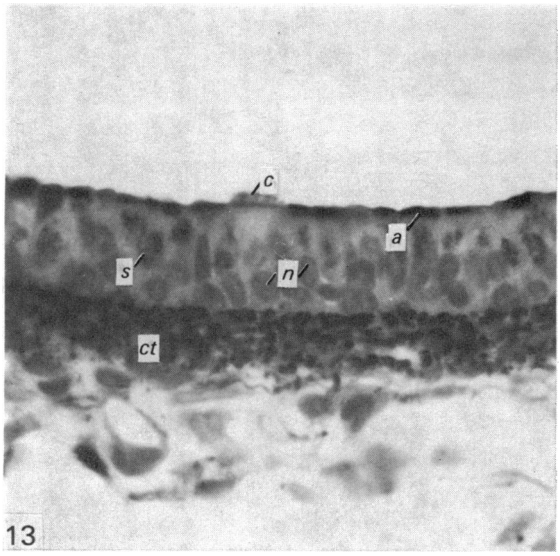
The mucin-containing cells, seen at various stages of accumulation of secretory products, were polygonal, oval or circular, between 3×5 μm and 5×7 μm in size, with domed apical surfaces populated by various combinations of elongated and spheroidal high electron responsive microvilli, and smooth, rounded, granular protrusions 0·5–1 μm in diameter. The brush cells, more numerous than in the early postnatal period, also differed in the morphology of their brushes, which were of a low to medium, rather than of a high electron response (Figs. 14, 15). These cells were 2×2 μm to 2×3 μm in size and often showed adherent, multiple, rough-surfaced granules, 0·2–1 μm in diameter, not regularly found on other cells of the respiratory epithelium (Fig. 15).

On occasion, within a cartilaginous area, large irregular epithelial patches with few ciliated or brush cells occurred: they exhibited numerous flattened cells with a low electron response and dimensions of 4×7 μm to 8×12 μm, interspersed with groups of smaller, medium electron responsive cells with dimensions between 3×5 μm and 4×7 μm (Fig. 16).

Least change was seen in the membranous zone. Intersecting longitudinal corrugations had become prominent and divided this region into epithelial folds which, in general, appeared to contain a lesser proportion of ciliated cells than in the early postnatal period (Fig. 17).

Late phase: The late phase demonstrated various pathways of epithelial maturation. As in the early phase, the normal epithelium consisted of the three main cell types: ciliated, mucin-containing and brush cells. However, on the basis of the variation in the ratio of ciliated to non-ciliated cells, four types of epithelium were distinguished in the laryngeal, cartilaginous and intercartilaginous areas. The first, which was most prominent in the specimens examined, contained a preponderance of ciliated cells, forming a carpet punctuated by an occasional non-ciliated cell (Fig. 18). The second was again dominated by ciliated cells, but non-ciliated cells occurred in larger numbers and brush cells were common among these (Fig. 19). The third consisted of nearly equal numbers of ciliated and non-ciliated cells. Most of the non-ciliated cells were mucin-containing cells active in the synthesis and storage of granules, and the remainder were brush cells (Fig. 20). The fourth type of epithelium was found adjacent to irregular areas of squamous metaplasia characterized by large, flat, low electron responsive cells, some with remnants of cilia, and occasional asymmetrical domed cells. Mucin-containing and brush cells far outnumbered ciliated cells, on which the cilia showed a variable electron response ranging from low to high levels (Fig. 21).

Corrugations persisted in many areas of the membranous zone but the composition of the epithelium, as in the other zones, was variable. Most often, all visible



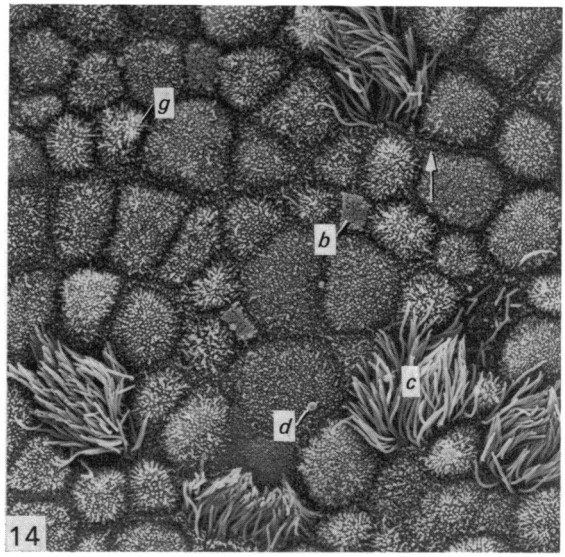
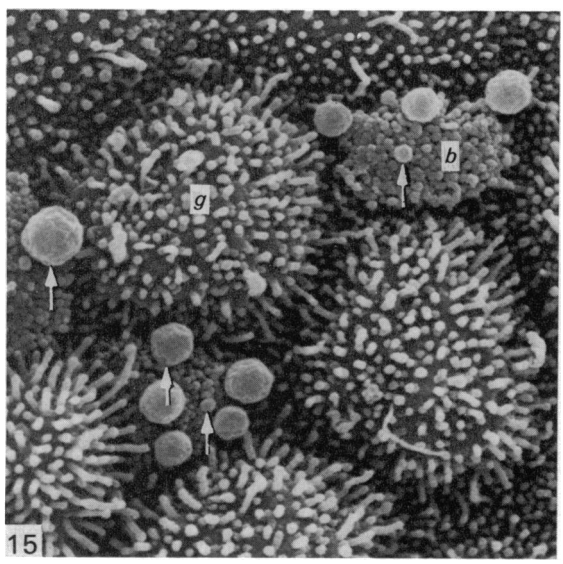


Fig. 13. Light micrograph of the early postnatal epithelium stained for acidic mucosubstances. As in Fig. 12 supranuclear (*s*) and subapical (*a*) regions show a positive reaction whilst ciliated cells (*c*) do not react. A layer of connective tissue (*ct*) with an intense reaction for aldehyde fuchsin underlies the epithelium. The two types of staining in the nuclei (*n*) cannot be distinguished. Age: 1 day. Stain: aldehyde fuchsin–alcian blue, Halmi counterstain. ×960.

Fig. 14. Scanning electron micrograph of the typical epithelium of the laryngeal, cartilaginous and intercartilaginous regions during the early phase of the mature period. Ciliated (*c*), mucin-containing (*g*) and brush (*b*) cells are present. Mucous granule (*d*). Cell border (↑). Age: 5 weeks. ×2000.



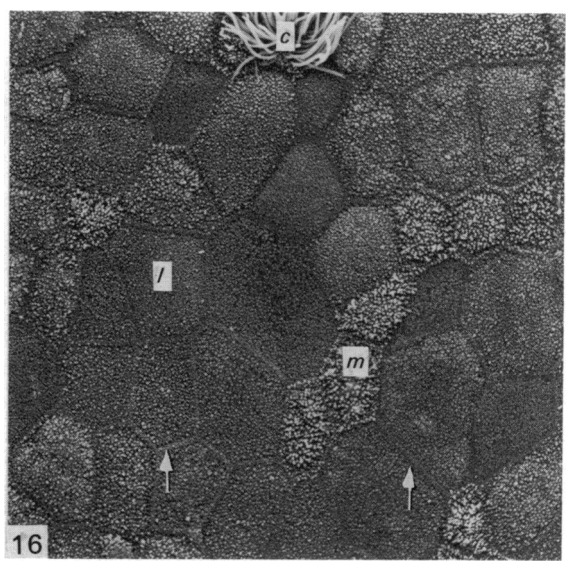
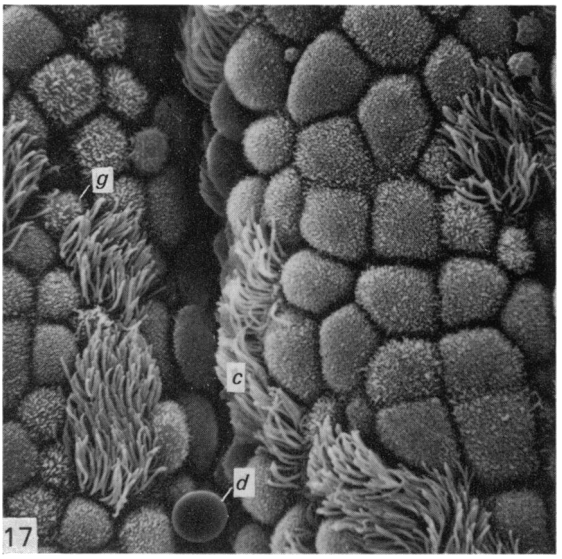


Fig. 15. Scanning electron micrograph of brush cells (*b*) during the early phase of the mature period with adherent granules of various sizes (↑) situated adjacent to mucin-containing cells (*g*). Age: 4 weeks. ×8000.

Fig. 16. Scanning electron micrograph of a cartilaginous zone during the early phase of the mature period in which large cells with a low electron response (*l*) and smaller medium to high electron responsive cells (*m*) predominate. Many cell borders (↑) resemble those of the early postnatal period. Ciliated cell (*c*). Age: 5 weeks. ×2000.



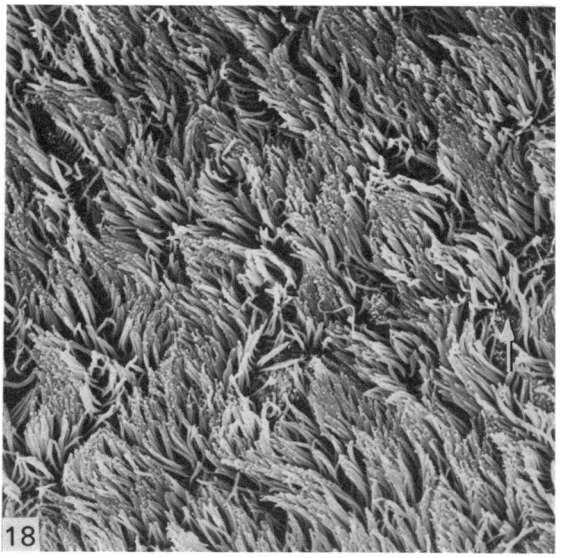


Fig. 17. Scanning electron micrograph of the membranous zone during the early phase of the mature period showing non-ciliated cells, some with protruding intracellular granules (*g*); ciliated cells (*c*) and a large mucous granule (*d*). Age: 5 weeks. ×2000.

Fig. 18. Scanning electron micrograph of the most common type of epithelium during the late phase of the mature period. Ciliated cells form a carpet punctuated by occasional non-ciliated cells (↑). Age: 5 months. ×2000.

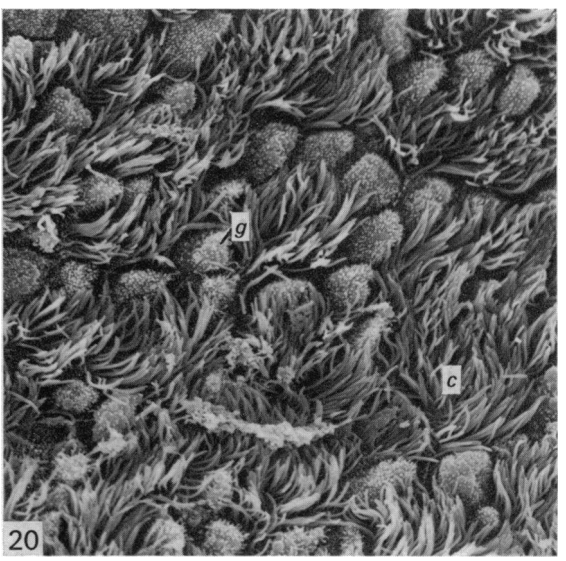
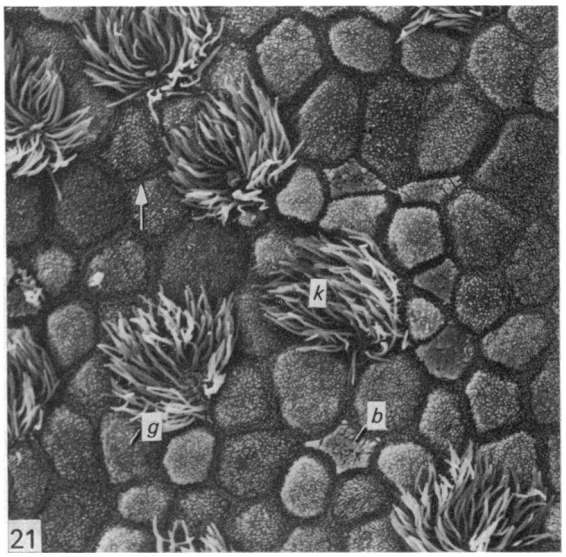


Fig. 19. Scanning electron micrograph of the second type of epithelium found during the late phase of the mature period. Ciliated cells (*c*) outnumber brush (*b*) and other non-ciliated cells (*n*). Age: 5 months. ×2000.

Fig. 20. Scanning electron micrograph of the third type of epithelium evident during the late phase of the mature period. Similar numbers of ciliated (*c*) and non-ciliated (mainly mucin-containing) cells (*g*) are present. Age: 5 months. ×2000.



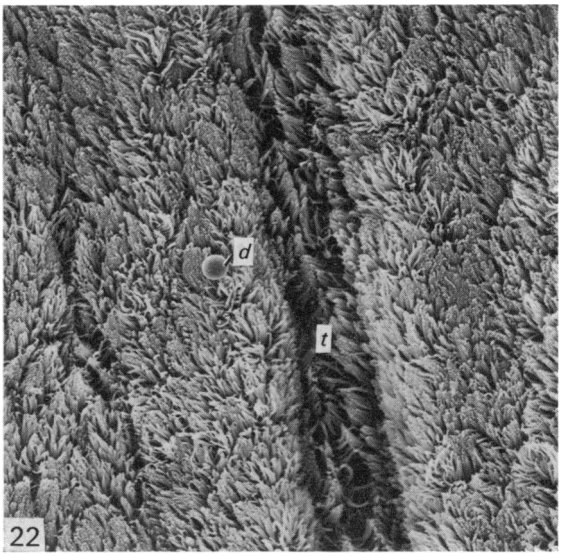


Fig. 21. Scanning electron micrograph of the fourth type of epithelium found in the late phase of the mature period. Mucin-containing cells (*g*) predominate and brush cells (*b*) are conspicuous. Cilia (*k*) show various degrees of electron response. Cell border (↑). Age: 5 months. × 2000.

Fig. 22. Scanning electron micrograph of the first type of membranous zone seen in the late phase of the mature period. Ciliated cells, a central trough (*t*) and a mucous granule (*d*) are evident. Age: 5 months. × 960.

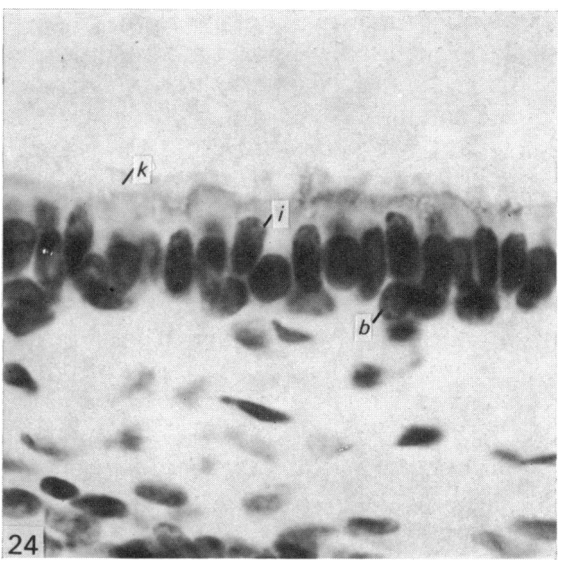


Fig. 23. Scanning electron micrograph of the second type of membranous zone evident during the late phase of the mature period. Ridges are formed by large cells with a high electron response (*h*) and a trough by smaller cells with a low or sometimes high electron response (*s*). Ciliated cell (*c*). Age: 5 months. × 960.

Fig. 24. Light micrograph of freeze-dried, mature epithelium. Basal (*b*) and intermediate (*i*) nuclei show a distinct chromatin pattern. Cilia (*k*). Age: 10 weeks. Stain: haematoxylin and eosin. × 960.

surfaces, ridges and troughs were covered by ciliated cells (Fig. 22). Sometimes, however, ciliated cells were few, or almost absent. The ridges were then covered by irregular, bulging, medium to high electron responsive cells. Intervening troughs were lined with smaller, flattened, low electron responsive cells often marked by high electron responsive elevations (Fig. 23). Brush cells, on occasion, occurred within this latter type of epithelium.

Mature period: light microscopy

The tissue prepared by freeze–drying and vapour fixation (Tock & Pearse, 1965) was superior to that prepared by aqueous fixation in both nuclear detail and staining properties.

The columnar epithelium, which contained basal cells, displayed one or two layers of rounded nuclei (Fig. 24). The mucous granules in the mucin-containing cells stained mainly for neutral mucosubstances, although occasional cells contained additional acidic mucosubstances. The distribution of these granules differed from the early postnatal period in that they were diffusely scattered throughout the cytoplasm, or concentrated in a hemispherical region immediately beneath the apical membrane, which was itself defined by a thin line of acidic mucosubstances (the glycocalyx) (Fig. 25). Occasionally, a dense accumulation of granules largely filled a mucin-containing cell, which then had the typical appearance of a goblet cell.

Elements of the epithelium: scanning electron microscopy

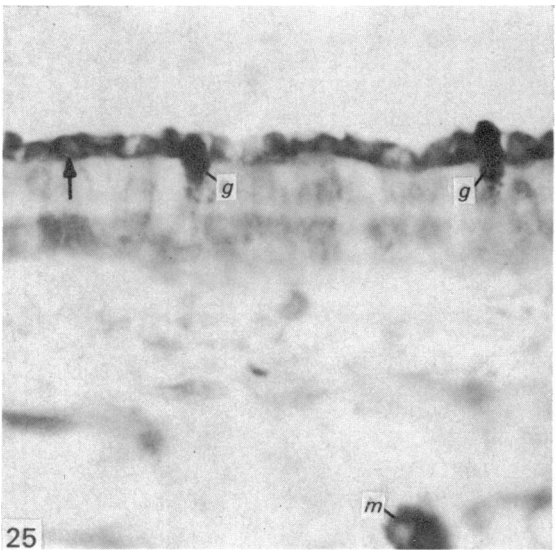
Cell borders

The peripheral limit of a cell was delineated by a cell border. This structure differed in appearance according to the cell type. With cells other than brush and ciliated cells it usually formed a continuous boundary composed of 4–6 short, linear segments, each in apposition to one or more surrounding cells (Figs. 1, 2, 10, 14, 16, 21). The morphology of these segments differed during development. In the early postnatal period they were of a uniform width and covered with a random aggregation, or less frequently with two rows, of microvilli. In the cartilaginous and intercartilaginous areas the segments formed a ridge higher than the rest of the apical surface of the cell (Figs. 2, 10). In the mature period the segments were of a less constant width and were often expanded at their ends (Figs. 14, 21). In the main they consisted of a central, linear band of high electron responsive microvilli flanked on either side by a low electron responsive zone having lesser numbers of similar microvilli. The central band of microvilli was observed to separate in cell dissociations. In common with the laryngeal and membranous zones of the early postnatal period, the cell borders seldom extended up to or beyond the level of the enclosed upper surface of the cell.

Brush and ciliated cells did not show any characteristic arrangement of surface projections at their periphery.

Filamentous strands

Fine strands, 0·1–0·2 μm in length and 0·02–0·04 μm in diameter, were present in abundance on all cell types of the early postnatal period (Figs. 5, 7). On most non-ciliated cells these strands connected adjacent microvilli (or brushes in the case of the brush cell) and usually reflected the electron response of these microvilli. However, in occasional cells they were so prolific that they formed an intricate trabecular meshwork interspersed with spheroidal structures of medium to high electron



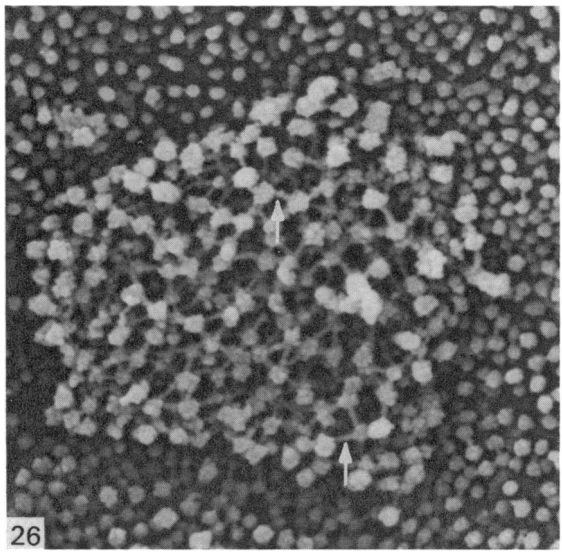


Fig. 25. Light micrograph of freeze–dried, mature epithelium stained for acidic and neutral mucosubstances. In most mucin-containing cells secretory granules occur mainly in the hemispherical subapical zone (↑). However, in the two goblet cells (*g*) the granules occupy a much greater proportion of the cell volume. Mast cell (*m*). Age: 10 weeks. Stain: PAS–alcian blue. ×960.

Fig. 26. Scanning electron micrograph of a cell in the latter part of the early postnatal period covered with numerous filamentous strands (↑) linking irregular spheroidal structures. Age: 2 weeks. ×12000.

response (Fig. 26). On ciliated cells the strands occurred between adjacent microvilli or cilia and between a microvillus and a cilium. Filamentous strands still occurred in relation to the cells of the mature period. However, they were seldom evident on mucin-containing cells advanced in their accumulation of granules.

DISCUSSION

Scanning electron microscopy is an invaluable addition to light and transmission electron microscopy in the study of cell development and structure. The main practical benefits are a better appreciation of the morphology and the distribution of cells. Thus it has been possible in this investigation to divide the development of the epithelium of the larynx and trachea of the rat into two distinct phases, arbitrarily termed the early postnatal and mature periods. In the transition between these periods adult mucin-containing cells appear, 'low electron responsive' cells disappear, and the morphology of the non-ciliated cells is modified. This change in the appearance of the epithelium after birth has not been observed in other laboratory animals. The epithelium of the fetal rabbit, for example, already resembles that of the adult (Leeson, 1961; Kanda & Hilding, 1968). However, De Haller (1969) reports that, in the human fetus, goblet cells become more prominent closer to term whilst 'precursor cells' containing fine granules of neutral mucosubstances and an occasional apical fringe of acidic mucosubstances, decline in number. The rat, because of its relatively short gestation, may exhibit an acceleration of a similar type of process after birth. The respiratory epithelium of the rat does, for example,

resemble that of man in the pattern of cellular changes seen in chronic bronchitis (Reid, 1973).

The absence of adult mucin-containing cells, the relative scarcity of ciliated cells, and the widespread occurrence of primary cilia, which, according to Sorokin (1968), form after cell replication and indicate cell differentiation, suggest that the early postnatal period is essentially a time of proliferation and differentiation. Furthermore, the distinctive features of the various anatomical areas imply that the epithelium is divided into compartments with different functions and patterns of maturation.

The presence of common surface specializations, and of cells with microvilli having a variable electron response, suggests a link between low and medium electron responsive cells. 'Low electron responsive' cells are not recognizable with light microscopy, and further study is required to ascertain their function. One possibility is that they are undergoing division and that their apical protrusions represent part of the mechanism of production of daughter cells. The medium electron responsive cells and the secretory cells of light microscopy appear to be one and the same.

Ciliated cells appear close to term in the trachea and bronchi of the fetal rat (Sorokin, 1968), and therefore the question of where ciliated cells first develop cannot be resolved in this study. They may initially differentiate in the posterior and distal parts of the trachea and then extend to other regions; or alternatively, their formation could begin in the larynx or upper trachea but be most prolific towards the posterior and distal areas. In the rabbit ciliated cells first appear at upper levels of the respiratory tract and progress distally (Kanda & Hilding, 1968).

The structure of the respiratory epithelium in the mature period of the present study correlates with the results of past investigations (Rhodin & Dalhamn, 1956; Rhodin, 1959; Andrews, 1974; Alexander *et al.* 1975). From the early to the late phase there appear to be unequal, localized alterations in the proportion of ciliated cells. This is responsible for the great variation in the ciliated cell to non-ciliated cell ratio between rats (also reported by Andrews, 1974), and between different regions of the same trachea. In transmission studies this ratio has been assessed as 4:1 (Rhodin & Dalhamn, 1956) and 3–5:1 (Dalhamn, 1956).

The brush cell has been assigned a number of possible functions: absorption (Rhodin & Dalhman, 1956; Rhodin, 1959; Jeffery & Reid, 1975), ciliogenesis (Rhodin, 1963), chemoreception (Luciano, Reale & Ruska, 1968) and stretch reception (Meyrick & Reid, 1968) activity: it has also been regarded as a discharged mucin-containing cell (Sorokin, 1973).

An absorptive function has been postulated because of the similarity in structure between the projections of the brush cell and those of the brush border of intestinal cells (Rhodin & Dalhamn, 1956). In addition, Jeffery & Reid (1975) have described pinocytotic vesicles in the apical portion of this cell. Such a function appears to be compatible with the regular presence of granules of varying dimensions on brush cells during the early phase of the mature period which was observed in the present study. These granules are almost certainly mucous in nature. The largest are similar in size to the granular protrusions of the mucin-containing cells and the mucous granules on other cells. Also, all have a similar electron response to that of mucous granules. It is proposed that, in the early phase of the mature period, a portion of the mucous granules, after discharge from a mucin-containing cell, become attached to and are absorbed by brush cells. This absorptive activity may be important in clearing away mucus at this particular time when active mucin-containing cells are

abundant but ciliated cells are not yet present in the density associated with mucus transport in the adult animal.

It is difficult to reconcile some results of this study with the observations of Luciano *et al.* (1968) who postulate a chemoreceptive role for the brush cell. In particular, the pairing of brush cells in mature epithelium emphasized by these workers, though often, is not always, present. In the early postnatal period all brush cells are present as single cells, and it is only in the mature period with the increase in the numbers of brush cells that these cells are observed in pairs. This suggests that pairing is an incidental, rather than a constant, feature of this cell type. Indeed, the initial description of the brush cell by Rhodin & Dalhamn (1956) was that of a single cell usually surrounded by four to six goblet cells. Furthermore, brush cells, although infrequent, are definitely present in the epithelium from at least the second day after birth. The failure of Luciano *et al.* (1968) to find brush-bordered cells at 2 weeks with transmission electron microscopy is probably due to their being present in only small numbers in the epithelium at this time.

The proposition that brush cells are discharged mucin-containing cells may also be challenged on a number of points. In the present study brush cells occurred during the early postnatal period, but typical adult mucin-containing cells did not. Furthermore, transition stages between the brush cell and either a discharging or a replenishing mucin-containing cell were not observed. In addition, although mucin-containing cells occur in the respiratory tracts of many mammals, brush cells are rare in man (Watson & Brinkman, 1964) and are absent from the monkey respiratory tract (Castleman *et al.* 1974). Moreover, brush cells occur in the rat alveolar epithelium (Meyrick & Reid, 1968) in the absence of mucin-containing cells.

None of our observations suggested that the brush cell is involved in ciliogenesis (Rhodin, 1963).

Filamentous strands were first described by Meyer *et al.* (1971) between all the types of projections of ciliated cells, and later by Andrews (1974) between the microvilli of goblet cells. It has been proposed that these strands may be the residue of unremoved mucus (Meyer *et al.* 1971), or extensions of the surface glycocalyx (cell coat) (Andrews, 1974). In the present study filamentous strands were found to occur on all the cell types of the epithelium throughout life, but especially on the non-ciliated cells other than brush cells, of the early postnatal period. We propose that these strands do, in fact, constitute an extension of the glycocalyx because (1) they react with stains demonstrating this structure (colloidal iron in Meyer *et al.* 1971 and Ruthenium red in Andrews, 1974), and (2) they are not of a random or constant electron response but instead usually reflect the electron response of the cell projections to which they are adherent. As a corollary, we also postulate (1) that on tissue surfaces, it is the glycocalyx, and not the cell membrane, which is observed with scanning electron microscopy: it is of interest that the variations in the intensity of the colloidal iron staining in the glycocalyx of the tracheal epithelium reported by Meyer *et al.* (1971) are similar to the variations in electron response seen by scanning electron microscopy; and (2) that the electron response of a particular site of a biological specimen may be related to the presence of potential acid radicals within that region of the glycocalyx. In this connection it is of interest that the staining by Ruthenium red of the glycocalyx is reduced following digestion by the enzyme neuraminidase (Kilburn, 1974). The action of this enzyme is specific and results in the release of a neuraminic acid moiety containing a carboxyl radical (Gottschalk & Drzeniek, 1972).

SUMMARY

The development of the epithelium of the larynx and trachea of the rat was investigated from birth onwards by means of light and scanning electron microscopy.

Two periods, the *early postnatal* and the *mature*, were recognized. The early postnatal period, the first 3 weeks after birth, was characterized by the presence of abundant and atypical mucin-containing cells, lesser numbers of 'low electron responsive' and ciliated cells, infrequent brush cells and primary cilia. Regional differences in the morphology and distribution of the different cell types also occurred. The mature period was divided into *early* and *late phases*. In the early phase adult mucin-containing cells appeared for the first time in development, while brush cells increased in number and displayed adherent mucous granules of various sizes. In the late phase four types of epithelium were identified in the laryngeal, cartilaginous and intercartilaginous (tracheal) zones, based on differences in the ciliated to non-ciliated cell ratio and in the nature of the non-ciliated cells. Most often the epithelium appeared to be formed mainly by ciliated cells, but variations occurred both between and within animals. The membranous zone was corrugated in many regions and, in general, ciliated cells predominated. However, areas where these cells were few or absent also occurred.

All cell types of the epithelium displayed filamentous strands between their apical projections. These appeared to be extensions of the glycocalyx. Cell borders separated the cells of the epithelium and, on non-ciliated cells other than brush cells, had a characteristic structure which altered with maturation.

It is proposed that (1) brush cells may be active in the absorption of mucus, (2) scanning electron microscopy of biological tissue surfaces in reality demonstrates the glycocalyx and (3) the electron response of a tissue surface may be related to the density of exposed, potential acid radicals in the glycocalyx.

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