RESPIRATORY TRACT EPITHELIUM IN PRIMARY CULTURE: CONCURRENT GROWTH AND DIFFERENTIATION DURING ESTABLISHMENT

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SUMMARY

Studies of cellular function in the respiratory tract lining have traditionally been limited by the small tissue mass and functional diversity of the epithelium. Recent improvements in culture conditions have permitted long-term maintenance of epithelial cells derived from the upper respiratory tract of rats. The present study determined the extent to which proliferation and differentiation took place in such epithelial cell cultures. The labelling index obtained after ³H-dThd administration was approximately 100-fold higher than that of the quiescent epithelium in vivo; therefore, a large proportion of the cells were probably in the cycling population.

Ultrastructural studies showed that features unique to the specialized mucous secretory cells and ciliated cells were lost rapidly with entry of these cells into the in vitro environment. With long-term maintenance, the cultures were reorganized into a stratified epithelium containing large, squamous, apical cells and small basal cells. The ultrastructural appearance of basal cells in vitro was nearly identical to that of basal cells in vivo. Squamous cells were frequently joined by tight junctions. Because hemicysts originated by detachment of squamous cells from the basal layers but not from adjacent squamous cells, they were considered to indicate stratification in the cultures. The proliferative and differentiative status of the mucociliary epithelium was altered by in vitro conditions, and came to resemble that of early squamous metaplasia in the respiratory tract epithelium.

INTRODUCTION

In recent years, attempts to initiate primary cultures of epithelial cells from various organs have met with considerable success (Hay, 1975; Flaxman, Lutzner & Van Scott, 1967; Nevo, Weisman & Sadé, 1975; Wilbanks, 1975; Bissell, Hammaker & Meyer, 1973; Flaxman & Van Scott, 1972; Walker & Potter, 1972; Pickett, Pitelka, Hamamoto & Misfeldt, 1975). In many of these epithelial cell cultures, loss of differentiated characteristics has been reported. Cultured mammary gland (Pickett et al. 1975; Emerman & Pitelka, 1977; Flaxman & Van Scott, 1972) and pancreatic epithelial cells (Hay, 1975) showed ultrastructural evidence of decreased secretory function. However, differentiation into ciliated and secretory cells continued to occur in short-term cultures derived from mucociliary epithelium (Nevo et al. 1975).

Maintaining primary cultures of epithelial cells for more than a few weeks has proved difficult in many previous studies. In a few instances, more prolonged growth
of epithelial cells has been obtained by enriching culture media with hormones and other additives (Wigley & Franks, 1976; Owens, Smith & Hackett, 1974). In preliminary studies of respiratory tract epithelium in culture, it was found that the epithelium obtained by outgrowth from explants in suboptimal media sloughed off the substratum within 2–3 weeks after initiation into in vitro conditions. Under optimal media conditions, however, most cultures could be maintained well beyond 3 weeks (Marchok, Rhoton & Nettesheim, 1976). Since long-term growth of epithelial cells from adult animals has not been achieved routinely, it was important to determine the characteristics of respiratory tract epithelial cultures which might contribute to their maintenance. The present report describes the manner in which such cultures were generated, as determined by 3H-dThd incorporation and autoradiographic identification of S-phase cells. So that factors contributing to the establishment of cell cultures could be studied, early (4–7 day) outgrowths were compared with 3-week cultures, with reference to their organization and morphology.

MATERIALS AND METHODS

Cell culture

Tracheas were obtained from 10- to 12-week-old, specific-pathogen-free Fischer-344 female rats. Explants were prepared under sterile conditions as previously described (Marchok, Cone & Nettesheim, 1975). The culture medium was an enriched Waymouth's MB 752/1 containing 10% aseptically collected foetal calf serum (Microbiological Associates) and additional components, linoleic acid, lipoic acid, sodium pyruvate, putrescine, amino acids, insulin (I), and hydrocortisone (HC). The medium used contained 100 µg/ml of insulin and 0.1 µg/ml of hydrocortisone and was designated WR 100 I, 0.1 HC. In one experiment, insulin limitation was tested by use of the same formulation containing only 0.1 µg/ml of insulin (designated WR 0.1 I, 0.1 HC).

The cells were cultured at 37°C in humidified 5% carbon dioxide in air. To establish primary cultures, the explants were removed at 4–7 days after the initiation of cultures. Treatment with 0.07% trypsin and 0.07% EDTA in Ca²⁺-Mg²⁺-free Hanks' solution (Grand Island Biological Co.) was used to remove fibroblasts when necessary.

Cell kinetics

For studies of proliferation, explants and cell cultures derived as above were used at times of 24 h, 4 days, and 7 days. Cultures were pulse-labelled for 45 min with 4 µCi/ml of 3H-dThd (Schwarz/Mann; sp. act. 6 Ci/mmol). They were fixed for 20 min with 3% glutaraldehyde (Ladd) in 0.05 M sodium cacodylate buffer (pH 7.3). After a brief rinse with 0.1 M cacodylate buffer, the cultures were postfixed in 1% OsO₄ buffered with 0.05 M phosphate (pH 7.4) for 1–2 h at room temperature. After dehydration with graded concentrations of hexylene glycol (Eastman Organic Co.), cultures were embedded in an epoxy mixture (Coulter, 1967). Sections 2 µm thick were cut at 40-µm intervals on a Sorvall JB-4 microtome. These were mounted on glass slides and layered with a 1:1 dilution of NTB-2 emulsion (Kodak) for autoradiography. After exposure at 4°C and development in D-19 (Kodak), the sections were stained with 0.05% toluidine blue by standard procedures (Trump, Smuckler & Benditt, 1961). Counts of labelled and total nuclei were determined at a magnification of 800 x on a Zeiss light microscope. Three sites were indexed on each section: the luminal surface of the explant, the side of the explant, and the cell outgrowth. Sectioned nuclei showing more than 4 grains were counted as labelled. From each explant, 10–18 sections were analysed.

The labelling index of cell cultures was confirmed by preparing autoradiograms directly on Petri dishes after fixation of the cultures with methanol. Counts of labelled and total nuclei were obtained at a magnification of 400 x for contiguous areas of 0.0338 mm² with
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an ocular reticle, along 4 axes extending from the explant at 90° from each other. At each time point, 6 outgrowths were sampled. The areas occupied by each outgrowth were determined by counting the numbers of reticle squares, 0.00484 mm² in area, occupied by the outgrowths.

Electron microscopy

Explants and cell cultures were studied by transmission electron microscopy (TEM) at 4 days (8 samples), 7 days (5 samples), and 3 weeks (6 samples) after initiation of the cultures. The results of these studies were correlated with scanning electron microscopy (SEM) observations of a few samples at each of the above time points.

For TEM studies, cultures fixed as above were observed by phase microscopy, and selected areas were photographed and marked for examination (Maxwell & Heckman, 1977). The cultures were postfixed and embedded as above. Thin sections were cut with a diamond knife, mounted on copper grids, and stained with uranyl acetate and lead citrate (Reynolds, 1963). Some sections were mounted on gold grids and stained for polysaccharides by a slight modification of the Thiery procedure (Thiery, 1967). The grids were viewed on a Hitachi HU-11-B or Siemens 102 electron microscope. Additional cultures, prepared for SEM as previously described (Maxwell & Heckman, 1977), were viewed on an ETEC scanning electron microscope.

RESULTS

When pieces of trachea were explanted into culture dishes, epithelial cells grew out on to the surfaces of the dishes within 3 days. The organization of the explant-outgrowth system is shown in Fig. 1. It was apparent that the epithelial cells formed a continuous sheet extending from the lumenal surface of the explant over its cut side on to the culture dish surface (outgrowth). The lumenal surface of the explant had a mucociliary epithelium with 3 major cell types: basal, secretory, and ciliated (Fig. 2). Although the cells of the lumenal epithelium appeared densely packed, the cells on the sides of the explant and in the outgrowth appeared progressively flattened and spread out (Fig. 1). In addition, the sides and outgrowth appeared to have fewer ciliated and mucous cells than the lumenal epithelium.

Distribution of labelled cells on explants and outgrowths

The mechanism by which outgrowths were generated was studied by comparing ³H-dThd labelling in epithelial cells of explants and outgrowths. For indexing of labelled nuclei, zones were designated on the explant outgrowth, with the centre of the lumenal surface as zone 1, the portion of the lumenal surface near the side as zones 2 and 3, the side of the explant as zone 4, and the outgrowth as zone 5 (Fig. 1). The end of the elastic lamina was used to define the end of the lumenal surface. The final 40 cells at this site (zones 2 and 3) comprised about 20% of the total cells on the lumenal surface and were indexed separately because the incidence of labelled nuclei seemed higher than in the central portion of the lumenal surface.

At day 1, zones 2, 3, and 4 showed high levels of labelling: 10.6, 17.7, and 17.2%, respectively. The labelling index in zone 1 was much lower, and the labelling indices of zone 1 and zone 3 were significantly different at the $P = 0.01$ level (Table 1). No cells had reached the Petri dish surface at this time. On day 4, the index in zones 1-4 had decreased 2- to 4-fold from the level observed at day 1. A high percentage (18.8%) of cells in zone 5 were labelled, however.
Fig. 1. Cross-section of a tracheal explant and outgrowth after 4 days in culture, 2-μm epoxy section stained with toluidine blue. The epithelium extends from the luminal surface to the culture dish (d). In labelling index determinations, zone 5 comprises the outgrowth and zone 4 the side of the explant. Zone 3 comprises the 20 cells extending from the end of the elastic lamina (arrow) on to the luminal surface. Zones 2 and 1 are not shown, but include the next 20 cells and central portion of the luminal surface, respectively. Although the cells on the explant are columnar, progressive flattening occurs in cells on the side and in the outgrowth. × 270.

Fig. 2. Differentiated cells on an explant after 4 days in culture. Normal ciliated (c), mucous (m), and basal (b) cells are present. In some areas of the epithelium, the intercellular spaces appear broadened (arrow). × 1000.

On day 7, the labelling index in zones 1–3 had decreased to levels below 0.1%. Zone 4 had a labelling index of approximately 5%, while the outgrowth, zone 5, remained at a high level of labelling (18±0.3%), similar to that observed in 4-day growths. The labelling indices of the outgrowth and zones 1–3 were significantly different at the $P = 0.01$ level (Table 1). Throughout the time course, mitotic figures were observed frequently in sites showing high labelling indices. On the luminal surface, labelled columnar cells were observed rarely. At extra-lumenal sites basal cell nuclei could not always be distinguished from those of overlying cells because of the extreme flattening of the cells. However, in stratified portions of 7-day outgrowths (see below), most labelled nuclei were basal in position.

In control experiments, labelled and total nuclei were recorded from autoradiograms prepared directly on the outgrowths. Labelling indices, ± s.e.m., near the explants were $14.0 ± 4.3\%$ at 4 days and $23.0 ± 2.3\%$ at 7 days. At the advancing edges of the outgrowths, the values were $30.2 ± 4.2$ and $18.3 ± 2.2\%$, respectively. Analysis of cell densities in the outgrowths showed that the density decreased 3-fold with distance from the explant in 7-day outgrowths (Fig. 3A). The cell density, however,
Table 1. Percentage of $^3$H-dThd-labelled cells at sites on tracheal explants and outgrowths*

<table>
<thead>
<tr>
<th>Medium</th>
<th>Time after initiation, days</th>
<th>No. of explants sampled</th>
<th>Percentage labelled cells at given site</th>
<th>Lumenal surface</th>
<th>Side, Zone 4</th>
<th>Outgrowth, Zone 5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Zone 1</td>
<td>Zone 2</td>
<td>Zone 3</td>
<td>Zone 4</td>
</tr>
<tr>
<td>WR 1001, 0:1 HC</td>
<td>1</td>
<td>6</td>
<td>2.3 ± 0.4†</td>
<td>10.6 ± 1.9</td>
<td>17.7 ± 2.6†</td>
<td>17.2 ± 2.7</td>
</tr>
<tr>
<td>WR 1001, 0:1 HC</td>
<td>4</td>
<td>6</td>
<td>1.4 ± 1.1</td>
<td>2.7 ± 1.7</td>
<td>4.0 ± 1.2†</td>
<td>6.8 ± 1.4</td>
</tr>
<tr>
<td>WR 1001, 0:1 HC</td>
<td>7</td>
<td>5</td>
<td>0.06 ± 0.03</td>
<td>0.06 ± 0.06</td>
<td>0.08 ± 0.05§</td>
<td>4.8 ± 1.6</td>
</tr>
<tr>
<td>WR 0:1, 0:1 HC</td>
<td>5</td>
<td>4</td>
<td>0.24 ± 0.23</td>
<td></td>
<td>N.D.†</td>
<td>N.D.†</td>
</tr>
</tbody>
</table>

* At intervals after initiation into culture, tracheal explants were prepared for autoradiography as described in Materials and methods. Counts of labelled and total cells present at each site on the explant and outgrowth were obtained from the autoradiograms. Cells in the outgrowth were very elongated, so that only 2000-4000 could be counted in serially sectioned explants at each time point. At other sites, 2500-30000 cells were counted. Labelled cells were expressed as a percentage of total cells at each site ± S.E.M.

† Closest zones showing differences significant at the $P = 0.01$ level at 1 day.

§ Closest zones showing differences significant at the $P = 0.01$ level at 4 days.

§ Closest zones showing differences significant at the $P = 0.01$ level at 7 days.

|| No significant differences among these levels.

§ Not determined.
Fig. 3. A, cell densities in epithelial outgrowths at 4 (○) and 7 days (▲) after initiation of explants into culture. Cell densities were determined directly on epithelial outgrowths as detailed in Materials and methods. The average cell density was higher at 7 than at 4 days of in vitro culture. B, fraction of cells which incorporated 3H-dThd in 7-day outgrowth cultures as a function of distance from the explant. The labelling index did not vary in a consistent way with the density of the cultured cells.

did not appear well correlated with the variations in labelling index across the outgrowth (Fig. 3B). The average cell densities, ± S.E.M., calculated from the counts across individual outgrowths were 1170 ± 110 cells/mm² at 4 days and 1860 ± 110 cells/mm² at 7 days. The average areas calculated were 1.9 ± 1.5 mm² at 4 days and 14.3 ± 8.4 mm² at 7 days. Thus the population of cells in an average outgrowth increased from approximately 2200 to 26600 in the period of 4–7 days.

Our preliminary studies indicated that few respiratory tract epithelial cultures could be maintained beyond 3 weeks in suboptimal media formulations. If a decrease in the growth fraction was associated with this growth failure, a lower labelling index would be seen. Culture media containing 100-fold lower insulin concentration
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(WR 0.1 I, 0.1 HC) supported outgrowths similar in size to those obtained in the usual medium (WR 10.0 I, 0.1 HC). However, the labelling index was low at all sites of the explant-outgrowth system under insulin-limited media conditions (Table 1).

Distribution of specialized cells on explants and outgrowths

Specialized cells were indexed at various sites on explants and in outgrowths in order to determine the distribution of these cells in the newly generated epithelium. Counts of mucous cells showed that a stable population was maintained on the lumenal surface of the explant over 1–7 days of in vitro culture (Table 2). As observed by TEM, mucous droplets became fewer and smaller in mucous cells on the sides of the explants and in the outgrowths over the time of culture. Since the droplets decreased to sizes below the resolving power of the light microscope, not all mucous cells could be identified reliably at extra-lumenal sites, and particularly in the most peripheral, highly elongated epithelial cells. Mucous-cell densities in the regenerated epithelium at the sides (zone 4) of the explants were estimated to be lower than in the lumenal epithelium (Table 2). Although counts could not be obtained in the outgrowth epithelium, TEM observations indicated that mucous droplets were present in many or most large cells of 4-day outgrowths. Mucous droplets were found less frequently in large cells of 7-day outgrowths (see below). The fractions of total mucous cells found at extra-lumenal sites were 0, 21, and 30% at 1, 4, and 7 days, respectively, after initiation of the explants into culture (data not shown).

Although ciliated cells at extra-lumenal sites usually had fewer cilia than those of the lumenal epithelium (see below), the cilia could always be resolved at the light-microscopic level in epoxy sections. For this reason, the ciliated cells were considered more reliable markers of the specialized cell populations than the mucous cells. The percentage of ciliated cells present in the lumenal epithelium remained stable over the period of time the explants were maintained in culture. At 1 day after initiation of the explants into culture, the epithelium at the sides of the explants contained a much smaller proportion of ciliated cells than the lumenal epithelium. A similar percentage (1.7 vs. 2.0%) of ciliated cells was found in the 4-day outgrowths (Table 2).

The proportion of ciliated cells on the sides of the explants was higher at 4 and 7 days of culture than at 1 day. In the outgrowths, however, the percentage of ciliated cells decreased nearly 3-fold between 4 and 7 days (Table 2). The fractions of total ciliated cells in the explant-outgrowth system which appeared at extra-lumenal sites were 2, 21, and 14% at 1, 4, and 7 days, respectively (data not shown).

Morphological characteristics of tracheal explant epithelium in culture

Ciliated and mucous cells could be recognized readily in the lumenal epithelium of the explants (Fig. 2), and few ultrastructural alterations in the cells were found. By 4 days after initiation of the explants into culture, the secretory cells contained fewer mucous droplets than usual in vivo. On the sides of the explants, in particular, the mucous droplets were fewer and smaller within the cells. Ciliated cells at this
Table 2. Percentage of specialized cells present at sites on tracheal explants and outgrowths.

<table>
<thead>
<tr>
<th>Time after initiation, days</th>
<th>No. of explants sampled</th>
<th>Percentage specialized cells at given site</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lumenal surface</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ciliated Mucous</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Side</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ciliated Mucous</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Outgrowth</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ciliated Mucous</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6</td>
<td>10.4±0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13±0.14</td>
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<tr>
<td></td>
<td></td>
<td>13±0.14</td>
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<tr>
<td></td>
<td></td>
<td>N.D.†</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>9.3±1.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12±1.4</td>
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<td></td>
<td></td>
<td>10.9±1.8</td>
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<tr>
<td></td>
<td></td>
<td>6.3±0.9</td>
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<tr>
<td></td>
<td></td>
<td>7.0±1.2</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>11.5±1.8</td>
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<td>13±1.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12±1.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10.6±1.2</td>
</tr>
</tbody>
</table>

*Counts of specialized and total cells were prepared for sectioning as described in Materials and methods. Counts of specialized and total cells were counted at each site on the explant and outgrowth. Specialized cells were expressed as a percentage of total cells at each site. Not included in the tabulation are other cell populations in the tracheal epithelium and specialized cells which could not be identified due to variations in the plane of sectioning.
†Not determined.
Morphological characteristics of epithelial outgrowths at 4–7 days of culture

Typically, the newly initiated outgrowths contained areas of small, densely packed cells and areas of larger, spread-out cells (Fig. 6). While the areas of densely packed cells were frequently found near the explants, some outgrowths lacked such areas altogether. Large cells were found in all outgrowths and were particularly numerous at the periphery of the outgrowths. The cells with beating cilia were typically elongated and were most frequently seen near the explants. When examined in
transverse sections by TEM, such cells showed only a few cilia and microvilli on their surfaces. Elongated cells without cilia were also seen in the outgrowths (Fig. 6). By SEM the outgrowths appeared to contain many large, flat cells, which were thin enough to be penetrated by the electron beam. Points at which these cells were in contact with the culture-dish surface appeared as black areas. At some points, the outlines of smaller cells could be detected beneath the large cells (Fig. 7) indicating a pseudostratified layer. Most cells appeared very long when viewed in transverse sections by TEM, overlapping other cells as well as making contact with the surface of the culture dish. Cells at the edges of the outgrowths did not overlap as extensively, so that monolayers were formed in these areas.

Fig. 6. Epithelial cells of a 7-day outgrowth by phase microscopy. Areas containing large (l) cells and small (s) cells in compact groups are present. Elongated cells, some of which are ciliated (arrows), are also seen. The explant is at the left. × 190.

Thin sections taken in the plane of the cell layer were used to study subcellular characteristics of the cultured cells. The cells were closely adherent to each other, so that little extracellular space was visible. The smaller cells in the outgrowths were characterized by extensive lateral interdigitations and desmosomes at the cell surfaces. They contained many tonofilaments and profiles of rough endoplasmic reticulum. Mucous droplets, bundles of intermediate (10-nm) filaments, and relatively inactive Golgi bodies were found in many of the cells (Fig. 8). In addition, gap junctions were frequently seen between small cells (Fig. 9).

Although the characteristics of small cells did not appear to vary in different areas of the outgrowths, those of larger cells varied with their distance from the explant. Near the explants, large cells were sometimes elongated, with clear or dense cytoplasm. The cells contained tonofibrils and were joined by desmosomes at some
points. Bundles of fine filaments dissimilar to tonofibrils were occasionally found in a perinuclear location, and lysosomes were sometimes apparent. The cells frequently contained mucous droplets and had straight lateral borders (Fig. 10). The large cells more distant from the explant showed mucous droplets frequently associated with large, clear vacuoles. The nuclei contained little condensed chromatin (Fig. 11). Although profiles of rough endoplasmic reticulum, Golgi bodies, and polyribosomes were present in the cells, smooth membranous vesicles and lysosomes were not seen. The basal specializations present in vivo, i.e. hemidesmosomes and basal lamina, were not found in transverse sections of the cells from early outgrowths.

In certain areas of 7-day outgrowths, 3 layers of cells were present, as shown in transverse sections (Fig. 12). These cells were separated by broad intercellular spaces but were connected at intervals by desmosomal junctions. Similar areas were viewed in sections taken in the plane of the cell layer. A characteristic organization of the cells was found in which a number of small cells were encircled by cytoplasmic extensions of the larger cells (Fig. 13). Broad intercellular spaces were apparent between the cells. The large cells of these areas still contained mucous droplets, as confirmed by their reactivity in the Thiery procedure, which identifies 1,2-glycol groups (Fig. 14).

Cultures obtained under suboptimal media conditions showed similar morphological features in pseudostratified areas of the cultures. Although stratified areas were also observed in these cultures, the basal cells generally appeared larger than those found under optimal media conditions.

Morphological characteristics of cultures 3 weeks after initiation of outgrowths

Well established cultures viewed with the phase microscope consisted of pavement-like arrays of polygonal cells, which appeared visibly separated from one another. The cells contained many clear vacuoles and smaller, refractile granules. Hemicysts, i.e. thin, dome-like structures, were particularly common near the central part of the cultures (Fig. 16). Large, spread-out cells were seen at the periphery of the cultures. When viewed by TEM, the cell layer of established outgrowths was organized as shown in Fig. 13. As in early outgrowths, the small cells were compact and appeared denser than the larger cells because of the abundance of cytoplasmic polyribosomes and tonofilaments. The nuclei of small cells, unlike those of the larger cells, contained some peripheral condensed chromatin. Both large and small cells had desmosomal junctions and finger-like microvilli at their surfaces (Fig. 15). As at early times after initiation of the cultures, rough endoplasmic reticulum and Golgi bodies were present subcellularly, while lysosomes and smooth endoplasmic reticulum were not seen. Mucous droplets could no longer be identified by the Thiery procedure. The mitochondria contained a matrix of exceptionally low electron density (Fig. 15). The large cells of established outgrowths had osmiophilic lipid inclusions and vacuoles containing diffuse, proteinaceous material. Osmiophilic, perinuclear lipid inclusions were also common in the small cells (Fig. 15).

The stratified organization of established outgrowths was seen by SEM. The cell borders of large, polygonal, or elongated cells were apparent. Two to eight smaller,
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rounded cells were covered by each large cell. Although hemicysts were usually formed by the detachment of a number of squames (Fig. 17), they were also occasionally produced by detachment of a single squame. In some areas of the cultures, particularly near the periphery, an overlapping organization of cells similar to that of early outgrowths was indicated by black spots where large cells contacted the Petri dish surface. Hemicysts were absent from these areas.

In transverse sections, it was apparent that the cultures contained 2 or 3 overlapping layers of epithelial cells. The uppermost cells of these layers were thin and contained large vacuoles. In most of these cells, no site of attachment to the Petri dish was found. Small cells were found only beneath the larger cells. Frequently, very thin profiles of other cells extended beneath the smaller, rounded cells. Hemicysts were apparent as areas in which an upper cell had separated from the underlying cells to form a dome-like structure (Fig. 18). Except at hemicysts, desmosomal junctions joined the squamous cells to the underlying strata. Rarely, tonofibril condensation typical of early stages of keratinization was observed in the squames. Apical junctional complexes, including tight junctions, were seen occasionally (Fig. 19). In addition, hemidesmosome-like structures were present at the basal borders of both large and small cells, where they were in contact with the Petri dish surface (data not shown). Between cytoplasmic extensions of small cells, gap junctions were seen. However, basal lamina-like specializations were not present.

Discussion

Cell kinetics of the explant outgrowths

During the derivation of outgrowth epithelium from explants of respiratory tract tissue, there was a tendency for the highest labelling indices to occur in a broad margin behind the epithelial periphery. Early after initiation of the explants to in vitro conditions, a small area of the lumenal epithelium, comprising about 20% of the lumenal cells, was included in this margin. It seemed likely that this area (zones 2 and 3), near the sides of the explants, was largely responsible for the formation of the outgrowths. Highly proliferating populations were also found in the epithelium of submucosal glands, as noted in a previous study of regeneration (Wilhelm, 1953). We believe that the glands may be a source of newly generated cells for repopulation of the lumenal epithelium during the process of epithelial outgrowth.

Fig. 7. Cells of a 7-day outgrowth. Small cells (arrows) can be seen under the edges of larger (l) cells. Black areas (b) are visible where thin portions of the large cells are in contact with the culture dish surface. x 600.

Fig. 8. Small cells from the outgrowth shown in Fig. 6. The cells have extensive lateral interdigitation (i) and desmosomal junctions (d) at their surfaces. They contain prominent tonofilaments (t) and mucous droplets (m). Gap junctions (arrows), Golgi bodies (g), and intracellular filament bundles (f), containing 10-nm filaments, were also present. x 11300.

Fig. 9. Gap junction between 2 small cells in a 7-day outgrowth. Gap junctions extend for distances up to 600 nm between the cells. x 70000.
Light- and electron-microscopic examination showed that the number of cilia on ciliated cells appeared to decrease progressively with the distance of the cells from the lumenal epithelium. It was unlikely, therefore, that continual differentiation of uncommitted cells into ciliated cells occurred in general at extra-lumenal sites. The stability of the ciliated cell population in the lumenal epithelium, however, suggested that differentiation of uncommitted cells into ciliated cells may have occurred at this site. Since low percentages of ciliated cells were found in areas having high labelling indices, ciliated cell specialization may have been suppressed in highly proliferating populations. The proportion of mucous secretory cells was higher than that of ciliated cells both on the sides of the explants and in the outgrowths (as estimated by electron microscopy). Since the proportions of mucous and ciliated cells observed in the lumenal epithelium were quite similar, the data suggested that continued specialization and longer maintenance of mucous cells occurred under *in vitro* conditions. Morphological observations of mixed cytodifferentiation (see below) also suggested that continued specialization of mucous cell characteristics was possible in outgrowth cultures.

Labelling indices in most areas of newly initiated outgrowths were in the range of 14–30%, indicating that a large proportion of the population was capable of proliferation. Preliminary studies of established cultures indicated that a 3–6-fold depression of the labelling index occurred in comparison to newly initiated cultures (A. C. Marchok, unpublished data). While our present findings have shown no strict correlation between labelling index and cell density within epithelial cultures (Fig. 3), the possibility that the growth status of the culture as a whole was regulated by density-dependent phenomena could not be ruled out. Since electrotonic communication of basal cells through gap junctions would permit equilibration of low-molecular-weight growth promoters and inhibitors in the cultured cells, density-dependent accumulation or depletion of such effectors could regulate growth in the culture as a whole. This possibility should be further explored.

The labelling index of primary outgrowth cultures was approximately 100-fold higher than that of the quiescent tracheal epithelium *in vivo*. The latter was 0.4% in adult Sherman rats (Messier & Leblond, 1960). These results suggested that environmental alterations provided by *in vitro* culture could induce a marked proliferative response accompanied by only minimal pathological changes at the cellular

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Fig. 10. Elongated cells from the outgrowth shown in Fig. 6. Clear and denser cells with straight lateral borders are observed. Perinuclear filament bundles (f) and invaginations of the nuclear envelope (e) are sometimes seen. Both cell types contain mucous droplets (arrow). × 5600.

Fig. 11. Large cells from the outgrowth shown in Fig. 6. The cells have straight lateral borders at some areas and are joined by desmosomes (d). Prominent mucous droplets (m) and tonofilaments (t) are present intracellularly. The mucous droplets occasionally adjoin clear vacuoles (arrow). × 2800.

Fig. 12. Cells of a 7-day outgrowth. The cells form 3 layers and are separated by broad intercellular spaces. The bottom cell is closely apposed to the surface of the culture dish (arrow). Desmosomes (d) with typical ultrastructure join the cells to one another. × 22 000.
level (see below). In both magnitude and timing, the proliferative response of respiratory tract epithelium in vitro resembled that obtained after wounding in mouse epidermis (Potten & Allen, 1975). Elevation of labelling indices of tracheal epithelium in conjunction with squamous metaplasia induced by vitamin A deficiency in vivo has also been described (Harris et al. 1973).

Morphological characteristics of newly initiated epithelial cultures

The prevalent cell types in the tracheal mucosa, i.e. ciliated, mucous, and basal cells, could be recognized in newly initiated cultures by ultrastructural features described previously (Rhodin & Dalhamn, 1956; Pavelka, Ronge & Stockinger, 1976; Jeffery & Reid, 1975). While certain ultrastructural features associated with specialized functions of the mucous and ciliated cells were lost rapidly, other features...
were retained up to 7 days in vitro. The long microvilli, cilia, and nuclear indentations typical of ciliated cells (Rhodin & Dalhamn, 1956) were found only in cells near the explants. Likewise, the abundant rough endoplasmic reticulum and free ribosomes characteristic of mucous cells in vivo were rarely seen in the cells in vitro. The presence of mucous droplets, fine-filament bundles, and straight lateral borders in many large cells of 7-day cultures indicated the persistence of some subcellular features of the specialized cells. In addition, the pseudostratified organization and closely apposed lateral borders which typified the epithelial cells in vivo were retained in newly initiated cultures.

By 7 days in vitro, individual cells could no longer be identified as mucous or ciliated cells on the basis of their cytoplasmic features. Although cells recently derived from the explants still showed specialized features, the large cells farther from the explants all appeared similar, constituting one cell type within the cultures. These cells contained mucous droplets but also had electron-lucent cytoplasm and fine-filament bundles characteristic of ciliated cells in vivo (Jeffery & Reid, 1975; C. A. Heckman, unpublished data). The large cells also appeared to compose the apical cells in stratified areas of 7-day cultures.

A second, smaller cell type in the epithelial cultures was characterized by gap junctions and lateral interdigitations similar to basal cells of the rat tracheal epithelium. These cells were usually, but not always, basally located in the cultures. Anomalous findings of mucous droplets and 10-nm-filament bundles in the small cells, neither of which was found in basal cells in vivo, suggested that mixed cytodifferentiation could occur. However, the cell of origin could not be identified on the basis of morphological criteria.

**Characteristics of cells in established cultures**

In long-term cultures, 2 cell types could again be distinguished. Large cells were characterized by a flat, squamous shape, intracellular vacuoles, large nuclei with no heterochromatin, and tight junctions (zonulae occcludentes). It is noteworthy that tight junctions were the only subcellular characteristic of the in vivo columnar cell population to be retained in long-term proliferating cultures. These cells were typically apical in stratified areas of the cultures (see below).

Small cells were characterized by their basal location, rounded profiles, and nuclei containing areas of condensed chromatin. Desmosomes were found in all

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Fig. 17. Cells of a 3-week culture. By SEM, small cells (s) appear evenly arrayed underneath larger cells. The outlines of the uppermost, squamous cells can be seen as fine lines (arrows), both on the cell layer and in the hemicyst (h). × 600.

Fig. 18. Cells of a 3-week culture. In transverse sections, 3 overlapping layers of cells are frequently seen (1–3). Desmosomes (arrows) are found between the cells. The hemicyst (h) is formed by the separation of the uppermost squama of the cell layer. Note the punctate appearance of microvilli on the apical surfaces of squamous cells. × 6300.

Fig. 19. Junction between 2 cells in hemidesmosome of a 3-week culture. The junction is 11 nm wide, indicating a zonula occcludens. × 111,200.
cells except those participating in hemicyst formation. Since the distribution of mitotic figures in established cultures appeared to be restricted to basal cells, the maintenance of a basal small cell population may be required for long-term proliferation in epithelial cultures.

**Squamous metaplastic changes**

Long-term primary cultures showed broad intercellular spaces in conjunction with true stratification, except at the periphery of the cultures. Because nuclear overlap rarely occurred, the degree of stratification could not be appreciated by phase microscopy. Since initial pathological squamous metaplastic changes in the respiratory tract in vivo were accompanied by stratification of the cell layer and broadening of intercellular spaces (Wong & Buck, 1971), similar organizational changes observed in epithelial cultures may be interpreted as mild squamous metaplastic changes. To some extent, the establishment of stratified organization may be necessary for continuing proliferation in normal cells (see below).

Hemicysts, like the domes previously described in cultures of skin cells (Fusenig & Worst, 1975), were formed by detachment of the uppermost squamous cells from the lower cells, and were thus another indication of stratification in the cultures. Pseudostratified areas in both new and established cultures contained mainly cells which adhered to the surface of the culture dish, so that hemicysts were not formed in these areas. Hemicysts in respiratory tract epithelial cultures, although composed of fewer cells, appeared similar to domes found in mammary cell cultures (Pickett et al. 1975). Domes were presumed to arise as a result of epithelial impermeability and transport function (Miesfeldt, Hamamoto & Betelka, 1976).

Although they contained fewer layers, the long-term cultures originating from the tracheal epithelium were similar in organization to outgrowth cultures of skin and buccal mucosa cells (Flaxman et al. 1967). They differed from the cultured skin cells in major respects, in that the squames rarely contained increased numbers of tonofilaments and membrane-coating granules were not seen.

The basal cells of long-term respiratory tract epithelial cultures were quite similar to basal cells of skin and buccal mucosal outgrowths, which had abundant ribosomes and tonofilaments, numerous microvilli, and large intercellular spaces (Flaxman et al. 1967). With few exceptions, the subcellular fine structure of basal cells in vitro was identical with that of basal cells in vivo. While the basal lamina is known to be synthesized by epithelial cells in vivo (Meier & Hay, 1974; Briggaman & Wheeler, 1975), it was not produced by tracheal epithelial cells or, apparently, by breast epithelial cells (Pickett et al. 1975) in vitro. An additional feature, not found in basal cells in vivo, was the accumulation of cytoplasmic lipids. Lipid accumulation was also reported in cultured normal cervical squamous epithelium (Wilbanks & Shingleton, 1970), but has not been noted in skin epithelial cultures (Flaxman et al. 1967). Lipid metabolism in normal and neoplastic epithelia in vitro is under further investigation in this laboratory.

In separate studies of the growth potential of normal respiratory tract epithelium, we have attempted to grow primary cultures of dissociated cells under conditions
identical to those in which primary outgrowth cultures have been obtained. These cultures have not become established and appeared to lack the basal, small cell populations which may be essential for long-term growth of the epithelium. The cultures have survived for a few weeks, however, before sloughing off the culture substrata. Similarly, secondary subculture of dissociated primary cultures has not been possible. Since epithelial outgrowths derived from carcinogen-treated tracheal tissues underwent successful subculture (Marchok et al. 1977), the effect of dissociation on the growth potential of normal epithelial cells is of particular interest.

Our findings indicated that rapid loss of most specialized features of the respiratory tract epithelium occurred under conditions favouring proliferation of the epithelial cells. On the other hand, ultrastructural characterization of normal liver (Alexander & Grisham, 1970; Chapman, Jones, Meyer & Bissell, 1973) and breast (Pickett et al. 1975; Russo et al. 1976) epithelial cells has shown that specialized features of these epithelia were retained in primary cultures. A major difference between the present and previous studies is that the conditions presently used permitted expanding epithelial populations to be maintained for several months and promoted squamous metaplastic alterations in the cultures. While the latter changes did not depend on prior reversal of specialized mucociliary features, as indicated by the presence of mucous droplets in stratified squamous cells, reversal of many specialized features apparently occurred concomitantly with squamous differentiation. However, tight junctions were retained over the time of culture studied and were an important indicator of residual mucociliary specialization.

As a working hypothesis, we consider the proliferative and differentiative status of respiratory tract epithelial cells in culture to reflect squamous metaplastic modifications induced by in vitro conditions. Since disruption of squamous organization by dissociation of the cultures led to cessation of growth, we believe this organization may contribute to the maintenance of the basal cell population. Further research is in progress to determine the possible role of vitamin A in reversing squamous metaplasia in vitro and to elucidate the control mechanisms mediating normal epithelial growth.

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Respiratory tract epithelium in culture


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