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Mucus and Surfactant Synthesis and Secretion by Cultured Hamster Respiratory Cells

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Procedures for the selective isolation and cultivation in monolayer of respiratory cells have been developed. This technique requires repeated protease treatment and gradient centrifugation of hamster tracheal or lung tissues and permits the establishment of proliferating cultures of epithelial cells with biologic specialization.

Mucus synthesis was monitored in cultured tracheal cells by incorporation of ^{3}H-labeled *N*-acetyl-D-galactosamine and ^{14}C-serine into glycoprotein as determined by trichloroacetic acid precipitation of growth medium followed by acrylamide gel electrophoresis. For comparative purposes tracheal explants and several established cell lines were also examined. Synthesis and secretion of the glycoprotein macromolecule by tracheal cell monolayers appeared to be regulated by vitamin A since its addition to the culture medium significantly increased both the number of cell-associated granules and glycoprotein secretion.

Lung-originated cell cultures were grown to confluence and radio-labeled with ^{3}H-choline in serum-free medium for 24 hr to examine surfactant synthesis. Cell monolayers and growth medium were then extracted by the Folch method, and total radioactive phosphatidylcholine as well as disaturated phosphatidylcholine were determined by thin-layer chromatography and alumina gel fractionation of osmium tetroxide-reactive phospholipid, respectively. Data indicate that these cultures have a marked ability to synthesize and secrete surfactant when compared to other established cell lines. In addition, naturally transformed cells that arose during passage and senescence of the primary cultures were analyzed for their biosynthetic capabilities.

Introduction

The establishment of eucaryotic cells in monolayer culture as experimental models has contributed significantly to our current understanding of growth control and regulation of specialized functions in mammalian cells. The possibility that single cell types can be examined free from the heterogeneity of host tissue and yet retain their biologic function is a major advantage of tissue culture techniques. Unfortunately, the mammalian lung is a complex structure with different cells possessing numerous physiologic roles (*1*) and therefore provides a difficult primary source of tissue from which to selectively dissociate particular cell types. Of special interest are two classes of respiratory epithelial cells whose key functions are the synthesis and secretion of mucus glycoprotein or pulmonary surfactant, each essential to normal lung maintenance. Mucus is a viscous material which protects the tracheobronchial epithelium from inhaled particles and pathogenic microorganisms by trapping them and permitting their removal via ciliary activity. Surfactant or disaturated (primarily dipalmitoyl) phosphatidylcholine is released onto alveolar surfaces apparently by type II alveolar cells to reduce the surface tension at the air-liquid interface, preventing alveolar collapse. Mucus-secreting cells have until recently (*2*) not been isolated and grown in pure culture, restricting basic studies on the biology of the cell as well as properties of mucus production under normal and abnormal conditions. Attempts at cultivation of

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type II alveolar cells, the major *in vivo* contributor of surfactant, have met with limited success (*3-5*). Type II cells in culture lose their specialized function and characteristic multilamellar bodies during early passage and these cultures are often contaminated with fibroblasts. Transformed cells which apparently arise during passage of the type II cell cultures have been investigated (*6*) but these cells are clearly different from the original parent cell type. In this paper we discuss selected properties of the mucus-secreting cell which we have isolated and passaged in culture for the first time (*2*) and report on the application of similar methodology to derive from hamster lung tissue proliferating cells which maintain a specialized capacity to synthesize and secrete surfactant.

Materials and Methods

Respiratory Cell Cultures

Hamster tracheal cells were isolated by the Dissociated Cell Rescue technique previously described (*2*) and maintained as monolayer cultures in nutrient mixture F-12 (GIBCO, Grand Island, N. Y.) with 10% fetal calf serum (FCS, GIBCO) plus penicillin G (100 units/ml) and streptomycin sulfate (100 μg/ml). This isolation procedure requires repeated protease treatment of tracheal rings and collection of dissociated cells by sequential gradient centrifugation.

Primary cultures of hamster lung epithelial cells were prepared by a similar isolation procedure using tissue from a newborn litter of Syrian Golden hamsters (Engle Laboratory Animals, Farmerburg, Ind.) less than 1 day of age. Lungs were pooled, rinsed several times in nutrient mixture F-12 containing 10% FCS and antibiotics, minced into smaller fragments prior to repeated protease treatment and gradient centrifugation. Dissociated cells were suspended in F-12 medium containing 10% FCS and supplemented with vitamin A (2 μg/ml, Sigma Chemical Co., St. Louis, Mo.) and epidermal growth factor (10 ng/ml, Collaborative Research Inc., Waltham, Mass.) during initial growth. These lung-originated cells as well as the initial tracheal cells were seeded in appropriate tissue culture vessels and grown to confluence with half-medium changes at 2-day intervals. The latter procedure which provided cells with half-conditioned and half-fresh medium during the earliest stages of growth was beneficial for monolayer formation. Cloning of lung cells was accomplished by releasing cells from the vessel surface during primary culture with 0.02% ethylenediaminetetraacetate (EDTA) in phosphate-buffered saline (PBS, pH 7.8) and plating them at low density in 100 mm petri dishes. Individual penicylinders were placed over isolated cell growth and sealed with silicone grease. Cells were washed in PBS and treated with EDTA-PBS prior to transfer to culture vessels containing fresh growth medium. All subsequent growth and passage of cells occurred in nutrient mixture F-12 containing 15% FCS plus antibiotics. These lung epithelial cell cultures were maintained for five to seven passages after which cells increased in size, became more granular and failed to divide. On one occasion, a transformed cell clone spontaneously emerged during this senescence of the epithelial cultures. Established lines of lung cells from rat (L2), cat (AKD), and human (A549) reported to be of type II alveolar cell origin were obtained from the American Type Culture Collection, Rockville, Md. These cells were routinely grown in F-12 containing 10% FCS with antibiotics. Generally all cultures were kept at 37°C in an atmosphere of 5% CO_2 in air.

Other Cell Cultures:

Primary cultures of chick embryo cells were prepared in Minimum Essential Medium (MEM, GIBCO) plus 10% FCS as previously described (*7*) and used during the third passage. Baby hamster kidney (BHK) and mouse (L25) established cell lines were also maintained in this medium. These cells were examined for their ability to synthesize phosphatidylcholine and derivatives.

Radioisotopes:

N-Acetyl-D-galactosamine (acetyl-^{3}H, 1.5 Ci/mmole) and L-serine (U-^{14}C, 156 mCi/mmole) were purchased from ICN Radioisotope Division, Irvine, Calif. Choline chloride (methyl-^{3}H, 69.5 Ci/mmole) was supplied by New England Nuclear, Boston, Mass.

Detection of Glycoprotein Secretion

Hamster tracheal cells suspended in MEM with 10% FCS were seeded in MultiWell (Falcon; Oxnard, CA) plates (0.5 ml volume/well, 150,000 cells/ml), and monolayer cultures were later radiolabeled with [^{3}H]*N*-acetyl-D-galactosamine (2.4 μCi/well) and [^{14}C]L-serine (0.8 μCi/well) for 24 hr. The culture fluid was removed from each well, clarified by centrifugation, and precipitated with 10% trichloroacetic acid prior to sodium dodecyl sulfate-polyacrylamide gel electrophoresis as earlier detailed (*2*). Gels were sliced into 1 mm fractions and radioactivity determined by liquid scintillation spectrometry.

Phospholipid Bioassay

Synthesis of lecithin (phospholipid) and its derivatives was measured by incorporation of radioactive choline into phosphatidylcholine. For this biosynthetic determination, all cell types including lung cell cultures normally passaged in F-12 medium were grown to confluence in MEM containing 10% FCS. Cell monolayers were washed once with serum-free MEM and incubated under the latter conditions for 20 hr with 1.0 μCi ^{3}H-choline per 75 cm^{2}flask. Monolayers were then washed twice with PBS, gently trypsinized and total cell numbers determined by counting with a hemacytometer. Cells were centrifuged at 400*g* for 10 min and each pellet transferred to a Potter Elvehjem tube and homogenized for 10 min at room temperature in a 2:1 chloroform-methanol mixture (v/v) according to the Folch method (*8*). The extract was mixed with one-fifth its volume of 0.05% $CaCl_2$ and allowed to separate into two phases at 4°C overnight. The upper phase and interphase were discarded. The lower phase was carefully washed three times with upper phase solvent. The lipid sample was evaporated to dryness under a stream of nitrogen and redissolved in 2:1 chloroform-methanol mixture (v/v). All samples were stored at −20°C.

To compare total phospholipid synthesis with secretion among the various lung cell cultures, each cell type was seeded in 50 cm^2 Petri dishes and grown as described above. At 24 hr after addition of radioactive choline, cells were separated from the culture fluid and lipids extracted from the cell fraction by the Folch procedure. The fluid containing secreted phospholipid was mixed with 1 volume of methanol and 2 volumes of chloroform, shaken well and placed at −20°C overnight. Lipids in the lower phase were isolated as previously indicated.

Phospholipid Separation by Thin-Layer Chromatography

Lecithin was identified by one-dimensional thin-layer chromatography (TLC). Radioactive samples were applied to a 20 × 20 cm precoated Silica Gel 60 TLC plate (0.25 mm thickness). Dipalmitoyl phosphatidylcholine (Sigma Chemical Co., St. Louis, Mo.) and a mixture of lecithin and sphingomyelin (Sigma Chemical Co., St. Louis, Mo.) were routinely included as standards. Plates were developed for 90 min with a 65:25:4 chloroform-methanol-water mixture (v/v) and dried for 30 min at room temperature. Spots were stained by 2,7-dichlorofluorescein (Sigma Chemical Co., St. Louis, Mo.) dissolved in acetone and detected by ultraviolet light illumination. The spot correlating with the R_f value of lecithin was scraped into ACS scintillation fluid (Amersham, Arlington Heights, Ill.) for radioactive determination.

Isolation of Disaturated Phosphatidylcholine

Since lecithin and its derivatives have similar R_f values and are difficult to separate by TLC, the method of Mason et al. (*9*) was employed to isolate disaturated phosphatidylcholine (DPC). In general, the lipid sample was reacted with 3.1 mg osmium tetroxide (Sigma Chemical Co., St. Louis, Mo.) in 0.5 ml of carbon tetrachloride for 15 min at room temperature. This material was evaporated to dryness under a stream of nitrogen and redissolved in 0.5 ml of 20:1 chloroform-methanol (v/v). Individual samples were then placed on an aluminum oxide (100-200 mesh, Bio-Rad Laboratories, Richmond, Calif.) column and neutral lipids were eluted from the column with 20:1 chloroform-methanol (v/v). DPC was then released with 70:30:2 chloroform-methanol-7*M* ammonium hydroxide (v/v). The total amount of DPC in the original sample was measured by counting an aliquot in ACS scintillation fluid. Values were expressed as cpm per 10^6 cells.

Fluorescence Microscopy

Confluent cell monolayers were established on 12 mm cover slips in MultiWell vessels. Cell preparations were washed once with PBS and stained for 2 to 5 min in the dark with phosphine 3R at a concentration of 1 μg/ml of PBS. Cover slips were drained and placed on slides with the cell side facing downward. Cells were examined for the presence of fluorescent inclusion bodies in the cytoplasm, typically observed in type II alveolar epithelial cells (*10*).

Transmission Electron Microscopy

Monolayer cultures grown to confluence in MEM supplemented with 10% FCS were fixed and processed by the method of Stratton (*11*) to observe multilamellar bodies. Cells were fixed with 3% glutaraldehyde in 0.1*M* cacodylate buffer, pH 7.3 for 3 hr at room temperature. Cells were then scraped directly into the fixative without rinsing and centrifuged at 400 *g* for 10 min. The cell pellet was resuspended in 1% tannic acid and 1% glutaraldehyde in 0.1*M* cacodylate buffer and treated for 2 hr at room temperature. Cells were then centrifuged, treated with 1% osmium tetroxide in 0.1*M* cacodylate buffer for 2 hr, and exposed to a series of acetone dehydration steps prior to embedding in Epon and sectioning.

Results

Morphology of Hamster Tracheal Cells

After selective dissociation of tracheal tissue as previously indicated, specific cells attach to the vessel surface and appear to undergo extensive structural modulation during the first week of growth. Figure 1 is representative of the homogeneous cell population that arises during *in vitro* incubation. Note the considerable network of cytoplasmic granules (Fig. 1*a*). When the outer surfaces of individual cells are observed by interference contrast microscopy, prominent packets which apparently derive from active cell secretion are detected (Fig. 1*b*). These packets selectively react with histochemical stains for acidic mucins (*2*).

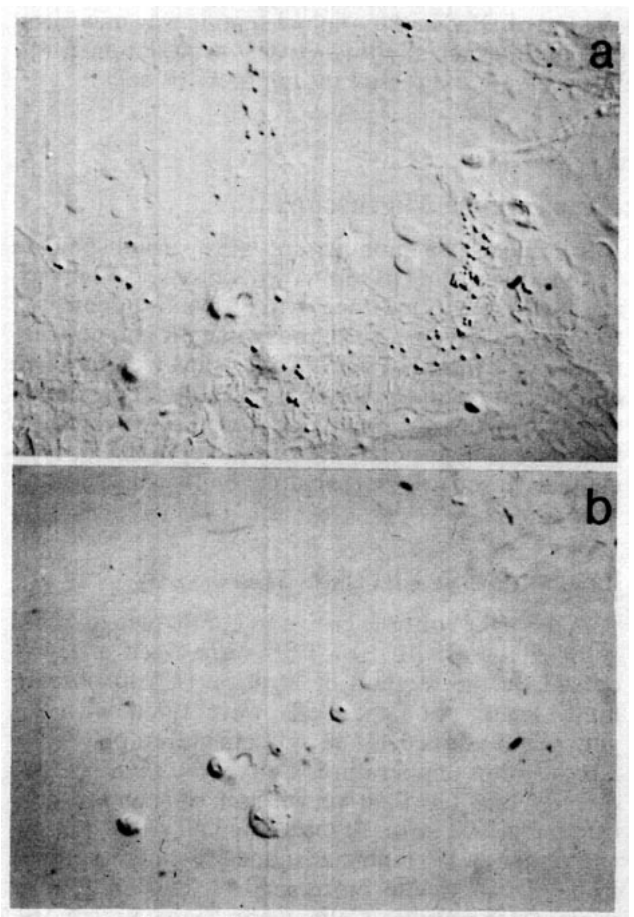

FIGURE 1. Cytoplasmic granules and secretory packets associated with monolayer cultures of hamster tracheal cells. Packets are visualized in cells by focusing in different planes using interference contrast microscopy; (*a*) the image in focal plane of the cytoplasm; (*b*) a view just above the cell surface. × 850.

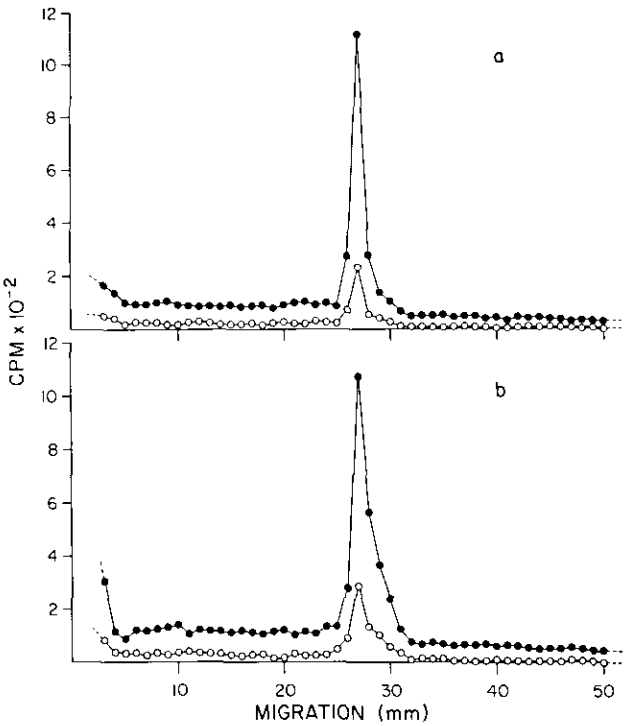

FIGURE 2. Glycoprotein synthesis by hamster tracheal rings and cultured tracheal cells. Profiles display the radioactivity from (●) ^{14}C-L-serine and (○) ^{3}H-*N*-acetyl-D-galactosamine in 1 mm slices of SDS-polyacrylamide gels: (*a*) secretion collected from a pair of hamster tracheal rings in organ culture after 24 hr of incubation with radiolabeled precursors; (*b*) secretion collected from a monolayer culture of tracheal cells incubated under similar conditions.

Glycoprotein Synthesis by Tracheal Cell Monolayers

Consistent with the histochemistry and tissue origin of these monolayer cultures, cells were readily capable of incorporating radioactive amino acid and sugar precursors into macromolecules which were then secreted into the medium. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of concentrated culture medium revealed a single radioactive peak with a molecular weight of about 18,500 daltons (Fig. 2*b*). Tracheal ring organ cultures were radiolabeled under similar conditions, and an identical gel profile was obtained (Fig. 2*a*). The influence of vitamin A on the morphological and biochemical properties of this cell-type was also examined. Absence of the vitamin from culture medium dramatically decreased both the number of cytoplasmic granules (Fig. 3) and glycoprotein secretion (Table 1) in monolayer cultures correlating with the putative *in vivo* role of vitamin A (*12*).

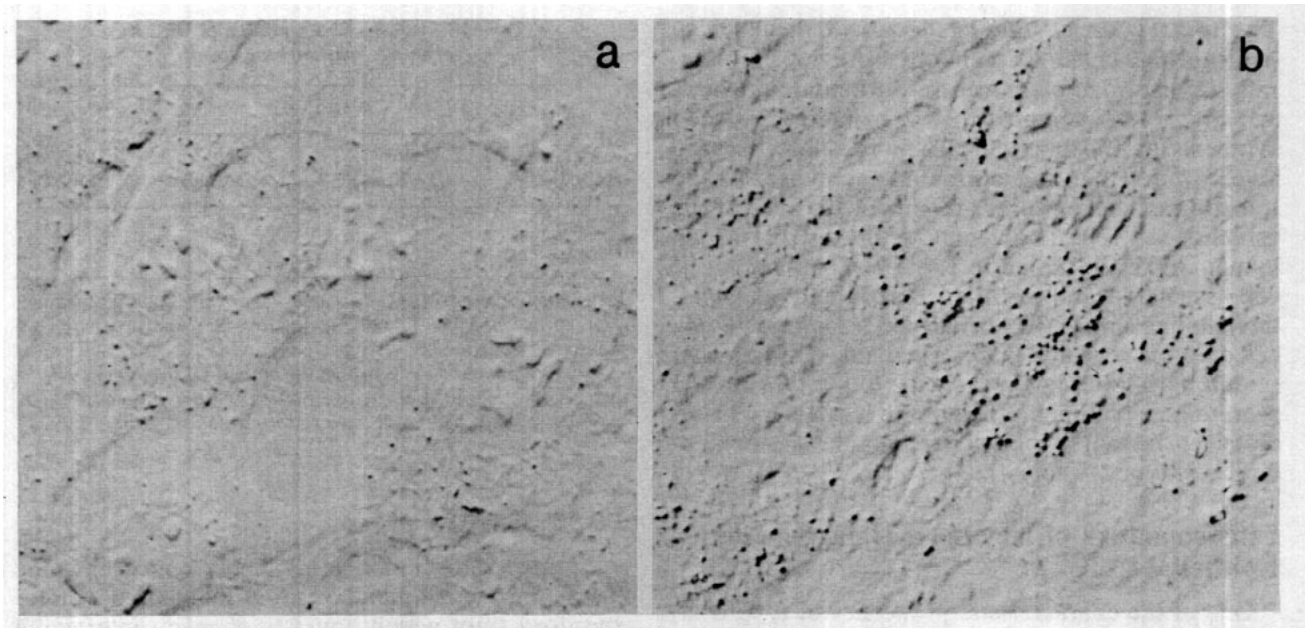

FIGURE 3. Effect of vitamin A on numbers of intracellular granules in cultured hamster tracheal cells: (*a*) cells grown for 48 hr after removal of vitamin A from culture medium; (*b*) cells maintained continuously in medium containing vitamin A (2 μg/ml). Interference contrast microscopy. × 675.

Table 1. Stimulation of glycoprotein secretion by vitamin A.

Test system[a]	Radioactivity, per 50 μg cell protein[b]	
	^{3}H	^{14}C
− Vitamin A	291.0 ± 16.4	2313.7 ± 192.5
+ Vitamin A	835.3 ± 120.6	4108.9 ± 286.2

[a]Cells were grown in MEM + 10% FCS with or without vitamin A for 72 hr prior to addition of radioisotope for 24 hr.
[b]Based upon incorporation of *N*-acetyl-D-galactosamine (acetyl-^{3}H) and L-serine (U-^{14}C) into a secreted glycoprotein. Values were calculated geometrically from cpm in the single radioactive peak on SDS-polyacrylamide gel, and are reported as the mean ± 1 standard deviation from the mean.

Phospholipid Synthesis Among Various Cell Types

Cell monolayers of newborn hamster lung epithelium were monitored for their ability to synthesize lecithin and disaturated phosphatidylcholine. Most of the biochemical measurements were performed on cultures during the third to seventh *in vitro* passage. As seen in Table 2, these cells readily incorporated ^{3}H-choline into phospholipid. Other animal cell cultures were also included in this study since it was important to establish the biologic specialization of the newborn lung epithelial cells. A significant range in the capacity of the various cultures to synthesize lecithin and disaturated phosphatidylcholine was observed. However, it was clear that the hamster lung cultures were most proficient in this bioassay.

Table 2. Incorporation of choline chloride (methyl-^{3}H) into lecithin and disaturated phosphatidylcholine (DPC) by different animal cells.

Cell type[a]	Activity, cpm × $10^{-4}/10^{6}$ cells	
	Lecithin[b]	DPC[c]
Newborn hamster lung epithelium	114.0	19.6
Rat (L2)	68.2	11.6
Mouse (L25)	33.2	4.0
Cat (AKD)	30.0	3.3
Baby hamster kidney	24.9	4.7
Human A549	15.9	2.7
Chick embryo fibroblast	12.3	2.1

[a]Confluent cell cultures were radiolabeled for 20 hr in serum-free MEM.
[b]Lipids extracted from cells were separated by thin-layer chromatography. The appropriate spot correlating with the R_f value of lecithin was removed and counted by scintillation spectrometry.
[c]DPC was isolated from the lipid mixture by aluminum oxide chromatography after treatment of sample with osmium tetroxide.

Phospholipid Synthesis and Secretion by Normal and Transformed Lung Cells

During early cultivation (three to five passages) of hamster lung epithelial cells, increased ability to synthesize total phospholipid and disaturated phosphatidylcholine was demonstrated (Table 3). This observation appeared consistent with increases in both cell size and numbers of cytoplasmic granules. All cells examined in Table 2 with the exception of chick embryo fibroblasts stained with phosphine 3R.

The most intense staining occurred in newborn hamster lung cells. As mentioned previously, naturally-transformed clones arose during late passage, and these cells demonstrated a markedly reduced synthesis of phospholipid (Table 3). Note that the levels of lecithin and surfactant synthesis in the transformed cell cultures are similar to those of the established "type II alveolar" cultures (cat-AKD, human-A549) presented in Table 2. Comparisons of cell-associated and secreted disaturated phosphatidylcholine among the normal and transformed lung cell cultures revealed that normal lung cultures possessed a 10-fold higher capacity to synthesize and secrete phospholipid although the percentage of secreted surfactant to total phospholipid was similar in both cell-types (Table 4).

Ultrastructure of Newborn Hamster Lung Epithelial Cells

One of the characteristic properties of type II alveolar cells is the existence of multilamellar bodies randomly distributed throughout the cytoplasm. These organelles serve as the source of surfactant, and alveolus function is dependent upon their formation and secretion into the alveolar space. In Figure 4*a*, a classical membrane-limited lamellar body is observed in close association with Golgi vesicles, the latter probably being responsible for the generation of the lamellar structures. A lamellar body with its highly ordered structure appears at the cell surface (Fig. 4*b*) apparently after its release from the cytoplasm by a specialized type of exocytosis (*13*). Transformed lung cells derived from the hamster epithelial cultures also possessed similar multilamellar structures which were readily secreted. It is of interest that all other cell types (Table 2) examined by electron microscopy demonstrated cytoplasmic multilamellar bodies similar to those of the hamster lung epithelial cells.

Table 3. Phospholipid synthesis by normal and transformed newborn hamster lung cells.[a]

Lung epithelium	Passage no.	Activity, cpm × $10^{-4}/10^6$ cells	
		Lecithin	DPC
Normal	3	46.9	6.1
	4	57.0	11.6
	5	110.4	16.0
Transformed	9	35.0	4.2
	40	24.7	4.7

[a]Experimental conditions are as in legend of Table 2.

Table 4. Synthesis and secretion of DPC by newborn hamster lung cells.

Lung epithelium	DPC, %[a]		Total cpm[b]
	Cell-associated	Cell-secreted	
Normal	71	29	13.0
Transformed	67	33	1.4

[a]Lipids were extracted separately from cells and culture fluid after 24 hr exposure to choline (methyl-^{3}H) and processed as previously described.

[b]Total radioactivity represents the sum of DPC counts ($\times 10^{-4}$) in the cells and culture media fractions after aluminum oxide chromatography. All values are reported per 10^6 cells.

Discussion

Limited information is available concerning the biochemistry and metabolism of the mammalian respiratory tract which consists of many interacting cell types and related organs. The cellular heterogeneity and biologic complexity of the tracheobronchial epithelium have prevented the establishment in culture of pure populations of normal differentiated cells for monitoring respiratory function under various experimental conditions. Tissue slices, organ explants, primary and organotypic cultures, and selected cell purification procedures have offered useful models for examining pulmonary function. However these systems do not provide homogeneous populations of epithelial cells that replicate *in vitro* with retained biochemical and ultrastructural specialization.

In this report we define selected properties of hamster respiratory cells readily passaged *in vitro*. Replicating monolayer cultures comprised of homogeneous populations of epithelial cells were obtained from trachea and lung tissues by a reliable technique previously described (*2*) and examined for their ability to synthesize and secrete mucus and surfactant respectively. Evidence is presented to support the biosynthetic proficiency and uniqueness of these cell types.

Tracheal cell monolayers incorporated radioactive serine and *N*-acetyl-D-galactosamine into macromolecules with a molecular weight of approximately 18,500 daltons based upon SDS-polyacrylamide gel electrophoresis. Exposure of tracheal cell cultures to vitamin A stimulated cytoplasmic granule content and enhanced glycoprotein secretion. This information, along with previously described biochemical and histochemical data, suggests the potential application of this model system for investigating fundamental properties of mucus-secreting cells.

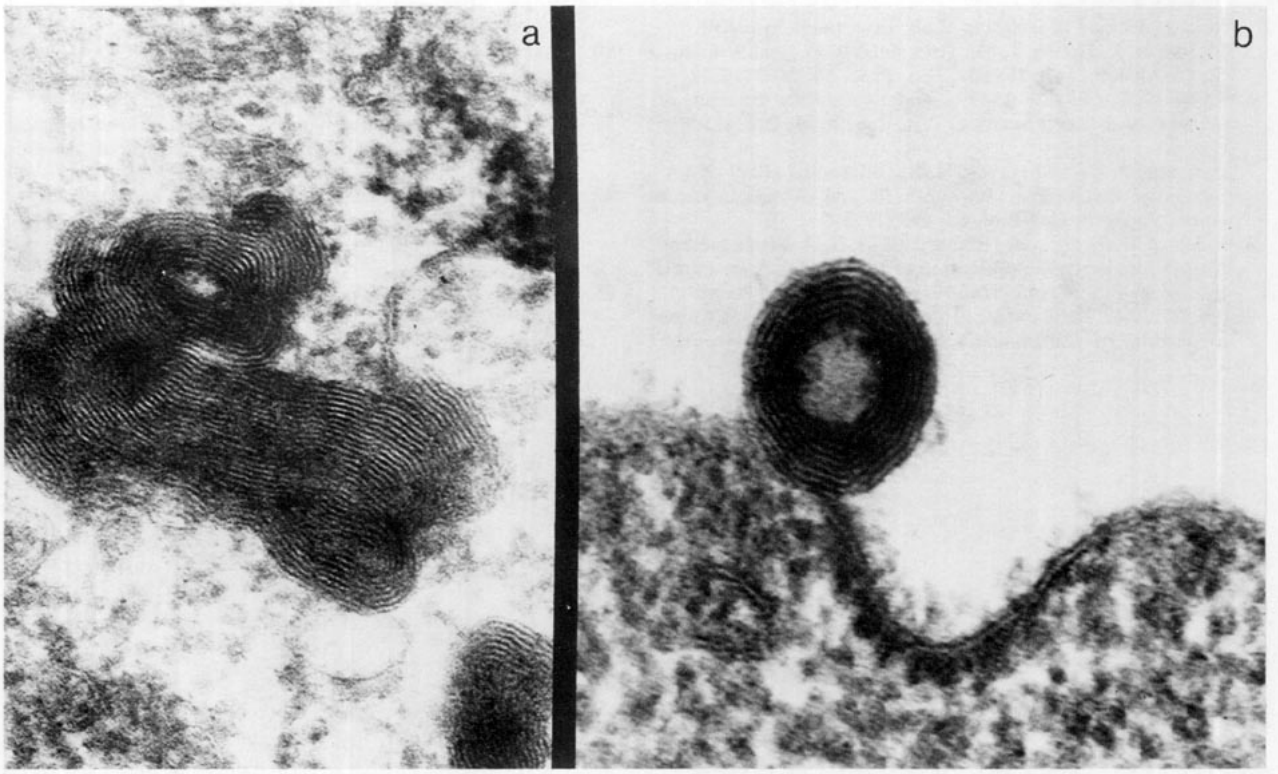

FIGURE 4. Transmission electron photomicrograph of newborn hamster lung epithelial cells during *in vitro* passage: (*a*) thin section a mature multilamellar body; note the close association of Golgi vesicles (× 166,870); (*b*) a small compact multilamellar structure is associated with the cell surface, apparently originating via exocytosis (× 207,570). Similar ultrastructure was observed in all hamster lung cell passages including transformed cell cultures.

Characteristics of newborn lung epithelial cell monolayers described here reinforce their metabolic specialization. Synthesis of phospholipid and more specifically disaturated phosphatidylcholine, considered a major component of lung surface-active material, was greatest in these cultures when compared to a variety of other animal cell types. Electron microscopy of newborn hamster lung cells passaged in monolayer for 4 to 8 weeks demonstrated the classical multilamellar bodies of type II alveolar cells. The proximity of these organelles to Golgi vesicles and the intense phosphine 3R staining of the cells supported their biologic identity with type II cells. The relationship between early passage lung cultures and late transformed cell clones revealed significant differences in capacity to synthesize surfactant. Transformed cells were much less biosynthetically active in terms of phosphatidylcholine synthesis although prominent cytoplasmic multilamellar bodies were still visualized.

Homogeneous cell populations of respiratory epithelium with retained capacity to synthesize and secrete mucus or surfactant can be experimentally manipulated providing useful models for pulmonary research. Growth regulation and cellular differentiation can be measured in the presence of various stimuli or insults such as hormones and drugs, infectious agents, nutritional deficiencies, carcinogens, and pollutants. Chemical and physical properties of mucus glycoproteins and surfactant can be analyzed under a range of test conditions. Such studies should clarify the biologic competence of the tracheobronchial epithelium in maintaining pulmonary function and permit the identification of factors that alter the apparent normalcy of these respiratory cells.

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