

Pathways of Differentiation of Airway Epithelial Cells

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The question being examined is whether one or more morphologically distinct cell types can be identified in the conducting airways of adult rabbits possessing stem cell functions. The term "stem cell" is used to denote cells with extensive self-replicating potential and the ability to produce differentiated progeny. According to various models of cell renewal in the conducting airways that have been proposed over the years, two different cell types have to be regarded as primary candidates for the stem cell: basal cells and some type of secretory cells. The question is complicated by the fact that significant differences exist between species in the distribution and morphological characteristics of airway cell types. In addition, different airway segments may or may not be occupied by different populations of stem cells. Previously, investigators have addressed the problem by studying normal cell regeneration or injury induced cell regeneration *in vivo* in the whole animal. We decided to attempt a different approach, namely, to separate specific cell types and to study the proliferation and differentiation capacity of such cell isolates using *in vitro* and *in vivo* cell culture techniques. Our studies lead us to conclude that the conducting airways of adult rabbits contain at least two distinct cell populations endowed with stem cell potential, namely basal cells and bronchiolar Clara cells. From that it follows that the trachea and bronchi, on one hand, and the bronchioles, on the other hand, are occupied by two different stem cell populations governing renewal of the epithelial lining.

Introduction

The epithelium of the conducting airways normally has a rather low cell turnover rate when compared with other surface epithelia such as the epidermis and the lining of the intestinal tract. It is composed of five to six major cell types: basal cells, mucous and serous cells, Clara cells which in most species are confined to the bronchioles, neuroendocrine cells (also called Kulchitsky cells), and ciliated cells. The latter have rarely been observed to undergo cell division and are usually regarded as being terminally differentiated, analogous to the fully differentiated keratinocytes of the epidermis.

The tracheobronchial mucosa is a target for a host of airborne toxic agents: bacterial and viral agents, allergens, reactive gases, aerosols, and irritant particles. Exposure to such agents commonly results in marked increases in cell proliferation and in transient or long-lasting changes in differentiation, so-called mucous or squamous metaplasias. The latter are typically seen in

wound healing after exposure to carcinogens and during severe retinoid deficiency.

Our laboratory is engaged in studies concerning the regulation of proliferation and differentiation of airway epithelium and also cellular pathways of differentiation that determine the progenitor-progeny relationship between the different cell types (1-3). It is hoped that these investigations will provide information needed for an understanding of the responses of airway epithelium to toxic agents as well as of the events leading to neoplastic transformation. It is of particular importance to determine which cells in the respiratory epithelium have stem cell properties, since stem cells may be the major transformable target for carcinogens (4). Thus it is conceivable that all of the different histological types of bronchogenic carcinoma originate from one cell type, provided the respiratory epithelium contains only one cell type with stem cell potential.

Currently, two simple models of cell renewal in mature tracheobronchial epithelium exist. The older model postulates that the basal cell acts as the principle stem cell in the conducting airways, giving rise to the various secretory cells and ciliated cells. It is largely based on cell turnover studies of normal respiratory epithelium using ^3H -thymidine as a marker of cell proliferation (5-7). A new model proposed by McDowell et al. and supported by others (8-11) postulates the secretory cell to

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be the pivotal cell from which most other cells develop, including squamous cells arising in the bronchial mucosa during severe retinoid deficiency (10). The evidence on which this model is based stems from studies of regeneration of chemically or physically injured epithelium (and from vitamin A deficiency studies) which suggested that basal cells only generate basal cells and that the secretory cells of the trachea and bronchus are responsible for regeneration of the epithelium (and for generating squamous cells during vitamin A deficiency). The function assigned to basal cells that are anchored to the basement membrane with hemidesmosomes is to provide attachment for secretory and ciliated cells (12). How Clara cells and neuroendocrine cells fit into either one of these two models is not clear.

In this presentation we will address the following three interrelated questions: *a*) Which cells in the adult tracheobronchial mucosa have stem cell properties? *b*) Is there more than one morphologically distinct cell type that possesses stem cell potential? *c*) Are the different airway segments populated by one and the same stem cell population? The term "stem cell" is used to designate cells having extensive self-renewal capacity and the ability to generate differentiated progeny. To date, our studies have concentrated on the differentiation potential of two cell types obtained from respiratory tract tissues of adult rabbits: tracheal basal cells and bronchiolar Clara cells (2,3,13,14). The pertinent findings will be summarized and discussed below.

Experimental Approach

The experimental approach we chose for our studies (2,3,13,14) consisted essentially of two parts. First, specific epithelial cell populations were isolated from rabbit tracheas and lungs, respectively. This was done by enzymatic dissociation of tracheal epithelium or whole lungs, followed by centrifugal elutriation, yielding tracheal basal cell fractions containing > 90% basal cells and pulmonary Clara cell fractions containing 85% Clara cells. (Separation of Clara cells involves first Percoll density gradient centrifugation and then centrifugal elutriation.) The cellular composition of these fractions was established by light and electron microscopy. In the second step the purified cell populations were tested in an *in vitro* culture system for their ability to proliferate and in an *in vivo* culture system for their ability to differentiate. The *in vivo* test was necessary since the available *in vitro* systems only support partial expression of differentiation of rabbit airway cells.

The *in vivo* culture system consists of tracheal grafts stripped of their own epithelium by repeated freezing and thawing; the tracheas were inoculated with the cell suspension to be tested and were then transplanted subcutaneously into nude mice (15). The tracheal grafts, which served as *in vivo* culture vessels, were examined at different time intervals after cell inoculation and transplantation for establishment of an epithelial lining resembling tracheobronchial or bronchiolar epithelium.

The cell types present in such reepithelialized tracheas are identified by their histochemical and ultrastructural characteristics.

In several experiments an additional cell purification step was inserted between part one and part two of the experimental design. Instead of inoculating aliquots of cell fractions into the tracheal grafts, single cell clones were derived *in vitro* from the cell fractions, and the clones were inoculated into the grafts. In this way the entire epithelial lining established in such tracheas was derived from a single cell.

Differentiation Capacity of Basal Cells

The purpose of these studies was to examine the potential of tracheal basal cells to proliferate and differentiate. Based on the newer model of airway cell differentiation proposed by McDowell and her colleagues (8-10), it was predicted that basal cells are able to proliferate and establish in tracheal grafts an epithelial lining consisting solely of basal cells.

Production and Characterization of Basal Cell Fractions and Basal Cell Clones

These experiments were conducted either with basal cell fractions or with single cell clones derived from basal cell fractions. Figure 1 shows that the cell separation procedure used in these studies very effectively isolated a fraction of small cells from tracheal cell suspensions. Electron micrographs made from pellets of such cell fractions showed small cells with high nuclear to cytoplasmic ratios (Plate 1). The scanty cytoplasm contained few organelles, tonofilament bundles were prominent, and the nuclei were indented and showed prominent heterochromatin. Differential cell counts made using electron micrographs and Papanicolaou-stained cytospin preparations showed that approximately 90% of the isolated cells were basal cells (Table 1).

We tested the ability of such basal cell fractions to grow in tissue culture and to form colonies when cultured at low clonal cell densities (determination of colony forming efficiency). This was done to examine the proliferative potential of basal cells, to compare it with that of unseparated tracheal cell suspensions, and to determine whether it is possible to raise single cell clones from basal cell fractions.

These experiments showed that basal cell fractions grew well in cell culture and had about the same colony forming efficiency as unseparated tracheal cells, indicating that basal cells were not the only proliferative tracheal cell type. Since the colony forming efficiency of basal cell fractions was as good as that of unseparated tracheal cell suspensions, namely, about 2 to 5% in most experiments, it seemed feasible to establish basal cell clones in culture and to use such clones in the tracheal reepithelialization studies to test the differentiation potential of basal cells. Since contamination of basal cell

Morphological Evaluation of Tracheal Grafts Repopulated with Basal Cell Fractions and Basal Cell Clones

The initial studies were performed with basal cell fractions (rather than basal cell clones) which were inoculated into tracheal grafts. Of 17 tracheas inoculated, 13 (76%) were reepithelialized with an epithelium containing basal cells, secretory cells, and ciliated cells, as determined by light and electron microscopy. The morphological findings of these experiments will be discussed together with the clonal studies, because the results of the two studies were the same in all respects.

A total of 39 basal cell clones and 25 clones derived from unseparated tracheal cells were inoculated into denuded tracheal grafts. Four weeks after transplantation, the grafts were examined by light and electron microscopy. As is evident from the data summarized in Table 2, there were no significant morphological differences between the two groups of tracheas inoculated with clones derived either from basal cell fractions or from unseparated tracheal cell suspensions. Seventy-two percent of the transplants were successfully reepithelialized. Secretory cells with small PAS-positive granules and typical basal cells were present in all of the reepithelialized grafts. Approximately 40% of the grafts also contained mucous goblet cells, i.e., cells with large, confluent, PAS-positive secretory granules (Plate 3). The light microscopic diagnoses were confirmed by electron microscopy (Plates 4 and 5), which demonstrated the presence of goblet cells and a variety of secretory cells rich in rough endoplasmic reticulum and sometimes in smooth endoplasmic reticulum and which contained mostly small electron lucent mucous granules. These granules were positive with the PA-TCH-SP reaction, which stains neutral glycoproteins and typical goblet cells (Plate 6). Roughly 80% of the reepithelialized tracheal transplants showed ciliated cells as well. We con-

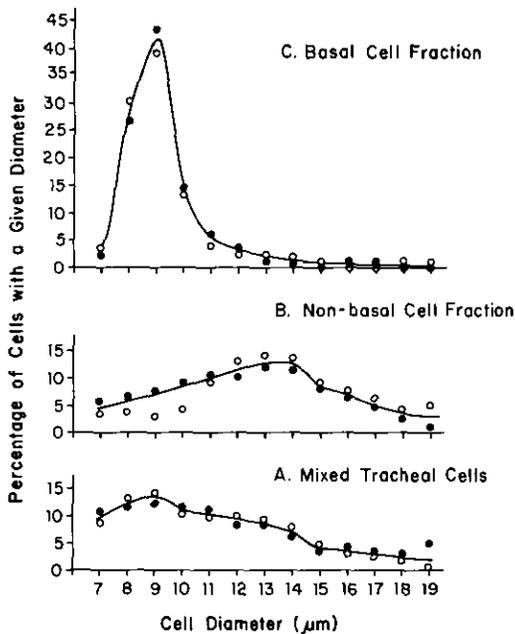


FIGURE 1. Cell size profiles of (A) unseparated tracheal cells, (B) a nonbasal cell fraction (i.e., a cell fraction not enriched in basal cells), and (C) a basal cell fraction. Channelized cell counts were grouped into 1- μ m diameter size classes, totaled and normalized to 100%. Two separate experiments (○, ●). From Inayama et al. (14).

fractions with nonbasal cells was $\leq 10\%$, we could be confident that 9 out of 10 clones were derived from a basal cell.

Plate 2 shows several basal cell clones that have been grown in culture for 2 weeks. Clones established from basal cell fractions and from unseparated tracheal cell suspensions grew equally well (Fig. 2) and underwent 15 to 20 population doublings. The clones were isolated and inoculated individually into tracheal grafts 9 to 22 days after starting the cultures.

Table 1. Differential cell counts of suspensions inoculated into tracheal grafts.^a

Cell types	Basal cell fractions			Mixed tracheal cell suspension	
	LM ^b		TEM ^b	LM	TEM
	Experiment 1a	Experiment 1b	Experiment 1b	Experiment 1a	
Ciliated	0.4	1.2	0.0	44.0	25.0
Mucous	0.0	0.0	0.9	9.1	10.8
Clara-like	2.4	2.9	1.4	7.0	10.8
Basal	94.0	91.8	83.1	34.0	35.8
Undetermined epithelial	3.2	4.1	7.5	5.9	14.9
Inflammatory	0.0	0.0	7.1	0.0	2.7
Number of cells counted	513	486	213	341	148

^aDifferential cell counts are given in percent. In experiment 1a the basal cell fraction was examined by light microscopy and the mixed tracheal cell suspension by light as well as by electron microscopy. In experiment 1b only the basal cell fraction was studied; it was examined by light as well as by electron microscopy.

^bLM, light microscopy cell count made on cytocentrifuge preparation stained with either Papanicolaou stain or NBT stain. TEM, cell count made on sections of cell pellets embedded in Epon and viewed with transmission electron microscope. From Inayama et al. (3).

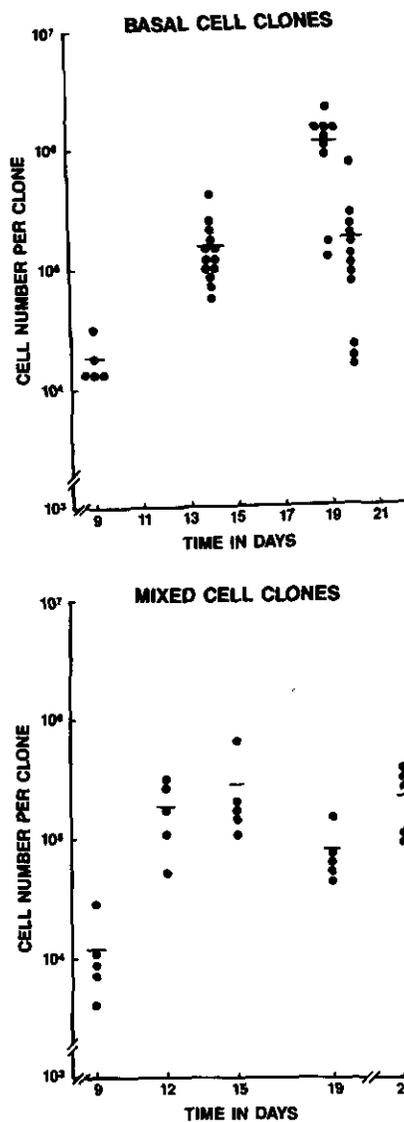


FIGURE 2. Growth of basal cell clones and clones derived from mixed tracheal cells. A total of 39 basal cell clones (A) and 25 tracheal cell clones (B) were obtained by seeding 2×10^2 cells of either type into culture dishes containing irradiated feeder cells. At the indicated times the feeder cells were removed, the clones were dispersed, and the number of cells per clone was determined. Points indicate the size of individual clones in terms of number of cells per clone; horizontal bars indicate the mean cell number per clone for all clones at the given time points. From Inayama et al. (14).

cluded that basal cells isolated from tracheas of rabbits have the potential to self-generate and to produce ciliated and secretory cell progeny.

Squamous Differentiation of Basal Cells

The cells of the tracheobronchial epithelium can differentiate along two distinct pathways, namely, the pathways of mucociliary differentiation and squamous differentiation. So-called squamous metaplasias can develop as a result of injury or during severe vitamin A deficiency (8-10). The cellular evolution of such metaplasias is not well understood. One possibility is that squamous cells originate from basal cells. Previous studies (1,16) showed that tracheal cells of rabbits undergo squamous differentiation *in vitro* when cultured in vitamin A-free media. They become flat, stratified, and form cross-linked envelopes—the end product of terminal differentiation of keratinocytes. Their keratin profile changes and two biochemical markers of squamous differentiation, namely, epidermal transglutaminase (17) and cholesterol sulfate (18) are dramatically increased. We wanted to determine whether highly purified tracheal basal cells, when cultured under the same conditions, undergo similar changes characteristic of squamous differentiation. The results of these studies are summarized in Tables 3 and 4. Early basal cell cultures showed very little in the way of expressing squamous cell markers. However, after several days of culture in retinoid-free media, the cultures produced a large number of cross-linked envelopes; in addition, cholesterol sulfate levels and transglutaminase activity increased markedly.

Similar to cultures of unfractonated tracheal cells (1,16), the vitamin A-depleted basal cell cultures showed characteristic changes in keratin profiles. In addition to the keratins with molecular weights of 40, 50, and 58 kD, two keratins with molecular weights of 48 and 56 kD were expressed. Importantly, Clara cells did not show this change in keratin expression. These experiments clearly showed that in the absence of vitamin A, basal cells can undergo squamous differentiation. Whether other cells such as secretory cells can undergo similar changes in differentiation remains to be tested.

Table 2. Histological findings in tracheal grafts inoculated with cell clones derived either from basal cells or from unseparated tracheal cells.^a

	No. of tracheas reepithelialized	No. of tracheas inoculated	Percentage of reepithelialized tracheal grafts revealing		
			Ciliated cells	Secretory cells ^b	
				Cells with small secretory granules	Goblet cells
Basal cell clones	28/39	71.8%	82.1	100	39.3
Tracheal cell clones	18/25	72.0%	77.8	100	38.9

^aCells from each clone, 2 to 5×10^4 , were inoculated per tracheal graft. Four weeks after transplantation the grafts were removed from the hosts, processed, and stained with Alcian blue (pH 2.5)-PAS. Two to three different tracheal rings were examined per trachea.

^bTwo types of secretory cells were recognized by light microscopy: cells with small discrete Alcian blue and/or PAS positive granules and/or a thick apical rim of purple or magenta staining cytoplasm; cells with abundant large granules, typical goblet cells. From Inayama et al. (14).

Table 3. Expression of differentiation markers in cultured tracheal basal cells during the exponential and confluent growth phase.

Parameter tested	Exponential phase	Confluent phase
CFE ^a	14.8	< 0.1
Transglutaminase Type I, dpm/hr/ μ g protein	2.1	57.2
Cholesterol sulfate, dpm/ μ g protein	0.5	78.3
Cross-linked envelope formation, % of total cells	1	81

^aCFE, colony forming efficiency.

Table 4. Comparison of keratin expression in tracheal basal cells and bronchiolar Clara cells isolated from the rabbit.

Keratin, mw	Immunoreactivity	Basal cell ^a		Clara cell ^a	
		A	B	A	B
58	AE3	+	+	-	-
56	AE3	-	+	-	-
54	AE3	-	-	+	+
50	AE3	-	-	+	+
50	AE1	+	+	-	-
48	AE1	-	+	-	-
46		-	-	+	+
45		-	-	+	+
40	AE1	+	+	+	+

^aA and B designate freshly isolated and cultured cells, respectively.

Differentiation Capacity of Pulmonary Clara Cells

Clara cells are a unique pulmonary cell type which, in most mammalian species, occurs only in the small bronchioles (7,19). However, in rabbits and hamsters, Clara cells or Claralike cells have been reported to occur also in the large airways (20). For the sake of clarity we will refer to bronchiolar, nonciliated secretory cells as Clara cells and to tracheal cells with morphological features similar to those of Clara cells as Claralike cells. The most characteristic morphological features of Clara cells are as follows: abundant smooth endoplasmic reticulum, small electron-dense secretory granules that do not react with mucous stains, and a low cuboidal shape—as opposed to the tall columnar shape of secretory cells in the tracheobronchial tree (7,19,21). The Clara cell has been shown to be rich in enzymes involved in drug metabolism (22-24). In addition, this cell appears to play a key role in maintaining the integrity of the bronchiolar epithelium and has been reported to be responsible for regeneration of that epithelium following toxic injury (25).

The purpose of the studies described in this paper was to examine the proliferation and differentiation capacity of the Clara cells. Since Clara cells do not grow sufficiently *in vitro* to derive single cell clones, these studies were conducted with Clara cell fractions (2,13).

Characterization of Clara Cell Fractions

Clara cell fractions obtained from whole lung digests contained between 80 to 85% Clara cells, which were

Table 5. Composition of cell preparations.

Cell type	Lung cells	Clara cell fraction
Clara ^a	4.1 \pm 2.3	84.1 \pm 4.7
Type II ^b	33.0 \pm 5.1	4.4 \pm 2.3
Basal ^b	1.5 \pm 0.7	0.6 \pm 0.7
Ciliated ^b	1.2 \pm 0.6	0.4 \pm 0.2
Goblet ^b	0.8 \pm 0.0	1.3 \pm 1.3
Macrophages ^c	2.7 \pm 1.25	0.6 \pm 0.7
Granulocytes ^c	18.4 \pm 1.2	9.0 \pm 2.8
Miscellaneous ^d	38.4 \pm 7.8	0.3 \pm 0.8

^aCells identified by NBT stain.

^bCells identified by Papanicolaou stain.

^cCells identified by Diff-Quick-Wright-Giemsa stain.

^dUnidentified. From Hook et al. (13).

identified at the light microscopic level either with the nitro-blue tetrazolium reductase reaction or by electron microscopy (Table 5 and Plate 7). The isolated Clara cells showed the typical oval electron-dense granules and abundant smooth endoplasmic reticulum characteristic of Clara cells (Plate 8). We believe that these cells are bronchiolar Clara cells and not tracheal Claralike cells, which are tall columnar in shape, rather than low cuboidal, because the elutriation procedure employed did not separate out Claralike cells from tracheal cell suspensions.

Evaluation of Tracheal Grafts Repopulated with Clara Cells

Tracheal grafts repopulated with Clara cell fractions or with unseparated tracheal cells (which were used for comparative purposes) were examined morphologically at several different time points after transplantation. Both cell preparations regularly reepithelialized denuded tracheal grafts. The epithelia established with tracheal cells were strikingly different from epithelia established with bronchiolar Clara cells at 2 weeks, and even more so at 4 and 14 weeks after transplantation (2). Grafts inoculated with tracheal cells were lined with a tall columnar, pseudostratified epithelium (Plate 9A,B); basal cells, mucous cells, and ciliated cells were abundant and could be identified at the light and electron microscopic level. In sharp contrast, the epithelium established with Clara cell fractions was low columnar and showed a single layer of low cuboidal cells at all time points (Plate 10A,B). Only two cell types could be identified in this epithelium, which was very reminiscent, even at low magnification, of bronchiolar epithelium, namely Clara cells with abundant smooth endoplasmic reticulum, small electron-dense secretory granules, and ciliated cells. Both cell types were low cuboidal in shape. No mucous cells or basal cells could be identified in any of the sections.

We concluded that Clara cells had sufficient proliferative capacity to reepithelialize tracheal grafts, and were able to self-generate and to produce ciliated cells. However, under the conditions tested, they were unable to generate either basal or mucous cells.

Discussion

Before discussing the possible implications of this work, we should point out two significant limitations of the information presented. The first and most obvious is the absence of data on tracheal secretory cells. With the techniques used at present, we have not been able to produce cell fractions highly enriched with tracheal secretory cells. There seems little doubt that tracheal secretory cells are highly proliferative and that they can produce ciliated cells (8–11). What is not known is whether they can give rise to basal cells. A second limitation of the work presented here is due to the definition of cell types solely by morphological criteria. It is essential that in the future, antigenic, biochemical, and/or molecular markers be developed to define the different cell populations and the different stages of differentiation in a precise, quantifiable, and functionally meaningful way. It is conceivable that the criteria used to define the tracheobronchial cell types are, in some cases, misleading, since the morphological markers which we are currently using may not always reflect the functional commitment at early stages of differentiation.

What have we learned from our experiments about differentiation pathways in the conducting airways, and which questions remain unresolved? We will first discuss the role of basal cells in tracheal cell differentiation. Our data strongly suggest that, at least in the rabbit, tracheal basal cells have stem cell potential, they are self-replicating and can generate secretory as well as ciliated progeny. However, it is conceivable that the basal cell compartment is functionally nonhomogeneous. Even at the morphological level, some heterogeneity can be observed. As described previously (14), approximately 10% of the basal cells are slightly larger than the rest of the basal cells, have more abundant cytoplasm, and are less electron dense. Whether this variation in basal cell morphology is of functional significance is not known. Biochemical and antigenic markers are needed to define the cell types in a biologically meaningful way.

The tracheal epithelium of the rabbit contains two morphologically distinct secretory cells (20). The most common one is a cell resembling in many respects bronchiolar Clara cells, the Claralike cell. The other secretory cells are typical mucous goblet cells. Whether these two tracheal cell types represent different functional states of the same secretory cell, whether they are two separate cell types derived from a common progenitor, or whether they belong to two separate cell lineages is not known.

Our studies do not provide an unequivocal answer to this puzzle. However, it may be significant that the spectrum of secretory cell morphologies in tracheas repopulated with unseparated tracheal cells did not differ from that of tracheas repopulated with basal cell inocula. In both cases, the reestablished epithelium contained a small number of typical goblet cells and cells with small discrete secretory granules, cytochemically and ultrastructurally identified as mucous granules. These cells exhibited varying amounts of smooth and rough endoplasmic reticulum.

It is not clear why typical Claralike cells, as described in rabbit tracheas by Plopper (20), were not found. We interpret these findings to suggest that perhaps all tracheal secretory cells, including Claralike cells, are derived from a common progenitor and are variations of the same secretory cell phenotype. Obviously, other interpretations are possible and new experimental tools need to be applied to resolve this problem.

What do our studies reveal about the differentiation capacity of bronchiolar Clara cells? It needs to be emphasized that the tracheal *in vivo* culture system was found to support the typical differentiation pattern of bronchiolar Clara cells, but not of tracheal Claralike cells described by Plopper et al. (20). This is important because tracheal Claralike cells did not develop in tracheas repopulated with cells of tracheal origin, even though Claralike cells must have been present in the inoculum. Our studies suggest that tracheal Claralike cells and bronchiolar Clara cells are not the same cell type. Bronchiolar Clara cells were found to be self-replicating and to give rise to ciliated cells. Thus, we consider them to have stem cell properties. Tracheas inoculated with bronchiolar Clara cells did not contain basal cells or mucous cells (2). We conclude therefore, that in the adult rabbit, bronchiolar Clara cells and their ciliated progeny represent a cell lineage separate from the epithelial cells of the large conducting airways. (We want to emphasize that these experiments do not address the question of embryologic derivation of cell types.) It appears that the epithelium of the large airways and the epithelium of the bronchioles are maintained by two separate stem cell populations: basal cells on one hand and Clara cells on the other. These findings are surprising in view of the apparent anatomical continuity of the large and small airways. We had expected that the bronchiolar Clara cell might simply be a modified tracheobronchial secretory cell endowed with a differentiation potential similar to that of tracheal secretory cells, if placed in the same environment. That, however, clearly is not the case.

There are other findings that support the view that, in the adult rabbit, tracheal cells and bronchiolar cells are separate populations endowed with different growth and differentiation potentials (Jetten et al., unpublished observations). Unseparated tracheal cells and tracheal basal cells have different *in vitro* growth requirements than bronchiolar Clara cells, and the *in vitro* growth capacity of the latter is far inferior to that of the former. Furthermore, tracheal cells, as well as tracheal basal cells, exhibit a cytokeratin profile different from that of Clara cells grown in culture (Table 4).

Clearly, Clara cells are expressing a different differentiation program than tracheal cells. This is not to deny, however, that there are important similarities between bronchiolar Clara cells and tracheal Claralike cells (20,24). Both are rich in drug-metabolizing enzymes and contain electron-dense secretory granules that do not react with mucous stains. In spite of these similarities, our data suggest that Claralike cells of the rabbit trachea and bronchiolar Clara cells belong to two different stem lines.

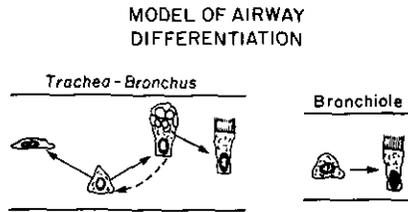


FIGURE 3. Model of cell differentiation in the conducting airways. The highly simplified model depicts the tracheobronchial and bronchiolar epithelium as being occupied by two separate cell populations. *Left:* In the trachea and bronchus, basal cells give rise to secretory cells (of various subtypes), which in turn give rise to ciliated cells. Basal cells can also give rise to squamous cells. The broken arrow suggests that, under certain conditions, some secretory cells may convert to basal cells (27). *Right:* In the bronchiole, Clara cells give rise to basal cells. In this hypothetical model basal cells and bronchiolar Clara cells are considered to have stem cell properties.

For the purpose of guiding future studies, we propose the following model of cell differentiation in the conducting airways of the rabbit (Fig. 3): *a)* The tracheobronchial epithelium and the bronchiolar epithelium are occupied by two different epithelial cell populations belonging to separate stem lines. *b)* Clara cells are the stem cells of the bronchiolar epithelium and give rise to Clara cells and bronchiolar ciliated cells. They belong to a cell lineage independent of tracheobronchial basal and Clara-like cells. *c)* Tracheobronchial basal cells are stem cells and can give rise to basal cells, secretory cells of different types, and ciliated cells. This may or may not involve morphologically intermediate cell types (6, 7). Basal cells may, furthermore, serve as anchorage for other cells in the large airways (12). *d)* Basal cells can give rise to squamous cells in vitamin A-deficient animals. *e)* The different secretory cells in the large airways either represent different functional states or different stages of differentiation of one principle secretory cell type. Differentiation into secretory cells may be a reversible process. *f)* Tracheobronchial secretory cells may also have stem cell potential; however, we prefer to think that secretory cells are responsible for the expansion of basal cell-derived clones during normal or injury-induced regeneration (26).

It is hoped that future studies will help to separate facts from fiction in this hypothetical model of airway cell differentiation.

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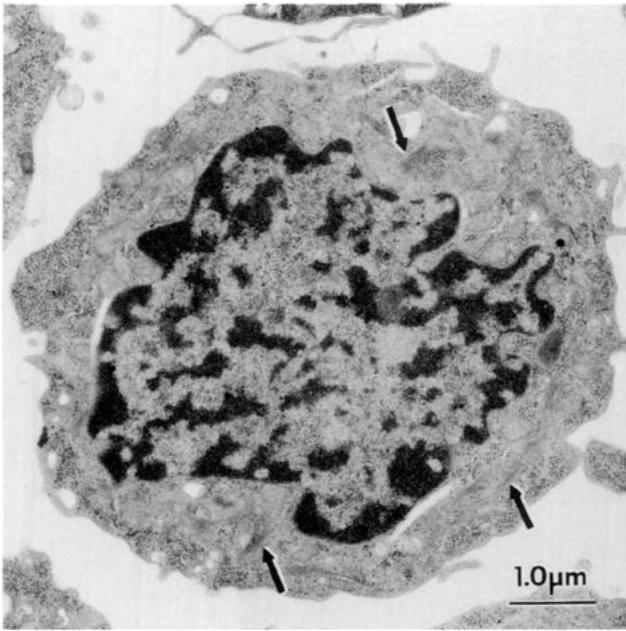


PLATE 1. TEM of isolated basal cells. Illustrated is the most common type (91%) of basal cell. It is small ($6.7 \pm 0.6 \mu\text{m} \pm \text{SD}$, $n = 30$) and exhibits high N/C ratio, round to oval shaped nuclei with well-developed nuclear indentation, and prominent heterochromatin. It has an electron-dense cytoplasm, conspicuous perinuclear tonofilaments (arrows), few mitochondria, and abundant free ribosomes. From Inayama et al. (3).

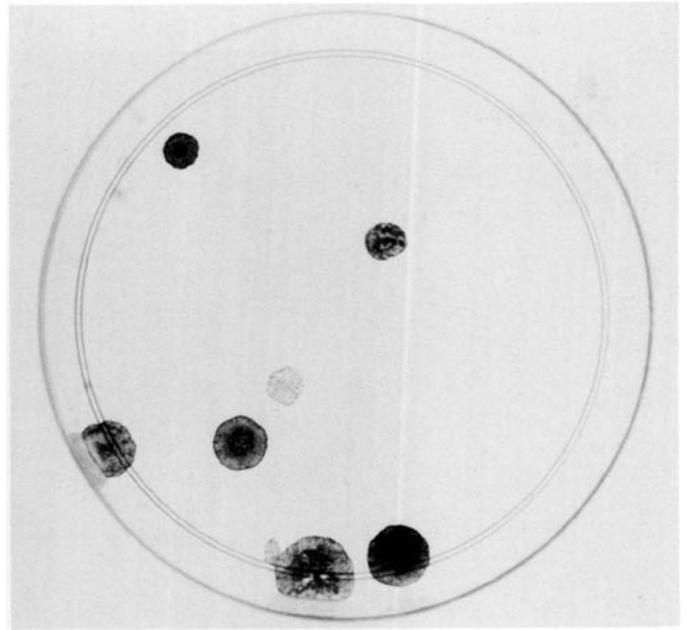
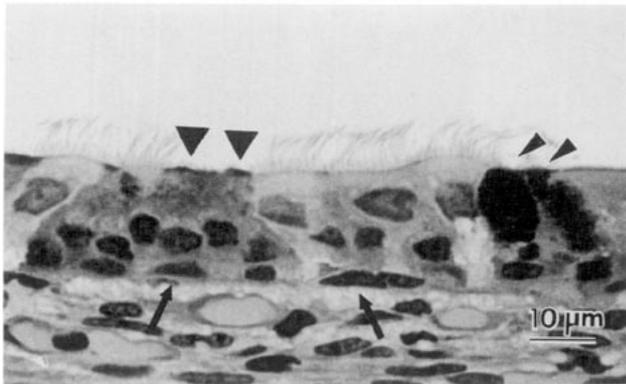
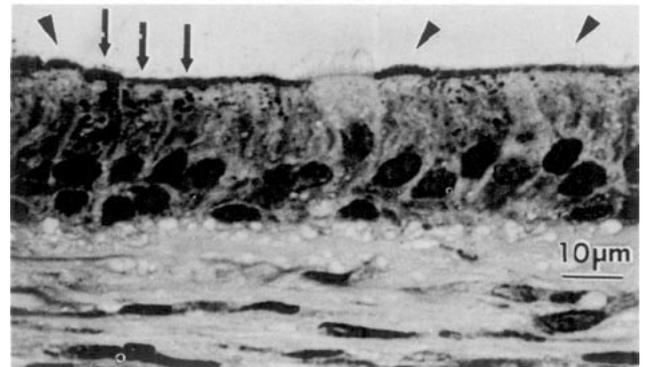


PLATE 2. Fourteen-day-old basal cell clones. Giemsa stain, $\times 0.8$. From Inayama et al. (14).



A



B

PLATE 3. Light micrographs of epithelial lining of tracheal grafts repopulated with basal cells; (A) Pseudostratified columnar epithelium, 4 weeks after implantation. Mucous goblet cells (small arrowheads), cells with small secretory granules (large arrowheads), and ciliated cells, as well as basal cells (arrows) are seen. (B) Pseudostratified columnar epithelium, 4 weeks after implantation. Cells show an alcian blue and/or PAS-positive surface rim (arrowheads); also visible are cells with few small secretory granules (arrows). Alcian blue (pH 2.5)-PAS-hematoxylin stain. From Inayama et al. (3).

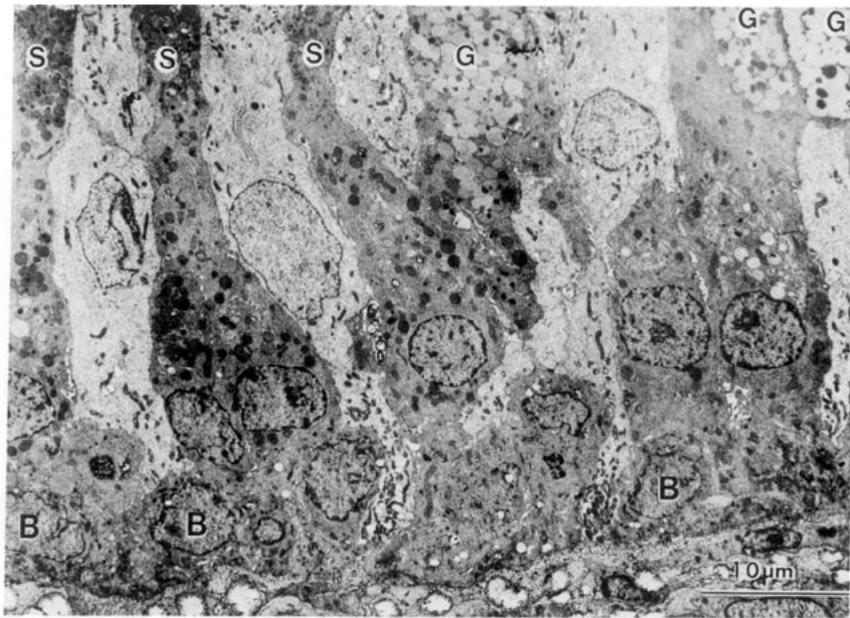


PLATE 4. TEM of columnar epithelium of tracheas inoculated with basal cells, 4 weeks after implantation. The following cell types are seen; mucous goblet cells (G), secretory cells (S) with small granules (arrows), ciliated cells and basal cells (B). The electron density of the secretory cells is variable. Uranyl acetate and lead citrate. $\times 2400$. From Inayama et al. (3).

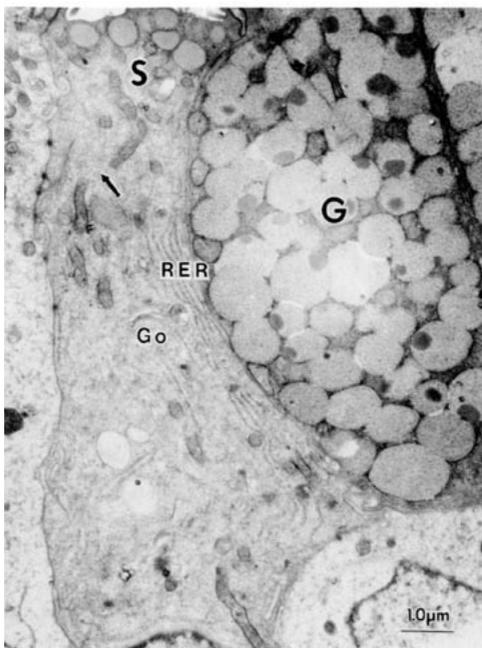
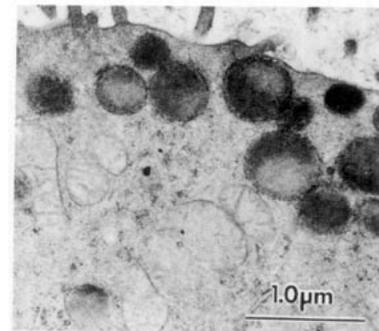


PLATE 5. TEM of mucous goblet cells, 4 weeks after inoculation of grafts with basal cells. Goblet cell (G) filled with secretory granules that are electron lucent have a meshlike structure and electron-dense cores. Secretory cell (S) with small secretory granules and moderate amounts of SER in the apical cytoplasm (arrow). Go, Golgi apparatus; RER, rough endoplasmic reticulum. Uranyl acetate and lead citrate. From Inayama et al. (3).



A



B

PLATE 6. TEM of cells with small secretory granules, 4 weeks after inoculation of grafts with basal cells. (A) The granules as well as the surface of the microvilli are positive with PA-TCH-SP stain. (B) Electron-dense secretory granules. Uranyl acetate and lead citrate. From Inayama et al. (3).

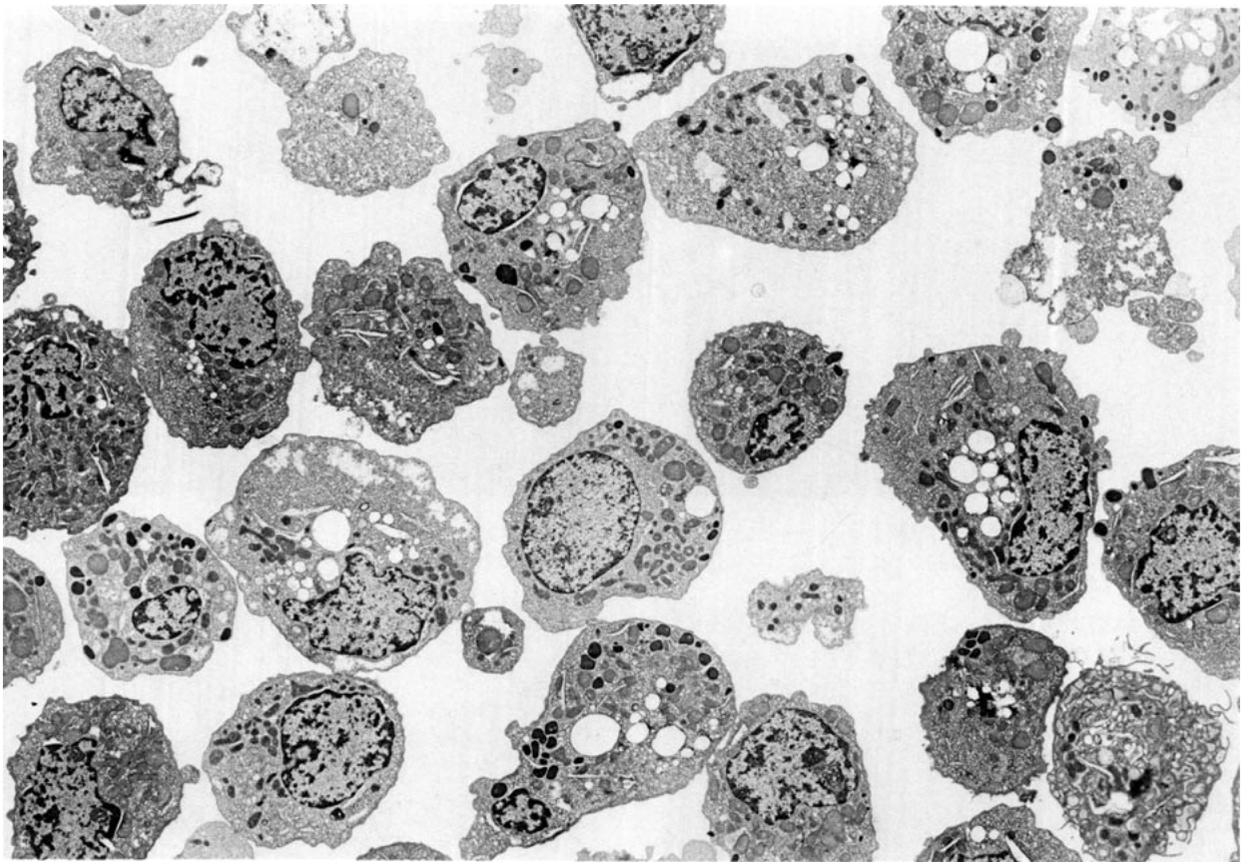


PLATE 7. Electron micrograph of Clara cell preparations from the lungs of rabbits. Most of the cells are identified as Clara cells based on their abundance of smooth endoplasmic reticulum and cytoplasmic osmiophilic granules. $\times 2970$. From Hook et al. (13).

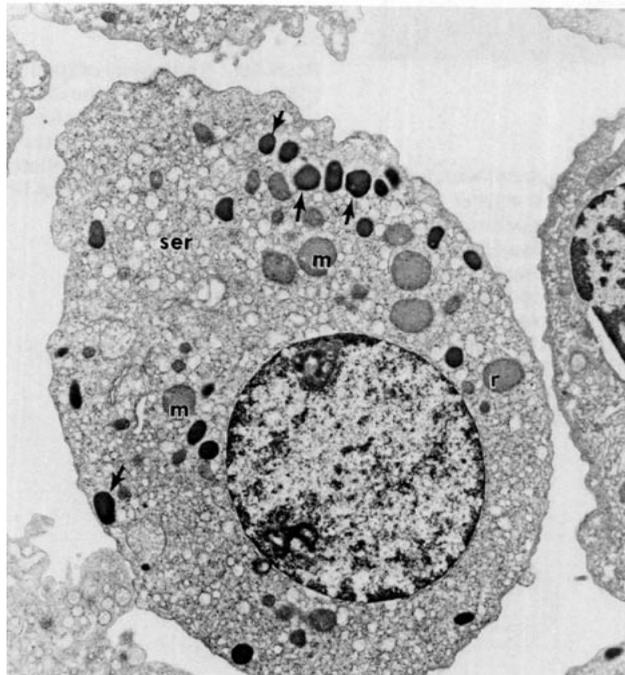
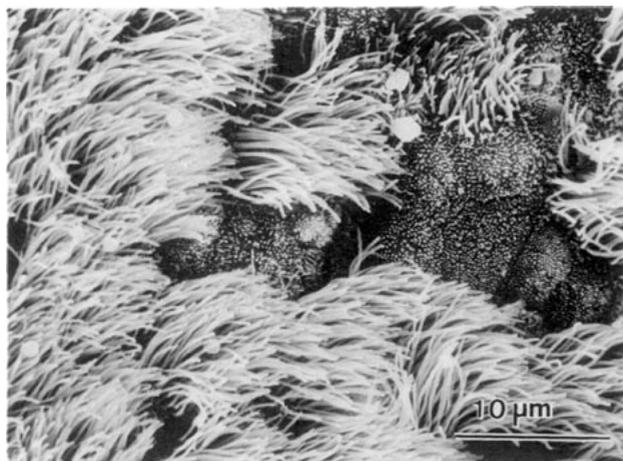
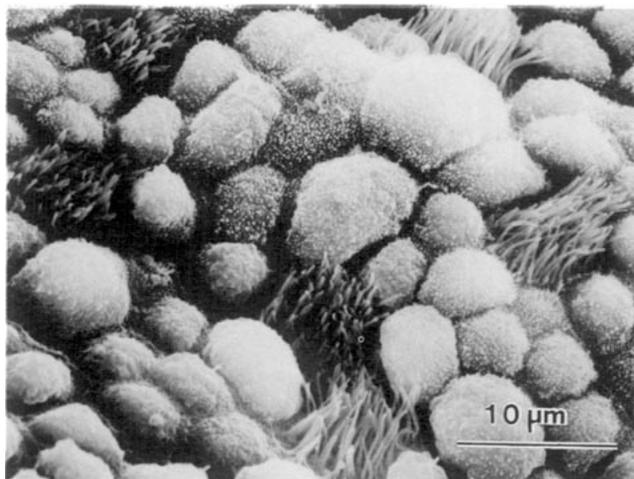


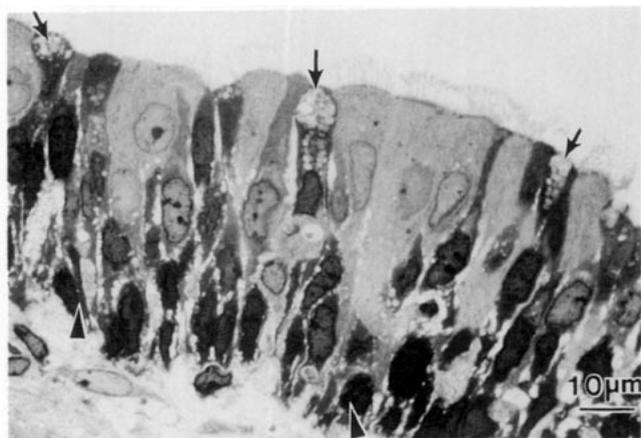
PLATE 8. Electron micrograph of a typical Clara cell isolated from the lungs of rabbits. The cytoplasm of the cell contains abundant smooth endoplasmic reticulum (SER), irregularly shaped osmiophilic granules (arrows), and mitochondria (m). $\times 7500$.



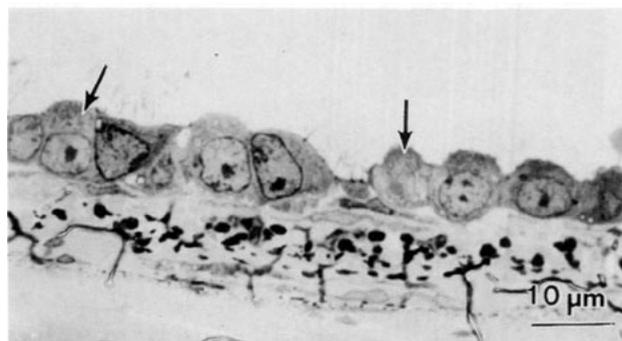
A



A



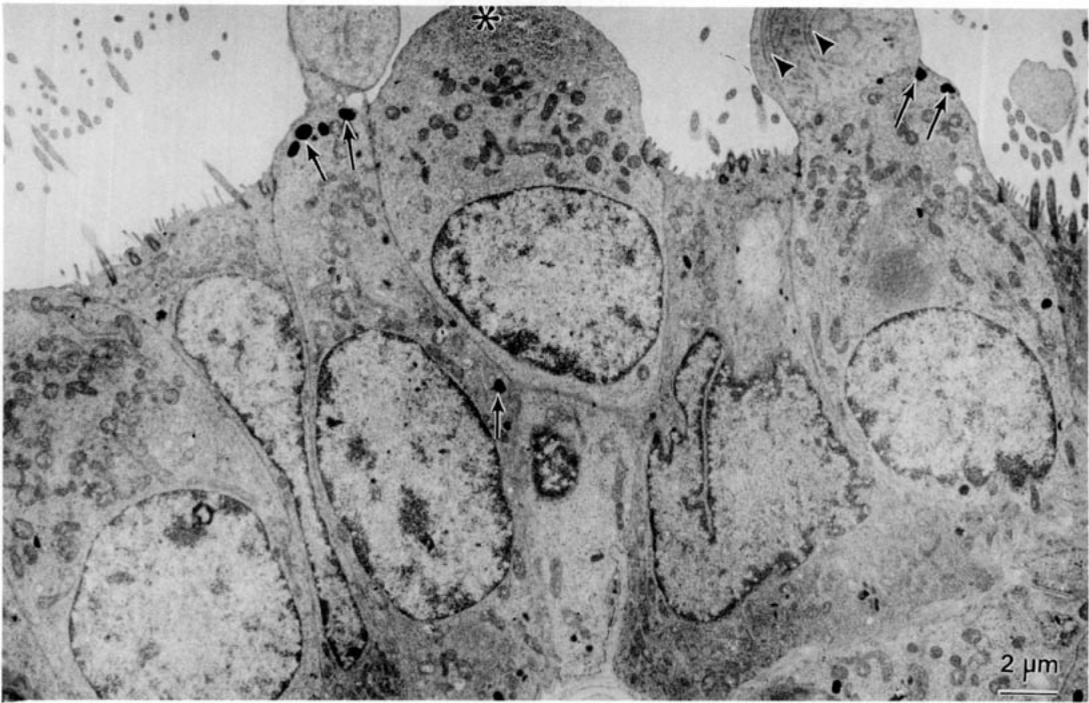
B



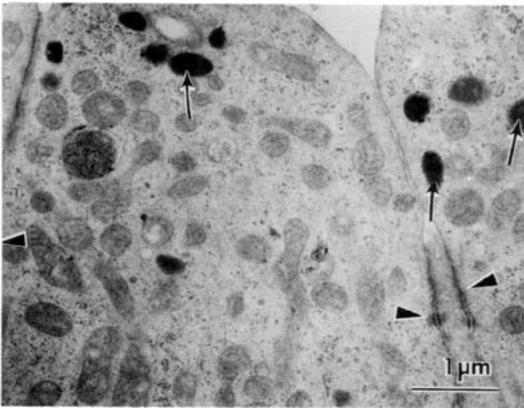
B

PLATE 9. Epithelial lining of tracheal graft repopulated with unseparated tracheal cells. (A) SEM of mixed tracheal cells at 4 weeks postimplantation. A large proportion of the unseparated tracheal surface is covered by well-developed ciliated cells and nonciliated cells with short microvilli. (B) LM of the 4-week-implanted unseparated tracheal cells showing a fully differentiated, pseudostratified mucociliary epithelium. Two types of tall columnar cells were readily identified by light microscopy: ciliated cells and cells containing mucous granules (arrows). Other unidentified columnar cells are apparent. The basal cell layer (arrowheads) was quite prominent at this time point. From Brody et al. (2).

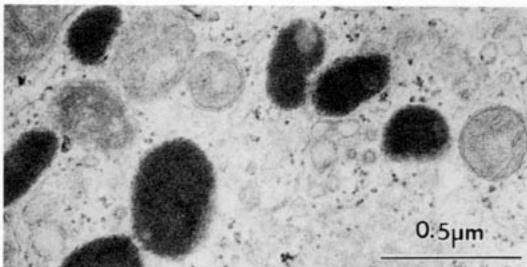
PLATE 10. Epithelium of tracheal grafts repopulated with Clara cell fraction. (A) SEM of the Clara cell implants at 4 weeks postimplantation show a predominance of cells with apical domes and ciliated cells. (B) LM demonstrates the simple cuboidal epithelium composed of ciliated cells and nonciliated dome-shaped Clara cells. No basal cells were observed. From Brody et al. (2).



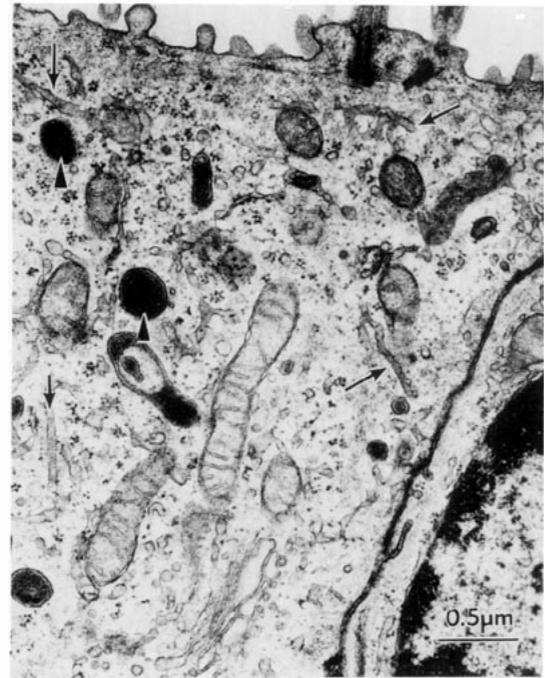
A



B



C



D

PLATE 11. Ultrastructural features of epithelium lining tracheal graft repopulated with Clara cell fraction. (A) TEM of cuboidal cell epithelium from a Clara cell preparation at 4 weeks postimplantation. Ciliated cells are interspersed among domed cells that contain smooth ER (arrowheads) and apical electron-dense ovoid granules (arrows) typical of Clara cells. Some cells, apparently undifferentiated, contained glycogen (*). (B,C) Some cells appeared similar to the "type B" Clara cells (25) exhibiting ovoid electron-dense granules (arrows) but little or no smooth ER. All the Clara cells had well-developed tight junctions and desmosomes (arrowheads). The high magnification view (C) demonstrates the membrane-bound oval granules typical of Clara cells. (D) Many ciliated cells in the Clara cell population at 4 weeks contained smooth ER (arrows) and dense granules (arrowheads). From Brody et al. (2).