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Long-Term Maintenance of Differentiated Respiratory Epithelium in Organ Culture I. Medium Composition[1] (38778)

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Organ cultures of respiratory tract tissue have been employed in a wide variety of experimental studies during the past two decades (1–3). Tissues maintained *in vitro* have proven useful in studies of experimental carcinogenesis (4, 5). Recent investigations have shown that polycyclic hydrocarbons interact with differentiated cells of the respiratory epithelium (6–10). Thus, the long-term maintenance of these tissues in organ culture affords a means for evaluating the morphological effects of oncogenic chemicals and the occurrence of malignant transformation in differentiated epithelial cells.

Regrettably, studies with carcinogens have been compromised thus far by technical limitations, inasmuch as investigators have found it difficult to maintain organ cultures of differentiated respiratory tract tissue in a viable state for extended periods of time (7, 11). The studies recorded here were undertaken to assess the nutritional factors and culture conditions that might make this possible.

Materials and Methods. Culture preparation. Tracheal tissue was obtained from 2- to 3-mo-old female hamsters of the 87.20 strain (TELACO, Bar Harbor, Maine). The animals were administered tetracycline-HCl (ca 8.6 mg/kg) at 24-hr intervals for 5 days; they were then killed using a lethal dose of Nembutal. The trachea was excised by sharp dissection from the larynx to the bifurcation and immediately immersed in Hanks' Balanced Salt Solution containing 5% chicken serum and 100 μg gentamicin, 50 μg kanamycin, 50 μg nystatin per ml. The external surface was trimmed of extraneous tissue and the trachea opened with sharp scissors along the dorsal aspect at the site of the anatomic defect in the cartilage rings. Using a sharp scalpel, approximately 1-mm tracheal rings were cut and halved, yielding tissues with epithelial surfaces approximately 1 x 2 mm. One ring from each animal was fixed in Bouin's solution for determination of morphology before culturing.

Remaining explants were then transferred to 35-mm diam Falcon plastic dishes (three to four specimens/dish) that had been scored to provide a roughened surface to permit adherence of the nonepithelial surface (12); 0.5 ml of medium was added to each dish so that the epithelial surface was moistened but not immersed in fluid. Dishes were placed in a 95% air–5% CO_2 water-saturated environment at 37°. Medium was changed on a routine basis twice weekly.

Media. The following chemically defined media were tested in sequential studies: Waymouth's MAB 87/3 Medium, GIBCO Lot A232924; Parker's Medium 199, HEPES buffered, GIBCO Lot A631612; CMRL-1066, GIBCO Lot R241307; Dulbecco's Modified Eagle's Medium, HEPES buffered, GIBCO Lot R833311; Eagle's Minimum Essential Medium, HEPES buffered, GIBCO Lot A833314; Serumless Medium, GIBCO Lot A33412. Chicken serum (198 IU Vitamin A/100 ml) was employed in concentrations ranging from 2% to 10%. All media contained 50 μg/ml gentamicin.

Histological techniques. At biweekly intervals 0.5 μCi[^{3}H]thymidine was added to each of several culture dishes. Eighteen hours later the explants were fixed in Bouin's solution (13). Alternate 5-μm sections were stained with hematoxylin and eosin or prepared for autoradiography using the emulsion-dipping technique (14). All autoradiograms were developed after 1 wk exposure in light-tight boxes at 4°C. Two hundred epithelial cells (basal and suprabasal) on each side of the approximate center of the explant in each of five serial histologic sections (a total of 2000 epithelial

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cells per specimen) were counted to determine the labeling index.

Specimens were harvested on a biweekly basis throughout an 8-wk period. From these observations we selected several media to be tested over longer time periods (10–24 wk).

Results. Normal morphology. The epithelial lining of the normal hamster trachea is comprised of ciliated, mucin-producing and basal cells; the relative number of each type depends in part upon anatomical location (15). The arrangement of the mucosal cells varies from simple columnar (Fig. 1a) to pseudostratified columnar (Fig. 1b). Ciliated and mucin-producing cells extend from the basement membrane to the luminal surface. They have vertical boundaries and are tapered slightly adjacent to the basement membrane. Nuclei of these cells generally are oval in outline and located in the mid-region of the cell. Basal cells are polygonal in outline; they exhibit a scant cytoplasm and a spherical, darkly stained nucleus.

Cultures in serum-containing media. Striking alterations were observed in organ cultures maintained in some media whereas the columnar structure of the normal hamster tracheal epithelium was preserved with others.

Proliferation of differentiated epithelial cells was prominent in specimens maintained in Waymouth's medium after 1 wk *in vitro*. Prominent excrescences of newly-formed ciliated cells appeared to accumulate above the mucosal surface and "buckle over" onto the adjacent epithelium. This feature seemed to result from the luxuriant multiplication of epithelial cells at localized sites in the mucosa (Fig. 2a, 2b), and persisted for as long as 8 wk in this medium. After 8 wk these proliferative changes were no longer evident for the epithelium of most specimens became cuboidal and basal cells often appeared elongated (Fig. 2c).

Medium 199 encouraged a more diffuse proliferation of epithelial cells at 1 wk in culture (Fig. 3a, 3b). Ciliated, mucin and basal cells were intensely labeled as compared to nuclear labeling of epithelia maintained in other test media. The mucosa seemed stratified and four to five cells in thickness. These proliferative alterations were transient; the epithelium of explants maintained in this medium for 2 wk and longer progressively decreased in height, becoming only one to two cell layers thick at 8 wk *in vitro* (Fig. 3c).

Specimens maintained in CMRL medium exhibited a pseudostratified high columnar epithelium for as long as 4 wk in culture (Fig. 4a). Labeling indices were elevated in comparison to those of explants maintained in other serum-containing media and decreased almost linearly from 1 to 4 wk *in vitro* (Fig. 7a). At 8 wk the epithelium was simple columnar and well-differentiated (Fig. 4b).

The mucosa of specimens maintained both in Eagle's and in Dulbecco's medium was similar to the epithelium of the intact animal. Explants demonstrated a well-differentiated pseudostratified epithelium with an abundance of ciliated cells (Fig. 5a). After 8 wk *in vitro*, columnar cells were decreased slightly in height (Fig. 5b).

Sequential studies with Eagle's and 199 media at serum concentrations of 2% indicated that the tracheal mucosa remained viable and in a differentiated state for as long as 20 wk in culture (Fig. 5c).

Cultures in serum-free media. Striking metaplastic changes were observed in the epithelium of cultures maintained without serum. These alterations often were evident after 1 wk *in vitro* and usually were prominent by 4 wk (Table I). With some media epithelial changes were focal, whereas with other media they were extensive, and the normal respiratory mucosa was largely replaced by bizarre squamous-like cells.

Localized areas of basal cell proliferation interspersed with a normal-appearing columnar epithelium were observed in explants maintained in 199, CMRL, Dulbecco's, and Eagle's media. These foci consisted of nodular accumulations of polygonal cells exhibiting an abundant eosinophilic cytoplasm and large nuclei that lacked polarity (Fig. 6a). Keratin formation was not observed in these specimens with the exception of a single explant maintained in CMRL medium for 8 wk *in vitro*. This tissue revealed a focal lesion of keratinizing cells.

Extensive metaplasia appeared as early as 1 wk in explants maintained in Waymouth's medium. The surface layer of columnar ciliated and mucin cells in these tissues was dis-

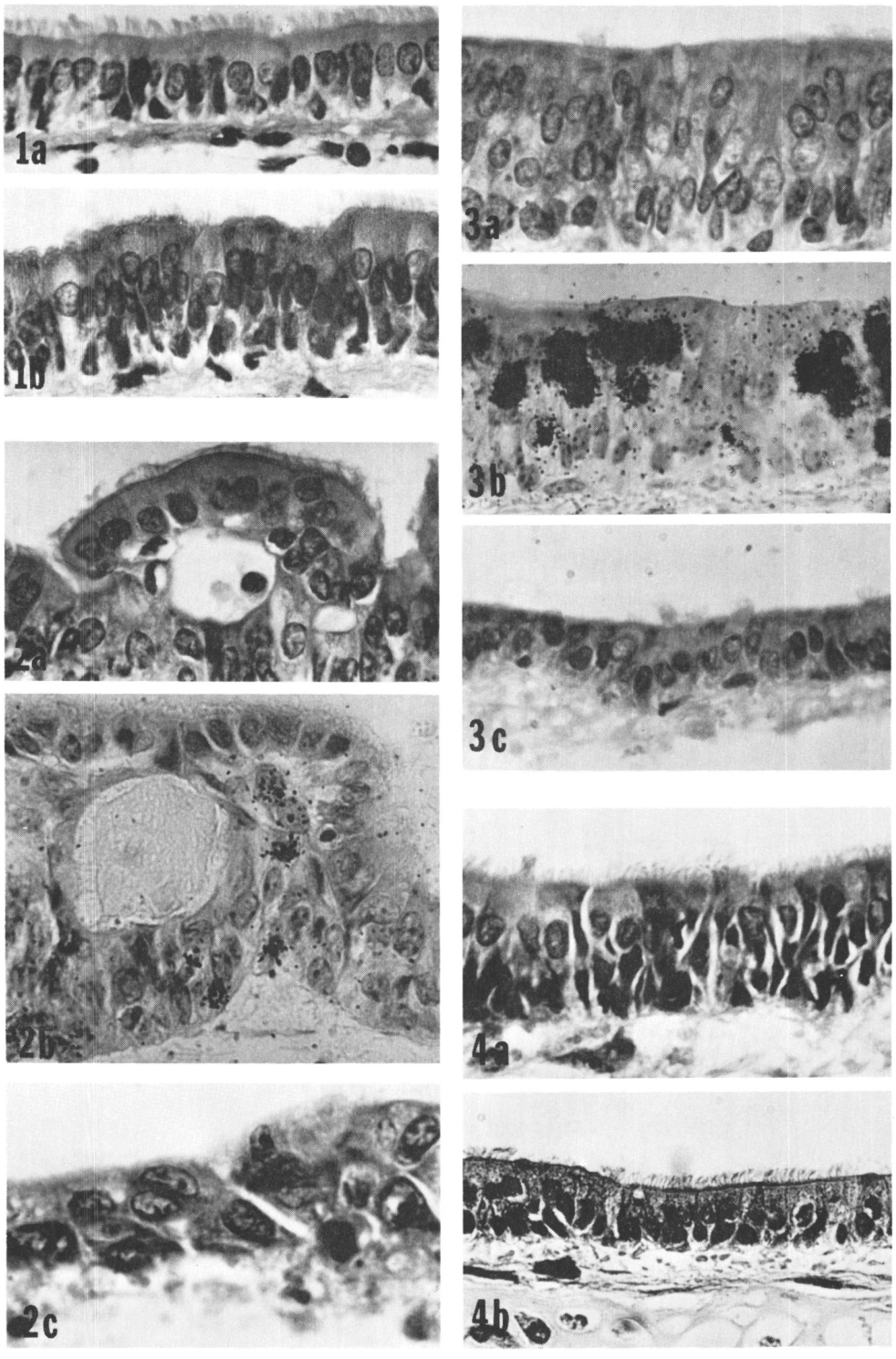
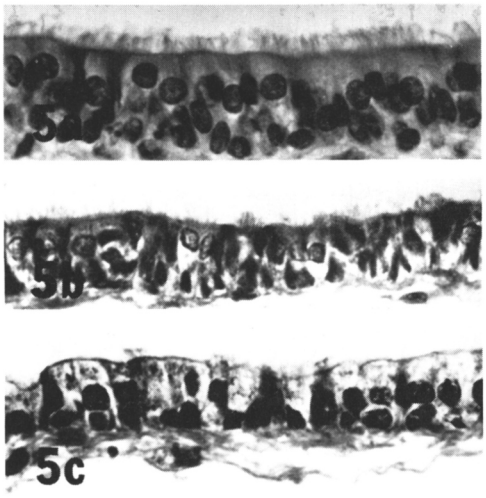


FIG. 1. Hamster trachea before incubation. (a) Simple columnar epithelium, 1360×. (b) Pseudostratified columnar epithelium, 1360×.

FIG. 2. Explants cultured in Waymouth's medium, 5% chicken serum concentration. (a) Four weeks *in vitro*, 1360×. (b) Autoradiogram, 4 weeks *in vitro*, 1360×. (c) Eight weeks *in vitro*, 1840×.

FIG. 3. Explants maintained in Medium 199, 2% chicken serum concentration. (a) One week *in vitro*, 1400×. (b) Autoradiogram, 1 week *in vitro*, 1300×. (c) Eight weeks *in vitro*, 1300×.

FIG. 4. Specimens cultured in CMRL medium, 5% chicken serum concentration. (a) Four weeks *in vitro*, 1500×. (b) Eight weeks *in vitro*, 1320×.



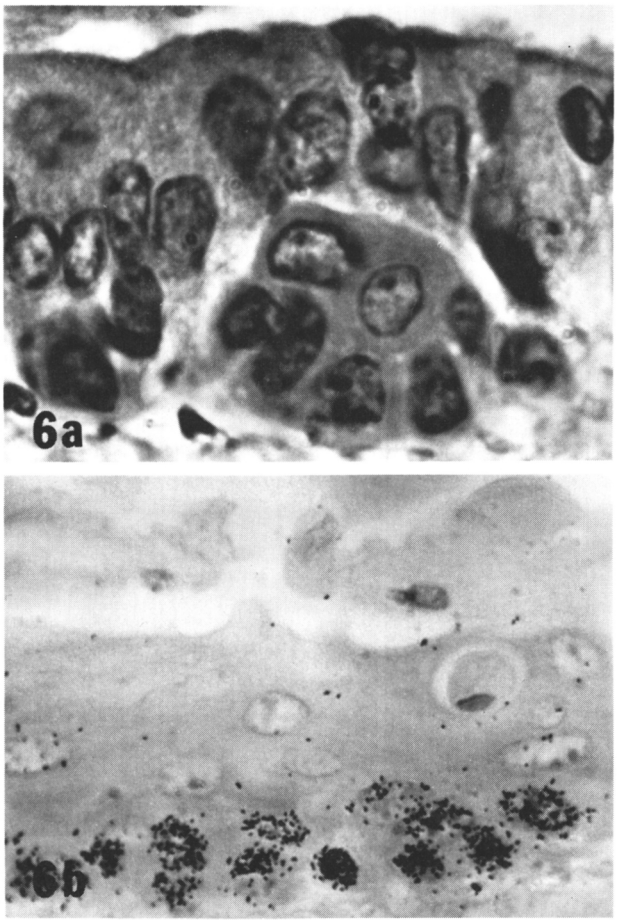


FIG. 5. Explants cultured in minimum essential media with serum addition. (a) Eagle's medium, 5% chicken serum concentration, 4 weeks *in vitro*, 1200×. (b) Eagle's medium, 5% chicken serum, 8 weeks *in vitro*, 1200×. (c) Eagle's medium, 2% chicken serum, 20 weeks *in vitro*, 1300×.

FIG. 6. Specimens cultured in media without serum addition. (a) Eagle's medium, 4 weeks *in vitro*, 3600×. (b) Autoradiogram, Waymouth's medium, 4 weeks *in vitro*, 1640×.

TABLE I. PERCENT OF EPITHELIAL SURFACE EXHIBITING INDICATED CHANGE IN EXPLANTS MAINTAINED IN MEDIUM WITHOUT SERUM.[a]

	Waymouth's				Medium 199				CMRL				Dulbecco's				Eagle's				Serumless			
Weeks *in vitro*	1	2	4	8	1	2	4	8[b]	1	2[b]	4[b]	8[b]	1	2	4	8	1	2	4	8	1	2	4	8[b]
No. specimens examined	6	6	13	14	5	7	7	4	4	4	4	5	3	3	3		5	4	3	4		6	6	3
Differentiated respiratory		8			100	71	42		100	75	25		66	33	33		80	75	60			50	17	
Focal basal cell metaplasia (Fig. 7a)	17	23	8			29	29				25	20	33	66	66				40	100		17		
Moderate squamous metaplasia	33	38	23				29	25									20	25				33		
Extensive squamous metaplasia	50	31	69	100																			83	33
Keratin-producing (Fig. 7b)	17		15	43							20												17	33

[a] The percentage of epithelium showing metaplasia was graded on observance of five or more serial sections. "Focal basal cell metaplasia" was defined as the presence of nodules of basal cells (Fig. 6a) or metaplasia involving less than 10% of the total length of the tracheal half-ring epithelium. Specimens were graded "Moderate" if between 10 and 50% of the epithelium was squamous, and "Extensive" if greater than 50% was squamous. Keratin-producing lesions were also noted.

[b] Other specimens exhibited a spindly epithelium suggesting a degenerative change.

placed by cells which were squamous-like in configuration. Autoradiograms showed intense labeling of pleomorphic basal cells (Fig. 6b). At 8 wk all of the specimens maintained in Waymouth's medium showed extensive metaplasia, and keratinization was noted in six of the 14 explants studied microscopically at this time. Most of the explants having a squamous, keratinizing epithelium remained viable for as long as 20 wk in culture.

The differentiated epithelium of specimens maintained in "Serumless" medium was replaced by squamous-like cells after 4 wk *in vitro*, yet the epithelium of most specimens had degenerated after 8 wk in culture.

The numbers of cells incorporating [^{3}H]-thymidine were increased in the epithelium of specimens maintained in serum-free media as compared to specimens maintained in serum-containing media. Labeling indices of explants in media containing serum decreased strikingly after 1 wk in culture and remained relatively constant for 8 wk *in vitro* (Fig. 7a).

Labeling in explants maintained in serum-free media was elevated with respect to these values (Fig. 7b); labeling indices of specimens maintained in "Serumless" or Waymouth's media increased dramatically from 2 to 4 wk *in vitro*. This increment paralleled the intensity of extensive metaplasia and keratinization in these systems.

Specimens maintained in CMRL and "Serumless" media exhibited a spindly epithelium at 4 and 8 wk making nuclei difficult to discern. Although labeling was noted, an accurate count of labeled versus unlabeled cells was not possible.

Discussion. The studies recorded here were carried out to assess comparatively the effects of synthetic media on the long-term growth properties and differentiation of the respiratory tract epithelium in organ culture. It is clear from our findings that differences in the composition of media and the presence or absence of serum have profound effects on the physiologic state of the tracheal mucosa maintained *in vitro*.

Respiratory mucosa of most specimens maintained in media containing serum exhibited a relatively normal appearing epithelium for extended periods of time *in vitro*. In contrast, proliferative changes were observed in specimens maintained in serum-containing complex media such as Waymouth's, 199, and CMRL. These "rich" media contain nutritional factors lacking in less complex media such as Dulbecco's or Eagle's.

The proliferative changes referred to above were of two types. Cultures maintained in

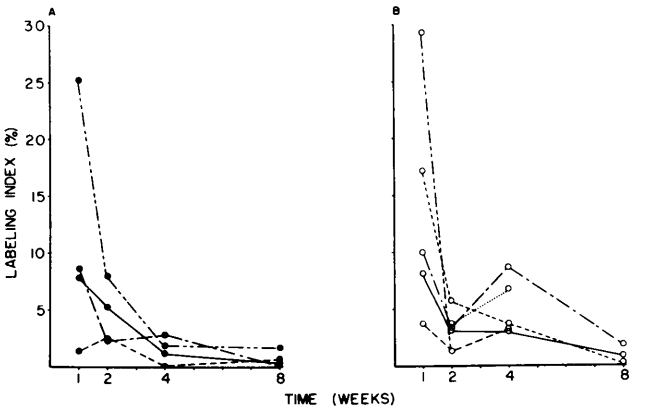


FIG. 7. Percentage labeling of hamster tracheal epithelial cells *in vitro*. Each point is comprised of the mean labeling index of 2–12 experimental specimens maintained under identical environmental conditions. Specimens were harvested at 1, 2, 4 and 8 wk after exposure to [^{3}H]thymidine for 18 hr. Autoradiograms were prepared and 2000 epithelial cells (basal and suprabasal) per specimen were counted for determination of the labeling index. (A) Percentage labeling of tracheal epithelia in media containing 5% serum concentration (●——●). Specimens in Waymouth's, CMRL, and Eagle's media exhibit a decline in labeling after 1 wk *in vitro*. This decline is not as abrupt and occurs after 4 wk in specimens maintained in Dulbecco's medium. (B) Percentage labeling of tracheal epithelium in media without serum (○——○). The number of labeled cells decreases between 1 and 2 wk in culture irrespective of test medium. Labeling indices are higher than those of epithelia maintained in media containing serum. Note: Waymouth's (—·—·); Parker's 199 (-------); CMRL-1066 (—·—·); Dulbecco's (———); Eagle's (——); Serumless (.....).

199 and CMRL media exhibited a diffuse stimulation of cell multiplication affecting both the basal and suprabasal cells. In cultures maintained in Waymouth's medium, the proliferative changes were localized to dispersed clusters of cells. This phenomenon suggested selective stimulation of scattered cells in the respiratory mucosa.

It is of interest that epithelial cell proliferation was observed for periods of only 1–4 wk in cultures maintained in Waymouth's, 199, and CMRL media containing serum. This observation suggests that the replicative capacity of the differentiated respiratory epithelial cell is limited. A similar "aging" effect has been observed in trypsin-dispersed diploid cells maintained in monolayer cultures (16–18).

Squamous metaplasia consistently was observed in cultures maintained for periods of several weeks in "Serumless" and Waymouth's media in the absence of serum. Since Vitamin A is present in commercially available chicken serum this change may be consequent to its absence. If so, our findings suggest that the metaplastic effects of the deficiency are enhanced when the differentiated epithelium is stimulated by other nutritional constituents of the medium.

The experiments of Lasnitzki using rat prostatic tissue indicate that epithelial differentiation *in vitro* is associated with relatively low mitotic activity whereas the loss of differentiation is accompanied by an increase in mitoses (19). Our findings are consistent with these observations.

An intriguing result of our studies is the ability to establish a focally metaplastic or extensively squamous epithelium *in vitro* by use of selected media without serum addition. The chemical constitution of these media is complex, and the presence of one or more of these elements may act as co-factors involved in proliferation or in the progression of metaplasia and keratinization in these systems. Maintenance of the mucosa in a proliferative or squamous state may enhance carcinogen interaction with the epithelium and may prove to be a valuable tool in experimental respiratory carcinogenesis. We are currently investigating these possibilities.

Summary. Six commercially prepared, chemically defined media were tested in the presence and absence of serum to assess their

influence on the maintenance of viable hamster respiratory epithelium over extended periods of time *in vitro*. Unique proliferation of epithelial elements was observed in organ cultures maintained in "complex" media containing serum, whereas use of these media in the absence of serum produced disorganized epithelial changes resembling squamous metaplasia. Minimum essential media at low serum concentrations preserved the columnar structure of the normal tracheal epithelium for 8 wk and longer *in vitro*.

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