Environmental Health Perspectives
Vol. 16, pp. 89-98, 1976

Use of Tracheal Organ Cultures in Toxicity Testing

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Fragments of tracheal epithelium, alone or in continuity with connective tissues, can be maintained in culture medium and used for short term or long term studies of toxicity of a variety of chemicals. Large numbers of uniform cultures are prepared with the aid of a slicing device or by application of simple method for dissecting sheets of epithelium free from underlying cartilage. The cultures may be placed in an exposure chamber-incubator mounted on a microscope stage and monitored continually for ciliostasis and exfoliation of cells. Morphology is further studied by fixation of selected specimens and preparation for light microscopy and electron microscopy.

Synthetic functions are evaluated by autoradiographic measurement of incorporation of radioactive precursors into macromolecules and other dynamic features are indirectly assessed by histochemical and histoenzymatic methods. Short-term studies using these several techniques have shown that ciliostasis does not correlate with cell injury in all instances, and a long-term study has demonstrated dose dependence of a cytotoxic agent when duration of culture viability is measured. The method lends itself to a broad range of investigations in which dose, period of exposure, and role of cofactors must be independently and quantitatively assessed.

The effects of toxic materials on the tracheobronchial epithelium is usually studied in intact laboratory animals which are exposed by instillation of the agent through endotracheal tube (*1*) or by placement in an inhalation chamber containing the agent in an airborne form (*2*). These methods place vital respiratory function of the experimental animal at risk and limit the types and dosages of materials which can be tested. Further, they do not exclude immune and inflammatory responses which may interfere with evaluation of the direct effect of the materials under study.

The removal of tracheobronchial epithelium to tissue culture eliminated these problems while preserving tissue and cellular specialization. Organ or tissue culture shares with cell culture the advantages of complete control of the environment, easy manipulation of the model, and continuous or continual monitoring for effect. Cell culture, however, does not reflect cellular specialization and therefore, at best, gives information on the general effects of a given agent, while organ cultures permit assessment of tissue-specific effects.

Methods are now available for preparation of uniform trachea-derived tissue or organ cultures in large numbers and for their long term maintenance. This technology permits extensive and fully controlled experimental approaches to studying respiratory tract toxicity.

Preparation of Cultures

Tracheas are excised aseptically from adult, in-bred, barrier-raised, pathogen-free rats. They are rinsed in one of several media which have been found to sustain growth *(31)* and cut longitudinally into strips or transversely into rings. The preparation of uniform fragments is facilitated by sectioning with a mechanical device. The trachea is pinned to a wax board and drawn across 5-15 razor blades which are aligned

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and held at a 20°, angle to the horizontal by two bolts and are separated by plastic spacers of equal thickness (Fig. 1). The fragments thus prepared do not exhibit injury of the epithelium beyond the first three layers of cells nearest the sharply incised margin, and there is little fraying or disruption of connective tissues (Fig. 2). The procedure may be performed rapidly and repeatedly so that batches of several hundred cultures can be prepared for each experimental protocol.

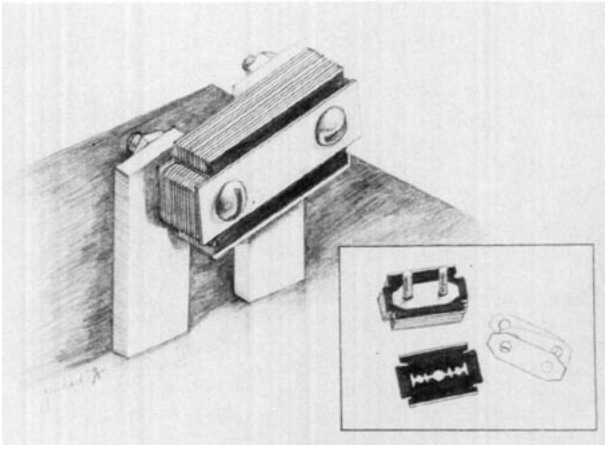

FIGURE 1. The device for sectioning tracheas into rings of uniform width consists of double-edged razor blades, separated by punched plastic spacers and held in register by two bolts. The bolts are attached to posts of unequal height so the razor blades are held at an angle to the base plate. Tracheas pinned firmly to a paraffin board are pressed against the sharp edges and the rings of tissue retrieved by flushing or by gently lifting with a pin from the spaces between blades.

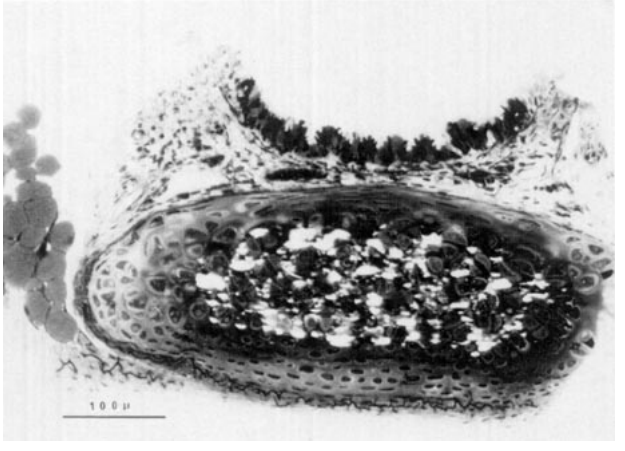

FIGURE 2. Fragments cut with the slicing device are minimally traumatized. Only two or three cell layers from the cut edge are injured and the tissues are not disrupted. Elastic fibers in the lamina propria account for the slight retraction of epithelium from the incised margins.

As an alternative type of culture, the mucosa alone is dissected from underlying cartilage and muscle by inserting a pin and undermining the layer. The process is hastened by immersion in saline prior to dissection or by introduction of saline with or without trypsin into the subepithelial space with the aid of needle and syringe (Fig. 3).

Both kinds of tissue fragments are cultured by the same technique. The specimen is submerged in medium which may be completely defined or may include serum (4). McCoy's 5a (modified), CMRL 1066, and Eagle's minimal essential medium all support differentiation and long-term viability. Strips or rings cut from rat trachea exhibit migration of the epithelium over the entire

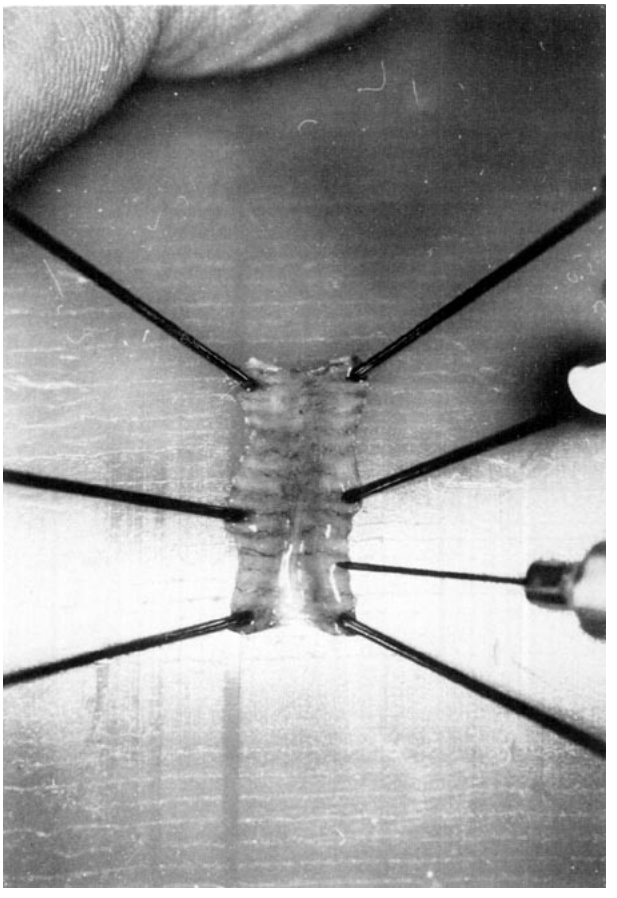

FIGURE 3. The epithelium can be removed from underlying cartilage by splitting the trachea along the ventral surface, pinning it to a board and inserting a sharp pin at a very small angle beneath the epithelium. The tissues dissect easily if the trachea is soaked in saline or if saline is injected beneath the epithelium before insertion of the pin. Proteolytic enzymes, added to the saline to be injected, further reduce adherence if necessary.

surface in 2-4 days (Fig. 4). The process is more uniform and rapid in the presence of calf serum but will occur without serum (*4*).

The mucosal fragments become rounded and the constituent cells remain well differentiated (Fig. 5), but no mitotic activity is evident; over a period of weeks, cells are exfoliated from the edge of the fragments. These floating masses, consisting of little more than a single epithelial cell layer, can be transilluminated and the entire ciliated surface assessed by phase or bright field microscopy.

Studies on the Cultures

Specimens can be observed or photographed intermittently by using an inverted phase microscope (Fig. 6) or continuously by using a miniature incubator mounted on the stage of the microscope (Fig. 7). Replicate cultures are fixed when changes are observed in specimens or after selected intervals and are examined with light microscope, scanning electron microscope, and transmission electron microscope. The ultrastructure of the epithelium of untreated cultures (Fig. 8) conforms to previous descriptions (*5*).

Assessment of cellular synthetic function is made autoradiographically. After incubation in medium containing 5.0 μCi/ml of ^{3}H-thymidine, 0.25 μCi/ml of ^{3}H-uridine or 0.25 μCi/ml of ^{3}H-leucine for 20-30 min, the tissue is exposed for 30 min to the same macromolecular precursor in nonradioactive form and at a tenfold higher concentration. This last step serves to displace unincorporated labelled molecules from binding sites in the cell. The tissues are then fixed and

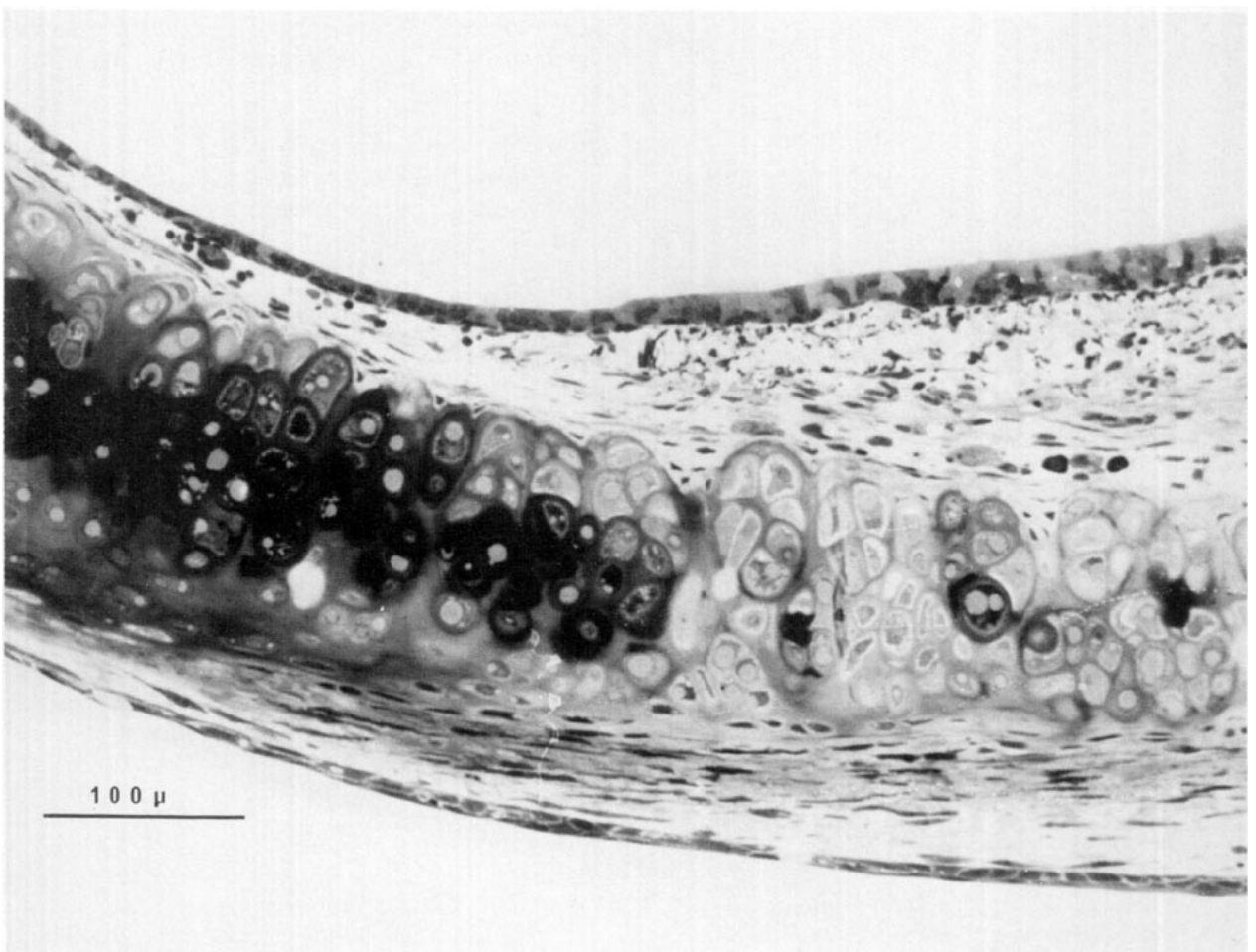

FIGURE 4. Epithelial cells have migrated from the original mucosal surface to cover the entire surface of the culture by 4 days. The cells on the outer surface at this stage are flat but they subsequently become arrayed as a well-differentiated ciliated pseudostratified layer.

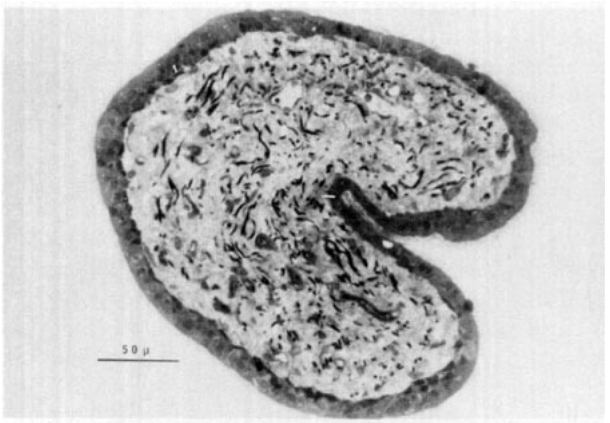

FIGURE 5. The fragments of mucosa dissected free of cartilage form a ball with the remnants of connective tissues covered by highly differentiated and well preserved epithelium.

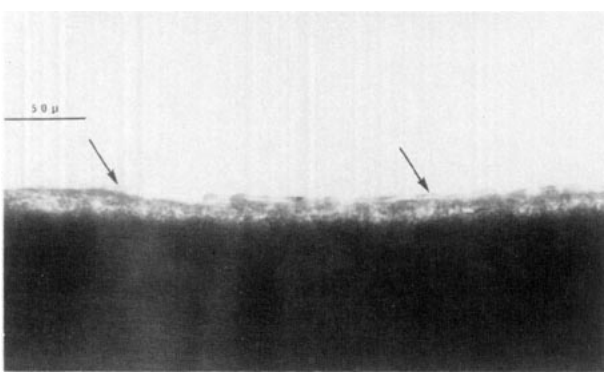

FIGURE 6. The inner and outer surfaces of the living ring cultures can be visualized with an inverted phase microscope and ciliary beat (arrows) or exfoliation of cells assessed.

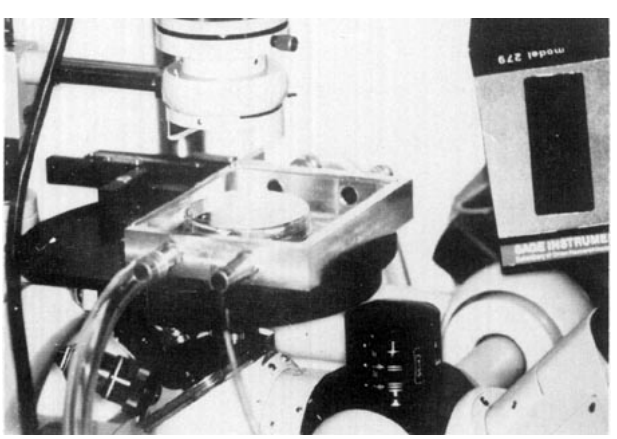

FIGURE 7. Cultures may be maintained in a miniature incubator mounted on an inverted microscope stage and observed through the glass window that forms the top surface. There are four gasketed ports for introduction and withdrawal of liquids or gases. The assembly is composed of milled aluminum which is maintained at constant temperature with a heater controlled by thermoprobe.

prepared for light and electron microscopy. Two thick sections from each block are coated with Kodak NTB2 photographic emulsion, exposed at 4°C in the dark for 14 days and developed with Kodak D19. For electron microscopy, Ilford 4 emulsion is substituted. Autoradiography reveals RNA synthesis limited to basal and ciliated cells in normal epithelium (Fig. 9) and diffuse leucine incorporation into proteins primarily in goblet cells and to a lesser extent in all other cell types. DNA synthesis, presumably in preparation for cell division, is very active during the first few days in culture but subsides after 14 days to a level three or four times that seen *in vivo* in unstimulated trachea.

Changes in cell surfaces are measured by staining en bloc with lanthanum nitrate (*6*) or by demonstration of membrane associated alkaline phosphatase (*7*). Cytoplasmic components are evaluated by histoenzymatic assay of mitochondrial succinic dehydrogenase (*8*) and lysosomal acid phosphatase (*9*).

Short-Term Toxicity Studies

Cultures are exposed to noxious chemicals dissolved in the medium at varying concentrations and over different periods of time. Chromates in concentrations of 5.0 μg/ml to 1.0 g/ml, cytochalasin B at levels of 10^{-6}-10^{-9} g/ml, sulfurous acid, generated by dissolving metabisulfite, and hydrochloric acid sufficient to adjust the pH to 3.0 and 4.5 are among the substances which have been studied. The cultures are transferred to medium containing the agent to be evaluated and observed with the phase microscope for ciliostasis or gross exfoliation. The ability of specimens to recover from brief exposures when returned to normal medium is assessed as are the effects of continuous exposure and repeated brief exposure. Replicate cultures are tested autoradiographically for impairment of DNA, RNA or protein synthesis and histochemically for enzyme function or cell surface changes. Histologic (Fig. 10) and ultrastructural features are also examined, using three or more specimens for each set of conditions.

Following this protocol, 20 min of exposure of tracheal epithelium to 100 μg/ml of sodium or calcium chromate was shown to result in ciliostasis (*10*). Lower concentrations of the salt did not produce acute ciliary arrest or reduction in RNA or DNA synthesis but ciliated cell degeneration (Fig. 11) or death was evident at 24 hr even if the tissue was removed from chromate-containing medium after brief exposure.

FIGURE 8. The epithelium of the cultures is pseudostratified with narrow processes of the tall columnar ciliated and goblet cells abutting on basal lamina between the squamous or cuboidal basal cells. Cytologic differentiation is well maintained with a continuous line of basal bodies at the apical surface and cilia with intervening microvilli projecting into the lumen.

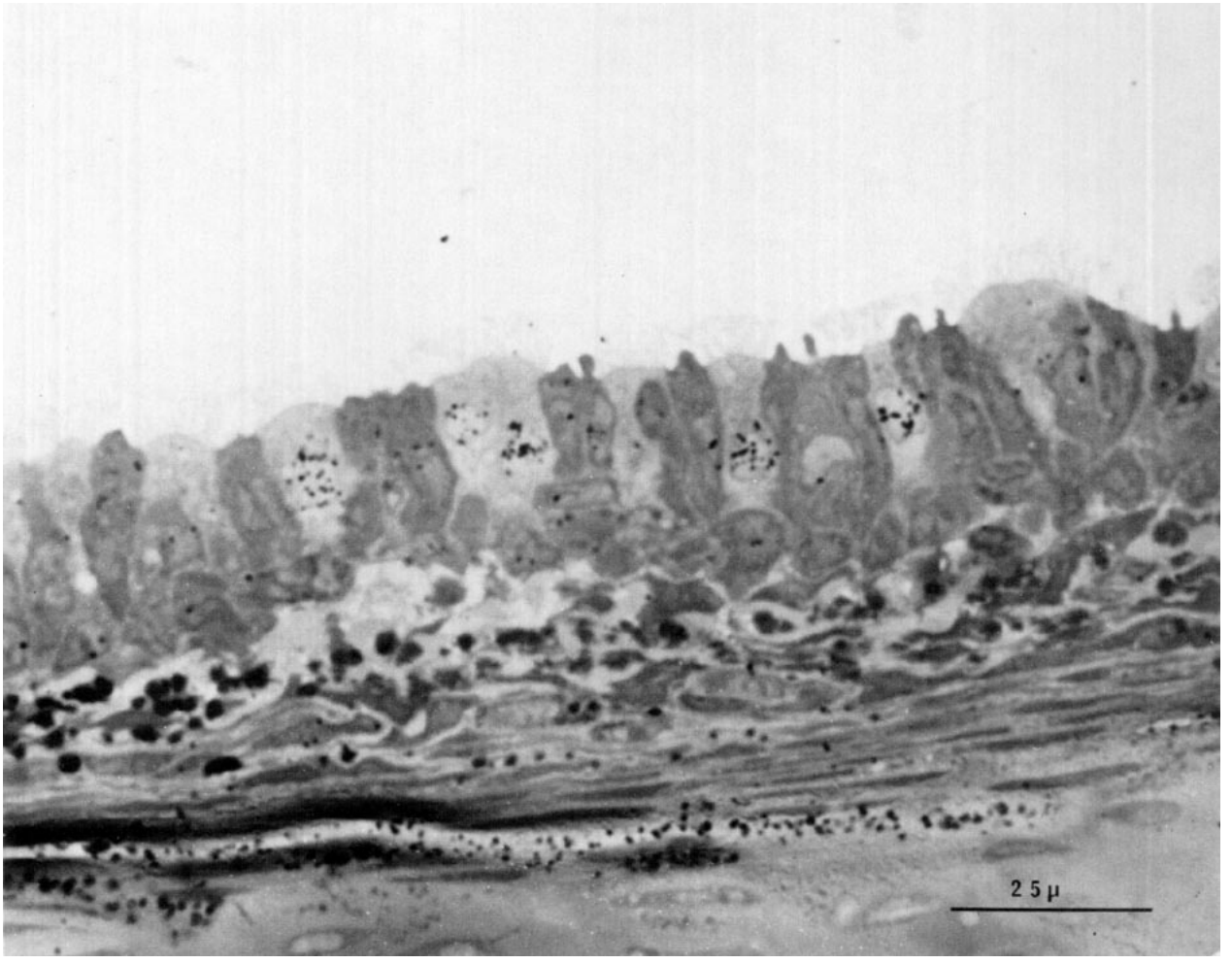

FIGURE 9. Autoradiograph of the epithelium of a normal culture incubated briefly in ^{3}H-uridine reveals incorporation of the RNA precursor into nuclei of ciliated cells with limited incorporation into those of basal cells.

Hydrochloric and sulfurous acids sufficient to reduce the pH to 4.0 also caused ciliary arrest in 20 min but did not interfere with other cellular functions. Further, on removal from the medium which contained the toxic agent, the cilia resumed beating. Only slight dilation of Golgi apparatus and endoplasmic reticulum was noted in ciliated cells fixed during the period of ciliostasis and cells were normal by the end of the ensuing 24 hr.

Long-Term Studies

Toxic effects over a period of days, weeks, and months can be studied by using protocols in which cultures are exposed continuously or repeatedly. In one such investigation (*11*), viability and DNA synthethic activity as a measure of cell turnover were evaluated when cultures were incubated continuously in medium containing serum and various concentrations of benzo [a] pyrene.

Continuous exposure to 1 μg/ml of benzo[a]-pyrene for one week induced extensive epithelial cell degeneration and cell death. Hyperplasia with high rates of DNA synthesis (Fig. 12) was seen in cultures which remained in carcinogen-containing medium and the epithelium was dead by three months after onset of exposure. Untreated cultures are viable for more than 6 months. Incubation in benzo[a]pyrene at concentrations lower than 1 μg/ml shortens the life of the culture to 4 or 5 months, and exposure to concentrations greater than 2 μg/ml reduces the period of culture to less than a month.

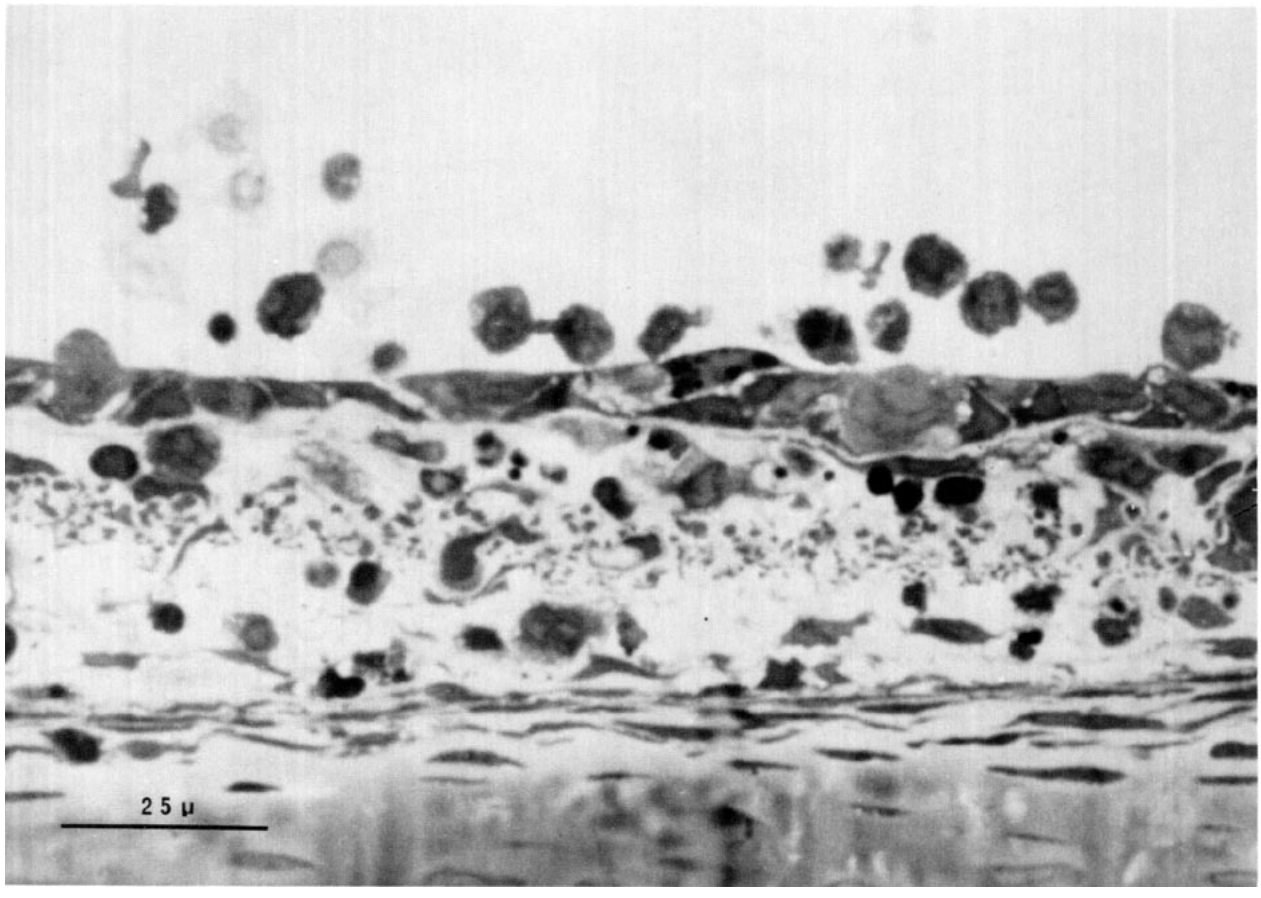

FIGURE 10. Treatment with cytochalasin B at a concentration of 1×10^{-6} g/ml for 1 hr has resulted in detachment of many of the columnar cells from the epithelium. The exfoliating cells are, in instances, still tenuously attached to the basal cells.

Conclusion

Large-scale preparation of uniform tissue or organ cultures consisting in part of pseudostratified, ciliated tracheal epithelium permits study with replicate specimens of the effects of toxic agents on this tissue. Cultures can be maintained for periods of months under completely defined conditions and can be continuously monitored while being exposed for any desired length of time. The ability to produce hundreds of cultures allows multiple specimens to be taken for each experimental point and a wide range of concentrations and modifying conditions to be evaluated as part of each testing protocol. The small amount of tissue in a small volume of fluid facilitates pulse labelling techniques for either autoradiographic or scintillation counting measurements of synthetic functions. The preparations which consist of mucosa alone are particularly well suited to determination of ciliostasis since the entire surface can be evaluated. Rings and strips which include large amounts of cartilage and connective tissue are not easily transilluminated and only the edges can be adequately assessed. On the other hand, ultrastructural and cytologic studies are more easily performed with fragments in which the normal histologic organization is retained. The mucosal fragments are not suitable where regenerative capability is necessary, because mitotic activity or DNA synthesis, normally present in the epithelium of the rings or strips, cannot be easily stimulated in the isolated mucosa.

We have used this system for studying both acute and long term effects of noxious agents. Two of the short-term exposures protocols assessed cytolytic effects of different ciliostatic chemicals. We showed that at concentrations below those resulting in ciliostasis, immersion for 20 min in chromate-containing medium produced irreversible changes leading to cell death (*10*). In

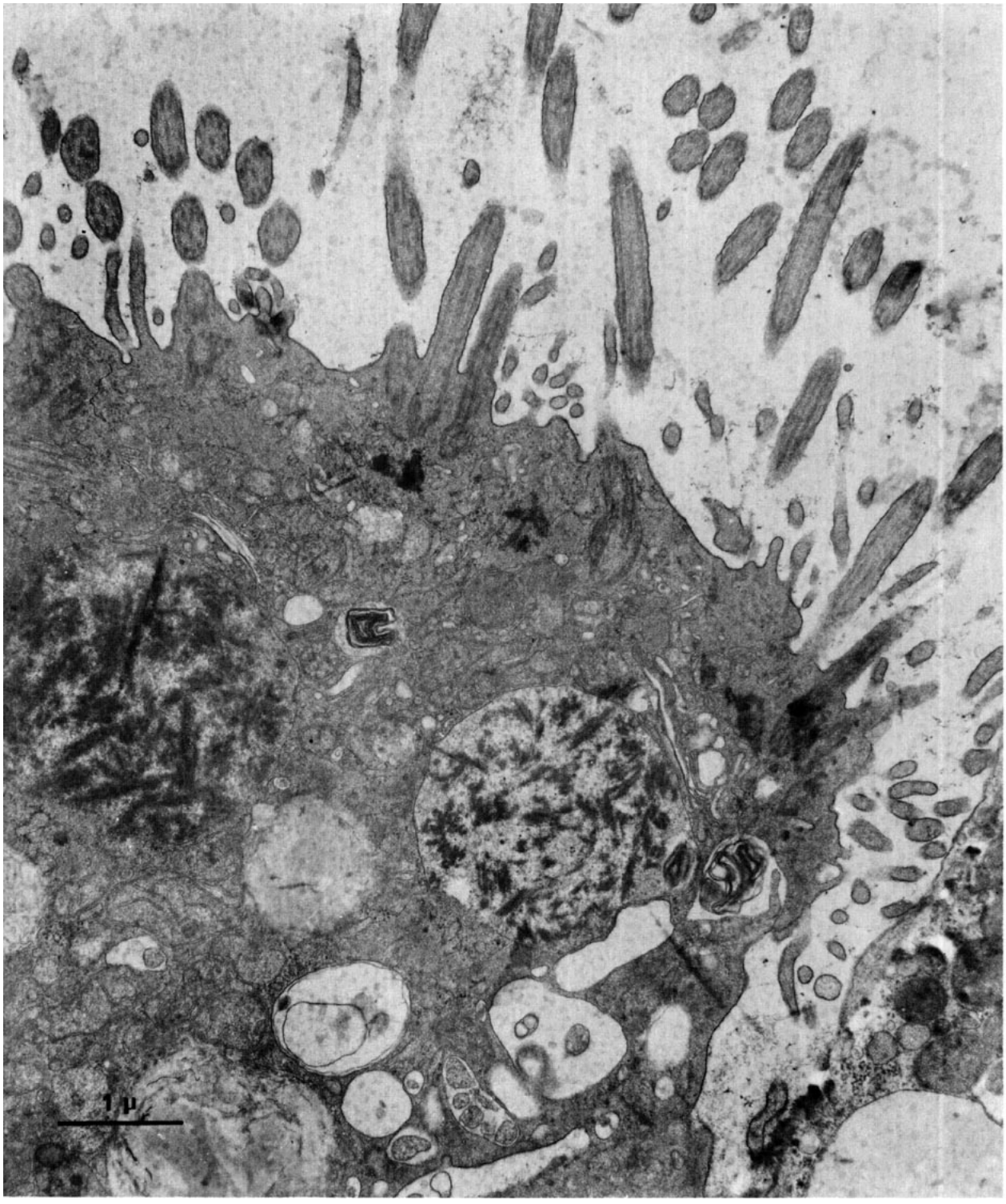

FIGURE 11. At 24 hr after a brief treatment with 100 mg/ml of sodium chromate, the apical end of this ciliated cell is disrupted. Cilia and basal bodies are distorted and are no longer aligned. The filamentous interbasal body apparatus is absent and various organelles intrude into the subplasmalemmal zone normally occupied by this network. Lipid droplets and vacuoles containing partially degraded materials are also present.

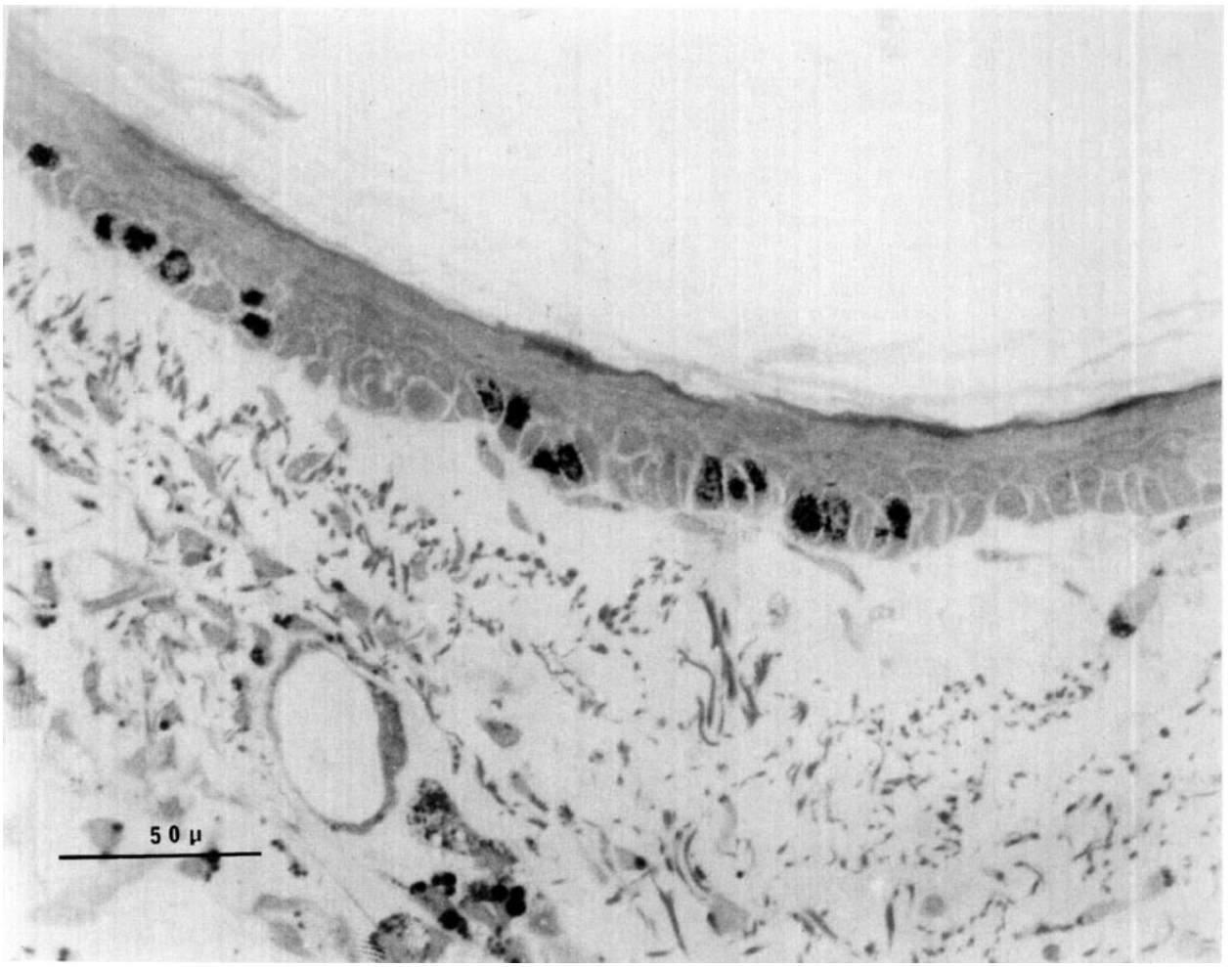

FIGURE 12. After 2 weeks of exposure of a ring culture to benzo[a]pyrene at a concentration of 2 μg/ml, the hyperplastic epithelium exhibits extensive DNA synthesis as reflected in this autoradiograph demonstrating ^{3}H-thymidine incorporation.

contrast, sulfurous and hydrochloric acids, even at ciliostatic levels, produce no lasting morphologic effect after a similarly short exposure. These results indicate that ciliostasis alone is an insufficient indicator of the hazards of intermittent exposure to toxic agents and should be coupled with other measures. A longer term study was concerned with the cytotoxic effects of benzo[a]pyrene, a polycyclic hydrocarbon which is a complete respiratory carcinogen (*12*). Continuous exposure to benzo[a]pyrene was shown to result in premature death of the culture with the average reduction in viability proportional to the concentration of the carcinogenic chemical (*11*).

Tracheal organ cultures have been used in the past to grow respiratory viruses (*13*) and to study early events in carcinogenesis (*14*). Broader application of this technique can be envisioned in identification of toxic agents and of factors affecting toxicity of such agents, in the study of mechanisms of toxic action and in development of methods for modifying or avoiding toxicity. The long-term large-scale culture techniques and criteria for assessment of injury which we describe will facilitate these investigations.

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