

Studies on the Mechanism of Mucin Secretion by Cells of the Porcine Tracheal Epithelium (41030)

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Abstract. The effects of selected pharmacological agents on mucin secretion by the tracheal epithelium of piglets were studied using organ culture. Mucin release into the culture medium was reduced, and mucin retention by secretory cells of the mucosa and submucosal glands was increased, by colchicine, vinblastine sulfate, and cytochalasin B. Dibutyryl cyclic AMP had no significant effect. Although these agents have diverse and poorly understood influences on cell functions, the results suggest a possible role for microtubules and microfilaments in the intracellular movement and release of mucin.

The mechanism of mucin secretion by cells of the mammalian respiratory tract remains to be elucidated. The process whereby the constituents of mucin are synthesized, assembled, and released by tracheal secretory cells has not been defined. Information on the regulation of mucin production and secretion in health and disease could serve as a basis for understanding the pathogenesis of certain respiratory diseases and the development of pharmacological agents to modify abnormal responses to infectious and environmental agents. In this report, we describe studies concerned with the secretion of mucin by explants of porcine trachea exposed in organ culture to pharmacological agents known to affect microtubules and microfilaments.

Materials and Methods. *Organ cultures.* Outbred female piglets (seven) from a local source were killed within 5 days of birth by intraperitoneal injection of sodium pentobarbital. Trachea were excised aseptically from the larynx to carina, immersed in Hanks' balanced salt solution, and opened along the dorsal and ventral aspects. Explants, consisting of half-rings, were placed in 35-mm plastic Petri dishes and incubated at 35° in a humid, 95% air–5% CO₂ environment as described

previously (1). Waymouth's MAB 87/3 (GIBCO, Grand Island, N.Y.) containing 100 µg/ml gentamicin and 25 units/ml nystatin was the culture medium used.

Organ cultures were incubated for 18 hr in medium containing 25 µCi/ml glucosamine–HCl (D-6-³H(N)), sp act 5–15 Ci/mole; (New England Nuclear, Boston, Mass). The explants were washed four times with 2 ml of Hanks' balanced salt solution containing 0.1% dithiothreitol to remove unincorporated labeled glucosamine. At this time, the explants were placed in new Petri dishes, four per dish, and 0.5 ml of fresh Waymouth's medium with no labeled glucosamine was added. This allowed the mucosal surfaces of the explants to be moistened by the fluid, but did not permit immersion. A total of 35 dishes were prepared. They were incubated at 35° for a period of 5 hr. The media then were removed and saved separately. The cultures were divided into five groups of 7 dishes, each of which contained four explants. One group served as control, and 0.5 ml of fresh Waymouth's medium was added to each of the 7 dishes in this group. To the dishes in the second group, medium containing 500 µg/ml N⁶,O₂–dibutyryl adenosine 3':5'-cyclic monophosphoric acid (BtcAMP) was added. To the third, fourth, and fifth groups of 7 dishes, medium with 400 µg/ml colchicine (COL), 200 µg/ml vinblastine sulfate (VB), and 10 µg/ml cytochalasin B (CB), respectively, was added (Sigma Chemical Co., St. Louis, Mo). Preliminary experiments have demonstrated that these

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concentrations are not toxic to tracheal explants (2). In addition, similar concentrations of these agents have been shown to be nontoxic in other experimental cellular systems (3–6).

Analysis of mucin secretion. The five groups of tracheal organ cultures were incubated at 35° for an additional 18 hr. At that time, the medium was removed and saved separately. These specimens and the samples recovered after incubation for 5 hr were analyzed for mucin glycoproteins using the method described by Jennings *et al.* (7). Ten milliliters of cold 10% trichloroacetic acid (TCA) was added to each of the samples (0.5 ml) to precipitate the glycoproteins. After storage for 18 hr at 4°, the precipitate was collected on a Millipore glass fiber filter, using a vacuum funnel. Two additional washes were carried out using 15 ml cold TCA. The filters were placed in scintillation vials and allowed to air dry. After the addition of 10 ml of Instagel (New England Nuclear), the radioactivity of the precipitate was determined in a Packard scintillation counter. Medium that had not been used to maintain organ cultures was employed as a further control. Mucin secretion by the explants in each dish was expressed as the percentage difference between the 5-hr baseline and the 18-hr values (see Fig. 1).

Histochemical studies. Explants were removed from the culture dishes after exposure to the drugs for 18 hr and fixed in 10% buffered Formalin. Histological sections were prepared by standard techniques at an approximate thickness of 6 μ m and stained with both alcian blue (pH 2.5) and the periodic acid-Schiff reaction (AB/PAS) to identify acidic and neutral mucosubstances (8). The percentage of the area of surface mucosa and of submucosal glands that stained with AB/PAS was quantitated using an automated image analyzer (9) (Optomax Corp., London, England). Slides were examined with a Zeiss light microscope using a 40 $\times$ objective, and the image was transferred to a cathode ray screen by a closed-circuit camera attached to the ocular. On the screen, the total area of the mucosa in the section, as well as the mucosal area consisting of AB/PAS-staining material, was determined (Fig. 2). The same procedure was applied to submucosal gland cells, making certain that the AB/PAS-staining material in the glandular lumen was not included. Hence, a quantitative measure of mucin retained in the mucosal and submucosal gland cells was obtained.

A second means of analyzing the mucin content of mucosal goblet cells also was applied. One hundred goblet cells in each direction from the approximate center of

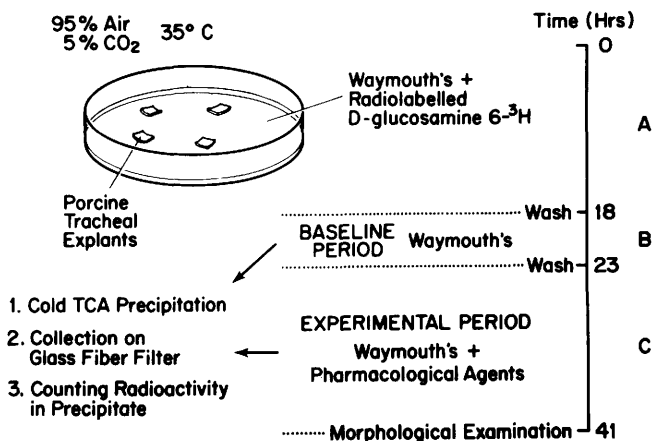


FIG. 1. Procedure for analysis of effects of agents on mucin secretion and secretory cells. The percentage change in release of TCA-precipitable labeled glycoproteins in 18-hr (experimental) period after drug addition is compared to the 5-hr predrug (baseline) values of secretion for each separate dish of explants (Radioactivity in (C) $\div$ radioactivity in (B)).

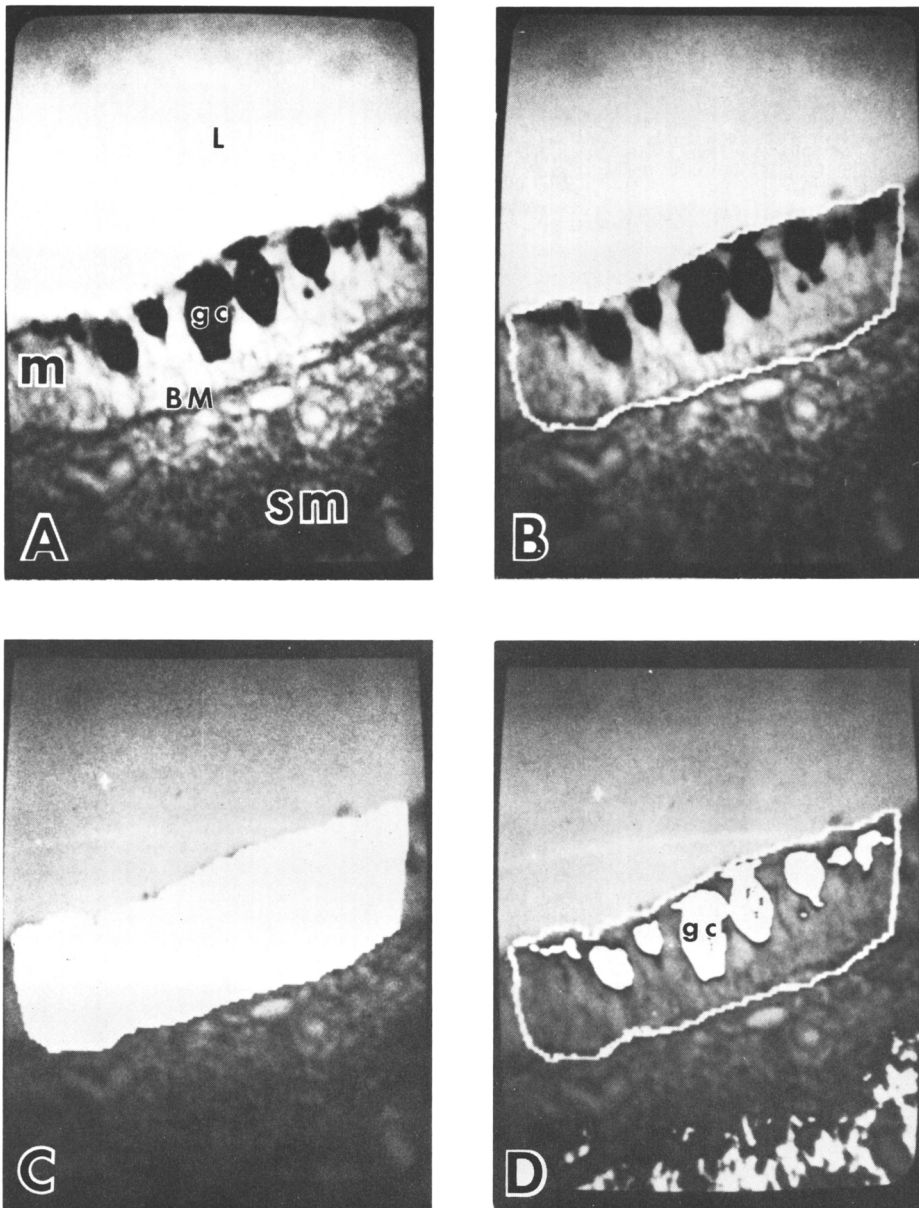


FIG. 2. Use of image analyzer to quantitate retention of mucin (AB/PAS stain) in the tracheal mucosa. Illustration taken from cathode ray screen. (A) Mucosa at $400\times$ magnification projected on screen (L = lumen, m = mucosa, BM = basement membrane, sm = submucosa, gc = goblet cell). (B) Entire mucosa is outlined in white by variable framing mechanism. (C) Total mucosal area (white) encompassed by outline is determined. (D) Area inside (C) that stains for mucin is measured and the percentage of surface epithelial area containing mucin is calculated. $1050\times$.

the explant were examined at 100 times magnification. As illustrated in Fig. 3, the mucin granules in the cells were characterized as representing one of three differ-

ent states of mucin secretion: (A) secreting, with granules entering the lumen; (B) luminal, with granules abutting the apical surface of the cell; and (C) subluminal, with

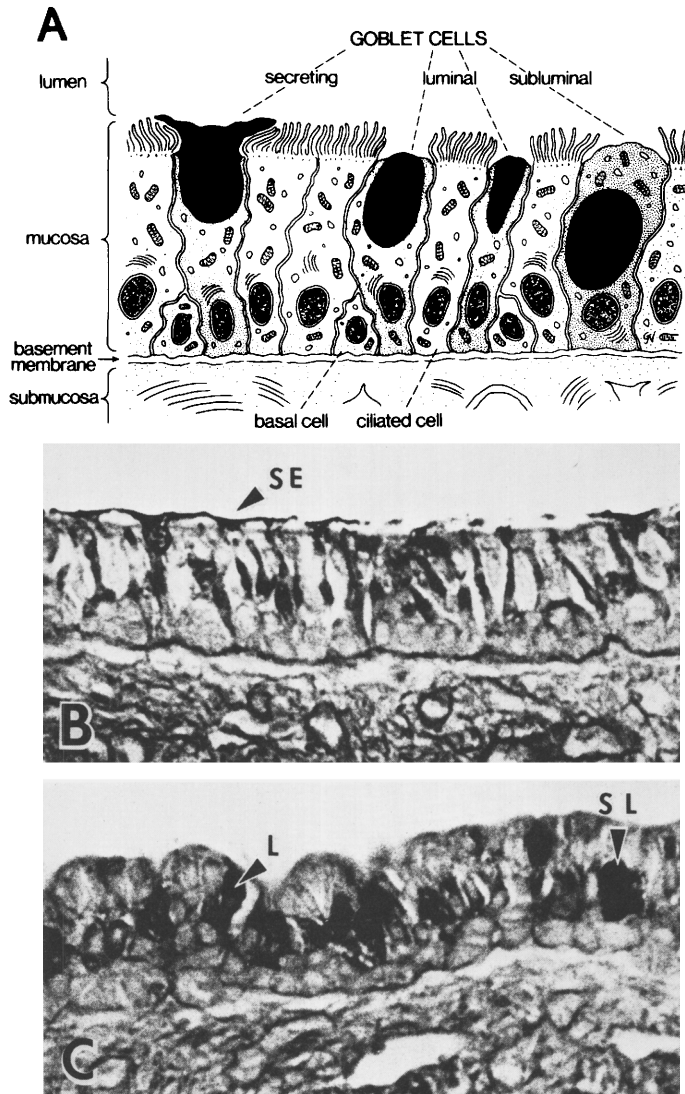


FIG. 3. (A) Schematic illustration of goblet cells with granules representing three morphologically distinct states of secretion as observed in AB/PAS-stained sections of explants. Secreting cells had mucin overflowing into the lumen; other goblet cells contained mucin at the cell apex (luminal) and others contained discrete groups of mucin granules in the cytoplasm distinctly below the cell apex (subluminal). Representative mucosa from (B) control and (C) colchicine-treated explants illustrate secreting (SE), luminal (L) and subluminal (SL) configurations. AB/PAS stain, 800 $\times$.

granules topographically removed from the luminal surface. The percentage of goblet cells with mucin granules in each of the three configurations then was calculated.

The mucin content of submucosal gland cells also was analyzed by an additional method. The cells were graded, based on their content of mucin, on an index of 0 (not

containing mucin) to 4 (filled with mucin) as illustrated in Fig. 4. This was done for each submucosal gland in sections from six separate explants, and a quantitative index of the mucin content of submucosal glands was calculated.

Ultrastructural studies. Goblet cells of the mucosa and submucosal glands in the



tracheal explants were examined by transmission electron microscopy (TEM) at selected intervals during the experiments described above. Since COL and VB have been described as inhibitors of microtubule (MT) formation (10, 11) and CB inhibits the formation of actin microfilaments (MF) (12–15), attention was focused on these organelles. Glutaraldehyde prepared with “PM buffer” (16), believed to stabilize cytoskeletal elements, was used for fixation. Samples of trachea were fixed for 24 hr at room temperature, postfixed in cold 1% OsO_4 in 0.1 M Na cacodylate buffer for 60 min, stained *en bloc* with cold 2% uranyl acetate in sodium hydrogen maleate buffer, pH 5.2, for 2 hr, dehydrated in a graded series of ethyl alcohols, and embedded in Epon. Ultrathin sections were mounted on copper grids and stained with uranyl acetate in 50% ethanol for 10 min and basic lead citrate for 5 min. The sections were examined at 60 kV in a Philips 201 transmission electron microscope.

Results. Figure 5 shows an autoradiograph of an explant incubated with labeled glucosamine for 1 hr. It can be seen that the glycoprotein precursor was incorporated into mucins of the goblet cells and submucosal glands. This observation is consistent with the findings of others (17).

The release of labeled mucins by explants of porcine trachea after exposure to the battery of pharmacological agents is summarized in Fig. 6. BtcAMP failed to increase the secretion of mucin by tracheal explants, whereas the amounts of glucosamine released by tissues treated with COL, CB, and VB were significantly less than both control and nucleotide-treated cultures.

Microscopic examination revealed an apparent retention of AB/PAS-positive mucin in goblet cells and submucosal

FIG. 4. Illustration of typical submucosal glands and their mucin content. The amount of mucin in the gland cells (but not in the lumen) ranged from none (0) to “filled” (4). Every gland from sections of six explants exposed to each agent was evaluated, and a mean index of submucosal gland mucin content calculated (see Table II). AB/PAS stain, 1600 $\times$.

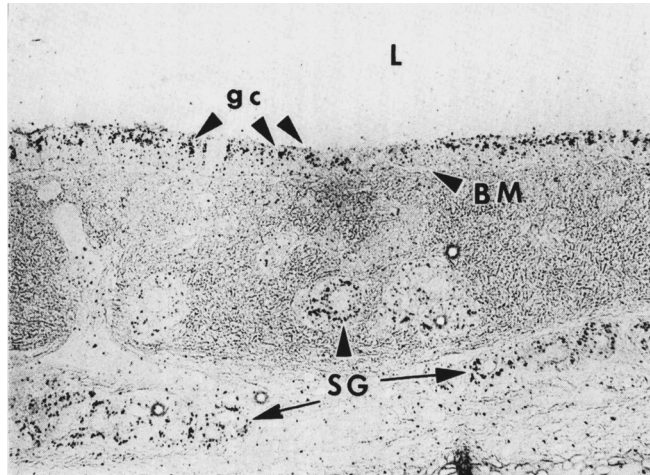


FIG. 5. Autoradiogram of explant exposed to [^3H]-glucosamine for 1 hr in organ culture, then fixed and sectioned. The slides were dipped in NTB₃ emulsion and developed after storage at 4° for 5 days. Label apparently is concentrated in epithelial goblet cells (gc) and submucosal glands (SG), indicating incorporation of the glycoprotein precursor into mucin. L = lumen, BM = basement membrane. Unstained section, 330 $\times$.

glands of tissues incubated with COL, VB, and CB. This visual impression was confirmed by quantitative evaluation and image analysis of mucosal and submucosal secretory cells. As shown in Table I, the amounts of mucin retained in the mucosal goblet cells and submucosal glands of

explants incubated with COL, VB, or CB were significantly greater than in control cultures and those treated with BtcAMP. Moreover, the mucin granules in the majority of goblet cells of the mucosa were located subjacent to the lumen, and retention of mucin in the submucosal glands was

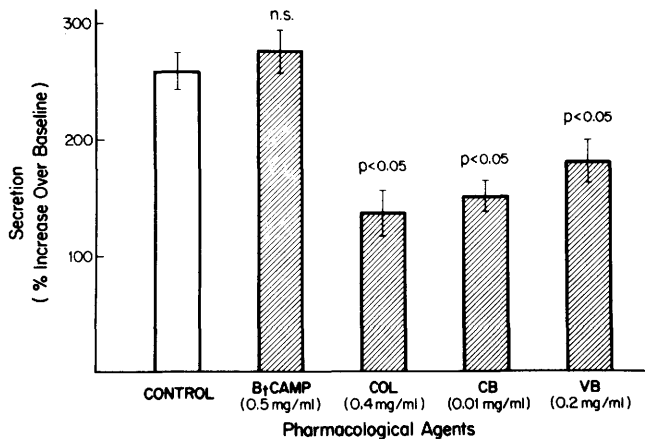


FIG. 6. Secretion of labeled mucin by cultures after incubation with pharmacological agents. Cultures were incubated with labeled [^3H]-glucosamine for 18 hr then washed. After a 5-hr baseline secretion period, culture medium with the pharmacological agents was added for 18 hr (see Fig. 1). Mucin secretion is expressed on the ordinate as the percentage increase over the 5-hr baseline values for each separate culture. Each bar represents the mean $\pm$ 1 SEM for seven cultures exposed to each agent. Significance compared to the control value is based on Bonferroni *t* confidence intervals (40).

TABLE I. AB/PAS-STAINING MUCIN IN MUCOSA AND SUBMUCOSA AFTER INCUBATION WITH PHARMACOLOGICAL AGENTS FOR 18 hr^a

Agent	No. of explants examined	Percent of mucosal area AB/PAS (+) ^b	Percentage of submucosal gland nonluminal area AB/PAS (+) ^b
Control	6	2.66 ± 0.54	4.35 ± 0.77
BtcAMP	6	2.71 ± 0.41	6.07 ± 1.62
COL	6	6.48 ± 0.57 ^c	10.40 ± 2.11 ^c
CB	6	5.89 ± 0.71 ^c	8.67 ± 1.28 ^c
VB	6	5.82 ± 0.55 ^c	9.98 ± 1.82 ^c

^a Image analysis. See text and Fig. 2.^b All values mean ± 1 SE of mean.^c $P < 0.05$ (significantly different from controls based on Bonferroni t confidence intervals (40)).

increased (Table II). The mucin content of cells in BtcAMP-treated cultures did not differ from controls.

TEM of the goblet cells in control cultures revealed numerous membrane-bound secretory granules in the luminal aspect of the cell adjacent to a prominent Golgi apparatus. Extensive rough endoplasmic reticula imparted an electron-dense appearance to the cytoplasm of the secretory cells. Large numbers of intracellular MT and MF also were observed. MT often were located in close association with secretory granules, aligned in the long axis of the cell (Figs. 7A, B). MF, measuring 50–60 Å in diameter, also were observed in proximity to secretory granules. Often they appeared to abut the membranes of the granules (Figs. 7C–F). TEM of explants maintained in organ culture with BtcAMP, COL, and CB revealed no morphological evidence of cytotoxicity. Membrane blebbing, mito-

chondrial swelling, and disrupted intercellular junctions were not observed (Fig. 8). In goblet cells exposed to VB, however, cytoplasmic crystals of MT protein were observed, often in close proximity to secretory granules (Figs. 7G, H). The realignment of MT into characteristic crystal-line arrays by VB has been described previously (11).

Discussion. In these studies, the effects of selected pharmacological agents on the functioning of mucin-secreting cells in the respiratory airways was evaluated quantitatively. Mucin release was decreased by treatment of tracheal explants with CB, COL, and VB (Fig. 6). This was associated with retention of mucin in secretory cells of both the mucosa and the submucosa (Tables I and II). The data strongly suggest that these agents affected the intracellular transport and release of mucin synthesized in these cells.

TABLE II. AB/PAS-STAINING MUCIN IN MUCOSAL GOBLET CELLS AND SUBMUCOSAL GLANDS AFTER INCUBATION WITH PHARMACOLOGICAL AGENTS FOR 18 hr

Agent	No. of explants examined	Percentage of AB/PAS (+) goblet cells with "subluminal mucin" ^{a,b}	"Mucin content index" of submucosal glands ^{b,c}
Control	6	38.7 ± 4.8	1.23 ± 0.24
BtcAMP	6	46.9 ± 7.1	1.20 ± 0.12
COL	6	88.4 ± 3.1 ^d	2.02 ± 0.19 ^d
CB	6	61.4 ± 5.6 ^d	2.06 ± 0.32 ^d
VB	6	84.4 ± 4.5 ^d	2.12 ± 0.10 ^d

^a See text and Fig. 3.^b All values mean ± 1 SE of mean.^c See text and Fig. 4.^d $P < 0.05$ (significantly different from controls based on Bonferroni t confidence intervals (40)).

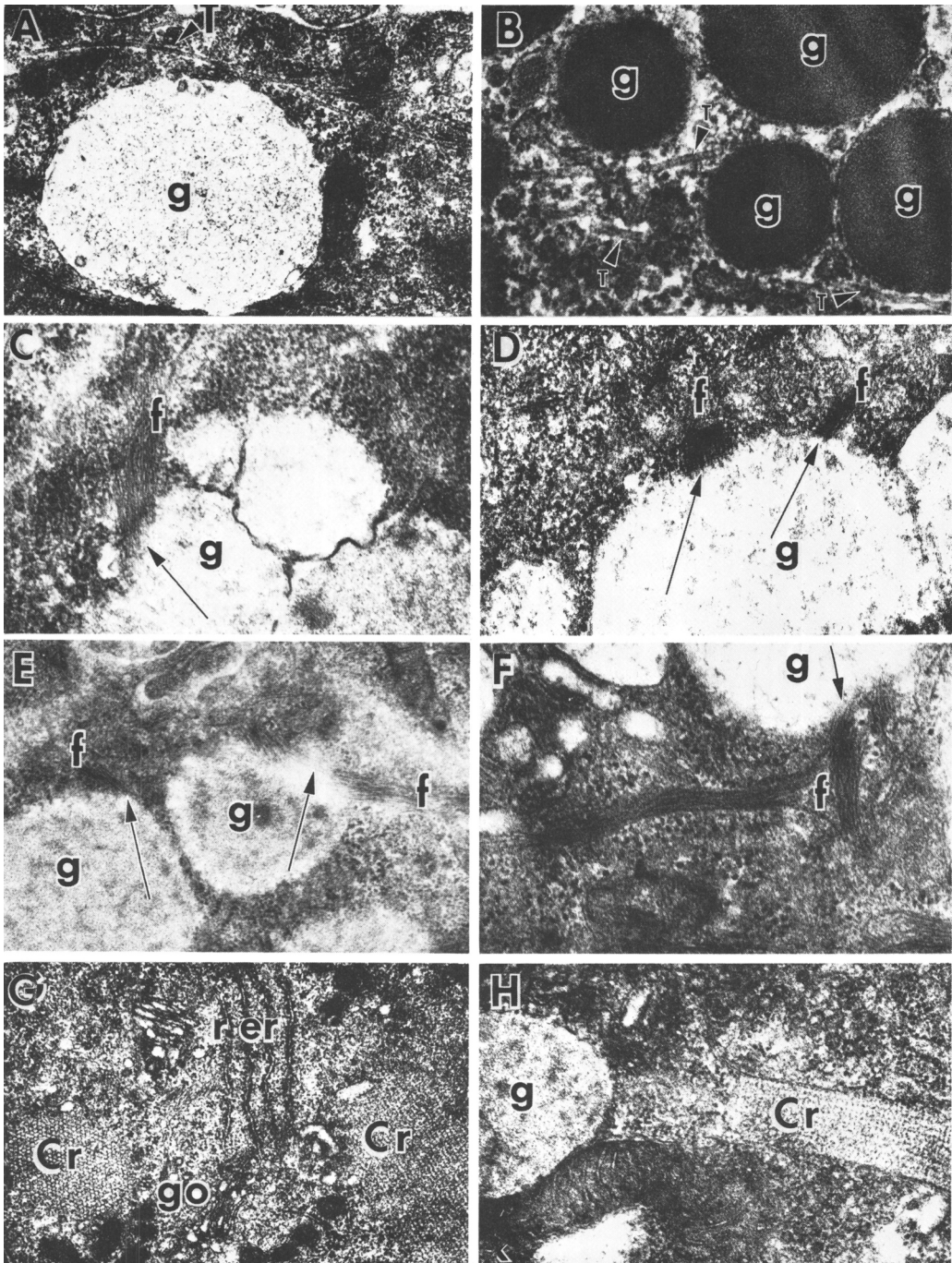


FIG. 7. Ultrastructural association of microtubules and microfilaments with secretory granules. (A) A long, curved microtubule (T) associated with a large secretory granule (g) in a goblet cell (29,280 $\times$). (B) Microtubules and electron-dense mucin granules in close proximity in submucosal gland cell (56,800 $\times$). (C–F) Microfilament bundles (f) in contact with secretory granules (arrows) in four different goblet cells (c, 31,040 $\times$; d, 46,080 $\times$; e, 28,000 $\times$; f, 46,080 $\times$). (G) Two crystals (Cr) of microtubular protein in a tracheal ciliated cell induced by exposure to vinblastine sulfate for 2 hr *in vitro*. The left crystal is seen in cross section; the right, in longitudinal section. go = golgi; rer = rough endoplasmic reticulum (24,000 $\times$). (H) A microtubule crystal induced by vinblastine in a neighboring goblet cell. The close association with a secretory granule (g) is apparent (29,280 $\times$).

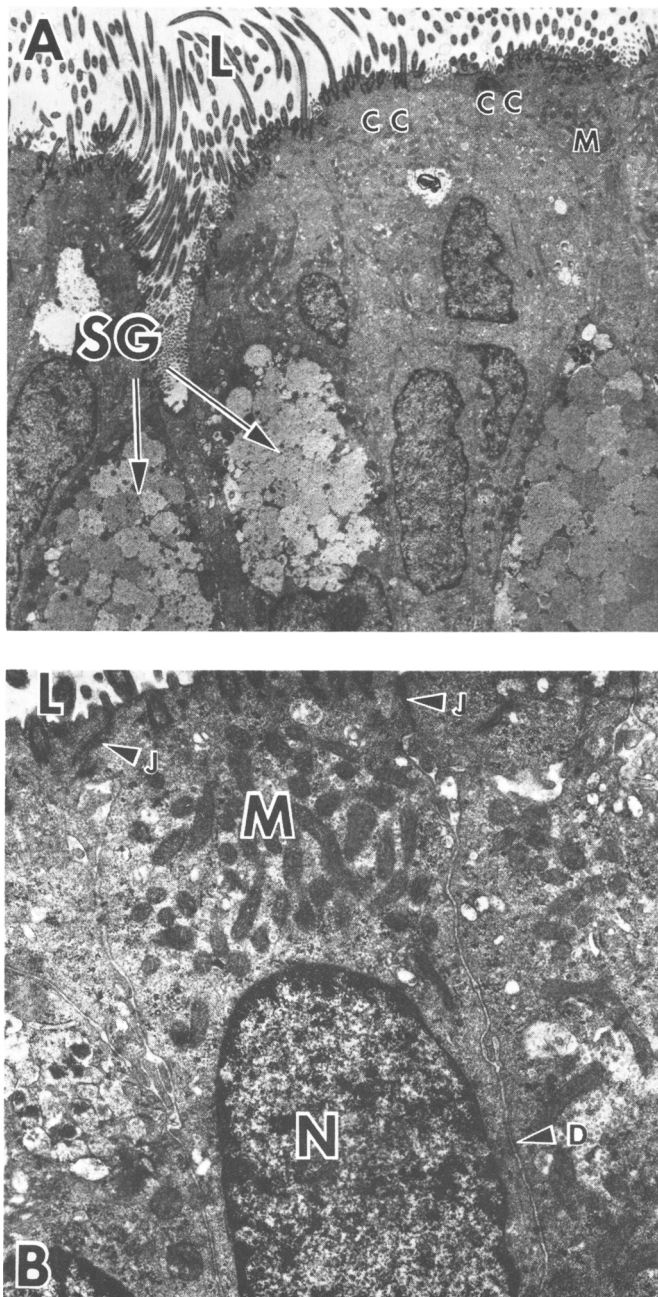


FIG. 8. Typical epithelial cells from mucosa of tracheal explants exposed for 24 hr to (a) colchicine ($400 \mu\text{g/ml}$, $10^{-3} M$) and (b) cytochalasin B ($10 \mu\text{g/ml}$, $2 \times 10^{-8} M$). Cellular and organellar membranes are intact, and intercellular junctions appear normal, indicating no toxicity. (A) $12,000\times$; (B) $20,000\times$. L = Lumen, SG = secretory granules, CC = ciliated cell, M = mitochondria, D = desmosome, J = tight junction. Uranyl acetate and lead citrate stain.

The pharmacological agents employed in these studies have a number of effects on cells and tissues. COL binds to MT protein (tubulin) subunits, blocking further polymerization (and therefore formation) of MT. As MT are in a constant state of polymerization-depolymerization, the net result is continual depolymerization and thus, loss of MT inside cells (10). Vinblastine-induced crystallization of MT protein (11) has been theorized to occur via a similar mechanism (10). In addition, COL binds to membranes and, at relatively high concentrations, causes structural changes and impaired membrane mobility (18). COL also has been shown to decrease the uptake and oxidation of 2-deoxy-D-glucose (19) and to inhibit glucose metabolism (20, 21) and nucleoside transport (22). Vinca alkaloids aggregate polyribosomes (23) and inhibit the synthesis of RNA, DNA (5), and proteins (24). Interaction with acidic membrane proteins also has been reported (5). Cytochalasin B is one of a group of fungal metabolites (cytochalasins A-E) that act to disrupt the formation of actin filaments. Recent reports (12-15) have described this effect as a blocking of polymerization of actin monomers ("G" actin) into a filamentous form ("F" actin) that comprises microfilaments observed in the electron microscope. Since cellular actin is undergoing continuous polymerization-depolymerization, in a manner similar to MT formation, the net effect of CB is a loss of cellular MF. CB also suppresses glycolysis (25) and glucose metabolism, probably by interfering with the transport of sugars across membranes (25, 26).

The experimental data do not demonstrate clearly the site of action of the pharmacological agents in tracheal secretory cells; several of the effects described above, alone or in combination, could result in decreased mucin secretion. The effects also could be due to some other indirect, perhaps unsuspected mechanism. However, we believe the most likely explanation, in terms of a cellular mechanism of secretion, relates to the inhibition of MT and MF. Disruption of these organelles in secretory cells could interfere with the intracellular translocation and release of mucin granules, resulting in decreased se-

cretion and increased retention of mucin, as observed in our experiments.

The cytoplasm of tracheal secretory cells contains many MT and MF that often are observed in close association with secretory granules (Fig. 7). In addition, a plethora of other investigators have demonstrated, using these same agents, a functional role for MT and MF in secretion by a variety of cell types. These include endocrine (4) and exocrine (27) pancreas, connective tissue (28), liver (29), thyroid (5, 6, 30), anterior pituitary (31), parotid (3), lacrimal (32), and lung (33). We believe the results of our study support the view that MT and MF also are involved in the process of mucin secretion. However, we recognize that other effects of the pharmacological inhibitors may have contributed to the results obtained.

Hypothetical systems have been suggested in which MT and MF act in concert to move granules out of export cells. Erlich and Bornstein (28) theorized that MT and MF control the movement of procollagen vesicles from polysomes to the cell membrane, where conversion to collagen and secretion by connective tissue cells occur. These organelles are believed to function in concert to move chromaffin granules from the Golgi apparatus to the surface of cells of the adrenal medulla (34). Orr *et al.* (35) and Neve *et al.* (6) suggested a supportive role for MT in secretion by mast cells and thyroid tissue, respectively, with MF providing the actual contractile force. MT act as an internal skeleton in pancreatic beta cells, guiding insulin granules to the apical plasmalemma, while contraction of cytoplasmic MF appears to supply the force for movement of these granules (36). Complementary involvement of MT and MF in secretion by cultured rat anterior pituitary cells (31) and hepatocytes (29) has been indicated. Evidence for MT-MF interaction has been provided recently by Griffith and Pollard (37), who demonstrated specific binding sites for actin filaments on MT, and Fujii and Tanaka (38), who showed that MT protein was capable of binding actomyosin.

Exposure of tracheal explants to BtcAMP did not affect mucin secretion. These data conflict with the findings of Whimster and Reid (39), who reported an increase in se-

cretion of mucin by human bronchial glands *in vitro* after exposure to comparable concentrations of BtcAMP. However, their data were based on interpretation of autoradiographs prepared after exposure of human tissue to the cyclic nucleotide for 1 hr, whereas our conclusions were derived from studies of porcine tissue carried out after 18 hr. The discrepancy could relate to these differences.

The quantitative results of our study suggest that MT and MF could be involved in the intracellular transport and release of mucin by mammalian airway cells. Ultrastructural examination of these cells revealed a close association of MT, MF, and secretory granules, supporting strongly the biochemical and histochemical findings. These data suggest cellular targets for management of respiratory disease characterized by altered mucin secretion. Furthermore, they elucidate a significant aspect of the cellular biology of the secretory process in respiratory tract epithelium.

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