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SYNTHESIS OF MUCIN GLYCOPROTEINS BY EPITHELIAL CELLS ISOLATED FROM SWINE TRACHEA BY SPECIFIC PROTEOLYSIS

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SUMMARY

Mucus-producing cells were isolated from swine trachea mucosa by a method that included enzymatic digestion of the epithelial surface with Dispase, a neutral protease from *Bacillus polymyxa*, and differential attachment of the washed cells to culture flasks coated with collagen. Epithelial cells were the major cell type isolated by these procedures. Ciliated cells that did not attach to the flasks were removed by decantation, and fibroblasts were destroyed by the bacterial protease. The isolated cells synthesized respiratory mucins and the rate of secretion was increased about threefold when tracheas were exposed to sulfur dioxide. The cultured cells incorporated both [^{35}S]O_4 and [I-^{14}C]*N*-acetylglucosamine into secreted mucin glycoproteins. The secretion of glycoprotein increased for about 3 d until the cells became confluent, and then a constant rate was observed for a period of at least 7 d. This increase in the output of mucin glycoprotein during the initial 3 d of culture was accompanied by a corresponding increase in the number of mucus-producing cells in the flasks. The results obtained in these and subsequent studies suggest that the rate of formation of mucus-producing cells may be a rate limiting step in the regulation of mucin glycoprotein synthesis in tracheal epithelium.

The chemical, physical, and immunological properties of the glycoprotein secreted by isolated tracheal epithelial cells were very similar to the mucin glycoprotein purified from washes of swine trachea epithelium. The purified mucin glycoproteins showed complete cross-reaction with antibodies to trachea mucin glycoprotein. They were eluted near the void volume during gel filtration on Sepharose CL-6B columns. The glycoprotein isolated from culture media under the standard assay conditions had nearly the same carbohydrate composition as samples purified from washes of trachea epithelium. Reduced oligosaccharides released by β-elimination with dilute alkaline borohydride showed similar elution profiles during chromatography on Bio Gel P-6 columns. Taken collectively, these results suggest that the isolated epithelial cells secreted mucin glycoproteins that were very similar to those synthesized by the intact trachea epithelium under standard incubation conditions.

Key words: trachea; epithelial cells; mucin glycoprotein; Dispase; bromhexine.

INTRODUCTION

Mucin glycoprotein secretions that protect the respiratory tract are synthesized by epithelial cells on the surface epithelium and in the acinotubular glands in the submucosa layer of the tracheobronchial system (22). Mucous and serous cells line the submucosal glands, and goblet cells are present on the tracheobronchial epithelium (22). There are more than 10 different cell types in the tracheobronchial system. However only the goblet, mucous, and serous cells contain secretory granules. The mucous cells of the submucosal gland also secrete acidic mucin glycoproteins. Autoradiographic and histochemical studies have shown that labeled sulfate, sugars, and amino acids are incorporated into granules in these mucus-producing cells and into acidic glycoproteins secreted by these cells (17,36,39).

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Most studies of mucus secretion in respiratory tissue have been conducted with organ explants (11,25,26,41). Extensive studies by Ellis and Stahl (8,38), with canine trachea explants have shown conclusively that labeled sulfate, glucosamine, and L-fucose are incorporated into secreted acidic mucin glycoproteins. These studies further showed that denuded explants, prepared by removing surface cells from the epithelium leaving a viable submucosa, also incorporated radioactive presursors into acidic mucin glycoproteins. The glycoproteins isolated from epithelial goblet cell secretions were more sulfated than secretions from submucosal glands. However, the cellular heterogeneity of the trachea mucosal membrane makes the assignment of specific functions to individual cell types very difficult. This tissue secretes a number of different glycoproteins, including mucin glycoproteins and secretory IgA, and it is likely that these glycoproteins are formed by different cells in the intact tissue. To circumvent this problem of cellular heterogeneity and to study the mechanisms involved in the synthesis and secretion of mucin glycoproteins, we have developed a procedure for the isolation of an enriched population of mucus-producing cells from swine trachea.

Cultures of normal epithelial cells have been prepared from lung (6,27,29) and numerous other tissues. The elimination of contaminating fibroblasts from cultured epithelial cells usually required tedious, careful dissection of the epithelial layer from connective tissue, the cloning of cells growing out of explants, or the control of fibroblast proliferation. The fibroblasts present in primary cultures tend to grow more rapidly than epithelial cells and usually become the dominant cell type in cultures. Indeed, when conventional procedures are used to release epithelial cells from trachea, the principal cells that proliferate after several days in culture are fibroblasts. In a recent study Goldman and Baseman (13) used repeated treatments with thermolysin and gradient centrifugation to prepare epithelial cells free of fibroblasts from hamster trachea. The molecular weight of the glycoprotein synthesized by these cultured epithelial cells was surprisingly low, 18,500 daltons (12).

The present communication describes a unique and simple procedure which has been developed to obtain large amounts of mucus-producing cells from swine trachea. These preparations are completely free of connective tissue elements and the primary cultures produced mucin glycoproteins for periods of at least 7 d. The metabolic studies reported here show the cells actively incorporate radioactive glucosamine and sulfate into secreted acidic mucin glycoproteins, which can be isolated from the culture medium and compared with mucin glycoproteins synthesized in vivo. The mucin glycoprotein synthesized by isolated tracheal epithelial cells in culture was purified and characterized. Its chemical and immunological properties were compared with the glycoprotein synthesized by swine trachea and deposited on the epithelial surface. Several immunological procedures employing antibody to purified swine trachea mucin glycoprotein were developed for the quantitation and characterization of mucin glycoprotein secreted by the isolated cells and for establishing the antigenic identity between this glycoprotein and the corresponding macromolecule that is synthesized in vivo.

Studies with these isolated epithelial cells suggest that the increased synthesis of mucus is accompanied by a rapid increase in the number of mucus-producing cells. However, it is not presently known whether this increase is due to increased mitosis of mucus-producing cells or to increased differentiation of precursor cells to mucus-producing epithelial cells. The studies described in this report, employing trachea epithelial cells with well-defined characteristics and growth requirements, should enable us to examine the relationship between precursor cells and mucus-producing cells. It is hoped that the information gained with these isolated cell systems will allow the development of an assay for the differentiation of precursor cells or the transformation of inactive secretory cells to active mucus-producing cells. An understanding of the mechanism of formation of mucus-producing cells in the tracheobronchial epithelium and the effects of various stimuli on the regulation of the rate of this transformation would greatly aid efforts directed toward the management of chronic obstructive lung disease and reduction of the hypersection of mucus glycoproteins characteristic of these diseases. Our original observations on the selective isolation of mucus-producing cells from swine trachea have been communicated in a preliminary report (4).

MATERIALS AND METHODS

Isolation of epithelial cells. Trachea, about 15 cm long from the larynx to the bifurcation of

the bronchi, were removed by aseptic technique from small pigs, 40 to 60 lb, immediately after slaughter. The excised tracheas were washed three times with 20 ml of Hanks' balanced salt solution (HBSS) containing 200 U/ml penicillin, 200 μg/ml streptomycin sulfate, 5 μg/ml Fungizone (GIBCO, Grand Island, NY), and 1 mg/ml gentamicin sulfate. Washing was carried out by vigorous shaking in an Erlenmeyer flask. Afterwards the lower end of the trachea was clamped with a hemostat and the lumen was filled with 20 ml of HBSS containing antibiotics and 0.08 mg/ml of an expectorant, bromhexine (*N*-cyclohexyl-*N*-methyl-2 amino-3,5-dibromobenzyl) ammonium chloride in order to remove mucus and adhering bacteria. The upper end of the trachea was closed with a hemostat and it was incubated at 37° C for 20 min with intermittent shaking. The solution was then removed and the lumen was washed twice with 5 ml of HBSS. The trachea was filled with 20 ml of HBSS containing 2 mg/ml of Dispase and closed with hemostats. It was incubated at 37° C for 30 min with intermittent gentle shaking. The resulting opaque solution was passed through sterile 80 mesh nylon screens into conical shaped tubes and centrifuged at 200 ×*g* for 20 min. The sedimented cells were resuspended in sterile HBSS and washed three times with buffer. More enzyme (20 ml HBSS containing 2 mg/ml Dispase) was added to the trachea, and it was incubated for an additional 60 min. The cells released after this second treatment were isolated by the same procedure.

Washed cells from each preparation were suspended at a concentration of 4×10^5 cells/ml in Medium 199 containing 15% fetal bovine serum (Gray Industries, Inc., FL), 0.29 mg/ml L-glutamine, 1.0 μg/ml bovine insulin (Eli Lilly Co., Indianapolis, IN), 0.1 μg/ml hydrocortisone, 0.1 μg/ml β-retinyl acetate (Hoffman-LaRoche, Nutley, NJ), 100 U/ml penicillin G, 100 μg/ml streptomycin, 2.2 mg/ml $NaHCO_3$ and 20 m*M* Tricine-HCl (Sigma Chemical Co., St. Louis, MO). The medium was adjusted to pH 7.4 with 1 *N* NaOH. Aliquots of 10 ml were seeded in 75-cm^2 tissue culture flasks, which were previously coated with collagen. The suspensions were incubated at 37° C for 8 h at which time the medium containing unattached cells was removed and 10 ml of fresh medium was added. The medium was removed every 12 h and fresh medium was added. At intervals some of the cells were fixed and stained for histological examination.

Trachea epithelial cells were also prepared from detached trachea mucosa. In this case, the trachea were cut into halves longitudinally and pinned to a sterile board. The membrane was removed by dissection and minced with scissors in 10 ml of HBSS. The tissue was collected by decantation of the medium and resuspended in 50 ml of HBSS containing 2 mg/ml of Dispase. The minced tissue was stirred at 37° C for 30 min. Then the suspension was passed through a 80-mesh nylon screen and the cells were collected and washed as described previously.

Cell culture and cell number. All of the cells were cultured in supplemented Medium 199 (GIBCO, Grand Island, NY) containing 15% fetal bovine serum as described in the previous section. Both the medium and serum were free of mycoplasma contamination when examined by a direct culture assay. The flasks were coated with a 1% solution of collagen, and cells were cultured in a controlled chamber at 37° C, 95% humidity, and in an atmosphere of 95% air:5% CO_2. Under these conditions the secretion of mucin glycoprotein by attached epithelial cells continued for at least 7 d. When incubations were carried out with labeled precursors, 5 μCi/ml [I-^{14}C]GlcNAc (9.5 mCi/mmol) or $Na_2[^{35}S]O_4$ (346 mCi/mmol) were added to the medium.

The increase in the number of viable cells during the course of incubation was determined by the following procedure. At each time point three flasks were washed twice with HBSS and the cells were then released by incubation with 1 ml of 0.25% Trypsin-EDTA for 10 min at 37° C. The resulting suspensions were centrifuged at 500 ×*g* for 5 min and the pelleted cells were washed twice with 2 ml of HBSS. The final pellets were resuspended in 1 ml of salt solution and the number of viable cells was determined by trypan blue dye, 0.02%, exclusion in a hemocytometer. The number of cells was calculated from the average of three determinations.

Characterization of cells in culture. Microscopic and histochemical monitoring indicated that no apparent morphological changes occurred in the attached epithelial cells during the 7-d period. Cells grown over cover slips were used in morphological studies. The plates were washed and fixed in 10% phosphate buffered (pH 7) formalin for 24 h. The samples were then treated with alcian blue dye 8GX (pH 2.6) periodic acid-Schiff base, which stains acidic mucin glycoproteins (23). For electron microscopy, cells were fixed in 3% glutaraldehyde in 0.1 *M* phosphate

buffer (pH 7.2) for 1 h at room temperature. Cells were rinsed in buffer and postfixed in 2% osmium tetroxide in 0.1 *M* phosphate buffer (pH 7.2), dehydrated, and embedded in Epon 812. Sections were cut on a Riechert OMU-3 ultratome with diamond knives, stained with uranyl acetate and lead citrate, and examined in a Hitachi H-600 or a Zeiss 9S microscope. For scanning electron microscopy, tissue samples were fixed as above, dehydrated, and critical point dried in a Bomar apparatus. Samples were coated with gold:palladium (60:40) and examined in a ETEC Autoscan microscope.

Assay for the synthesis of mucin glycoproteins. In the standard assay a 0.1-ml aliquot of the incubation medium was added to 0.5 mg of carrier Cowper's gland mucin (34). The glycoproteins were precipitated by the addition of 3 ml of 2.8 *N* perchloric acid containing 2% phosphotungstic acid. The precipitate was collected by centrifugation, dissolved in 0.5 ml of 1 m*M* NaOH, and reprecipitated with 3 ml of the acid solution. This washing procedure was repeated twice and the final pellet was dissolved in 0.6 ml of 0.5 *M* NaOH, added to a scintillation vial containing 0.4 ml 1 *M* HCl and 10 ml scintilation fluid, and counted (33).

More than 80% of the radioactivity present in the washed acid precipitate was mucin glycoprotein. The isolated cells incorporated [^{3}H]GlcNAc mainly into this secreted glycoprotein. Aliquots of the same samples that were purified by chromatography on DEAE-cellulose columns or by gel filtration on Sepharose CL-4B columns contained 90% of the counts detected by the acid precipitation method. The ion exchange procedure described by Havez et al. (17) was used at 3° C. Samples were applied to a DEAE-cellulose column (5 × 20 cm), which was equilibrated with a solution containing 0.02 *M* KH_2PO_4, pH 6.5, -6 *M* urea. The column was eluted with a stepwise gradient. It was washed with 100 ml of 0.02 *M* KH_2PO_4, pH 6.5, 6 *M* urea and then with 100 ml of 0.2 *M* KH_2PO_4, pH 6.5,-6 *M* urea. The mucin glycoprotein was then eluted with 150 ml of 0.3 *M* Na_2CO_3-0.1 *N* NaOH - 6 *M* urea. This last fraction was neutralized immediately, dialyzed, concentrated by lyophilization, and applied to a Sepharose CL-4B column. All of the radioactivity eluted from DEAE-cellulose with 0.3 *M* $NaCO_3$-0.1 *N* NaOH was recovered in excluded high molecular weight fractions during gel filtration on Sepharose CL-4B columns. Hydrolysis with 2 *N* HCl for 4 h and subsequent analysis by paper chromatography in several solvent systems showed that GlcN was the principal radioactive component present in the isolated mucin glycoproteins.

Purification of mucin glycoprotein. Mucin glycoprotein, which contains more than 80% of the bound carbohydrate present in washings from swine trachea epithelium, was solubilized by reduction and carboxymethylation and purified as described in a previous study (34). Tracheas were removed from pigs after slaughter and were washed with modified phosphate buffered saline (PBS) containing 8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, 1.2 g KH_2PO_4, 0.9 m*M* $CaCl_2$ and 0.5 m*M* $MgCl_2$/l. The solution was adjusted to pH 7.4 with NaOH. The gelatinous wash solutions from about 100 trachea were combined and concentrated by lyophilization. The resulting gel was dissolved in 6 *M* guanidine-HCl, reduced with 30 m*M* dithiothreitol, and alkylated twice with 90 m*M* iodoacetamide to solubilize the mucin glycoprotein. The mucin glycoprotein (4 mg/ml) was then purified by gel filtration on a Sepharose CL-6B column (5 × 100 cm) in the presence of 2 *M* guanidium chloride, pH 7.5. A high molecular weight mucin glycoprotein was eluted just after the void volume in a broad peak. Fractions containing mucin glycoprotein were combined, concentrated, dialyzed, and rechromatographed on a second Sepharose CL-6B column. The high molecular weight mucin glycoprotein peak, which again eluted near the void volume, was collected and dialyzed against 0.03 *M* potassium phosphate, pH 8.0. The solution was adjusted to 6 *M* urea and it was applied to a DEAE-cellulose 5 × 20 cm column previously equilibrated against the same buffer. The column was washed with 200 ml of 0.2 *M* potassium phosphate, pH 6.5, 6 *M* urea, and a mucin glycoprotein fraction was then eluted with 0.3 *M* sodium carbonate-0.1 NaOH-6 *M* urea. This fraction was neutralized immediately with 1 *M* acetic acid, concentrated by lyophilization, and dialyzed against 90 m*M* Tris-borate, pH 8.2-2 m*M* EDTA. The isolated glycoprotein showed a single diffuse band after electrophoresis in 1% agarose gels and it banded at the top of polyacrylamide gels even after heating at 100° C for 3 min in 1% 2-mercaptoethanol and 1% dodecyl sulfate. The preparation was completely free of other proteins and stained weakly with protein reagents and very strongly with periodate Schiff base and alcian blue. The purified mucin glycoprotein was completely free of human lacto-

transferrin, immunoglobulins, and serum proteins, which are present in the crude mucus preparations.

Mucin glycoprotein labeled with [^{14}C]GlcNAc or [^{35}S]O_4 was isolated from the medium of epithelial cell cultures by the same procedures on a smaller scale. The medium was removed from culture flasks every 12 h and the attached cells obtained from 10 trachea were shaken with 5 ml of Medium 199 to dislodge all of the secreted mucus. Fresh medium at 37° C was then added and the incubation was continued for another 12 h. Radioactive mucin glycoprotein was isolated from the combined spent culture media and washes were obtained after 5 d of incubation.

Preparation of antibodies to porcine trachea mucin glycoprotein. An emulsion of the purified trachea mucin glycoprotein, 2 mg, in Freund's complete adjuvant was injected intradermally into young White New Zealand adult rabbits. After 3 wk each animal received booster injections of 1 mg of mucin glycoprotein and a week later serum was collected. The antibodies were partially purified from 40 ml of serum by precipitation with ammonium sulfate (0.4 saturation at 4° C). The precipitate was dissolved in PBS and dialyzed against this buffer. The solution was then passed through a small DEAE-cellulose column (2.2 × 10 cm) and the column was washed with 50 ml of PBS. The effluent and wash were combined and passed into a protein A-Sepharose 4B column (2.2 × 7 cm). The column was washed with PBS until the effluent was free of protein, and bound IgG was then eluted with 40 ml of 0.1 *M* glycine-HCl, pH 3.0. The eluent was adjusted immediately to pH 7.0 with Tris base. The IgG fraction was concentrated and dialyzed against PBS. The purified immunoglobulin fraction contained 1.5 mg of protein/ml and it was sterilized by ultrafiltration and stored in the presence of 1:10^5 merthiolate. Examination by immunodiffusion against the purified mucin glycoprotein yielded only one precipitin line. Recovery of the antibody was measured in a standardized radioimmunoassay employing ^{125}I-labeled mucin glycoprotein. Serum at a dilution of 1:500 and 1:1000 precipitated 27 and 21% of the labeled antigen. The purified IgG at a dilution of 1:500 and 1:1000 precipitated 30 and 25% of the antigen under identical conditions. These results indicate that nearly all of the antibody was recovered in the purified IgG fraction.

Immunoassays for mucin glycoproteins. Double immunodiffusion in agar gel was carried out by the Ouchterlony procedure. Antibody was added to the center well and mucin glycoprotein samples in 0.05 *M* phosphate buffer, pH 7.0, containing 1 *M* urea to increase solubility were added to the outer wells. The concentration of mucin glycoprotein was measured by a quantitative double antibody radioimmunoassay employing ^{125}I-labeled antigen (20,21). Purified mucin glycoprotein samples were labeled with [^{125}I]NaI by the procedure of Greenwood et al. (14). The specific radioactivity of the purified trachea wash mucin glycoprotein reisolated by two successive passages through Sephadex G-100 columns was 5×10^6 cpm/μg of protein. The columns were equilibrated and run with buffer containing 0.02 *M* potassium phosphate, pH 7.0, 0.85% NaCl, and 0.01 *M* potassium iodide. The second antibody used in these assays, goat antibody to rabbit IgG, was obtained from Calbiochem Behring Corp., San Diego CA, and rabbit anti-porcine serum used as a control in these experiments was obtained from GIBCO.

Procedures and materials. The expectorant, bromhexine (*N*-cyclohexyl-*N*-methyl-2-amino-3,5-dibromobenzyl) ammonium chloride was provided by Dr. Karl Thomae, Gm BH, Chemischpharmazeutische, Fabrik, Biberbach an der Riss, West Germany. Trypsin was obtained from Difco, and Dispase, a neutral protease from *Bacillus polymyxal*, was supplied by Boehringer Mannheim (Indianapolis, IN). The concentration of protein was measured by several methods as described previously (34) and total carbohydrate was determined by the anthrone procedure (7). Analysis of isolated mucin glycoproteins was carried out by specific colorimetric and enzymatic methods as described in our previous studies (33,37). Bound and free sulfate were determined by the methods of Spencer (40) and Dodgson (5). Electrophoresis was carried out as described by Holden et al. (18,19). Bands containing protein were located with Coomassie blue dye and carbohydrate was detected with the periodic acid Schiff reagent. Radioactivity in the bands containing labeled glucosamine and sulfate was measured by slicing the gel into 4-mm discs and dissolving them in scintillation vials containing Bray's mixture as described previously (32).

RESULTS

Identification and morphology of isolated tracheal epithelial cells. The viability of unat-

tached cell preparations was examined by trypan blue dye exclusion. The majority of the cells, 85 ± 5%, isolated after 30 min of incubation with Dispase excluded trypan blue dye and most of the cells had an elliptical epithelial structure. After treating the denuded membranes for an additional 60 min, only about 70 ±5% of the isolated cells excluded trypan blue dye. There were no apparent differences between cell preparations obtained from intact trachea or dissected membranes. The yield ranged from 2×10^6 to 6×10^6 cells/trachea. Epithelial cells that synthesize

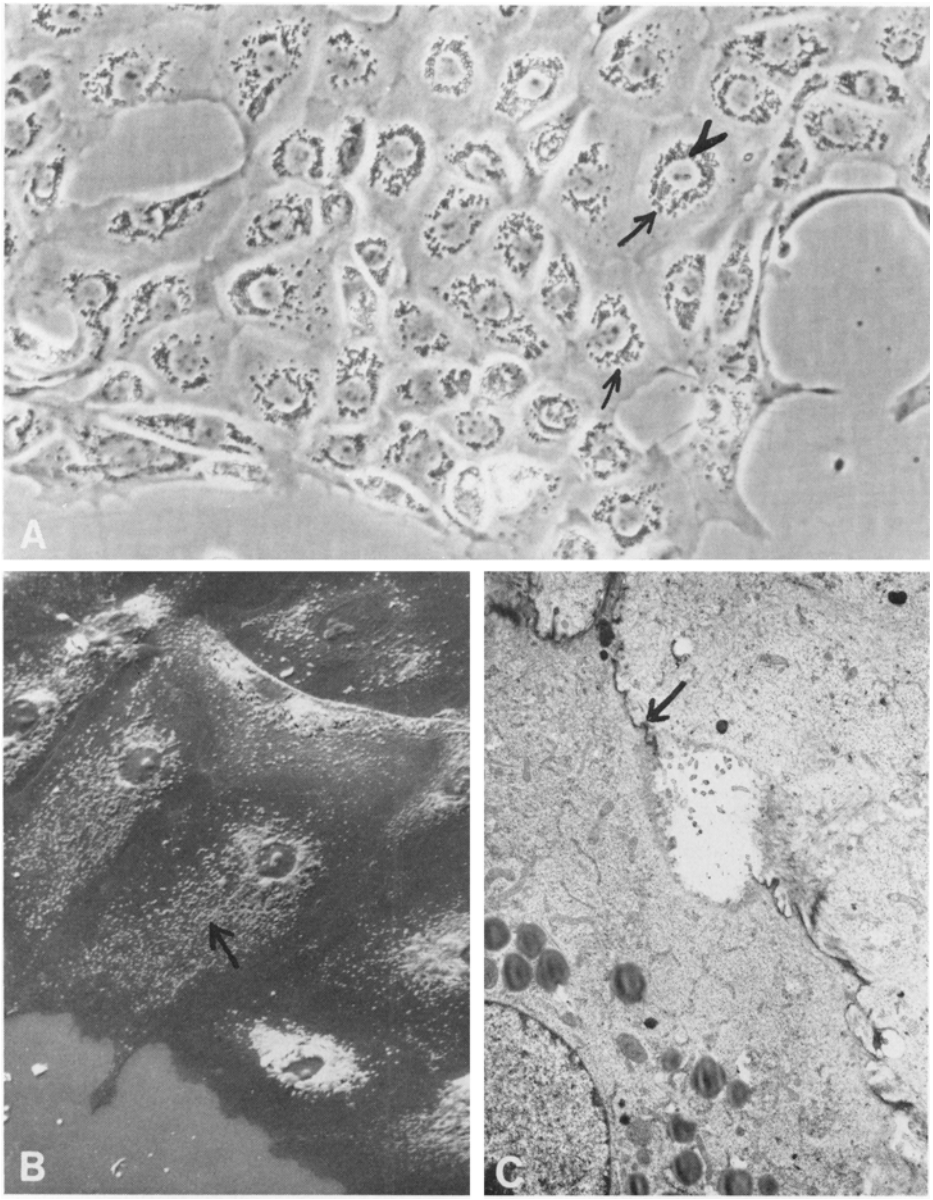


FIG. 1. *A*, Photomicrograph of a sheet of mucus-secreting swine tracheal epithelial cells in culture. The cells contain a nucleus with prominent nucleoli (*arrowhead*). Cytoplasmic granules (*arrow*) surround the nucleus. ×208. *B*, Scanning electron microscopy demonstrates the epithelial nature of the sheets of mucus-secreting trachea cells. The apical surface is covered with short microvilli (*arrow*). ×370. *C*, Thin sections through junctional complexes of adjacent cells demonstrate the epithelial characteristics of desmosomes (*arrow*). ×3,330.

mucin glycoprotein were further purified by differential adherence to flasks coated with collagen. After incubating suspensions in primary culture for 8 h most of the ciliated cells aggregated along their lateral surfaces to form elliptical clusters with cilia directed outward. Ciliated cells did not attach to the collagen surface, and almost all of these cells were removed by decantation after incubation for 8 h at 37° C. Precoating the tissue culture flasks with collagen increased the number of attached epithelial cells by at least 50%. The cell cultures prepared by treatment with Dispase contained epithelial-type cells. The cells that attached after 24 h (Fig. 1 *A*) formed clusters containing 10 to 50 cells that seemed to form tight junctions. The number of cells and the size of the clusters increased over the next several days until cells proliferated over the entire surface of the flask. One major cell type containing numerous small, dense granules was observed during this period of proliferation.

Scanning electron microscope preparations of cells (Fig. 1 *B*) also showed the epithelial sheet characteristic of tightly joined cells. The surface of these cells were covered with fine microvillar processes. Examination of cells in section revealed the typical junctional complexes of epithelial cells (Fig. 1 *C*). Mitochondria, secretory granules, and fine tonofilaments were also present.

The characteristic polarity of secretion in goblet cells is lost in cells in culture. The nucleus, centrally placed in the cell (Figs. 1 *A*; 2 *A*), is surrounded by secretory granules varying in size (Fig. 2 *A*). However, the large granules typically

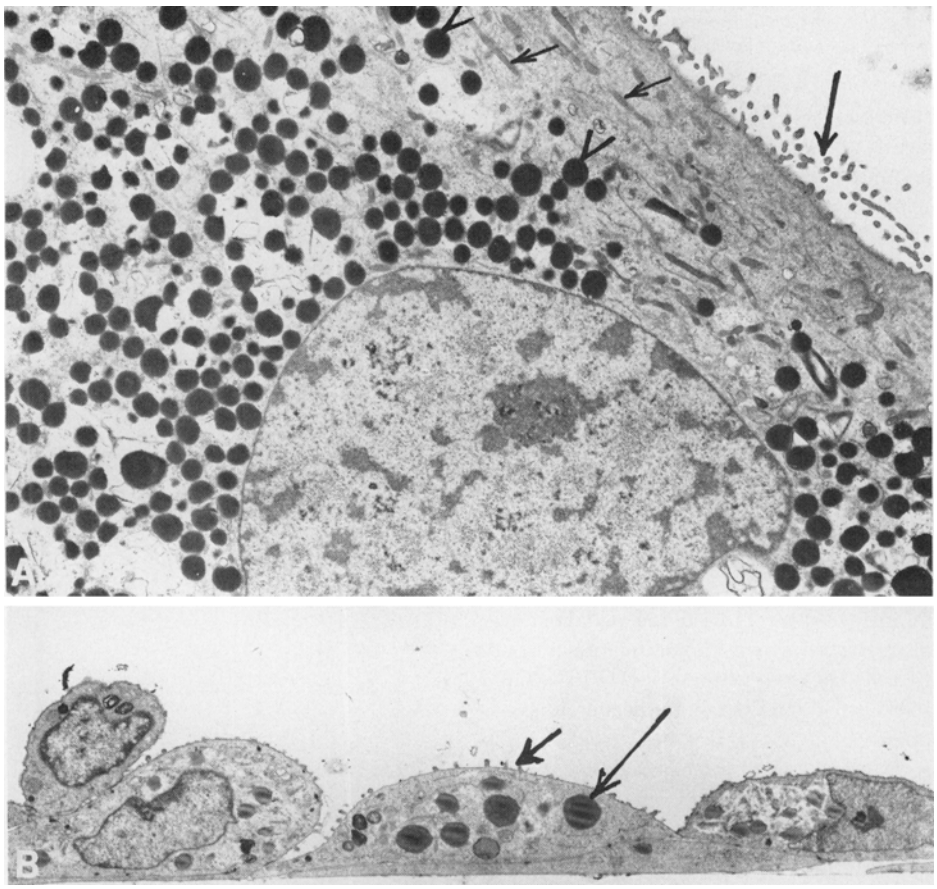


FIG. 2. *A*, This epithelial cell in culture shows the heavy distribution of secretory granules (*arrowhead*) in the cytoplasm, mitochondria (*short arrow*), and microvilli (*long arrow*) on the cell surface. ×5280. *B*, When cultures are cut in a plane perpendicular to the cell sheet it is evident that the cells overlap in some regions to form a bilayer. Secretory granules (*long arrow*) and short microvilli (*short arrow*) on the surface cells are also evident. ×3,340.

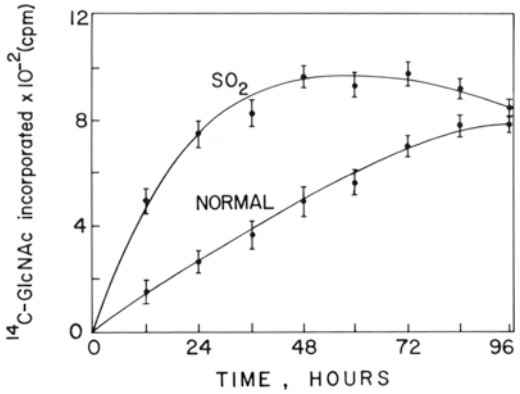


FIG. 3. Time course for the incorporation of [^{14}C]GlcNAc into mucin glycoproteins by isolated swine trachea epithelial cells. The cells were cultured under the standard conditions described in Materials and Methods and 5 Ci/ml of [I-^{14}C]GlcNAc (9.5 mCi/mmol) was added to 2.5 ml of medium. The medium was changed at 12-h intervals and 0.1-ml aliquots were assayed by the procedure described in Materials and Methods. Each point represents the mean of four experiments and the *bars* represent ± standard deviation.

found in the apical region of goblet cells are only infrequently observed in cells in culture. Loss of cellular apical-basal cell orientation may account for the failure of these cells to form larger secretory granules. Filamentous mitochondria and fine surface microvilli (Fig. 2 *A*) can also be seen in thin sections. Sections perpendicular to the culture surface (Fig. 2 *B*) indicate that the cultures may be bilayers in certain regions. Secretory granules seem to accumulate in large vacuolated regions in the cytoplasm before release from the cell. However, the mechanism of secretion has not yet been established.

Long strands of mucus originating from clusters of cells floated in the medium, and one end of the strand was usually still attached to the cells. The secretion of mucin glycoprotein into the medium increased for 2 to 3 d and remained constant or decreased slowly thereafter. After about 10 d the cells began to increase slowly in size and some cells were found floating in the medium.

Comparison of the cell types released from trachea by the neutral protease, Dispase, with those released by trypsin and other mammalian proteases revealed some striking differences. The cells obtained by treatment with various concentrations of trypsin-EDTA contained more fibroblasts, and after 24 h of incubation these flasks contain fibroblasts with only small islands of granulated mucin-producing cells. After 3 d, very few mucin-producing cells remained, whereas the fibroblasts proliferated over the entire surface of the flask. The medium from these flasks did not contain labeled mucin glycoprotein when they were incubated with [^{35}S]O_4 or [^{14}C]GlcNAc. The preparation obtained by treating the trachea a second time with trypsin for 60 min contained even more fibroblasts. In marked contrast, cell preparations isolated by disruption with Dispase showed no proliferation of fibroblasts even though the initial unattached primary suspension contained an appreciable number of fibroblasts. Under these conditions, fibroblasts did not attach well to the surface of the flask, and those few that did were unable to proliferate.

Metabolic properties and the synthesis of mucin glycoprotein by isolated epithelial cells. The exclusion of vital dye and utilization of glucose were used to evaluate the physiological viability of the isolated cells. Most of the attached cells, 90%, excluded trypan blue dye. The rate of glucose utilization was 7 nmol/10^6 cells per hour. The isolated cells were also completely functional and synthesized mucin glycoproteins for at least 7 d. The influence of the time of incubation on the rate of incorporation of [^{35}S]O_4 and [I-^{14}C]GlcNAc into secreted mucin glycoprotein is shown in Figs. 3 and 4, respectively. The rate of synthesis and secretion of mucin glycoprotein increases for about 72 h and then levels off. Cells isolated from trachea treated with 500 ppm SO_2 (1) showed a much higher rate of synthesis and secretion of

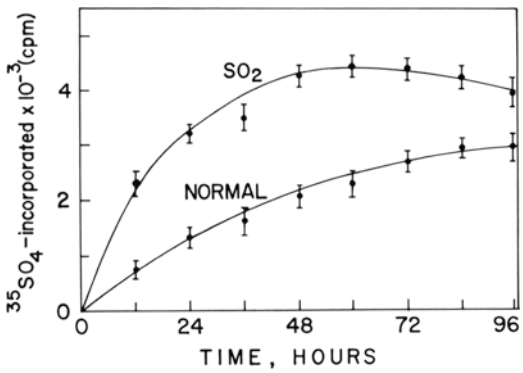


FIG. 4. Time course for the incorporation of [^{35}S]O_4 into secreted mucin glycoprotein by isolated swine trachea epithelial cells. The cells derived from four swine trachea as described in Materials and Methods were incubated in supplemented Medium 199 containing 5 μCi/ml of [^{35}S]Na_2SO_4 (346 mCi/mmol) in a total volume of 2.5 ml. The medium was changed at 12-h intervals and 0.1-ml aliquots were assayed by the acid precipitation method. Each point represents the mean of four determinations and the *bars* represent ± standard deviation.

labeled mucin glycoprotein at earlier times, as seen in Figs. 3 and 4. However, both SO_2 and normal preparations showed similar output after 96 h. It should be noted in this regard that preparations treated with SO_2 intially contained a larger number of attached cells containing large translucent granules that stained strongly for mucin glycoproteins. After 12, 24, and 48 h of incubation, flasks with cells from normal trachea (Fig. 3) contained $3.1 \pm 0.5 \times 10^5$, $6.0 \pm 1.1 \times 10^5$, and $1.1 \pm 0.2 \times 10^6$ cells/flask, respectively. The corresponding flasks containing cells from animals exposed to SO_2 contained $1.6 \pm 0.3 \times 10^6$, $2.9 \pm 0.5 \times 10^6$, and $3.8 \pm 0.6 \times 10^6$ cells/flask after 12, 24, and 48 h in culture. The incorporation of [^{3}H]GlcNAc into secreted mucin glycoprotein by normal control cells during these same time periods was about 150, 250, and 475 cpm/0.1 ml medium for the 0 to 12, 12 to 24, and 24 to 48-h interval. The corresponding values for the cells isolated from trachea exposed to SO_2 were 500, 750, and 950 cpm/0.1 ml medium. A slower rate of incorporation per cell was observed with SO_2 treated cells. These results suggest that the increased synthesis of mucin glycoprotein with time of incubation is accompanied by a corresponding increase in the number of mucus-secreting cells. These data indicate clearly that cultured swine trachea epithelial cells are able to synthesize mucin glycoproteins and transport them into the extracellular medium and that the rate of synthesis is related to the number of mucus-secreting cells in the preparation.

Purification of mucin glycoprotein synthesized by isolated epithelial cells in culture. The mucin glycoprotein secreted by epithelial cells in large scale incubation mixtures, 30 flasks (75 cm^2) containing 5×10^6 cells/flask, was purified and compared with the mucin glycoprotein obtained from washes of normal swine trachea. Cells were cultured with [^{35}S]O_4 or [I-^{14}C]GlcNAc for periods of 5 d. The labeled mucin glycoprotein in the culture medium was collected, reduced with dithiothreitol, alkylated, and purified by gel filtration on Sepharose CL-6B and chromatography on DEAE-cellulose as described in Materials and Methods.

The elution profiles of the mucin glycoprotein obtained from the medium of cells incubated with [^{35}S]O_4 or [^{14}C]GlcNAc are shown in Fig. 5. These glycoproteins were eluted in the same high molecular weight fraction as authentic mucin glycoprotein purified from the surface of swine trachea epithelium. Both of the samples from Peak I (Fig. 5) were further purified by chromatography on DEAE-cellulose columns. More than 90% of the radioactive mucin glycoprotein, in each case, was eluted in the 0.3 *M* sodium carbonate-0.1 *N* NaOH-6 *M* urea fraction. These eluents were immediately neutralized, dialyzed against 0.02 *M* Tris-HCl, pH 8.0,-1 m*M* EDTA and concentrated by lyophilization.

Properties of purified mucin glycoproteins. Aliquots from the purified mucin glycoprotein samples were reduced with 1% 2-mercaptoethanol in the presence of 1% dodecylsulfate and examined by electrophoresis in 1% agarose gels. As seen in Fig. 6 (*Frame A*) the profiles obtained with mucin glycoprotein purified from the culture medium of epithelial cell preparations resembled the pattern of mucin glycoprotein purified from washes of swine trachea epithelium. In each case, a typical band, which stained weakly for protein and intensely for carbohydrate, was observed. The purified mucin glycoproteins did not enter polyacrylamide gels even when electrophoresis was performed in the presence of 1% 2-mercaptoethanol and 1% dodecyl sulfate. No included bands were observed after electrophoresis on polyacrylamide gels, which suggested that the

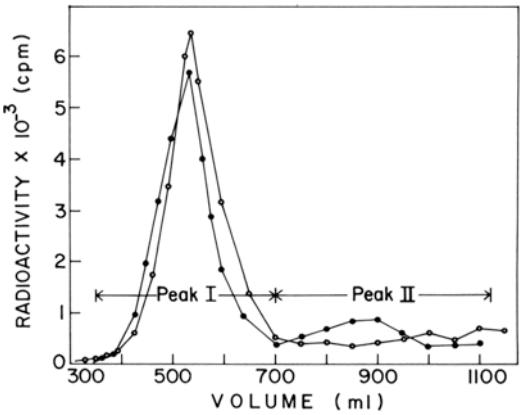
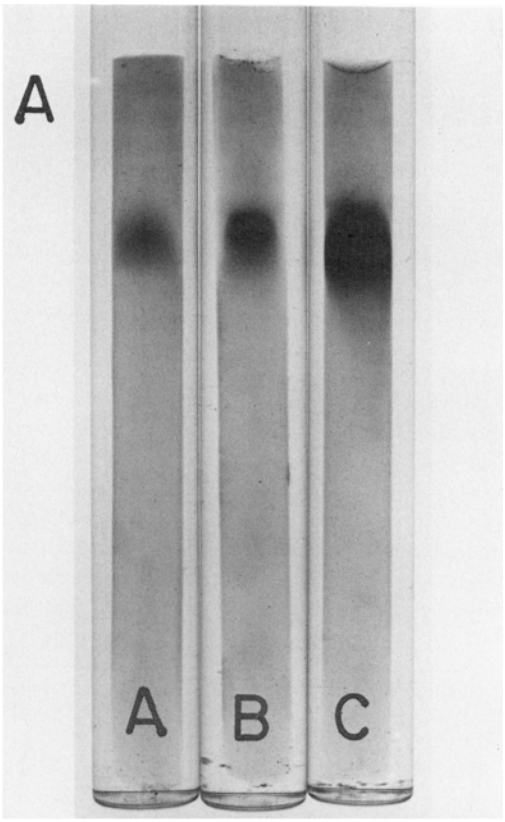
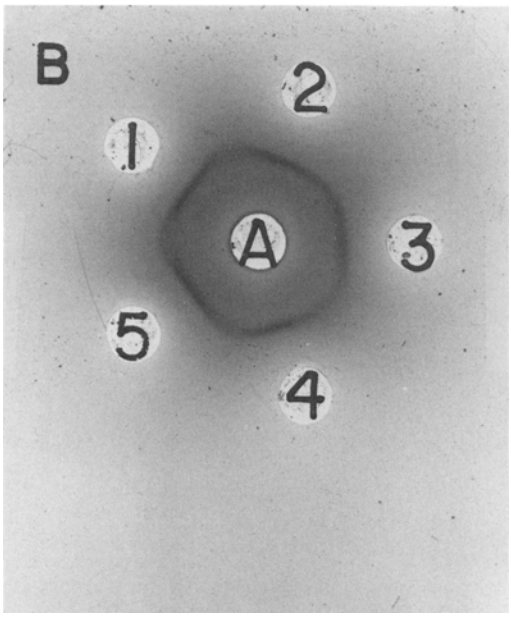


FIG. 5. Gel filtration of mucin glycoproteins metabolically labeled with [^{14}C]GlcNAc or [^{35}S]O_4 by isolated epithelial cells in culture. The concentrated media, 25 ml, were dialyzed against 0.02 *M* Tris-HCl, pH 8.0-2 *M* guanidine-HCl-0.1% sodium azide reduced and applied to Sepharose CL-6B columns (5 × 100 cm), which were equilibrated with the same buffer. Fractions of 15 ml were collected at a flow rate of 28 ml/h. Aliquots of 1 ml were assayed for radioactivity. *Curve* (O--O) was obtained with medium from cells incubated with [^{35}]SO_4, and *Curve* (●--●) was obtained with medium from cells incubated with [^{14}C]GlcNAc. Duplicate anthrone assays for carbohydrate showed similar profiles and a single major peak between 400 and 600 ml for both samples.

purified mucin glycoproteins were completely free of lactotransferrin, immunoglobulins, and serum proteins present in the crude mucus preparations.





The electrophoretic behavior of mucin glycoproteins isolated from cell cultures containing [^{35}S]O_4 or [I-^{14}C]GlcNAc is shown in Fig. 7. The radioactive profile of mucin glycoprotein labeled with [^{35}S]O_4 or [I-^{14}C]GlcNAc were nearly identical and coincided with a single band, which stained for both carbohydrate and protein after gel electrophoresis (*Curve A* and *Curve B*).

The identity of these glycoproteins was further demonstrated by immunodiffusion analysis using a partially purified antibody to mucin glycoprotein isolated from washes of normal swine trachea epithelium. As seen in Fig. 6 (*Frame B*) each of the glycoprotein samples formed single confluent precipitin lines with antibody and these lines fused completely in each case. Mucin glycoproteins purified from cell cultures and labeled with ^{125}I reacted quantitatively with antibodies to mucin glycoprotein purified from washes of swine trachea, as seen in Fig. 8. The radioactivity remained in the immunoprecipitate after extensive washing with 10 m*M* sodium phosphate buffer, pH 7.5,-0.15 *M* NaCl, and the addition of unlabeled normal swine trachea mucin glycoprotein resulted in a proportional decrease in the amount of ^{125}I-mucin glycoprotein found in the immunoprecipitate. These results suggest that the mucin glycoprotein synthesized by isolated epithelial cells and normal swine trachea have very similar antigenic determinants.

FIG. 6. Gel electrophoresis and immunodiffusion patterns of purified mucin glycoprotein synthesized by isolated epithelial cells. *Frame A*, Samples were incubated in 1% dodecyl sulfate-1% 2-mercaptoethanol-0.02 *M* sodium phosphate, pH 7.5, for 3 min at 100° C before electrophoresis on 1% agarose gels. *Gel A* contains 50 μg of authentic mucin glycoprotein purified from washes of swine trachea epithelium. *Gel B* contains 50 μg of mucin glycoprotein, which was purified from medium of cells incubated with [^{35}S]O_4 and *Gel C* contains 100 μg of mucin glycoprotein purified from medium of cells incubated with [^{14}C]GlcNAc. The gels were stained for carbohydrate with periodic acid Schiff reagent. Duplicate gels stained with Coomassie blue for protein showed single bands at the same position as those seen in the figure. *Frame B*, Immunodiffusion patterns were obtained with the same samples. The center well contains a purified IgG fraction isolated from antiserum to purified swine trachea mucin glycoprotein. *Well 1* contains mucin glycoprotein purified from washes of swine trachea epithelium. Wells 2 and 4 contain mucin glycoprotein purified from the medium of cultured cells isolated from two different trachea and incubated with [^{35}S]O_4. *Wells 3* and 5 contain mucin glycoprotein secreted by cells incubated with [^{14}C]GlcNAc.

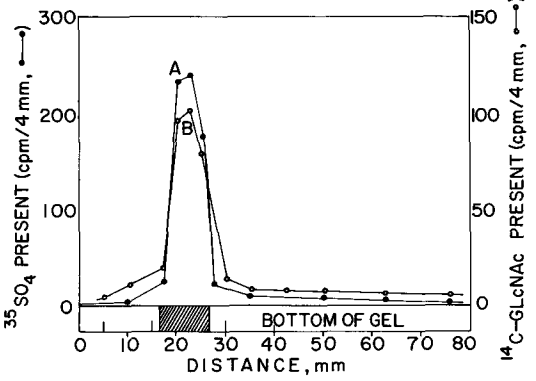


FIG. 7. Electrophoresis of $[^{35}S]O_4$ and [^{14}C]GlcNAc labeled purified mucin glycoprotein on 1% agarose gels containing 0.1% dodecyl sulfate in 0.09 *M* Tris-2.5 m*M* EDTA-0.09 *M* sodium borate buffer at pH 8.0. The samples were solubilized with 1% 2-mercaptoethanol and 1% dodecyl sulfate and ran for 2 h at 8 mA/tube. A single diffuse glycoprotein band was located by staining with the periodic acid Schiff reagent. Radioactivity profiles were obtained by cutting the gels into slices of 4 mm. *Curve A* (●--●) was obtained with a mucin glycoprotein sample labeled with $[^{35}S]O_4$, and *Curve B* (○--○) was obtained with a sample labeled with [I-^{14}C]GlcNAc.

Carbohydrate composition of trachea mucin glycoprotein. The carbohydrate composition of mucin glycoproteins synthesized by isolated epithelial cells and those formed by normal swine trachea were compared. Carbohydrate analysis showed that the glycoprotein purified from washes of swine trachea mucosa contained GalNAc, GlcNAc, galactose, fucose, sialic acid, and sulfate in a molar ratio of 0.80:0.87:1.00:0.85:0.15:0.12 based on the galactose present. The purified glycoprotein did not contain mannose or uronic acids, which suggested that it was free of serum glycoproteins and proteoglycans, respectively. The sample purified from epithelial cell cultures had a similar carbohydrate composition. The molar ratio of GalNAc, GlcNAc, galactose, fucose, sialic acid, and sulfate was 0.83:0.80:1.00:0.92:0.21:0.10.

The principal amino acids present in both glycoproteins were serine, threonine, and proline, and more than 90% of the oligosaccharide chains were released by β-elimination with dilute alkaline borohydride. Alkali labile oligosaccharides attached to serine or threonine are released from glycoproteins by treatment with 0.05 *M* KOH and 1 *M* sodium borohydride for 20 h at 50° C. This treatment released nearly all of the covalently bound $[^{32}S]O_4$ and [I-^{14}C]glucosamine from the isolated mucin glycoprotein samples. These results further demonstrate that the mucin glycoproteins synthesized by the isolated epithelial cells contain carbohydrate attached almost exclusively to seryl and threonyl residues through an *O*-glycosidic linkage. These properties are characteristic of mucin glycoproteins. The reduced oligosaccharides were separated on a Bio Gel P-6 column and their elution profiles are shown in Fig. 9. Two major oligosaccharide peaks were obtained with both preparations, and the molecular weights and relative proportions of the reduced oligosaccharides were remarkably similar. Taken collectively, these results indicate that the isolated epithelial cells synthesize mucin glycoproteins, which are very similar in structure to those found on the epithelial surface of swine trachea epithelium.

DISCUSSION

The successful isolation and culture of normal mucus-secreting epithelial cells from swine trachea required the use of the mucolytic agent, bromhexine, and a neutral protease, Dispase. Treatment with bromhexine removed most of the adhering mucus from the epithelial cell surface. It

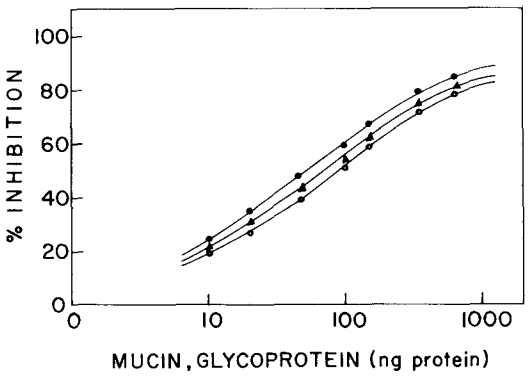


FIG. 8. Inhibition curves for radioimmune assays with antitrachea mucin glycoprotein. *Curve* (●--●) ^{125}I-trachea mucin glycoprotein (5×10^4 cpm, 5×10^3 cpm/ng) from trachea washes with unlabeled trachea wash mucin glycoprotein as indicated in the figure. *Curve* (▲-▲) ^{125}I-trachea mucin glycoprotein (5×10^4 cpm, 2×10^3 cpm/ng) from medium of cultured epithelial cels incubated with [^{14}C]GlcNAc. Unlabeled trachea wash mucin glycoprotein as indicated in the figure. *Curve* (○-○) ^{125}I-trachea mucin glycoprotein (2.5×10^4 cpm, 0.8×10^3 cpm/ng) from medium of cultured epithelial cells incubated with $[^{35}S]O_4$. Unlabeled trachea wash mucin glycoprotein as shown in the figure. About 100 μl of antiswine trachea mucin glycoprotein antibody (purified IgG fraction) was used in these experiments.

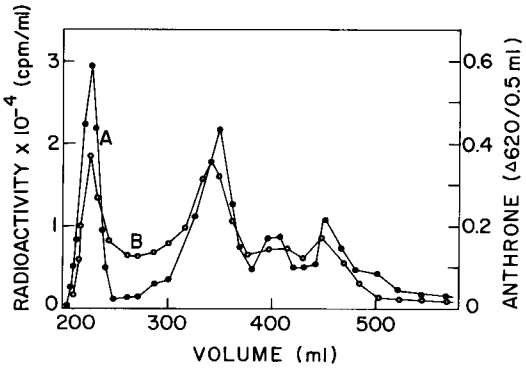


FIG. 9. Elution profiles of reduced oligosaccharides released from mucin glycoprotein purified from washes of swine trachea (*Curve A*) and the medium of epithelial cell cultures (*Curve B*). The reduced oligosaccharides were released from the glycoproteins by incubation with 0.1 NaOH and 2 *M* sodium borohydride at 45° C for 36 h. They were isolated and concentrated as described in a previous report (42). The samples were applied to Bio Gel P-6 columns (2.2 × 200 cm) and eluted with 0.1 *M* pyridinium acetate, pH 5.5. The fractions in *Curve A* (●--●) were assayed by anthrone determination. The labeled reduced oligosaccharides isolated from purified mucin glycoprotein synthesized by epithelial cells in the presence of [^{14}C]GlcNAc, *Curve B* (O--O), were assayed by determination of radioactivity.

did not have a toxic effect on the cells at the concentrations and times of exposure used in these studies. In preliminary trials it was difficult to make the tracheal surface aseptic because bacteria bound tightly to the mucus at the membrane surface. By removing the mucus from the epithelial surface with bromhexide, bacteria, which could later contaminate the cell culture, were eliminated before digestion with proteases. Furthermore, removal of the mucus layer made the surface epithelial cells more accessible to the proteases. This treatment also probably eliminated the need for careful aseptic handling of fresh trachea, inasmuch as extensive precautions were not necessary to obtain sterile cultures. This mucolytic agent has also been used in the isolation of bovine colon epithelial cells (28).

The mechanism by which bromhexine promotes the release of mucus is not known. However, it has been shown that bromhexine increases the volume of mucus secretion apparently by a direct action on cells secreting mucus (9,35). In a more recent study an increase in the secretion of mucus in the trachea was observed 15 min after the intravenous injection of 4 mg of bromhexine into anesthetized dogs (15). Goblet and serous cells in the epithelial layer and the seromucus glands in the submucosa contributed to this rapid tracheal discharge. Numerous spherical mucous granules derived from goblet cells and submucosal glands were observed on the epithelial surface after the administration of bromhexine. A fine flocular serous discharge or transudate was also formed 15 min after treatment with the expectorant. After 60 min no mucous granules were observed on the surface and the amount of serous secretion was reduced. Many goblet cells were present but they contained only a few secretory granules and the mucous glands in the submucosa seemed empty. The serous glands were shrunken and contained only a small amount of granular material. No significant differences in the glycoprotein composition of sputum from patients receiving bromhexine or placebo have been observed (3). These observations suggest that the expectorant may act by stimulating the discharge of both serous and mucus secretions from granules without influencing the biosynthesis of acidic mucus glycoproteins and thereby promote the removal of surface mucus and attached bacteria during agitation and incubation of the closed trachea.

The isolation of epithelial cells from trachea in good yield also required the use of a neutral bacterial protease, Dispase. This enzyme promoted the survival of trachea epithelial cells, while suppressing the proliferation of fibroblasts. In contrast, disruption of the membrane with trypsin-EDTA resulted in the isolation of a large number of viable, rapidly dividing fibroblasts and very few epithelial cells. Matsumura et al. (30) have used Dispase to detach mammalian cells growing in tissue culture. Fibroblasts are detached from culture flasks and dissociated when this protease is used. However, epithelial cells of strains HeLa-53, JTC-16, and RLC-10 are detached from the surface of the flask, but they are not extensively dissociated. In other studies these workers further showed that the enzyme did not damage epithelial cells nor interfere with growth when it was added to suspension cultures (31). These observations indicate that the neutral protease probably does not destroy membrane receptors or recognition sites on the epithelial cell surface. However, Dispase does attack fibroblasts and other connective tissue cells. The epithelium of the trachea can be removed and dispersed by mechanical disruption, therefore it is possible that Dispase only loosens the upper layer of epithelial cells and the freed cells may then be dispersed by shaking. Although the mechanism of action of

Dispase is not presently completely understood, the results obtained in the present study clearly indicate that the use of this enzyme for the disruption of the trachea epithelium permits the reproducible isolation of viable mucus-producing epithelial cells essentially free of fibroblasts.

The tendency of tracheal epithelial cells to form clusters suggests that they may indeed still contain recognition sites and that the rate of binding of unattached cells to those already attached may be much greater than the rate of binding to the collagen surface. Precoating the flasks with 1% collagen increased the number of epithelial cells attached to the surface. However, this increase was due mainly to an increase in the size of the clusters and not to the number of clusters present. The groups of cells expanded to form confluent layers, and this process was accompanied by an increase in the output of mucin glycoproteins over a 3-d period. When confluency was attained, the rate of synthesis of mucin glycoprotein leveled off and remained constant or decreased slightly. Under the standard conditions used to culture cells in the present studies, increase in the rate of secretion of mucin glycoprotein was accompanied by an increase in the number of mucus-producing cells in the culture. The origin of the new mucus-secreting cells has not yet been established. These cells may arise by mitosis of mucus-producing cells in the culture, by transformation of serous cells to mucus-producing cells, or by differentiation of basal cells to mucus-producing cells. Evidence obtained in ultrastructural studies (2) and the observed incorporation of [^{3}H]thymidine into goblet cells in the trachea (16,24) suggest that mucus-producing cells can undergo mitotic division. The results obtained in other histological studies further suggest that serous type cells containing electron dense granules may be converted to mucus-secreting goblet cells (10,22).

The structure and immunological properties of the mucin glycoproteins synthesized by the isolated epithelial cells over a 5-d period were nearly identical to the glycoprotein purified from washes of normal swine trachea. The high molecular weight glycoprotein in both cases eluted near the void volume during chromatography on Sepharose CL-6B, and the molar ratio of monosaccharides found in the mucin glycoprotein purified from the medium of cell cultures was very similar to that of mucin glycoprotein formed in vivo. The reduced oligosaccharides obtained from both glycoproteins showed similar profiles when examined by gel filtration on Bio Gel P-6 columns. The mucin glycoprotein synthesized by epithelial cells in culture had nearly the same immunological properties as authentic swine trachea mucin glycoprotein, and it showed complete cross-reaction with antibodies to the normal swine trachea glycoprotein. These results further suggest strongly that the cultured swine trachea epithelial cells synthesized and secrete a mucin glycoprotein that is very similar to the corresponding glycoprotein synthesize in normal swine trachea.

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