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ESTABLISHMENT AND CHARACTERIZATION OF A CELL LINE DERIVED FROM BOVINE TRACHEAL GLANDS

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SUMMARY

Bovine tracheal submucosal gland cells have been isolated by enzymatic digestion and serially propagated in tissue culture for more than 12 mo. (40 passages). The cells exhibit an epithelioid appearance at confluence and contain alcian blue (pH 2.5)/periodic acid-Schiff-positive material within cytoplasmic granules. By electron microscopy numerous osmiophilic secretory granules are seen. Maximal growth is observed when the cells are grown on human placental collagen-coated culture vessels in medium supplemented with 20% fetal bovine serum. Scintillation spectrometry revealed that radiolabeled precursor ($^{35}SO_4$) was incorporated into high molecular weight molecules and released from cells. Isoproterenol (10^{-6} to 10^{-3} *M*) stimulated the release of $^{35}SO_4$. The maximal response to isoproterenol was completely inhibited by the β-adrenergic antagonist propranolol. It is concluded that the cultured cells retain features of tracheal gland cells and may serve as a useful model of synthesis and secretion of macromolecules by tracheal gland cells.

Key words: cell culture; trachea; glands; isoproterenol; airway (mucus) secretion.

INTRODUCTION

Tracheobronchial submucosal glands are composed of two major types of secretory cells, serous and mucous. Together these cells make up approximately 60% of the secretory cell volume of the human airways (23). As such, these cells are important contributors to the complex mixture of water, ions, and macromolecules that make up tracheobronchial secretions. Although differences have been noted in the morphology and histochemistry of serous and mucous cells, surprisingly little is known about the biochemical composition of their respective secretory products. Previous attempts to investigate this subject have relied on histochemical methods, including stains for sulfated and sialylated glycoproteins (15,16,27), affinity for certain lectins (20), and immunohistochemistry (2,6,29). Histochemical techniques are, however, limited to the study of materials for which appropriate probes exist, such as lectins, antibodies, or specific stains. A more comprehensive biochemical analysis of serous and mucous cells awaits the development of preparations composed of single cell types.

Extensive biochemical studies have already been performed on tracheobronchial secretions, using as starting material human sputum samples, bronchial lavage fluid, or medium harvested from organ culture of tracheobronchial biopsies. Such studies have provided detailed information concerning the amino acid and sugar composition of the glycoprotein components (9,24,26), analysis of oligosaccharide chain length by HPLC (5), and the sequencing of sugars by NMR spectroscopy (17). From such studies, however, it has not been possible to determine the biochemical contribution of the different cell types. The objective of this study was to establish a cell line representing a single cell type from the submucosal glands of bovine trachea. Morphological, immunological, biochemical, histochemical, and physiologic characterization indicates that the cells in culture retain features of submucosal gland serous cells. A preliminary report of this work has appeared (11).

MATERIALS AND METHODS

Materials. All media, phosphate buffered saline (PBS), and antibiotics were obtained from the Cell Culture Facility, University of California, San Francisco. Fetal bovine serum lots (HyClone Laboratories, Logan, UT) were tested by the Cell Culture Facility for the ability to support colony formation and growth of selected standard cell lines. All other chemicals were obtained from Sigma Chemical, St. Louis, MO.

Isolation of tracheal submucosal gland cells. Bovine tracheas, obtained from a local abattoir, were rinsed twice in sterile PBS containing penicillin (10^6 U/l), streptomycin (100 mg/l), gentamicin (100 mg/l), and amphotericin B (2.5 mg/l) at 4° C. The tracheas were opened longitudinally along the posterior membrane and mounted in a stretched position on a sterile dissecting tray. The epithelium at one end of the tracheal segment was lifted free of the underlying submucosa by sharp dissection,

and the epithelium was removed in strips, exposing the underlying submucosal tissues. With a fine scalpel and forceps, the gland-rich submucosal tissues were removed from the tracheal cartilage and adventitia and placed in Hanks' balanced salt solution (HBSS) containing 20 m*M* HEPES buffer (pH 7.4), bovine serum albumin (1 mg/ml), and penicillin, streptomycin, gentamicin, and amphotericin B at the same concentrations used in the rinse solution. The tissue was minced finely with scissors and transferred to trypsinizing flasks (Wheaton) containing disaggregation medium (2 ml/mg tissue) composed of HBSS, HEPES buffer, bovine serum albumin, and antimicrobial agents as described above but, in addition, containing crude collagenase type IV (500 U/l), elastase (6 U/ml), hyaluronidase (200 U/ml), and DNase (10 U/ml). The flasks were agitated on an orbital shaker (200 rpm) for 12 to 18 h. After this procedure, the disaggregation medium was replaced (4 ml/mg tissue). Agitation was continued, but the medium was replaced every 30 min for 120 min. The removed medium was centrifuged (150 × *g*, 10 min) to obtain cell pellets of isolated gland cells. Isolated cells were resuspended in a 1:1 mixture of Dulbecco's modified Eagles' medium (4.5 g glucose/l) and Ham's F12 nutrient medium (DF12) supplemented with antibiotics and fetal bovine serum (20%), hereafter referred to as DF12/20% FBS. Cell yield and viability at each collection was assessed. Erythrosin B, made up as a 0.4% solution, was diluted 1:10 with cell suspension. This mixture was placed in a hemacytometer, and counts of viable and nonviable cells were obtained. After the last 30-min collection, the cells were pooled, and the cell yield and viability of the total sample were determined.

Initiation of cell culture, growth kinetics, and related techniques. Freshly isolated cells in DF12/20% FBS were preplated for 1 h to remove fibroblasts and then plated at 5×10^6 cells/cm^3 onto T-25 culture flasks coated with human placental collagen (15 μg/cm^3) as described by Coleman et al. (8). Flasks were incubated at 37° C in 5% CO_2:95% air. The cells were observed daily in a phase contrast inverted microscope. Medium was changed at 24 and 48 h and every 3 d thereafter. At the time of the first change of medium (24 h), attachment of numerous epithelioid cells and a few fibroblastic cells were apparent. When the monolayer of mixed cells reached 70 to 80% confluence, cells were removed from the flasks by gentle trypsinization with STV (0.9% NaCl, 0.1% trypsin, 0.02% EDTA) in two stages: the first stage removed easily detached fibroblasts, and the second stage removed the epithelioid cells. Fibroblastic cells were discarded. Epithelioid cells were resuspended in complete medium and subcultured. The two-step trypsinization procedure was repeated for 4 serial passages until a homogeneous population of epithelioid cells was obtained, hereafter referred to as bovine tracheal gland or BTG cells. After the 2nd passage, antibacterial additives to the medium consisted only of gentamicin (50 mg/l). Cells were maintained by routine subculturing at approximately weekly intervals.

To study growth kinetics of the BTG cells grown in serum-free DF12 or DF12 supplemented with either 10 or 20% fetal bovine serum, cells (5×10^6) in their 25th passage were seeded onto human placental collagen-coated (15 μg/cm^3) or uncoated T-25 flasks. Triplicate cell counts from three flasks of each culture condition were made using a hemacytometer and a light microscope.

Chromosome preparations were prepared from cells in the 18th passage by the method of Worton et al. (31). Slides were stained with Giemsa before examination. Cultures were negative for mycoplasma contamination when tested by culture method (18) and by the FA and DNA fluorochrome staining procedures (1,7).

Characterization of cultured cells.

Ultrastructure. Electron microscopy was performed on cells grown on glass cover slips or on the surfaces of the culture vessels. For fixation, culture medium was replaced with a solution of 2.5% glutaraldehyde, 0.08 *M* Na cacodylate, 5 m*M* $CaCl_2$, and 1% sucrose (pH 7.4, 4° C). After 12 to 18 h, the cells were postfixed with 1.5% osmium tetroxide in 0.2 *M* phosphate buffer (pH 7.4) for 2 h. The cells were then rinsed in 0.025 *M* sodium maleate buffer (pH 6.0) and stained with uranyl acetate (1.5% in 0.025 *M* sodium maleate buffer, pH 5.2). Cells were either dehydrated and then infiltrated with Epon 812 on the culture vessel surface or scraped from the culture vessels and transferred into microcentrifuge tubes for processing. The cells grown on cover slips were embedded by placing embedding capsules on top of the monolayers and filling them with Epon 812. After polymerization was complete, the resin and the cells were separated from the cover slip surfaces. Semithin (0.5 μm) sections were cut with glass knives on an LKB Nova ultramicrotome, mounted on microscope slides, and stained with toluidine blue before examination with a light microscope. Specific areas were selected, and thin sections having a silver interference color were cut with a diamond knife and mounted on Formvar-coated copper slot grids. These sections were stained with uranyl acetate and lead citrate before examination in a JEOL 100S electron microscope.

Histochemistry. Cells grown on glass cover slips or trypsinized culture cells spun onto glass slides with a cytospin centrifuge (Shandon Scientific) were stained with alcian blue (pH 2.5)/periodic acid-Schiff (AB/PAS) and oil red 0. Staining was performed both with and without prior formalin (10%) fixation. The percentage of cells containing AB or PAS staining granules or both was determined (300 cells counted from two cytospin preparations). The number of AB/PAS granules per unit cytoplasmic area was determined for at least 100 stimulated and unstimulated cells using an ocular grid in a light microscope with a 100× oil immersion objective. Acid phosphatase staining was performed using the method of Beckstead (4).

Immunocytochemistry. Monoclonal antibodies directed against tracheal antigens were produced by methods similar to those described previously (2). Briefly, sputum from a patient with cystic fibrosis was dialyzed for 2 h at 4° C (Spectrapor tubing; MW cutoff, 12 000 to 14 000

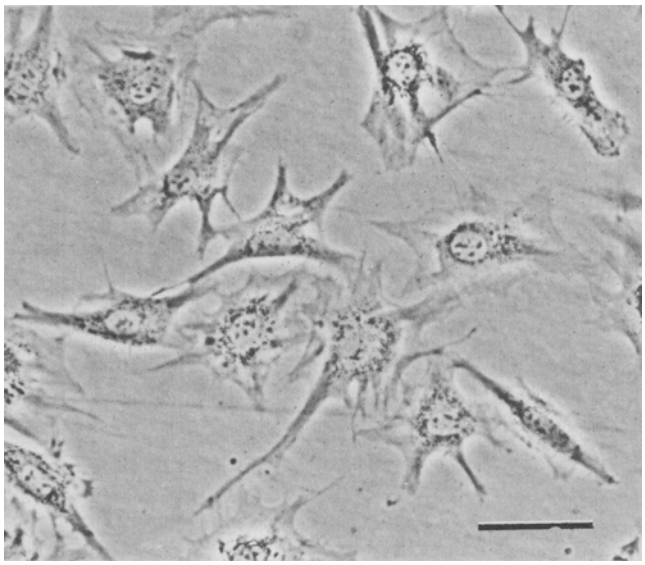

FIG. 1. Phase contrast micrograph of bovine tracheal gland cells grown on a collagen-coated culture dish. The cells have a polygonal or fusiform shape. *Bar* = 50 μm.

daltons) against 25 V of PBS and concentrated overnight by centrifugal evaporation (Speed Vac). An aliquot of the desiccated material was assayed for protein by the method of Lowry et al. (19). Balb/c mice were given 2 i.p. injections of 100 μg of the desiccated secretions dissolved in 100 μl of PBS and emulsified in an equal volume of complete Freund's adjuvant. The intraperitoneal injections were separated by 2 wk and followed 2 wk later by an intravenous injection of 100 μg dissolved antigen. Three days later spleens were removed from immunized mice and the cells fused with SP2/0 myeloma cells. Preliminary results of two fusions are reported elsewhere (10). An antibody directed against human tracheal submucosal gland serous cells (B-7E5) that cross-reacts with bovine tracheal submucosal gland serous cells was used to stain BTG cells. Unfixed cytospin preparations were stored at −20° C before staining. Immunofluorescent staining was carried out using the double antibody method as described previously (2). Cytospins were examined and photographed using a Zeiss fluorescence microscope. Controls in which primary and secondary antibodies were omitted were negative.

Radiolabeling of glycoproteins, pulse chase, and pharmacologic stimulation. Cells in their 20th to 30th passage were studied. Tissue culture flasks (75 cm^2) were seeded with 1.5 × 10^5 cells/cm^2. Medium was changed every 3rd d. On Day 9, medium was replaced with 10 ml of DF12/20% FBS containing $Na_2(^{35}SO_4)$ (20 μCi/ml). After 24 h, the radiolabeled medium was removed, and flasks were washed with PBS (three times). Serum-free medium (DF12, 10 ml) was added to each flask; every 30 min for 210 min the medium was collected and renewed. At 210 min DF12 was replaced with DF12 containing isoproterenol (10^{-9} to 10^{-3} *M*), isoproterenol and propranolol (both 10^{-5} *M*), or DF12 without additives. At 240 min cells were trypsinized from the culture flasks and counted, and viability was assessed by exclusion of erythrosin B. All 30-min samples were dialyzed against 25 vol of distilled water containing 0.1% Na azide (Spectrapor tubing; MW cutoff, 12 000 to 14 000 daltons). Aliquots (1 ml) of the dialysates from each collection were prepared for scintillation spectrometry by addition of 2 ml scintillant (Hydrofluor, National Diagnostics, Somerville, NJ). Samples were counted on a scintillation counter (Beckman Instruments, Inc., Irvine, CA).

Statistical treatment of data. We evaluated the degree of stimulation-induced secretion by comparing the release of nondialyzable radiolabel (cpm/10^6 cells) for the periods during and immediately preceding drug exposure. In previous experiments (data not shown) we determined that baseline secretion was stable or slightly decreasing between 210 and 240 min. Ratios comparing release during and before stimulation, when significantly greater than 1.0, indicated the presence of a secretory response. The mean ratio for six flasks in each condition was obtained. Relative secretory rate (RSR) represents the value obtained by dividing each of these mean values by mean ratios obtained from six control flasks receiving no drugs. We tested for pharmacologic effects in dose response experiments using a Newman-Keuls test.

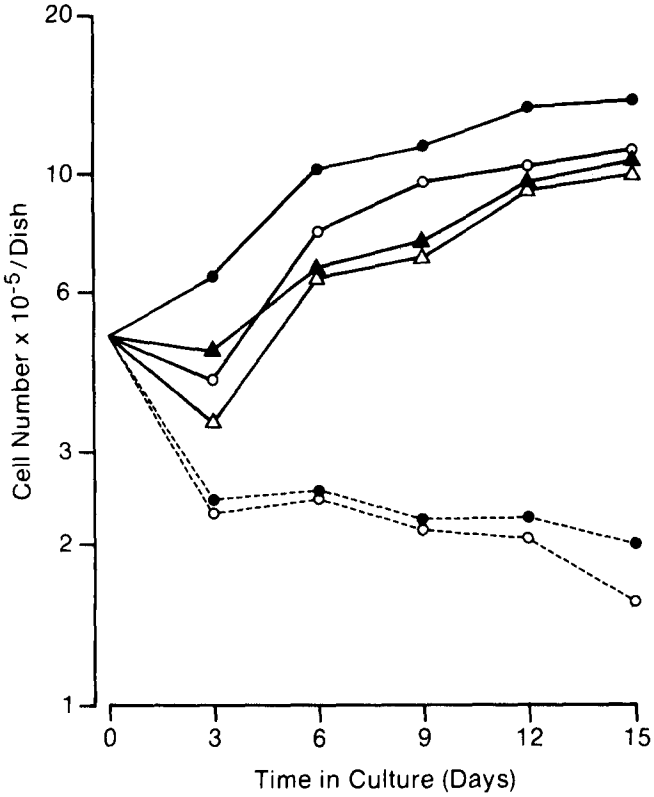

FIG. 2. Growth kinetics of bovine tracheal gland cells. Cells (5 × 10^5) were seeded onto collagen-coated or uncoated (T-25 flasks and grown in serum-free DF12 or DF12 supplemented with 10 or 20% FBS. Triplicate cell counts were made using a hemacytometer. Values shown are the mean of three experiments. o---o = DF12, uncoated; ●---● = DF12, collagen; △_____△ = DF12/10% FBS, uncoated; ▲_____▲ = DF12/10% FBS, collagen; o_____o = DF12/20% FBS, uncoated; ●_____● = DF12/20% FBS, collagen.

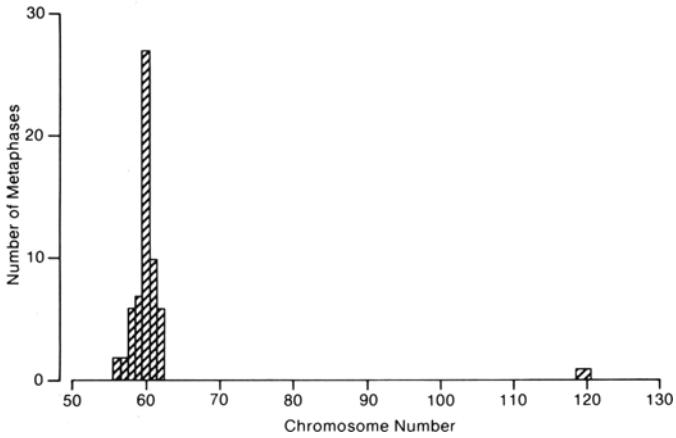

FIG. 3. Chromosome numbers of bovine tracheal gland cells in the 18th passage. The diploid bovine chromosome number is maintained.

Growth curve experiments were analyzed using the same test. The numbers of granules present in control and stimulated cells were analyzed by two-sample *t*-test.

RESULTS

Establishment of the cell line, growth kinetics. Cell yield varied considerably with the amount of starting material ($58 \times 10^6 \pm 23 \times 10^6$, $n = 4$). However, viability as assessed by exclusion of vital dye was consistent among preparations ($62 \pm 1.9\%$, $n = 4$). Microscopic examination revealed that the isolated cell preparations contained numerous red and white blood cells. The majority of nonhematogenous cells were epitheloid. These cells, as well as contaminating fibroblastic cells, adhered to the culture vessels. Fibroblasts were reduced by the preplating step (*see* Methods). Blood cells, nonviable cells, and other cells that failed to attach were removed in the first few changes of media. The rapid growth of BTG cells after subculturing and their greater resistance than fibroblasts to trypsinization permitted the development of an apparently homogeneous cell line by the 4th passage. Examination of cultures using an inverted phase microscope revealed that the BTG cell line consists of cells having a polygonal or fusiform shape at confluence. The cells have large, oval nuclei and moderately abundant cytoplasm (Fig. 1).

Cell growth in 0, 10, or 20% FBS was compared when cells were grown on collagen-coated vs. uncoated flasks (Fig. 2). Most rapid growth ocurred when BTG cells were grown in the presence of collagen and 20% FBS. In the absence of collagen, cells grew better in medium containing 20 than 10% FBS ($P < 0.05$). Serum-free medium supported cell growth poorly, regardless of the presence of collagen. The bovine diploid chromosome number of 60 (13) was maintained (Fig. 3).

Ultrastructure. Ultrastructural examination revealed cells with discrete electron-dense granules (Fig. 4 *A*). Granular morphology was comparable to that seen in bovine tracheal gland serous cells (Fig. 4 *b*). Fusion of adjacent granular membranes was not seen. Nuclei were round or oval with dispersed chromatin and often contained dense nucleolar structures. Rough endoplasmic reticulum, free ribosomes, and Golgi complexes were present. Numerous large mitochondria, scattered lysosomes, and lipid droplets could be identified adjacent to the secretory granules. Junctional complexes connected the lateral membranes of confluent cells, and apical membranes contained microvilli (Fig. 5).

Histochemistry. Cells grown on glass cover slips with or without collagen were identical by phase and electron microscopy to cells grown on collagen-coated plastic. On glass, however, the cells grew more slowly.

Virtually all cells (98%) contained AB- or PAS-positive granules or both (Fig. 6). The ratio of alcianophilic to

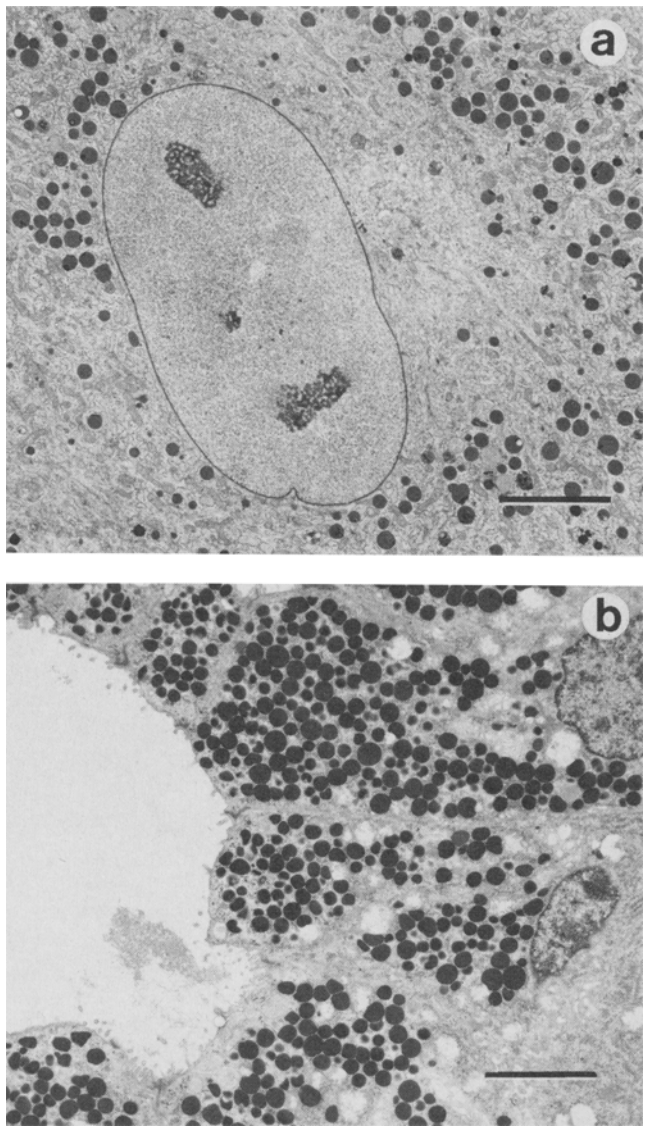

FIG. 4. *a*, Electron micrograph of bovine tracheal gland cell in culture. Cell contains discrete electron-dense granules; *b*, electron micrograph of a bovine tracheal submucosal gland acinus. *Note* the similarity between the granules in (*a*) and (*b*). *Bar* = 5 μm.

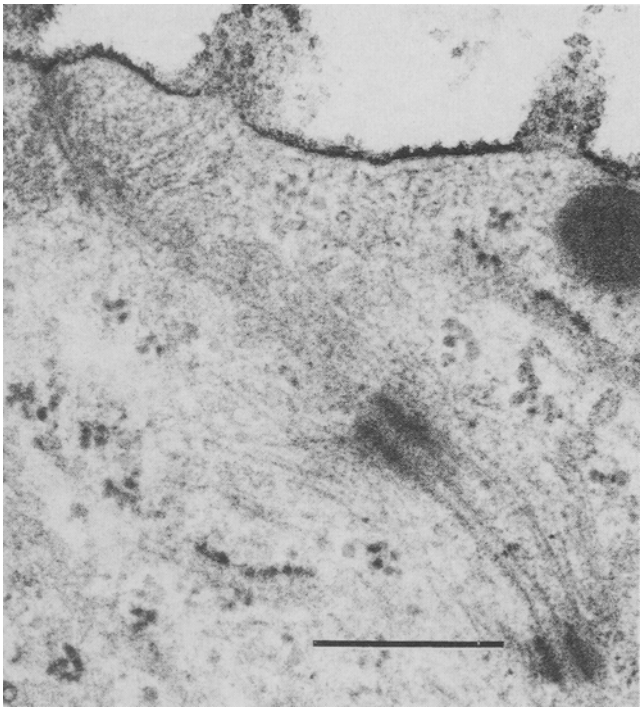

FIG. 5. Electron micrograph of bovine tracheal gland cells in culture. A junctional complex connects the lateral membranes of adjacent cells. *Note* the presence of microvilli on the apical membrane. *Bar* = 1 μm.

PAS-positive granules was reduced by prior formalin fixation. Fixation also led to apparent granule aggregation. The proportion of granules showing alcianophilia varied from cell to cell under both fixed and nonfixed conditions. At pH 2.5, the presence of alcian blue positivity indicates the presence of polysaccharides carrying a negative charge which could be conferred either by sulfate or sialic acid residues. Under the conditions of our experiments, PAS-positive, alcian blue-negative granules indicate the presence of neutral polysaccharides.

The cells also contained droplets of triglyceride as indicated by a positive staining reaction with oil red 0. These droplets could be discriminated from the glycoconjugate-containing granules at the level of the phase microscope by their extremely refractile appearance. In the electron microscope, lipid droplets could be discriminated from secretory granules by reduced osmiophilia in the lipid droplet interior (due to extraction during processing). Many cells contained lysosomes as determined by staining for the presence of acid phosphatase. Lysosomal distribution was confined to the perinuclear region, whereas AB/PAS-positive secretory granules were distributed extensively throughout the cells.

Immunocytochemistry. The monoclonal antibody B-7E5, which stains bovine tracheal serous cells, also stained the BTG cells. Fluorescence was detected in nearly all cells. The pattern of staining was punctate, suggesting localization within granules (Fig. 7).

Physiology. Cell viability at the termination of each experiment was greater than 90% and did not differ between control and drug-treated samples. The concentration of AB/PAS-staining granules was reduced after stimulation (control: 2.05 ± 0.08 granules/μm^2, n = 101; stimulated: 1.55 ± 0.07 granules/μm^2, n = 107; mean ± SEM; $P < 0.001$). Statistical analysis of nondialyzable radioactivity released from six flasks per condition showed that cells incubated in the presence of 10^{-6} to 10^{-4} M isoproterenol released increased quantities of ^{35}sulfate-labeled macromolecules ($P < 0.05$). The RSR calculated for propranolol-blocked, isoproterenol-stimulated flasks was not different from controls (Fig. 8).

DISCUSSION

In this report we present a technique for the isolation and culture of submucosal gland cells from the bovine trachea. Although similar methods have been used previously to obtain cultures of cells from the tracheal surface epithelium [e.g., (8,12,21,32)], the results of this study represent the first characterization of a cell line derived from tracheal glands.

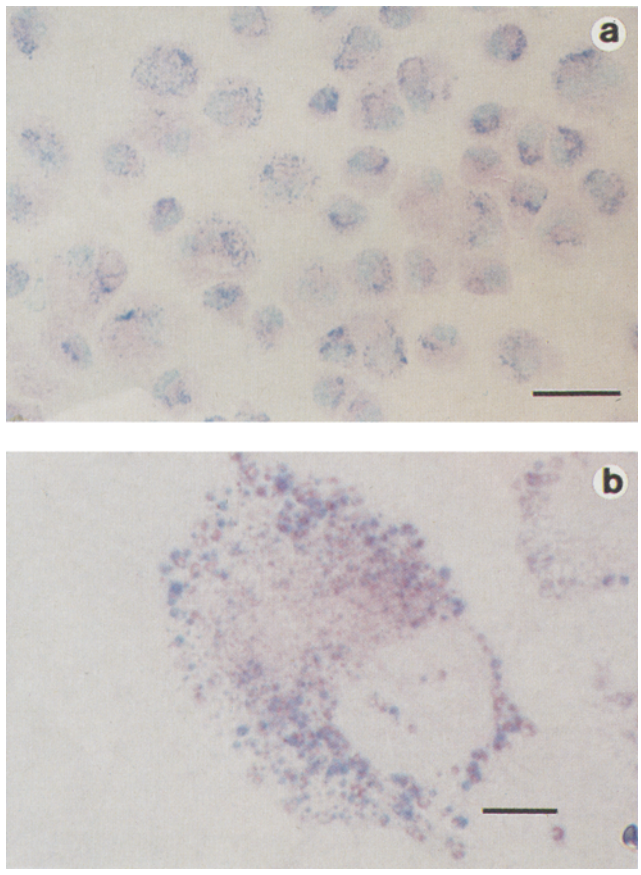

FIG. 6. Alcian blue pH 2.5/periodic acid-Schiff staining of bovine tracheal gland cells. *a*, Cytospin preparation of freshly trypsinized cells. Nearly all cells contain AB- or PAS-positive granules or both. *Bar* = 30 μm. *b*, Direct staining of cell grown on a collagen-coated glass cover slip. Both AB- and PAS-positive granules are seen. *Bar* = 2 μm.

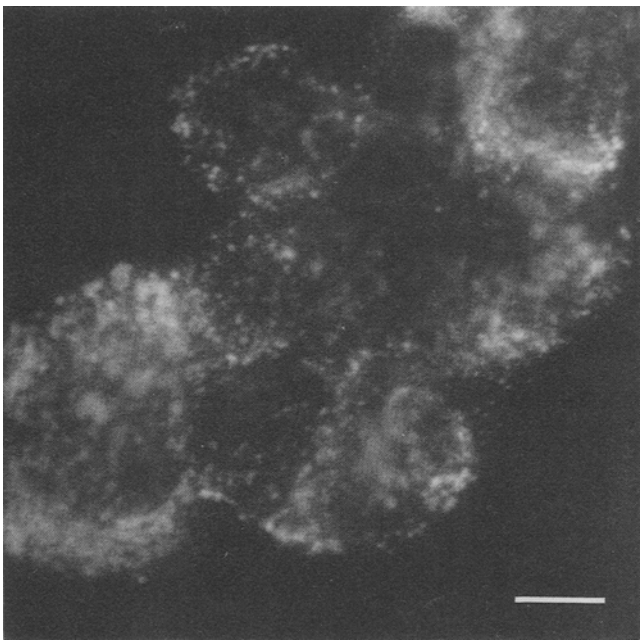

FIG. 7. Fluorescence micrograph showing staining pattern obtained using antibody B-7E5 on a cytospin preparation of bovine tracheal gland cells. The fluorescence is punctate, suggesting localization within granules. *Bar* = 10 μm.

The cells of the tracheal glands derive from plaques of the surface epithelium that invaginate into the submucosa before birth (28,30). These cells differentiate to form the acinar and duct cells that remain in continuity with the tracheal lumen throughout life, and from whose secretions the mucous blanket covering the epithelial surface is continuously maintained and renewed.

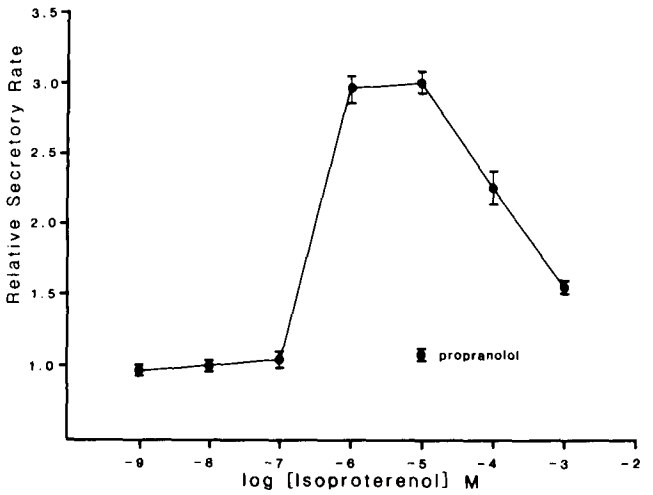

FIG. 8. Response of bovine tracheal gland cells to isoproterenol. Cells were incubated with ^{35}S for 24 h. The radiolabeled medium was then removed, and the flasks were washed with PBS. Serum-free medium (DF12) was added to each flask, and every 30 min for 210 min this medium was collected and replaced. At 210 min, DF12 containing isoproterenol (10^{-9} to 10^{-3} *M*) was added. Control flasks received DF12 alone. Samples were dialyzed and analyzed by scintillation spectrometry. Data are expressed as relative secretory rate (mean ± SEM) for six flasks for each condition. The effect of isoproterenol was prevented by the addition of propranolol when both drugs were administered at a concentration of 10^{-5} *M*.

The gland cells are morphologically distinct from the goblet cells of the surface epithelium and have different responses to secretagogues (25). Therefore, it is not possible to extend findings obtained from surface epithelial cultures (even when a subpopulation of the cells are mucin secreting) to questions involving secretion from the submucosal glands.

The cells described in this study are derived from bovine tracheal glands, which contain mainly serous cells (i.e. bovine cells in vivo contain dense osmiophilic granules and show a predominant histochemical reaction for neutral as opposed to acidic mucosubstances; W. E. Finkbeiner, unpublished observation). In culture, the cells retain many features of submucosal gland serous cells. By electron microscopy, the cultures are found to be composed of epitheloid cells having junctional complexes. The secretory granules are intensely osmiophilic and clearly demarcated from adjacent granules. Histochemically, the granules are found to contain large quantities of neutral and also acidic polysaccharides, a feature consistent with their identification as serous cells (16). Polysaccharide-containing granules (as revealed by AB/PAS staining) were present throughout the cytoplasm of virtually all BTG cells. Although the cells also contain lysosomes, their distribution (as revealed by acid phosphatase staining) was confined to the perinuclear region. This is consistent with observations made on cultures of hamster tracheal epithelial cells (21). The presence of serous cell-specific antigen (defined by monoclonal antibody B-7E5) in BTG cells corroborates the localization of the AB/PAS-positive material to specific secretory granules. Furthermore, recently obtained findings indicate that BTG cells release the serous cell specific marker (6) lysozyme into the culture medium (14).

The cells in culture retain not only the morphological and histochemical features of tracheal gland cells, but also the ability to take up radiolabeled sulfate, incorporate it into macromolecules, and release it upon physiological stimulation. The cells respond physiologically to stimulation with the β-adrenoceptor agonist isoproterenol [described for human bronchial explants by Phipps et al. (22)]. Evidence that the cell response to isoproterenol is physiological and not due to toxicity is provided by dye exclusion and the fact that the effects are blocked at the level of the adrenergic receptor by the presence of propranolol. The sulfated macromolecules in serous cells have not been previously identified. Biochemical analysis of the sulfated material released by BTG cells indicates that a large portion of the material is chondroitin sulfate proteoglycan (3).

In summary, we have placed in culture and characterized for the first time a cell line derived from bovine tracheal glands. The cells should provide a useful model for studies of synthesis and secretion of macromolecules by tracheal gland cells.

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