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Growth Requirements of Ferret Tracheal Epithelial Cells in Primary Culture[1] (42102)

JOHN W. GROELKE, JACQUELINE J. COALSON, AND JOEL B. BASEMAN

Departments of Microbiology and Pathology, The University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284

Abstract. In mass cell culture conditions, protease dissociated ferret tracheal epithelial cells (FTE) proliferated in growth factor-supplemented F12 medium to high cell densities (0.5×10^5 cells/cm^2) with an average population doubling time of 24 hr. The growth factor constituents of the F12 medium included epidermal growth factor (25 ng/ml), insulin (1 μg/ml), transferrin (10 μg/ml), hydrocortisone (18 ng/ml), hypothalamus extract (30–100 μg/ml), and conditioned medium from mouse 3T3 fibroblasts. Growth of these cells under clonal conditions was achieved by the partial replacement of F12 medium with M199 medium which was attributed, in part, to the presence of vitamin A in M199 medium. Serum did not stimulate the growth of FTE cells. The epithelial cell nature of these cells in culture was confirmed by ultrastructural features and by immunofluorescent staining for fibronectin.

Recent successes have been reported in the culture of normal airway epithelial cells from laboratory animals (1, 2) and humans (3, 4). In the case of human bronchial cells, serum has been reported to either facilitate (3) or inhibit (4) the growth of epithelial cells from explants. In the latter instance, a hormone-growth factor mixture was developed to stimulate cell multiplication. Serum in high concentrations (e.g., 10%) stimulated the growth of hamster tracheal cells (1) while profoundly inhibiting the growth of rabbit tracheal cells at concentrations of 1% or greater (2).

These observations may be explained, in part, as species differences. In addition, it is also possible that they represent the selective growth of one subpopulation of cells over another since at least several cell types [e.g., Clara, basal, mucus secreting (5–7)] having proliferative capacity exist in the airway epithelium.

We report here on the growth of dissociated ferret tracheal epithelial (FTE) cells in primary culture in both clonal and mass cell culture conditions. The multiplication of these cells was facilitated by certain specific growth factors. In addition, conditioned medium from 3T3 cells and an extract of bovine hypothalamus markedly enhanced FTE cell growth as has been previously reported for airway epithelial cells (2, 12) and other epithelial cells (10). The epithelial nature of FTE cells was confirmed by ultrastructure and immunofluorescent staining for fibronectin.

Methods. *Reagents.* The following were purchased from Sigma Chemical Company, St. Louis, Missouri: bovine insulin (INS), human transferrin (TF), hydrocortisone (HC), protease Type XIV, 3,3′,5-triiodo-L-thyronine (T_3), sodium pyruvate, bovine serum albumin Fraction V (BSA), *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes), gentamycin sulfate (GS), tetrasodium ethylenediaminetetraacetic acid (EDTA), progesterone, luteinizing hormone, cholesterol, vasopressin (Arg), prostaglandin E_2, bovine serum albumin, retinoic acid (all *trans*), and sodium selenite. Ham's F12 medium (F12), Eagle's minimal essential medium (MEM), Dulbecco's MEM (D-MEM), M199 medium, Hanks' balanced salt solution (HBSS), 2.5% trypsin, 50× MEM essential amino acids solution (EAA), 100× nonessential amino acids solution (NEAA), 100× MEM vitamin solution, penicillin (PEN)–streptomycin solution (STR), and fetal bovine serum (FBS) were obtained from GIBCO, Grand Island, New York. Culture grade epidermal growth factor (EGF) was from Collaborative Research, Lexington, Massachusetts. All media were prepared with water purified by the

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Milli-Q System (Millipore Corp., Bedford, Mass.).

Cell isolation and culture. Ferrets, 12–14 weeks old and not having received the mink dystemper virus vaccination, were obtained from Marshall Research Animals, North Rose, New York. Animals were killed by intraperitoneal injection of 0.5 ml T-61 euthanasia solution (Taylor Pharmaceutical Co., Decatur, Ill.) followed by cardiac bleeding. Tracheas were excised from just below the larynx to the bronchial bifurcation (8–10 cm) and washed extensively in cold MEM with PEN (100 units/ml) and STR (100 μg/ml). Each trachea was clamped with a sterile hemostat at the bifurcation and filled from the open end with 0.25% Type XIV protease in F12 medium plus 5 μg/ml INS, 2 mg/ml Hepes, and antibiotics (100 units/ml PEN, 100 μg/ml STR, and 50 μg/ml GS). A second hemostat closed the open end and the trachea was placed in a large sterile beaker with cover at 4°C for 15–18 hr. During this interval sufficient MEM was added to the beaker to bathe the tracheas. The cells from each trachea were collected by allowing the enzyme solution to run into a 50-ml tube. The luminal surface of the fully opened trachea was then flushed with 7 ml of F12 medium containing 10% FBS. The cell–enzyme solution plus washes were pooled, and centrifuged (400*g* for 5 min). The cell pellet was dispersed with pipetting in 10 ml serum-free F12, centrifuged, and resuspended in protein-free F12 medium. An aliquot from this preparation was then counted in a Coulter Counter (Model F).

Primary FTE cells were seeded in culture vessels at an appropriate concentration and grown in a CO_2 incubator containing humidified 5% CO_2–95% air. After an attachment period of 24 hr, the cells were washed with HBSS and refed 2 ml F12 medium (35-mm dishes) containing the desired combination of growth factors, FBS and/or conditioned medium to be checked for growth stimulation. The medium was changed at 2- to 3-day intervals. For cell counting (Coulter Counter, Model F), dishes were washed three times with NaCl (0.14 *M*)–EDTA (0.5 m*M*), pH 7.5, and the cells dispersed with 0.20% trypsin–0.01% Type XIV protease in NaCl–EDTA.

Preparation of hypothalamus extract (HE) and conditioned medium (CM). The HE was prepared using a modified procedure of Maciag and co-workers (8). Bovine hypothalamus (150 g; Pel-Freeze, Rogers, Ark.) was homogenized in 250 ml phosphate-buffered saline (PBS), pH 7.5, at 4°C in a Waring blender. The homogenate was stirred at 4°C for 10 min and then centrifuged at 11,200*g* for 45 min. The pooled supernatant was further centrifuged at 56,500*g* for 1 hr. The supernatant was sequentially filtered through 0.8- and 0.2-μm disposable filter units (Nalgene, Rochester, N.Y.) and stored in aliquots at −70°C. The protein concentration of the HE was determined to be 10 mg/ml using the method of Lowry and co-workers (9).

Swiss 3T3 cells (CCL-92) (10) were obtained from the American Type Culture Collection and routinely grown in D-MEM with 10% FBS. Near-confluent cells (passage 128–131) in T75 flasks were washed one time with HBSS and then overlaid with a modified D-MEM medium to permit reduction in the Ca^{2+} ion. Calcium at the concentration present in D-MEM (1.8 m*M*) has been reported to inhibit epithelial cell growth (11, 12). Constituents of this medium included the normal concentrations of D-MEM inorganic

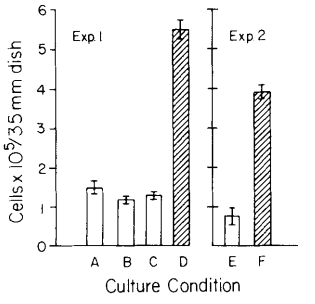

FIG. 1. Effect of 3T3 mouse fibroblast CM on the growth of FTE cells. Cells were seeded at 5.0×10^4 cells (Experiment 1) or 6.0×10^4 cells (Experiment 2)/dish. Conditions A, B, C, E: F12 medium (3 parts) combined with nonconditioned modified D-MEM (1 part) to which FBS was added to a 0 (A, E), 0.5% (B) or 10% (C) concentration. Each point is the mean of three separate determinations (±SD) after 7 days in culture. Conditions D, F (dashed bars): F12 medium (3 parts) combined with 3T3 cell-CM (1 part) (final FBS concentration 0.5%). All of the above (A–F) also contained 25 ng/ml EGF, 5 μg/ml INS, 10 μg/ml TF, 0.18 μg/ml HC, and 20 μg/ml HE.

salts (except that $CaCl_2$ was reduced to 30 mg/l), glucose (1000 mg/l), sodium pyruvate (110 mg/l), phenol red (15 mg/l), 50× EAA (40 ml/l), 100× NEAA (10 ml/l), and 100× vitamins (10 ml/liter). The medium (10 ml/flask) containing 2% dialyzed FBS was 3T3 cell-conditioned for 48 hr in a CO_2 incubator, sterilized by filtration through a 0.2-μm Nalgene filter, and stored in aliquots at −20°C.

Cell ultrastructure. Culture dishes containing cell monolayers were flooded with 2.5% glutaraldehyde in 0.05 *M* cacodylate buffer with 6% sucrose and fixed for 1–2 hr. Following several rinses in 0.05 *M* cacodylate buffer with 12% sucrose, monolayers were postfixed with Zetterqvist's osmium fixative for 1 hr. Following a rinse in the Zetterqvist's barbitol buffer, cells were stained with 2% uranyl acetate followed by dehydration in ascending grades of ethanol. Propylene oxide was flooded onto the cells and the monolayer lifted from the dish. The monolayer was pelleted in a microfuge tube, and embedded with Polybed 812. Sections were stained with uranyl acetate and lead citrate and examined with a Phillips 301 or Jeol 100-CX electron microscope.

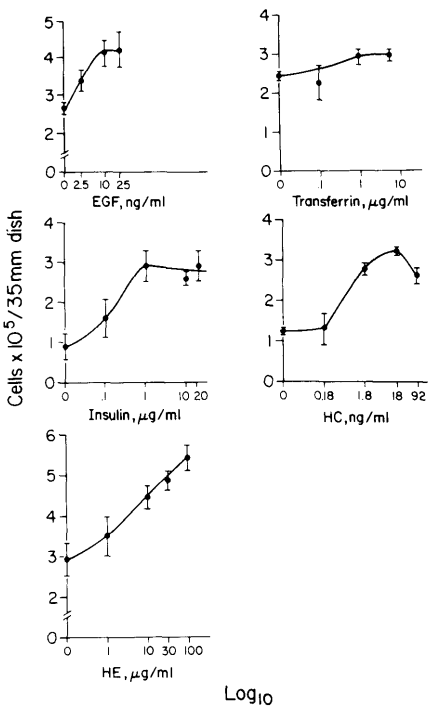

FIG. 2. Titration of individual growth factors in combination with 1 part CM/4 parts F12. Each growth factor concentration was varied while the other four test reagents remained constant at the levels described in Fig. 1 (except that HE was reduced to 10 μg/ml). Cells were counted on Day 7 (EGF, insulin, transferrin, HC) or Day 8 (HE, HC) (±SD of three separate determinations).

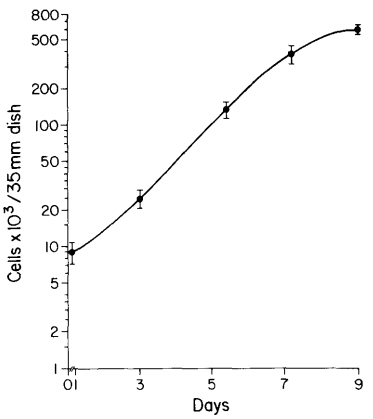

FIG. 3. Growth of FTE cells with optimized concentrations of growth factors (25 ng/ml EGF, 1 μg/ml INS, 10 μg/ml TF, 0.5 × 10^{-7} *M* HC, 30 μg/ml HE) and 1 part CM/4 parts F12 medium. Cells were seeded at 3.0 × 10^4 cells/dish (Day 0) and selected dishes counted or refed at Days 1, 3, 5, 7, and 9.

Immunofluorescent staining for fibronectin. Primary FTE cells were grown to near confluence on 12-mm-diameter glass coverslips, rinsed in PBS, fixed in 3.7% formaldehyde, and stored at 4°C in PBS with 0.1% sodium azide. For comparative purposes additional cells (MDCK, ATCC-CCL34; normal mouse skin fibroblasts; hamster lung fibroblasts, ATCC-CC1 195; normal baboon lung fibroblasts) were prepared using 10% FBS–MEM for growth stimulation. Prior to staining, fixed cells were washed in prechilled (−20°C) methanol and acetone, and then rinsed in H_2O and PBS (13). Rabbit anti-human fibronectin antiserum (Cappel Laboratories, West Chester, Pa.) was diluted 1/7.5 in PBS as was normal rabbit serum; fluorescein (FITC)-conjugated goat anti-rabbit IgG (Miles Laboratories, Elkhart Ind.) was diluted 1/5. All reagents were centrifuged at 8730*g* for 5 min prior to use. Coverslips were treated with normal serum or anti-fibronectin antiserum for 45 min at 37°C. After extensive washing, the cells were stained with FITC conjugate for 45 min, washed, and viewed with epifluorescence.

Clonal growth. Freshly isolated FTE cells were filtered through an 46-μm nylon mesh (Tetko Inc., Elmsford, N.Y.) and seeded in 35-mm dishes at $2.5–4.0 \times 10^3$ cells/dish. Ten days later the cells were fixed in 10% Formalin and stained with Giemsa. Retinoic acid was prepared as a 1000× stock solution in 95% ethanol.

Results. The FTE cells used for these studies were isolated over a 9-month period with a mean cell yield/trachea of 3.9×10^6 cells ($\pm$SD 1.4×10^6; $N = 25$). Initial growth experiments attempted to culture FTE cells in either serum-supplemented F12 medium or growth factor-supplemented F12 medium. Serum did not support the growth of FTE cells while the mixture of EGF, INS, TF, and HC stimulated growth to a limited extent (data not shown). The addition of other potentially growth promoting substances (e.g., progesterone, luteinizing hormone, cholesterol, T_3, prostaglandin E_2, sodium selenite, vasopressin, glucagon) to this growth factor mixture did not further enhance the proliferation of FTE cells (data not shown).

Further improvement in FTE cell prolif-

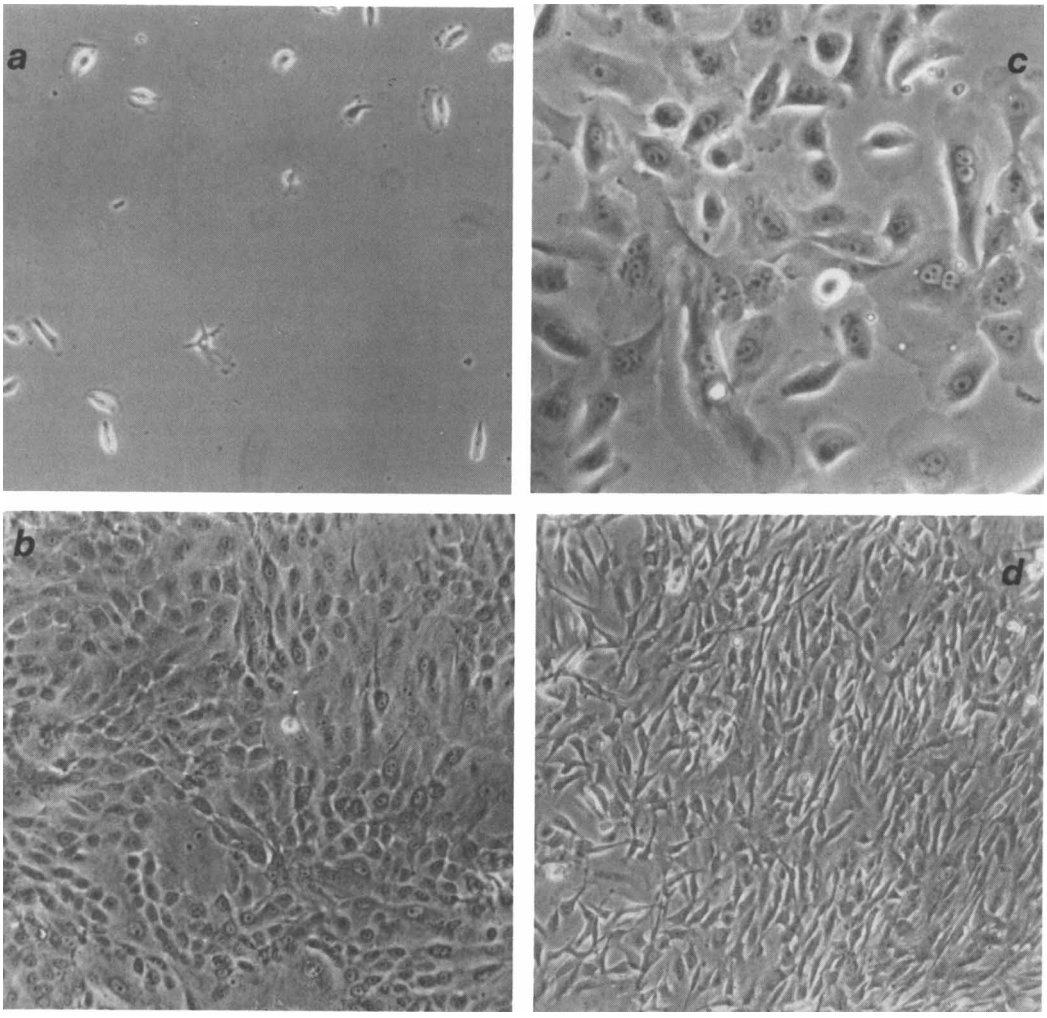

FIG. 4. Light microscopy of FTE cells in culture. Cells were seeded at 3.0×10^4 cells/dish as described in Fig. 3. Photographs taken after 1 (a) and 7 days (b, c) in culture. Culture of fibroblast-like cells (d) was prepared by trypsinization of ferret lung tissue and culture of dissociated cells in 10% FBS–MEM. Magnification a, b, d 70×; c 140×.

eration was dependent on the use of CM and HE. As shown in Fig. 1, CM in combination with growth factors (EGF, INS, TF, HC, HE) dramatically enhanced FTE cell proliferation (Figs. 1D, F). For these experiments CM (1 part) containing the usual 2% dialyzed FBS (see Materials and Methods) was combined with F12 medium (3 parts) to give a final FBS concentration of 0.5%. Nonconditioned D-MEM (1 part), F12 medium (3 parts) and growth factors plus FBS added to 0.5 and 10% concentrations (Figs. 1B, C) failed to stimulate cell growth above levels seen in controls without serum (Figs. 1A, E). Titration of CM demonstrated that combinations of one part CM to 1–4 parts F12 medium (plus other growth factors) optimally enhanced cell growth and that further dilution of CM progressively diminished the growth response until at 1 part CM/79 parts F12 medium the growth was essentially equal to that seen without CM (data not shown).

In Fig. 2 the titration of each of the growth factors for optimal stimulation of cell multi-

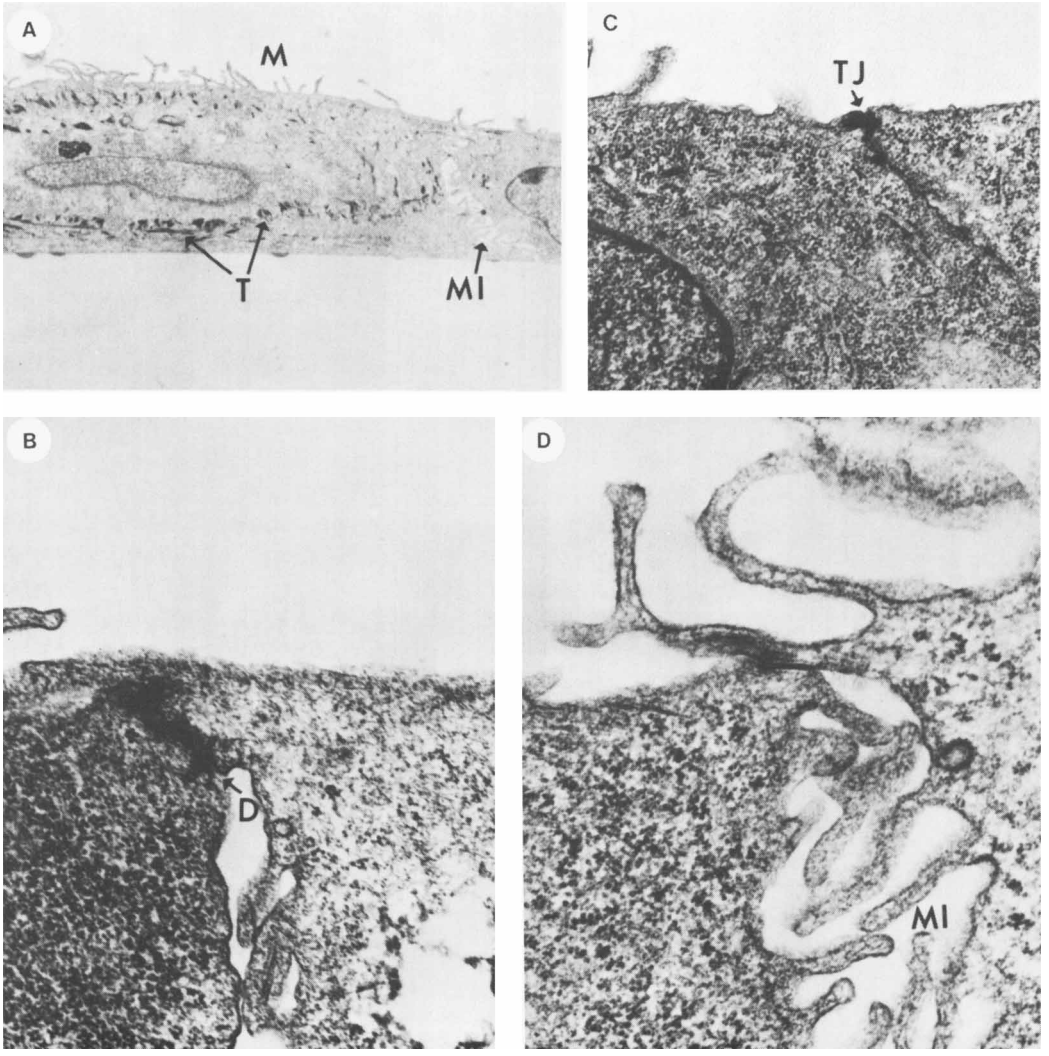

FIG. 5. Transmission electron microscopy of FTE cells. Abbreviations: M, microvilli; T, tonofilaments; MI, membrane interdigitation; D, desmosome; TJ, tight junction. Magnification: A, 6950×; B, 57,650×; C, 45,500×; D, 83,250×.

plication is presented. Using a CM/F12 medium mixture of 1/4 (final FBS concentration of 0.4%), EGF stimulated optimal growth at 25 ng/ml, INS at 1 μg/ml, HC at 18 ng/ml, and HE at or greater than 100 μg/ml. Transferrin appeared only slightly stimulatory at a concentration of 1–10 μg/ml (Fig. 2). Using the growth factor concentrations determined in Fig. 2, FTE cells seeded at 3.0–6.0 × 10^4 cells/dish grew rapidly from sparse to confluent cultures in 7–9 days with an average population doubling time of 24 hr (Fig. 3) and displayed a definite epithelial cell morphology (Fig. 4).

Ultrastructural features of FTE cells were highly indicative of their epithelial nature. Surface microvilli, membrane interdigitation, tonofilaments, tight junctions, and desmosomes were consistent features of these cells (Fig. 5).

We have found that immunofluorescent staining of epithelial cells for fibronectin gives

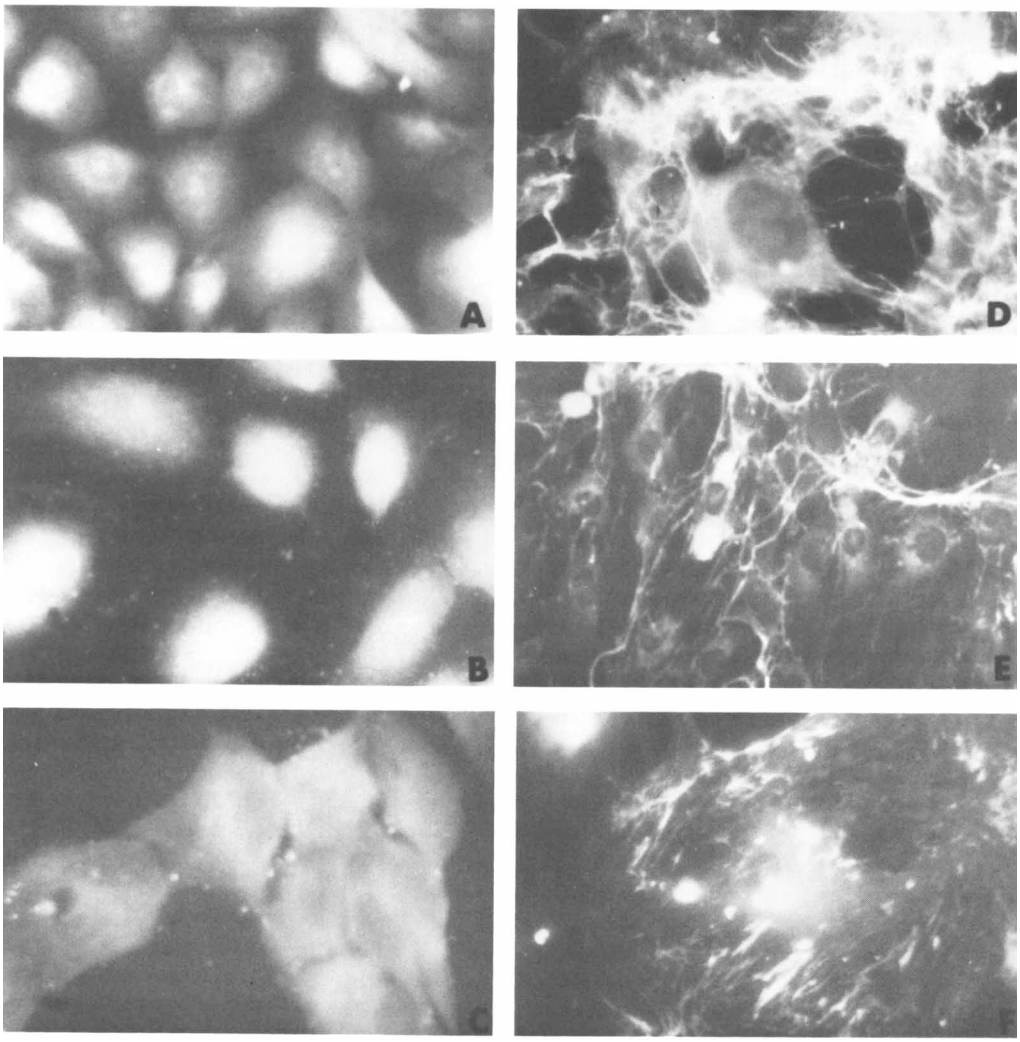

FIG. 6. Immunofluorescent staining of FTE cells for fibronectin. Fixed cultures were treated with normal rabbit serum (A) or rabbit anti-human fibronectin (B–F) followed by treatment with FITC-conjugated goat anti-rabbit IgG (Methods). FTE cells, A, B; MDCK cells, C; normal mouse skin fibroblasts, D; hamster lung fibroblasts, E; baboon lung fibroblasts, F. Magnification: A–D, 150×; E, 60×; F, 96×.

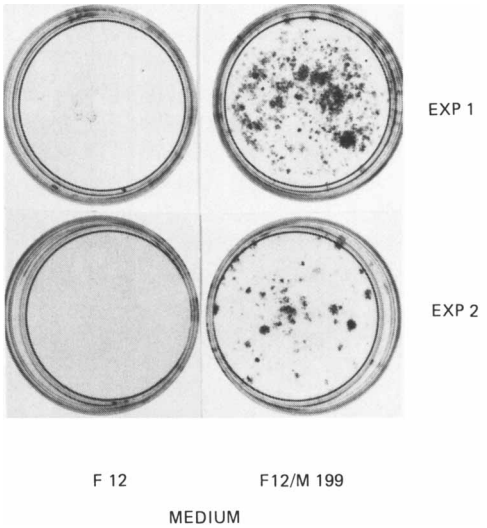

FIG. 7. Clonal growth of FTE cells. Clonal growth of FTE cells. Cells were seeded at 4.0×10^3 cells/dish (Experiment 1) or 2.5×10^3 cells/dish (Experiment 2). In each case, cultures received 1 part CM/3 parts F12 medium (F12) *or* 1 part CM/1.5 parts F12 medium/1.5 parts M199 medium (F12/M199). Additional growth factors (Fig. 3) were also present.

a pattern distinct from that seen with fibroblasts. As shown in Fig. 6, FTE cells stained moderately for surface-associated packets of fibronectin, the pattern indicative of epithelial cells. MDCK cells stained in a similar fashion (Fig. 6C). In contrast, fibroblasts formed a network of extracellular fibrils or matrices (Figs. 6D–F). The usefulness of fibronectin immunofluorescence for distinguishing epithelial cells from fibroblasts has been previously reported (16, 17).

The ability of a growth factor mixture to support cell growth is more stringently tested in clonal growth conditions (e.g., 250–400 cells/cm^2). When examined in such a manner, FTE cells failed to grow when cultured with growth factors (EGF, INS, TF, HC, HE), CM (1 part) and F12 medium (3 parts) (Fig. 7). Clonal growth was obtained with the above mixture, however, when half the volume of F12 medium was replaced by M199 medium (Fig. 7). Since M199 medium contains vitamin A which has been reported to enhance human bronchial epithelial cell growth (4), retinoic acid (10 ng/ml, 3.3×10^{-8} *M*) was investigated for stimulation of FTE cell growth by its addition to F12 medium. As shown in Fig. 8, retinoic acid was required for cell growth in a density-dependent fashion. When seeded at 2.0×10^4 cells/dish (2×10^3 cells/cm^2) FTE cells proliferated at essentially equal rates with or without retinoic acid, while at $0.5–1.0 \times 10^4$ cells/dish retinoic acid was required for cell multiplication (Fig. 8). FTE cells seeded at 0.25×10^4 cells/dish failed to proliferate even with retinoic acid (Fig. 8).

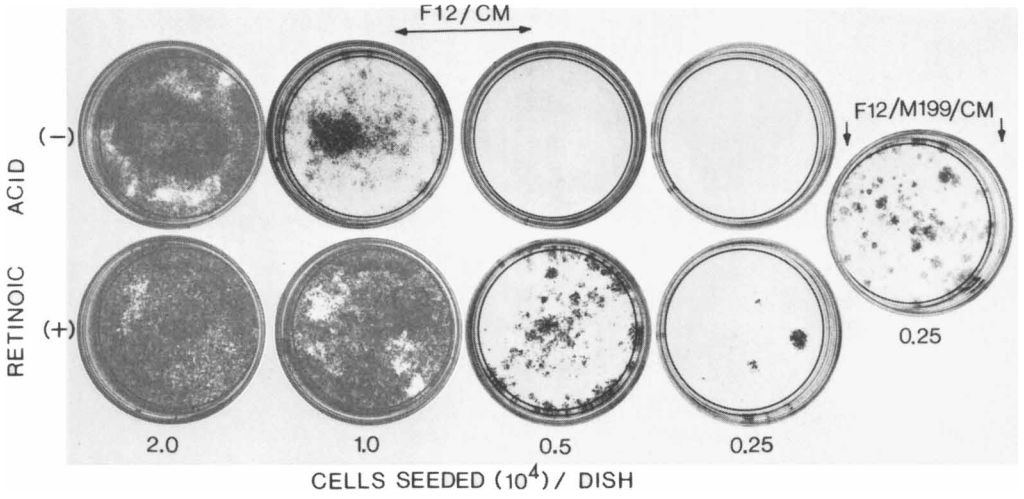

FIG. 8. Effect of retinoic acid (10 ng/ml) on FTE cell proliferation. The indicated number of cells were seeded with growth factors in either an F12/CM mixture or F12/M199/CM mixture as described in Fig. 7. To one series of dishes containing F12/CM retinoic acid (+) was also added.

Thus, the requirement by FTE cells for M199 medium to achieve clonal growth would appear partially attributable to the presence of vitamin A in this medium. At present we are examining additional M199 components for enhancement of FTE cell clonal growth.

Discussion. Much interest in the culture of normal airway epithelial cells has stemmed from the potential usefulness of such systems in the study of carcinogenesis (18, 19), pulmonary disease (20), and microbial pathogenicity (21). In the present study the isolation and growth in culture of airway epithelial cells from the ferret are described. Previously, the culture of normal hamster (1), rabbit (2), and human (3, 4) airway epithelial cells has been reported. In mass cell culture conditions, primary FTE cells with an average population doubling time of 24 hr required F12 medium with 2 mg/ml Hepes plus the addition of EGF (25 ng/ml), INS (1 μg/ml), TF (10 μg/ml), HC (18 ng/ml), HE 30–100 μg/ml, and CM from 3T3 fibroblasts (Fig. 3). Fibroblast contamination of the epithelial cell cultures was rare and only observed in cultures exposed to 10% FBS. The absence of fibroblast contamination can probably be attributed to (a) the method of cell isolation which used whole trachea and not minced tissue and (b) the use of low levels of serum which favors the growth of epithelial cells over fibroblasts (22).

Although the ferret is relatively unknown in animal cell investigations, its recent use in airway morphologic (23) and secretory (24) studies suggests that a ferret tracheal cell culture model is suitable. In one of these investigations (24), the researchers took advantage of the fact that ferrets have few surface airway goblet cells and, thus were able to investigate macromolecular secretion from submucosal glands. It may also be of interest that the growth factor requirements of FTE cells appear to be similar to those reported for human bronchial epithelial cells. Thus, in their initial report Lechner and coworkers (12) showed that the growth of human bronchial cells required cocultivation with 3T3 feeder cells as well as HC, INS, and EGF in M199 medium. In serum-free conditions (4), HE and retinoic acid also enhanced the growth of these cells. The stringent growth requirement of FTE cells for 3T3-cell CM (Fig. 1) may also aid in the identification of growth and attachment factors required by specialized epithelial cells.

In summary, the culture of tracheal epithelial cells from the ferret is reported by what now might be called conventional methods. The epithelial nature of these cells in culture was confirmed by their ultrastructural features and immunofluorescent staining for fibronectin (Figs. 5 and 6). The ease of manipulation of the relatively large trachea, and good cell yield with little fibroblast contamination in culture should facilitate the use of these cells for studies of normal and pathologic respiratory cell biology in cell culture.

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