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CLONAL GROWTH OF NORMAL ADULT HUMAN BRONCHIAL EPITHELIAL CELLS IN A SERUM-FREE MEDIUM

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SUMMARY

Defined culture conditions for routine clonal growth of normal human adult bronchial epithelial cells have been developed. Serum and feeder cell requirements were abrogated by: (a) optimizing the calcium concentration in nutrient medium, MCDB 151; (b) supplementing with purified factors (epidermal growth factor, 5 ng/ml; insulin, 5 μg/ml; transferrin, 10 μg/ml; hydrocortisone, phosphoethanolamine and ethanolamine, each at 5×10^{-7} *M*; and trace elements); and (c) coating the surface of the culture dish with a mixture of fibronectin, collagen, and bovine serum albumin. Endothelial cell growth supplement (100 μg/ml) and retinoic acid (3×10^{-10} *M*) further enhanced growth, whereas cholera toxin was nonmitogenic and serum supplementation (> 2%) markedly reduced the growth rate. Using the defined system, dissociated cultures of bronchial epithelial cells, obtained from more than 15 donors, have been subcultured at clonal densities with a colony forming efficiency of 3 to 4%. In addition, high density cultures have been subcultured more than five times with four to six population doublings per passage. The features of this system permit pathobiologic investigations of bronchial epithelial cells, e.g., aging, differentiation, and carcinogenesis using conditions that isolate the results from the influence of serum, feeder cells, and other undefined factors.

Key words: serum-free; clonal growth; epithelial.

INTRODUCTION

Recently, we reported culture conditions for growing normal human bronchial epithelial cells (1). This method, cocultivation with growth arrested Swiss mouse 3T3 feeder cells, was optimized for rapid clonal growth by adjusting the calcium concentration of Medium 199 (M199) and adding specific hormones, growth factors, and 1.25% serum. The method, however, is not optimal for ascertaining the response of the epithelial cells to exogenous agents, e.g., nutrients, growth factors, and carcinogens, because the influence of the feeder cells cannot be determined. Further, continual propagation and mitotic inactivation of the 3T3 cells is time consuming and feeder cell introduced viral contamination of the bronchial epithelial cells is a potential problem. Thus, we sought a method to grow these epithelial cells without feeder cells. Several serum-free media formulations for growing neoplastic epithelial cells have been described (2,3) and, recently, serum-free media formulations for growing normal human epidermal keratinocytes without feeder cells have been published (4,5). We report here conditions for routine clonal growth of normal adult bronchial epithelial cells without serum or feeder cells in fibronectin-collagen coated dishes.

MATERIALS AND METHODS

Culture conditions. Human bronchial tissue was obtained from donors at the time of autopsy or surgery. The tissue was dissected free of peripheral lung tissue, cut with a scalpel into 2 × 3 mm fragments, and placed into culture on a rocking platform for 4 to 6 d to promote reversal

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TABLE 1

INFLUENCE OF SURFACE PREPARATION ON CELL ATTACHMENT, COLONY FORMING EFFICIENCY, AND GROWTH RATE[a]

	Surface			
	None	FN	Vitrogen	FN + Vitrogen[b]
Attachment %[c]	10	20	35	80
CFE %[d]	0.05	0.3	1.0	3.8
R[e]	0.20	0.37	0.52	0.91

[a] 5,000 Cells were inoculated per dish.

[b] The FN and Vitrogen concentrations were 10 and 30 μg/ml, respectively; the coating procedure was as described in Materials and Methods.

[c] Number of cells attached after 24 h of incubation divided by the number of cells inoculated.

[d] Number of colonies after 12 d of incubation divided by the number of cells inoculated.

[e] Population doublings per day.

of ischemia (6). The bronchi were then cut into smaller (0.5 cm^2) pieces and incubated in explant growth medium (1). Explant growth medium was M199 nutrient medium that was modified by lowering the calcium concentration to 0.6 m*M* and adding HEPES buffer, 20 m*M*, pH 7.6; epidermal growth factor (EGF), 20 ng/ml, 3.3×10^{-9} *M*; hydrocortisone (HC), 5×10^{-7} *M*; insulin (ISN), 0.2 μg/ml, 3.3×10^{-8} *M*; trace elements (1); gentamicin, 50 μg/ml and 1.25% Sephadex G 10 filtered fetal bovine serum (FBS). [Sephadex filtration (1,7) reduced the calcium content of the serum more than 90-fold to less than 70 μ*M* Ca^{2+}, data not shown.] The medium was replaced every 3 to 4 d. After 8 to 11 d of incubation, outgrowths of epithelial cells radiated outward from the tissue more than 1.5 cm. At this time the explants were transferred to a new culture dish to reestablish primary cultures (1).

The epithelial cell outgrowth cultures were incubated in LHC-1 medium. This was prepared by adjusting the Ca^{2+} concentration of Medium MCDB 151 (8) to 110 μ*M* and supplementing with EGF, 5 ng/ml, 8.25×10^{-10} *M*; ISN, 5 μg/ml, 8.25×10^{-7} *M*; transferrin (TF) 10 μg/ml, 1.25×10^{-7} *M*; phosphoethanolamine and ethanolamine (P-E), each at 5×10^{-7} *M*; HC, 5×10^{-7} *M*; trace elements (1), and gentamicin, 50 μg/ml.

After 3 to 5 d of incubation the primary outgrowth cultures were dissociated into single cells using trypsin. First, the cells were washed twice with HEPES buffered saline (HBS) (9) and then incubated at 4° C for 5 to 15 min in a trypsin solution [1% polyvinylpyrrolidine (PVP) and 0.02% ethylene glycol bis (β-aminoethyl ether) *N,N,N',N'* tetraacetic acid (EGTA) and 0.02% crystalline trypsin prepared in HBS]. Dissociated single cells were suspended in 5 ml of medium containing 10% FBS or soybean trypsin inhibitor (0.1 mg/ml), pelleted (5 min, 125 ×*g*), resuspended in serum-free LHC-1 and used to inoculate experimental culture dishes, or cryopreserved in liquid N_2 (9). Mycoplasma contamination was monitored by scanning electron microscopy (10); none was detected.

Culture dish surfaces were coated using a mixture of human fibronectin (FN), 10 μg/ml; collagen, Vitrogen, 30 μg/ml; crystallized bovine serum albumin (BSA), 10 μg/ml dissolved in LHC-1 medium. Two milliliters of this mixture were pipetted into a 60 mm culture dish (1 ml/10 cm^2 of surface area) and the plates were incubated at 37° C for 2 to 6 h. The mixture was vacuum aspirated; the plate was filled with 4 ml of medium and inoculated with cells.

Bovine ISN was a gift of the Eli Lilly Co., Indianapolis, IN; EGF, endothelial cell growth supplement (ECGS), and FN were purchased from Collaborative Research, Waltham, MA; HC from Steraloids, Inc., Wilton, NH; Cholera toxin (CT), crystalline trypsin, soybean trypsin inhibitor, and TF from Sigma Chemical Co., St. Louis, MO; PVP from Calbiochem Behring Corp., La Jolla, CA; BSA preparations from Miles Biochemicals, Elkhorn, IN, and Vitrogen from The Collagen Corp., Palo Alto, CA. Retinoic acid (RA) was a gift of Dr. Anton M. Jetten, NIH, Bethesda, MD. Antikeratin antibody was a gift of

TABLE 2

EFFECT OF SERUM ON THE CLONAL RATE OF NORMAL HUMAN BRONCHIAL EPITHELIAL CELLS

Serum Concentration %						
0	0.25	0.5	1	2	4	8
0.86 (0.09)[a]	0.90 (0.07)	0.83 (0.11)	0.77 (0.13)	0.52 (0.09)	0.46 (0.10)	0.37 (0.11)

[a] Population doublings per day (SE).

Dr. Howard Baden, Department of Dermatology, Harvard Medical School, Boston, MA. Media were obtained from the NIH Media Unit and FBS was purchased from Biofluids Inc., Rockville, MD.

Clonal growth assay. The mitogenic potency of medium supplements was measured using clonal growth dose response experiments according to methods described previously (1,9,11). Five thousand cells were inoculated per dish. After 8 to 11 d of incubation the colonies were fixed with formalin and stained with 0.25% aqueous crystal violet. The mean number of cells per clone in 18 randomly selected colonies (6 to 9/replicate dish) was determined for each mitogen concentration. To derive the growth rate (R, population doublings per day) the $\log_2$ of the average number of cells per clone was divided by the number of days of incubation.

Keratin detection. Cultures were fixed and processed using procedures published previously (12,13); immunoperoxidase staining was used to detect keratin.

Results

Surface preparation and plating efficiency. Our previous work (1) had shown that coating the surface of the culture dish with FN enhanced both the colony forming efficiency (CFE) and growth rate of bronchial epithelial cells cocultivated with Mitomycin C growth arrested Swiss mouse 3T3 feeder cells. Serum-free LHC-1 medium was tested for growth of adult human bronchial epithelial cells both with and without FN surface coating (Table 1). Fibronectin coating alone increased CFE and R. Fibronectin was significantly more effective when used in combination with 1% collagen (Vitrogen) that had been diluted in LHC-1 containing 10 μg/ml crystalline BSA. Omission of the BSA from the FN-collagen mixture resulted in slower growing colonies (0.75 population doublings/day).

Effect of serum. The Ca^{2+} concentration of the medium was adjusted to 110 μM and the effect of serum supplementation on the growth rate of the epithelial cells was ascertained using clonal growth dose response experiments. The data (Table 2; Fig. 1) showed that the rate of cell division decreased as the serum concentration was increased. Less than 1% FBS resulted in some growth inhibition; progressively greater inhibition was noted as the serum concentration was increased. Serum also changed cellular morphology and cell-to-cell arrangements within colonies (Fig. 2, *A–D*). In serum-free medium, the cells were small and migratory, whereas with 1% serum supplementation pleomorphic cells were seen. The cells became squamous and tended to be multilayered in higher serum concentrations and an increasing proportion of the cells became vacuolated.

Peptide and hormone supplements. The mitogenic potencies of the peptide and hormone additives incorporated into Medium MCDB 151 were

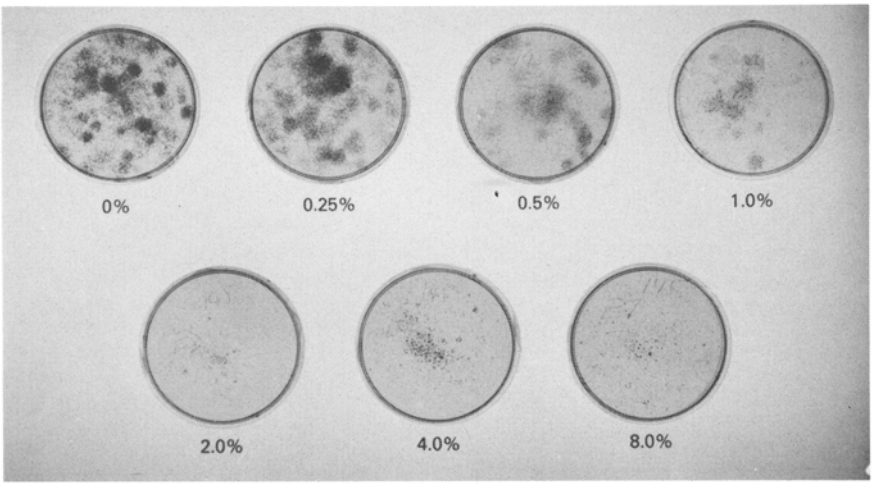


FIG. 1. Crystal violet stained colonies of normal human bronchial epithelial cells incubated for 12 d in LHC-1 medium without serum and supplemented with increasing concentrations of serum. Five thousand cells were inoculated per dish.

ascertained. Cholera toxin (14) was also assessed because this factor had been shown previously to stimulate the growth of bronchial epithelial cells when propagated with Mitomycin C growth arrested 3T3 feeder cells (1). All additives were tested either alone or in combination. Only those combinations that caused a significant increase in growth rate are shown (Fig. 3). The effect of omitting each additive singly was also assessed (Fig. 3). These experiments indicated that: (a) alone, CT, TF, or HC were not mitogenic; (b) slight stimulation was obtained with P-E supplementation ($P < 0.10$); (c) both EGF and ISN were each stimulatory, and (d) enhanced (synergistic) stimulation was noted with HC when combined with ISN, EGF, and P-E.

Optimal additive concentrations were established using dose response experiments (Fig. 4). For these experiments only one mitogen was varied whereas the others were present at the concentration listed for medium LHC-1 (*see* Materials and Methods). The optimal concentrations were determined to be 5 μg/ml, 5 ng/ml, 5×10^{-7} *M*, and 5×10^{-7} *M* for ISN, EGF, HC, and P-E, respectively. Using this defined medium (LHC-1), cultures of normal adult human bronchial epithelial cells developed from more than 15 donors have been subcultured more than five times with four to six population doublings per passage.

Effect of calcium. The optimal Ca^{2+} concentration for bronchial epithelial cells in LHC-1 medium was determined using dose response experi-

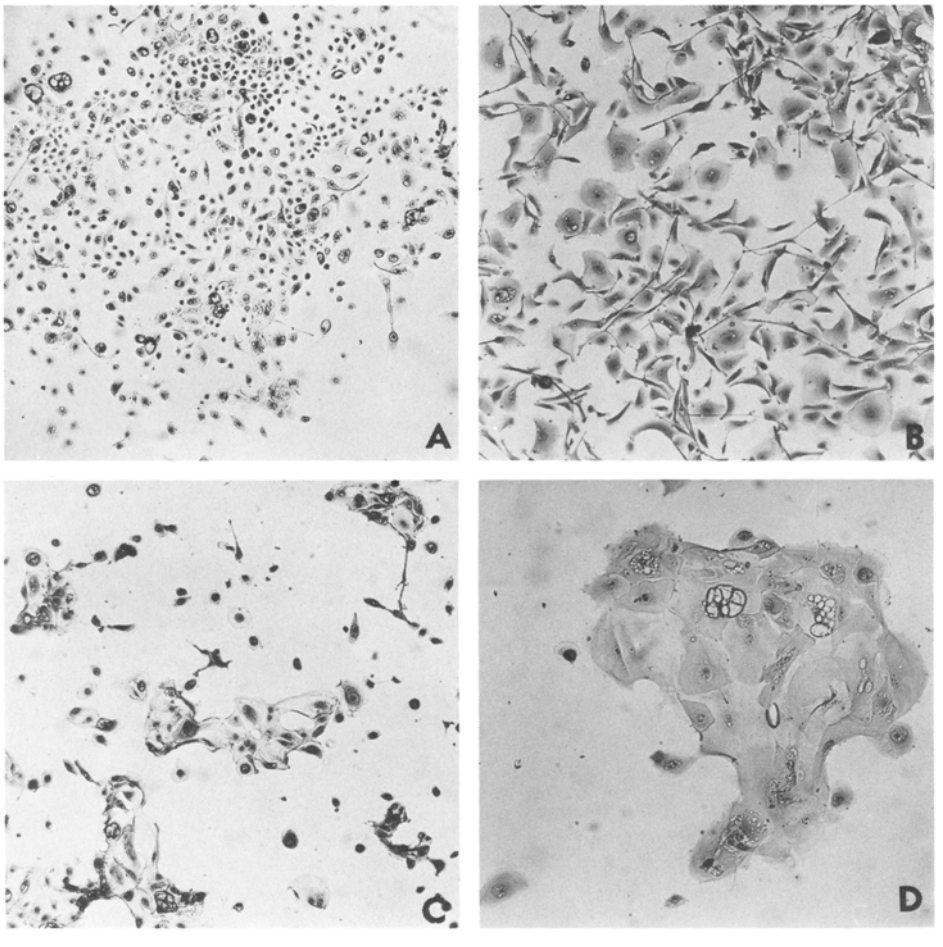


FIG. 2. Photomicrographs of crystal violet stained colonies of normal human bronchial cells after 12 d of incubation in LHC-1 medium without serum (*A*) and supplemented with (*B*) 1% serum (*C*) 2% serum and (*D*) 8% serum. ×96.

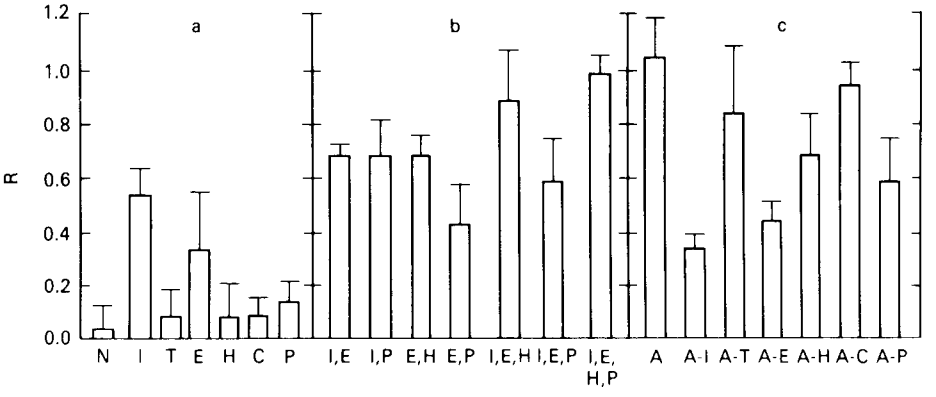


FIG. 3. Effect of putative growth factors and hormones on growth rate. Each additive was incorporated into modified MCDB 151 medium with trace elements and 100 μM Ca^{2+} either: (*A*) alone, or (*B*) as combinations (only those combinations that caused a significant increase in growth rate are shown) or (*C*) individually omitted. Symbols and concentration of additive: *N*, none; *I*, 5 μg/ml ISN; *T*, 10 μg/ml TF; *E*, 5 ng/ml EGF; *H*, 5×10^{-7} *M* HC; *C*, 10 ng/ml cholera toxin; *P*, 5×10^{-7} *M* each of phosphoethanolamine and ethanolamine; *A*, all additives. *Bars* denote SE.

ments. The growth rate increased as the Ca^{2+} concentration was increased from 30 to 110 μM Ca^{2+}, then remained constant up to 1000 μM Ca^{2+} (Table 3). Further, the cells became less migratory and the cell-to-cell arrangement became more "epithelial-like" as the Ca^{2+} concentration was increased (Fig. 5, *A*). This change in cellular morphology was noticeable 20 h after increasing the Ca^{2+} concentration. Calcium did not alter the extent of keratin staining. Cells grown in either high (1000 μM) or low (110 μM) Ca^{2+} containing media stained equally positively for keratin.

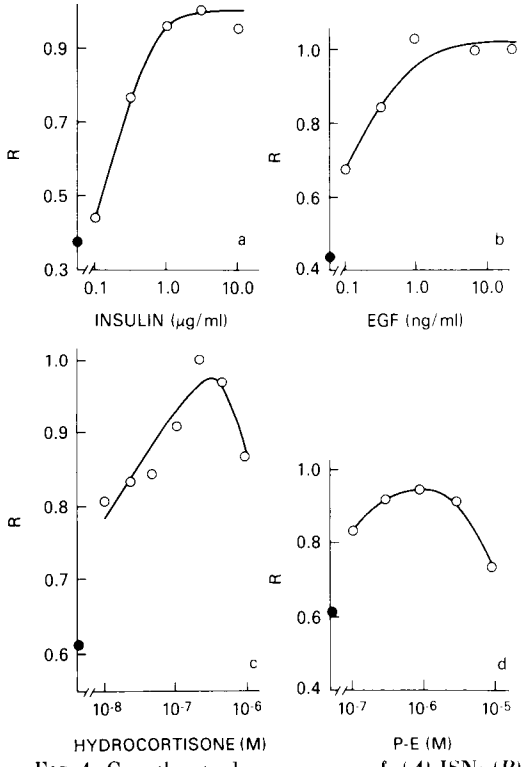


FIG. 4. Growth rate dose responses of: (*A*) ISN; (*B*) EGF; (*C*) HC, and (*D*) P-E. Five thousand cells were inoculated per dish. For each dose response the concentrations of the nonvaried additives were as stated for medium LHC-1. (●), Growth rate without varied additive.

Effect of retinoic acid and endothelial cell growth supplement. Vitamin A related compounds and ECGS have been reported to promote the growth of human epidermal keratinocytes (15–17) and prostatic epithelial cells (7,18). Thus, the effect of incorporating these compounds into basal LHC-1 medium on the growth rate of bronchial epithelial cells was investigated. Alone, both retinoic acid (3×10^{-10}) and ECGS (100 μg/ml) proved to be growth stimulatory; the growth rate was further enhanced by incorporating both factors into the medium (Table 4). The cell-to-cell arrangements within the colonies were also affected. The cells remained close to one another in colonies that developed in ECGS supplemented media, whereas marked cell migration was noted when the media contained RA or RA and ECGS (Fig. 5, *B*, *C*).

Effect of serum-free medium and bronchial explant longevity. The explant culture steps of our earlier method (1) were not changed. This work had shown that by transferring sequentially a single 0.5 cm^2 piece of bronchus over a period of 1 yr, more than 20 successive outgrowth cultures each with more than 200,000 epithelial cells could

TABLE 3

EFFECT OF CALCIUM ON THE CLONAL GROWTH RATE OF NORMAL HUMAN BRONCHIAL EPITHELIAL CELLS

Calcium Concentration μM				
30	110	235	475	1000
0.46 (0.16)[a]	0.93 (0.13)	0.85 (0.10)	0.91 (0.09)	0.93 (0.14)

[a] Population doublings per day (SE).

be obtained using explant culture medium (modified M199 supplemented with 1.25% FBS, *see* Materials and Methods). Tissue explanted in serum-free LHC-1 medium rapidly produced outgrowth cultures of epithelial cells. However, the longevity of tissue maintained in LHC-1 medium was markedly short; only small outgrowths slowly emerged from less than 10% of the explants after three transfers of the tissue. Histological examination of explants maintained in explant growth medium revealed healthy stromal tissue surrounded by multilayered epithelium. On the other hand, tissue maintained in LHC-1 medium exhibited deterioration of the stroma and extensive sloughing of the epithelium. Supplementation of LHC-1 medium with 250 μg/ml Fraction V BSA, which contains 0.5% lipids, improved the appearance of the bronchial mucosa (Fig. 6). Tissue pieces that had been incubated in each of these media for 2 wk were minced with scissors and subsequently reexplanted using conditions previously shown to support outgrowths of both fibroblastic and epithelial cells [serum supplemented nutrient medium CMRL 1066 (12)]. Only a few small epithelial outgrowths developed from tissue previously explanted in LHC-1 medium, whereas both epithelial and fibroblastic outgrowths emerged from tissue that had been previously incubated in either explant growth medium or LHC-1 medium supplemented with BSA.

TABLE 4

EFFECT OF RETINOIC ACID AND ENDOTHELIAL CELL GROWTH SUPPLEMENT OF MEDIUM LHC-1 ON THE CLONAL GROWTH RATE OF NORMAL HUMAN BRONCHIAL EPITHELIAL CELLS

Additive	R
None	0.91 (0.09)[a]
RA[b]	1.31 (0.09)
ECGS[b]	1.21 (0.10)
RA + ECGS	1.48 (0.15)

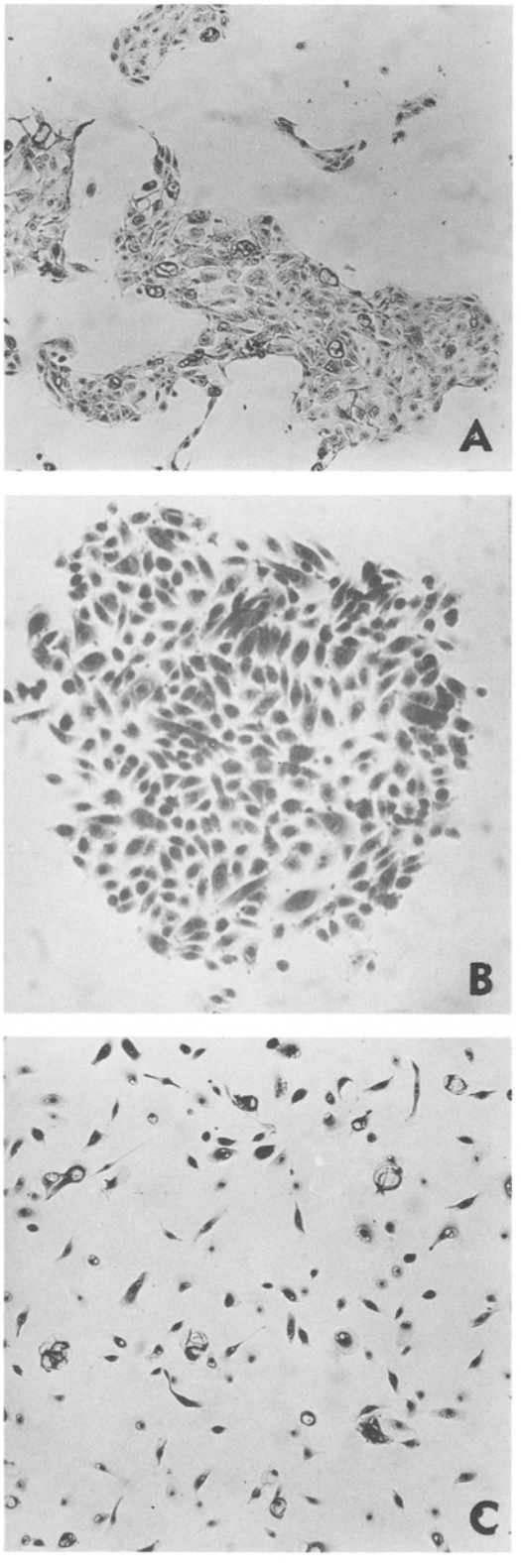
[a] Population doublings/day (SE).
[b] RA = 3×10^{-10} *M*, ECGS = 100 μg/ml.

DISCUSSION

Cultured normal epithelial cells often exhibit a strong tendency to differentiate terminally into populations of nondividing cells. Controlling this potential to differentiate terminally has been a major problem for establishing replicative cultures of epithelial cells. Selecting an optimal nutrient formulation (5,8,15); adjusting the Ca^{2+} concentration (1,8,16,19,20); lowering the pH of the medium (16,19,21); collagen coating the culture dish surfaces (1,22,23); supplementing with specific growth factors, hormones, and conditioned media (1,2,4,7,14,18,19,24-26); and cocultivating with feeder cells (1,10,27-29) are examples of techniques that have been used to maintain a population of growing cells. This report shows that a replicating pool of normal adult bronchial epithelial cells can be maintained using a specifically formulated, low Ca^{2+} containing, serum-free medium supplemented with selected growth factors and hormones and that these cells will grow as colonies when inoculated at low cell numbers.

Coating the culture dish surface contributed to the success of these culture methods. There are several reports describing enhanced cell growth by specifically treating the culture dish surface (30,31), especially when cells were grown in low serum containing (1,32) and serum-free (33) media. These components are associated with basement membranes and are known to affect cytoskeleton, motility, and growth pattern of cells in culture (30). The combination of collagen, BSA, and FN may mimic those basement membrane characteristics that stimulate cell proliferation (31,34).

Although trypsin dissociated and subcultured cells rapidly attached with high efficiency (80%) to the surface of the culture dish, less than 5% of the attached cells grew into colonies composed of more than 12 cells after 10 d of incubation. Stratified epithelial cells exhibit a strong tendency to become differentiated terminally by suspension (27). Although the rate of differentiation is re-



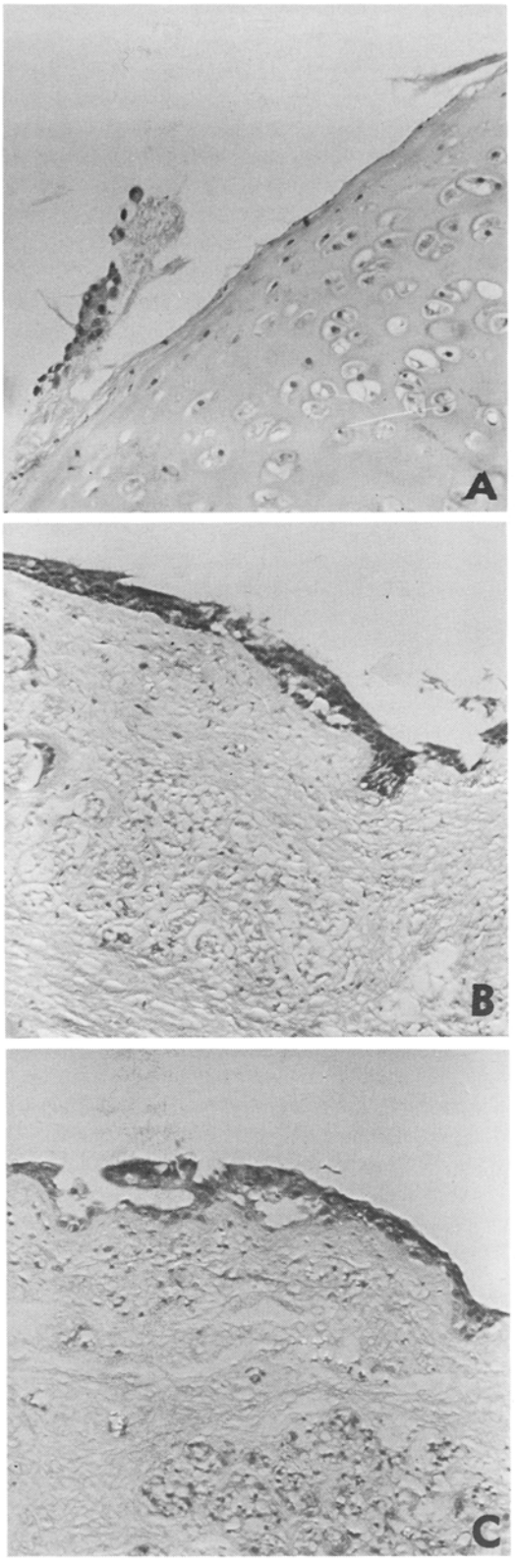
duced by lowering the temperature during suspension, and a slight improvement in CFE was obtained by reducing the temperature to 4° C during subculturing, a significant number of the cells still polymerize envelope precursors and differentiate terminally. Thus, the low CFE is not unexpected.

It is probable that the nutrient requirements of normal adult human bronchial epithelial cells are similar to those delineated for human neonatal epidermal keratinocytes (4,8) inasmuch as nutrient mixture MCDB 151 will support the growth of both cell types. However, differences were noted regarding the response of the epithelial cells to supplemental factors, e.g., P-E, Ca^{2+}, ECGS, RA, and serum. The optimal P-E concentration was found to be 5% of that reported for epidermal keratinocytes (4). Further, although some epidermal keratinocyte stratification and growth inhibition were noted at 100 μM Ca^{2+}, colonies of bronchial epithelial cells grew rapidly with little squamouslike differentiation at Ca^{2+} concentrations greater than 600 μM.

Peehl and Ham (8) and Tsao (4) reported that an extract of bovine pituitary increased the clonal growth rate of human epidermal keratinocytes. This nondefined mixture of hormones and growth factors also is growth stimulatory for bronchial epithelial cells (data not shown). Therefore ECGS, a partially characterized extract of brain (35), was tested in an effort to define further the active factor(s). This growth factor preparation was found to promote the growth of bronchial epithelial cells, suggesting that at least part of the pituitary extract activity may be due to ECGS. Further research using ECGS with other epithelial cell systems also may prove to be fruitful inasmuch as this growth factor has been shown to be mitogenic for human prostatic epithelial cells as well (7).

The effect of RA on epidermal keratinocytes was not evaluated by Taso et al. (4). However, Kubilus et al. (29) noted that this retinoid increased the distances between cells and prolonged

FIG. 5. Photomicrographs of crystal violet stained colony of normal bronchial epithelial cells after 10 d of incubation; (*A*) LHC-1 with 1,000 μM Ca^{2+}; (*B*) LHC-1 with 100 μg/ml ECGS; and (*C*) LHC1 with 3×10^{-10} *M* RA (compare to clonal morphology depicted in Fig. 2, *A*). Clonal morphology of cells grown in medium LHC-1 supplemented with both RA and ECGS similar to (*C*).



the population doubling potential without changing the growth rate of human epidermal keratinocytes cultured with Mitomycin C growth arrested 3T3 feeder cells. Retinoic acid also increased the migratory activity of bronchial epithelial cells, but in addition was markedly growth stimulatory. The surface of the culture dish and medium nutrient composition have been shown to influence serum and growth factor requirements and vice versa (25,30,36). Thus, growth conditions (feeder cells versus defined) may account for these differences in growth promoting activity inasmuch as RA has been reported to be mitogenic for epidermal keratinocytes by other investigators using feeder cell free conditions (15,17). The observation that CT stimulates the growth of bronchial epithelial cells cultured with Mitomycin C growth arrested Swiss mouse 3T3 feeder cells but is not mitogenic in a serum-free medium is an additional example of these phenomena.

The serum dose response of the bronchial epithelial cells was markedly different from epidermal keratinocytes. Tsao et al. (4) reported a direct relationship between the serum concentration and the growth rate of epidermal keratinocytes. In contrast, serum progressively decreased the growth rate of the bronchial epithelial cells. Serum toxicity per se cannot account for this inverse relationship. Serum dose response experiments with the same lot of serum showed progressive growth stimulation of bronchial epithelial cells when grown using the Swiss mouse 3T3 feeder cell cocultivation system (1). The squamous appearance of the colonies (Fig. 2, *D*) suggests that the combination of serum and LHC-1 medium depresses the growth of bronchial cells by stimulating terminal (squamous) differentiation.

Cell-to-cell interactions may explain the extensive longevity of the epithelium when explants are maintained in serum supplemented medium. We suggest that a viable fibroblastic cell population is necessary to maintain conditions for both division and preservation of a basal epithelial cell population. (Release of toxic substances during necrosis of the stromal cells is an alternative explanation.) Medium MCDB 151 does not support the growth of human foreskin fibroblasts (8), and bronchial

FIG. 6. Histological sections of normal bronchial tissue explants after 2 wk of incubation in: (*A*) Medium LHC-1; (*B*) explant growth medium; and (*C*) Medium LHC-1 supplemented with BSA (250 μg/ml). ×73.

fibroblastic cells grow poorly in LHC-1 medium; CFE of bronchial fibroblastic cells <0.01% (data not shown). Further, fibroblastic cells did not emerge from minced explants that had been maintained for 2 wk in LHC-1 medium unless the medium was supplemented with fatty acid containing BSA. Human fibroblastic cells require lipids for growth (37); normal and transformed epithelial cells grow readily without lipids (2,3,25). Therefore, a possible explanation of why the epithelium undergoes squamous differentiation and sloughs from tissue incubated in serum-free LHC-1 medium is that the stromal cells die without lipids and epithelial-stromal cell interactions (perhaps analogous to those that occur between epithelial cells and growth inactivated Swiss mouse 3T3 feeder cells) are disrupted.

REFERENCES

1. Lechner, J. F.; Haugen, A.; Autrup, H.; McClendon, I. A.; Trump, B. F.; Harris, C. C. Clonal growth of epithelial cells from normal adult human bronchus. Cancer Res. 41: 2294-2304; 1981.
2. Barnes, D.; Sato, G. Methods for growth of cultured cells in serum-free medium. Anal. Biochem. 120: 255-270; 1980.
3. Kaighn, M. E.; Kirk, D.; Szalay, M.; Lechner, J. F. Growth control of prostatic carcinoma cells in serum-free media: Interrelationships of hormone response, cell density, and nutrient media. Proc. Natl. Acad. Sci. USA 78: 5673-5676; 1981.
4. Tsao, M. C.; Walthall, B. J.; Ham, R. G. Clonal growth of normal human epidermal keratinocytes in a defined medium. J. Cell. Physiol. 110: 219-229; 1982.
5. Maciag, T.; Nemore, R. E.; Weinstein, R.; Gilchrest, B. A. An endocrine approach to the control of epidermal growth: Serum-free cultivation of human keratinocytes. Science 211: 1452-1454; 1981.
6. Harris, C. C.; Autrup, H.; Stoner, G. D. Carcinogenesis studies in human respiratory epithelium: An experimental model system. Harris, C. C. ed. Pathogenesis and therapy of lung cancer. New York: Marcel Dekker; 1978: 559-607.
7. Kaighn, M. E.; Naragan, K. S.; Ohnuki, Y.; Jones, L. W.; Lechner, J. F. Differential properties among clones of simian virus 40-transformed human epithelial cells. Carcinogenesis 1: 635-645; 1980.
8. Peehl, D. M.; Ham, R. G. Clonal growth of human keratinocytes with small amounts of dialyzed serum. In Vitro 16: 526-538; 1980.
9. Lechner, J. F.; Babcock, M. S.; Marnell, M.; Narayan, K. S.; Kaighn, M. E. Normal human prostatic epithelial cell cultures. Methods Cell Biol. 21B: 195-225; 1980.
10. Brown, S.; Teplitz, M.; Revel, J. P. Interaction of mycoplasmas with cell cultures, as visualized by electron microscopy. Proc. Natl. Acad. Sci. USA 71: 464-468; 1974.
11. Lechner, J. F.; Kaign, M. E. Application of the principles of enzyme kinetics to clonal growth assays: An approach for delineating interactions among growth promoting agents. J. Cell Physiol. 100: 519-530; 1979.
12. Stoner, G. D.; Katoh, Y.; Foidart, J.; Myers, G. A.; Harris, C. C. Identification and culture of human bronchial epithelial cells. Methods Cell Biol. 21A: 15-35; 1980.
13. Katoh, Y.; Stoner, G. D.; McIntire, K. R.; Hill, T. A.; Anthony, R.; McDowell, E. M.; Trump, B. F.; Harris, C. C. Immunologic markers of human bronchial epithelial cells in tissue sections and in culture. J. Natl. Cancer Inst. 62: 1177-1185; 1979.
14. Green, H. Cyclic AMP in relation to proliferation of the epidermal cells: A new view. Cell 15: 801-811; 1978.
15. Price, F. M.; Camalier, R. F.; Gantt, R.; Taylor, W. G.; Smith, G. H.; Sanford, K. K. A new culture medium for human skin epithelial cells. In Vitro 16: 147-158; 1980.
16. Hawley-Nelson, P.; Sullivan, J. E.; Klug, M.; Hennings, H.; Yuspa, S. H. Optimized conditions for the growth of human epidermal cells in culture. J. Invest. Dermatol. 75: 176-182; 1980.
17. Chopra, D. P.; Flaxman, B. A. The effect of vitamin A on growth and differentiation of human keratinocytes in vitro. J. Invest. Dermatol. 64: 19-22; 1975.
18. Webber, M. Growth and maintenance of normal prostatic epithelium in vitro—A human cell model. Prog. Clin. Biol. Res. 37: 181-216; 1980.
19. Peehl, D. M.; Ham, R. G. Growth and differentiation of human keratinocytes without a feeder layer or conditioned medium. In Vitro 16: 516-525; 1980.
20. Hennings, H.; Michael, D.; Cheng, C.; Steinert, P.; Holbrook, K.; Yuspa, S. H. Calcium regulation of growth and differentiation of mouse epidermal cells in culture. Cell 19: 245-254; 1980.
21. Eisinger, M.; Lee, J. S.; Hefton, J. M.; Darzynekiewicz, Z.; Chiao, J. W.; De Harven, E. Human epidermal cell cultures: Growth and differentiation in the absence of dermal components or medium supplements. Proc. Natl. Acad. Sci. USA 76: 5340-5344; 1979.
22. Lu, S-C., Karasek, M. Isolation and growth of adult human epidermal keratinocytes in cell culture. J. Invest. Dermatol. 71: 157-162; 1978.
23. Yang, N-S.; Kube, D.; Park, C.; Furmanski, P. Growth of human mammary epithelial cells on collagen gel surface. Cancer Res. 41: 4093-4100; 1981.
24. Ambesi-Impiombato, F. S.; Parks, L. A. M.; Coon, H. G. Culture of hormone-dependent functional epithelial cells from rat thyroids. Proc. Natl. Acad. Sci. USA 77: 3455-3459; 1980.
25. Wu, R.; Sato, G. H. Replacement of serum in cell culture by hormones: A study of hormonal regulation of cell growth and specific gene expression. J. Toxicol. Environ. Health [Suppl.] 4: 427-448; 1978.

26. Stampfer, M.; Hallowes, R. C.; Hackett, A. J. Growth of normal mammary cells in culture. In Vitro 16: 415-425; 1980.
27. Rheinwald, J. G. Serial cultivation of normal human epidermal keratinocytes. Methods Cell Biol. 22A: 229-254; 1980.
28. Taylor-Papadimitriou, J.; Shearer, M.; Stoker, M. G. P. Growth requirements for human mammary epithelial cells in culture. Int. J. Cancer 20: 903-908; 1977.
29. Kubilus, J.; Rand, R.; Baden, H. P. Effects of retinoic acid and other retinoids on the growth and differentiation of 3T3 supported human keratinocytes. In Vitro 17: 786-795; 1981.
30. Reid, L. M.; Rojkind, M. New techniques for culturing differentiated cells: Reconstituted basement membrane rafts. Methods Enzymol. 58: 263-278; 1979.
31. Gospodarowicz, D.; Vlodavsky, I.; Greenburg, G.; Johnson, L. K. Cell shape is determined by the extracellular matrix and is responsible for the control of cellular growth and function. Sato, G. H.; Ross, R. eds. Hormones and cell culture. Book B. Cold Spring Harbor: Cold Spring Harbor Laboratory; 1979: 561-592.
32. McKeehan, W. L.; Ham, R. G. Stimulation of clonal growth of normal fibroblasts with substrata control with basic polymers. J. Cell Biol. 71: 727-734; 1976.
33. Orly, J.; Sato, G. Fibronectin mediates cytokinesis and growth of rat follicular cells in serum-free medium. Cell 17: 295-305; 1979.
34. Salomon, D. S.; Liotta, L. A.; Kidwell, W. R. Differential response to growth factor by rat mammary epithelium plated on different collagen substrata in serum-free medium. Proc. Natl. Acad. Sci. USA 78: 382-386; 1981.
35. Maciag, T.; Cerandolo, J.; Ilsley, S.; Kelley, P. R.; Forand, R. An endothelial cell growth factor from bovine hypothalamus: Identification and partial characterization. Proc. Natl. Acad. Sci. USA 76: 5674-5678; 1979.
36. Lechner, J. F.; Kaighn, M. E. Reduction in the calcium requirement of normal human epithelial cells by EGF. Exp. Cell Res. 121: 432-435; 1979.
37. Bettger, W. J.; Boyce, S. T.; Walthall, B. J.; Ham, R. G. Rapid clonal growth and serial passage of human diploid fibroblasts in a lipid-enriched synthetic medium supplemented with epidermal growth factor, insulin and dexamethasone. Proc. Natl. Acad. Sci. USA 78: 5588-5592; 1981.

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