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EPITHELIAL CELL CULTURES FROM THE COLON OF THE SUCKLING RAT

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SUMMARY

Epithelial cells from the colon of suckling rats have been propagated in vitro. The colons were excised and cut longitudinally. The epithelial sheets were peeled off and dissociated in 0.1% trypsin solution at 25° C for 10 min. The first cell suspension was discarded and the remaining fragments trypsinized again for an additional 20 min. The dissociated cells were washed and cultured. Forty-eight hours later, several epithelial colonies consisting of closely packed polygonal cells were formed. Transmission and scanning electron microscope examination of the colonies showed numerous regularly spaced microvilli on the surface and tight junctions and desmosomes between adjacent cells. Immunocytochemical studies with antiserum prepared against the brush-border membrane of the colonic epithelium showed specific staining of the epithelial colonies.

Epithelial colonies were subcultured by the penicylinder method. Although the subcultured cells retained their epithelial characteristics, the proliferative activity of the cells gradually decreased. Currently, efforts are being made to determine the optimum nutritional requirements of the primary and low-passage cultures.

Key words: colon epithelial cell cultures; suckling rat.

INTRODUCTION

Colon carcinoma is the second leading cause of cancer-related deaths in the United States. Epidemiologic studies indicate that environmental factors are associated with development of this cancer (1). Although chemical carcinogens, i.e., aromatic amines, polycyclic hydrocarbons, and nitrosamines, have been shown to induce colon cancer in experimental animals (2-4), the nature of environmental factors and mechanisms by which these agents cause neoplastic transformation remain to be clarified. In order to bypass complexities of the in vivo systems, development of a colonic epithelial cell culture model would be highly desirable for the study of carcinogenesis. Moreover, the primary cell cultures or cell lines of the colonic epithelium will provide suitable models for elucidating factors that regulate cell proliferation and differentiation. This area of research in the colon has received little attention, although such studies have been performed in the small intestine (5-9).

There are a number of reports dealing with the isolation of epithelial cells from the normal colon (10-13). Little success, however, has been achieved to culture the isolated cells. Recently, proliferative cells separated from the basal crypts of the adult rat colon have been cultured for almost 3 weeks (14), although the plating efficiency and proliferative potential of the dissociated cells were low. Since the division potential of the cultured cells progressively decreases with increasing age of the donor (15,16) and the proliferative rate of colonic epithelial cells in vivo is higher in the suckling than in adult mice (17), the cells of suckling rat may be more suitable for in vitro propagation. Therefore, we have attempted to isolate and culture colonic epithelial cells from the suckling rats. The results show that the isolated epithelial cells remain highly proliferative in vitro, although the proliferative activity in different colonies is variable. Cultured cells retain typical epithelial morphology characterized by apical brush borders including numerous microvilli and glycocalyx on the outer surface and tight junctions

and desmosomes between cells. The cells retain epithelioid morphology for six passages over a period of 4 months.

MATERIALS AND METHODS

Tissue preparation and culture methods. Newborn Sprague-Dawley rats were purchased from the Southern Animal Farms, Prattville, AL. Two- to sixteen-day-old suckling rats were sacrificed and their colons dissected out. Each colon was washed in Ca^{++}- Mg^{++}-free Hanks' balanced salt solution (BSS) and cut longitudinally. The epithelial sheet was carefully peeled off from the underlying lamina propria by using a dissecting microscope and two pairs of forceps. Epithelial sheets were pooled, cut into small pieces, and incubated in 5 ml of BSS containing 0.1% trypsin (GIBCO, Grand Island, NY) for 10 min at room temperature with gentle stirring. The first dissociated cells were discarded by filtering through a layer of 48-μm Nitex (Tetko, Elmsford, NY). The cell clumps remaining on the Nitex were resuspended in 5 ml of 0.1% trypsin and incubated for an additional 20 min. This second cell suspension was filtered through a layer of 100-μm Nitex. The filtrate was combined with 2 *V* Eagle's minimum essential medium (MEM, GIBCO) supplemented with 15% fetal bovine serum (FBS, GIBCO), and centrifuged at 100 ×*g* for 7 min in a DPR-6000 centrifuge (IEC, Needham Heights, MA). The cell pellet was resuspended and washed twice in the above medium. The final pellet was resuspended and washed twice in the above medium. The final pellet was resuspended in the culture medium and cells were counted in a hemocytometer. Each cell clump consisting of 2 or more cells was counted as 1 cell. Approximately 2×10^5 cells were cultured in 60-mm tissue culture dishes (Falcon Plastics, Oxnard, CA) containing MEM supplemented with 15% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml Fungizone (Squibb & Sons, Inc., Princeton, NJ). In some experiments, glass cover slips were placed in the culture dishes before plating cells. The cultures were maintained at 37° C (pH 7.2 to 7.4) in a humidified gas containing 5% CO_2 and 95% air. The medium was changed twice a week.

Cultures were observed for the formation and growth of epithelial cell colonies. Although epithelial colonies were predominant during the 1st week after plating, fibroblasts were also present in the cultures. Attempts were made to remove or eliminate fibroblasts from the primary cultures by either using the ethylenediaminetetraacetic acid (EDTA)-trypsin method (18) or by substituting culture medium with D-valine medium (19) at 7 or 10 days after plating. When the EDTA-trypsin method was used, cultures were rinsed with BSS twice and then incubated in BSS containing 0.01% trypsin (Worthington, Freehold, NJ) and 0.01% EDTA (Sigma, St. Louis, MO) for 1 to 2 min at room temperature. The cells remaining on the dishes were rinsed twice and finally incubated in the culture medium. When D-valine medium was used, culture dishes were rinsed twice and incubated in D-valine medium (GIBCO) supplemented with 15% dialyzed FBS and antibiotics. In some experiments, culture medium was supplemented with either collagenase (Types I or IV, 20 to 50 μg/ml, Sigma) or *cis*-4-hydroxy-L-proline (200 μg/ml, Sigma) to inhibit fibroblast growth (20–22).

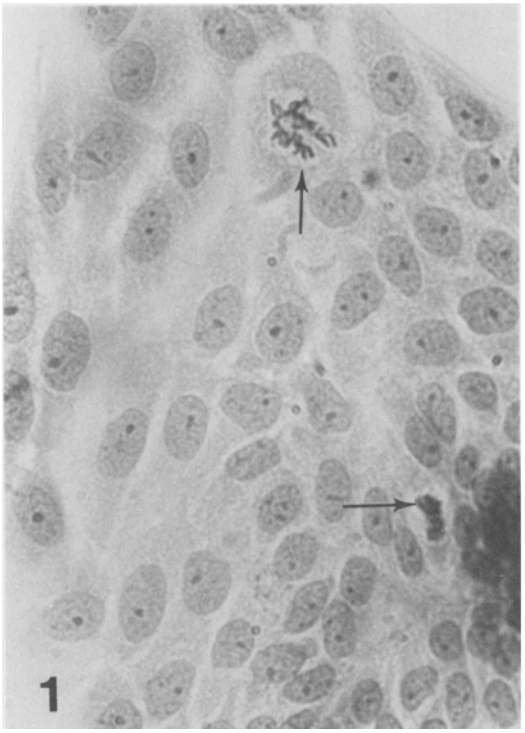
Serial passage of epithelial colonies. Primary epithelial colonies consisting of 50 to 100 cells were dissociated by using a stainless steel penicylinder (Fisher Scientific Co., Philadelphia, PA). The colony to be subcultured was separated from the adjacent colonies or cells by scraping the area around the colony with a curved needle. The colony was capped with a penicylinder, washed with BSS, and incubated in dissociation solution (BSS containing 0.01% EDTA and 0.1% trypsin) at 37° C for 1 to 2 min. After the cells began to round up, dissociation solution was withdrawn, and the colony carefully rinsed and covered with the culture medium. Subsequently cells were dislodged by repeated pipetting and suspended in the culture medium. Aliquots of the cell suspension were cultured in several 60-mm culture dishes containing 2.5 ml medium.

Morphology of the cultured cells. The cell cultures were examined every other day after plating. For histochemical study, the colonies that grew on the cover slips were fixed in a mixture of alcohol and acetic acid (3:1 v/v) and stained in Periodic Acid Schiff reagent (PAS) (23). The cover slips were mounted on slides.

For electron microscopy, cell cultures were fixed in situ with 4% glutaraldehyde in 0.1 *M* cacodylate buffer, pH 7.4, for 1 to 2 hr. They were postfixed in buffered 1% osmium tetroxide and dehydrated through a series of graded alcohol. For scanning electron microscopy, cultures were transferred from alcohol into liquid CO_2 in a critical-point drying apparatus. After several

rinses in CO_2, the temperature of the closed system was raised to 45° C according to the critical-point drying method of Anderson (24). Cells were then coated with Gold-palladium alloy and observed in an AMR model-1000 scanning electron microscope. For transmission electron microscopy, dehydrated cells were stained with toluidine (0.1% in absolute alcohol) and embedded in situ with Epon 812. Embedded cells were sectioned (800–900 Å) with a Porter-Blum MT-2B ultramicrotome (DuPont Co., Newtown, CT). Sections were stained in uranyl acetate followed by lead citrate and examined in a Phillips-200 electron microscope.

Immunocytochemical techniques. Since the surface components of most cell types contain specific antigens (25,26), the brush-border membranes (BBM) of colonic epithelial cells was used to obtain anti-BBM antiserum for this study. The method of Schmitz et al. (27) was used to purify BBM. Colons from six adult rats were excised, cut longitudinally, and flushed with normal saline. The mucous layer was removed by scraping the colon with a glass slide. A 1% homogenate was made in 50 m *M* mannitol-2 m *M* Tris (pH 7.1). The homogenate was filtered through a layer of 48-μm Nitex. Solid $CaCl_2$ was added with stirring to obtain a final concentration of 10 m*M*. The homogenate was centrifuged at 2000 ×*g* for 10 min in a Sorvall centrifuge. The supernatant fluid (S_1) was centrifuged again at 20,000 ×*g* for 15 min to yield a pellet containing brush-border fragments. The pellet was resuspended in 0.8 *M* Tris buffer and stirred for 1 hr to disrupt brush-border fragments into BBM and core material. The mixture was layered on the top of a 37, 40, 42, 45, and 60% glycerol gradient (with 0.05 *M* $MgCl_2$) and centrifuged in an ultracentrifuge (L2-65B, Beckman, Palo Alto, CA) with SW 27.1 rotor for 15 min. The BBM was in the second band from the top of the gradient and was pipetted out. The BBM was washed twice by 3 *V* of distilled water, and the final BBM pellet was obtained by centrifugation at 200,000 × *g* with SW 50.1 rotor for 60 min. Since there is no suitable enzyme marker found in the BBM of colonic epithelial cells in adult rats (28,29), electron microscopy seems to be the only means for assessing the



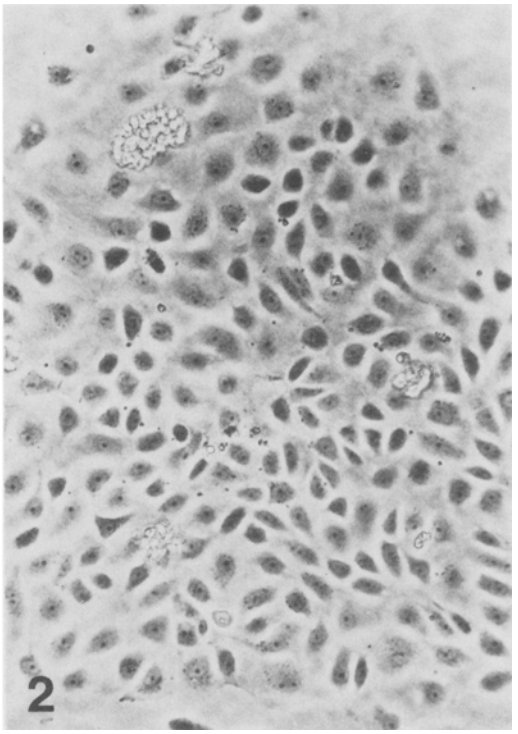


FIG. 1. Primary epithelial culture stained with PAS and hematoxylin at 2 days after plating. Two mitotic figures (*arrows*) were present. ×720.

FIG. 2. Primary epithelial culture at 6 days after plating. Secretory products were seen on the top of some cells. ×180.

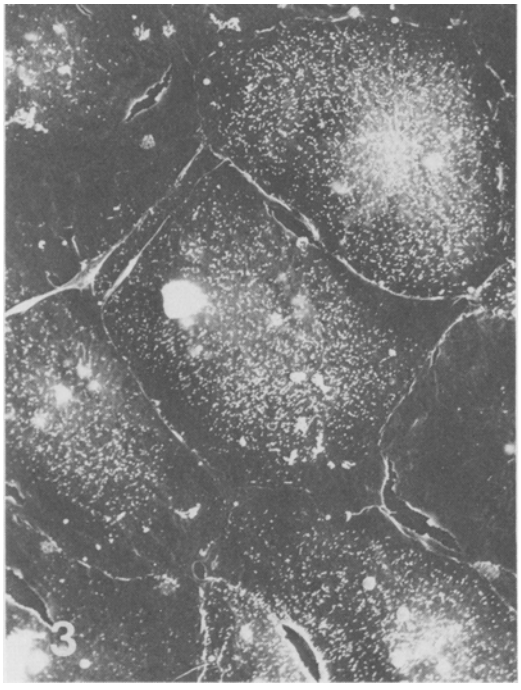
purity of BBM preparation. A portion of BBM pellets was fixed and processed for electron microscopy.

Immunization. Two white male New Zealand rabbits (about 4 months old) were used. The control serum was obtained by bleeding from the ear vein of rabbits 2 days before the injection of BBM. Before inoculation, BBM (920 μg of protein in 1.0 ml of H_2O) was emulsified with 9 ml of complete Freund's adjuvant (GIBCO). Five milliliters of the mixture was injected into each rabbit at multiple intramuscular sites. Each rabbit was boosted with 360 μg BBM protein in Freund's complete adjuvant at Days 14 and 26 after the first inoculation. Seven days after the final injection, 20 ml of blood was collected from the ear vein of each rabbit and clotted for 5 hr at room temperature. The serum was collected with a Pasteur pipette and stored at −20° C. Since the anti-BBM antiserum specifically bound to epithelial cells of colon sections and cultured epithelial cells (14), no further purification was attempted.

Immunocytochemistry. Living cultures and cultures fixed in 85% alcohol for 20 min were washed with phosphate buffered saline (PBS; pH 7.2) three times over a 30 min interval. They were then incubated with control or anti-BBM antiserum for 2 hr at room temperature, washed four times over a 30 min period with PBS, fixed with 4% glutaraldehyde for 20 min, washed again four times with PBS, and finally incubated with goat antirabbit IgG conjugated with fluorescein isothiocyanate (GIBCO) for 1 hr. Cultures were washed four times within a 1-hr period, embedded in phosphate buffered glycerol, and observed in a Leitz Dialux fluorescence microscope.

RESULTS

Structural characteristics and maintenance of epithelial cell cultures. Epithelial cells were able to attach and flatten on both the glass and plastic surfaces. Cells isolated from 2- to 16-day-old rats exhibited similar plating efficiencies of 0.05 to 0.5%, when colonies consisting of four or more cells were counted. Forty-eight hours after plating, epithelial colonies consisting of monolayers of closely packed polygonal cells were formed (Fig. 1). Each cell contained a large round



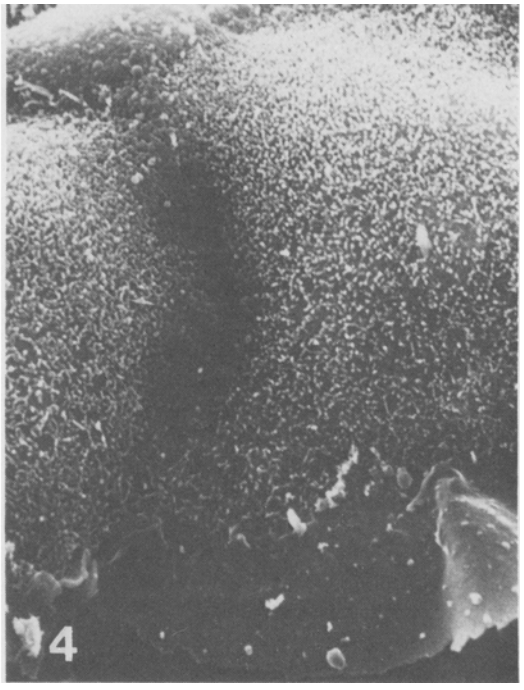
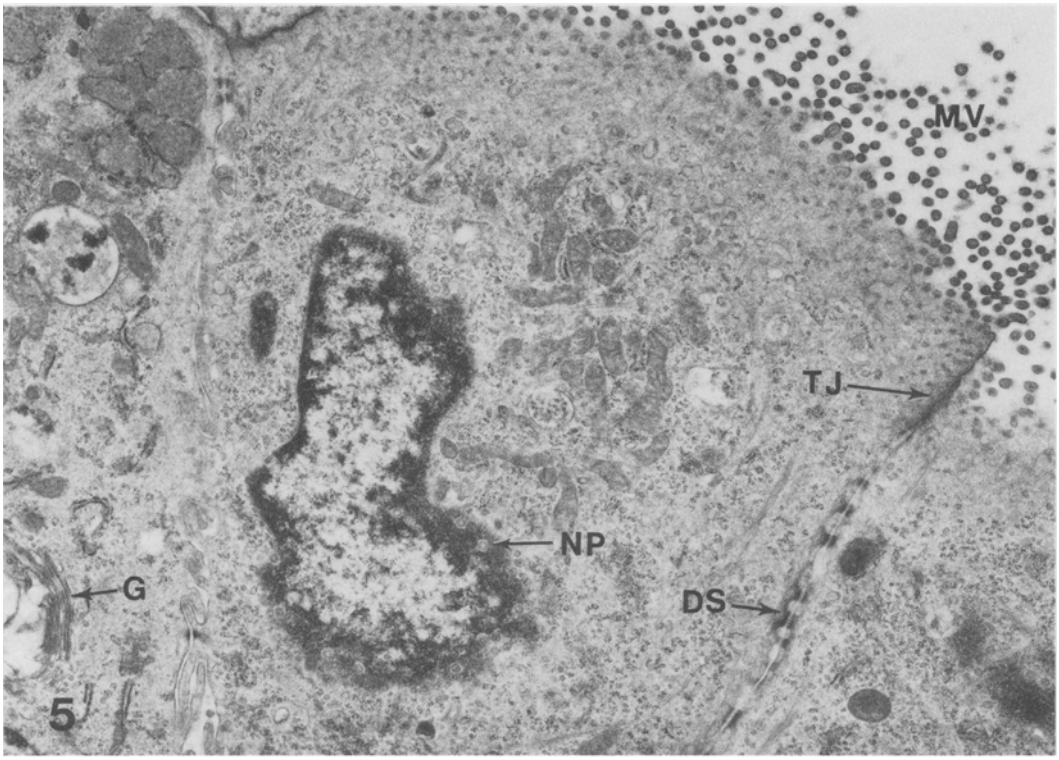


FIG. 3. Scanning electron micrograph of a primary culture at 8 days after plating. Squamous-like cells were closely contacting and cement-like substances were present on the contact surface. ×1,250.

FIG. 4. Scanning electron micrograph of a primary culture at 2 days after plating. There were numerous microvilli on the cell surface with the exception of ruffles. Instead of microvilli, a few blebs were present on ruffles. ×2,810.

nucleus with one or two nucleoli. The cytoplasm of some cells was highly vacuolated and appeared to release PAS-positive mucus-like substances into the culture medium (Fig. 2). Upon ultrastructural examination, these secretory cells showed characteristics of mucus cells (*see* Fig. 6). The majority of the cells in the epithelial colonies showed weak PAS-positive cytoplasm. The absorptive cells, which are highly vacuolated and located on the tip of crypts, were rarely seen.

Scanning electron microscopic observations showed that the epithelial cells were intimately connected with each other (Fig. 3). Cell boundaries were conspicuous by the presence of cement-like substances between the cells. Numerous microvilli generally located on the supranuclear



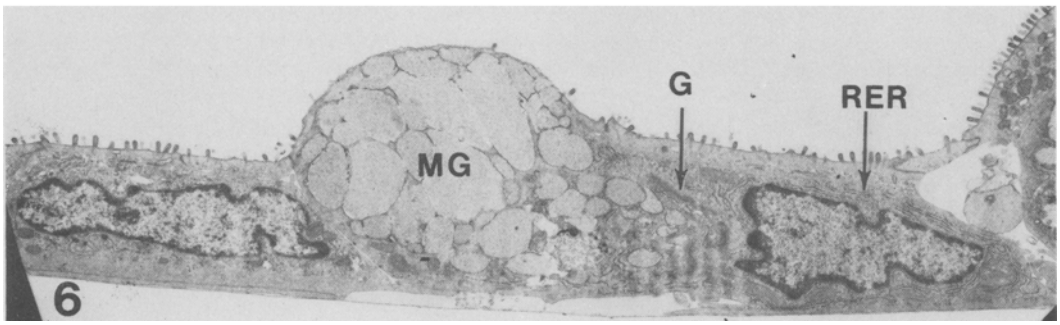


FIG. 5. Transmission electron micrograph of a primary culture. Cells were sectioned in parallel to the culture surface. Nuclear pores (NP) were prominent on the nuclear envelope. Cells showed numerous microvilli (MV), tight junction (TJ), desmosomes (DS), and a well-developed Golgi complex (G). Glycocalyx was present on the outer surface of microvilli. ×14,000.

FIG. 6. Transmission electron micrograph of a primary culture. Cultures were sectioned at a right angle to the culture surface. A flattening goblet cell showed mucogen granules (MG) loaded cytoplasm at one side, and the nucleus at the other. Well-developed Golgi complexes (G) and rough endoplasmic reticulum (RER) were conspicuous. ×10,000.

surface were present on most cells (Figs. 3 and 4). The number of microvilli, however, was variable in different cells and decreased with aging of the cell cultures. Cells at the outer periphery of colonies formed ruffles at the free edges (Fig. 4). The surfaces of the ruffles were smooth and were studded with a few small surface blebs. With the transmission electron microscope, epithelial cells showed numerous microvilli covered with glycocalyx on the outer surface (Fig. 5). Junctional complexes, including tight junction and desmosomes, were conspicuous between cells. The cytoplasm contained a large number of mitochondria and well-formed Golgi complexes. A small proportion of the epithelial cells could be identified as differentiated goblet cells since they showed an irregular nucleus on one side and mucogen-loaded supranuclear cytoplasm at the other (Fig. 6). The cell membrane on the supranuclear region but not on the mucus-secreting region showed numerous microvilli. Goblet cells contained long and dilated rough endoplasmic reticulum (RER) and highly developed Golgi complexes. Occasionally cells with numerous small vacuoles in the cytoplasm were seen (Fig. 7). These cells were morphologically different from goblet cells since they had well-developed microvilli on the outer surface and no dilated RER in the cytoplasm. Enteroendocrine cells were rarely seen. They contained numerous secretory granules in the cytoplasm (*not shown*).

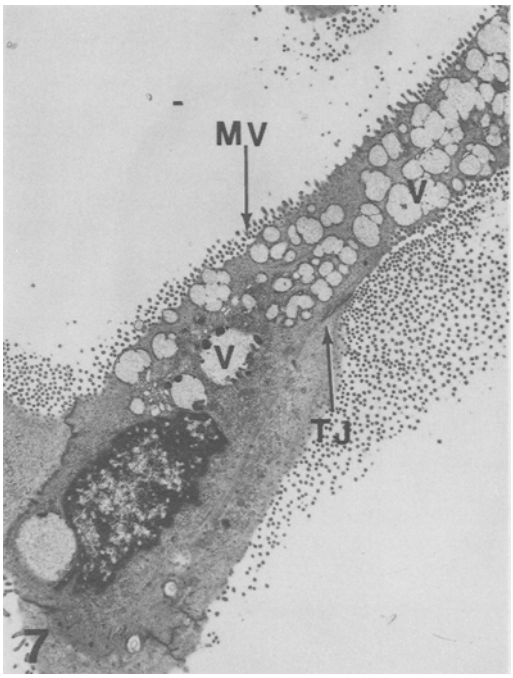


FIG. 7. Transmission electron micrograph of the vacuolated cell in the primary culture. A highly vacuolated cell showed numerous vacuoles (V) in the cytoplasm. There were numerous microvilli on the surface of the cell. Tight junction (TJ) was present in contact with other cells. ×4,100.

Based on the size and the number of mitotic figures, the epithelial colonies showed variable degrees of proliferative activity. In certain colonies, active proliferation was conspicuous by the presence of mitotic figures (Fig. 1). The proliferation continued for about 2 weeks, thereafter the mitotic activity decreased and cells began to flatten (Fig. 8). Cells of some epithelial colonies, however, stopped dividing soon after attachment and flattened; subsequently, these cells migrated away from each other and finally degenerated within 3 weeks after plating.

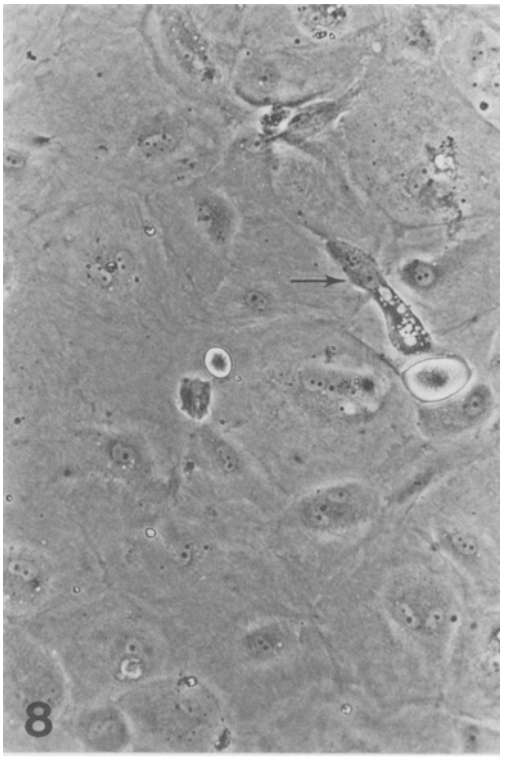
Selective removal of contaminating fibroblasts from the cultures. During the 1st week after plating, epithelial colonies were predominant although some fibroblasts were also present in the cultures. Attempts to reduce the fibroblast contamination by their selective removal with the EDTA-trypsin method was not successful because epithelial cells and fibroblasts had similar sensitivity to this treatment. When collagenase was included in the culture medium (25 or 50 μg/ml), the proliferative activity of both the epithelial cells and fibroblasts was adversely affected. After this treatment, most cells ceased to proliferate and subsequently degenerated. The addition of *cis*-4-hydroxy-L-proline to the medium (200 μg/ml) had no inhibitory effect on the proliferation of fibroblasts. When culture medium was replaced by D-valine medium, colonic epithelial cells showed signs of degeneration within 2 days after the replacement of the regular medium.

The epithelial colonies, however, were separated by successfully subculturing them with the penicylinder method. Although the proliferative activity of subcultured cells was extremely variable, they retained epithelioid morphology and formed islets of closely packed polygonal cells (Fig. 9). Of the 37 epithelial colonies that were subcultured, 34 degenerated within two passages. Two other colonies degenerated at the fourth and fifth passage, respectively. The one remaining colony was subcultured six times over a period of 4 months.

Subculturing of epithelial colonies resulted in a reduction in the number of microvilli on the cell surface. However, microvilli were still present on

the surface of some cells even after the fifth passage (Figs. 10 and 11). Tight junction and desmosomal-like structures were present between cells (Fig. 11). The goblet and vacuolated cells were not seen in the subcultures.

Immunofluorescent staining. Electron microscopic study revealed that the BBM preparations used to immunize rabbits were homogenous and consisted of vesiculated membranes (Fig. 12).



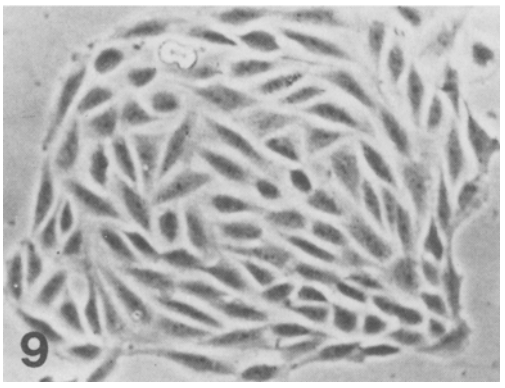


FIG. 8. Primary epithelial culture at 26 days after plating. A mucus-producing cell (*arrow*) was present in the midst of flattened epithelial cells. ×175.

FIG. 9. Subcultured epithelial cells at Passage 5. ×175.

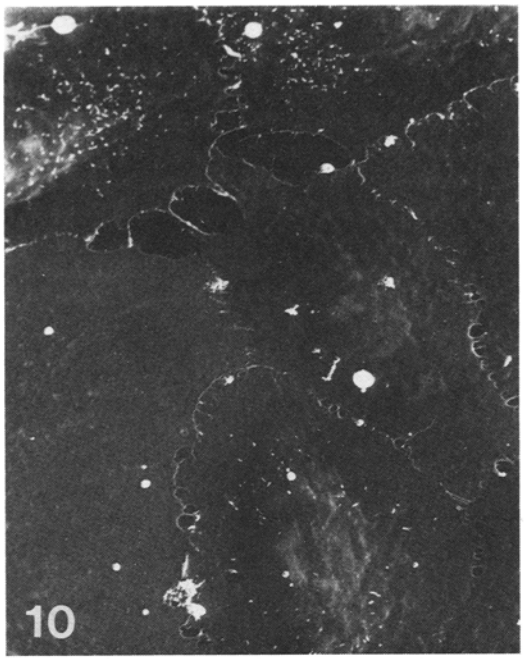
However, a few vesicles filled with electron-dense materials were present. These dense vesicles were probably derived from the core of microvilli. Organelles other than electron-dense vesicles were not seen.

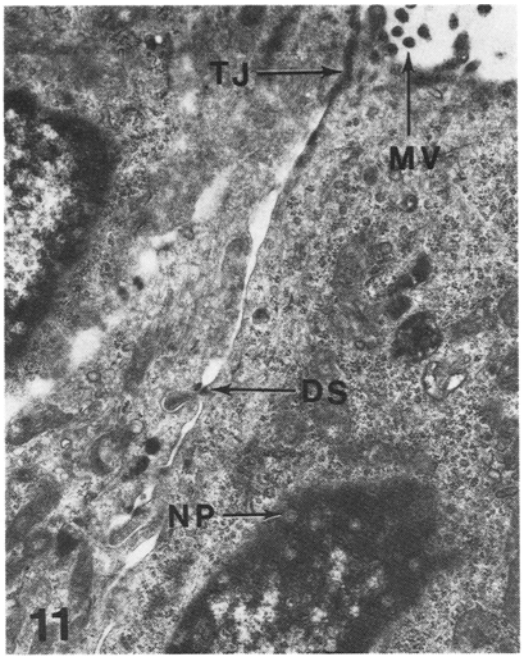
When examined with the indirect immunofluorescent staining method, primary epithelial cells contained antigen determinants that were specifically stained by anti-BBM antiserum (Fig. 13). The fluorescence was mostly confined to the cell surface or perinuclear region, or both, and was absent from the cell ruffles (Fig. 13, *a*). When living cells were stained with the antiserum, fluorescence was also present on the cell surface, although cells began to dislodge during staining (Fig. 13, *b*). No fluorescence was seen when the cells were stained with the control serum. The anti-BBM antiserum was specific for colonic epithelial cells, since no fluorescence was observed on fibroblasts (Fig. 13, *c*). Subcultured epithelial cells at the 5th passage showed no fluorescence when they were stained with the anti-BBM antiserum (Fig. 13, *d*).

DISCUSSION

The present study shows that primary cultures of colonic epithelial cells retain their structural characteristics. Numerous microvilli are present on the surface of epithelial cells. In addition to the cement-like substances that hold cells together, the outer layers of cell membrances are fused to form tight junction. In addition to the proliferative cells, other cell types such as goblet, vacuolated, and enteroendocrine cells are also present occasionally in the epithelial colonies. Whether these specialized cell types are differentiated from a common stem cell in vitro remains to be clarified, although this precursor-progeny relationship has been proposed in the in vivo system (30).

Although the enzymic components of the BBM change drastically in both quantity and quality in the small intestine of developing rats (5–9), these changes are less conspicuous and only quantitative in the colon of rats (28,29). Apparently the changes in enzyme levels in BBM during development reflect functional modifications of the colon. Nevertheless, present results that the antiserum prepared against BBM isolated from the epithelium of adult colons binds to cultured epithelial cells of suckling rats indicate the presence of antigenic determinants in these cells. The presence of immunofluorescence in cultured epithelial cells but not in fibroblasts further confirms that





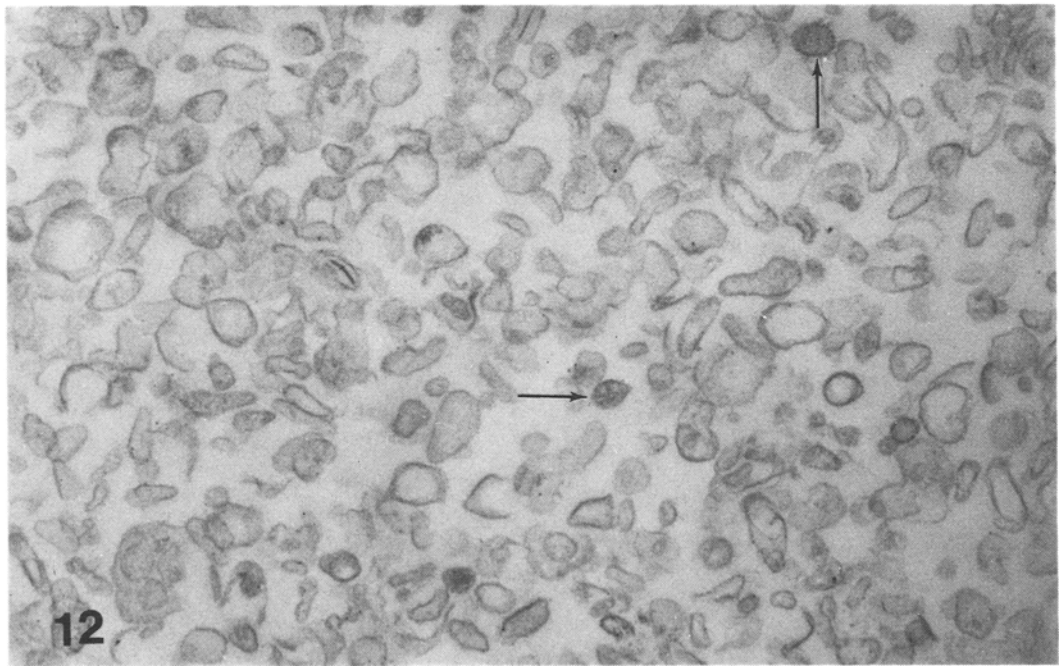


FIG. 10. Scanning electron micrograph of the subcultured cells at Passage 5. Cells were closely contacting, but the cement-like substances were less conspicuous. Microvilli were also present on some cells, but they were less abundant as compared with cells in the primary cultures.

FIG. 11. Transmission electron micrograph of subcultured cells. Numerous nuclear pores (NP) were present on the nuclear envelope. Cells showed microvilli on the surface. Tight junction (TJ) and desmosomes were present between cells. ×12,000.

FIG. 12. Electron micrograph of the BBM isolated from the colons of adult rats. This photograph represents a typical appearance of two BBM preparations. The BBM preparations consisted of homogeneous membrane vesicles. A few electron dense vesicles (*arrows*) were present. Other organelles were not seen. ×50,000.

the epithelial cell cultures originated from the colonic epithelium.

The colonic epithelial cells are in a state of constant renewal; new cells are produced in the lower half of the crypts, migrate up to the luminal surface, and are finally extruded into the lumen. The cell turnover rate is 2 to 3 days in rodents (30,31) and 3 to 8 days in humans (32-34). This rapid turnover rate of the colon cells may present an inherent difficulty for the long-term maintenance of the cells in vitro. Thus epithelial cells isolated from the adult rat colon exhibited low plating efficiency and a limited proliferative potential (14). The colonic epithelial cells of the suckling rats studied in this paper showed comparatively higher plating efficiency and proliferative activity, although only 1 out of 37 epithelial colonies could be subcultured 6 times. This is consistent with the evidence that the cells of younger donors exhibit greater proliferative activity than those of

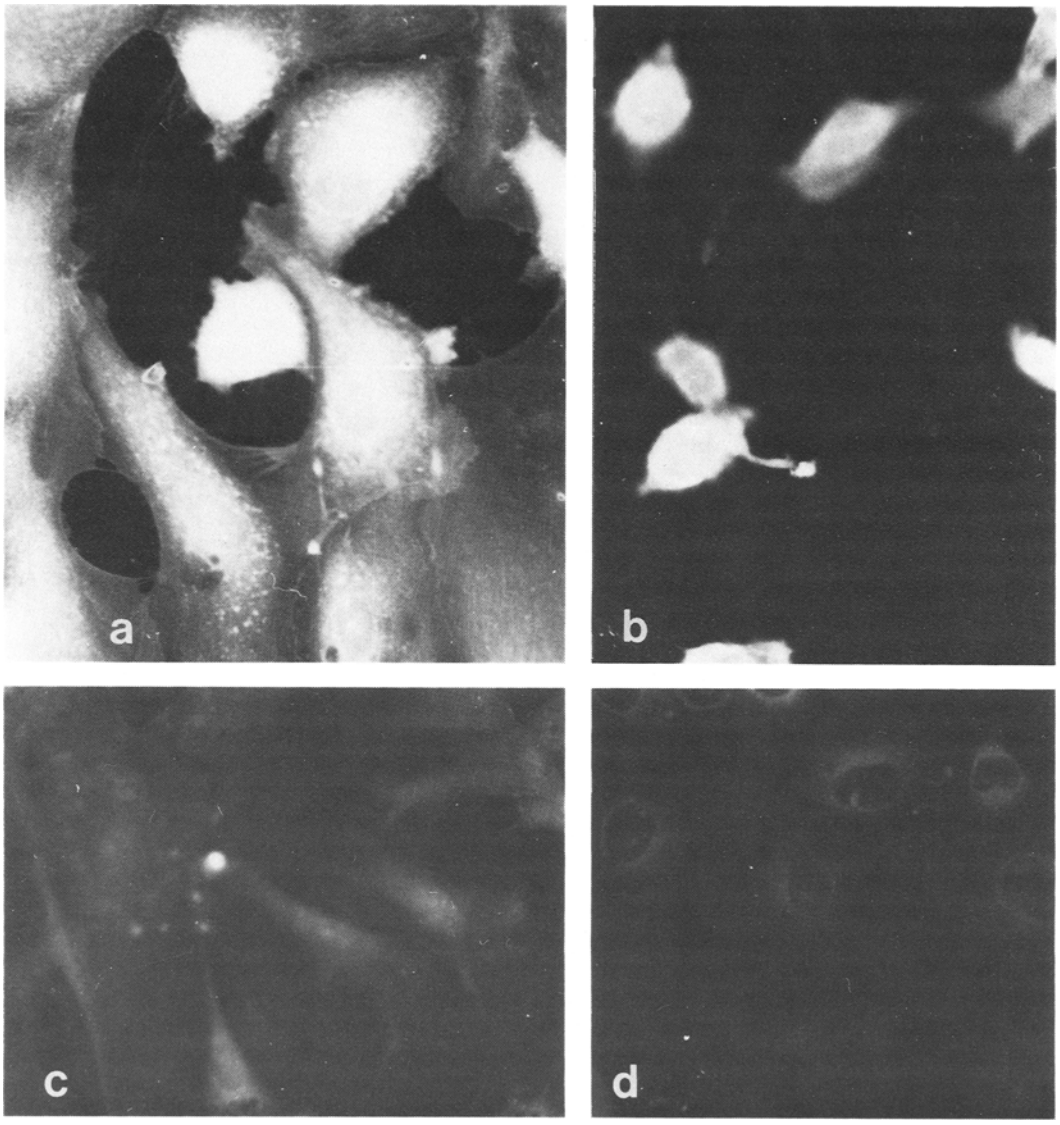


FIG. 13. Immunochemical studies of cultured cells stained with the anti-BBM antiserum. ×1,400. *a*, Primary culture of epithelial cells fixed in 85% alcohol before staining. Fluorescence was present on the cell membrane and in the cytoplasm of perinuclear region. *b*, Unfixed epithelial cells, fluorescence was also present on the cell surface of cells. *c*, Fibroblasts fixed before staining, no fluorescence was seen. *d*, Subcultured (5th passage) epithelial cells fixed before staining, no fluorescence was present.

the older donors (15-16). However, further improvements in the cell dissociation procedures and nutritional conditions may yield colonic epithelial cell lines that can be maintained for longer periods.

One of the critical requirements for the successful long-term maintenance of epithelial cell cultures is to inhibit or eliminate the growth of contaminating fibroblasts because they adapt to the culture environment rapidly and subsequently overgrow epithelial cells. Although there are reports demonstrating that fibroblast contamination can be eliminated or reduced by collagenase treatment (20,22) or by sequential trypsinization (18), these methods were not successful in eliminating fibroblasts from the colonic epithelial cell culture. The colonic epithelial cells appeared to be as sensitive as fibroblasts to the collagenase and trypsin treatments. Furthermore, the colonic epithelial cells failed to divide in D-valine medium, although this medium had been used to inhibit the growth of fibroblasts without preventing proliferation of the epithelial cells of renal, lung, and mammary tissues (19,35). The utilization of D-valine, however, depends on the presence of D-amino acid oxidase, and the intestinal epithelium had been shown to be devoid of this enzyme activity (36).

The subculture of epithelial colonies by the penicylinder technique proved to be an appropriate method to solve the problem of fibroblast contamination. However, the epithelial cells became refractory to proliferation after being subcultured several times. Improvements of the culture medium may overcome this situation. Nevertheless, we have successfully subcultured one epithelial colony for six passages over a period of 4 months. The presence of tight junctions and desmosomes between the tightly packed squamous-shaped cells suggested that the subcultured cells were epithelial. In comparison with the cells of the primary cultures, the subcultured cells showed less microvilli on the surface, lacked specialized cell differentiation, and contained no antigenic determinant for anti-BBM antiserum. The search for a specific colonic epithelial cell marker is under way to establish the colonic origin of the subcultured epithelial cells.

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