EPITHELIOID CELL CULTURES FROM RAT SMALL INTESTINE

Characterization by Morphologic and Immunologic Criteria

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ABSTRACT

Rat small intestinal epithelial cell lines have been established in vitro and subcultured serially for periods up to 6 mo. These cells have an epithelioid morphology, grow as monolayers of closely opposed polygonal cells, and during the logarithmic phase of growth have a population doubling time of 19–22 h. Ultrastructural studies revealed the presence of microvilli, tight junctions, an extensive Golgi complex, and the presence of extracellular amorphous material similar in appearance to isolated basement membrane. These cells exhibit a number of features characteristic of normal cells in culture; namely, a normal rat diploid karyotype, strong density inhibition of growth, lack of growth in soft agar, and a low plating efficiency when seeded at low density. They did not produce tumors when injected in syngeneic animals. Immunochemical studies were performed to determine their origin using antisera prepared against rat small intestinal crypt cell plasma membrane, brush border membrane of villus cells and isolated sucrase-isomaltase complex. Antigenic determinants specific for small intestinal epithelial (crypt and villus) cells were demonstrated on the surface of the epithelioid cells, but they lacked immunological determinants specific for differentiated villus cells. An antiserum specifically staining extracellular material surrounding the cells cultured in vitro demonstrated cross-reactivity to basement membrane in rat intestinal frozen sections. It is concluded that the cultured epithelioid cells have features of undifferentiated small intestinal crypt cells.

KEY WORDS   small intestine · epithelioid cell cultures · cell-specific antigens

Small intestinal epithelial cells represent a rapidly renewing cell population characterized by a precise segregation between mitotically active cells, present in the crypt region, and mature differentiated villus cells, mitotically inactive. Intestinal cells have a rapid cell turnover, with a mean cell duration time of 2–3 d in most animals (5, 6, 8, 27). The differentiation of the mitotically active crypt cells is accompanied by dramatic changes in enzyme and transport activities and in cell morphology, including the appearance of a well-organized brush border at the luminal surface and a more columnar cell shape. It is of interest that, although intestinal crypt cells have one of the shortest cell cycle times in vivo (5), the occurrence

of malignancy in these cells is rare. In spite of numerous studies (1, 7, 24, 19, 48, 43, 49, 50), the factors regulating intestinal crypt cell proliferation and differentiation are still unknown. Indeed, the cellular complexity of the intestine, with the possibility of extensive interactions among different cell types (which has led to the suggestion of the existence of an intestinal "proliferon" [51]), is a major obstacle to the interpretation of in vivo studies. Thus, the ability to culture intestinal epithelial cells in vitro would be of major importance in studies aimed at investigating the factors involved in their proliferation and differentiation. Although intestinal epithelial cells can be isolated readily by a number of established techniques (37, 16, 45) and although they appear, shortly after isolation, to retain a satisfactory viability, their survival time in vitro is very limited, and extensive cell death occurs within a few hours after plating. This problem probably accounts for the lack of success in efforts to obtain short- or long-term cultures of intestinal epithelial cells. Henle and Deinhardt (18) reported the establishment of an epithelial cell line from fetal human intestine, but no attempt was made to determine the identity of these cells. More recently, it has been reported that pentagastrin selectively induced in vitro growth of epithelioid cells from adult rat duodenum (28); however, these cells were only characterized morphologically with no definitive evidence to indicate that they were indeed intestinal epithelial cells. We now report the establishment of epithelioid cell lines from germ-free adult rat small intestine. Extensive morphologic and immunochemical characterization strongly suggests that these cells originated from intestinal crypt cells and retain features of small intestinal epithelial cells.

MATERIALS AND METHODS

Materials

Male germ-free rats (Charles River CD (SD) GN rats), 18- to 24-d-old, obtained from Charles River Breeding Laboratories (Wilmington, Mass.) were used for all in vitro culture experiments. They were sacrificed within 24 h after arrival. Isolated crypt and villus cells and intestinal mucosal scrapings for immunization and for immunochemical staining, and for isolation of subcellular fractions, were prepared from Sprague-Dawley rats, CD strain, weighing 170-225 g of either sex.

Insulin (Iletin, 100 U/ml) was obtained from Eli Lilly and Co. (Indianapolis, Ind.). Collagenase (types I and III) and mitomycin C were obtained from Sigma Chem-

ical Co. (St. Louis, Mo.). Dulbecco's Modified Eagle Medium (DMEM),[1] fetal bovine serum (irradiated), chicken serum, penicillin-streptomycin mixture, and trypsin (2.5% in Hanks' balanced salt solution without calcium and magnesium) were obtained from Microbiological Associates (Walkersville, Md.).

Fluorescein-conjugated goat anti-rabbit immunoglobulins (molar F/P ratio 4.0-5.0) was obtained from Behring Diagnostics, American Hoechst Corp., (Somerville, N. J.); goat anti-rabbit IgG, normal rabbit serum, goat serum, peroxidase-anti-peroxidase soluble complex and concanavalin A (3× crystallized) were obtained from Miles Laboratories Inc., Miles Research Products (Elkhart, Ind.).

All other chemicals were obtained from commercial sources and were of the highest available purity.

Cell Culture Conditions and General Methods

Monolayer cultures were grown in plastic petri dishes (10 cm Diam, purchased from Lux Scientific Corp., Newbury Park, Calif.), at 37°C in an atmosphere of 90% air, 10% CO_2. The complete medium (10 ml/dish) routinely used consisted of DMEM (with 4.5 g/l of glucose), containing 5% fetal bovine serum, 10 μg/ml insulin, 50 U/ml penicillin, 50 μg/ml streptomycin, and 4 mM glutamine.

The cultures were refed twice weekly with 10 ml of fresh medium. The intestinal epithelioid cells, cell line No. 6 (IEC-6) were routinely subcultured every 5 d, as follows: the medium was removed, and the cell layer rinsed with 10 ml (for 100-mm dishes) of an EDTA solution (0.02% in phosphate-buffered saline, 0.01 M phosphate buffer, pH 7.2, 0.154 M NaCl [PBS]); the dishes were incubated at 37°C for 20-30 min, then 10 ml of complete medium was added and the cells were suspended into the medium by gentle scraping with a rubber-tipped spatula. The cell suspension was spun (at 900 rpm for 10 min at 4°C in a refrigerated centrifuge), and the cell pellet was resuspended in fresh medium and transferred to new dishes. The intestinal.epithelioid cells, cell line No. 14 (IEC-14) and rat intestinal fibroblasts (RIF) were subcultured as follows: Culture medium was removed and cell layers were rinsed with 10 ml of PBS containing 0.02% EDTA and 0.6 mg/ml trypsin and incubated at 37°C for 5 min; the cells were then suspended in 10 ml of complete medium by gentle shaking, spun as above, and finally resuspended in fresh medium and transferred to new dishes.

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[1] Abbreviations used in this paper: CFA, complete Freund's adjuvant; Con A, concanavalin A; DMEM, Dulbecco's modified Eagle medium; DTT, dithiothreitol; IEC-6, intestinal epithelioid cells, cell line No. 6; IEC-14, intestinal epithelioid cells, cell line No. 14; PAP, peroxidase-anti-peroxidase soluble complex; PBS, phosphate-buffered saline, 0.01 M phosphate buffer, pH 7.2, 0.154 M NaCl; RIF, rat intestinal fibroblasts.

All cell lines were stored in liquid nitrogen in 1-ml aliquots (2–4 × 10⁶ cells/ml in DMEM containing 7.5% dimethyl sulfoxide and 20% fetal bovine serum.

Cells were counted and sized with a Coulter ZBI counter (Coulter Electronics Inc., Hialeah, Fla.) equipped with channelyzer. (Coulter counter settings: lower threshold, 5; upper threshold, 100. Cell volumes were derived from frequency distribution curves.) Cell viability was estimated by the trypan blue exclusion method: cells were suspended in PBS containing 0.04% trypan blue for 1 min, then the percentage of viable cells (excluding the dye) was determined by hemocytometer counting. Cell cultures were routinely screened for mycoplasma contamination by tritiated thymidine labeling and autoradiography (39). Solutions were sterilized by Millipore filtration, using Millex filter units (Millipore Corp., Bedford, Mass.; 0.22-μm pores).

Growth rates were determined by plating cells in complete medium at 0.2–0.3 × 10⁵ cells/ml in 60-mm dishes (4 ml/dish). After 1 d the medium was changed, and cell counts were performed at 24-h intervals in duplicate or triplicate. Plating efficiencies were determined by plating 10²–10⁴ cells/100-mm dishes in 10 ml of complete medium. Before plating, cell suspensions were checked microscopically to exclude cell clumping. The medium was first changed after 1 wk. After the following week the dishes were rinsed twice with PBS, the cells were fixed with absolute methanol, and stained with Giemsa's (diluted 1:50). Colonies of at least 10 cells were counted. Cultures of IEC-6, RIF, and IEC-14 cells were tested for their ability to grow in agar suspension (33); 10³–10⁴ cells were seeded in 60-mm dishes, and incubated at 37°C for 2 wk. Colonies of at least five cells were examined and counted. RIF and baby hamster kidney fibroblasts were treated with mitomycin C (29) before being used as fibroblast feeder layers.

Collagen-coated dishes were prepared by treating plastic dishes with rat tail tendon collagen as described by Hauschka and Konigsberg (17).

For light microscope examination, cells were grown on glass cover slips, washed with PBS, fixed in absolute methanol, and stained with Giemsa's. For chromosome analysis, cells were exposed to colchicine (1 μg/ml) for 2 h at 37°C and then trypsinized, treated with a hypotonic (0.075 M) KCl solution, and fixed in a 3:1 mixture of cold absolute methanol and glacial acetic acid; several drops of the cell suspensions were placed on a glass slide, air dried, and stained with Giemsa's (1:50) buffered to pH 6.8 with citric-phosphate buffer.

Agglutinability by concanavalin A (Con A) was determined as described by Burger (4).

Electron Microscopy

Samples of cells for electron microscopy were grown on plastic dishes, fixed at room temperature for 1 h in 4% paraformaldehyde-2.5% glutaraldehyde buffered with 0.1 M sodium cacodylate at pH 7.4, rinsed, and

postfixed in 1.3% osmium tetroxide buffered with 0.1 M collidine at pH 7.4. The cells were then dehydrated and embedded in a mixture of Epon-Araldite, after which the cured resin and cells were separated from the plastic dish. Sections were cut on a Porter-Blum MT2 ultramicrotome (DuPont Instruments-Sorvall, DuPont Co., Wilmington, Del.), stained with uranyl acetate and lead citrate, and examined in a Philips 300 electron microscope.

Scanning Electron Microscopy

Cells grown on glass cover slips were extensively washed with PBS and fixed for 1 h at 4°C with 1.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. The cover slips were then stored in cacodylate buffer. The cells were dehydrated by passage through graded dilutions of ethanol. They were then dried in a Polaron E 3000 Critical Point Drying Apparatus (Polaron Instruments Inc., Line Lexington, Pa.), using CO_2 as the transition fluid. After drying, the cover slips were attached to an aluminum stub using conductive paint, and were coated with gold using a Polaron E 5000 sputter coater. The coated specimen was examined in an ISI-60 scanning electron microscope at 30 kV potential.

Establishment of Small Intestinal Cell Lines

CELL SUSPENSIONS: Intestinal epithelial cell suspensions were prepared by three different techniques: (a) by the Harrison-Webster method (16); (b) by the Weiser technique (45) (attempts to culture the isolated cells were made both with pooled villus and crypt cell fractions and with the fractions enriched in crypt cells alone); and (c) by collagenase digestion. This method involved flushing the small intestine with 1 mM dithiothreitol (DTT) in saline (100 ml) followed by 1% chicken serum in DMEM; the intestine was then filled with a solution of collagenase (type I, Sigma Chemical Co.), 0.1% in Moscona saline (31) containing 1% chicken serum. The intestine was then placed in Moscona's saline at 37°C for 10 min; the released cells were collected by centrifugation, washed twice with complete medium, and placed in culture.

CELL CULTURES: Successful cultures were established using small fragments of small intestine. The protocol which gave the most consistent results will be described in detail. The entire small intestines of two germ-free rats were thoroughly rinsed with 1 mM DTT in normal saline and then flushed with 100 ml of Moscona's saline containing 1% chicken serum. While kept in cold complete medium, they were cut into 4- to 5-mm fragments, which were then rinsed three to four times with complete medium and finally divided among 18 dishes (10 cm Diam, 10 ml of medium/dish) and placed in the incubator. Histological examination of the tissue fragments before incubation showed preservation

of a normal morphology. After 24 h, the medium was carefully removed and the intestinal fragments, resuspended in complete medium containing 100 μg/ml collagenase (type I, Sigma Chemical Co.), were transferred to new dishes and again incubated. After 24 h the medium and the tissue fragments were carefully removed, and fresh medium (containing 20 μg/ml collagenase) was added to the dishes which were left undisturbed in the incubator for 1 wk. The medium (always containing collagenase) was afterwards changed at biweekly intervals. After 3-4 wk, epithelioid colonies had developed in the majority of the dishes, and colonies of at least 10^3 cells showing no visible contamination with fibroblasts were encircled with stainless steel cylinders (1-cm Diam) kept in place with sterile silicon grease. The selected colonies were washed with EDTA in PBS, kept for 30 min at 37°C, and the cells were suspended in complete medium by gentle scraping with a rubber-tipped spatula. Each colony was separately transferred to a well of a Micro test II plate (Falcon); 20-30 colonies were usually selected for each preparation. The colonies which grew to confluence (2-3 × 10^4 cells/well) were transferred to 35-mm dishes in 2 ml of complete medium without collagenase. When confluent, cultures were transferred to 100-mm dishes. The first confluent culture in a 100-mm dish obtained from each original colony was defined "passage No. 1."

Immunochemical Techniques

PREPARATION AND CHARACTERIZATION OF ANTIGENS: Papain-solubilized sucrase-isomaltase complex was isolated as described by Cogoli et al. (9). The isolated enzymic complex had a specific activity of 14 sucrase U/mg and migrated as a single band on polyacrylamide gels. Brush border membranes were purified from intestinal mucosal scrapings (21) and monitored for purification by following the increase in sucrase specific activity, which was 40 to 50 times higher in the final microvillus membrane fraction than in the homogenate.

Crypt cell plasma membranes (from crypt cell fractions) were prepared according to Weiser et al. (46); the Na^+,K^+-ATPase specific activity in the final membrane fractions was 12-15 times higher than in the homogenate. Suspensions of IEC-6 cells were obtained from confluent dishes of 10th-15th passage cells. Cells were suspended in PBS after EDTA treatment, collected by centrifugation, and washed twice with PBS. They were finally resuspended in the same buffer at a concentration of 5-10 × 10^6 cells/ml.

IMMUNIZATION PROTOCOL: All antisera were produced in white male New Zealand rabbits (2 kg). The route of inoculation varied and depended in part on the sensitizing antigens.

(a) 2 mg of sucrase-isomaltase complex was suspended in 1 ml of PBS and combined with 9 ml of complete Freund's adjuvant (CFA). Two rabbits were inoculated at multiple subcutaneous sites. They were each boosted intramuscularly with 100 μg of enzyme on days 14 and 21 and bled on day 28.

(b) Brush border membranes (1.83 mg of protein in 1 ml of PBS) were similarly combined with 5 ml of CFA and injected subcutaneously at multiple sites. The intramuscular booster dose consisted of 1.4 mg of membrane protein suspended in Freund's incomplete adjuvant at days 14 and 21, with bleeding on day 38.

(c) Immunization with crypt cell plasma membrane and villus cell lateral-basal membranes followed a protocol identical to that for brush border membranes.

(d) Intact IEC-6 cells were injected intraperitoneally (1.5 × 10^7 cells/injection) twice a week for a month. The rabbit was bled 1 wk after the last injection.

PREPARATION OF ANTISERA FOR IMMUNOFLUORESCENCE STUDIES: Antisera to the various immunizing antigens were obtained from each animal by the following procedure: 20-50 ml of venous blood (ear vein) were collected and left to clot for 2 h at 20°C and then overnight at 4°C. The serum was collected and ammonium sulfate was added to 40% saturation. The precipitate was dissolved in PBS, exhaustively dialyzed against PBS, sterilized by Millipore filtration, and stored in 2-ml aliquots. In selected instances, the IgG fraction was further purified by diethylaminoethyl cellulose chromatography (13).

IMMUNOFLUORESCENT STAINING: Isolated intestinal crypt and villus cells, suspended in saline, were placed on glass slides, allowed to air dry, and fixed with ethanol (10 min at −40°C) followed by acetone (10 min at −40°C). Cells cultured in vitro on glass cover slips were washed two to three times with saline and fixed as described above. Cryostat sections (2-4 μm) of rat small intestine, liver, kidney, and heart were placed on glass slides, allowed to air dry, and used shortly after preparation.

The double antibody fluorescence technique was used for staining: the first antiserum (or normal rabbit serum of PBS serving as controls) at a 1:10 to 1:50 dilution was placed over the slides for 30 min at room temperature, followed by three 10-min washes with PBS. Thereafter, fluorescein-conjugated goat-anti-rabbit immunoglobulins (at a 1:25 dilution) were placed over the slides for 30 min at room temperature, followed again by three 10-min PBS washes. In some cases, cells and tissues were counterstained with Evans Blue (0.01% in PBS); in all cases, they were mounted in a 9:1 mixture of glycerol-PBS and examined under a Zeiss model 260 fluorescence microscope. Fluorescence was excited with the output of an Osram HBO 200 lamp filtered through a Zeiss BG_{12} interference filter. Samples were observed with a barrier filter 50. Pictures were recorded on Agfachrome 64 films with exposure times of 15-60 s. In some cases, the peroxidase-anti-peroxidase (PAP) staining technique (38) was used instead of immunofluorescent staining.

CHARACTERIZATION OF ANTISERA: ABSORPTION AND SPECIFICITY STUDIES: Antiserum to rat intestinal sucrase-isomaltase complex formed a single precipitin line with the purified antigen in agar gel diffusion. Negative controls consisted of papain-solubilized villus cell protein fractions devoid of sucrase activity. Isolated villus cells and small intestine, liver, kidney, and heart sections were evaluated by immunofluorescent staining to determine the specificity of the antiserum.

The antisera against crypt cell plasma membrane and brush border membranes, and the antiserum produced by i.p. injection of IEC-6 cells, were tested for specificity by immunofluorescent staining of cultured cells (RIF, IEC-6, IEC-14), isolated villus cells, and small intestine, liver, kidney, and heart frozen sections. The specificity of the antiserum against crypt cell plasma membrane was increased by sequential absorptions as follows: 1 ml of antiserum was treated five times with a suspension of liver cells prepared by the collagenase perfusion technique of Witters et al. (47); for each absorption, 2×10^7 liver cells were used; the cell suspension was kept at 37°C for 30 min with shaking, spun, and the absorbed serum was removed and then added to a fresh pellet of liver cells. The serum was absorbed further with kidney glomeruli, isolated as described by Quadracci and Striker (34), and then three times with suspensions of spleen cells, using 2×10^7 cells/absorption.

RESULTS

Establishment of Intestinal Cell Lines

Initial attempts to establish cultures of small intestinal epithelial cells involved the use of freshly isolated suspensions of crypt and villus cells obtained by a number of different techniques such as mechanical agitation (37), the use of citrate- or EDTA-containing buffers (16, 45), or brief collagenase digestion (see Materials and Methods). All of these techniques yielded intestinal cells (mostly in large clumps, but also as single cells) with varying degrees of viability. Collagenase appeared to give the greatest number of viable cells; in most cases this was >90%, based on trypan blue exclusion. Irrespective of the isolation technique, however, the intestinal epithelial cells quickly lost their viability in culture, and within 2-3 h most of the cells were found floating on the surface of the medium. In some experiments, a small fraction of the cells initially attached to the dishes and appeared to remain viable for a few days; in no instance, however, were viable cultures found after 1 wk.

To improve cell viability, various hormones were added to the culture medium (e.g., insulin, glucocorticoids, gastrin [28], etc.) and a number of manipulations of the culture conditions were also tried (e.g., collagen-coated dishes, fibroblast feeder layers) but these approaches were not successful. We, therefore, employed small fragments of intestine as the starting material, and this approach permitted us to obtain cultures containing distinct colonies of epithelial cells. The finally adopted protocol is the one described in detail under Materials and Methods.

Comments on the Steps Establishing the Epithelial Cell Lines

STARTING INTESTINAL FRAGMENTS: To limit the trauma to the epithelial cells, small fragments of intestine were used. When placed in culture, they tended to remain near the surface of the medium, apparently retaining good viability. Because a large amount of debris accumulated on the surface of the dishes in the first 24 h, the intestinal fragments were preincubated in standard medium overnight before transferring them to new dishes containing fresh medium and collagenase.

COLLAGENASE DIGESTION: While still suspended in the medium, the intestinal fragments were digested with collagenase for varying periods of time, after which they were discarded, and the cells that were released and loosely attached to the dish were maintained in culture. Different concentrations of collagenase (100-500 μg/ml) and different type I collagenase preparations were used with essentially identical results. It should be noted that when the collagenase treatment was performed for >48 h, only cells with a fibroblastic morphology were observed. However, fibroblastic contamination was limited when the tissue fragments were incubated with collagenase for 24 h, and therefore this period of digestion was selected. Finally, when collagenase was omitted, few cells were released from the intestinal fragments and final cell growth was either very limited or absent, unless the tissue fragments were left in culture for at least a week. Under the latter conditions, however, again only cells with a fibroblastic morphology were obtained.

ADDITION OF COLLAGENASE TO THE CULTURE MEDIUM: During the first 2 mo in culture, and until pure epithelioid colonies were selected with cloning cylinders, a small amount of collagenase (type I, Sigma Chemical Co., 20 μg/ml) was present in the culture medium. The presence of collagenase had a dramatic effect. In

its absence, fibroblastic colonies were mostly obtained; the few colonies with epithelioid morphology were much smaller than when collagenase was present and the cells were large and pleomorphic. When collagenase was present in the medium, the rate of success in obtaining epithelioid type colonies was very variable from culture to culture; some dishes had no colonies whereas others had a confluent monolayer with very limited fibroblastic contamination. However, to date, every preparation, using the procedure outlined and starting with the intestinal segments of two rats, has been successful in yielding pure cultures of epithelial cells.

SELECTION OF EPITHELIOID COLONIES: In most cases, the primary cultures consisted of separate discrete colonies with epithelioid or fibroblastic morphology in different proportions. In a limited number of cases, we obtained confluent cultures of epithelioid cells with no more than 10% fibroblastic contamination, based on morphologic criteria. When such cultures were subcultured without selection of the epithelial cells, they could be carried through a few passages without significant morphological change. After four to five passages, however, the distinct epitheliod morphology was lost, and different cell types appeared. Selective detachment of fibroblasts by brief EDTA treatment (32) was attempted, and was somewhat successful in reducing contamination by fibroblastic-appearing cells, but did not solve the problem. On the contrary, when single epithelioid colonies were selected with cloning cylinders from the primary cultures and serially propagated, the cell lines were morphologically stable for the whole period of their lifespan in culture (40–50 passages). A cell line obtained in this way, subsequently referred to as IEC-6, was the one selected for morphological and immunochemical characterization.

OTHER CELL LINES: In addition to the epithelioid cell lines, cultures of fibroblasts were easily obtained. One confluent dish was randomly chosen and serially propagated; this cell line was called RIF. From a dish containing mostly fibroblasts, which had been in culture for 3 mo, cells with epithelioid morphology suddenly appeared and showed rapid growth. Isolated colonies were selected and serially propagated; they were termed IEC-14. Both RIF and IEC-14 cell lines have been only partly characterized, but their properties were of great interest in comparison to those of the IEC-6 cells.

Morphologic Characterization

The IEC-6 cell line consists of a homogeneous population of epithelial-like cells with large, oval nuclei and growing as tight colonies of polygonal, closely opposed cells (Figs. 1 and 2). RIF cells (Fig. 1) are morphologically quite different from IEC-6 cells; the former, when confluent, form tightly packed monolayers of parallel arrays of spindle-shaped cells. IEC-14 cells are also epithe-
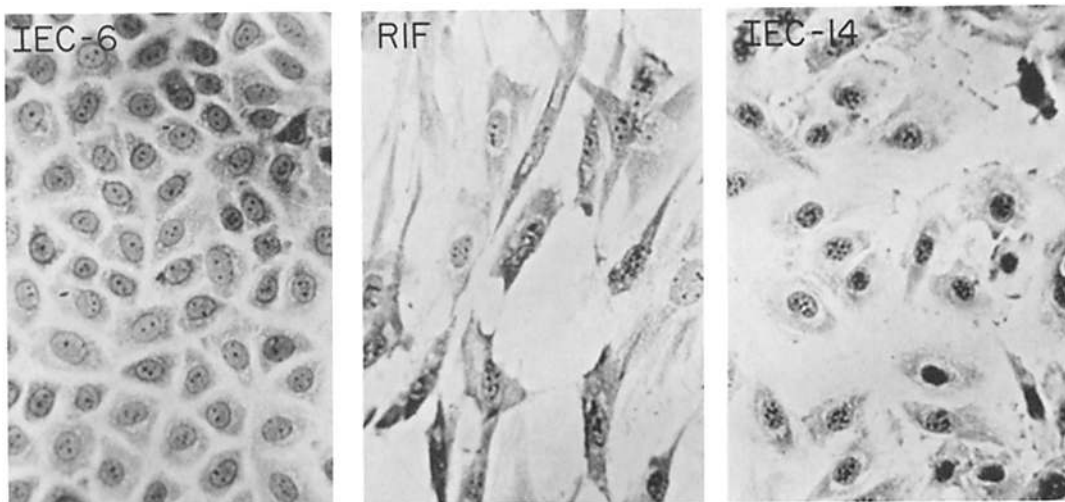

FIGURE 1 Morphology of epithelioid and fibroblastic cell lines derived from rat small intestine. Giemsa stained. × 250.
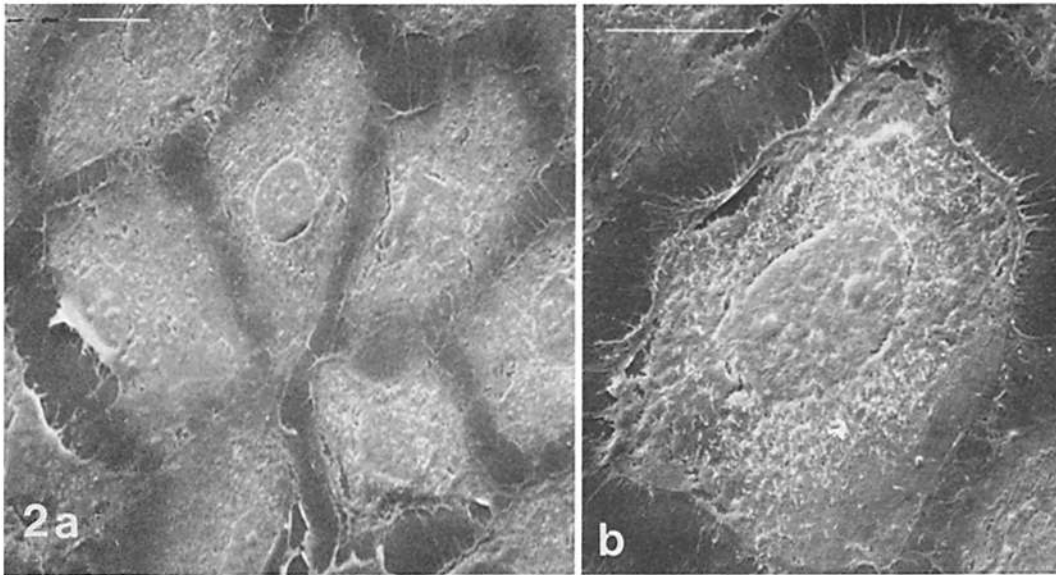
FIGURE 2 Scanning electron micrographs of IEC-6 cells, illustrating their epithelioid morphology and the presence of numerous microvilli on their surface, concentrated in a perinuclear region. Frequent pseudopods extend from the cell borders to the surface of the culture dish and to neighboring cells, establishing frequent cell-cell contacts. Bar, 10 μm. (a) × 1000. (b) × 2000.

lial-like (Fig. 1), but they have a clearly different appearance than the IEC-6 cells.

The morphologic characteristics of the IEC-6 cells were examined by scanning electron microscopy. The epithelioid appearance of these cells can be clearly seen in Fig. 2. Long, slender microvilli (Fig. 3a and b) were numerous over the cell surface. However, these tended to be localized to the perinuclear region; they were not observed in the supranuclear region, and were very sparse at the cell margins, which had a relatively smooth surface (Fig. 3a). As seen in Figs. 2b and 3a, frequent pseudopodia seem to extend from the cell to the growth surface and to surrounding cells, often forming intercellular contacts.

Ultrastructural Characteristics of IEC-6 Cells

The flattened cells in culture retained a number of cytological features typical of intestinal crypt cells. The Golgi apparatus was well-developed and a number of membrane-limited granules were present in the cytoplasm (Figs. 4 and 5a-c). Microvilli were often prominent on one surface or pole of the cell, which also included the centriole, Golgi complex, and a junctional complex (Fig. 5a and b). Bundles of microfilaments were prominent throughout the cells, but especially near the cell

surface and at sites of cell-cell contact. Ribosomes, both free and associated with the endoplasmic reticulum, were abundant. The nuclear chromatin was generally dispersed, with a subnuclear membrane condensation common and numerous nuclear pores apparent. The mitochondria had well-defined cristae. The extracellular matrix consisted of microfilamentous material, semiflattened globular aggregates, and cell surface coats resembling basement membrane (Fig. 5c). Characteristic 67-nm cross-striated collagen fibrils were not observed. The basement membrane-like material was present as a discontinuous surface coat and often merged from the surface of thin cellular processes with the rest of the extracellular matrix.

Karyotyping

The IEC-6 cells possess a normal rat karyotype, even after 6 mo in culture. The modal number, 42, corresponds to the diploid state. No metaphases with a higher chromosome number were observed; a small percentage of cells showed fewer than 42 chromsomes, presumably because of loss of chromosomes during fixation.

Con A Agglutination

The IEC-6 cells were agglutinated by Con A only at relatively high concentrations ($\geq$100 μg/
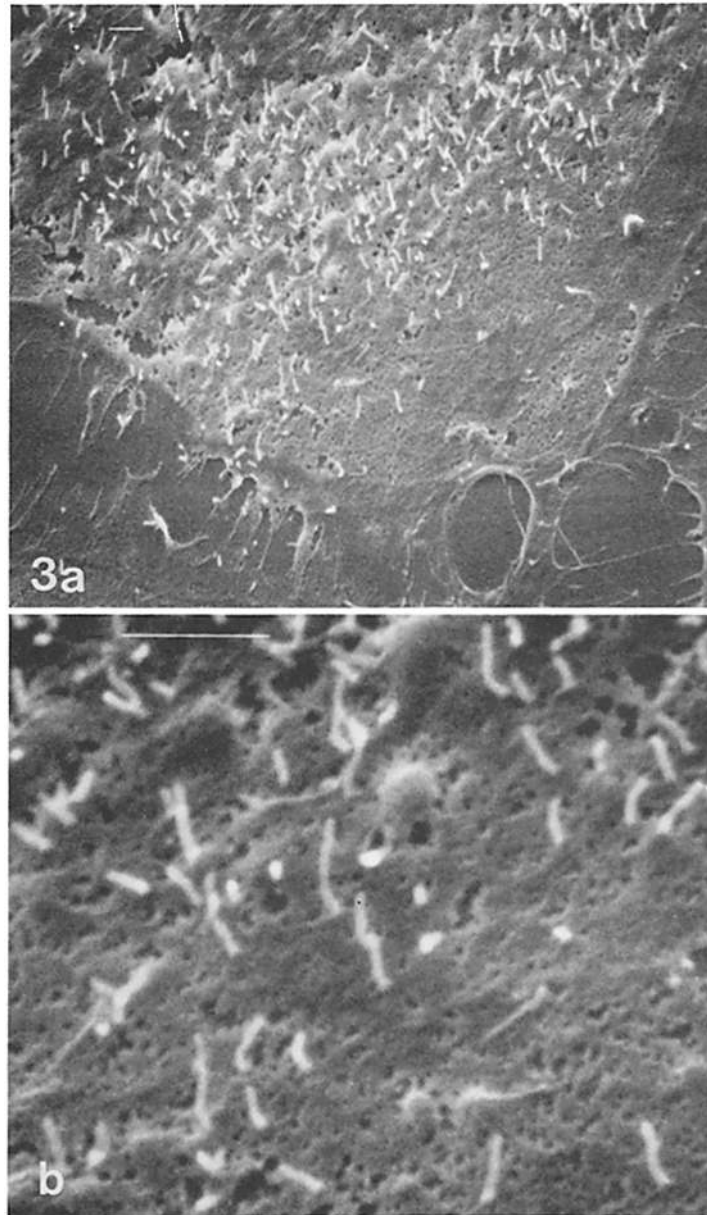
FIGURE 3 (a) A detail of the same cell as in Fig. 2b, illustrating the uneven distribution of the microvilli and the relatively smooth appearance of the cell surface close to the cell borders. Frequent pseudopods are apparent. Bar, 1 μm. × 5,000. (b) At higher magnification the thin, slender microvilli can be seen protruding from the surface of the cell facing the medium. Bar, 1 μm. × 20,000.

ml). RIF cells were partially agglutinated but only at even higher Con A concentrations ($\geq$200 μg/ ml); IEC-14 cells were strongly agglutinated even at the lowest Con A concentration tested (20 μg/ ml). In all cases, agglutination was prevented by the addition of α-methyl-mannopyranoside, a competitive inhibitor of con A binding (4).

Growth Characteristics of IEC-6, RIF, and IEC-14 Cells

The IEC-6 cells, when grown in presence of >2% fetal calf serum, were very tightly adherent to the culture plate. This phenomenon has previously been observed in the case of cultured epithe-
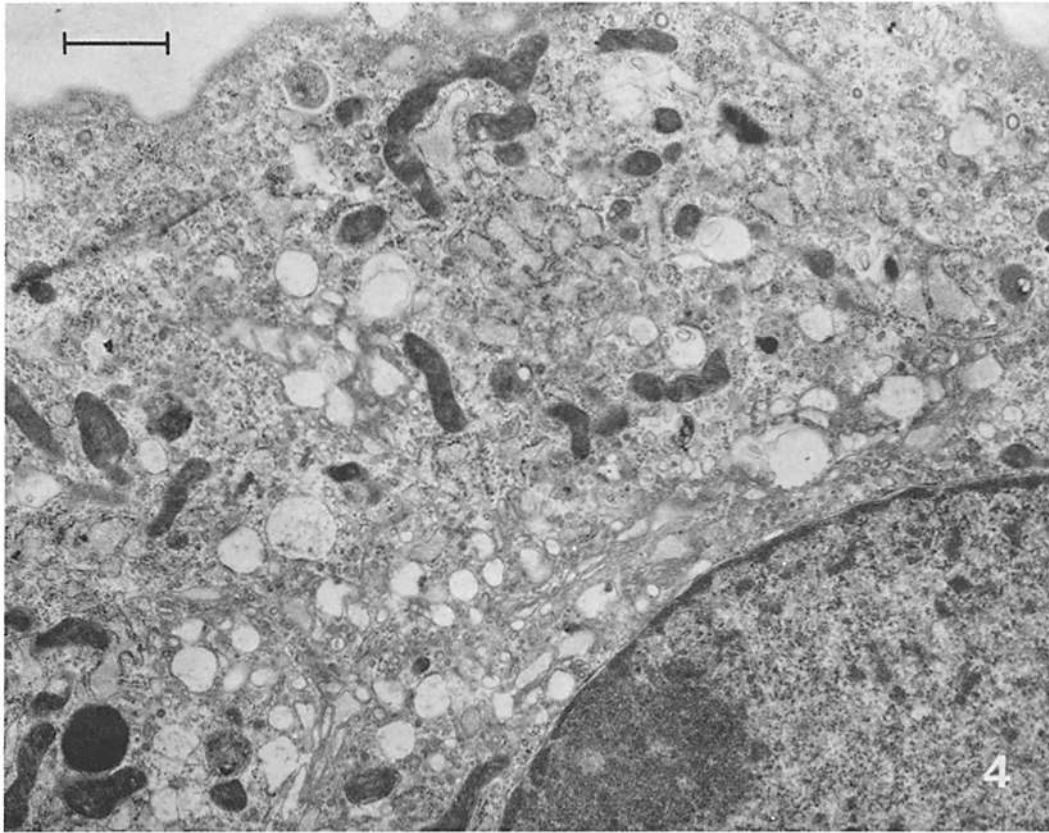
FIGURE 4   An overview of a typical epithelioid cell (IEC-6). Bar, 1 μm. × 13,700.

lial cells, and is the basis of an established technique for their purification (32). All three cell lines (IEC-6, IEC-14, and RIF) could be stored in liquid nitrogen, and when recovered were perfectly viable with no observed changes in their morphological and immunological characteristics.

The IEC-6 cells could be serially passaged (1:2) 30–40 times, after which time the rate of growth considerably diminished. In contrast, RIF and IEC-14 cells have been subcultured >100 times without apparent change in their growth rate.

IEC-6 cells, from early passage, appeared quite homogeneous in size, having a mean vol of 600 μm³ at confluence. Cell growth was rapid in low density cultures, and during the logarithmic phase of growth (Fig. 6) a population doubling time of ~20 h was observed. At higher cell density, however, growth was much slower.

The saturation density of the IEC-6 cells remained constant with subculture (Table I) but tended to decrease slightly in older cultures. No viable cells were ever detected floating in the

FIGURE 5   (a) Cultured cell (IEC-6) containing a well-developed cilium, dense granules, and a band of microfilaments. Numerous nuclear pores are present on the obliquely sectioned face of the nucleus. Bar, 1 μm. × 13,700. (b) The apparent "apical" surface of two IEC-6 cells in culture with microvilli present on both cells. A junctional complex is present at the site of cell-cell contact. Dense granules and microfilaments are present. Bar, 1 μm. × 12,740. (c) Cellular processes and a portion of a cell body from a number of IEC-6 cells. Basement membrane-like material covers the surface of a process and a region of the cell body (arrows). The extracellular matrix contains fibrillar material <10 nm in diameter. No cross-striated fibrils are apparent. Dense globular material, ~30 nm in diameter, is distributed in a number of patches. Dense granules and microfilaments are apparent. Bar, 1 μm. × 19,180.

Daily
medium change

Medium
unchanged

CELLS / DISH × 10^{-5}
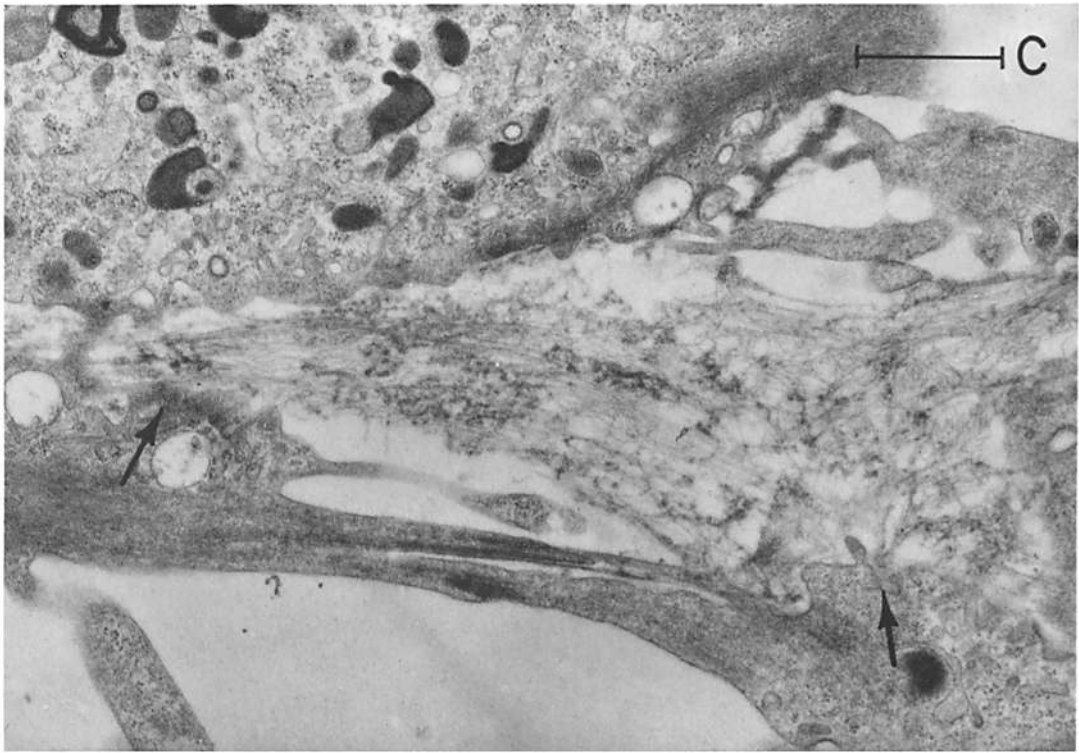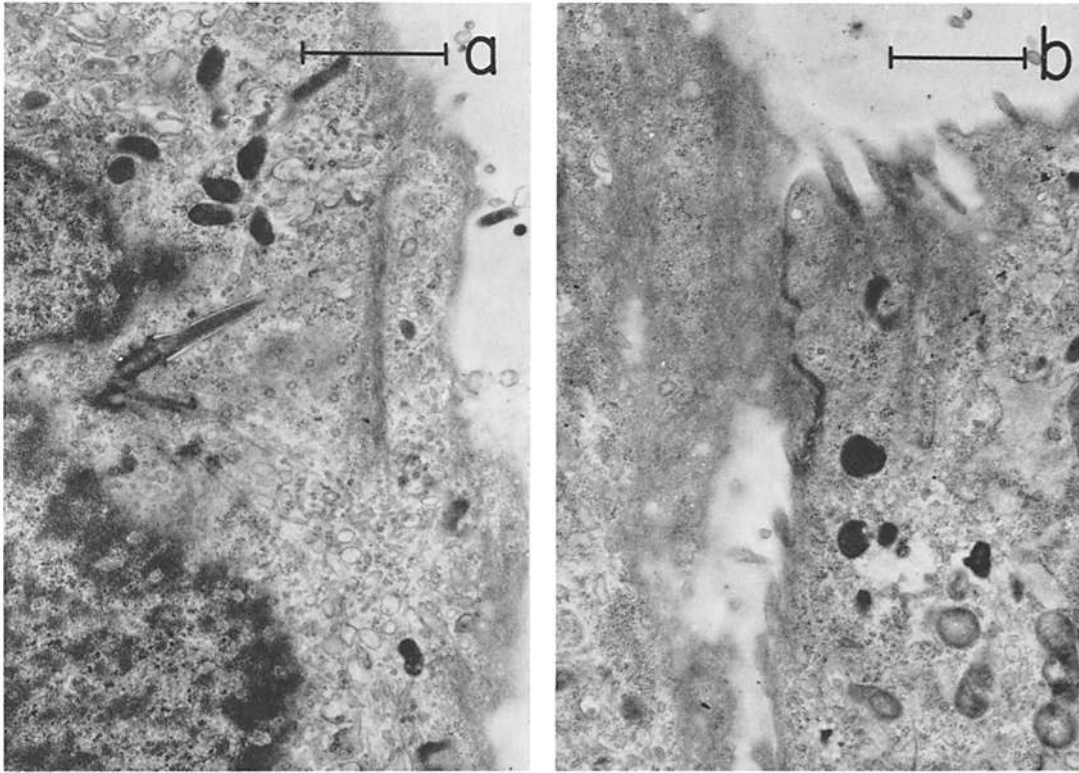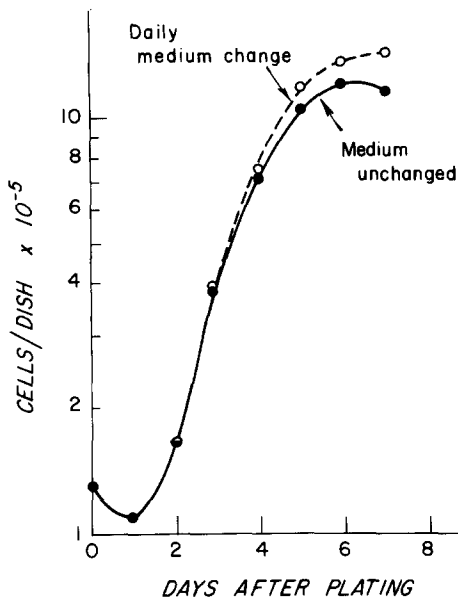
DAYS AFTER PLATING

FIGURE 6 Growth curves of IEC-6 cells. The culture medium was either unchanged after plating (continuous line, closed circles) or changed daily (broken line, open circles).

medium; in contrast, dense cultures of IEC-14 cells had many free-floating cells, indicating continued growth even after reaching an apparently constant saturation density (Table I). Growth in suspension (agar) was not observed with IEC-6 and RIF cells; however, the IEC-14 cells easily formed colonies in soft agar (Table I).

When seeded at very low densities (10^3–10^4 cells/100-mm dish), IEC-6 and RIF cells showed poor growth, and their plating efficiency under these conditions was very low (Table I). IEC-14 cells easily formed large colonies when plated at densities as low as 100 cells/100-mm dish (Table I).

All three cell lines, when injected subcutaneously into 21-d-old Sprague-Dawley rats, CD strain (3–4 × 10^6 cells/rat), failed to produce tumors up to 6 mo of observation.

Immunologic Observations

Because cell membrane surfaces appear to contain specific antigens (2, 26, 40), it should be possible to develop antisera to identify specific cell types in culture. For the characterization of the IEC-6 cells, rabbits were immunized with purified subcellular fractions of intestinal epithelial cells, isolated crypt cells, and whole IEC-6 cells. The

antisera thus obtained were evaluated for specificity by staining frozen sections of small intestine, heart, kidney, and liver before and after absorption with suspensions of isolated rat liver and/or spleen cells. By the use of these different sera, the presence of specific antigenic sites on the IEC-6 cells was evaluated. The preparation of the different sera is described under Materials and Methods.

ANTI-CRYPT CELL PLASMA MEMBRANE ANTISERUM: The unabsorbed antiserum strongly stained the intestinal sections (Fig. 7a). The fluorescence was strongest at the luminal membrane of crypt and villus cells, but it was not confined to the epithelial cells, only the muscularis mucosa being totally unreactive. Heart, kidney, and liver sections were also fairly strongly stained, but nonspecifically. Therefore this antiserum was extensively absorbed with isolated liver cells. The absorbed antiserum now showed different prop-

TABLE I

Characteristics of Cell Lines Established from Rat Small Intestine

Morphology	IEC-6 epithelioid	RIF fibroblastic	IEC-14 epithelioid
Saturation density (cells × 10^4/cm²)	4.0	12.3	5.7
Plating efficiency (%)*	2.3	2.4	17.5
Efficiency of colony formation in soft agar (%)‡	0	0	12
Cell doubling time (hours)§	20–24	25	18
Con A agglutination concentration‖			
20 μg/ml	−	−	+ +
40 μg/ml	+	−	+ + +
100 μg/ml	+ +	+	+ + + +

* IEC-6 and RIF lines: 10^3 cells plated/100-mm dish; IEC-14 line: 100 cells plated/100-mm dish. Cultures were fixed after 2 wk from plating and stained with Giemsa's. Colonies of at least 10 cells were counted.
‡ IEC-6 and RIF lines: 10^4 cells plated in 4 ml medium/60-mm dish; IEC-14: 10^3 cells plated/60-mm dish. After 2 wk in culture, colonies of at least five cells were examined and counted.
§ Determined during the logarithmic phase of growth. The complete medium contained 10 μg/ml insulin and 5% fetal calf serum.
‖ Agglutination was scored on a scale from − (no agglutination) to + + + + (no single cells detected). In all cases, agglutination was prevented by the addition of 100 mM α-methyl-mannopyranoside.

erties. In intestinal sections, it only stained the luminal membrane of crypt and villus epithelial cells (Fig. 7b–e); no fluorescence was detected in the cytoplasm and no other cell types present in the intestine appeared to cross-react with the antiserum. The absorbed serum no longer stained liver, kidney, and heart sections, thus demonstrating specificity to intestinal crypt and villus cells. When evaluated with the cells in culture, it specifically stained the IEC-6 cells, the fluorescence being mostly confined to a perinuclear region (Fig. 7g); this was also evident by the PAP-staining technique at higher magnification (Fig. 7h). When the IEC-6 cells were briefly treated with EDTA and fixed as round cells, fluorescence staining was confined to the cell periphery (Fig. 7i); treatment of the cells with trypsin (30 min at 37°C) abolished staining (Fig. 7k), demonstrating that the antigenic sites common to crypt cell plasma membrane are present on the surface of the cultured epithelioid cells. Absorption of the antiserum with isolated crypt cell suspensions totally abolished immunofluorescent staining of both intestinal frozen sections (Fig. 7f) and IEC-6 cells (Fig. 7l). It appears, therefore, that specific antigenic sites are present both on intestinal crypt and villus cells and on the IEC-6 cells in culture.

ANTI-VILLUS CELL LATERAL-BASAL MEMBRANE ANTISERUM: This antiserum, obtained by immunizing rabbits with partially purified lateral-basal membranes, appeared rather nonspecific when tested on intestinal sections. It did, however, specifically react with the surface (lateral-basal and brush border) membrane of isolated villus cells (46), no fluorescence being detected in the cytoplasm. It stained the IEC-6 cells, but to date no attempt has been made to increase its specificity by appropriate absorptions.

ANTI-BRUSH BORDER MEMBRANE ANTISERUM: The brush border membrane of differentiated villus cells can be isolated by established techniques (21) and is characterized by enzymatic activities specific for mature intestinal villus cells. Immunization with brush border membranes produced an antiserum of unusual specificity. Without absorption and when tested on whole intestinal sections, it reacted only with the luminal membrane of villus cells (Fig. 8a). Crypts were stained only in the upper portion, corresponding approximately with the region where cell differentiation appears to occur (35) (Fig. 8b). Absorption of this antiserum with purified brush border membranes completely abolished fluorescent

staining (not shown). This antiserum, reacting primarily with mature, differentiated intestinal epithelial cells, did not stain the IEC-6 cells.

ANTI-SUCRASE-ISOMALTASE COMPLEX: The sucrase-isomaltase complex consists of a pair of digestive disaccharidases localized in the brush border membrane of the enterocytes (14). A catalytically inactive sucrase antigen has been detected on the surface of small intestinal crypt cells by immunological techniques (10, 11, 36) and is believed to represent an enzyme precursor. An antiserum prepared against papain-solubilized sucrase-isomaltase complex confirmed the presence of cross-reactive antigens on intestinal crypt cells (Fig. 8c); absorption with purified antigen completely abolished staining (Fig. 8d). This serum, however, did not react with the IEC-6 cells. It should be noted that the serum prepared against purified brush border membranes cross-reacted only very weakly with solublized sucrase-isomaltase complex, as judged by double-diffusion in agar, explaining the different staining pattern of these two sera on intestinal sections.

ANTI-IEC-6 CELL ANTISERUM: Intraperitoneal injection of IEC-6 cells into rabbits produced an antiserum which, when tested on the same cells in culture, specifically stained extracellular material present between cells (Fig. 9a and b). When tested on intestinal sections, the fluorescence was confined to the basal layer of the epithelial cells and to structures present in the lamina propria (Fig. 9c). The immunofluorescent staining of these structures was much more prominent in the crypt than in the villus region. When the IEC-6 cells were cultured in the presence of collagenase (type III, Sigma Chemcial Co., 20 μg/ml), no immunofluorescent staining was detected with this antiserum (Fig. 9d). Kidney sections were also specifically stained, but the exact localization of the reactive regions was not determined.

DISCUSSION

The intestinal epithelial cells represent a cell population characterized by continuous growth and differentiation in vivo (5, 6). Cell replication is limited to the lower two-thirds of the crypts and is balanced by continuous loss of differentiated cells at the villus tip (8, 35). Intestinal crypt cells have been compared to fetal or tumor cells because of their rapid growth in vivo (33, 44). Because crypt cells can be readily isolated by a number of techniques, yielding cell suspensions retaining a
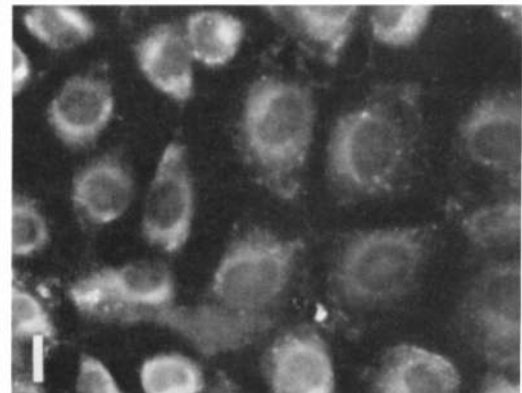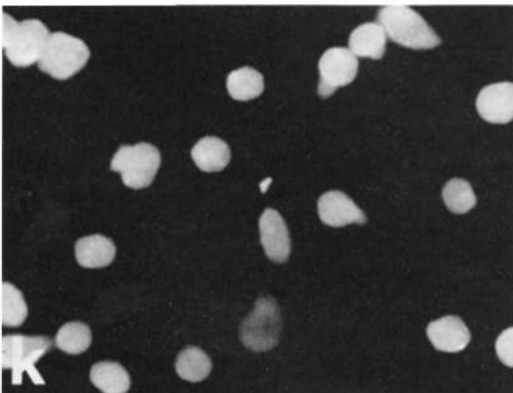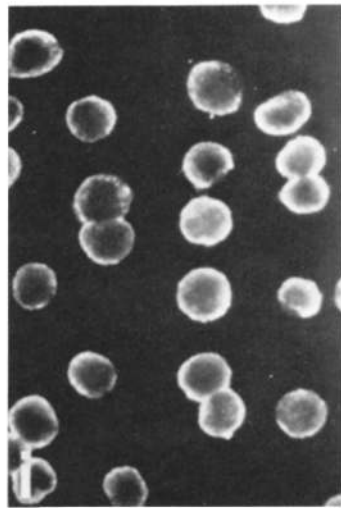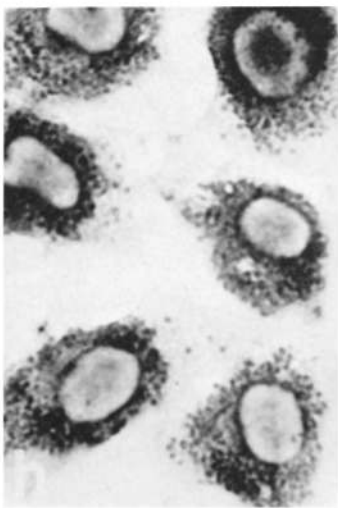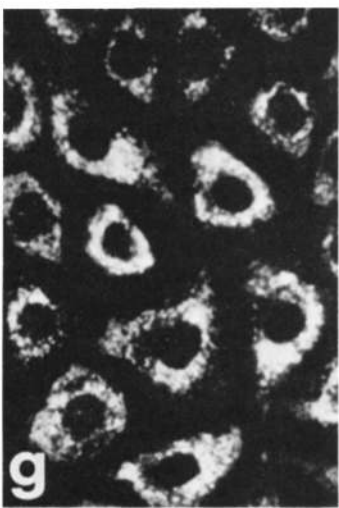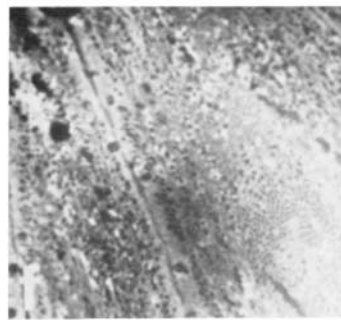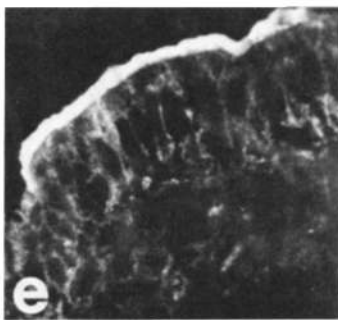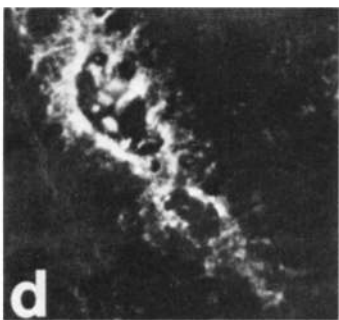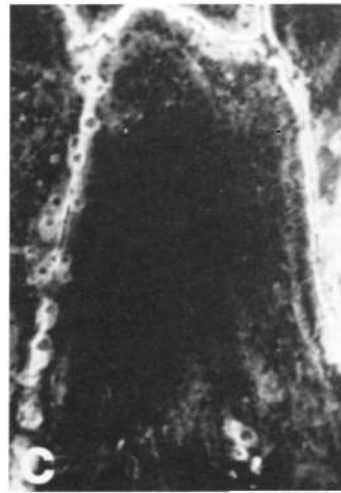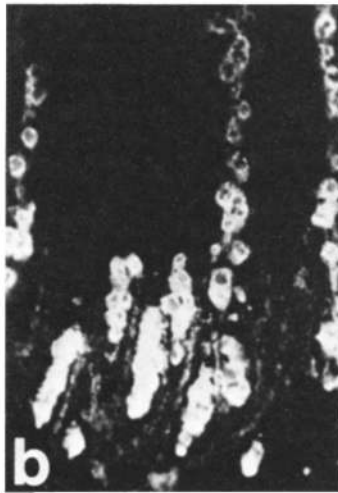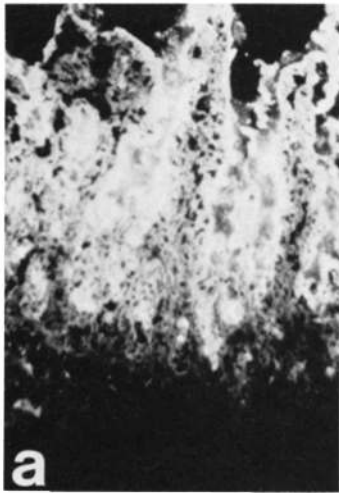
good viability (16, 37, 45), the problem of their culture in vitro seems, in principle, limited to the choice of a proper culture medium. Unfortunately, isolated cell suspensions of intestinal epithelial cells, irrespective of the method used for their isolation, undergo rapid autolysis in vitro, precluding any significant study of their properties in short or long-term culture. In contrast, organ cultures of small intestinal fragments or explants have been shown to remain well-preserved for up to 24 and 48 h (42).

In the present work, our initial approach was that used in organ culture. Intact fragments of small intestine, kept floating in the culture medium, were first exposed to collagenase; it is possible that under these conditions the epithelial cells can more gradually adapt to the in vitro conditions. In spite of these precautions, few of the cells released by collagenase were able to survive and establish colonies in vitro. The conditions we have chosen for primary cultures have, therefore, approached cloning conditions, which have been suggested to be essential for the establishment of monolayer cultures of other differentiated cell types (22). Two additional conditions proved critical for the establishment of intestinal epithelial cell cultures. Firstly, the addition of a relatively small amount of collagenase to the culture medium during primary culture was essential. It is not possible at present to explain this requirement, because commercial preparation of collagenase are contaminated with other enzymes and proteins (15); in particular, the crude type I collagenase used may contain a proteolytic mitogen. It is of interest, however, that addition to the culture medium of cis-4-hydroxy-L-proline (23), a proline analogue known to interfere with collagen

biosynthesis by preventing triple helix formation, has been effective in removal of contamination of fibroblasts from mixed cell cultures, without affecting growth of epithelial cells.

Secondly, the selection of colonies of epithelial cells from the primary cultures, uncontaminated by different cell types, appeared essential to obtain morphologically stable, homogeneous cultures.

This approach presented two major problems. Because a relatively long time (1-2 mo) was required for the establishment of sizable colonies of epithelial cells, these cells could possibly be the result of spontaneous transformation of cells originally released from the intestinal fragment, but themselves incapable of growth in vitro. It was therefore important to demonstrate that the cultured epithelial cells had properties of "normal" and not transformed cells.

The second problem arose from the use of intact fragments of small intestine as source of cells subsequently grown as monolayer cutures; the epithelial cells could then possibly be derived from any of the many cell types present in the small intestine. It was therefore essential to study thoroughly the IEC-6 cells in order to identify and characterize them as intestinal epithelial cells. In this regard, it is unfortunate that most of the known properties of intestinal epithelial cells, described in vivo, apply to the mature, differentiated villus cells; no specific marker is known for the undifferentiated, proliferating stem cells present in the crypts.

The IEC-6 cells exhibit a number of characteristics of normal cells grown in vitro (12, 20). Thus, (a) they have a normal rat diploid karyotype, without evidence of genetic heterogeneity; (b)

FIGURE 7 Immunochemical staining of small intestinal frozen sections (a-f) and cultured IEC-6 cells (g-l) with anti-crypt cells' plasma membrane antiserum. (a) Unabsorbed antiserum (× 200). (b-e) After absorption of antiserum with liver cell suspensions, immunofluorescent staining is limited to the epithelial cells both in the crypts (b) and on the villus (c). At higher magnification, it is apparent that the luminal membrane of both crypt (d) and villus (e) cells is specifically stained. (b and c) × 500. (d and e) × 800. (f) Absorption of the antiserum with isolated crypt cell suspensions totally abolished the immunofluorescent staining (× 500). (g) Immunofluorescent staining of a confluent monolayer of IEC-6 cells with the antiserum absorbed with liver cell suspensions. Fluorescence is confined to a perinuclear region, with the supranuclear region and cell borders negative (× 800). (h) The uneven distribution of the specific antigens and the patchy appearance of the stain is also apparent after immunochemical staining by the PAP technique (× 1,200). (i) Peripheral staining of IEC-6 cells pretreated with EDTA before fixation (× 800). (k) Trypsin treatment of the IEC-6 cells abolished fluorescent staining (× 1,200). (l) No staining of IEC-6 cells was observed after absorption of the antiserum with isolated crypt cell suspensions (× 800). (f, i, k, and l) Counterstained with Evans blue; (h) lightly counterstained with hematoxylin; (a-e and g) not counterstained.
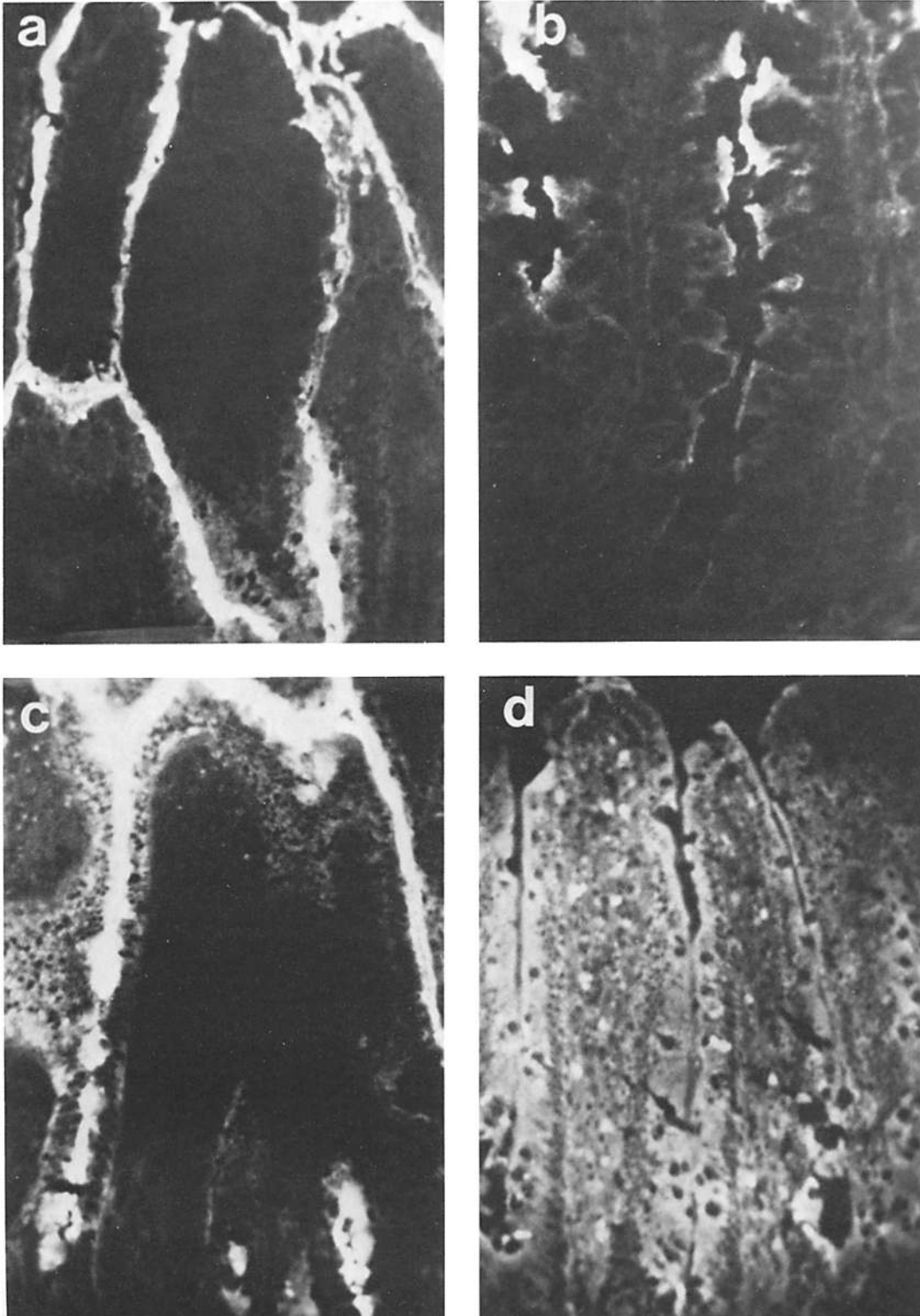
FIGURE 8 (a and b) Immunofluorescent staining of small intestinal frozen sections with anti-brush-border membrane antiserum. (a) Fluorescence is confined to the luminal membrane of the epithelial cells lining the villi; (b) in the crypts, only the upper portion (top of the figure) is stained. (a) × 700. (b) × 1,100 (not counterstained). (c) Immunofluorescent staining of intestinal section with an antiserum specific for sucrase-isomaltase complex; fluorescence is present both on the villi and in the crypts (bottom of the figure); absorption with purified antigen completely abolished staining (d). (c) × 700 (not counter-stained). (d) × 700 (counterstained with Evans blue).
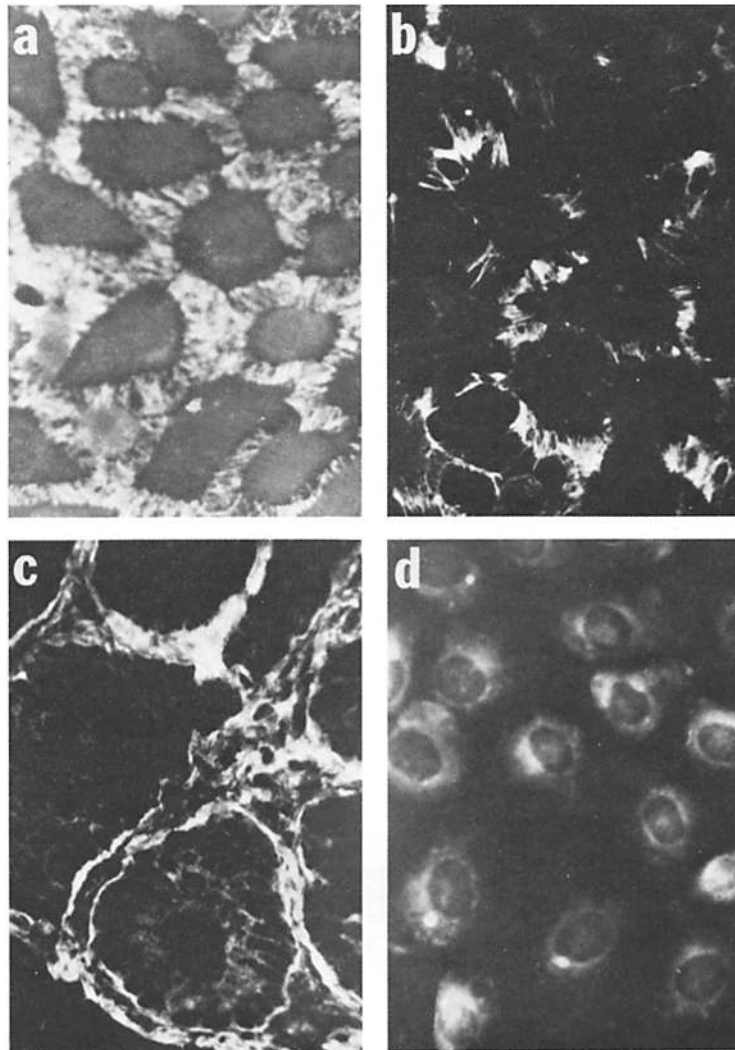
FIGURE 9 Immunofluorescent staining of intestinal frozen section and monolayers of IEC-6 cells with the antiserum prepared by intraperitoneal injection of IEC-6 cell suspensions. (*a*) Confluent monolayer of IEC-6 cells: fluorescence is confined to regions of cell-cell contact; (*b*) subconfluent monolayer of IEC-6 cells illustrating the patchy appearance of fluorescence, corresponding to cell projections; (*c*) intestinal crypt stained in correspondence with the basal side of epithelial cells and in the lamina propria; and (*d*) type III collagenase digestion of IEC-6 cells monolayer completely abolished fluorescence. (*a*, *b*, and *d*) × 800; (*c*) × 500. (*a* and *d*) Counterstained with Evans blue; (*b* and *c*) not counterstained.

they have a finite lifespan in vitro; (*c*) they have a fairly constant saturation density (Table I); a daily change of culture medium (Fig. 6) or an increase in serum concentration (not shown) produced only minor and not reproducible changes in the saturation density; (*d*) no colony formation was observed in soft agar (Table I), nor were tumors produced after injection in syngeneic animals. These results seem to demonstrate convincingly

that the IEC-6 cells have characteristics of nontransformed cells.

Podolsky and Weiser (33) and Weiser (44) have reported that intestinal crypt and fetal cells, but not villus cells, are agglutinated by Con A (33, 44). The IEC-6 cells agglutinated in the presence of Con A but only at relatively high lectin concentrations (Table I). Although the latter finding is not strong evidence that these cells are crypt cells,

it further suggests that they are not transformed cells. Virus-transformed and tumor cells have been shown to be readily agglutinated at much lower Con A concentrations (3).

A number of different approaches were used for the characterization of the epithelioid cells grown in vitro, each one providing some evidence for their origin from intestinal crypt cells.

Morphologically, the IEC-6 cells are clearly epithelioid (Figs. 1 and 2); SEM examination revealed the presence of numerous, thin microvilli concentrated in a perinuclear region (Figs. 2 and 3); both the cell surface covering the nuclear regions and the regions close to the cell periphery were almost devoid of microvilli, suggesting an in-plane polarization of the epithelioid cells. Ultrastructurally, a number of characteristics typical of crypt cells could be recognized (Figs. 4 and 5), such as tight junctions linking adjacent cells, numerous mitochondria, a well-developed endoplasmic reticulum, an extensive Golgi complex (suggestive of active secretory activity), and cytoplasmic dense bodies similar in appearance to the ones described as characteristic for intestinal crypt stem cells (41).

Although these morphologic and ultrastructural characteristics are common to many cells present in the small intestine, they do not conform to the structure of specialized cells such as intestinal Goblet and Paneth cells. Also, Weibel-Palade bodies, characteristic of endothelial cells both in vivo and in vitro (25), were not observed.

Further characterization of the IEC-6 cells has been directed towards the demonstration of the presence of cell- or tissue-specific antigens on their surface membrane. This approach has been fruitful in the characterization of different subpopulations of lymphocytes and has often provided important information in determining the identity of cells culture in vitro (2, 26, 40). We therefore prepared a number of different antisera against different membrane fractions of small intestinal crypt and villus cells, and also against intact IEC-6 cells. Three of these antisera have been particularly useful in the characterization of the IEC-6 cells. The antiserum raised against crypt cell plasma membrane, after absorption with isolated liver cells, has been demonstrated to recognize antigens specific for intestinal epithelial cells, because: (a) intestinal crypt and villus cells were specifically stained at the luminal membrane (Fig. 7b–e), no other cells or structures present in intestinal sections being reactive; (b) further ab-

sorption of the antiserum with kidney glomeruli and spleen cells did not abolish staining; (c) liver, kidney, and heart sections were totally negative when tested with the absorbed serum; (d) absorption with isolated crypt cell suspensions totally abolished the immunofluorescent staining. Positive staining of the cultured epithelioid cells with the absorbed antiserum has demonstrated that these cells express antigens specific for intestinal epithelial cells: the localization of the fluorescence in a perinuclear region of the IEC-6 cells, where microvilli were most numerous as seen by SEM (Fig. 3), suggests that the specific antigens may be present on the surface of the microvilli.

In contrast, the cultured epithelioid cells were not stained by the antiserum prepared against purified brush border membranes, apparently specific for differentiated intestinal villus cells (Fig. 8a and b), and by an antiserum specific for the sucrase isomaltase complex. These results therefore suggest that the cultured IEC-6 cells are derived from the intestinal crypt cells, and do not express differentiation-specific antigens. The antiserum raised by intraperitoneal injection of suspensions of IEC-6 cells showed a somewhat surprising specificity. This antiserum appeared to react primarily with material present extracellularly in monolayers of IEC-6 cells (corresponding with areas of cell-cell contact) and was totally unreactive against the cell surface membranes (Fig. 9a and b). In the same regions of cell-cell contact, the EM studies have revealed amorphous material similar in appearance to basement membrane (Fig. 5c). Because this antiserum, when tested on intestinal sections, specifically stained basement membrane and unidentified material present in the lamina propria (Fig. 9c), it is possible that basement membrane components are synthesized by the IEC-6 cells in vitro. Work is in progress to substantiate this possibility.

In conclusion, monolayer cultures of epithelioid cells have been established from rat small intestine. These cells have characteristics of normal cells cultured in vitro and exhibit a number of features suggesting that they are derived from undifferentiated small intestinal crypt cells.

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