Absorptive and Mucus-secreting Subclones Isolated from a Multipotent Intestinal Cell Line (HT-29) Provide New Models for Cell Polarity and Terminal Differentiation

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Abstract. A clone HT29-18 has been isolated from the parent cell line HT-29, which derived from a human colon adenocarcinoma (Fogh, J., and G. Trempe, 1975, Human Tumor Cells In Vitro, J. Fogh, editor, Plenum Publishing Corp., New York, 115–141). This clone is able to differentiate as the parent cell line does. Differentiation occurs when glucose is replaced by galactose in the culture medium (Pinto, M., M. D. Appay, P. Simon-Assman, G. Chevalier, N. Dracopoli, J. Fogh, and A. Zweibaum, 1982, *Biol. Cell.*, 44:193–196).

We demonstrate here that the differentiated cloned population HT29-18/gal is heterogenous: although 90% of the cells show morphological characteristics of "absorptive cells", only 20–30% of them display sucrase-isomaltase in their apical microvillar membranes. About 10% of the entire cell population consists of cells containing mucous granules similar to intestinal goblet cells.

We have isolated two subclones, HT29-18-C_1 and HT29-18-N_2, from the differentiated HT29-18/gal cells. HT29-18-C_1 cells show morphological characteristics of polarized absorptive cells, when growing either in glucose- or in galactose-containing media, but the sucrase-isomaltase is not expressed in the cells grown in glucose-containing medium. The clone HT29-18-N_2 is also polarized in both culture conditions and is similar to goblet cells in vivo. It grows as a monolayer, exhibits tight junctions, and contains numerous mucous granules whose exocytosis can be triggered by carbachol, a parasympathomimetic drug.

We conclude that the clone HT29-18 first isolated was a multipotent cell population from which we isolated several subclones that differentiate either as absorptive (HT29-18-C_1) or as mucous (HT29-18-N_2) cells. In contrast to the parent HT-29 cell line, the subclones retain most of their differentiated properties in glucose-containing medium.

In the small intestinal mucosa, a monolayer of epithelial cells is distributed along the crypt-villus axis. Undifferentiated cells derived from stem cells rapidly divide in the crypts and give rise to differentiated cells that are seen along the villi. There is a continuous renewal of cells during the life span of the individual, with cells originating in the crypts gradually migrating to the tip of the villus where dead cells are shed (Leblond and Messier, 1958; Chang and Leblond, 1974; van Dongen et al., 1976). The major cell types are: absorptive cells displaying a brush border, goblet cells containing mucous granules, Paneth, and enteroendocrine cells containing dense secretory granules.

Numerous biochemical and morphological studies have shown the intestinal epithelium to be an attractive model for the study of basic questions in cell biology. However, such studies have been impeded by its complex structure, its cellular heterogeneity, and its tight adhesion to underlying nonepithelial tissues. Cultured cells from such an epithelium could provide a useful tool for studying cellular metabolism, cell differentiation, and the formation and maintenance of cell surface polarity.

It has not been possible yet to establish epithelial cell lines displaying the structural and functional properties of differentiated intestinal cells using normal adult or fetal rat intestine. The few cell lines derived from the small intestinal mucosa did not exhibit the morphological features of differentiated enterocytes (Quaroni et al., 1979; Quaroni and May, 1980; Quaroni and Isselbacher, 1981; Quaroni, 1985; Negrel et al., 1983).

In 1972, Fogh (Fogh and Trempe, 1975) isolated and cultured cells from human colonic adenocarcinomas. It has been shown recently that one of these, the HT-29 line was able to undergo differentiation and express an enterocytic-like phenotype when grown in glucose-free medium. (Pinto et al., 1982; Zweibaum et al., 1985; Chastre et al., 1985). This is the only cell line of intestinal origin known so far that can be induced to display reversibly most structural and functional features of well-differentiated intestinal cells. In the absence of glucose, the HT-29 cells can form polarized monolayers with well-developed junctional complexes and well-defined apical brush borders. Increased activity of brush border–associated enzymes such as alkaline phospha-

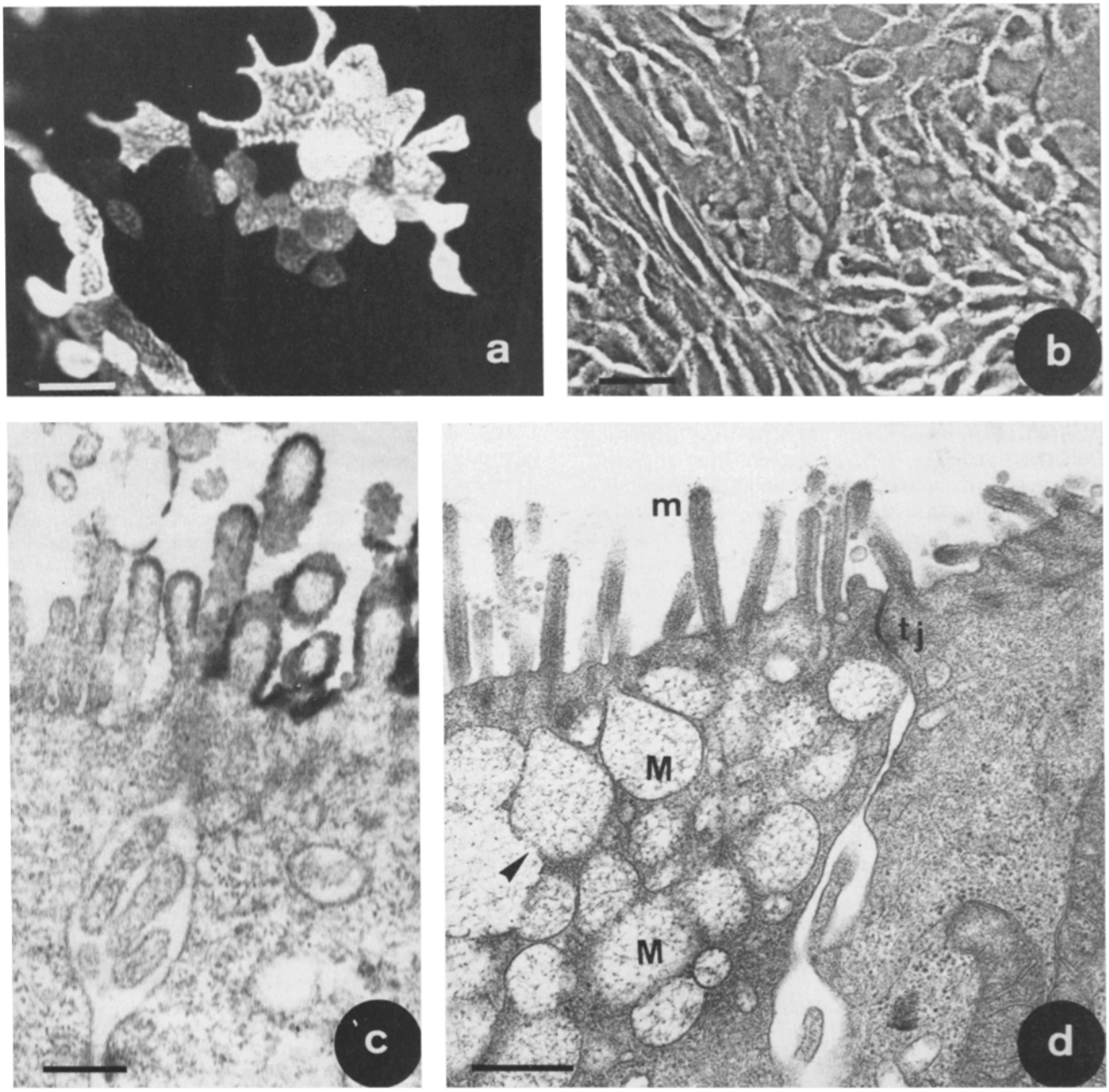

Figure 1. Multipotency of HT29-18 clone. The HT29-18 clone adapted in galactose for 1 wk displays several cell types. The presence of sucrase-isomaltase was demonstrated on the apical membranes of some cells by indirect immunofluorescence and cells containing mucus granules were detected by electron microscopy. (*a*) When the culture is stained with anti-sucrase-isomaltase antibodies, small clusters of cells scattered randomly throughout the culture express sucrase-isomaltase at their apical borders; 20–30% of the cells express this enzyme when confluent, 1-wk cultures are stained. (*b*) A field corresponding to *a* in phase contrast microscopy, showing the typical appearance of confluent cells grown in galactose medium. (*c*) At the ultrastructural level, we observe cells positive for sucrase-isomaltase (*right*) adjacent to well-differentiated cells that are negative for this marker (*left*) (immunoperoxidase staining). (*d*) At the ultrastructural level, cells containing granules filled with a fuzzy material (*M*) are found. These granules are located at the apical pole. They clearly tend to fuse together (*arrow*). Cells containing granules also form tight junctions (*tj*) with adjacent absorptive cells. These have microvilli (*m*) on their apical membrane as do intestinal goblet cells before they release their mucus content into the intestinal lumen. Bars: (*a* and *b*) 10 μm; (*c*) 0.2 μm; (*d*) 0.5 μm.

tase, aminopeptidase, and disaccharidases is found. Cell surface protein markers, such as polymeric IgA receptors (Mostov and Blobel, 1982) and HLA antigens (Louvard, D., unpublished results) are expressed heterogeneously when the cells are grown in media containing glucose. Moreover, sucrase-isomaltase, often used as a marker for differentiated enterocytes, was shown to be expressed only at the apical brush borders of a subpopulation of differentiated cells (30–50%) obtained in sugar-free media (Zweibaum et al., 1985).

In an effort to obtain a homogeneous population of cells we have cloned the HT-29 parent cell line by the limiting di-

lution technique and selected from 30 others the clone HT29-18. The clone HT29-18 behaves very similarly to the original uncloned HT-29 cell line. In particular it can undergo an enterocytic-like differentiation in a medium that lacks glucose. The cells in galactose are characterized by a well-developed brush border and by the presence of apical or basolateral surface markers such as sucrase-isomaltase, aminopeptidase, transferrin receptor, IgA receptor, and histocompatibility antigens, respectively (Godefroy, O., C. Huet, L. Blair, and D. Louvard, manuscript in preparation). After three successive clonings of the original cell line in glucose-containing medium, the undifferentiated cells appeared homogeneous. However, the differentiated cultures, obtained after transfer to galactose medium, still displayed heterogeneity. Two major distinct phenotypes could be identified: "absorptive cells" having a well-organized brush border and cells containing mucous granules. When differentiated HT29-18 cells were transferred back to glucose medium distinct polarized phenotypes were still observed. This last property enabled us to subclone in glucose medium the differentiated cells and to separate absorptive cells from those containing mucous granules. Several properties of these subclones are reported here.

Materials and Methods

Cell Cultures

The cell line HT-29 (Fogh and Trempe, 1975) was obtained from Dr. Zweibaum (Institut National de la Santé et de la Recherche Médicale U-178, Villejuif, France). Cells were seeded in plastic petri dishes (Falcon Labware, Becton, Dickinson & Co., Oxnard, CA) at 5×10^4 cells/cm^2 and cultured at 37°C in a 10% CO_2/air incubator. Glucose- and pyruvate-free DME was obtained from Eurobio (Paris, France). The medium was supplemented with FCS (10%) that had been dialyzed 3 d in the cold against 0.9% NaCl. Also added to the culture medium were glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and human transferrin (10 μg/ml).

Glucose-containing medium was supplemented with 20 mM glucose in addition to the 5 mM already present in DME. When these cells are maintained in glucose-containing medium, it was essential to change the medium once a day, since high glucose content is required to maintain the undifferentiated state of the cells.

Transfer of undifferentiated cells grown in glucose into galactose medium could not be done in one step but required progressive adaptation of cells to avoid massive death. The following schedule was used in our laboratory to adapt the cells to galactose-containing media. Cells were suspended by treatment with trypsin/EDTA (0.05%/0.02%; seromed; Biochrom KG, D800 Berlin, Federal Republic of Germany) and seeded in a medium containing 20 mM glucose plus 1 mM galactose (day 0). Medium was changed every day over a period of 4 d and cells were grown in graded series of galactose-containing medium (day 1, 15 mM glucose plus 1 mM galactose; day 2, 10 mM glucose plus 3 mM galactose; day 3, 5 mM glucose plus 4 mM galactose; day 4, 5 mM galactose). In these conditions, no significant cell death was observed.

Galactose-containing medium was supplemented with 5 mM galactose and was changed twice a week. Once adapted to galactose the culture could be replated in galactose-containing medium for at least 30 passages. Cells were passaged before reaching confluency, since confluent cultures were difficult to transfer with a good plating efficiency. In addition, passage at low density did not produce rapidly growing healthy cultures. Stocks of cells were stored in liquid nitrogen after slow freezing at −80°C in media containing 20% FCS and 10% DMSO (Sigma Chemical Co., St. Louis, MO) as a cryoprotectant.

The following abbreviations will be used here for the different clones: HT-29, the original line isolated by Fogh (Fogh and Trempe, 1975); HT29-18/glu, a clone isolated from original HT-29 growing in a glucose medium; HT29-18/gal, a clone after adaptation of HT29-18/glu in this medium growing in a galactose medium; HT29-18-C_1/glu, a subclone isolated from a HT29-18/gal culture with absorptive characteristics, growing in glucose medium; HT29-18-C_1/gal, subclone HT29-18-C_1 growing in galactose-containing medium; HT29-18-N_2/glu, a subclone isolated from HT29-18/gal culture with mucous granules, growing in glucose medium; HT29-18-N_2/gal, subclone HT29-18-N_2 growing in galactose-containing medium.

Subcloning of HT29-18/gal Differentiated Cells

Differentiated HT29-18/gal cells grown in galactose were replated back into glucose-containing medium and carefully trypsinized to obtain a suspension of individual cells. Cells were seeded at a density of $2–3 \times 10^3$ cells in a 10-cm diam petri dish (Falcon Labware). After 3 wk, 100–200 colonies were obtained on each petri dish. Single clones were isolated by trypsinization in a stainless steel ring sealed around the colony by means of silicone grease. The cell suspension was then transferred to a 24-well plate (Linbro, Flow Laboratories, Hamden CT), grown for a week, and then transferred to a 6-cm petri dish. About 2–3 wk later, when cells reached confluency, they were transferred in 25 mM glucose medium and grown as described above. This cloning procedure was repeated twice after the isolation of any clone of interest. The selection of subclones was based on careful examination with an inverted microscope equipped with phase contrast. Cloning efficiency of the subclones was equivalent to that of the original HT29-18 cells. (These successive cloning steps are summarized in Fig. 4.)

Immunocytochemistry at the Light Microscope Level

Light microscopy was used to follow the growth of living cells, using an inverted Olympus microscope (IMT2) equipped with phase contrast and an Olympus OM.1 camera. Semithin sections (2 μm) of cultures embedded in Epon as described below were used to characterize the general organization of the cell layers and were observed with a Zeiss photomicroscope III equipped with regular or interference phase-contrast optics.

Fluorescence immunocytochemistry was used with cultured cells on glass coverslips to demonstrate the expression of specific antigens. The detailed protocols for the preparation of immunofluorescent reagents and for immunocytochemical procedures have been described previously by Reggio et al. (1983). Briefly, cultures were fixed with 2% paraformaldehyde, incubated in a humid chamber on a drop of primary antibody, and anti-mouse IgG coupled to fluorescein, rhodamine, or peroxidase was used as secondary antibody. Coverslips were mounted either with glycerol (90% glycerol, 10% PBS) or in Mowiol 4-88 mounting medium (Hoechst Aktiengesellschaft, Frankfurt, FRG). Monoclonal antibodies against sucrase-isomaltase and aminopeptidase (diluted 1:5 to 1:10) were generous gifts from Dr. H.-P. Hauri (Hauri et al., 1985). Anti-mucus monoclonal antibodies were a generous gift from Dr. J. Bara (Bara et al., 1984). A Zeiss photomicroscope III equipped with appropriate UV filters and interferential phase-contrast optics was used for observations.

Ultrastructural Immunocytochemistry and Electron Microscopic Procedures

For ultrastructural studies, cells were fixed for 1 h at room temperature in situ in the petri dish with a fixative consisting of 2% paraformaldehyde, 2% glutaraldehyde, 4 mM Ca^{++}, 2 mM Mg^{++}, and 0.1 M sodium cacodylate, pH 7.4. After subsequent washes with cacodylate buffer (sodium cacodylate 0.1 M, pH 7.4), cultures were postfixed with OsO_4 (2% in sodium cacodylate 0.1 M pH 7.4), stained en bloc with uranyl acetate (1% in acetate 0.1 M, pH 5.5), dehydrated in a graded series of alcohols, and embedded with a thin layer of Epon in the dish. After polymerization, the Epon was separated from the culture dish, and two pieces (2×4 mm) were placed together and were flat embedded. Sections were cut perpendicular to the plane of the cellular monolayer with a microtome (Ultracut; Reichert Jung S. A., Paris). Samples were stained with uranyl acetate, lead citrate (Reynolds, 1963), and visualized in a Siemens 101 electron microscope.

To demonstrate the presence and impermeability of occluding junctions at the ultrastructural level, ruthenium red was used as a permeation dye. Cells were incubated with ruthenium red/glutaraldehyde and with ruthenium red/OsO_4 mixtures as described by Luft (1972).

For ultrastructural immunocytochemistry, cells grown on glass coverslips were fixed with paraformaldehyde (2% in cacodylate buffer) for 30 min at room temperature. Cultures were then washed with cacodylate buffer (three times, 15 min), incubated first with 50 mM NH_4Cl (in the same buffer) for 10 min, and then with cacodylate-buffered gelatin solution (0.2% gelatin in cacodylate buffer) for 15 min. Antibodies were diluted in cacodylate-gelatin solution and briefly centrifuged before use. Coverslips were inverted on 80 μl of antibody solution for 20 min in a humid chamber at room temperature. Cultures were subsequently washed (three times for 15 min) with cacodylate-gelatin solution and inverted in a humid chamber on 80 μl of anti-mouse IgG coupled to peroxidase (Biosys, Compiègne, France). Con-

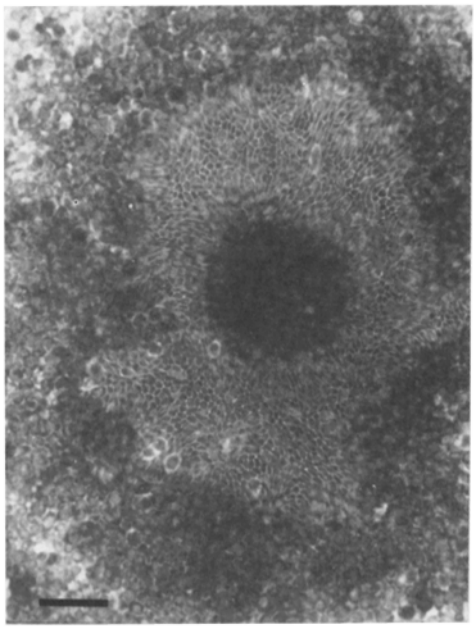

Figure 2. Differentiated epithelial cells in glucose medium. A confluent culture examined by phase-contrast light microscopy after replating HT29-18/gal in a glucose-containing medium. A foci of cells forming a typical epithelial sheet with bright cellular contacts is seen surrounded by cells poorly organized growing in multilayers with poorly visible cell–cell contacts. The dark spot in the middle of these differentiated cells is an island of undifferentiated cells protruding out of the focal plane. Bar, 30 μm.

trol experiments were performed omitting the primary antibody. After washing in cacodylate buffer, cells were postfixed with 2% glutaraldehyde in cacodylate buffer for 30–60 min. After extensive rinsing in Tris-HCl buffer (50 mM, pH 7.4), the peroxidase was revealed by diaminobenzidine and hydrogen peroxide according to Graham and Karnovsky (1966). Samples were postfixed in 2% osmium tetroxide and dehydrated in a graded series of alcohol. After dehydration, the coverslip was taken out of the petri dish and glued with a drop of Epon to a glass slide, the cell monolayer facing up. The culture layer was covered with 4–5 drops of Epon and allowed to polymerize 9 h at 60°C. Subsequent cooling on a piece of dry ice caused the thin layer of Epon to separate from the glass. Small square pieces were flat embedded as described above for tissue cultures in situ.

Scanning Electron Microscopy

Scanning electron microscopy was carried out on cultures grown on coverslips. After fixation with glutaraldehyde (2% in cacodylate buffer) cultures were dehydrated in a graded series of ethanol, from 70 to 100%. Wet coverslips were transferred into Freon 113 and dried after substitution with liquid CO_2 in a critical point dryer (model CPD 020; Balzers, Hudson, NH). Dried cultures were coated with gold, using a coating unit (model E51 SEM; Polaron Instruments, Inc., Hatfield, PA). Samples were viewed either with a Cameca or with a Jeol 35CS scanning electron microscope.

Results

Multipotentiality of the HT29-18 Clone

The HT29-18 clone, which grows as a multilayer of undifferentiated cells in a glucose-containing medium, forms a monolayer of polarized cells at confluency when galactose replaces glucose in the culture medium. The differentiated cells display a characteristic enterocytic differentiation. The HT29-18/glu cells appear homogeneous and undifferentiated (Godefroy, O., C. Huet, L. Blair, and D. Louvard, manuscript in preparation), but careful examination of the differentiated HT29-18/gal culture detected some diversity in the expressed phenotype revealed by immunocytochemistry at the light and electron microscope levels.

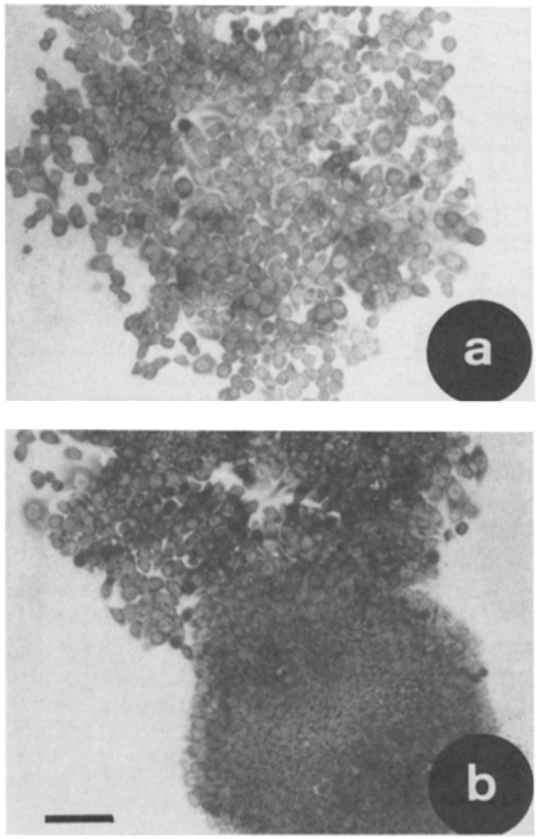

Figure 3. Morphological features of different colonies obtained during subcloning. (*a*) A typical colony of HT29-18/glu is shown. At low density these small cells grow in a monolayer and have poor cellular contacts. (*b*) Two adjacent colonies obtained after replating HT29-18/gal at low density in medium containing 25 mM glucose are shown: cells remain segregated and form distinguishable colonies; a colony of cells at the top of the figure whose phenotype is similar to the original HT29-18 clone is touching a colony of cells that clearly is different and displays a typical epithelial sheet arrangement with close cell–cell contact. Bar, 30 μm.

In preliminary experiments, we found that the culture cells were homogeneously expressing aminopeptidase at their apical membrane (data not shown). With a monoclonal antibody against sucrase-isomaltase (generous gift from Dr. H.-P. Hauri) revealed by immunofluorescence (Fig. 1, *a* and *b*) or immunoperoxidase (Fig. 1 *c*), we found that 20–30% of the cells are strongly immunoreactive on their apical surface. However the presence of the enzyme could not be correlated directly with the existence of an organized brush border. This was demonstrated by ultrastructural analysis after immunoperoxidase staining, which showed positive cells with microvilli heavily stained next to cells with unstained microvilli. These observations apply to adjacent neighbor cells with equally well-developed apical membranes (Fig. 1 *c*).

In the HT29-18/gal cultures that have been confluent for 1 wk most of the cellular population (~90–95%) consisted of columnar absorptive cells. However, a small number (5–10%) of cells contained large granules under the apical

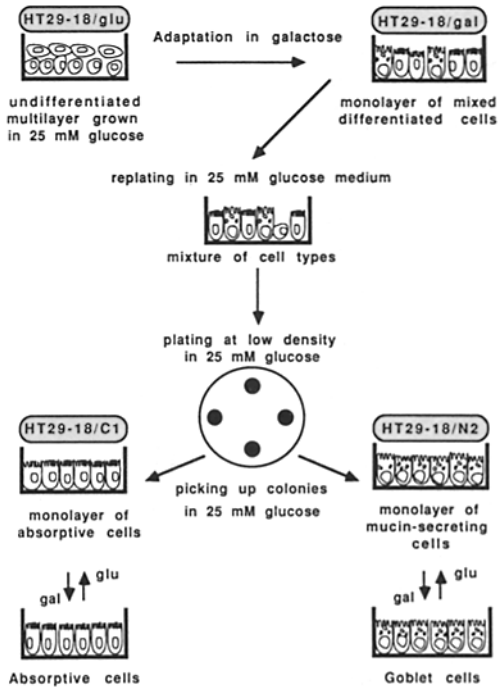

Figure 4. Schematic diagram HT29-18 subclones isolation. After three successive clonings of the HT-29 parent cell line the HT29-18 clone was selected. It grows well and looks homogeneous in glucose medium. When adapted to galactose medium, the HT29-18/gal culture is well differentiated into enterocyte-like cells with at least two major phenotypes: absorptive and mucous cells. After replating in 25 mM glucose medium, differentiated phenotypes were still observed and colonies were selected for subcloning using the limiting dilution method. Two subclones of special interest were selected: HT29-18-C_1, a population similar to absorptive cells and HT29-18-N_2, a population of mucous-secreting cells very homologous to intestinal goblet cells. These subclones keep a differentiated phenotype when grown in glucose medium. They can be transferred to galactose medium without any prior adaptation, and they can be seeded back similarly from galactose medium into glucose medium.

membrane (Fig. 1 *d*) filled with a fuzzy material resembling the mucus seen in secretory granules of intestinal goblet cells. In the HT29-N_2 subclone derived from these cells, the content of these granules was shown to be mucin, as described below.

Persistence of Differentiated Characters in Glucose Medium

When the differentiated parent HT-29 cell line grown in galactose is transferred back into glucose media, it loses its differentiated phenotype as measured both biochemically and morphologically (Pinto et al., 1982). To see if these observations also apply to our clone, an HT29-18/gal culture maintained for >20 passages in galactose was replated into a glucose medium. To our surprise the entire culture did not return to its former undifferentiated state, but instead a cellular heterogeneity was revealed. This heterogeneity could be observed clearly with an inverted light microscope. Numerous foci organized as typical epithelial sheets (up to 20% of cells on the dish) were surrounded by cells without distinct intercellular contacts (Fig. 2). The clonal origin of this heterogeneity was illustrated when this mixed cell population was plated at low density. Each developing colony (or clone) was homogeneous. Furthermore when two clones came into contact with each other, the cells of each clone remained segregated and a clear border between them was maintained (Fig. 3 *b*). Colonies of cells having poor contacts could be seen next to colonies of cells with close contacts organized as an epithelium; the former phenotype resembled equivalent colonies obtained by seeding HT29-18 cells at low density in a glucose-containing medium (Fig. 3 *a*). The presence of an epithelium-like phenotype suggested that some cells retained an intermediate level of differentiation as the HT29-18/gal culture was returned to glucose medium. Subclones that retained their ability to form epithelial sheets were isolated because this phenotype is unusual for the HT-29 cell line in a glucose-containing medium.

Subcloning of the HT29-18 Clone

Since such a differentiated phenotype had not been reported previously in a glucose medium, we decided to isolate colonies exhibiting these unusual characteristics. At least 30 clones exhibiting monolayer organization in glucose medium were isolated and the cultures were expanded in glucose medium. During this procedure no change in the characteristics of the cells could be observed by inverted light microscopy. When enough cells were obtained, we tested their ability to be grown in a galactose-containing medium and examined their properties under these conditions. In contrast to the cloned HT29-18 cells, these subclones could be easily grown in the galactose medium without progressive adaptation as required for the parent cell line or for the HT29-18 clone, and further morphological changes were observed in response to these culture conditions. Two subclones were studied in detail because they represented stable cultures of the two different phenotypes typical of cells of normal intestinal mucosa present in differentiated HT29-18/gal cultures: the absorptive enterocyte-like subclone HT29-18-C_1, and the mucin-containing subclone HT29-18-N_2. This procedure and the results are schematically represented in Fig. 4.

Characterization of the Absorptive Enterocyte-like Subclone HT29-18-C_1

When HT29-18-C_1 cultures are kept in glucose (HT29-18/glu), they grow mainly as a monolayer with occasional multilayer areas (10–20%). Scanning electron microscopy (Fig. 5 *a*) demonstrated the presence of straight, ordered microvilli typical of enterocytes. In a cross section through the monolayer, the cells were polarized, tall (25 μm), and bore a row of short, straight microvilli seen at the apical surface (Fig. 5 *b*). The presence of junctional complexes at the apical membrane boundaries was clearly visible at higher magnification and after apical application of ruthenium red the occluding junctions prevented the dye from penetrating into intercellular spaces (Fig. 5 *c*).

Immunocytochemistry at both the light and electron microscope levels failed to detect sucrase-isomaltase on the microvilli of these polarized cells in glucose-containing media (Fig. 5 *d*). These observations were confirmed using a sensitive enzymatic assay for sucrase-isomaltase (data not shown). However, when a monoclonal antibody against aminopeptidase was used, positive staining was seen on all the apical membranes of the cells (Fig. 5 *e*). This observation

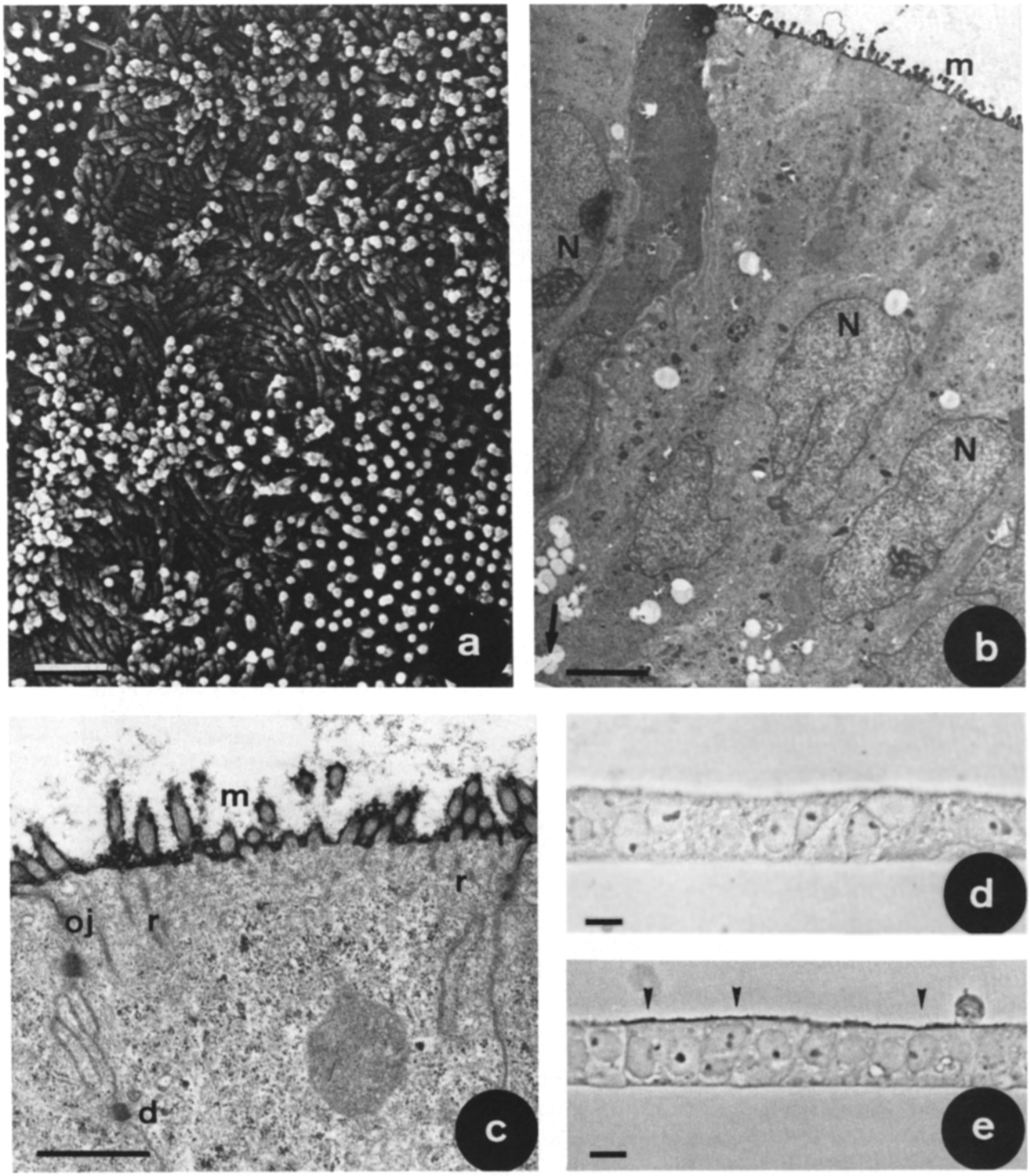

Figure 5. HT29-C_1 subclone in glucose. (*a*) The upper surface of the culture appears covered by numerous organized microvilli (scanning electron micrograph). (*b*) Longitudinal section of the culture showing a monolayer of tall columnar cells, 20–25 μm high. The apical membranes are covered by numerous microvilli (*m*). The nuclei (*N*) are generally located in the center of the cellular body. The arrow shows the bottom of the culture (transmission electron micrograph). (*c*) At higher magnification, the cytoskeletal rootlets (*r*) anchoring the microvilli (*m*) are clearly visible. The dark ruthenium red precipitate is only seen on the apical membrane. The occluding junction (*oj*) divides the cellular membrane in two domains: the apical membrane and the basolateral membrane. Desmosomes (*d*) join the lateral membranes (transmission electron micrograph; ruthenium red staining). (*d*) Longitudinal section through a HT29-C_1 glucose culture. After incubation with anti-sucrase-isomaltase antibodies to detect the presence of sucrase-isomaltase, no positive reaction (immunoperoxidase) could be observed on the apical membranes (phase contrast microscopy). (*e*) Immunostaining of HT29-C_1 glucose cells with monoclonal antibody against aminopeptidase. The entire culture is positive (*arrows*), and all cells express the enzyme on their apical membrane (notice midposition of the nuclei along the section) (phase contrast microscopy). Bars: (*a*) 1 μm; (*b*) 2 μm; (*c*) 0.5 μm; (*d* and *e*) 10 μm.

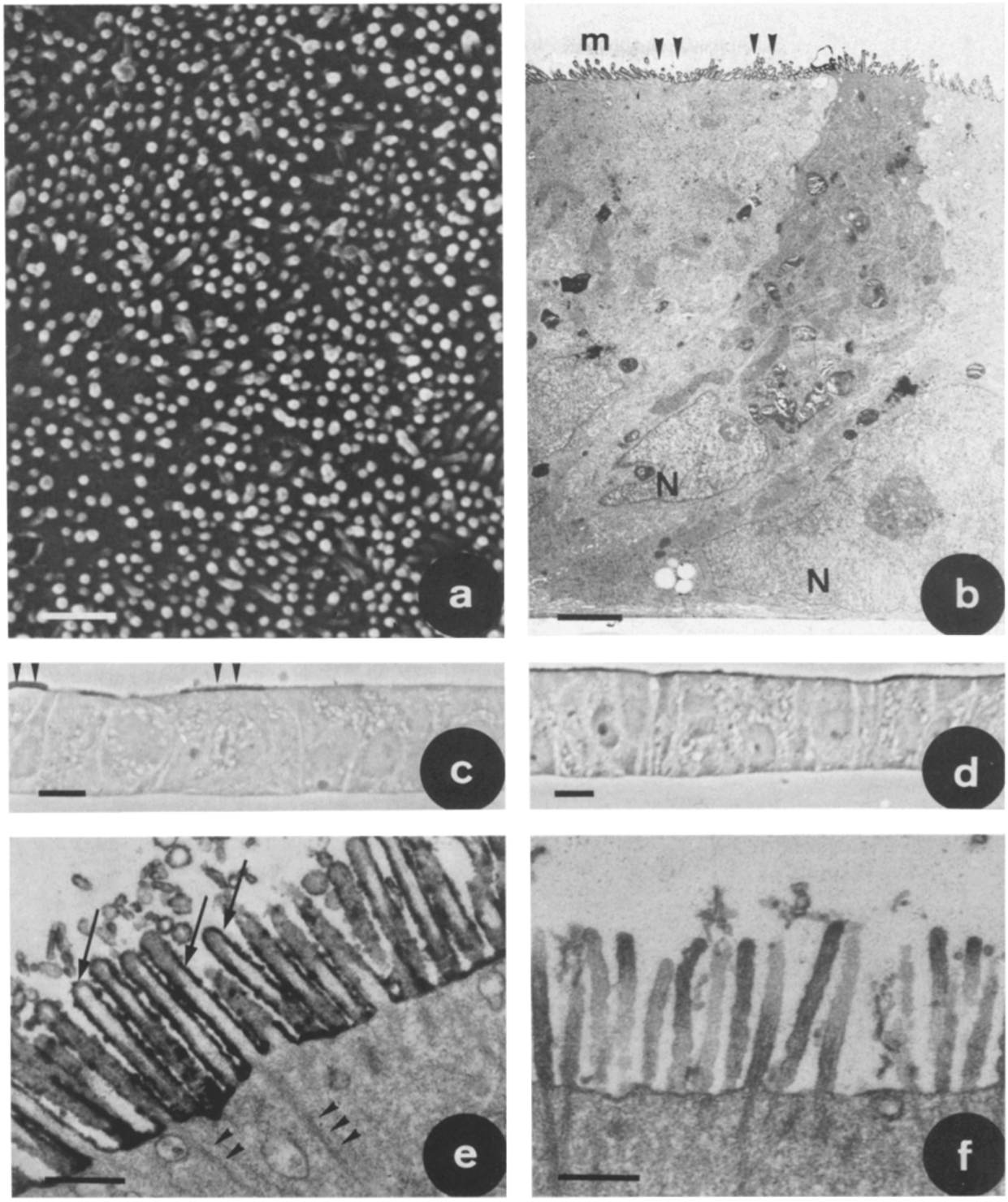

Figure 6. HT29-C_1 subclone in galactose. (*a*) The apical surface of the culture is covered by numerous and homogenous microvilli (scanning electron micrograph). (*b*) The tall columnar cells form a highly polarized monolayer, with microvilli (*m*) at the apical membrane and interdigitating cell processes at their lateral surfaces. Nuclei (*N*) are localized close to the basal membrane of the cell. Ruthenium red dye added to the apical face of the culture stains the apical membranes (*arrowheads*) but does not penetrate through the occluding junctions joining the cells at the apex (transmission electron micrograph). (*c*) At the optical level, sucrase-isomaltase can be revealed (*arrowheads*) by peroxidase immunocytochemistry with antibody against the enzyme. Only some cells display a positive reaction on their apical surface (phase contrast microscopy). (*d*) The entire cell monolayer is positively stained by the immunoperoxidase reaction with antibody directed against aminopeptidase (phase contrast microscopy). (*e*) At the ultrastructural level, well-organized membranes of microvilli are positively stained by the immunoperoxidase reaction (*arrows*) using antibody against sucrase-isomaltase. Notice the presence of filament bundles in the microvilli plunging into the underlaying cytoplasm (*arrowheads*) (transmission electron micrograph). (*f*) In the same culture and reaction conditions as in *e*, well-organized microvilli can also be found free of immunoreaction (transmission electron micrograph). Bars: (*a*) 1 μm; (*b*) 2 μm; (*c* and *d*) 10 μm; (*e* and *f*) 0.5 μm.

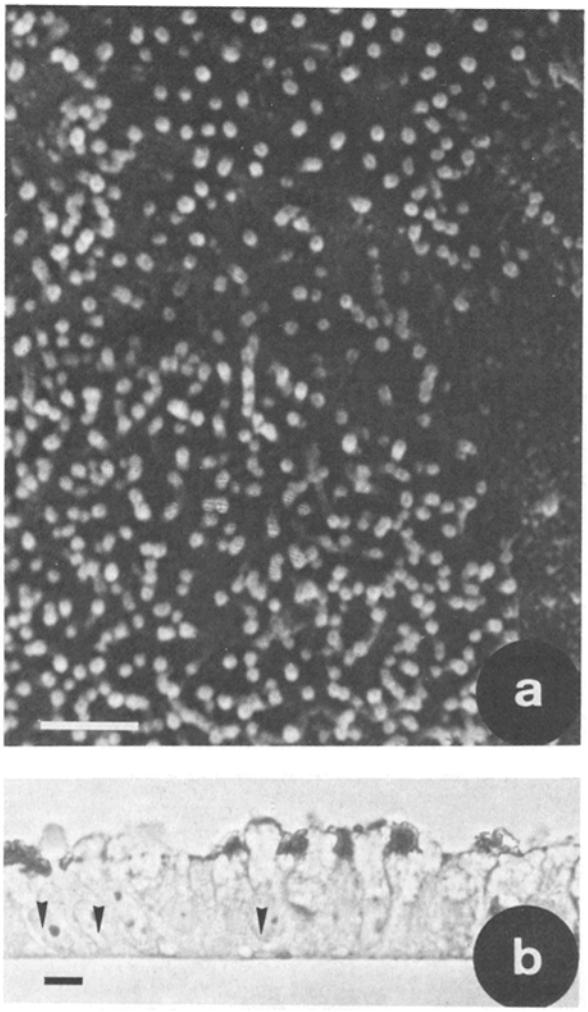

Figure 7. HT29-N_2 subclone in glucose. (*a*) The surfaces of these cells are covered by microvilli (scanning electron micrograph). (*b*) Longitudinal section through a confluent culture. The cells are growing as a monolayer which is formed mainly of mucus-containing cells. Their overall morphology with mucus granules accumulated underneath the apical membrane is very similar to intestinal goblet cells. Mucins are stained by the immunoperoxidase reaction using human fetal anti-mucin monoclonal antibodies. Notice that all the permeabilized cells are not positive although they obviously contain mucus (see text). Nuclei (*arrowheads*) are mostly located in the lower part of the cells (phase contrast microscopy). Bars: (*a*) 1 μm; (*b*) 10 μm.

was confirmed with an enzymatic assay for this enzyme (not shown).

Unlike the HT29-18 clone, the HT29-18-C_1/glu subclone could be transferred to galactose medium in one step, and did not require a step by step adaptation to galactose medium (Godefroy, O., C. Huet, L. Blair, and D. Louvard, manuscript in preparation). Using scanning electron microscopy, confluent HT29-18-C_1/gal cultures showed numerous surface microvilli (Fig. 6 *a*); with transmission electron microscopy they appeared as columnar cells (20–25 μm) growing as a monolayer with almost no multilayer areas (Fig. 6 *b*). Typical microvilli, junctional complexes, and numerous dense bodies (presumably secondary lysosomes) were present. Nuclei generally were located closer to the basal membrane than when this subclone was maintained in glucose-containing medium (compare Figs. 5 *b* and 6 *b*).

Monoclonal antibody against sucrase-isomaltase revealed enzyme-positive apical membranes in ~20% of the cells (Fig. 6 *c*), whereas all cells were stained with a monoclonal antibody against aminopeptidase (Fig. 6 *d*). The anti-sucrase-isomaltase antibody-binding sites appeared to cover the entire apical surface of individual cells (Fig. 6 *e*), although adjacent cells were unstained (Fig. 6 *f*).

Glucose or galactose can be exchanged repeatedly in the media to induce cyclic changes in phenotype without generating observable heterogenity in the cultures. In contrast to the clone HT29-18, cells containing mucous granules were never observed, a result that emphasizes the efficiency of our procedure to sort out absorptive cells from mucous cells. There are few clear differences between HT29-18-C_1/glu and HT29-18-C_1/gal: an enhanced polarized organization, the basal localization of the nuclei, the expression of sucrase-isomaltase on the brush borders of HT29-18-C_1/gal of ~20% of cells, and a higher villin content (see companion paper, Dudouet et al., 1987). It is likely that when other tools become available to analyze the properties of this subclone further differences will be identified.

Characterization of a Subclone Containing Mucus Granules HT29-18-N_2

The HT29-18-N_2 subclone also maintained a typical epithelial organization in a glucose medium. In this medium most of the cells displayed numerous microvilli on apical surface (Fig. 7 *a*), and grew as a monolayer (Fig. 7 *b*). The distinctive feature of this subclone was the presence of numerous cells having a typical goblet cell morphology. After permeabilization with Triton X-100, most of the cells (70–80%) were heavily stained by anti-mucus monoclonal antibodies (Fig. 7 *b*). Cells that were not stained either presumably were not permeabilized or the synthesized mucins were not recognized by the antibodies, since at the ultrastructural level typical granules were seen in all cells.

When such a culture was transferred into the galactose medium, the terminal differentiation of this subclone was more dramatic. At 10–15 d after confluency most of the cells displayed numerous microvilli as demonstrated by scanning electron microscopy (Fig. 8 *a*). With transmission electron microscopy, (Fig. 8 *b*), the HT29-N_2/gal cells displayed in addition to mucus granules the general features of differentiated cells (microvilli with actin bundles and junctional complexes at their apical boundaries, numerous undifferentiated cellular processes with desmosomes on basolateral membranes). No clear difference either in size or in content could be detected at the electron microscope level between the granules found in the HT29-18-N_2/glu and those in the HT29-18-N_2/gal cells. A few cells in the intact monolayer were stained by anti-mucin antibodies without permeabilization, suggesting that these cells were normally releasing mucus into the medium or that they were permeabilized spontaneously during the staining procedure (Fig. 8 *c*). After permeabilization with Triton X-100, many more cells appeared positive (Fig. 8 *d*). Higher magnification immunofluorescence illustrated the general distribution of granules and the presence of immunoreactive mucus granules (Fig. 8, *e* and *f*).

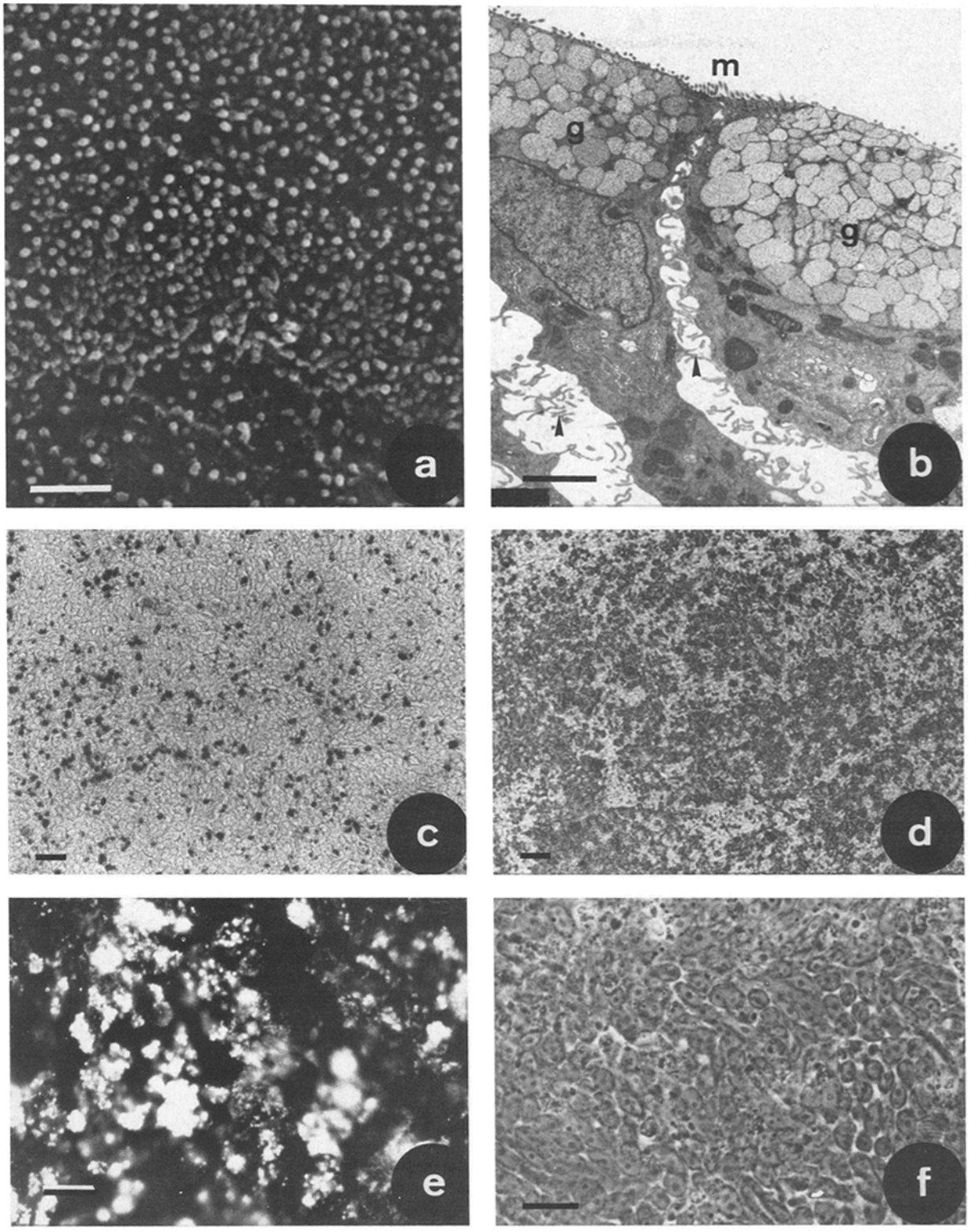

Figure 8. HT29-N_2 subclone in galactose. (*a*) The cells exhibit numerous microvilli on their apical membranes like intestinal cells (scanning electron micrograph). (*b*) The culture forms a cellular monolayer. Below the apical surface, numerous mucin granules (*g*) are packed together. Organized microvilli (*m*) are seen at the apex, whereas unordered intercellular processes are present on the basolateral membranes (*arrowheads*) (transmission electron micrograph). (*c*) Immunoperoxidase reaction on cells after incubation with anti-mucin monoclonal antibodies. The sparse but localized positive reaction indicates that only a few cells are open spontaneously without permeabilization and therefore may be releasing mucus into the culture medium. (*d*) After Triton X-100 treatment to permeabilize the membranes, most of the cells are positively labeled with anti-mucin monoclonal antibodies. This demonstrates the high level of differentiation and the homogeneity of this culture. (*e*) Immunofluorescence staining by anti-mucin monoclonal antibodies (see Materials and Methods). The granular aspect of the cellular content is well demonstrated after permeabilization. (*f*) Same field as in *e* but with phase-contrast microscopy. The focusing was done at a lower level demonstrating that the nuclei occupy a lower position in the cytoplasm underneath the mucus granules. Bars: (*a*) 1 μm; (*b*) 2 μm; (*c* and *d*) 100 μm; (*e* and *f*) 30 μm.

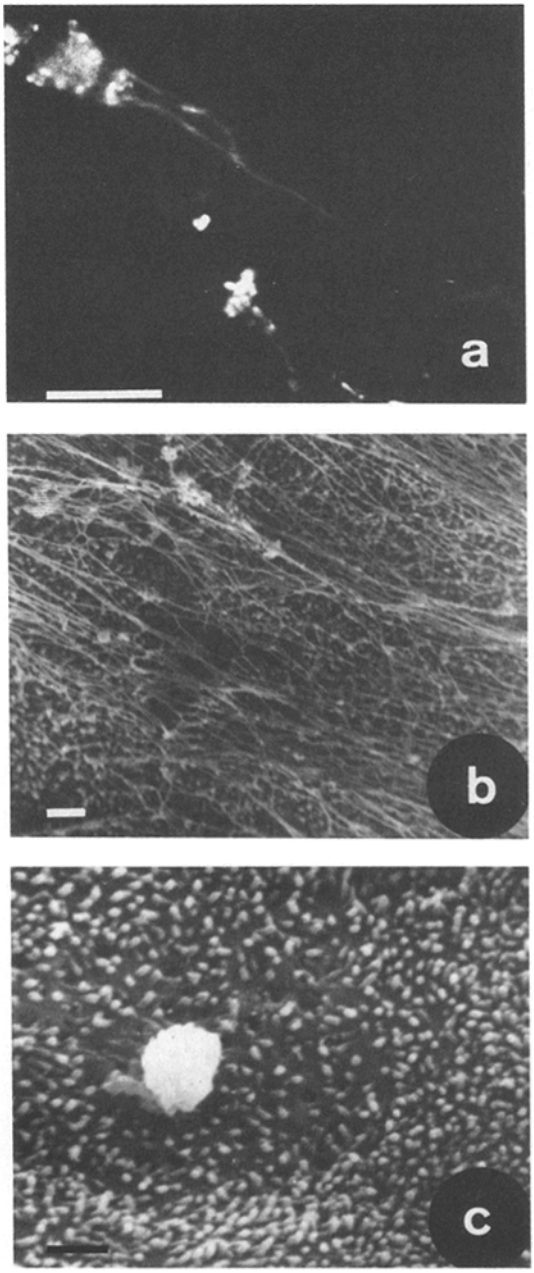

Figure 9. Carbachol effect on HT29-18-N_2 cells. (*a*) When these cultures maintained in glucose- or in galactose-containing media are incubated with carbachol (see Materials and Methods section), numerous mucus-secreting cells are seen. Long mucin filaments are detected by immunofluorescence on top of an HT29-18-N_2/glu or /gal cell monolayer with anti-mucin monoclonal antibodies. (*b*) In many areas, long filaments of mucus secretion are found on top of cells (scanning electron micrograph). (*c*) At higher magnification, the initial step of the secretion can be observed. A droplet of mucosal secretion is seen coming from a cell with less microvilli on its surface. Holes in the apical membrane are observed next to the mucus droplet, presumably representing other secreting apertures (scanning electron micrograph). Bars: (*a*) 30 μm; (*b* and *c*) 1 μm.

Parasympathetic stimulation is known to induce secretion of mucus from crypt goblet cells but not from those on small intestinal villi or the colonic mucosal surface (Specian and Neutra, 1980). To determine whether HT29-18-N_2 cells were sensitive to cholinergic stimulation, we incubated HT29-18-N_2/glu and HT29-18-N_2/gal cell monolayers in culture medium supplemented with 20 μM carbachol (carbamylcholine; Sigma Chemical Co.) for 15 or 30 min at 37°C in a CO_2/air incubator. In both cultures we observed a release of mucus from some cells at 15 min. This was detected by immunofluorescent techniques as large streams of immunoreactive material flowing from the cells (Fig. 9 *a*). After treatment of the culture with carbachol, a larger number of cell surfaces were stained with the anti-mucin antibody without permeabilization compared with control experiments (data not shown). The effect of carbachol on the secretion of mucins was illustrated with the scanning electron microscope in which a large amount of filamentous material was seen on top of the monolayer (Fig. 9 *b*), and mucous droplets could be seen occasionally after drug stimulation (Fig. 9 *c*). Exocytosis of mucous granules triggered by carbachol suggests the presence of a functional muscarinic acetylcholine receptor in both types of cultures (Neutra and Forstner, 1987; Phillips et al., 1984).

Since the gobletlike phenotype is the only one observed, it is worth stressing the homogeneity of the HT29-18-N_2 subclone. This clone also shows two other features in common with HT29-18-C_1: it can be transferred into galactose medium without requiring prior adaptation and replating within a given media, and a switch to glucose or galactose could be performed easily without appearance of heterogeneity.

Discussion

Several groups have tried to obtain stable and permanently differentiated intestinal cells. Many malignant cell lines were isolated from rat colon (Borman et al., 1982; Martin et al., 1975), rat small intestine (Martin et al., 1975), mouse colon (Brattain et al., 1980; Cleveland et al., 1979; Tan et al., 1976), and guinea pig colon (Rabito, 1981). In most of the cases, the expression of differentiated phenotypes and functional properties remains to be analyzed. Since the first human colon carcinoma cell line HT-29 was established by Fogh and Trempe (1975), many cultures have been derived from human colon cancers. Only recently three cell lines, $CaCO_2$, HT-29, and T-84, were found to exhibit either spontaneously or inductively permanent differentiated phenotypes of either mature absorptive cells or mature mucous-secreting cells (Pinto et al., 1983; Dharmsathophorn et al., 1984; Madara and Dharmsathaphorn, 1985). It was previously reported that the HT-29 adenocarcinoma cell line displayed an enterocytic terminal differentiation on manipulation of the nutritional conditions, such as the replacement of glucose by galactose (Pinto et al., 1982) or total sugar depletion (Zweibaum et al., 1985).

This cell line was suggested to be pluripotent as it induces tumors with a heterogeneous enterocyte-like pattern of differentiation (Zweibaum et al., 1984, 1983) as well as a large number of mucus-secreting colonies (Rousset et al., 1978). However, these observations could be due to an initially mixed population of cells since the HT-29 cell line had not

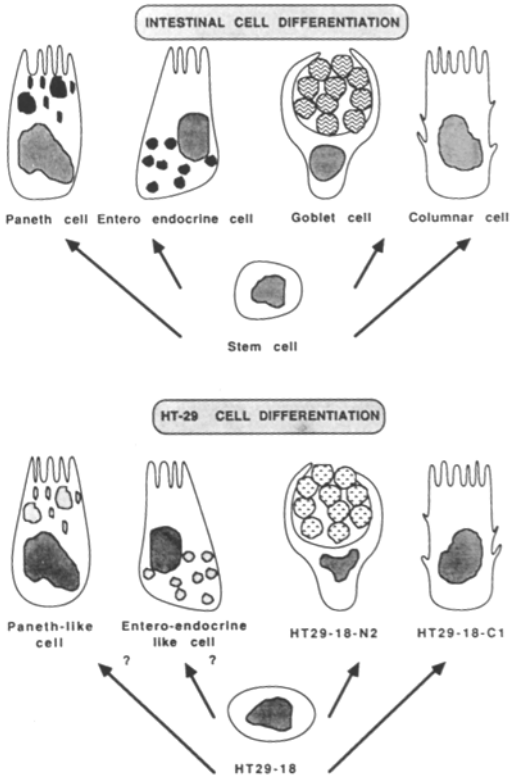

Figure 10. Unitary theory of the origin of epithelial cells. Four main cell types are present in the small intestine: Paneth cells with large granules, enteroendocrine cells with many secretion granules, goblet cells filled with mucins, and villus columnar cells. Immature crypt base cells should give rise to these four main kinds of intestinal cells. By analogy, the HT29-18 clone is proposed to be multipotent and to give rise to distinct differentiated cells with properties similar to the four main kinds of intestinal cells. HT-29 differentiation to Paneth-like cells or enteroendocrine-like cells remains to be demonstrated similar properties are proposed (see Discussion).

yet been cloned. Consequently, in an effort to obtain a homogeneous population of cells for further biological and biochemical studies on cell differentiation and cell polarity, the cloning of the HT-29 cell line was undertaken. The HT29-18 clone was selected from among several others.

The HT29-18 clone we have studied in the present work displays terminal enterocyte-like differentiation under the same nutritional conditions used to differentiate the original HT-29 cell line. However, after three successive clonings by the limiting dilution method, cellular heterogeneity was still indicated by morphological and biochemical criteria in the differentiated cultures in contrast to the undifferentiated cells that appeared homogenous. It is therefore unlikely that the heterogeneity of the differentiated cells is due to an initial mixture of cells. Furthermore, we observed that the relative amounts of the different cell types described here remained remarkably constant over a long period of time (i.e., after at least 30 successive transfers). Changes in the distribution of cell types would have been expected in the case of a mixed population because different clones are likely to have different growth rates and plating efficiencies. In a mixed population of cells, an enrichment in one cell type should have been observed at the expense of the others. From these observations it may be concluded that the HT29-18 clone is pluripotent.

It seems that the nutritional method used here to induce cellular differentiation, i.e., glucose depletion, triggers metabolic pathways that lead to the expression of different phenotypes, namely those of absorptive and/or mucosal enterocytes. This suggests that the conditions that allow the differentiation of the HT-29 cells in culture are not inductive but rather permissive conditions. When the cultures are grown under conventional nutritional conditions in the presence of glucose, the differentiation programs presumably are inhibited.

The differentiation of the uncloned HT-29 cell line that takes place under glucose depletion has been reported to be a reversible phenomenon. When a differentiated population of HT-29 cells was trypsinized and seeded back into a glucose-containing medium, the cell culture dedifferentiated (Pinto, 1982; Zweibaum, 1985). However our HT29-18 clone responded differently. After replating its differentiated form in the presence of glucose, we were able to sort from undifferentiated cells subclones that continuously display differentiated phenotypes: the HT29-18-C_1 subclone which has a villus columnar phenotype and the HT29-18-N_2 subclone which has a gobletlike phenotype. In these reversion experiments most of the differentiated HT29-18 cells exhibited complete dedifferentiation as reported above for the parent cell line, but a significant and apparently random fraction of the cell population (20%) underwent an incomplete and stochastic reversion of their enterocyte-like phenotypes. It is difficult to establish from our studies if the subclones have undergone an incomplete reversible dedifferentiation or if they are locked in a differentiated state allowing us to cultivate them either in glucose or in galactose-containing media without adaptation. It is clear that some if not most of the differentiated character of the clones is persistent. For example, in contrast to the parent cell line and to the HT29-18 clone it has to be pointed out that the subclones are polarized both in glucose- and in galactose-containing media, but the level of differentiation is enhanced in galactose-containing medium. The HT29-18 clone divides rapidly in glucose (doubling rate = 20 h) but very slowly in galactose culture media (doubling rate = 72 h). Interestingly, the subclones display a slow doubling rate (~50 h) in glucose, which is not modified in a galactose medium in case of HT29-18-N_2 subclone but is prolonged to 72 h for the HT29-18-C_1 subclone. Furthermore sucrase-isomaltase is only found in some HT29-18-C_1 cells in galactose-containing medium. The heterogeneity of this subclone in galactose obviously indicates that its ability to differentiate is limited to the absorptive phenotype but still can sustain the expression of at least two differentiation programs.

The use of the HT29-18 clone and related subclones is of special interest for further studies on cell differentiation and cell polarity. This system has two advantages. First the multipotency of the HT29-18 clone is strikingly reminiscent of the multipotency of the stem cell in the intestinal crypt as suggested by Cheng and Leblond (1974). In their hypothesis the stem cell can generate the four main enterocytic cell types: absorptive cells, mucous cell, endocrine cells, and Paneth cells. This is summarized in Fig. 10 where we also propose by analogy that the HT29-18/glucose clone could partially mimic the behavior of the intestinal stem cell. This

scheme could be further validated if, in the future, subclones exhibiting the phenotype and some properties of Paneth cells and/or enteroendocrine cells could be obtained from HT29-18 and induced to differentiate in culture. In this respect it is noteworthy that in serum-free medium the original HT-29 cell line can produce lysozyme (Alderman et al., 1985), a biochemical marker for Paneth cells. However, clear evidence in favor of the ability of HT-29 to synthesize and to secrete gastrointestinal hormones is still lacking. The existence of appropriate immunological and biochemical markers (Buckley et al., 1985; Alderman et al., 1985) for these quantitatively minor cell types of the intestinal mucosa together with the strategy presented here to isolate differentiated subclones of HT29-18 could allow us to document such subclones in the future. The second main advantage of these tissue culture lines is that they offer the possibility of studying the acquisition cell polarity. This process is very difficult to investigate in organs because of their cellular heterogeneity. With the different homogeneous and cloned populations we have isolated, it is possible to start from a totally undifferentiated state (i.e., the HT29-18/glu culture) and to study how cells undergo differentiation as absorptive cells (HT29-C_1 cultures) or as mucous-secreting cells (HT29-N_2 cultures). How cells acquire their brush borders and how proteins are assembled to build up this cellular organelle are questions pursued in our laboratory. Villin is one of the major actin-binding proteins of intestinal brush border that could play an important role in microvilli organization. In the following paper, results on villin synthesis and subcellular localization in the HT29-18 clone and its subclones are presented.

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