The Influence of Culture Time and Passage Number on the Morphological and Physiological Development of Caco-2 Cells (44093)

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Abstract. **The Caco-2 cell line is used by many investigators as a model of the intestinal epithelium to study nutrient uptake and transport. Our goal was to create an awareness of inherent variabilities in the Caco-2 cell line which may influence their suitability as a model or their application to specific problems. To study the influence of passage on the model, cultures were monitored from passage 20 to 109. Transepithelial electrical resistance (TEER) and sucrase activity (measured in 21-day-old cultures) increased through about passage 36. TEER values declined after about passage 60; sucrase remained elevated but variable. Cells at passage 22, 33, and 72 were grown simultaneously for 24 days. Older-passaged cells reached plateau phase sooner. Before Day 15, passage 72 cells had higher TEER and lower permeability to ^{14}C-mannitol than passages 22 and 33; however, after Day 15 all passages showed similar permeability. On Day 21, passage 72 cells had significantly lower alkaline phosphatase activity than did the other passages. Electron microscopy did not reveal any major morphological differences between the passages; however, it did show that some areas of cells grown on membranes were not monolayers but were several cells thick with varied morphology. Investigation of the formation of these multilayered areas showed them to be an inherent part of cell growth under the conditions used. These results emphasize the inherent variability in Caco-2 cell models and emphasize the need to monitor closely the culture characteristics during growth and differentiation under specific experimental conditions.** [P.S.E.B.M. 1997, Vol 214]

The colon carcinoma cell line, Caco-2, has received much attention in recent years for its use as an *in vitro* model of the intestinal epithelium. Monolayers of Caco-2 cells grown on permeable supports have been used to assess the transcellular movement of various substances including drugs (1–5), amino acids (6, 7), and metal ions (8–11). Our laboratory has used this technique to study the kinetics of transport of the essential trace nutrients zinc (12, 13) and manganese. (Finley JW, Monroe P, submitted).

In vitro models are usually chosen because they are simpler and have fewer inherent variations than the corresponding *in vivo* model. Caco-2 cells were originally established from a well-differentiated colon adenocarcinoma. In culture they spontaneously differentiate, both structurally and functionally, into cells resembling mature enterocytes (14). This differentiation occurs over a period of days (15), and it is possible that culture conditions and passage number may affect the length of this period. A survey of Caco-2 cell literature shows that, from laboratory to laboratory, there are inconsistencies in culture ages used for any given experiment. Our laboratory conducts transcellular movement experiments approximately 21 days after seeding. Other laboratories have used monolayers for nutrient transport studies after as few as 10 days (16) or as many as 30 days (6) of culture. There are also conflicting reports

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concerning the optimal range of passages at which cells should be used. Some investigators report using cells at passages less than 25 (17), whereas other investigators have used cells at passage 100 (18). A recent report showed that sucrase-isomaltase activity was maximized in cells beyond passage 100 (19).

The purpose of the present study was to monitor the structural and functional characteristics of our Caco-2 cells during the growth period and from different passages, to detect changes potentially significant to the use of these cells for transport studies. Our intent was to establish an optimal range of passages and identify the time frame within the differentiation process of our cells that would reduce variation between experiments. Also, Caco-2 cells are a preferred cell culture model of intestinal epithelium, compared with other cell types (e.g., HT29), because most investigators consider Caco-2 cells to be relatively free of the problem of multilayering. However, in our hands, multicellular protrusions are common in Caco-2 monolayers. The morphology of these protrusions is described here, and the influence they may have on the use of Caco-2 cells as a model of the gut epithelium is discussed. We also present data comparing phenol red diffusion and TEER to the traditional method for evaluating monolayer integrity, ^{14}C-mannitol diffusion.

Materials and Methods

Cell Culture. Caco-2 cells (passage 17) were obtained from ATCC and maintained as stock cells in Corning T flasks (Corning Costar Corp., Cambridge, MA).[3] Standard medium consisted of Dulbecco's modified Eagle's medium (DMEM, GIBCO Laboratories, Grand Island, NY) containing 25 m*M* glucose, 4 m*M* glutamine, 44 m*M* sodium bicarbonate, and 1% nonessential amino acids (GIBCO), supplemented with 10% fetal bovine serum (FBS). FBS was obtained from either GIBCO or Sigma Chemical Co. (St. Louis, MO). Batches of serum were assessed for their effect on Caco-2 growth curves and matched accordingly. The atmosphere was maintained at 36.5°C, 5% CO_2, and 90%–95% humidity. Cells were passaged weekly (0.25% trypsin [GIBCO], 5 m*M* EDTA, in Ca-Mg–free Hanks' balanced salt [Sigma]) and seeded onto new flasks at 15,000 cells/cm^2. Cultures were tested for mycoplasma according to the method of Chen (20) and found to be mycoplasma free.

Biochemical and Morphological Characteristics versus Passage. Cells were serially passaged from our working stock (passage 19) through passage 109. To monitor the biochemical and morphological characteristics of the monolayer at points during the passage series, cells at various passages were seeded onto each of six polyethylene terephthalate (PET) membranes (250,000 cells/membrane) with a 0.45-μm pore size (Falcon 3095; Becton Dickinson, Lincoln Park, NJ) and held in six-well plates (Falcon, Becton Dickinson). The membranes were previously coated with 50 μg of rat tail type 1 collagen (Collaborative Biomedical Products, Bedford, MA) to improve cell attachment and growth. Medium (apical:1.5 ml, basolateral:2.5 ml) was changed every two days. On Day 20 ± 1 day, transepithelial resistance (TEER) of each membrane was measured. Then two membranes were fixed for electron microscopy, and four prepared for sucrase activity.

Growth Curve. Cells were seeded onto collagen-coated membranes and cultured as described above. Cells were harvested by trypsinization on selected days (see legend for Fig. 3); estimates of cell numbers were made by using a hemacytometer.

Growth and Development of Caco-2 Cultures. The effect of serial passage on the growth and development of the monolayer throughout the 21-day culture period was investigated similarly. Cells from passage 20, 31, and 70 were thawed, cultured in flasks, then passaged onto PET membranes. The growth media for this experiment contained 50 μg of gentamicin/ml (Sigma). Membranes and/or cells were analyzed for TEER, transepithelial movement of ^{14}C-mannitol and phenol red, sucrase and alkaline phosphatase activity, and total protein at various days (see figure legends). Additionally, some membranes were prepared for transmission electron microscopy (TEM), scanning electron microscopy (SEM), and image analysis.

Measurements of Monolayer Integrity: Mannitol Flux and Phenol Red Diffusion. Growth medium was removed, both the monolayer and well were rinsed with phosphate-buffered saline (PBS), and 0.94 kBq of ^{14}C-mannitol (5.2 μ*M*) (ICN Radiochemicals, Irvine, CA) in DMEM and 10% FBS were added to the apical membrane. Basolateral medium (phenol red free) was supplemented with 5.2 μ*M* mannitol (Sigma). Monolayers were incubated for 60 min (37°C, 5% CO_2, 90%–95% humidity) on a Mistral Multimixer (Labline Instruments, Inc., Melrose Park, IL) at 150 rpm. Radioactivity of basolateral medium was determined in a liquid scintillation counter (Beckman LS-6800, Beckman Instruments, Inc., Fullerton, CA). The diffusion of phenol red across the monolayer was determined by measuring absorbance at 558 nm, after adjusting the pH of the basolateral medium to 11, incubating the medium for 1 hr, and pelleting the precipitate.

Measures of Membrane Integrity: Electrical Resistance. Monolayer integrity on six membranes was measured by using the Millicell Electrical Resistance System equipped with STX-2 electrodes (Millipore Corp., Bedford, MA). The electrodes were equilibrated

[3] Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.

in culture medium and the resistance meter allowed to stabilize for 1 hr before use, to minimize voltage drift. Monolayers with medium were allowed to cool to room temperature for 30 min before resistance measurements were taken. Four positions on each membrane were measured in duplicate. A lid to the six-well plate was modified to hold the electrodes securely in place during readings. The average resistance of two membranes without monolayers was subtracted from the average resistance of each membrane containing cells. The resistance of the monolayer was multiplied by the effective growing area to obtain the electrical resistance of the monolayer ($\Omega \cdot cm^2$).

Electron Microscopy.

Transmission electron microscopy. Cultures of Caco-2 cells from passages 22, 33 and 72 were preserved for transmission electron microscopy (TEM) examination on Days 1, 2, 3, 5, 7, 9, 12, 15, 18, and 21. Two duplicate cultures on membranes at each time point and from each passage were fixed with 1.8% glutaraldehyde in 0.1 *M* cacodylate buffer with 0.05% $CaCl_2$ at pH 7.4. The cells were washed in 0.1 *M* cacodylate buffer with 0.05% $CaCl_2$ and 0.18 *M* sucrose. The monolayers were treated with 1% osmium tetroxide in the same buffer but without sucrose. The cells were dehydrated with ethanol, washed in propylene oxide, and embedded in an Epon-812 substitute. The embedded cultures were examine with both stereo- and compound microscopes. Regions for sectioning were selected with regard to positional location (center, edge, etc.), culture homogeneity (high and low cell density), and for specific features (cell clumps and topographical protrusions). Selected areas were cut from the embedment discs with a jeweler's saw and mounted for sectioning in the apical-basal plane. Silver to gold thin sections of the embedded cells were mounted on bare grids (mesh 200), stained with uranyl acetate and lead citrate, and examined with a Philips EM300 transmission electron microscope at 60 kV.

Scanning electron microscopy. Support membranes were cut into quarters and processed as above through the ethanol dehydration stage. The pieces of membranes with cells were then critical-point-dried (Auto-Samdri 810; Tousimis Corp., Rockville, MD) and mounted on standard aluminium stubs with double-stick cellophane tape. The samples were sputter coated with a mixture of gold and palladium (Hummer V; Anatech, Alexandria, VA), and examined with a scanning electron microscope (AMR1000; Amray Corp., Bedford, MA) operated at 20 kV.

Image Analysis. Before sectioning, embedded cultures were examined by image analysis. Embedded disks were focused at 3× with a stereozoom microscope (Baush & Lomb), and the image transferred *via* a Chalnicon camera to a Cambridge Quantimet 970 Image Analyzer. Field area measurements were taken of binary images of the multilayered regions.

Enzyme Assays. Cultures were rinsed with PBS and scraped from the membranes. Cell suspensions in 1–2 ml of PBS were sonicated with three 5-second bursts at 75 watts (W-380 Ultrasonic Processor; Heat Systems-Ultrasonics, Inc., Farmingdale, NY) and assayed for sucrase activity using a modification of Messei & Dahlquist (21). Glucose production was quantified by using a Sigma Glucose Kit (Cat. #510-A). The cell suspension was assayed for protein by using bicinchoninic acid (Sigma).

Statistics. Biochemical and morphological changes over passage were analyzed by one-way analysis of variance (ANOVA) for each experiment (22). Effects of passage on growth and development of Caco-2 cultures were analyzed by one-way ANOVA for each day of the experiment. Pairwise comparisons were conducted following significant ANOVAs by using Tukey's contrasts. Logistic models of the form: y = Minimum + Range/{1 + exp[a − b*ln(day)]} were fit to the growth curve data. Indicator variables were used to determine if the coefficients (Minimum, Range, a, b) of the model differed significantly for each passage. Linear regression analysis was used to determine the relationships between TEER, ^{14}C-mannitol, and phenol red. Indicator variables were included in the models for TEER versus ^{14}C-mannitol and TEER versus phenol red to allow the direct estimation of the inflection points (23). All statistical analyses were performed by using the SAS statistical software package (24).

Results

Biochemical and Morphological Characteristics versus Passage. The change in TEER over passages 20 to 109 is shown in Figure 1. In Experiment 1, electrical resistance increased significantly ($P < 0.05$)

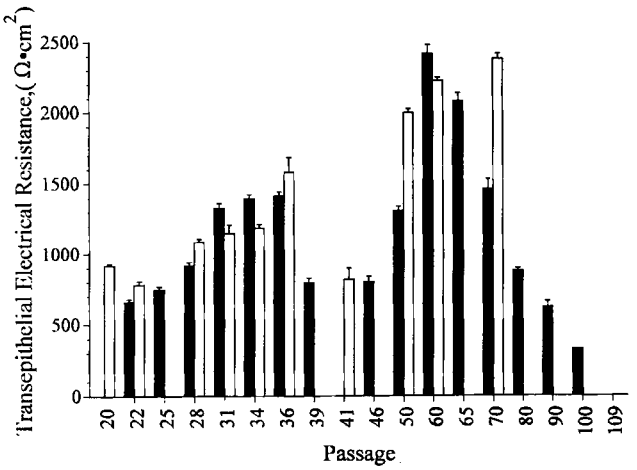

Figure 1. Effect of increased passage number on transepithelial electrical resistance in Caco-2 cell monolayers grown for 21 days on porous membranes. Six membranes measured at each time point for each experiment (mean ± SEM). Solid bars, Experiment 1; open bars, Experiment 2.

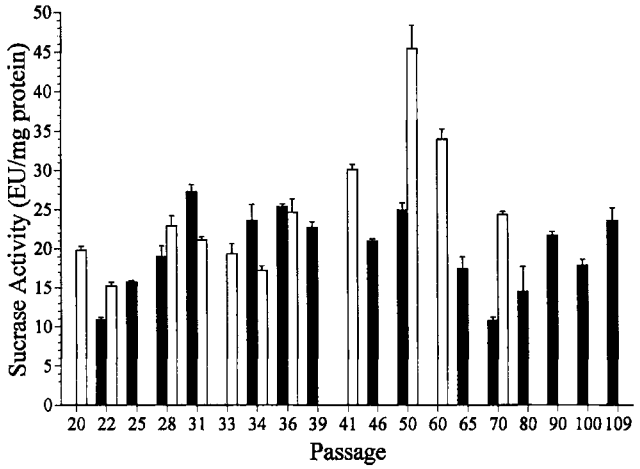

Figure 2. Effect of increased passage number on sucrase activity in Caco-2 cell monolayers grown for 21 days on porous membranes. Four membranes measured at each time point for each experiment (mean ± SEM). Solid bars, Experiment 1; open bars, Experiment 2.

from early passage 22 to passage 28. TEER continued to increase through passage 36. Following this initial increase, TEER was variable until around passage 70, after which there was a significant decline ($P < 0.05$), until passage 100. Essentially the same pattern was noted when the experiment was repeated one year after the original study (Experiment 2). Data past passage 70 from the second trial was not available at the time of this writing.

Sucrase activity (Fig. 2) also increased significantly ($P < 0.05$) from passage 22 to 28. The activity plateaued at passage 31 and remained at this level until passage 65. Experiment 2 showed greater variability but the same trend.

Growth Curve. Cells from passage 72 had a shorter lag period than those from passage 22. Population doubling times of passage 22 and 72 were not significantly different and averaged 1.5 days. Cells from passage 22 were in exponential phase longer than passage 72 cells, with passage 72 reaching plateau phase 6 days before passage 22. The extended exponential phase of passage 22 cells resulted in 1.9 times more cells than from passage 72.

Protein data from the experiment "Growth and Development of Caco-2 Cultures" (Fig. 3B), described below, also showed the same pattern; that is, total cellular protein from passage 72 reached a plateau faster (Day 8) than passage 22 (Day 12). Also, by Day 21 cultures of passage 22 contained 2-fold more total protein than those of passage 72.

Growth and Development of Caco-2 Cultures. Several passages (which had been frozen previously) were examined simultaneously to compare their biochemical characteristics under identical circumstances. Passage 22 was selected because it was the earliest passage that had been used in our experiments; passage 33 because that seemed to be the point at which cells had reached a plateau in TEER and sucrase activity (Figs. 1 and 2); and passage 72 because it was the oldest passage available at the time of the experiments.

Cultures from passage 72 exhibited a faster initial increase in TEER (Fig. 4) over the first 12 days than did cells from passage 33 or 22 (ANOVA, $P < 0.05$). After 12 days, TEER values began to plateau, but on day 24 passage 72 still had a significantly higher TEER than lower passages. The differences in TEER were also paralleled by differences in permeability of the monolayers to ^{14}C-mannitol (Fig. 5). Compared with lower passages, cells from passage 72 had significantly lower permeability to ^{14}C-mannitol on 6 of 8 days examined, including Days 12–26. However, it should be noted that ^{14}C-mannitol permeability was ≤0.5% for all passages from Day 15 onward.

Sucrase activity in cells from passage 72 increased more rapidly than did activity in cells from passage 22 or 33 (Fig. 6). However, activity in cells from passages 72 and 22 began either to plateau or to decrease after

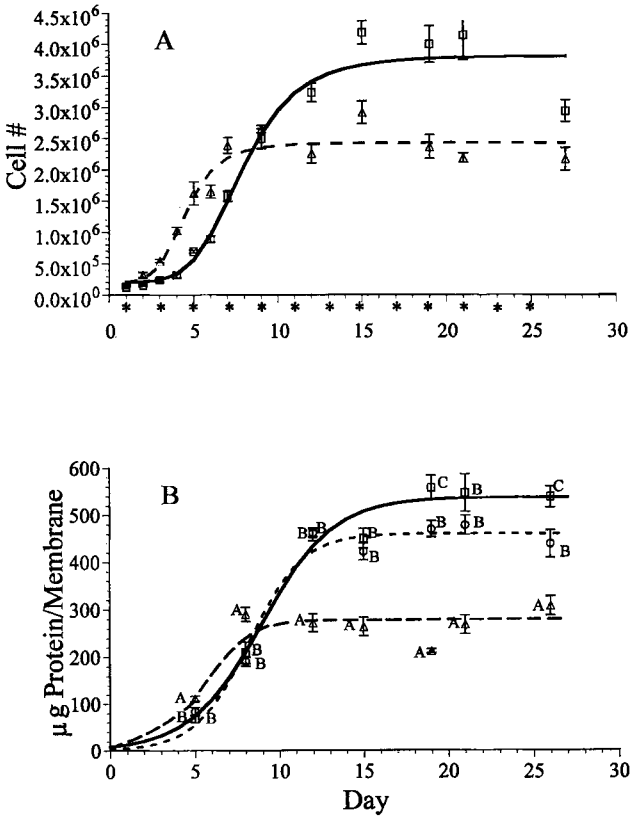

Figure 3. (A) Growth curve of Caco-2 cells from different passages. Open triangles, dashed line, passage 72; open squares, solid line, passage 22. Values are mean (n = 3) ± SEM. *Media changes. (B) Effect of passage and day of development on total protein per membrane of Caco-2 cells. Open squares, solid line, passage 22; open circles, dashed line, passage 33; open triangles, dashed line, passage 72. The values are mean ± SEM (n = 4–6) of one experiment. Another experiment confirmed these findings. Means within a single passage with the same letter are not significantly different ($P > 0.05$).

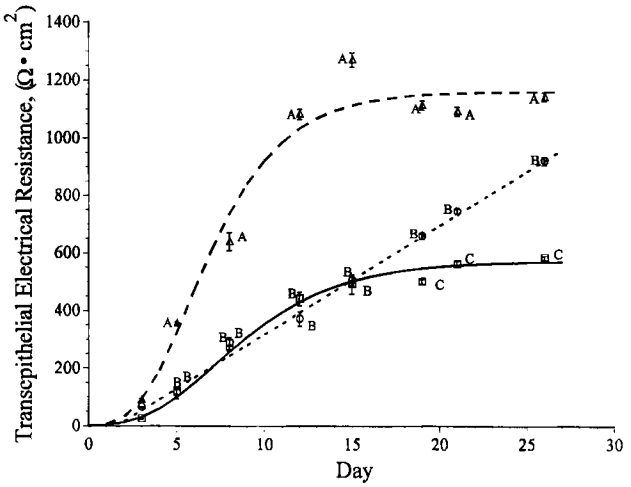

Figure 4. Effect of passage and day of development on transepithelial electrical resistance of Caco-2 cells. Open squares, solid line, passage 22; open circles, dashed line, passage 33; open triangles, dashed line, passage 72. The values are means ± SEM (n = 4–6) of one experiment. A second experiment confirmed these findings. Means within a single passage with the same letter are not significantly different ($P > 0.05$).

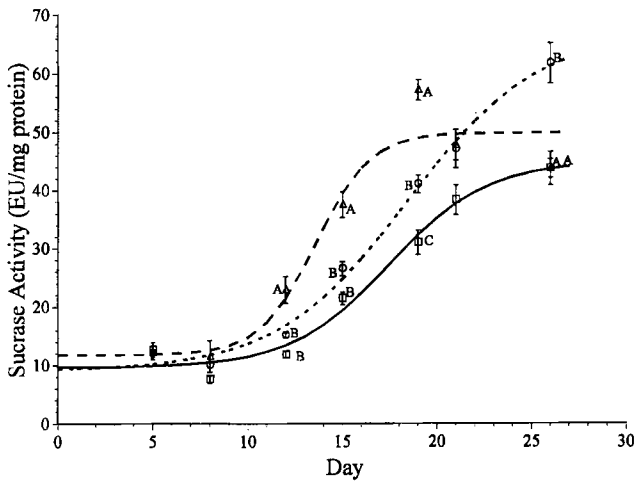

Figure 6. Effect of passage and day of development on sucrase activity of Caco-2 cells. Open squares, solid line, passage 22; open circles, dashed line, passage 33; open triangles, dashed line, passage 72. The values are means ± SEM (n = 4–6) of one experiment. Another experiment confirmed these findings. Means within a single passage with the same letter are not significantly different ($P > 0.05$).

Day 21, whereas passage 33 activity continued to increase linearly. Sucrase activity in cells from passage 22 was consistently lower than that from higher passages.

Alkaline phosphatase activity (Fig. 7) was the measured parameter most affected by passage. On 5 of the 6 days studied, cells from passage 72 had the lowest alkaline phosphatase activity, and at the end of the study activity from high-passaged cells was only 34% of the average activity of passage 33 or 22 cells.

Correlations between TEER, ^{14}C-Mannitol Flux and Phenol Red Flux. The standard method for evaluating monolayer integrity is the monitoring of ^{14}C-mannitol diffusion across the monolayer. Figure 8 shows the relationships between TEER, ^{14}C-mannitol, and phenol red diffusion. Phenol red diffusion correlates positively to ^{14}C-mannitol diffusion (Fig. 8A); this indicates that the diffusion of phenol red across the monolayer accurately predicts the integrity of the monolayer. TEER values correlate inversely with ^{14}C-mannitol diffusion (Fig. 8B) and phenol red diffusion (Fig. 8C) up to an inflection point at about 500 Ω · cm², as shown in Figure 8B. At the inflection point, ^{14}C-mannitol diffusion was

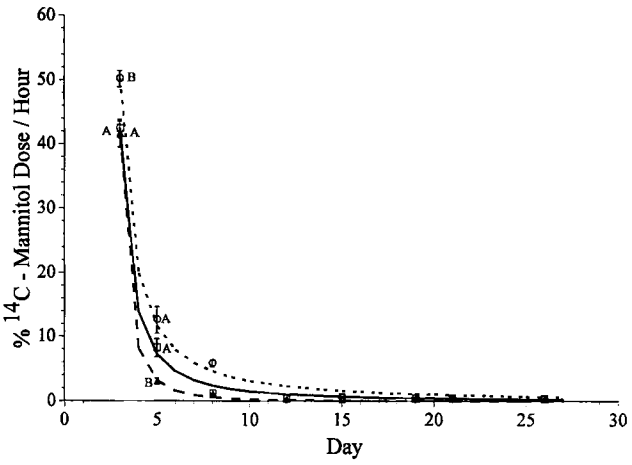

Figure 5. Effect of passage and day of development on diffusion of ^{14}C-labeled mannitol across monolayers of Caco-2 cells. Open squares, solid line, passage 22; open circles, dashed line, passage 33; open triangles, dashed line, passage 72. The values are means ± SEM (n = 4–6) of one experiment. A subsequent experiment confirmed these findings. Means within a single passage with the same letter are not significantly different ($P > 0.05$).

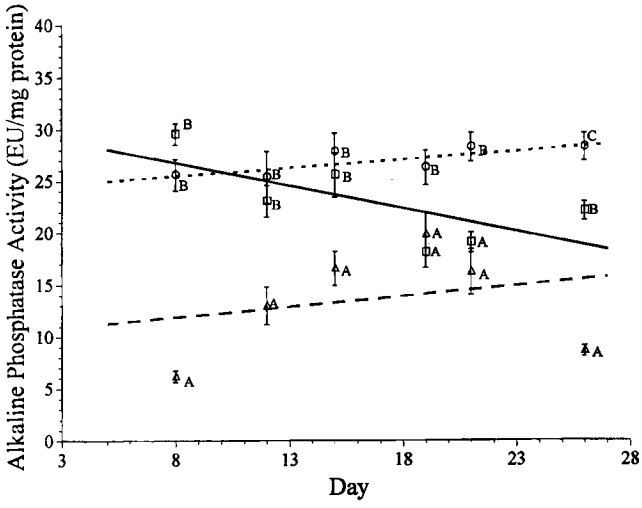

Figure 7. Effect of passage and day of development on alkaline phosphatase activity of Caco-2 cells. Open squares, solid line, passage 22; open circles, dashed line, passage 33; open triangles, dashed line, passage 72. The values are means ± SEM (n = 4–6) of one experiment. Another experiment confirmed these findings. Means within a single passage with the same letter are not significantly different ($P > 0.05$).

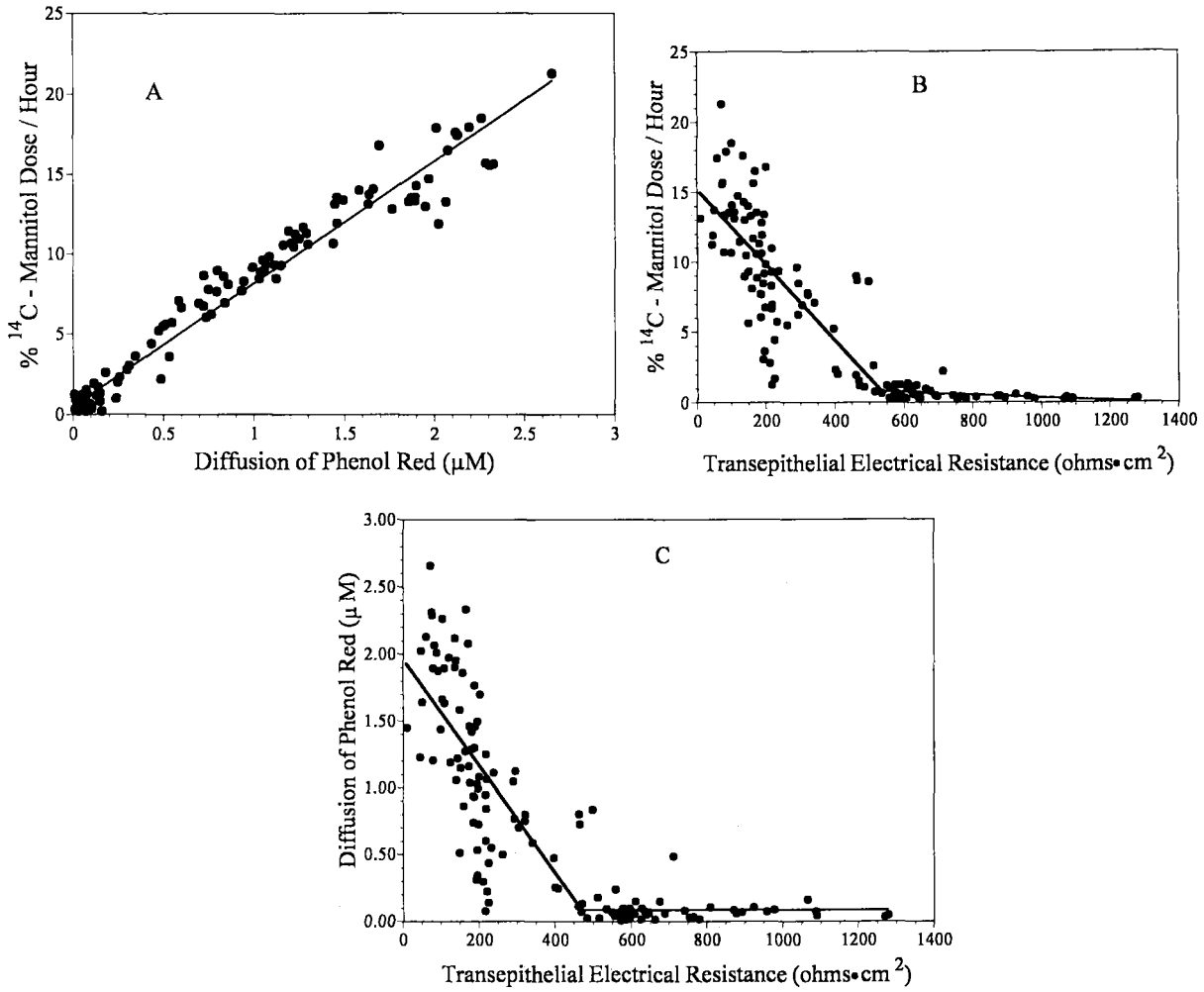

Figure 8. Correlations between transepithelial electrical resistance (TEER), diffusion of ^{14}C-mannitol and diffusion of phenol red across monolayers of Caco-2 cells. (A) Correlation between ^{14}C-mannitol and phenol red diffusion, $r^2 = 0.96$, $P < 0.05$. (B) Correlation between ^{14}C-mannitol diffusion and TEER, $r^2 = 0.77$, $P < 0.05$. (C) Correlation between phenol red diffusion and TEER, $r^2 = 0.74$, $P < 0.05$.

near zero. Therefore, increasing TEER values above 500 $\Omega \cdot cm^2$ are not related to ^{14}C-mannitol diffusion (i.e., cell layer integrity).

Electron Microscopy. The developmental status of cultures was evaluated from examination of several areas of each culture. The micrographs and descriptions presented are representative of the general overall condition of a culture at a particular time point.

The first day after seeding, cultures were composed of both rounded and flattened cells, as well as clumps of cells showing varying degrees of attachment to each other. Flattened cells exhibited extreme thinning, ruffled membranes, very long and narrow-filopodial extensions, and relatively large areas of non-contact with the substratum. These characteristics are consistent with cell motility; thus, the observed cell-cell associations imply that these cells can move over and around each other (Fig. 9A). By Day 2, cultures consisted almost entirely of flattened cells with many of the cells in layers up to six deep.

Initial indications of cell polarity appeared at Day 2 as apical junctional complexes (AJC) were apparent between many cells (Fig. 9B), and cytoplasmic inclusions and organelles were asymmetrically distributed. Cell polarity was well established by Day 5. AJCs, apical interdigitations, and typical intercellular spaces were consistently observed between adjacent cells. Early brush border development was apparent and some cells had begun to assume a columnar morphology (Fig. 9C).

By Day 7, very few flattened cells were present; cells had begun to take on a differentiated appearance, and most of the support membrane was covered by cells. Cells with columnar morphologies were common in cultures from both high and low passages at this time. In the interval of Days 7–21, cells showed increasing signs of differentiation and development of an enterocyte morphology. Cells became increasingly columnar in appearance, the brush border became more apparent and more uniform (Fig. 9, E–H), and intercellular spaces became more well defined. AJCs, which first appeared

as early as Day 2, were identifiable at every site of apical surface apposition by Day 21. They were seen in association with variable degrees of cell membrane interdigitation (Fig. 9D). After Day 15, no specific changes were observed, only a steady increase in the degree of differentiation within the culture.

The different cell passages were again compared on Day 21, because this has been the day on which we have routinely used monolayers for nutrient uptake and transport studies. No morphological differences between the cells from different passages were apparent on Day 21.

One aspect of Caco-2 cell development not previously described in the literature, but observed in this study, was the development of multicellular "bump-like" areas which protruded from the monolayer (Fig. 10, A and B). These multicellular protrusions within the monolayer appeared as various topographies, taking the form of thickened ridges, rounded masses, and, in extreme cases, polyp-like projections arising from the monolayer on a thin stalk (Fig. 10, A and C). Image analysis of these cultures revealed that approximately 3%–18% of any given insert membrane was covered by multilayered regions of undetermined thickness (data not shown). These structures were observed, in sections, to range in thickness from 2–3 cells to 20 or more cell profiles from the apical surface to the support membrane.

The surface of the multicellular protrusions were covered with cells bearing a typical brush border, attached to each other with apical junctional complexes (Fig. 10D). This uniform surface was continuous with and integrated into the monolayer proper of the Caco-2 cultures. Multicellular protrusions were frequently penetrated by channels or cavities, which were lined by characteristic brush border microvilli (Fig. 10E). Channels were observed to open onto the multicellular protrusion's apical surface, but never onto the support membrane. Within the multicellular protrusions, the intercellular spaces were not different in appearance and spatial configuration from the basolateral spaces of the monolayer (Fig. 10C). These protrusions were observed as early as Day 5, increased in number and size during the interval of Day 9–15, and were especially evident by Day 21.

The earliest multicellular protrusions were observed in areas of highest cell density, and it was ob-

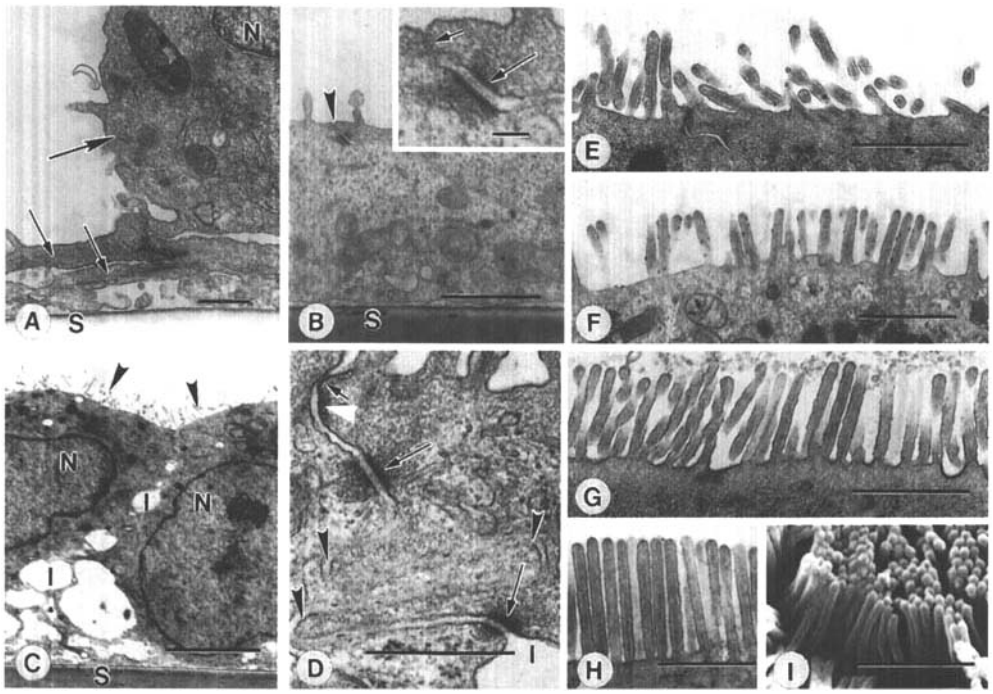

Figure 9. Development and maturation of Caco-2 cultures. (A) At 24 hr after seeding, cultures are composed of both flattened (small arrows) and rounded cells (large arrow). The flattened cells have features characteristic of motile cells. The support membrane (S) and a cell nucleus (N) are indicated. Bar, 0.5 μm. (B) As early as 2 days after seeding features resembling apical junctional complexes are present at points of contact between some of the flattened, undifferentiated cells (arrowhead). The area indicated by the arrowhead is shown at higher magnification (inset). The areas corresponding to the tight junction (short arrow) and the demosome (long arrow) are indicated. Compare with Figure 9D. The support membrane (S) is labeled. Bar, 1.0 μm; inset bar, 0.1 μm. (C) By day 5 areas of monolayer demonstrate the beginnings of characteristic intercellular spaces (I) and irregular brush border microvilli (arrowheads). Nuclei (N) and the support membrane (S) are indicated. Bar, 5.0 μm. (D) A typical apical junctional complex is shown between cells of a Day 21 culture. As a feature of differentiation, the apical junctional complex is composed of a region of tight junction (short arrow) and typical *zonula adherens* (white arrowhead), and is usually associated with desmosomal attachments (long arrows). The junctional complex is almost always associated with close interdigitations of the apical lateral cell membranes (arrowheads). The lateral intercellular space (I) is indicated. Bar, 0.5 μm. (E–H) The progression of differentiation of the microvillar brush border is shown by representative TEM images of the apical cell surface on Days 7, 9, 15, and 21 respectively. Bar, 1.0 μm. A surface view (by scanning electron microscopy) of the Day 21 brush border is shown in Panel I. Bar, 1.0 μm.

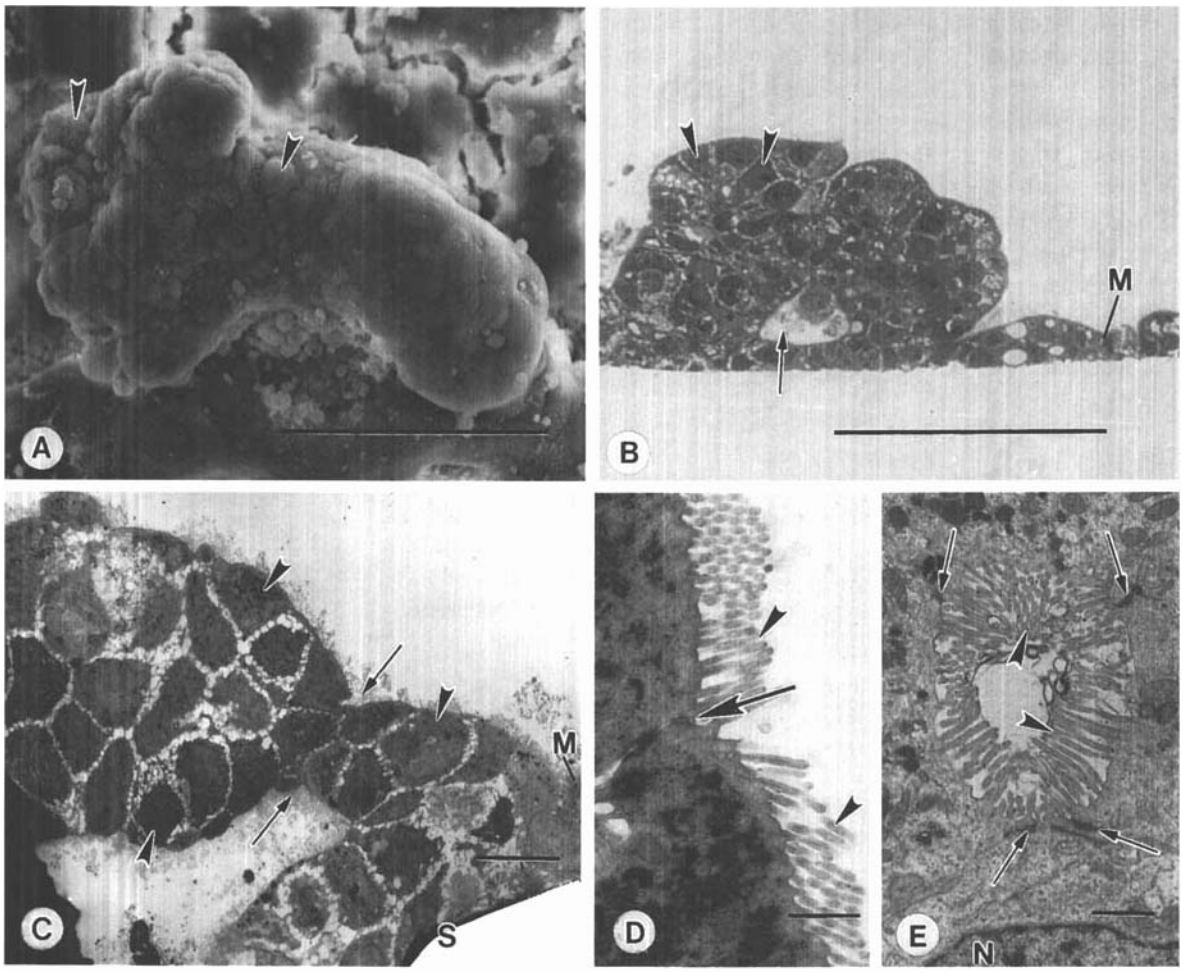

Figure 10. Multicellular masses (bumps) arising from the monolayer of Caco-2 cultures. (A) A multicellular bump is shown (by scanning electron microscopy) protruding from the monolayer of a day 21 culture. Individual cell surfaces are identifiable (arrowheads). Bar, 100 μm. (B) A section through a bump is shown in a light micrograph. The plane of section is perpendicular to the support membrane. Individual cells are apparent (arrowheads), the monolayer (M) of the Day 21 culture is indicated and an open space or channel (arrow) can be seen at the base of the bump. Bar, 100 μm. (C) The bumps present a variety of topographic forms; here a polyp-like profile at Day 15 is shown by TEM, with an approximately spherical mass attached to the monolayer (M) by a narrow connection (arrows). This polyp-like morphology is also identifiable in three dimensional surface views. Individual cells (arrowheads) and the position of the support membrane (S) are indicated. Bar, 10 μm. (D) A higher magnification view (TEM) of the surface of a typical bump, at Day 15, demonstrates well developed brush border microvilli (arrowheads) and a typical apical junctional complex (arrow). Bar, 1.0 μm. (E) A narrow channel is shown on the interior of a bump from a Day 9 culture (TEM). The channel is lined by brush border microvilli (arrowheads). Densities of intercellular attachments (arrows) typical of apical junctional complexes indicate that this channel is composed of the apical-like surfaces of at least four cells. A cell nucleus is indicated. Bar, 1.0 μm.

served that clumps of incompletely dissociated cells with differentiated characters were also more common in these regions immediately post-seeding. By Day 7 there were many more of these protrusions than there were clumps associated with seeding, and multicellular protrusions were observed in areas of the support membrane that had only recently become covered with cells (i.e., areas where cells did not initially adhere to the membrane at seeding).

Discussion

Changes in Caco-2 cell morphology and biochemistry, as a consequence of increased passage, have been reported (19, 25, 26). Results of the present study demonstrate increased growth rate, TEER, and sucrase activity as passage increases. We also observed, by phase contrast microscopy that as passage number increases, the morphology of Caco-2 stock cultures at confluence becomes more homogeneous. Our observation that alkaline phosphatase activity decreased in high passages in contradictory of a previous report. Based on our results, we believe that the optimum passage range for experimental purposes is 28–65.

Caco-2 cells are known to be a heterogeneous mixture of cell morphologies, and isolation of different clones of Caco-2 cells has shown that different biochemical characteristics can be selected from the general population (19, 26). An explanation for the passage-related changes described in this and other studies may lie in a selection process occurring at subculture. The com-

plexity of the *in vitro* requirements and the options available in manipulation of protocols allow for considerable variations in the system between different laboratories or even between passages in one laboratory. The degree of confluence and differentiation at passage, the density at which cells are seeded, the potential for seeding undissociated clumps, the efficiency of attachment (to the substratum), and variation in media are some factors that could favor the selection of a predominant clone over the course of passages. Variable conditions may result in an inconsistency in culture characteristics, with poor experimental reproducibility and ambiguous results.

Another aspect of the Caco-2 cell line that has the potential for introducing variation into experimental conditions is the course of growth and differentiation of a given culture. There is considerable variation in opinion in the literature as to the optimum time following seeding that provides a dependably differentiated culture for experimentation. Some investigators use Caco-2 cells as early as 6–9 days after seeding (27) and others feel that 30 days is better (6). Our data tell us that, under our conditions, the majority of the monolayer has differentiated by Days 15–18. At this time, sucrase and alkaline phosphatase activity are near maximum and cell numbers have stabilized, indicating that the proliferative stage is over. For purposes of uniformity in cell number and density, and for optimizing enzymatic properties, we routinely use our cells as enterocyte models at 18–21 days post-seeding.

Another criterion used widely to assess the development and integrity of Caco-2 cultures on porous supports is the TEER measurement. We found that TEER values correspond to ^{14}C-mannitol diffusion up to a TEER value of 500 $\Omega \cdot cm^2$. TEER values above this inflection point are related to something other than the functional tight junctions of the cell layer. This increase could be effected by the continued columnar development of the cells or differentiation of the brush border and/or its associated glycocalyx. If the tight junction is viewed as a sealing structure, the increased columnarization of the cells should result in an increase in the length of tight junction relative to the apical surface area. This type of change has been correlated with an increase in TEER (28).

Phenol red diffusion has been used previously to assess tight junction formation in renal epithelial cells (29). The method described in this study for measuring phenol red diffusion is shown to closely parallel the standard radioactive method for defining monolayer integrity. As phenol red is a normal constituent of most media, is not radioactive and can be collected without risking contamination of the culture, we find it a simplified and dependable alternative to radioactive mannitol for evaluation of monolayer integrity.

When Caco-2 cells are subcultured, some cells tend to remain in clumps which may still display differentiated characteristics. The fate of these clumped cells is not known, but phase contrast observations of developing cultures show that cells at the site of a clump go through changes involved in differentiation earlier than the rest of the culture.

The seeding of clumps has been of particular interest with regard to the development of multicellular protrusions or thickenings within the monolayer. Although other colon carcinoma cell lines have been reported to grow undifferentiated multilayers (30), there have been few reports of this phenomenon in Caco-2 cells (31). Riley *et al.* (31) reported that Caco-2 cells grown on floated filter assemblies, with changes of the basolateral but not the apical medium, formed multilayers with protrusions. The present study shows that these protrusions develop under standard culture conditions. During the development of a culture, these protrusions or bumps are first observed in the center and around the periphery of the support membrane which are the regions where single cells and cell clumps tend to aggregate during seeding. Protrusions, however, developed in all areas of the membrane as cell coverage increased and by Day 21 were found in areas that initially had low concentrations of cells. Therefore, protrusions do not seem to result from the seeding of clumps of attached cells, or as a consequence of seeded cells accumulating in one area of the membrane, and, under our conditions, are apparently an inherent feature of the cell line.

The consistent occurrence of thickened regions within cultures, at an incidence sometimes approaching 20% of support membrane surface area, may be of significance in the evaluation of transport dynamics. The surfaces of thickened areas are made up of typical polarized epithelial cells and there is no reason to suspect that they differ from the monolayer proper. In experimental situations that rely on comparative data among duplicated cultures, the thickened regions probably would not be a significant factor. Image analysis showed that cultures from a single seeding operation were very similar in regard to the amount of multicellular protrusions but cultures from separate seedings were less similar.

Experimental situations relying on a knowledge of surface area values, as in determining absolute rates of transport, might be more seriously affected. Image analysis shows that these multicellular features occupy 3%–18% of the surface area of a culture, but other factors related to surface area must also be considered. These areas are variable in thickness (2–20+ cells), they are of varied shape (bulbous, folded, ridges, polyp-like), and they are penetrated by channels that open onto the surface of the culture. These channels, which have not been seen to open onto the support membrane, are lined with typical polarized epithelial cells with a brush border, representing an extension of the culture surface;

consequently, an indeterminable amount of surface area exists additional to that measured by image analysis. As a result, rates based on the surface area of the support membrane would tend to be higher than actual cell surface based values, affecting not only the actual values but also reproducibility of data.

We have described several aspects of Caco-2 cell line behavior and development that can introduce variability into experimental condition. While it is not always possible to control every variable, the goal should be to optimize and simplify conditions appropriately, whenever possible. Even though *in vitro* systems are not perfect, with careful examination and manipulation they can provide the most controllable situations for the examination of cellular function.

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