The development of a method for the preparation of rat intestinal epithelial cell primary cultures

G. S. EVANS1, N. FLINT1, A. S. SOMERS1, B. EYDEN2 and C. S. POTTEN1

1Cancer Research Campaign Department of Epithelial Biology, Paterson Institute for Cancer Research, Christie Hospital and Holt Radium Institute, Christie Hospital, Withington, Manchester, M20 9BX, UK
2Department of Pathology, Christie Hospital and Holt Radium Institute, Withington, Manchester M20 9BX, UK

*Author for correspondence

Summary

We describe a reproducible method for growing small intestinal epithelium (derived from the suckling rat intestine) in short-term (primary) cultures. Optimal culture conditions were determined by quantitative assays of proliferation (i.e. changes in cellularity and DNA synthesis). Isolation of the epithelia and, significantly, preservation of its three-dimensional integrity was achieved using a collagenase/dispase digestion technique. Purification of the epithelium was also facilitated by the use of a simple differential sedimentation method.

The results presented below support the idea that proliferation of normal gut epithelium ex vivo is initially dependent upon the maintenance of the structural integrity of this tissue and upon factors produced by heterologous mesenchymal cells. Proliferation in vitro was also critically dependent upon the quality of the medium and constituents used.

Cultures reached confluence within 10-14 days and consisted of epithelial colonies together with varying amounts of smooth-muscle-like cells. Cultures have been maintained for periods up to one month, but the longer-term potential for growth by sub-culturing has not been examined. Strategies for reducing the proliferation of these non-epithelial cells are also described.

Key words: intestine, epithelial, in vitro, smooth muscle cells.

Introduction

The maintenance of proliferation of isolated intestinal epithelial cells ex vivo has proven to be a major limitation in the advancement of the understanding of growth factor function in this tissue. There are many epithelial cell lines derived from colonic adenocarcinoma (for recent review see Moyer et al., 1990) and there have been occasional successes in establishing permanent epithelial lines from the intestine of suckling animals (Quaroni and May, 1980; Blay and Brown, 1984; Négrel et al., 1983). In these instances, the epithelial cells were selected (using cloning cylinders) from cultures containing a mixture of cell types that formed over a period of weeks following the initial digestion of the intestine in collagenase. Normal or transformed cell lines cannot be considered adequate models of the intact epithelium, since these cells have undergone changes and selection to facilitate long-term growth in vitro. This does not preclude their use for experimental studies, but they clearly represent a different type of model to that of primary cultures. Consequently there is a need to develop methods for propagating freshly isolated epithelium on a short-term basis, allowing reproducible quantitative studies. Our ultimate aim is to identify growth factors and extracellular matrix components that are required to maintain cell proliferation, differentiation and ultimately the function of stem cells in the gut epithelium.

The suckling rat intestine was chosen for these experiments because this is the stage of development where there has been success in establishing cell lines (Quaroni and May, 1980). The in vitro requirements of this primary culture system that have been examined below include the procedures for isolating the cells, quality of the culture medium, optimal serum conditions, CO2 concentration and pH, and the use of biological substrata. Where possible we have tried to take a quantitative, rather than anecdotal, approach to studying the culture conditions for these cells. The isolated cells were plated into 24 multi-well dishes and changes in cell proliferation were determined by assays of cellularity (crystal violet staining, which preferentially binds to the nuclei of fixed cells) and DNA synthesis (tritiated thymidine incorporation). Using these methods reproducible culture of intestinal epi-
Animals
Male and female six-day-old Wistar rats were used and these were housed under a twelve-hour light/dark cycle (light: 0700 to 1900 hours, GMT), and given food and water ad libitum. The animals were killed by cervical dislocation and the complete length of the small intestine was removed (and cleaned free of mesentery).

Culture reagents
The culture media tested were Minimum Essential Medium (single strength and 10× concentrate, with Earle's salts, and L-valine; Gibco BRL), high-glucose formulation Dulbecco's Modified Eagle's Medium (single and 10× concentrates of DMEM; Gibco BRL). Foetal calf serum (Biological Industries) was chosen from batch tests for growth stimulation of two rat suckling intestinal epithelial cell lines (IEC 6 and IEC 18; Quaroni and May, 1980).

Additives to some of the media included sodium pyruvate (Sigma), glutamine (Gibco), non-essential amino acids (Gibco), insulin (Sigma and Collaborative Research) and epidermal growth factor (EGF; Sigma); penicillin (Crystapen, Glaxo at 100 U/ml) and streptomycin (Evans Medical Ltd, at 60 μg/ml). Porcine mucosal heparin (Sigma H8514) was added at concentrations of 1-200 μg/ml. All reagents were culture grade.

Tissue culture flasks (T25 and T75) were purchased from Falcon, and 24 multi-well plates from Costar, Corning, Falcon and Nunc; gas-permeable dishes were obtained from Herareus. All of the cultures were incubated at 37°C in a humidified atmosphere in either a 5%, 7.5 or 10% CO2 incubator. The attachment factors examined were; 1-100 μg/ml fibronec-tin (purified rat, Boehringer), laminin (Boehringer), 10-50 μg/ml collagen IV (Collaborative Research), 200 μg/ml bovine dermal collagens (Vitrogen, Collagen Corporation), 200 μg/ml rat tail tendon collagens I + III (McAteer and Cavanagh, 1983) in addition, dishes were prepared with Matrigel (Collaborative Research), bovine corneal endothelial cell matrix (Gospadarowicz et al. 1980) prepared by a modified procedure (Yvonne Barlow, personal communication) using 0.5% Triton X-100 (Sigma) and 0.0125 M ammonium hydroxide (BDH), and also mouse Swiss 3T3 fibroblast matrix (Hedman et al., 1979). Collagen gels were prepared by the method described by McAteer and Cavanagh (1983).

Cell isolation
Various techniques were employed for isolating the epithelial cells, these including a modified Weiser (Weiser, 1973) procedure at 4°C and 20°C (Flint et al. 1991), 1-5 mM EDTA (disodium salt, BDH), 0.01-0.1% trypsin (Worthington Biochemicals) at 4°C and 20°C, crude collagenase (Sigma; Clostridium histolyticum type XI), dispase (neutral protease type I, Boehringer) and a combination of the crude collagenase and dispase (Fig. 1); for details of these methods see Table 1. Apart from the Weiser solution, all of these other agents were dissolved in Hanks' buffered salt solution (HBSS-, low calcium formulation; Northumbria Biologicals Ltd).

Cell proliferation and growth assays
Owing to the difficulty of estimating cell yield when the epithelium was isolated in the form of organoids (villi and crypts), it was necessary to standardise the input of cells into each well. On average, the intestine from a single animal was used for each 24-well plate. To assess the growth-promoting effects of different media, additives etc, the number of cells was estimated by the crystal violet staining method (Bra-saemle and Attie, 1988). The important advantage of this assay is that it is non-destructive, and after solubilization of
the dye, the cell cultures can be restained with different histological/histochemical dyes so that the different cell populations of cells can be observed.

For this assay, the cells were seeded into 24 multi-well dishes, with triplicate wells for each variable, after appropriate periods of time, the dishes were fixed in methanol then they were stained in 0.1% crystal violet. The wells were washed thoroughly in distilled water and the bound dye (in the cell nuclei) solubilized and transferred to a 96 micro-well plate and read at 540 nm absorbency on an automated TitreTek Multiscan (MCC/340) plate reader. Using this procedure there is a linear relationship between cell number and dye concentration between 5 x 10^3 to 10^5 cells/well. Beyond this number there is an increase in dye concentration up to 2 x 10^5 but this is progressively non-linear. The minimum number of cells that can be distinguished by this assay is 5 x 10^3 cells/well. Following the extraction of the crystal violet the cells were re-stained for morphological examination.

The proliferation of cells was also assessed by continuous or pulse labelling with tritiated thymidine incorporation (Freshney, 1978). A dose of 0.025 μCi (0.925 kBq)/ml of [methyl-3H]thymidine (6.7 mCi/mmol specific activity; 24.9 GBq/mmol, NEM) was added to each well at the start of the culture and replenished every three days. The samples were counted on a Beckman LS 1801 scintillation counter.

**Immunohistochemistry**

To characterise the different cell types growing in these cultures a number of antibodies were used (see Table 2) for immunohistochemical staining. The primary culture cells (or control cell lines) were plated onto sterile (acid-cleaned) collagen-coated glass coverslips (1 cm diameter, BDH), and

<table>
<thead>
<tr>
<th>Isolation method</th>
<th>Temp. (°C)</th>
<th>Time</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-10 mM EDTA in HBSS</td>
<td>4</td>
<td>45 minutes</td>
<td>Pure epithelial cells, mostly single cells and clumps, no cells attached</td>
</tr>
<tr>
<td>1-10 mM EDTA in HBSS</td>
<td>20</td>
<td>15 minutes</td>
<td>Pure epithelial cells, mostly single cells, no cells attached</td>
</tr>
<tr>
<td>1-10 mM EDTA in HBSS</td>
<td>4</td>
<td>45 minutes</td>
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<td>20</td>
<td>15 minutes</td>
<td>Pure epithelial cells, mostly single cells, no cells attached</td>
</tr>
<tr>
<td>Weiser solution (modified)*</td>
<td>4</td>
<td>45 minutes</td>
<td>Pure epithelial cells, mostly clumps, some cells attached but no growth</td>
</tr>
<tr>
<td>Weiser solution (modified)*</td>
<td>20</td>
<td>15 minutes</td>
<td>Pure epithelial cells, mostly single cells and clumps, no cells attached</td>
</tr>
<tr>
<td>0.125% Trypsin† in HBSS</td>
<td>4</td>
<td>18 hours</td>
<td>Clumps of epithelial cells with some mesenchymal cells (low yield), some cells attached but no growth</td>
</tr>
<tr>
<td>0.125% Trypsin in HBSS</td>
<td>20</td>
<td>1 hour</td>
<td>Single epithelial cells, with some mesenchymal cells (low yield), a few cells attached but no growth</td>
</tr>
<tr>
<td>300-1500 U collagenase§ in HBSS</td>
<td>37</td>
<td>2 hours</td>
<td>Clumps of epithelial and mesenchymal cells (high yield), very good attachment and growth</td>
</tr>
<tr>
<td>1 mg/ml dispase§ in HBSS</td>
<td>20</td>
<td>2 hours</td>
<td>Single epithelial cells (low yield), these attached but did not grow</td>
</tr>
<tr>
<td>0.1 mg/ml dispase and 300 U/ml collagenase in HBSS</td>
<td>20</td>
<td>30 minutes</td>
<td>Intact organoids of epithelium with attached mesenchyme and single mesenchymal cells (high yield), very good attachment and growth</td>
</tr>
</tbody>
</table>

†Bovine pancreatic (Worthington Biochemicals).
§Collagenase type 1 (Sigma).
§Dispase (neutral protease) type 1 (Boehringer).

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**Table 1. Methods of isolating intestinal epithelium for culture**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Specifications and source</th>
<th>Neonate intestinal cells stained in vivo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-keratin(^{1})</td>
<td>Rabbit polyclonal; (Dako) 1/100</td>
<td>Epithelial and mesothelial cells</td>
</tr>
<tr>
<td>Anti-cytokeratin (^{18})</td>
<td>Mouse monoclonal LE-61 1/100</td>
<td>Simple epithelium and mesothelial cells</td>
</tr>
<tr>
<td>Anti-cytokeratin (^{18})</td>
<td>Mouse monoclonal LE-41 1/50</td>
<td>Simple epithelium and mesothelial cells</td>
</tr>
<tr>
<td>Anti-cytokeratin (^{19})</td>
<td>Mouse monoclonals LP 2K and BA-16 1/50</td>
<td>Simple epithelium and mesothelial cells</td>
</tr>
<tr>
<td>Anti-vimentin(^{3})</td>
<td>Mouse monoclonal V9 (I.C.N.) 1/100</td>
<td>Fibroblast, smooth muscle cells and blood vessels</td>
</tr>
<tr>
<td>Anti-desmin(^{5})</td>
<td>Mouse monoclonal D-ER-11 (Dako) 1/50</td>
<td>Muscle cells and myo-fibroblasts(^{2})</td>
</tr>
<tr>
<td>Anti-smooth muscle a actin(^{#})</td>
<td>Mouse monoclonal 1A-4 (Sigma) 1/250</td>
<td>Smooth muscle cells and myoepithelium</td>
</tr>
<tr>
<td>Anti-rat aminopeptidase(^{6})</td>
<td>Mouse monoclonal M233 1/50</td>
<td>Apical surface of villus and pre-crypt epithelium</td>
</tr>
<tr>
<td>Anti-rat Thy-1(^{7})</td>
<td>Mouse monoclonal M226 1/50</td>
<td>Fibroblasts, smooth muscle cells and blood vessels</td>
</tr>
<tr>
<td>Anti-rat endothelium(^{7})</td>
<td>Mouse monoclonal 0X-43 (Serotec) 1/50</td>
<td>Rat endothelium</td>
</tr>
</tbody>
</table>

\(^{1}\)Franke et al. (1978).
\(^{2}\)Donated by Dr B. Lane (I.C.R.F.).
\(^{3}\)Osborn et al. (1984).
\(^{\#}\)Debus et al. (1983).
\(^{5}\)Skalli et al. (1989).
\(^{6}\)Donated by Dr A. Ager (Department of Biochemistry, University of Manchester). This antibody cross-reacts with a putative rat Thy-1 antigen.
\(^{7}\)Barclay (1981).
grown in culture for various periods of time. The coverslips were then removed, washed twice in serum free medium, and fixed in 50:50 (v/v) methanol:acetone (at 4°C) for 10 min. The coverslips were then permanently mounted on to glass slides using Glass Bond (Loctite), and stored in a -20°C freezer under dry conditions until required.

For staining, the cultured cells were washed twice in buffer then incubated with a 10% goat non-immune serum in phosphate-buffered saline (PBS) for 20 min followed by the primary antibody (for dilutions see Table 2) in PBS and 0.5% BSA (Sigma) for 2 hours (at 20°C). The sections were then washed three times in PBS and fixed in cold acetone (4°C) for 5 minutes. After air drying, two further washes in PBS were followed by a 1 hour (at 20°C) incubation in goat anti-mouse peroxidase diluted at 1/75 in PBS plus 0.5% BSA. Following five brief washes in PBS, the sections were incubated in the chromogenic substrate (0.2 mg/ml diamino-benzidine HCl, Sigma; 0.8 mg/ml nickel chloride, Sigma; and 0.003% H2O2 BDH) in PBS for 5 minutes at 20°C. The slides were thoroughly washed in buffer and several changes of distilled water, dehydrated, cleared in xylene, and mounted under large glass coverslips with XAM (BDH).

Controls included the omission of the primary antibody, and both the primary and secondary antibodies, sections of intact 6-day gut and the inclusion of several indicator cell lines; IEC 6, 17 and 18; human breast carcinoma MCF-7 line; human smooth muscle cell line (from Dr A. Schor; Department of Oncology, Paterson Institute, Manchester), bovine corneal endothelial cells, and mouse Swiss 3T3 fibroblasts. In some cases verification of the antibody binding was also tested by Western blotting.

**Histology and electron microscopy**

Some material isolated during the enzymic digests was fixed in Carnoy's fluid, embedded in agar, and then dehydrated and embedded in Paraplast wax. Sections were cut at 4 μm and stained in haematoxylin and eosin. Specimens for electron microscopy were fixed in 2.5% glutaraldehyde in 0.15 M sodium cacodylate buffer, pH 7.4, followed by 0.1% osmium tetroxide, and then dehydrated and embedded in Epon-Araldite. The sections were stained with lead citrate and uranyl acetate.

**Results**

**Isolation of the intestinal epithelium**

The best isolation procedure (Table 1) was based upon a mixture of crude collagenase (type XI) and dispase (type 1). At low concentrations (300 U ml collagenase and 0.1 mg/ml dispase) the tissues could be rapidly disaggregated at 20°C. To ensure a maximal recovery of material, the tissues were placed on a shaking platform for 30 minutes during the enzymic digestion stage, followed by vigorous pipetting for 3 minutes using a wide-mouthed pipette. As a result of this procedure the epithelial cells detached as intact units (villi and small pre-crypt units; Fig. 2A,B) leaving behind large flaps of circular muscle (Fig. 2C). The epithelium in these organoid units remained as polarised intact layers (Fig. 2D), and attached readily to both plastic and collagen coated dishes within 24-48 hours (Fig. 2E,F), forming large coalescing colonies of epithelium. Higher concentrations of collagenase and dispase allowed a more rapid isolation, but resulted in the epithelial cells dissociating into single or clumps of cells. In this form the cells did not attach efficiently or grow well.

Crude collagenase digestion also released many clumps of epithelial cells and dissociated clumps of mesenchymal cells but this procedure took much longer (several hours) and gave rise to cultures that from the outset consisted of many cell types. All of the other isolation methods tested, i.e. trypsin or divalent ion chelating solutions (Table 1) generally released pure populations of dissociated epithelial cells, but these usually failed to attach or survive in culture longer than 24 hours. The deficiencies in these methods were not overcome by modifications in either the temperature or concentration of the reagents in these protocols.

**Culture medium**

The greatest difficulties with maintaining the proliferation of isolated gut epithelium were associated with the quality of the basal culture medium and the constituents that were added to this medium. The best results were always obtained when the cells were grown in medium prepared with fresh single-strength medium. In contrast, less growth was seen when cells were grown in medium prepared from concentrated stocks and this was seen both in reduced cellularity in these cultures (crystal violet assay), as well as reduced incorporation of triated thymidine.

An example of how important the quality of the medium is to the growth of these cultures is provided in Fig. 3. This shows the difference for two media formulations (DMEM and MEM) prepared either from 10× concentrated stocks or used as single-strength solutions. The media used in these experiments were all freshly manufactured. Very little growth was ever observed when medium was prepared from concentrated stocks that had been stored for more than one month (unpublished data).
pH and CO₂ concentration
In these studies we employed bicarbonate-buffered medium and varied the concentration of CO₂ to establish the effect of pH on the growth of the intestinal cultures. This indicated that at three different serum concentrations (10, 5 and 2.5% FCS) a CO₂ concentration between 5 and 7.5% was reproducibly the most suitable for growth (data not shown).
Fig. 3. (A-B) The effect of different medium on the growth of primary cultures of neonate rat intestinal epithelium. Epithelial organoids were plated in 24 multi-well dishes in the following medium; (A) 10x strength DMEM medium (high-glucose formulation reconstituted with laboratory supplied tissue culture water (LW, de-ionised, double-distilled and autoclaved); 10 x strength DMEM medium (high-glucose formulation) reconstituted with the manufacturer's supply of tissue culture water (GW, de-ionised double-distilled then heated to 65°C prior to flash cooling and bottling); single strength DMEM (high-glucose formulation). (B) 10x strength MEM medium reconstituted with laboratory supplied water (LW), 10x strength MEM medium reconstituted with the manufacturer's supply of tissue culture water (GW); single strength MEM. All six media were fresh batches from the manufacturer and contained 5% FCS, 10 ng/ml EGF and 2.5 μg/ml insulin, and other supplements and antibiotics as indicated in Materials and methods. The MEM medium was also supplemented with non-essential amino acids. Dishes were fixed at intervals up to day 14 and the cellularity measured by the crystal violet procedure where a stain density of 0.2 corresponds approximately to $2 \times 10^4$ to $3 \times 10^4$ cells/well and a value of 1.2 to $11 \times 10^4$ to $13 \times 10^4$ cells/well. Each data point represents the average reading (plus standard error of the mean) for triplicate wells.

Characterisation of the cultured cells

To establish which types of cells were present in the cultures, a battery of antibody probes and functional tests were applied. The results of this study indicated that after the initial isolation, less than 10% of the cells were non-epithelial, and these fell into three categories, smooth-muscle-like cells, smaller populations of myofibroblast-like cells and endothelial cells. In the presence of higher serum concentrations (i.e., >2.5% FCS) the smooth-muscle-like cells usually formed large colonies that surrounded the areas of epithelium.

The epithelial cells were identified using a combination of antisera against cytokeratins 8, 18 and 19 (see Table 2). In the sections of intact neonate epithelium the strongest reaction was to the cytokeratin 8 antiserum, which was localised throughout the epithelium (Fig. 4A), whereas the staining patterns were weaker and not uniform when antibodies to cytokeratin 18 and 19 were used. In the early stages of culture, the epithelial colonies were distinguished easily using these antisera (Fig. 4B), but with time as the epithelial cells spread out onto the substratum the reaction became weaker and many of these cells began to express vimentin (V9 monoclonal). Positive control epithelial lines also reacted with these cytokeratin antibodies, and the smooth muscle, endothelial and 3T3 cell lines were unreactive.

The smooth-muscle-like cells were identified by their reactivity with the smooth muscle α actin (1A-4) antibody in sections of intact neonate gut; the cells that reacted with this antiserum were present throughout the lamina propria (associated with the blood vessels) and the muscularis mucosa. Elements of the circular smooth muscle layer were also reactive (Fig. 4C). The antibody stained colonies of cells in the established primary cultures (days 7-14; Fig. 4D) and a positive control human pericyte smooth muscle line. A further feature of some of these smooth-muscle-like cells was their ability to invade thick collagen gels, an activity that is shared with foetal fibroblasts and some smooth-muscle-like cells (Schor, 1980). We have also been able to show that the growth of these smooth-muscle-like cells was inhibited by the addition of exogenous heparin (Table 3), like many other smooth muscle-like cells in vitro.

In addition, three other cell types were observed, usually as small colonies or single cells. The first type was reactive with the OX-43 antibody, which binds to an antigen expressed by rat endothelial cells, and showed uptake of fluorescently labelled acetylated low-density lipoprotein (LDLα; which is regarded as a functional marker of endothelial cells; Table 3). Another set of cells reacted with the anti-desmin antiserum (Fig. 5A), which in tissue sections reacted with the peri-cryptal fibroblasts and cells associated with the capillaries in the lamina propria. Finally, we have observed colonies of cells with morphologies analogous to developing nervous tissue, e.g. they exhibited extensive dendritic processes and only reacted with the anti-vimentin antibody (Fig. 5B).
Primary cultures of intestinal epithelium

Several batches of foetal calf serum were screened using the IEC 18 and 6 cell lines as an assay system, and the effect of different concentrations of the optimal batch of foetal calf serum on the growth of the primary cultures was assessed. Using single-strength DMEM and in the presence of insulin (2.5 μg/ml) and EGF (10 ng/ml) there was an increase in the growth of the intestinal cultures from 2.5% to 10% FCS (Fig. 6). Serum levels lower than 1% were not able to sustain the growth of these primary cultures beyond day 10.

Although growth was most rapid at high serum concentrations (Fig. 6), these conditions were not the most appropriate for the epithelial cells, since colonies of the smooth-muscle-like cells were also more abundant (Fig. 5C). At concentrations of FCS <2.5% the growth of these smooth-muscle-like cells was reduced so that they formed smaller colonies surrounding the epithelial cells (Fig. 5D), and hence the lower concentration of serum has been used for the further development of a method to grow mono-cultures of this epithelium.

Serum quality and concentration

Several batches of foetal calf serum were screened using the IEC 18 and 6 cell lines as an assay system, and the effect of different concentrations of the optimal batch of foetal calf serum on the growth of the primary cultures was assessed. Using single-strength DMEM and in the presence of insulin (2.5 μg/ml) and EGF (10 ng/ml) there was an increase in the growth of the intestinal cultures from 2.5% to 10% FCS (Fig. 6). Serum levels lower than 1% were not able to sustain the growth of these primary cultures beyond day 10.

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Table 3. Characterisation of gut cells by functional assays

<table>
<thead>
<tr>
<th>Assay for different cell types</th>
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</thead>
<tbody>
<tr>
<td>Attachment (within 20 minutes) of smooth-muscle-like cells to plastic and clean glass but not of epithelial cells</td>
</tr>
<tr>
<td>Specific internalisation of acetylated low density lipoprotein (LDL) by endothelial cells*</td>
</tr>
<tr>
<td>Ability to invade collagen gels a property of normal fetal like fibroblasts†</td>
</tr>
<tr>
<td>Smooth muscle cells inhibited by doses of heparin as low as 5 μg/ml‡</td>
</tr>
</tbody>
</table>

*Personal communication and gift from Dr D. West (Department of Paediatric Oncology, Christie Hospital, Manchester).
†Schor (1980).
‡Wright et al. (1988).

Plastic and biological substrata

To investigate the role of extracellular matrix interactions upon the epithelial cultures we have examined the requirements for different types of substrata, including plastic-coated with purified constituents of the extracellular matrix, or whole extracellular matrices. The results of these experiments suggest that the type of substratum is important, but that the effect is primarily upon the morphological appearance of the epithelial cells and not specifically upon proliferation.

The best results were obtained with complete biological matrices isolated from bovine corneal endothelial cells and Swiss Balb 3T3 cells, since these promoted attachment of most of the isolated cells. However, these matrices were less satisfactory for routine applications. Good attachment was also facilitated by the use of dried collagens (particularly Vitrogen and rat tail collagens), but did not affect the

Fig. 5. (A-D) Photographs of non-epithelial cells stained by: (A) anti-desmin antisera (1/50 dilution) 10 days after initiating primary cultures of rat intestinal epithelial cells; and (B) glia-like cells stained with anti-vimentin antisera (1/100 dilution) 10 days after initiating primary cultures of rat intestinal epithelial cells. The primary antibody layer was revealed with peroxidase-conjugated goat anti-mouse IgG (at 1/75 dilution) and diamino-benzidine/nickel chloride substrate.
(C,D) Photographs of cells fixed in methanol and stained with crystal violet and photographed under phase-contrast;
(C) smooth-muscle-like cells in primary cultures of neonate rat intestinal cells (day 10; ×150); (D) a colony of epithelium (E) surrounded by smooth-muscle-like cells (SM) (day 14; ×250).
Primary cultures of intestinal epithelium

Fig. 6. The effect of growing primary cultures of rat neonate intestinal epithelial cells in DMEM medium (bicarbonate-buffered, single-strength, high-glucose formulation) at five different foetal calf serum (FCS) concentrations (0, 0.5, 1.0, 2.5, 5.0 and 10% FCS). The epithelial organoids were plated out in 24 multi-wells and plates fixed at days 2, 7, 10 and 14. The changes in cellularity were measured by the crystal violet procedure where a stain density of 0.2 corresponds approximately to $2 \times 10^4$ to $3 \times 10^4$ cells/well and a value of 1.2 to $11 \times 10^4$ to $13 \times 10^4$ cells/well. Each data point represents the average reading (plus standard error of the mean) for triplicate wells.

Fig. 7. The effect of growing primary cultures of rat neonate intestinal epithelial cells in DMEM medium (bicarbonate-buffered, single-strength, high-glucose formulation) with 2.5% foetal calf serum, 10 ng/ml EGF, 2.5 μg/ml insulin and supplements as described in Materials and methods. The cells were grown in 24 multi-wells either with or without a dried coating of bovine dermal collagens I + III (Vitrogen; as described in Materials and methods) and plates fixed at days 1, 7, 14, 21 and 28. The changes in cellularity were measured by the crystal violet procedure where a stain density of 0.2 corresponds approximately to $2 \times 10^4$ to $3 \times 10^4$ cells/well and a value of 1.2 to $11 \times 10^4$ to $13 \times 10^4$ cells/well. Each data point represents the average reading (plus standard error of the mean) for triplicate wells.

Discussion

There have been many previous attempts to develop a method to grow in culture normal intestinal epithelial cells, and usually these reports have emphasised the difficulties of maintaining the proliferation of these cells when isolated from the gut (Kedinger et al., 1987a; Neutra and Louvard, 1989). There have been a few notable exceptions where the propagation of normal colonic cells for several months in culture has been reported (Chopra and Yeh, 1981). However, this has still not led to the widespread use of these methods. Without stimulation of proliferation there has been no objective way to identify the optimal in vitro conditions, and this paucity of hard data has inevitably led to a more anecdotal approach. Our aim has been, where possible, to use a quantitative approach to develop a primary culture method for normal gut epithelium.

Basic aspects of culture methodology have not received great attention in previous publications, but some of these conditions were found to be critical in this study and could well account for the observed difficulties in getting the isolated gut cells to proliferate in vitro. In this study the basic culture conditions that gave rise to the proliferation of epithelial and smooth-muscle-like cells are described.

The optimal method of isolating the gut epithelium was found to be a mixture of crude collagenase and dispase, a combination of enzymes that has been employed in some previous studies (Gibson et al., 1989). The success of this method may be associated with the low level of trypsin activity and the ability to maintain the integrity of the epithelium. In the six-day suckling rat gut, the epithelium forms small blunt villi but there are no well-developed crypts. The pre-crypt population (and hence proliferative cells) is present on the inter-villus plateau regions, or as small buds at the base of the villi (Fig. 2A). After incubation in this enzymic solution, gentle pipetting releases the villi and the pre-crypt cells, some of which remain attached to the base of the villi. These units have been termed epithelial organoids, since they retain their morphologi-
cal integrity on isolation and the closely associated mesenchymal cells remain attached at the core of the villi (Fig. 2D). Isolation of intact crypt units from the adult rat gut has not yet been achieved with this method to our satisfaction, but we have applied it successfully to the isolation of pure populations of colonic crypts from the adult human colon (Evans, de Silva and Reddel, unpublished data).

The isolation of other epithelial cells in the form of intact 'organoids' is also seen to be a key factor in the maintenance of growth and function in vitro (Barcellos-Hoff and Bissell, 1989). The apparent disadvantage of isolating epithelial cells as organoids is the contamination by attached mesenchymal cells. Collagenase/ dispase digestion releases large numbers of mesenchymal cells from the lamina propria, which are usually present as single cells and smaller clumps. The advantage of the gentle sedimentation steps employed in this method is that it has proved to be a very effective way of removing unwanted types of cells and microorganisms, without recourse to the use of graded meshes or cocktails of anti-biotic and anti-mycotic agents (Moyer et al., 1990). This differential sedimentation procedure can easily be adapted to enrich for single cells or very small cell clumps without a large loss of material.

The investigation of many other isolation methods (see Table 1) suggested that the maintenance of close cellular interactions is important if these cells are to be grown in culture. Methods that dissociated the epithelium by tryptic or chelating activity never yielded cells that would grow in culture (Table 1). Apart from possible toxic effects, this could support the idea that the maintenance and quality of the cellular interactions is most important for proliferation of gut epithelium.

Immunocytochemical characterisation of these early-stage cultures showed that >90% of the attached cells were positive for cytokeratin expression, whilst the remainder consisted of single cells and small clumps of smooth-muscle-like cells, myo-fibroblast-like cells and endothelial cells. Within a short period after the initial attachment and spread of these epithelial cells, they changed to a flat/cuboidal morphology and lost the supra-nuclear heterophagosomes and large Golgi bodies that typify the normal enterocyte in the neonate gut (Fig. 8A-C). These colonies of epithelium also became unreactive to an antibody (Mab 233) raised against surface aminopeptidase, suggesting further loss of functional activity. However, in the initial stages in the development of this culture technique no significant proliferation of any of these attached epithelial cells was observed, they often remained in culture for up to two weeks and gradually detached and died (in common with previous published observations; Gibson et al., 1989; Kondo et al., 1984).

The major contributory factor to the declining state of these cultures was found to depend upon the quality of the basal culture medium (Fig. 3), and the need for high-quality reagents in this primary culture system cannot be emphasised enough. The exquisite sensitivity of these primary cultures contrasted with that of the

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**Fig. 8.** (A) Photograph of an organoid of epithelium attaching in culture 24 hours after isolation, showing the central mass of the cells still unattached (T) and cells spreading on to the dish at the periphery (P). The cells were grown in DMEM (single-strength, high-glucose formulation) + 10% FCS (day 1). The cells were fixed in methanol and photographed under phase contrast (250 x magnification). (B) Ultrastructure of cells taken from the tip (T) of an attaching organoid showing large supra-nuclear heterophagosomes that result from the apical pinocytosis of milk proteins. The epithelial cells were grown under the same conditions as described above, but fixed in 2.5% glutaraldehyde and 0.1% osmium tetroxide and embedded in Epon-Araldite (x2500; bar, 4 μm). (C) Ultrastructure of cells taken from the edge (P) of an attaching organoid of epithelium grown under the same conditions (x7500; bar, 1.33 μm).

IEC 6 and 18 lines that were used to batch test many of the reagents that were initially chosen for these culture experiments (unpublished data). Storage of concentrated medium stocks may lead to sequestration of ions (e.g. on buffer salts) that are essential for gut cells in primary cultures. As short a period as 24 hours in a medium prepared from liquid concentrates is sufficient to suppress the proliferation of these primary cultures (unpublished data). These problems were overcome by the use of fresh batches of single-strength DMEM (high glucose formulation) medium and plastic disposables. However, even the plastic disposables may need to be selected carefully, since there is evidence that volatile constituents of some plastics can affect primary cultures of certain tissues (Knight, 1990).

With optimal conditions these intestinal primary cultures were able to proliferate very rapidly and would fill the multi-wells with cells within 10-14 days. In 10% FCS, this represents a starting population of about 25,000 cells/well (on day one) leading to 250,000 cells/well (on day 14). This enabled us to apply quantitative assays of growth and proliferation to assess the affects of other changes in the culture environment.

Close examination of cultures indicated the presence of heterogeneous populations of cells not all of which were epithelial in appearance (Fig. 5A-C). Most of these were smooth-muscle-like cells. Such smooth-muscle-like cells have been reported in a recent publication by Kéding et al. (1990) to be a heterogeneous population within the intestinal lamina propria. The presence of these heterologous cell types may be important for epithelial cell proliferation, since only in these mixed cultures have growing colonies of epithelium been observed previously (Kondo et al., 1984; Kéding et al., 1987a). This contrasts with isolations of purified gut epithelium that can attach but do not proliferate in vitro (Quaroni and May, 1980; Vidrich et al., 1988). It is significant that success in establishing epithelial cell lines from the normal gut has followed the use of crude collagenase digestion and spill culture techniques, with the colonies of epithelium being isolated (with cloning cylinders) from a mixed cell culture (Quaroni and May, 1980).
Reducing the growth of the smooth-muscle-like cells was an important objective if this method was to be used as a primary culture assay for epithelial cells. Lowering the serum level to concentrations <2.5% FCS was one way of keeping these cells in check. As a result, the reduced level of platelet-derived growth...
factor (present in the serum) may be one possible explanation for this effect upon the smooth-muscle-like cells. At low serum concentrations, the growth of the epithelial cells was still not expansive, and so the addition of supplements, e.g. transferrin, lipids, hormones etc, to enhance the growth of these cells is now being pursued. Initially EGF and insulin were added to the serum because of their general application in epithelial cell culture. Subsequently we have examined in greater detail the contribution of these two factors, and in their absence serum concentrations lower than 2.5% are less able to maintain the gut epithelium in culture. EGF also promotes the migration of the attaching cells in the early stages of the culture, and the promotion of growth by insulin and EGF is synergistic (unpublished data).

Heparin has been recognised as a potent inhibitor of the proliferation of many smooth muscle cells in vitro (Wright et al., 1988). At concentrations up to 100 μg/ml heparin was effective at preventing the proliferation of colonies of the smooth-muscle-like cells (unpublished observations). The role of the substratum in these cultures was another aspect which warranted attention. With collagen substrata, the morphological appearance of the epithelial colonies was much more organised, and the cells had a compact cobblestone appearance. In contrast the poor growth of the epithelial cells on the hydrated collagen gels suggests that this provided an insufficiently stable substratum, and we have observed similar results with Matrigel. There is good evidence that some of the more complex biological substratum, e.g. Matrigel, can induce differentiation of some epithelial cells in vitro (Montgomery, 1986). This is one aspect that we have not investigated, but there is already evidence to support this phenomenon in cultured intestinal epithelial cells (Schor, 1980; Kédinger et al., 1987b).

The development of a primary culture system for the gut epithelium is a worthwhile objective, particularly if it could be used as a reproducible assay to define further the in vivo requirements of the stem cell 'niche'. Although progress in this direction has been slow, methods to maintain other tissue stem cells have by necessity been complex and essentially uncharacterised, e.g. the use of long-term bone marrow cultures for haematopoietic cells and of 3T3 fibroblasts for embryonic stem cells (Dexter, 1979; Evans and Kaufman, 1981). The complexity of these systems, however, has not prevented the eventual identification of key regulatory molecules that maintain the haematopoietic and embryonic stem cells (Zsebo et al., 1990; Smith et al., 1988).

Apart from methodological considerations, the main outcome of this present work is the recognition that the epithelial cells need to be isolated in a way that maintains their integrity and organisation. Continued association with mesenchymal cells in vitro is also beneficial, but the major problem is the growth of the smooth-muscle-like cells at higher serum concentrations. This can be partly overcome by the procedures described above. The need for these heterologous interactions further supports the substantive evidence for epithelial-mesenchymal interactions in the gut, produced by Kédinger et al (1987a,b), and may indicate the involvement of paracrine growth factors such as those described for the epidermis (Finch et al., 1989).

Since there has been very limited success in inducing characteristic in vivo pathways of differentiation in the established epithelial lines derived from normal gut, it is important to determine if a primary culture model will be of more use for in vitro functional studies of this epithelium. The ability of monolayer cultures to recapitulate features such as cell polarity, maintenance of tight junctions and transmembrane electro-potential differences, transport functions, crypt-to-villus entero-cyte maturation, and the production of different cell lineages (e.g. Paneth and Goblet cells) are difficult and unresolved questions. We are currently using this primary culture model to investigate what effects extracellular matrix constituents, hormones, growth factors and known agents for inducing differentiation (e.g. retinoic acid and dimethyl sulphoxide) have upon these aspects of epithelial cell function in vitro. Preliminary evidence suggests that the epithelial cells remain polarized with junctional complex and apical micro-villi and retain expression of, e.g. alkaline phosphatase, which is seen strongly expressed in large growing epithelial colonies which develop between days 5 and 10. With respect to questions of cell lineage, the early stage of development (six days post-natal) of the gut must be borne in mind, since cell types such as entero-endocrine cells begin to appear only late in fetal development, and Paneth cells 5-6 days after birth. It will, therefore, be of interest to determine what effects differentiation-promoting agents have upon a population of cells undergoing commitment events.

A long-term aim is to identify factors that more specifically promote the proliferation of the epithelial cells, leading essentially to a monoculture. We have recently identified feeder cells (bovine corneal endothelial cells) that are able to promote selectively the proliferation of rat intestinal epithelium. This activity is due to a soluble factor found in the conditioned medium produced by this cell line (N. Flint and G. S. Evans, unpublished observations). In addition to the inhibitory effects upon the smooth-muscle-like cells, heparin has been found to stimulate significantly the proliferation of the epithelial cells independently of a biological feeder layer (N. Flint and G. S. Evans, unpublished observations). The mechanisms by which these signals stimulate the proliferation of the intestinal epithelial cells is being investigated.

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