

Simple method for the preparation of single cell suspensions from normal and tumorous rat colonic mucosa

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SUMMARY Viable single cell suspensions from rat colonic epithelium were obtained by using phosphate buffered saline containing 0.2 M mannitol. The method, which requires no prior enzyme treatment, provides undamaged cells in high yield within one hour. The procedure was also applied to neoplastic rat colonic tissue, which was induced by repeated intrarectal infusion of N-methyl-N-nitrosourea. Comparison between normal and neoplastic cells has shown that the latter have a higher nucleus: cytoplasm ratio and a higher metabolic activity.

In the course of the isolation of intestinal epithelium, it is advantageous to obtain viable single cells in order to carry out metabolic and structural studies pertaining to membrane glycoproteins. Also, such viable single cell preparations are a prerequisite for comparing normal cells with neoplastic ones.

Usually single cell suspensions are prepared by exposing biological tissue briefly to proteolytic enzymes, such as trypsin (Kruse and Patterson, 1973), pronase (Gwatkin and Thomson, 1964), collagenases (types I-IV) (Howard *et al.*, 1973), hyaluronidase (Perris, 1966), (Culling *et al.*, 1973), DNase (Madden and Burk, 1961), or a combination thereof. Other agents, such as EDTA (Culling *et al.*, 1973), dithiothreitol (LaMont *et al.*, 1974), citrate (Stern, 1966) have also been used for the disaggregation of cells.

In our study concerning the characterisation of the epithelial lining cells of the large intestine (caecum and colon), we first applied treatment with an enzyme or a combination of enzymes under a variety of conditions involving a large variety of simple and complex culture media. It was found that even mild treatment of cells with enzymes, individually or in combination, resulted in drastic morphological changes of the cell population, as revealed by microscopic examination. On the other hand, exposure to chelating agents, such as EDTA, dithiothreitol, and citrate, alone or in combination with enzymes such as hyaluronidase (Culling *et al.*, 1973), did not yield

single cell suspensions which were viable long enough for further studies. Equally unsuccessful were methods of mechanical disaggregation (shaking in a variety of media containing glucose at 37°C, gentle sonication, rubbing of tissue through a fine screen); the yield of intact cells in these instances was low and the amount of debris considerable. However, we found that mannitol (Kimmich, 1970; Evans *et al.*, 1971), used as outlined below, is an ideal agent for preparing viable single cell suspensions from colonic scrapings. This report describes the procedure used for obtaining such suspensions from the scraping of normal large intestinal mucosa and from colonic tumour of rats.

Methods

Young Fisher rats, weighing about 200-300 g, are decapitated and bled after being stunned by a blow on the head. The abdominal region of each rat is shaved, washed with 70% ethanol, and cut open. The colon is resected and carefully freed from all adjacent tissue, slit longitudinally with scissors, and the faeces pushed off gently with forceps. It is then washed three times with ice cold saline containing antibiotics, completely unfolded, slightly stretched out on a cold glass plate and the intestinal mucosa scraped off very gently with a blunt-edged glass slide. The scrapings are minced by 60 strokes of sharp scissors and weighed. The wet weight of the scrapings from one animal is about 500 mg. The minced mucosal scraping

is dropped into a 50 ml siliconised centrifuge tube containing 30 ml ice cold PBS-M (NaCl, 85.5 mM (5.0 g); KCl, 2.6 mM (0.2 g); Na₂HPO₄, 8.1 mM (1.15 g); KH₂PO₄, 1.5 mM (0.2 g); mannitol, 200 mM (36.4 g) in a total of one litre distilled water; 200U penicillin, 200 µg streptomycin, and 50 µg fungizone are added for each millilitre of medium; the pH of this solution is 7.4) and agitated by mild bubbling of oxygen through the suspension. At this point, the cells are in small clusters of tissue. Pipetting up and down facilitates disaggregation.

Within 30 minutes most of the cells are well dispersed. The suspension is now centrifuged at 75x g for two minutes. The supernatant is removed with a Pasteur pipette; 20 ml fresh PBS-M are added to the loosely packed cell sediment and the cells are resuspended by gentle oxygen bubbling through the medium for five minutes. The centrifugation and the washing with PBS-M is repeated once more and the cells are suspended in PBS-M.

Every operation is performed at 4°C and all of the glassware is siliconised. During the procedure, samples are examined microscopically; five drops of cell suspension are mixed with one drop of a 0.4% solution of trypan blue in PBS-M (Kruse and Patterson, 1973).

For measurements of oxygen uptake, 2 ml samples are withdrawn from the suspension at intervals of 30-60 minutes, and the rate of oxygen uptake from the extracellular fluid is measured at 37°C with a Clark oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio) attached to a Beckman gas analyser and a recorder. The instrument is calibrated by measuring the oxidation of a 0.2 µmolar glucose solution by glucose oxidase; results are expressed as microlitres of oxygen taken up per milligram cell protein per hour (Q_{O₂}).

For studying the incorporation of labelled metabolites, cells from one colonic mucosa were suspended in 20 ml PBS-M containing antibiotics and L-[4,5-³H]-leucine (10 µCi/ml; 5 mCi/mM) and D-[U-¹⁴C]-glucose (1 µCi/ml; 4.86 mCi/mM). The cells were shaken at 22°C. At various time intervals, 2-ml aliquots were precipitated with an equal volume of a mixture of 20% trichloroacetic acid and 2% phosphotungstic acid and centrifuged. The pellet was washed twice with 5% trichloroacetic acid, dissolved in 0.5 N NaOH and counted in Biofluor (New England Nuclear), using a Nuclear Chicago Scintillation counter.

Cell suspensions and paraffin-embedded tissues obtained from representative areas of normal mucosa and of colonic tumours were stained with haematoxylin and eosin, and periodic acid-Schiff for routine morphology. Some specimens were also stained with 1% alcian blue (pH 2.5 and 1.0), high

iron diamine, and 0.1% toluidine blue (pH 3.5) to differentiate the various acidic mucins.

Results

NORMAL CELLS

The time involved from killing the animal to suspending the mucosal scraping in the PBS-M is about 16 minutes. Dispersion of cells by means of bubbling oxygen through a Pasteur pipette into the medium takes about 30 minutes. The average cell density, as read in a haemocytometer, is about 4×10^6 ml. A typical cell suspension is shown in the Figure.

Under the microscope, the cell suspensions show a homogeneous population of columnar-shaped cells with some free floating nuclei. The cells are generally well dispersed, although, in a few instances, aggregates of epithelial cells were observed. These aggregated cells, however, are easily dispersed when reagitated in the presence of oxygen. Small sections of lamina propria are also occasionally seen. Microscopically, the cells look morphologically intact;

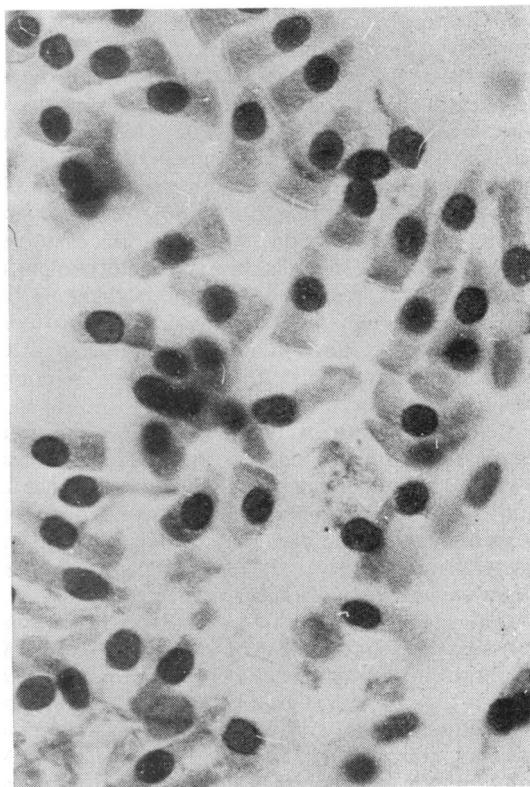


Figure Single cell suspension of rat colonic mucosa in PBS-M, stained with trypan blue. Preparation consists of columnar cells with basally situated nuclei. $\times 1200$.

their nuclei are slightly stained by trypan blue. When cells are kept for several hours at room temperature, trypan blue staining becomes considerably more intense, and occurs throughout the cell. When left overnight at room temperature, the cells are still well preserved. Incubated in the cold, most of the cells (90%) are still intact after 72 hours and show only faint trypan blue staining of their nuclei.

In order to determine contamination by connective tissue, cell suspensions (~1 g wet weight) were analysed for hydroxyproline (Stegemann, 1958) on acid hydrolysed samples and for uronic acid (Bitter and Muir, 1962) after papain and trypsin digestion of scrapings. These analytical methods, which detect a contamination greater than 4% of the macromolecular constituents of the corresponding connective tissue, were negative for hydroxyproline and showed only traces of uronic acid positive material.

In order to investigate the effects of time on the morphology of the PBS-M suspended cells, 5 ml aliquots of a colonic cell preparation were pipetted into buffered formalin at 30 minutes, 60 minutes, four hours, and 24 hours; paraffin-embedded sections were made which were then stained. Examination of the slides showed that the cells maintained their integrity to the extent of 80% even after 24 hours when kept in the cold. Single cell suspensions were also incubated at 37°C in Eagle's MEM (Gibco, catalogue No. 109 G) containing 10% fetal calf serum and penicillin (100 U/ml), streptomycin (100 µg/ml), and mycostatin (25 U/ml), in depression slides sealed with paraffin and periodically examined for cell integrity. The cells showed no morphological changes for at least 10 days without changing the medium, although they did not develop into confluency. When cultured in incubation flasks, at 37°C, cells became attached to the wall within 24 hours, but no growth occurred.

Cell suspensions were examined for bacterial contamination, using nutrient agar and blood agar plates. Both tests were negative in the presence of antibiotics, but if plating were performed on cell suspensions which did not contain antibiotics, bacterial growth was intense (*E. coli*, *Proteus*, *Pseudomonas*, *Staphylococcus albus*, etc.).

Cells suspended in PBS-M were layered upon a discontinuous Ficoll gradient of 1% to 6% in PBS-M solution and centrifuged for 10 minutes at 95 x g. With a 1, 3, 5% Ficoll gradient in PBS-M, all of the cells settled in the 3% layer. When the discontinuous gradient consisted of 2, 4, 6% Ficoll in PBS-M solution, the cells were layered at the interphase between the 2 and 4% Ficoll concentration. Microscopically, these cells did not show any difference in appearance, as compared with the original cell

suspension. The apparent density of singly suspended cells, as determined at ambient temperature by the Ficoll gradient, was 1.025 g/cm³, while copper sulphate and sucrose gradients both gave a value of 1.020 g/cm³.

Oxygen uptake, at 37°C, of cells suspended in PBS-M was negligible; in a PBS-M solution containing glucose (11 mM (2 mg/ml)), the Q_{O_2} was 11.25 µl O₂ × h⁻¹ × mg⁻¹ cell protein.

The rate of incorporation of labelled leucine and labelled glucose was found to be linear with time for at least 90 minutes. For the amino acid, the slope was 130 ± 18¹ and for ¹⁴C-glucose, 135 ± 21¹ cpm × mg⁻¹ cell protein × min⁻¹ of incubation.

TUMOUR CELLS

The above method was used to prepare suspensions from colonic tumour cells. For tumour induction, 40-day-old female Fisher rats (Charles River, North Willmington, MA), weighing about 100 g, with free access to Purina laboratory chow and tap water, were injected inter-rectally with *N*-methyl-*N*-nitrosourea (in 0.5 ml of saline, pH 6), essentially as described by Narisawa *et al.* (1976). The complete treatment consisted of 44 doses of 2.5 mg carcinogen each. At various times during the experimental period, animals were killed and their large intestine carefully examined for tumour growth.

Up to 32 injections, no gross changes were observed. In some instances, however, several foci of *in situ* carcinoma or smaller areas of epithelial atypia were found on histological examination of random sections of such colons. The *in situ* lesions consisted of cup-shaped aggregates of malignant glands composed of basophilic cells showing loss of nuclear polarity, enlarged hyperchromatic nuclei, increased numbers of mitoses, and reduced intracellular mucus.

All of the 12 rats which had received the complete treatment of 44 doses had developed large intestinal tumours.

Gross examination of colons obtained from these animals showed obvious tumours ranging from 0.1 to 1.7 cm in greatest dimension. They were found predominantly in the distal half of the colon, were polypoid for the most part, and showed a variable degree of luminal obstruction and penetration of the muscle wall of the colon. No metastases were found in the regional lymph nodes. Microscopic examination of the colonic tumours indicated that these were adenocarcinomas, some of which had a papillary pattern. In most cases, mucus production was reduced or absent in these tumours; where present, the mucus appeared as focal collections of atypical small intracytoplasmic mucin droplets. More rarely,

¹Values are means ± standard deviation of six experimental results.

increased amounts of mucus were found in the tumours in the form of large collections of extra-cellular mucus 'lakes' in which signet rings could occasionally be identified. Occasionally, patches of benign-looking columnar cells, resembling adenomatous epithelium found in benign colonic polyps in humans, were intermingled with the frankly malignant glands.

Disaggregation of cancerous tissue in PBS-M proved to be more difficult than normal colonic epithelial tissue. Nevertheless, if the minced tumour tissue was passed with a glass pestle through a 40 mesh stainless steel screen, well disaggregated cell suspensions were obtained in PBS-M within a 30 minute period. These suspensions showed a greyish-white colour, quite different from the pink shade of suspensions from normal tissue. About 30% of the cells were spherical in shape with a larger nucleus than that of normal colonic epithelial cells. The diameter of the nucleus of neoplastic epithelial cells was $4.35 \pm 0.254 \mu^2$ as compared with $3.72 \pm 0.133 \mu^2$ for cells derived from normal tissue. The ratio: $\frac{\text{area of nucleus}}{\text{total cell area}}$ was 0.784 and 0.332² for cancerous and normal animals, respectively.

Cell suspensions from colonic tumours were incubated, like the normal cells, with PBS-M medium containing antibiotics and 10 $\mu\text{Ci/ml}$ of ³H-leucine and 1 $\mu\text{Ci/ml}$ of ¹⁴C-glucose. In repeated experiments for which the standard deviations were similar to those for normal cells, the rate of incorporation was about twice as high as for suspensions of surrounding non-cancerous colonic tissue taken from the same animal.

Further details of these studies will be published in a separate paper.

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²Standard deviation, based on 12 measurements.

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