

Folate status of gastrointestinal epithelial cells is not predicted by serum and red cell folate values in replete subjects

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Abstract

Localised folate deficiency has been implicated in colonic carcinogenesis and supplementation has been proposed for certain populations at risk. However, identifying those groups that may benefit is difficult as the relation between blood folate and gut epithelial cell values is unknown. The aim of this study was to define this relation. Epithelial cells mean (SEM) (sigmoid: $5.35 (0.56) \times 10^6$ cells, caecum: $6.6 (0.71) \times 10^6$ cells, duodenum: $4.0 (0.62) \times 10^6$ cells) were isolated from four endoscopic mucosal biopsy specimens (n=25) by incubation with dithiothreitol (three hours) and EDTA (one hour). Lamina propria contamination was <1%, with <6% intraepithelial lymphocytes. Folate assay of isolates showed sigmoid colon folate content to be $20.1 (1.8) \text{ pg}/\mu\text{g DNA}$ ($10.2\text{--}46.6$). In the same subject, caecal folate concentrations were lower ($p < 0.01$, $n=11$) than sigmoid values, whereas duodenal isolates mirrored those of the sigmoid ($19.4 (2.9)$ v $20.5 (3.2)$, $n=5$). Sigmoid folate values were consistent over one to three weeks ($n=3$). In a single case with blood folate deficiency, colonic values were normal. Serum folate and red cell folate correlated poorly with sigmoid epithelial cell folate content ($r=0.41$, $p=0.063$ and $r=0.17$, $p>0.05$ respectively). This study reports a modified ion-chelation isolation method for colonic biopsy specimens that yields large numbers of viable epithelial cells. Cell folate values remain constant with time though vary with intestinal region. The inability of serum or red cell folate values to predict those of the sigmoid epithelium suggests that they cannot identify those patients that might benefit from folate supplements.

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tumours of several organs including the colon.⁶⁻⁹ Oral folate supplements have been advocated for the prevention of dysplasia in inflammatory bowel disease.¹⁰ There is no evidence, however, that such supplements have any influence on gut mucosal epithelial cell values. Indeed, the normal regional colonocyte folate content and its relation to serum and red cell folate and thus to folate status, remains to be defined.

Homogeneous populations of colonic epithelial cells obtained from normal large bowel are a prerequisite for establishing the normal range of colonocyte folate content. These conditions preclude the use of surgical specimens (cancer bearing or inflamed) as these cannot be considered to be normal. The use of whole mucosal biopsy specimens is precluded as they contain a heterogeneous cell population. Additionally, current ion-chelation and enzymatic methods when applied to large bowel endoscopic biopsy specimens, suffer from the disadvantage of poor cell yield or significant red cell contamination, rendering isolates unsuitable for accurate analysis.¹¹ The aim of this study was to develop a method for the isolation of large populations of epithelial cells from standard endoscopic biopsy specimens to determine the normal range for gastrointestinal epithelial cell folate stores. Using these preparations we wished to define the relation between such values and the established methods of determining folic acid status – that is, serum folate – or more importantly red cell folate values.

Methods

Tissue collection

Colonic biopsy specimens were obtained at endoscopy under a protocol approved by the ethics committee of the Federated Voluntary Dublin Hospitals. Twenty five patients were enrolled into this study (male: 12, female: 13) of median age 50 years (17-82). In all cases, samples obtained were suitable for processing.

Prior to endoscopy patient details including age, sex, medical history, and current medication were recorded. Patients using anti-folate medication and those with a current or past history of gluten sensitive enteropathy, colonic inflammation, tumour or polyp were excluded. Colonoscopy was considered to be normal if both the appearance of the mucosa to the caecum and rectal histology were normal. Small bowel biopsy specimens were taken from the second part of the duodenum at the same

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The ability of vitamins and micronutrients to influence cell proliferation suggests both an aetiological role in carcinogenesis and the possibility of therapeutic potential.^{1,2} Folate is of particular interest, as it is central to the one-carbon transfer reactions required for purine and pyrimidine synthesis, aminopropylation reactions, and the methylation both of proteins and of DNA.³⁻⁵

Localised folate deficiency has been implicated in the development of epithelial cell

Yield and viability of epithelial cells derived from differing areas of the gastrointestinal tract. Data shown as mean (SEM) and range

Tissue of origin	Yield ($\times 10^6$ cells)	Viability (%)
Duodenum (n=8)	4.0 (0.62) (2.15 to 7.25)	83 (72 to 98)
Caecum (n=11)	6.6 (0.71) (4.05 to 8.0)	76 (64 to 85)
Sigmoid (n=19)	5.35 (0.56) (2.0 to 11.2)	78.4 (67 to 90)

endoscopic session as those from the colon. Venous blood was drawn for the estimation of red blood cell folate, serum folate, and vitamin B-12 values. In three cases, sigmoidoscopy and mucosal biopsy was repeated after a period of several weeks.

Intestinal epithelial cell isolation

Four endoscopic biopsy specimens from each area of interest were taken into calcium and magnesium free Hank's buffered saline solution (Life Technologies, Paisley, Scotland) supplemented with 0.3% bovine serum albumin, penicillin and gentamicin (CMF HBSSsup) containing 0.75 mM dithiothreitol (Sigma, St Louis, USA). Specimens were allowed to stand in this solution for three hours at room temperature. Subsequently, the tissue was transferred to fresh CMF HBSSsup containing 2 mM EDTA and placed on an inclined (45°), rotating table at 37°C for one hour. The resulting cell suspension was washed and pelleted in Hank's buffered salt solution. After counting and assessment of viability using ethidium bromide/acridine orange,¹² cells were saved at -20°C in 500 μ l phosphate buffered saline containing 1% ascorbic acid (pH 6.5) until assayed. To determine the influence of cell viability on measured values of folate, three sets of specimens were divided equally and during EDTA treatment 5 mM DTT was added to one set of tubes. After treatment, cell viability in the 5 mM DTT treated fractions was <5%.

Epithelial cell isolates were assessed for lamina propria contamination and intraepithelial lymphocyte (IEL) content by flow cytometry (FACScan, Becton-Dickinson, Belgium) using the monoclonal antibodies: Ber-Ep-4 (epithelium) (Dako, Denmark); CD19 (B-cells) (Becton-Dickson), R155 (dendritic cells: a kind gift from Dr A Whelan, Department of Immunology, St James's Hospital, Dublin), and CD3 (T cells) (Becton-Dickinson). Digests of remnant lamina propria served as positive controls for staining. Haematoxylin and eosin staining of remnant biopsy tissue permitted estimation of crypt clearance by light microscopy.

Folate assay

Cell suspensions in ascorbate buffer (500 μ l) were sonicated for 10 seconds on ice at a power setting of 100 W (Cell Disruptor B15, Branson, USA) and deconjugated to permit microbiological assay. Three hundred microlitres of sonicated cell suspension were

placed in a water bath at 100°C for 10 minutes. Upon cooling, 10 μ l of chicken pancreas conjugase (a gift of Dr A Molloy, Department of Clinical Medicine, Trinity College, Dublin) was added and the suspension incubated at 37°C for two hours. After incubation, the suspension was once again boiled to quench conjugase activity. Aliquots of this suspension were assayed for folate content using a microtitre plate method previously described.¹³ Recovery of folate was estimated by use of tritium labelled pteroyl-monoglutamate 10 μ l (Amersham) added to each sample and compared with an external standard. Inclusion of a yeast extract (0.1%) control served to ensure uniform conjugase activity between assays. Blood folate and vitamin B-12 content were measured by the same method.

Folate results were standardised with respect to spectrophotometric determination of homogenate DNA content using bromodeoxyuridine.

Statistical analysis

Statistical analysis was performed using SPSS for Windows 6.0 (SPSS Inc, USA). Data were compared using Student's *t* test for paired data. Correlation values are given as the Pearson correlation coefficient. Data are expressed as mean (SEM). Alpha was set at 0.05.

Results

Mucosal epithelial cell isolation

The ion-chelation method applied, resulted in an epithelial cell population (duodenal and colonic) free of lamina propria contamination as assessed by B cell (<1%) and dendritic cell (<1%) content. Contamination with erythrocytes was not seen on light microscopy. Colonic tissue yielded epithelial populations with <6% IEL. The Table gives the yield and viability of cells recovered from various regions of the gastrointestinal tract. Suspensions derived from specimens of the small bowel and caecum contained fewer cell clumps (2-4 cells) than those of the distal colon. Colonic crypt clearance, as determined by microscopy, was complete and uniform between sigmoid and caecum.

Sigmoid epithelial cell folate content

Recovery of spiked folate was in the range 90% to 108%. Interassay and intra-assay variation for this assay were both <7%. The folate content of paired sets of four sigmoid mucosal specimens, from the same subject, processed separately correlated closely ($r=0.88$, $p<0.001$, $n=11$). Differences in degree of cell isolate viability had little influence on folate content (high viability: 17.8 (1.4) and low viability: 19.0 (1.0) μ g/ μ g DNA, $n=3$). Sigmoid epithelial homogenates ($n=22$) had a folate content of 20.1 (1.8) μ g/ μ g DNA (10.2-46.6). No folate could be detected in the isolation medium (CMF HBSSsup). In the three cases where sigmoid folate values were

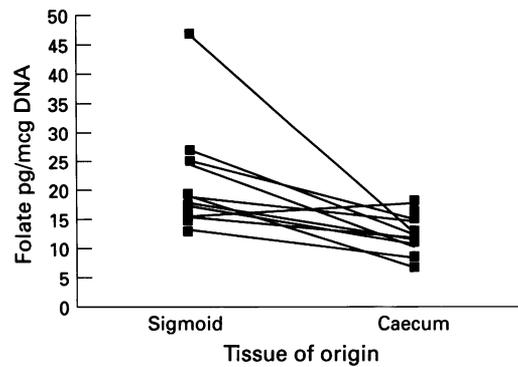


Figure 1: Comparison of sigmoid and caecal epithelial cell folate content from the same subject ($n=11$; $p<0.01$).

re-estimated following a repeat endoscopy at one to four weeks, results remained consistent (18.0 (1.0) v 19.0 (1.2) pg/ μ g DNA; $p>0.05$). In one patient where folate supplements were given for systemic folate deficiency, sigmoid colonocyte folate content rose from 20.0 to 41.8 pg/ μ g DNA in seven weeks. Red cell folate values rose from 140 ng/ml to 640 ng/ml over the same period.

Duodenal epithelial cell folate content

In five cases, folate values in sigmoid and duodenal epithelial cells were compared and found to correlate closely (19.4 (2.9) v 20.5 (3.2) pg/ μ g DNA; $r=0.93$; $p<0.001$).

Caecal epithelial cell folate content

Paired samples ($n=11$) from sigmoid (four specimens) and caecum (four specimens) of the same subject, showed the cells of the distal colon to have a greater folate content (Fig 1): 21.7 (2.8) v 13.3 (1.1) pg/ μ g DNA ($p<0.01$).

Serum folate, red cell folate, and vitamin B-12 values

Blood samples from the study population showed mean red cell and serum folate values of 340 (31) ng/ml (190–890 ng/ml) and 10.1 (0.9) ng/ml (4.4–20 ng/ml) respectively. Correlation between these values was significant ($r=0.46$; $p<0.03$). Mean serum vitamin

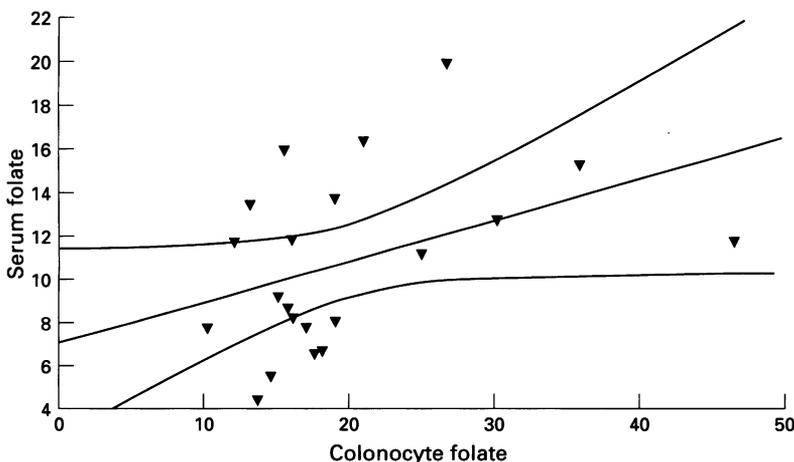


Figure 2: Scatter diagram showing correlation between sigmoid epithelial cell folate content and serum folate values ($r=0.41$, $p=0.063$). Curved lines represent 95% confidence limits.

B-12 content was 429 (38) μ g/ml (170–1000 μ g/ml). No significant correlation between sigmoid and red cell folate ($r=0.17$), or serum folate ($r=0.41$; $p=0.063$) values was seen (Fig 2). This picture was repeated with duodenal ($r=-0.04$ and $r=0.34$ respectively) and caecal ($r=0.06$ and $r=0.34$ respectively) tissue.

Discussion

Biopsy specimens of the intestinal mucosa contain a heterogeneous population of cells that includes fibroblasts and cells of blood, endothelial, and epithelial origin. To isolate colonic (colonocytes) and duodenal epithelial cells from small numbers of endoscopic biopsy specimens, a modified ion-chelation protocol was developed. Unlike enzymatic biopsy methods, this approach yields a large population of colonocytes with no erythrocyte contamination. The intact epithelial basement membrane seen on microscopy suggested that there would be little lamina propria contamination. This was confirmed through flow cytometry using dendritic and B cell markers. The initial prolonged exposure of whole biopsy specimens to DTT was of importance, as it permitted the removal of mucous without noticeably affecting cell viability and obviated the need for subsequent high concentrations of EDTA. Additionally, the use of the inclined, rotating table, enabled the specimens to tumble freely, exerting a shear force on the tissue and adding to cell yield.¹⁴

This study suggests that a standard range for colonocyte folate content can be defined. The range reported shows a fourfold difference between extremes, mimicking those seen with both serum and red cell folate values. This comparatively narrow range differs considerably from the wide range reported for human buccal mucosal cells though is consistent with that shown for whole colonic mucosal digests, perhaps reflecting differences in local conditions and cell function.^{15 16} In addition, we have found individual colonocyte folate content to be constant over a period ranging from one to four weeks, implying consistency of content in a rapidly proliferating cell population. We have shown that cell viability is unlikely to confound these results. This is in keeping with the steric effect predicted from the large intracellular polyglutamated storage form of folate.

The regional variation in folate content was an unexpected finding and may reflect a variable capacity for cellular folate transport. The proximal small bowel is the major site of absorption for dietary folates.¹⁷ This is reflected in the presence of specialised brush border mechanisms for dietary folate deconjugation and receptor mediated import. The ability of colonocytes to process luminal folate is less well defined. Explant studies show human colonic mucosa to possess a low affinity carrier permitting import through facilitated diffusion, greater uptake occurring with sigmoid than with caecal mucosa.¹⁸ Our finding that sigmoid colonocyte folate values are significantly greater than those of paired caecal

cells, may reflect these differences. It can be speculated that slower faecal transit in the distal colon contributes to this in permitting prolonged close approximation of faecal folate with the mucosal epithelium. Studies in rodents show the ability of colonic folate to contribute to hepatic reserves, this has never been shown in humans.¹⁹ Indeed, the common occurrence of systemic folate deficiency suggests that such a contribution does not occur, possibly showing the lack of a colonic epithelial cell folate export mechanism.

The lack of correlation between blood folate values and sigmoid colonocyte in folate replete subjects is not surprising as a similar discordance has been noted with human buccal epithelium.¹⁵ Though animal studies have reported a close association between gut and systemic values, experimental conditions were extreme, heterogeneous cell populations were assayed, and inter-species physiological differences exist.²⁰⁻²² Colonic epithelial cells are constantly exposed to a folate rich luminal flora. Coupled to the possession of a folate import mechanism, this may provide a degree of independence from circulating folates.

The displayed lack of correlation between measures of systemic folate reserve and colonic epithelial cell content may extend to folate deplete populations. It is of note that in our single case with folate deficiency, the colonocyte folate value was within our normal range. However, therapeutic doses of folic acid increased colonic intraepithelial values significantly. This is of clinical importance. Although it seems that oral folate supplements can increase colonocyte values, standard measures of folate reserve, such as red cell and serum content, possibly may not be depended upon to predict those that may benefit. Cell isolate contamination by unabsorbed oral folate is highly unlikely on account of the cell isolation procedure followed.

In conclusion, we report a modified ion-chelation method that permits the isolation of large numbers of viable gastrointestinal epithelial cells from standard endoscopic biopsy specimens, free of lamina propria contamination. Applying this technique, it has been shown that there is regional variation in gut epithelial cell folate content, that of the caecum being lower than those of both the duodenum and sigmoid colon. In subjects in folate balance, sigmoid colonocyte values remain constant with time and are independent of those found systemically. These findings are of

importance in considering (a) the relation between potential tissue (localised) folate deficiency, carcinogenesis and the use of folate supplements and (b) the relation between diets replete and deplete in folate and cancer.

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