The Role of the Colonic Flora in Maintaining a Healthy Large Bowel Mucosa

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The large bowel epithelium lives in a symbiotic relationship with millions of bacteria. These bacteria ferment dietary fibre to the short chain fatty acids, of which butyrate appears to be the most important. A technique was developed which allowed the rate of oxidation of fuel sources to be measured in colonic mucosal biopsies. This combined manometric methods with radiotracer technology. In normal colonic mucosal biopsies the rate of oxidation of butyrate was far greater than glutamine and in turn glucose. In patients with quiescent ulcerative colitis there was a significant defect in the mucosal ability to oxidize butyrate. Following on from this in vitro experiment, an in vivo experiment was performed, studying the rate of metabolism of a butyrate enema, in patients with quiescent colitis compared to controls. This showed no significant defect in whole body butyrate metabolism as measured by the production of $^{13}$CO$_2$ from $^{13}$C-butyrate. Hydrogen sulphide, a bacterial metabolite, was found to inhibit butyrate metabolism in the colonic cell culture line HT 29. However, this inhibition was prevented by the presence of glucose. Immunological studies found that butyrate, in physiological concentrations, inhibited phytohaemaglutinin induced proliferation of peripheral lymphocytes. Butyrate also induced expression of CD69 but not CD25. These studies show that butyrate, as well as being a major fuel source for the colonic epithelium, has an immunosuppressant effect on lymphocytes. This may play a major part in maintaining the homeostasis between the colonic epithelium and the colonizing bacteria.

Key words: butyrate; colonic mucosa; immunology

INTRODUCTION

The large bowel has been considered a source of sepsis and chronic ill health. Metchnikoff suggested, in the 1870's, that “the large intestine with its teeming myriads of bacteria was a source of chronic poisoning, the removal of which would indefinitely prolong life.” Arbuthnot Lane, a famous Guy’s surgeon, at the turn of the 19th century attributed a large number of diseases including diabetes mellitus and flat feet to “auto-intoxication arising from the chronic sepsis in the intestinal cesspool.” He advocated copious drafts of mineral oil or even colectomy to cure these ills. Consequently, at the turn of the century, the advertising columns of the popular press were full of the benefits of purgatives. In 1935 it was estimated that over 10 million pounds was spent on patent medicines, the majority being purgatives. Only in the last 15–20 years has the metabolic activity of the colon been considered of importance to its host (8).

At birth the colon is sterile, dark, warm, moist, anaerobic and rapidly fills with food. These are ideal conditions for bacterial growth and rapid colonisation occurs. A gram of caecal content may contain up to 2 billion organisms from 17 different bacterial families of 50 different genera comprising 400–500 species of bacterial. This leads to a microbial ecosystem, which is more numerous than the number of cells in a human. This contrasts with the relatively small number of colonic epithelial cells (colonocytes) and so on the basis of probability an epithelial cell abnormality is likely to reflect changes induced by the microbial flora. This ecosystem between the colonic flora and the epithelium has evolved over many millions of years and there are numerous inter-relationships; commensalism, mutualism, competition and predation all occur.

Disturbance of this balance, for example by antibiotic consumption, may result in diarrhoea because of a failure of the colonic epithelium to absorb sufficient quantities of water. If the host mounts an uncontrolled immune response to the colonising bacteria, the results are inflammation of the mucosa characterised by diseases such as ulcerative colitis. Conversely, should the bacteria start to invade across the colonic epithelium, the patient develops sepsis. Furthermore, bacteria can generate compounds that are toxic to the colonocyte. Hydrogen sulphide is produced as a consequence of bacteria using dietary sulphite ions as terminal ele-
tron acceptors in order to maintain an anaerobic environment. It has been suggested that this highly toxic compound inhibits the enzyme, butyryl co-enzyme A dehydrogenase, provoking mucosal inflammation.

Louis Pasteur at the age of 39 in 1861, whilst working at the Academy of Sciences in Paris, first discovered the process of anaerobic fermentation. Tappeiner at the close of the nineteenth century showed that micro-organisms from the rumen and equine large intestine fermented cellulose to yield methane, carbon dioxide and short chain fatty acids. However, it was more than 50 years before the nutritional importance of short chain fatty acids was realised. During the second world war, workers in Cambridge showed that most of a ruminants feed was fermented to short chain fatty acids, which act as the major energy source for these animals.

Fibre is an extremely complex collection of molecules comprising cellulose, inulin, guar and other plant gums and mucilages. During the past 15–20 years there has been an increasing body of evidence to suggest that there is considerable benefit from dietary fibre and it has been advocated for the treatment of patients with diverticular disease, irritable bowel syndrome and in the prevention of colorectal cancer (4).

In man, there is convincing evidence that the colon is the major site of fibre degradation. No human enzymes have been identified that are capable of breaking down fibre and so it enters the caecum unchanged from when it was ingested (5). Once inside the caecum, fibre is acted upon by polysaccharidases that are bound to bacterial cell walls. The end products of bacterial fermentation of fibre are the short chain fatty acids, the most important being acetate, propionate and butyrate (7).

Butyrate is a major energy source for the colonocyte and perturbations in its metabolism in health and disease is the focus of this study. The ability of normal and diseased colonocytes to metabolise butyrate in vivo and in vitro was studied, followed by the effects that sulphide has on butyrate metabolism. The final part of the study looked at the effects of butyrate on lymphocyte function.

**IN VITRO MUCOSAL STUDIES**

*Materials and Methods*

A technique was developed to quantify the rate of oxidation of glucose, glutamine and butyrate in “biopsy” sized samples. Glucose was used as it is the ubiquitous fuel source for mammalian cells, glutamine is the preferred fuel substance for the small bowel enterocyte and butyrate is believed to be the major fuel source for the large bowel epithelium. Briefly, mucosal biopsies were taken at endoscopy and placed into 2 ml of RPMI cell culture media. In the top of the reaction vial, a filter paper soaked in sodium hydroxide was suspended. To the vial, 20 µl of either radiolabelled glucose or glutamine or butyrate was added. After 2 hr incubation at 37°C, sulphuric acid was injected through the rubber stopper into the cell culture media displacing the dissolved carbon dioxide which was absorbed by the sodium hydroxide on the filter paper. The quantity of radioactive substrate that had been converted to carbon dioxide was calculated using liquid scintillation counting. The mucosal biopsy was retrieved and its protein content estimated by using the Lowry method. It was possible to calculate the rate of conversion of each of the 3 fuel substrates to carbon dioxide per microgram of mucosal protein/hr. Extensive validation of this assay found it to have an experimental error of about 11% (2).

**Assessment of regional variation of metabolism in normal large bowel mucosa.** Triplicate biopsies were taken from each of the 5 sites of the large bowel (ascending colon, transverse colon, descending colon, sigmoid colon and rectum) in 20 patients undergoing a colonoscopy in which no abnormal pathology was found.

**Mucosal metabolism in quiescent ulcerative colitis.** Triplicate biopsies were obtained from each of the 5 sites of the large bowel in 16 patients (10 male, median age 43, range 22–78) who had quiescent or mild ulcerative colitis. This was confirmed on histological assay.

**Results**

This study confirmed that butyrate is oxidised at a much faster rate than glutamine or in turn glucose (Fig. 1). There was no significant difference in the rate of oxidation between the 5 regions of the large bowel for each of the three substrates. Furthermore, there was no significant difference in the rate of metabolism for glucose or glutamine between the control group and patients with quiescent ulcerative colitis. However, there was a significant reduction in the ability of quiescent ulcerative colitic mucosa to oxidise butyrate when compared to the control population (Fig. 2). This defect was also found in the terminal ileal mucosa of patients with colitis (1, 3).

**IN VIVO STUDIES**

*Materials and Methods*

The above suggested that a failure of butyrate oxidation by the colonic mucosa is a factor in the develop-
ment of ulcerative colitis. Interestingly, the administration of short chain fatty acid enemas to patients with colitis has been found to decrease mucosal inflammation (10). The second part of this study assessed, in vivo, butyrate metabolism in patients with quiescent ulcerative colitis.

Eleven patients (mean age 48.8 ± 3.5 years) with quiescent colitis, as assessed by their symptoms and histological analysis of a rectal biopsy, and 10 control patients (mean age 59.8 ± 4.1 years) with no history of inflammatory bowel disease were recruited for the study.

A 100 ml $^{13}$C butyrate enema containing 1.25 mmol ($^{13}$C) sodium butyrate and 3.75 mmol unlabelled sodium butyrate in 10 mmol sodium chloride solution was administered. Breath samples were collected every 30 min for the next 4 hr. 13-Carbon dioxide ($^{13}$CO$_2$) enrichment in the expired breath samples was analysed using an isotope ratio mass spectrometer. The rate of metabolism of the butyrate enema to expired CO$_2$ was calculated. To control for variations in individual metabolic rates the baseline resting energy expenditure (REE), respiratory quotient (RQ) and CO$_2$ production (VCO$_2$) were measured using a ventilated hood indirect calorimetric system.

Results

The total quantity of $^{13}$CO$_2$ expired during the experiment was similar between the two groups ($p = 0.92$). Over the 4 hr of the experiment about 25% of the radio-labelled butyrate was recovered in the breath samples (9).

There was no significant difference between the two groups when the rate of the $^{13}$CO$_2$ production was standardised for body weight and metabolic rate ($p = 0.78$ and 0.88 respectively) (Fig. 3). However, there was a time x group interaction with regard to the $^{13}$CO$_2$ production and it appeared that the control group initially oxidised butyrate faster than the colitic group. Both groups reached similar peak levels of $^{13}$CO$_2$ production, although the control group’s rate of oxidation declined faster than the colitic group ($p < 0.05$).
EFFECTS OF SULPHIDE ON BUTYRATE METABOLISM IN THE HT29 CELL LINE

Materials and Methods
This part of the study investigated the effects of 0–5 mM sodium sulphide on the colonic cancer cell line HT29 and whether its effects could be overcome by increased butyrate concentrations. HT29 cells were cultured in 24 well tissue culture plates and grown until 90% confluent. They were then transferred to Dulbecco’s modified eagle medium containing amino acids and either 5 mM glucose or 3 mM butyrate or both together. Cultures were incubated for 3 hr, after which the cells were assayed for ATP and protein concentrations. ATP was quantified using a chemiluminescent method utilising firefly luciferase.

Results
As expected, HT29 cells oxidised significantly more butyrate than glucose. Co-incubation with butyrate and sulphide led to a significant decrease in the cellular ATP levels and this could not be overcome by increasing the butyrate concentration to 30 mM. However, the presence of glucose prevented this decrease, presumably as sulphide has no effect on glucose metabolism (Fig. 4) (6).

LYMPHOCYTE FUNCTION STUDIES

Materials and Methods
The final part of the study investigated the effects of butyrate on phytohaemagglutinin (PHA) induced lymphocyte proliferation and expression of CD69 (a marker of early cellular activation) and CD25 (a marker of late cellular activation). Venous blood was obtained from 6 normal volunteers (age range 20–27, median 21 years) and from 6 patients with quiescent ulcerative colitis (age range 20–70, median age 27). The blood samples were centrifuged and the lymphocyte population extracted. Aliquots of lymphocytes were placed into 96 well plates with different concentrations of butyrate. Five microgram per milliliter of PHA was added to each well to stimulate the lymphocytes to proliferate. Further samples were obtained from 7 patients (age range 19–25 years, mean 21 years). These lymphocytes were incubated for a variable time course with 2 mmol butyrate and using FACS analysis expression of CD69 and CD25 between butyrate exposed cells and control cells were established.

Results
The results showed that butyrate, in physiological concentrations, decreased the rate of proliferation of
peripheral blood lymphocytes, both in normal patients and those suffering from colitis (Fig. 5). Butyrate significantly increased the expression of CD69 after 4 hr but had no significant effect on CD25 expression.

CONCLUSION

This work has explored the intricate relationship between bacterial products of fermentation, short chain fatty acids and the effect that these have on the colonic epithelium and the immune system.

The in vitro experiments confirm previous work that butyrate metabolism is impaired in those with colitis, however this was not confirmed by the in vivo experiments. The explanation for this is not clear but would make it unlikely that there is a systemic metabolic defect in those with colitis. However, there does appear to be a perturbation in the pathway by which a butyrate load is handled in patients with ulcerative colitis.

Sulphide experiments suggest that butyrate metabolism is sensitive to poisoning by the bacterial production of hydrogen sulphide. The finding that glucose protects against this is intriguing and begs questions about the availability of glucose at the epithelium/gut lumen interface in those with colitis.

In view of the above findings and knowledge that butyrate enemas may decrease inflammation in sufferers of colitis we hypothesised that short chain fatty acids may play a role in modulating the colonic immune response to the colonising bacteria. The lymphocyte function studies would suggest that butyrate has such an “immuno-suppressant” effect and this is supported by the finding that butyrate induces expression of CD69. The expression of this marker appears to be at the crossroads in predicting lymphocyte behaviour. It is associated with activating responses such as production of IL2, IFN and TNF, alternatively, it can mediate apoptosis. The significance of this effect awaits further work.

Short chain fatty acids are ideally suited to modulating the host immune response to bacterial antigens. They are produced by the bacteria and are almost completely metabolised by the colonic epithelium so are unlikely to have any systemic effect. Further understanding of this complex relationship between the colonic flora and the epithelium will lead to alternative strategies to combat diseases of the large bowel.

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