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Hunterian lecture

The role of the colonic flora in maintaining a healthy large bowel mucosa

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This work explores the intricate relationships between bacterial products of fermentation, the short chain fatty acids and the effect that these have on the colonic epithelium and the immune system. It confirms that butyrate is a major energy source for the colonic epithelium and there may be a minor epithelial abnormality in the metabolism of butyrate in patients with ulcerative colitis. Immunological studies suggest that butyrate has an effect on lymphocyte activation and inhibits cell proliferation. Possibly, butyrate induces anergy in lymphocytes via an effect on the TCR receptor. This may represent a mechanism whereby colonic bacteria are able to regulate the host immune response. An abnormal response to butyrate may upset the homeostasis between the gut immune system and the colonising bacteria resulting in epithelial unrest and inflammation.

Key words: Colon – Bacteria – Butyrate – Lymphocytes – Colitis

Historically, the colon has been considered as a source of sepsis and chronic ill health. In the 1870s, Metchnikoff suggested 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 century took up this teaching and 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. This belief continues with the practise of colonic hydrotherapy where clients can have their colons washed out with copious quantities of water instilled via the anus. It is only in the last 15–20 years that the metabolic activity of the colon has been considered of importance to its host.[1]

At birth the colon is sterile, dark, warm, moist, anaerobic and rapidly fills with food. These are the ideal conditions for the growth of bacteria and so 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 bacteria. This leads to a microbial ecosystem, which is more numerous than the number of cells in a human. This contrasts with the relatively

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small number of colonic epithelial cells 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 which occur and an individual species can be involved in more than one type of inter-reaction.

If this balance between the colonic flora and the epithelium is disturbed, for example by the consumption of antibiotics, diarrhoea may result 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 as is frequently seen on surgical intensive care units.

While working at the Academy of Sciences in Paris, Louis Pasteur in 1861 discovered the process of anaerobic fermentation. During the Second World War, researchers in Cambridge realised the nutritional importance of fermentation. They discovered that ruminants obtain the majority of their cellular energy requirements from the end products of bacterial fermentation of their feed. The majority of these end products are the short chain fatty acids.

Fibre is an extremely complex collection of molecules, comprising cellulose, inulin, guar and other plant gums and mucilages. It is only in the last 20 years that the benefits of fibre in man has commanded any attention. However, now a considerable body of evidence has accumulated suggesting that there is a health benefit from ingesting dietary fibre.[2] Indeed, fibre has been advocated for the treatment of patients with diverticular disease, irritable bowel syndrome and for the prevention of colorectal cancer.[3]

In man, there is overwhelming evidence that the colon is the major site of fibre degradation. Of fibre administered to ileostomists, 90–100% was recovered in their ileostomy effluent. 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.[4] By a variety of biochemical pathways, bacteria are able to ferment fibre into the short chain fatty acids. The most important of these short chain fatty acids are acetate, propionate and butyrate.[5]

In humans, butyrate appears to be the major energy source for the large bowel epithelium (colonocyte). It has been suggested that ulcerative colitis is a disease characterised by a failure of the colonocyte to oxidise butyrate.[6] However, these experiments were performed on surgical resection specimens which obviously were severely diseased. Our further understanding of the role of short chain fatty acids in human mucosal metabolism has been limited by the lack of suitable experimental techniques.

Aim

The purpose of these studies was to investigate in normal patients and those with ulcerative colitis the effects of: (i) putative energy sources (glucose, glutamine and short chain fatty acids) on intestinal mucosal metabolism; and (ii) short chain fatty acids on lymphocyte function

In vitro mucosal studies

Experimental technique

A radio-isotope tracer technique was developed which allowed small numbers of colonic epithelial cells to be studied. Using this technique, the ability of large bowel mucosal biopsies to metabolise glucose, glutamine and butyrate were investigated. These were chosen as glucose 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.[7]

Briefly, mucosal biopsies were taken at endoscopy and placed into cell culture media. In the top of the reaction vial, a filter paper soaked in sodium hydroxide was suspended. To the reaction vial, radiolabeled glucose, glutamine or butyrate was added. The reaction vials were sealed and placed in a shaking water bath at 37°C for 2 h. At the end of this period, sulphuric acid was injected through the rubber stopper into the cell culture media so as to displace the dissolved carbon dioxide. This was absorbed by the sodium hydroxide and, by using liquid scintillation counting, the quantity of radioactive substrate converted to carbon dioxide was calculated. The mucosal biopsy was retrieved, its protein content estimated and the rate of conversion of each of the three fuel substrates to carbon dioxide per microgram of mucosal protein/hour calculated. This assay was found to have an experimental error of about 10%.[8]

Assessment of regional variation of metabolism in normal large bowel mucosa

Triplicate biopsies were taken from each of five sites of the large bowel (ascending colon, transverse colon,

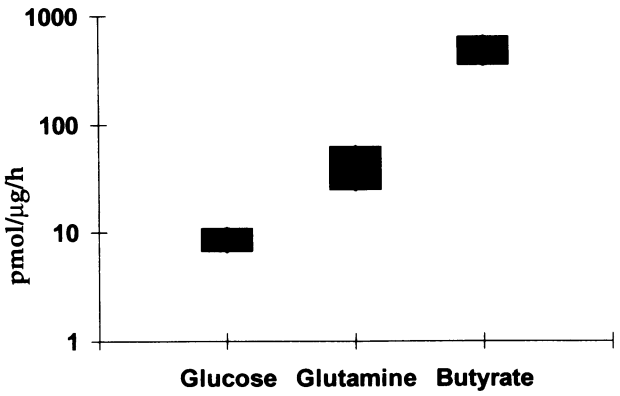

Figure 1 Rate of substrate oxidation in normal colonic mucosa.

descending colon, sigmoid colon and rectum) in 20 patients who were undergoing a colonoscopy in which no abnormal pathology was found. There was no significant difference in the rate of oxidation between these five regions ($P > 0.05$). This study confirmed that large bowel mucosal biopsies oxidised butyrate at a much faster rate than glutamine or in turn glucose (Fig. 1).

Mucosal metabolism in quiescent ulcerative colitis

Triplicate biopsies were obtained from each of the five sites of the large bowel in 16 patients who had total or sub-total quiescent or mild ulcerative colitis. Diagnosis was confirmed by histological assay. No difference in rate of metabolism of the three substrates was found between the five regions of the large bowel.

There was a significant reduction in the ability of quiescent ulcerative colitic mucosal biopsies to oxidise butyrate when compared to the control ones. However, there was no difference in the rate of metabolism of glucose or glutamine (Fig. 2). Furthermore, this metabolic defect was also present in the terminal ileum of people with colitis compared to the terminal ileum of patients with no evidence of colitis.[9,10]

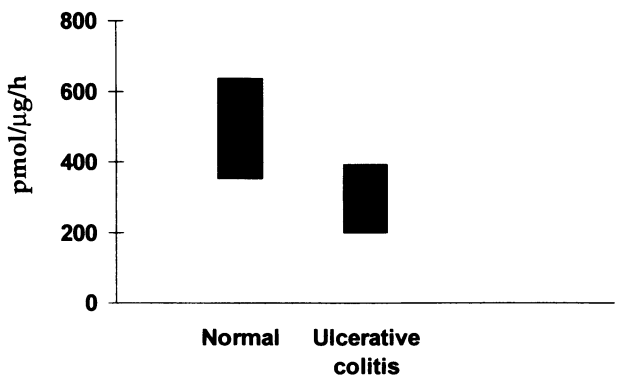

Figure 2 Rate of butyrate oxidation in colonic mucosa.

Mucosal metabolism in ileo-anal pouches

The construction of an ileo-anal pouch results in the ileal mucosa being exposed to a different luminal environment as a consequence of faecal stasis and bacterial colonisation. The aim of this experiment was to investigate whether this altered the rate of oxidation of the three energy sources.

Mucosal biopsies were taken in triplicate from the terminal ileal mucosa of 9 patients with ulcerative colitis. Further biopsies were taken in triplicate from 18 patients, who had mature ileo-anal pouches. These pouches have been constructed for ulcerative colitis and the covering ileostomies had been closed at least 6 months prior to biopsy.

There was no difference in the rate of butyrate or glucose oxidation. However, there was a significant reduction in the ability of the pouch mucosa to oxidise glutamine compared to the colitic terminal ileal mucosa.[11] This may be a consequence of bacterial consumption of glutamine. It interesting that glutamine has been used as an efficacious treatment of pouchitis.

In vivo measurement of butyrate enema metabolism

The above *in vitro* work suggested that a failure of butyrate oxidation by the colonic mucosa is a factor in the development of inflammation whether it be ulcerative colitis or pouchitis. Other groups have produced data, which support and refute this hypothesis.[6,12,13] These conflicting results may be as a consequence of different *in vitro* experimental techniques. Interestingly, short chain fatty acid enemas have been efficacious in treating patients with colitis and are beneficial in decreasing mucosal inflammation.[14] The aim of this part of the investigation was to use an *in vivo* technique to assess whether whole body butyrate metabolism was impaired in patients with quiescent ulcerative colitis.

Method

Eleven patients (mean age 49 years) with quiescent colitis as assessed by their symptoms and histological examination of a rectal biopsy and 10 control patients (mean age 60 years) with no history of inflammatory bowel disease were recruited for the study.

A 100 ml butyrate enema containing 1.25 mmol [1-^{13}C]-sodium butyrate and 3.75 mmol unlabelled sodium butyrate was administered. For the next 4 h, breath samples were collected every 30 min. [^{13}C]-Carbon dioxide (CO_2) enrichment in the expired breath samples were analysed using an isotope ratio mass spectrometer. This enabled the rate of metabolism of the butyrate enema to expired CO_2 to be calculated. During the time course of the experiment, the baseline resting energy expenditure (REE), respiratory quotient (RQ) and CO_2 production (VCO_2) was measured using a ventilated hood indirect calorimetric system.[15]

Results

The total quantity of [^{13}C]-CO_2 expired during the experiment was similar between the two groups (P = 0.92). Over the 4 h of the experiment, about 25% of the labelled butyrate was recovered in the breath samples.

There was no significant difference between the two groups when the rate of [^{13}C]-CO_2 production was standardised for body weight and metabolic rate (P = 0.78 and 0.88, respectively). However, there was a time x group interaction with regard to [^{13}C]-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}C]-CO_2 production although the control groups rate of oxidation declined faster than the control group (P <0.05 ANOVA: Fig. 3).

The respiratory quotient of the control group was significantly greater than that of the colitis group.

Discussion

This *in vivo* study has shown that patients with quiescent ulcerative colitis have no defect in the quantity or the peak rate of production of [^{13}C]-CO_2 obtained by oxidation of [^{13}C]-butyrate enema. The finding that the RQ of patients with colitis was significantly lower than the control group was an unexpected finding. This implies that the antecedent diet of the colitic group had a higher fat and a lower carbohydrate content than the control group. This may be due to the subjects in the colitis group selecting a low carbohydrate and possibly a low fibre diet so as to avoid provoking abdominal pain or exacerbating the colitis. Alternatively and intriguingly, it may be that a low fibre diet predisposes patients to the development of colitis and affects relapse rates.

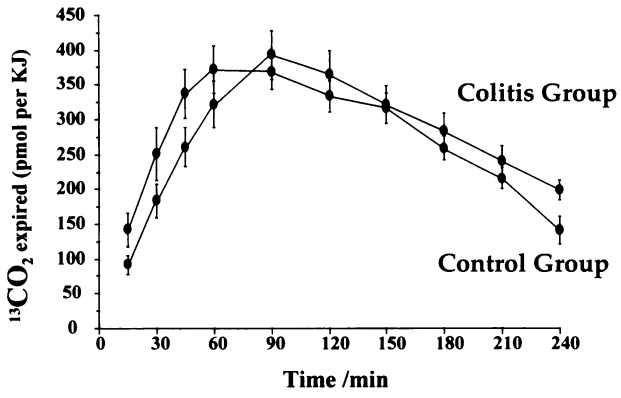

Figure 3 [^{13}C]-CO_2 expiration in ulcerative colitis patients and controls.

Conclusion of metabolic studies

It is difficult to reconcile these *in vivo* and *in vitro* findings. However, there does appear to be a perturbation in the metabolic handling of butyrate in patients with ulcerative colitis. This coupled with the clinical findings that butyrate enemas may decrease inflammation in patients with colitis led to the hypothesis that the short chain fatty acids may play a role in modulating the colonic immune response to the colonising bacteria. Short chain fatty acids would be ideally suited to this role being produced by bacteria and are almost completely metabolised by the colonic epithelium, so are unlikely to have any systemic effect.

Effect of short chain fatty acids on lymphocyte function

Phytohaemagglutinin (PHA) is a plant lectin, which induces rapid proliferation of the lymphocyte population. This proliferation can be measured by the addition of tritiated [^{3}H]-thymidine, which is taken up into the dividing cells' nuclei. The uptake of [^{3}H]-thymidine is quantified by liquid scintillation counting and is a surrogate marker of cell proliferation.

Method

Peripheral blood was obtained from 6 volunteers and from 6 patients with quiescent ulcerative colitis. The blood samples were centrifuged and the lymphocyte population extracted. Aliquots of lymphocytes were placed into 96-well plates to which 0–10 mmol/l butyrate concentrations were added. The lymphocytes were stimulated by the addition of 5 µg/ml PHA. Control wells were included to which no butyrate had been added.

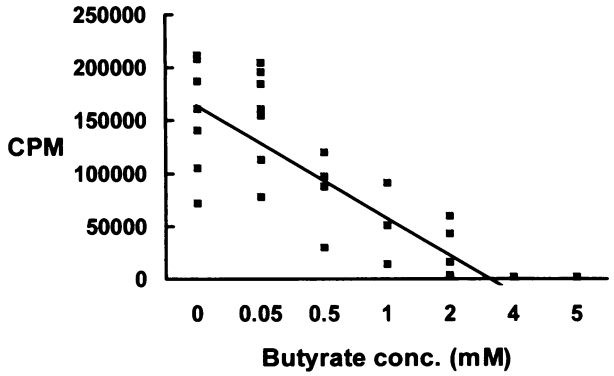

Figure 4 Effect of butyrate on PHA-stimulated lymphocyte proliferation.

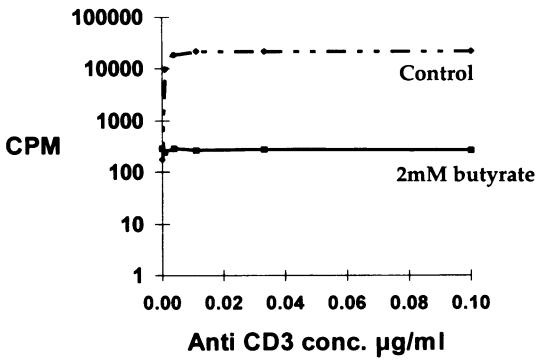

Figure 5 Effect of butyrate on anti-CD3 induced lymphocyte proliferation.

Results

Butyrate in physiological concentrations decreased the rate of proliferation of peripheral blood lymphocytes both in normal patients and those suffering from colitis (Fig. 4).

Discussion

This butyrate-induced inhibition of lymphocyte proliferation was not a toxic effect as when the butyrate was washed away and the lymphocytes re-stimulated with PHA they proliferated normally.

Elucidation of the mechanism of action of butyrate on lymphocyte proliferation

The mechanism by which butyrate inhibits lymphocyte function was explored by investigating its effect on CD expression. Cluster of determination (CD) nomenclature is used to characterise antigen complexes on lymphocytes. These are used as markers of lymphocyte subsets and by labelling with fluorescent antibodies can be quantified using flow cytometry (FACS scanning).

CD3 is the marker for the T-cell receptor (TCR) and this part of the work aimed to determine if the inhibitory effect of butyrate was mediated via the TCR receptor. Antibodies to the TCR receptor (OKT3) were prepared and used to stimulate lymphocytes. Butyrate (2 mM) significantly inhibited this proliferation indicating its effect is at the level of the T-cell receptor (Fig. 5). Acetate or propionate had no inhibitory effect.

Two further CD markers, CD69 and CD25, were studied to elucidate this mechanism. CD69 is one of the earliest markers of cellular activation. Its expression seems to mark the cross-roads in defining the behaviour of lymphocytes and can be associated with activating responses leading to the production of IL-2, IFN, and TNF, alternatively, it can mediate cell death via apoptosis. In contrast, CD25 (IL-2 receptor) is a late marker of activation and is essential for the continued proliferation of the lymphocyte population.

Peripheral blood lymphocytes were taken from 7 patients and incubated for a variable time with 2 mmol/l butyrate and the expression of CD69 and CD25 quantified using FACS analysis. Butyrate significantly increased the expression of CD69 after 4 h but had no effect on CD25 expression.

Conclusion

This work has explored the intricate relationships between bacterial products of fermentation, the short chain fatty acids and the effect that these have on the colonic epithelium and the immune system. It has been confirmed that butyrate is a major energy source for the colonic epithelium and there may be a minor epithelial abnormality in the metabolism of butyrate in patients with ulcerative colitis. Immunological studies suggest that butyrate has an effect on lymphocyte activation and inhibits cell proliferation. Possibly, butyrate induces anergy in lymphocytes via an effect on the TCR receptor. This may represent a mechanism whereby the colonic bacteria are able to regulate the host immune response. An abnormal response to butyrate may upset the homeostasis between the gut immune system and the colonising bacteria resulting in epithelial unrest and inflammation. Further work is needed to elucidate the role of butyrate on lymphocyte function.

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