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SHORT COMMUNICATION

Butyrate can act as a stimulator of growth or inducer of apoptosis in human colonic epithelial cell lines depending on the presence of alternative energy sources

Baldev Singh[1], Andrew P.Halestrap[2] and Christos Paraskeva[1,3]

Departments of [1]Pathology and Microbiology and [2]Biochemistry, School of Medical Sciences, University of Bristol, Bristol BS8 1TD, UK

[3]To whom correspondence should be addressed

***In vivo*, butyrate is a major energy source for the colonic epithelium and is thought to stimulate proliferation. In contrast, butyrate *in vitro* has been shown to inhibit proliferation and induce differentiation and apoptosis in colonic epithelial cells. Most colon cell cultures are grown in medium containing high concentrations of glucose, whereas *in vivo*, the main energy source used by the colon cells is butyrate. The aim of this study was to determine whether the apparent contrasting roles of butyrate *in vivo* and *in vitro* could be as a consequence of differences in glucose availability. The sensitivity of two human colorectal tumour cell lines, one adenoma (S/RG/C2) and one carcinoma (HT29) to butyrate-induced growth inhibition and apoptosis was investigated to determine whether these cellular effects were altered under glucose depleted culture conditions. Glucose depletion resulted in increased apoptosis in both cell lines in the absence of butyrate. Butyrate in standard culture conditions (containing 25 mM glucose and 1 mM pyruvate) inhibited growth and induced apoptosis in both cell lines. However, low concentrations of butyrate in glucose depleted culture conditions (i.e. standard growth medium without glucose and pyruvate supplements) were found to reduce apoptosis induced by glucose deprivation and increase cell yield in both cell lines. The results show that in glucose depleted culture conditions, butyrate at low concentrations (0.5 mM for S/RG/C2, and 0.5 and 2 mM for HT29 cells) was found to be growth stimulatory whereas in the presence of glucose, these same concentrations of butyrate induced apoptosis. Thus, whether butyrate is growth stimulatory or growth inhibitory may depend on the availability of other energy sources. These observations may, in part, provide an explanation for the apparent opposite effects of butyrate on proliferation reported *in vivo* and *in vitro*.**

Colorectal cancer is a common malignancy of western society and this high incidence is thought to be largely associated with dietary factors. A low fibre diet was proposed to contribute to an increased risk of colorectal cancer (1), a view supported by more recent epidemiological studies, which have provided strong support for this association (2). Carbohydrates which escape enzymatic digestion in the human small intestine undergo anaerobic fermentation in the colon. A major component of this class of carbohydrates is the non-starch polysaccharides (also known as dietary fibre) which is subjected to fermentation by the microflora of the large intestine, resulting in the production of acetic, propionic and butyric acid as major by-products (3). These short chain fatty acids (SCFA*), which occur in millimolar amounts, are rapidly absorbed in the colon, providing important energy supplies for the colorectal epithelium and maintaining the cellular electrolyte balance (4). Out of three SCFA, butyrate is considered to be the preferred energy source and it accounts for ~70% of the total energy consumed in rat colonocytes (4,5) and *in vivo* is reported to stimulate proliferation (6). This stimulation of proliferation is regarded as a physiological response rather than a pre-neoplastic associated phenomenon, since the dividing cells do not extend to the top of the crypt (7). Conditions which reduce the concentration of luminal SCFA have been shown to result in decreased epithelial cell proliferation. These conditions include colonic bypass surgery (8), feeding of a substrate-free diet (9) and germ-free conditions (10). The reduced level of SCFA results in metabolic starvation, accompanied by mucosal surface degeneration and acute inflammation of the colonic epithelium (11). Colonic instillation of SCFA has been shown to be beneficial in reducing this inflammation (12) and these studies have concluded that *in vivo*, butyrate is growth stimulatory and beneficial.

In contrast, butyrate *in vitro* has been shown to inhibit proliferation in a number of colorectal tumour cell lines (13,14). In addition, treatment of the colon carcinoma cell line HT29 with butyrate led to a permanently differentiated cell line (14). More recently, butyrate, propionate and acetate have been shown to induce apoptosis in a number of colonic tumour cell lines, with butyrate being the most effective inducer of apoptosis (15–17). These *in vitro* observations suggest a possible protective role for short-chain fatty acids in colorectal carcinogenesis and may explain, in part, the apparent protective role of dietary fibre. However, the effects of butyrate on proliferation, based on *in vivo* and *in vitro* studies, appear to give contradictory results. The preferred energy source for the colonic epithelium *in vivo* is butyrate (4,5), whereas most cell cultures are grown in tissue culture medium containing high levels of glucose for their energy source. Hence, differences in energy availability may, in part, provide an explanation for the observation that *in vivo* butyrate is reported to be growth stimulatory, whereas *in vitro* it is growth inhibitory.

In this study, since butyrate is the preferred energy source of the three SCFA present in the colon, we investigated the effects of butyrate on cell growth and apoptosis under two growth conditions: standard culture medium with high concentrations of glucose and culture medium without glucose supplementation. We asked whether under glucose-depleted culture conditions, butyrate may stimulate proliferation (as observed *in vivo*), and therefore whether the availability of alternative energy sources could alter the growth response of colonic cells to butyrate.

***Abbreviations:** DMEM, Dulbecco's modified Eagle medium; SCFA, short-chain fatty acids; $Glu^{+}Pyr^{+}$, medium supplemented with both glucose and pyruvate; $Glu^{-}Pyr^{-}$, medium deficient in both glucose and pyruvate; S/RG/C2, human colonic adenoma cell line; HT29, human colonic carcinoma cell line.

Two human colonic tumour cell lines, an adenoma cell line, S/RG/C2 (18), and the carcinoma cell line, HT29, were routinely grown on tissue culture plastic in Dulbecco's modified Eagle medium (DMEM; Life Technologies, Inc.) with 20 and 10% fetal bovine serum (batch selected), respectively, as described previously (19). Cells for the experiments were grown in either normal growth medium (DMEM), which had D(+) glucose (25 mM, Sigma) and sodium pyruvate (1 mM, Gibco BRL), referred to as Glu^+Pyr^+, or in DMEM medium which did not have added glucose and pyruvate. This was defined as glucose and pyruvate deficient medium referred to as Glu^-Pyr^- in the text. In these growth conditions, the other main potential energy sources available to the cells are glutamine and amino acids present in the medium and also the fetal bovine serum supplemented to the medium. For the experiments, 1×10^6 human colonic adenoma cell line (S/RG/C2) and 5×10^5 human colonic carcinoma cell line (HT29) cells were seeded in 25 cm^2 flasks and grown for 3 days in normal fully supplemented medium (Glu^+Pyr^+) to achieve ~50% confluent cultures. At this point, cells were grown in either Glu^+Pyr^+ or Glu^-Pyr^- medium in the presence of various concentrations (0–10 mM) of sodium butyrate (Sigma). After 4 days growth in the appropriate medium, the attached cells (those remaining adherent to the tissue culture flasks) and floating cells (those having detached from the tissue culture flasks) were counted separately.

As described previously, the level of apoptosis in cultured epithelial cell lines was assessed by measuring the proportion of the total cell population that had detached from the cell monolayers and was floating in the medium and determining the fraction of these floating cells that were apoptotic (15,16,20,21). Apoptotic cells were identified by their characteristically condensed chromatin stained by acridine orange (5 μg/ml in phosphate buffered saline) (15,17). The floating cells have previously been shown to have a high percentage of cells which display classical apoptotic cell morphology (80–90%), while the proportion of apoptotic cells in the attached compartment was low (<3%) in both control and butyrate treated cultures (15).

Effect of butyrate on growth inhibition

S/RG/C2 cells grown in Glu^+Pyr^+ showed a dose-dependent reduction in attached cell yield with increasing concentrations of butyrate (Figure 1A). HT29 cells were also growth inhibited by butyrate (Figure 2A) and showed an overall reduced sensitivity to butyrate in comparison to that observed in S/RG/C2 cells. To determine whether the growth inhibitory effects of butyrate are altered depending on the level of other potential energy sources present in the colon, we measured the effects of butyrate on cell growth in glucose/pyruvate (Glu^-Pyr^-) deprived culture conditions. For both cell lines, growth in Glu^-Pyr^- media (in the absence of butyrate) resulted in a reduction in the attached cell yield compared to the cell yields in Glu^+Pyr^+ media (Figures 1A,B and 2A,B). Interestingly, butyrate in Glu^-Pyr^- growth conditions increased cell yield at the lower butyrate concentration of 0.5 mM in S/RG/C2 (Figure 1B, $P < 0.05$), with a similar effect in HT29 cells at 0.5 and more significantly at 2 mM butyrate (Figure 2B, $P < 0.05$). Furthermore, both cell lines in Glu^-Pyr^- conditions were also found to be less sensitive to growth inhibition by butyrate at the higher doses compared to cells treated with butyrate in Glu^+Pyr^+ growth conditions (Figures 1 and 2).

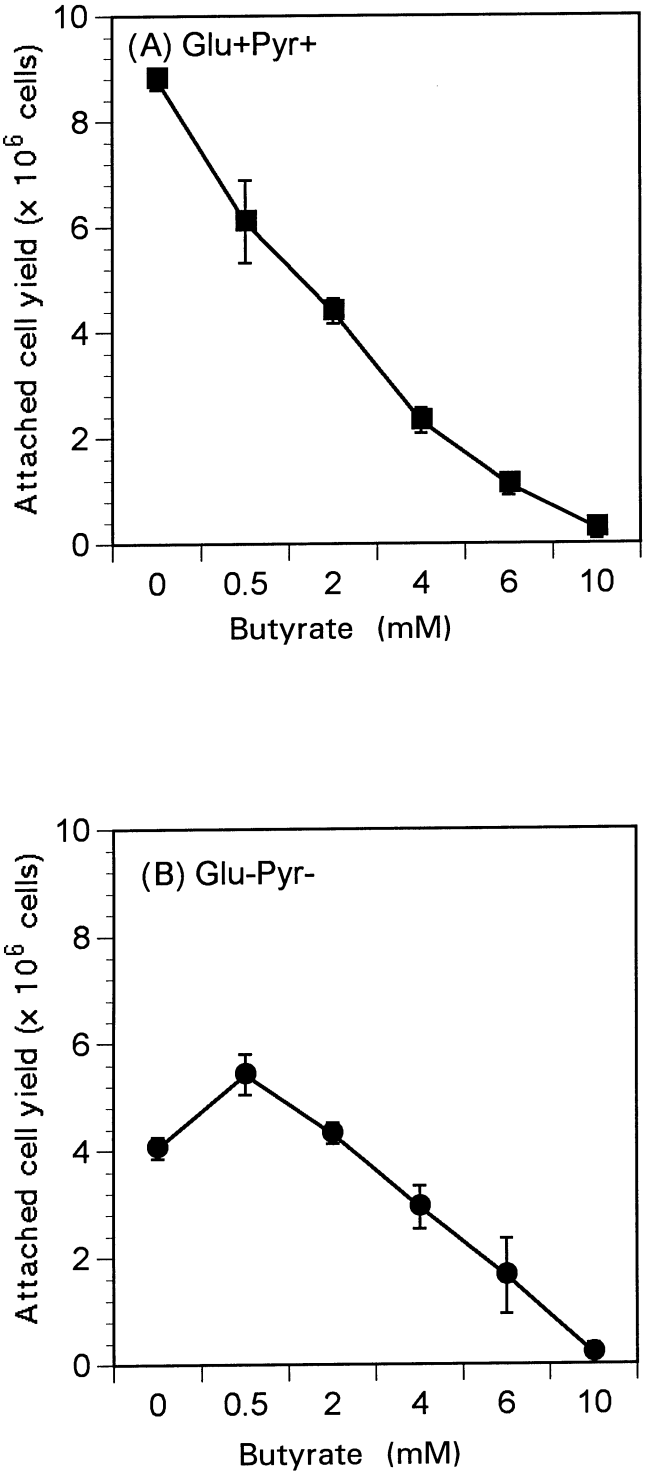


Fig. 1. The effects of increasing concentrations of butyrate on the attached cell yield in S/RG/C2 cells measured after 4 days incubation in (**A**) Glu^+Pyr^+ and (**B**) Glu^-Pyr^- growth medium. Each point represents the mean ± SEM of three independent experiments.

Therefore, to summarize, in the presence of glucose and pyruvate, butyrate is growth inhibitory in both cell lines. Withdrawal of glucose and pyruvate from the culture medium (in the absence of butyrate) reduced the attached cell yield in both cell lines, but the presence of butyrate in these glucose-

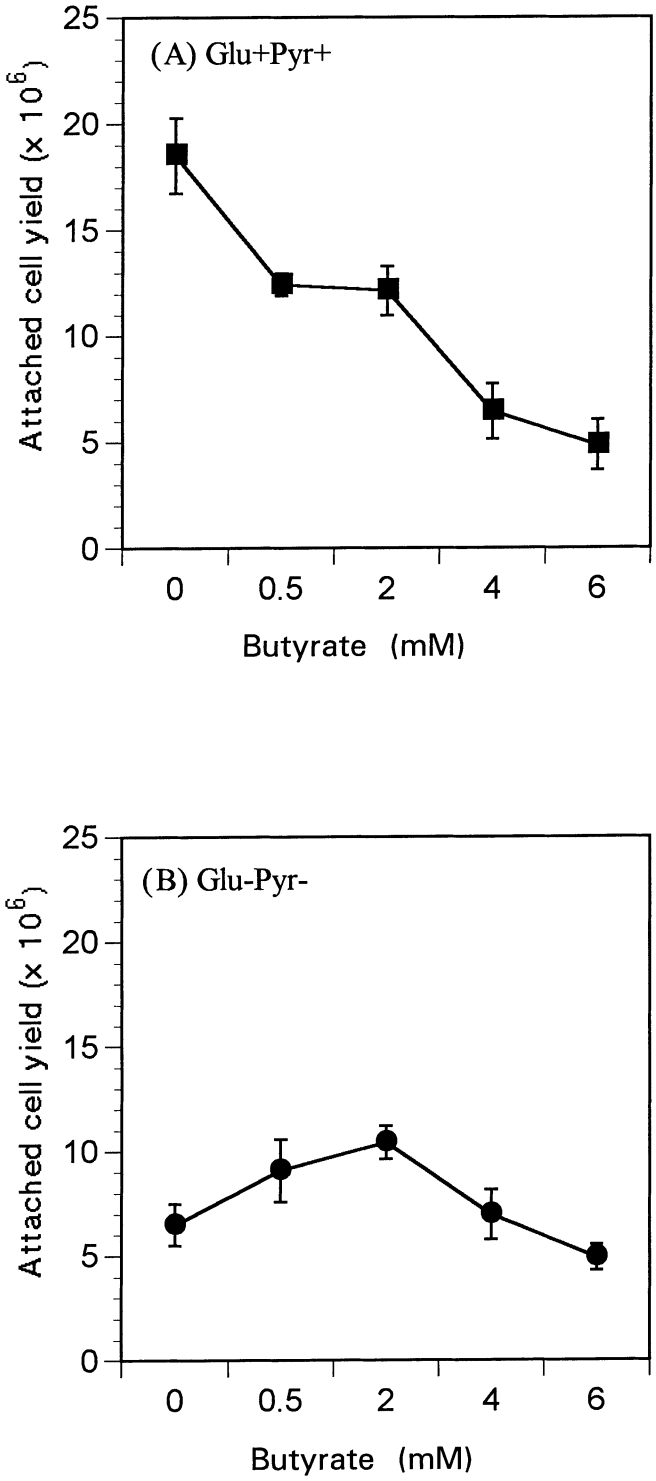


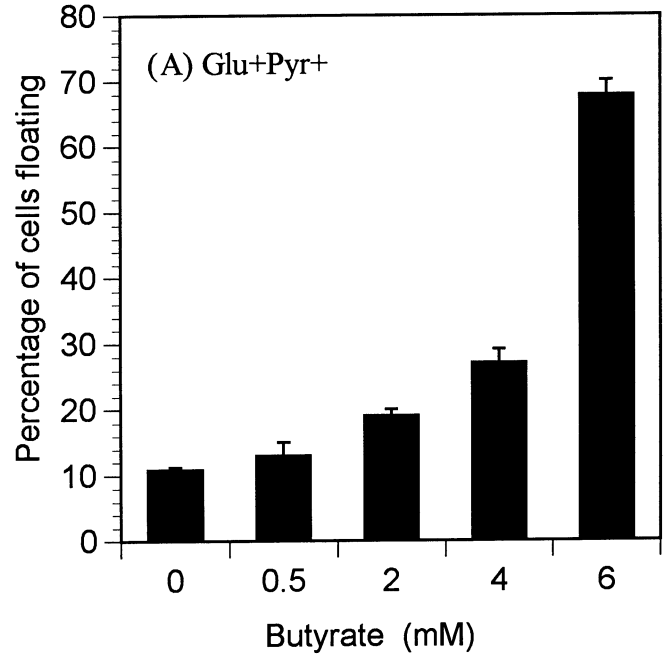
Fig. 2. The effects of increasing concentrations of butyrate on the attached cells yield in HT29 cells measured after 4 days incubation in (**A**) Glu^+Pyr^+ and (**B**) Glu^-Pyr^- growth medium. Each point represents the mean ± SEM of three independent experiments.

and pyruvate-depleted culture conditions did not result in an additive effect in terms of a reduction in attached cell yield. On the contrary, there was evidence of an enhanced cell yield at the lower butyrate concentrations in both cell lines in Glu^-Pyr^- growth conditions (0.5 mM in S/RG/C2 and 0.5 and 2 mM butyrate in HT29 cells). These observations that low concentrations of butyrate in glucose-depleted culture conditions can be growth stimulatory were also found in two other human colon tumour cell lines (results not shown). Furthermore, the apparent biphasic effect of butyrate with growth stimulation observed at the low concentrations (0.5 and 2 mM) compared to growth inhibition at the higher doses (4–6 mM) may have implications for the role of butyrate *in vivo*. An intracellular concentration gradient has been suggested to occur along the colonic crypt (22) with higher concentrations at the lumenal surface being growth inhibitory and lower concentrations of butyrate at the base of the crypt being growth stimulatory.

Effects of butyrate on apoptosis

Withdrawal of glucose and pyruvate (without addition of butyrate) from the cultures resulted in an increase of approximately 2- and 3.5-fold in the percentage of floating apoptotic cells in S/RG/C2 and HT29 cells, respectively, compared to cells grown in Glu^+Pyr^+ media (Figures 3B and 4B). The presence of butyrate in cultures grown in normal growth conditions (Glu^+Pyr^+) increased the percentage of floating apoptotic cells in both cell lines (Figures 3A and 4A). However, when cells were treated with butyrate in Glu^-Pyr^- growth conditions, there was a reduction in the percentage of floating apoptotic cells at the lower butyrate concentrations of 0.5 mM in both cell lines when compared to cells grown in Glu^-Pyr^- medium without butyrate (Figures 3B and 4B). Furthermore, the overall sensitivity of both cell lines to the induction of apoptosis at higher butyrate concentrations relative to controls was also reduced in Glu^-Pyr^- growth conditions.

One possible explanation for why butyrate is growth stimulatory *in vivo* is that colonocytes are dependent on the SCFA for their energy supply and metabolize it efficiently. Thus, as well as acting as an energy source *in vivo*, the efficient metabolism of butyrate may result in reduced intracellular concentration and thus reduces its growth inhibitory properties (23), whereas *in vitro*, cells in tissue culture medium with high glucose are adapted to using glucose as their energy source. Under conditions of glucose depletion, there was a reduction in cell yield and an increase in apoptosis in both the adenoma (S/RG/C2) and carcinoma cells (HT29). We then investigated whether the addition of butyrate to cell cultures in glucose depleted medium would enhance growth and reduce apoptosis. We reasoned that these conditions may be more similar to the *in vivo* situation where the preferred energy source is butyrate, and where glucose, although available through the vasculature, is not the main energy source for colonic epithelium. Interestingly, the addition of butyrate to glucose depleted medium at the lower butyrate concentration did not result in an additive effect in terms of a reduction in attached cell yield or induction of apoptosis. On the contrary, there was evidence of an enhanced cell yield and reduced apoptosis at the lower butyrate concentrations in both cell lines in Glu^-Pyr^- growth conditions. The addition of butyrate to cells under Glu^+Pyr^+ could be growth inhibitory because less butyrate is being metabolized as an energy source (due to the high levels of glucose), which may result in increased intracellular accumulation of butyrate ultimately leading to growth inhibition, differentiation and apoptosis. Increased intracellular accumulation of butyrate may lead to histone hyperacetylation which is one possible mechanism by which butyrate is thought to exert its growth inhibitory properties (reviewed in 24). However, when the energy availability is limiting for a particular tissue



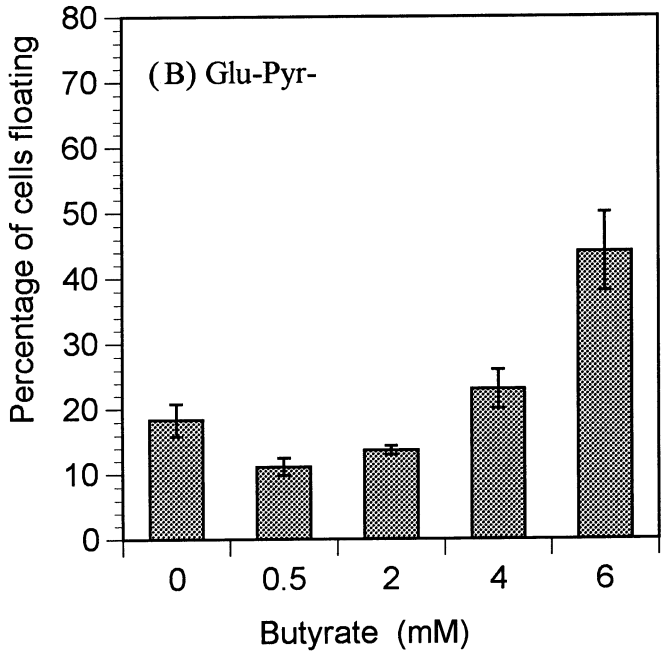
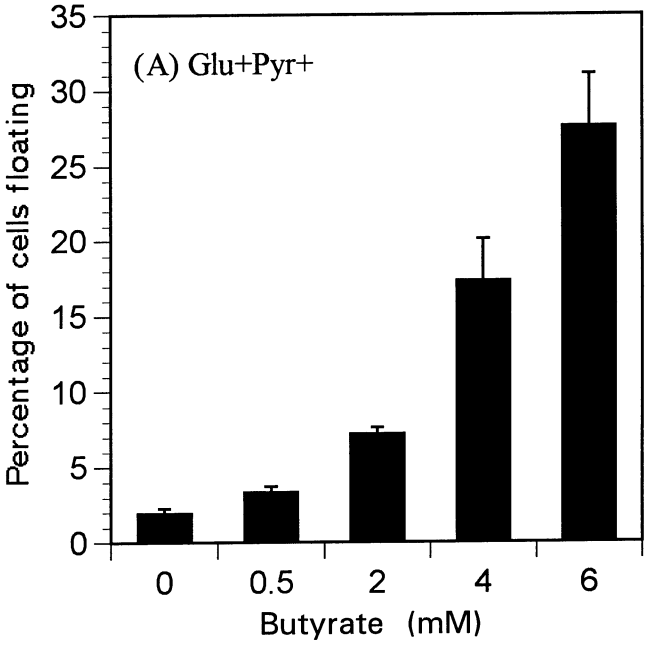


Fig. 3. Effect of butyrate treatment on the percentage of cells floating in S/RG/C2 cells measured after 4 days incubation in (**A**) Glu^+Pyr^+ and (**B**) Glu^-Pyr^- growth medium. A high percentage of floating cells stained with acridine orange were shown to display classic apoptotic cell morphology as described previously (15). Each bar represents the mean ± SEM of three independent experiments.



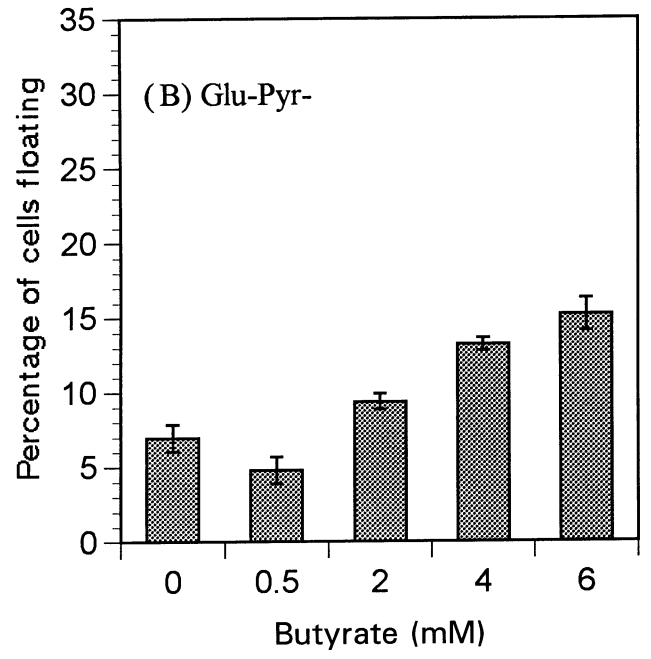


Fig. 4. Effect of butyrate treatment on the percentage of cells floating in HT29 cells measured after 4 days incubation in (**A**) Glu^+Pyr^+ and (**B**) Glu^-Pyr^- growth medium. A high percentage of floating cells stained with acridine orange were shown to display classic apoptotic cell morphology as described previously (15). Each bar represents the mean ± SEM of three independent experiments.

in vivo or cell culture, butyrate may be used as an energy source and can be growth stimulatory. To further address this possibility, preliminary metabolic labelling studies were conducted using S/RG/C2 cells. We found that [^{14}C]butyrate is more rapidly metabolized (producing more $^{14}CO_2$) in Glu^-Pyr^- medium than in Glu^+Pyr^+ medium (results not shown). This is evidence that butyrate is used as an energy source in glucose/pyruvate deficient medium. This could explain why, under these conditions, butyrate is not growth inhibitory. It is actively metabolized thus intracellular butyrate accumulation may be reduced.

An important conclusion from our studies is that the response of colonic epithelial cells to butyrate may depend in part on the other energy sources available to the epithelium. In conditions of low energy availability, butyrate could be growth stimulatory and/or trophic, but in the presence of high levels of alternative energy sources such as glucose, butyrate could switch from a growth stimulator to a growth inhibitor and/or an inducer of apoptosis. In view of the metabolic relationship between butyrate and glucose observed in colonocytes (25), it may well be that the 'switch to the oxidation' of butyrate in glucose-deprived culture may be a compensatory mechanism in response to a reduction in energy pools.

A remaining important question is whether colonic tumour cells are more sensitive to the growth inhibitory effects of butyrate than normal colon cells. The differential sensitivity of cells at different stages of tumour progression being potentially very important in determining the effectiveness of a potential chemopreventive agent (21). Most previous studies have compared normal cells *in vivo*, with tumour cell lines *in vitro*. Long-term normal adult human colonic epithelial cells have proved difficult to culture (26) and, therefore, it has been impossible to compare their responses to butyrate with tumour cell lines directly. Clearly, further work on this important area of research is necessary. Jass (23) in an attempt to explain the different growth inhibitory effects of butyrate has proposed an interesting hypothesis that butyrate metabolism in colonic tumours is impaired leading to a more rapid accumulation of intracellular butyrate compared to normal colon cells resulting in growth inhibition. In addition to possible impaired butyrate metabolism (23), the threshold above which butyrate might switch from being a growth stimulator to a growth inhibitor could be different according to the malignant potential of a cell. Hence, it is still possible that neoplastic cells are more sensitive to growth inhibition by butyrate than normal colonocytes.

The potential importance of considering the response of cells to butyrate in the context of overall energy availability and utilization is emphasized by the recent report that malignant human colonic tumours have been shown to have an increased glucose uptake and utilization when compared to their benign/normal counterparts (27). Furthermore, recent studies have shown a positive correlation between the expression of glucose transporter proteins in human colon cancers and incidence of lymph node metastases (28). In view of the possible difference in glucose requirements of normal and malignant colonic cells, this may alter the response of cells to butyrate-induced growth inhibition and apoptosis *in vivo*. One possible role of butyrate *in vivo* is that it can act as an energy source for normal cells, but induces apoptosis in neoplastic or damaged cells thus reducing the risk of cancer. The regulation of apoptosis in the colon is important since even low levels of apoptosis, if not balanced by proliferation, have been shown to result in significant tissue regression over time (29). Hence, small changes in butyrate-induced growth or apoptosis as a result of changes in energy requirements (e.g. glucose), thought to be associated with transition of cells from a normal to a transformed state, may have long-term implications regarding the protective role of butyrate against colon cancer. The response of cells to butyrate may depend on the overall energy pools available *in vivo* and *in vitro*. However, it should be noted that, at high concentrations, butyrate still retains its apoptotic potential in tumour cells (in both $Glu^{-}Pyr^{-}$ and $Glu^{+}Pyr^{+}$ conditions) and, hence, its potential protective effects against colorectal cancer.

To our knowledge, this is the first report of apoptosis in response to energy deprivation (glucose deprivation) in human colonic epithelial cell cultures. The apoptotic pathway in response to energy deprivation in these cells must be via a p53 independent pathway since both the S/RG/C2 and HT29 cells used in these studies lacked wild-type p53 (30). Our results also show that the response of cells to butyrate, i.e. whether growth inhibitory or stimulatory, may depend on the availability of other energy sources and that, in glucose-depleted conditions, butyrate at low concentration can be growth stimulatory to colon epithelial cells in culture. These observations may, in part, provide an explanation for the apparent opposite effects of butyrate on proliferation reported *in vivo* and *in vitro*. The present study also provides a model system to further our understanding of the possible importance of total energy availability, and utilization, whether through luminal dietary factors or through the vasculature, and how this may influence colonic carcinogenesis.

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