ABSTRACT

The actions of butyrate and related short-chain fatty acids were analyzed on the 9-1C retinoid-responsive rat prostatic adenocarcinoma cell. The 9-1C cells, which are inducible for alkaline phosphatase (AP) by retinoic acid, were also inducible for the enzyme by three- to six-carbon fatty acids. The most effective inducer was the four-carbon acid, butyrate, which caused an essentially linear increase in AP activity in the concentration range of 2 to 10 mM. A comparison of AP induction by butyrate and retinoic acid showed the retinoid to be a more potent inducer of the enzyme by several orders of magnitude.

Butyrate and related short-chain fatty acids also suppressed 9-1C cell growth, an effect which is not mediated by retinoic acid in these cells. Total growth suppression was achieved at butyrate concentrations of 5 mM and above; 1.5 mM caused 50% inhibition. As in the case of AP induction, all three- to six-carbon fatty acids suppressed growth to some extent, although butyrate was the most effective. The order of carbon chain length effectiveness was 4 > 5 > 3 > 6.

Butyrate appeared to be unique among the various fatty acids in causing an increase in cell protein. The protein content of 9-1C cells cultured in the presence of 4 mM butyrate for 72 h was more than 4-fold greater than that of control cells. This observation paralleled observations on cell volumes analyzed by forward-angle light-scatter flow cytometry, which showed a concentration-related increase in the cross-sectional areas of 9-1C cells following butyrate treatment. This effect has also been shown, in a recent study, to be mediated by retinoids.

One of the most striking effects of butyrate treatment was on cellular morphology. The fatty acid caused 9-1C cells, which normally grow in a disorganized array with no apparent affinity for each other, to spread out and become organized into parallel tracts through the monolayer.

INTRODUCTION

Cell line 9-1C (18) is a rapidly growing tumorigenic line which was derived, via cell line UMS-1541, from the G subline of the Dunning R-3327 rat prostatic adenocarcinoma (6). This line, which has a very low basal level of AP activity in vitro, was cloned solely on the basis of its strong inducibility for the enzyme by retinoic acid (18). This induction of AP can be detected as early as 3 to 4 h after the addition of 10 μM retinoid and is blocked by inhibitors of both RNA and protein synthesis (17). The induction of AP by retinoid has also been observed in urinary bladder transitional epithelium (19), epidermis (20), and cultured dermal fibroblasts. Although line 9-1C produces poorly differentiated prostatic adenocarcinomas when injected into male and female Copenhagen × Fischer F, rats, primitive prostatic acini are nevertheless formed. Furthermore, the apical surfaces of the cells which line the lumens of these acini are rich in AP activity, which is a characteristic feature of acini in the lateral lobe of the dorsolateral rat prostate (18), the source of the original Dunning R-3327 tumor (6).

In addition to inducing AP activity, retinoic acid also changes the growth and morphology of 9-1C cells. Although it does not significantly affect the rate at which the cells proliferate, retinoid (10 μM) causes cultures to saturate at cell densities which are approximately 40% lower than that of control cultures (18). This retinoid-induced lowering of the saturation density can be explained, in part, by the observation that retinoic acid (10 μM) also causes a 35 to 40% increase in the cross-sectional area of 9-1C cells as measured by light-scatter flow cytometry on dispersed cells.

In spite of considerable interest in retinoids in recent years as cancer-chemopreventive agents (see Ref. 11 for a review), their mechanism of action, especially at the molecular level, is still poorly understood. We are using the 9-1C line to characterize how retinoid acid controls AP activity as one approach to defining the molecular mechanism of retinoid action. We think that it might be useful in this endeavor if additional means of inducing the enzyme were also available. Numerous drugs and culture conditions have been reported to be effective inducers of AP activity in various cell types (see Ref. 12 for a review). We have initiated studies to investigate these additional means of inducing the enzyme in 9-1C cells, and of 3 investigated to date, glucocorticoids, 5-bromo-2'-deoxyuridine, and sodium butyrate, only the latter was found to induce AP activity. We also observed during the course of these studies that butyrate, like retinoic acid (18), affects the growth and morphology of 9-1C cells. However, very significant and interesting quantitative and qualitative differences between the 2 effectors were observed in all 3 parameters of differentiation measured: AP induction; growth suppression; and alteration of malignant cellular morphology. In this study, we have characterized these effects of butyrate on the 9-1C prostatic adenocarcinoma cell and show that butyrate and some of its closely related short-chain fatty acids cause the 9-1C cell to acquire some characteristics of the differentiated state.

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2 To whom requests for reprints should be addressed, at Department of Urology (D-1), University of Miami School of Medicine, P.O. Box 01617, Miami, FL 33101.
3 The abbreviation used is: AP, alkaline phosphatase.
4 D. H. Reese, unpublished observations.
EFFECT OF BUTYRATE ON PROSTATIC ADENOCARCINOMA CELLS

MATERIALS AND METHODS

The origin and characteristics of cell line 9-1C have been described previously (18). Cells were cultured in Connaught Medical Research Laboratories Medium 1415 (8) containing twice the prescribed sodium bicarbonate concentration and supplemented with 10% newborn calf serum. Penicillin (100 units/ml) and streptomycin (100 μg/ml) were included in all media. Cultures were maintained in a 10% carbon dioxide atmosphere at 37 °C. Cell dissociation was carried out in Dulbecco’s calcium- and magnesium-free phosphate-buffered saline (5) containing 0.25% trypsin and 0.02% EDTA. Counts of viable cells were made in a hemocytometer using the trypan blue dye exclusion method. The sodium salt of butyric acid was obtained from J. T. Baker Chemical Co. (Phillipsburg, NJ), while all other fatty acids were obtained from Sigma Chemical Co. (St. Louis, MO).

AP activity was measured on cell lysates using 16 mM p-nitrophenyl-phosphate as substrate in 0.75 M 2-amino-2-methyl-1-propanol buffer at pH 10.3 as described previously (17). Protein was measured by the method of Bradford (1) using plasma γ-globulin as standard.

Relative cell size measurements were made on ethanol-fixed cells in an EPICS IV cell sorter (Coulter Electronics, Inc., Hialeah, FL) exactly as described previously (18).

RESULTS

Effect of Butyrate on Growth. Butyrate was a potent inhibitor of 9-1C cell growth. At concentrations between 0 and 3 mM, growth decreased linearly (Chart 1). Fifty % inhibition occurred at 1.5 mM, and maximum growth inhibition was achieved at concentrations of 5 mM and above. At these concentrations, the number of viable cells per dish remained at initial values, indicating that butyrate was not cytotoxic to 9-1C cells, even at very high concentrations.

The onset of growth inhibition was rapid with near total suppression being evident by 24 h after the addition of 3 mM butyrate (Chart 2). With time, some cell growth did occur; however, by 5 days, the cell number in the butyrate-treated cultures increased only 2-fold over initial values, indicating that butyrate was not cytotoxic to 9-1C cells, even at very high concentrations.

The rate of growth was comparable to that of control cultures. During the subsequent 24-h period, however, the growth rate showed evidence of decreasing.

Chart 1. Dose response for the butyrate suppression of 9-1C cell growth. Cells were seeded at 2 × 10⁵ cells/60-mm culture dish. Twenty-four h later, the medium was replaced with sodium butyrate-containing medium at the indicated concentrations. Cell counts were also made on 2 dishes at this time to provide initial values (—). Cell counts were made after an additional 3 days of culture. Points, average cell count on duplicate cultures; bars, range of values.

Chart 2. Time course of the butyrate-induced growth inhibition and its reversal in 9-1C cells. Cells were seeded at 1 × 10⁵ cells/60-mm dish. Twenty-four h later (Day 1), the medium in all cultures was replaced with fresh medium without (•) or with 3 mM butyrate (O—O). At Day 3, the medium was removed from some butyrate-treated cultures and replaced with fresh butyrate-free medium (O—O). Points, average of cell counts on 3 cultures; bars, SE.

Chart 3. Dose response for the butyrate-induced increase in AP and cell protein. The experimental protocol is the same as that given in Chart 1. At the end of the culture period, the cell monolayers were lysed and processed for AP and protein determination as described previously (17). Cell counts and protein measurements were also made on an additional set of cultures. •, AP; O, mg protein per 10⁶ cells. Points, average of determinations on duplicate cultures; bars, range of values.
Effect of Butyrate on AP Activity and Cell Protein. Butyrate caused a significant increase in AP activity in 9-1C cells (Chart 3). At concentrations between 2 and 8 mM, the increase in AP activity was essentially linear. At butyrate concentrations higher than 8 mM, the increase in enzyme activity was even greater than that observed for concentrations between 2 and 8 mM. This sharp increase in the specific activity of the enzyme at concentrations greater than 8 mM may be related to some striking butyrate-induced changes in cell protein which were detected during the course of measuring the AP specific activities shown in Chart 3. We observed that, as the butyrate concentrations were increased, there was not a corresponding decrease in the total cell protein per culture as would be expected from the data in Chart 1. Instead, the protein content of cultures which were exposed to butyrate concentrations known to cause total growth suppression (see Chart 1) was found to be well above the protein content present at the time of butyrate addition (data not shown). This suggested that cell protein was increasing, in the absence of cell proliferation, as a result of butyrate treatment. This was found to be the case when protein and cell counts were determined on the same cultures (Chart 3). Butyrate caused a significant concentrated-related increase in cell protein. Cell protein increased greater than 4-fold at the higher butyrate concentrations (8 mM). Interestingly, cell protein was observed to decrease beyond 8 mM; this observation may explain, in part, the sharp increase in AP specific activity for this upper concentration range.

Inasmuch as one of our objectives in this study was to determine if butyrate, like retinoic acid, also induced AP activity in 9-1C cells, it was of interest, once we demonstrated that this was the case, to obtain some measure of how AP inducibility compared quantitatively between the 2 inducers. For this purpose, the time course of AP induction by 10 µM retinoic acid, a concentration found previously to induce the enzyme in various cell types and tissues without causing significant suppression of the growth rate or cytotoxicity (17-19), was compared with that of 5 mM butyrate, the lowest concentration shown here (Chart 1) to cause maximum growth inhibition. As shown in Chart 4, the amount of AP activity induced per mg cell protein during 24 h was 6-fold greater with 10 µM retinoic acid than in the presence of 5 mM butyrate.

Effect of Fatty Acid Chain Length on AP Activity, Growth, and Cell Protein Content. Some cellular responses to butyrate have been shown to have a high degree of specificity for the 4-carbon fatty acid (10), while others have shown that certain butyrate-induced responses can be obtained with additional short-chain fatty acids as well (2, 7, 9). In the present study, an analysis of various short-chain fatty acids revealed that the induction of AP activity and the suppression of growth were not unique to butyrate. It can be seen in Table 1 that 3-carbon (propionic), 5-carbon (valeric), and 6-carbon (caproic) acids at 4 mM caused significant enzyme induction and growth suppression. None of these fatty acids was as effective as butyric acid, however.

In contrast to the above-demonstrated absence of a stringent requirement for butyrate as an inducer of AP and as a growth suppressor, the ability to cause an increase in cellular protein content was found to be specific to the 4-carbon acid. The data in Table 1 show that only butyrate among the fatty acids analyzed caused a significant increase (4- to 5-fold) in cell protein content. In this experiment, the magnitude of cell protein increase, above control values, caused by 4 mM butyric acid was somewhat greater than that caused by 4 mM sodium butyrate shown in Chart 3.

Effect of Butyrate on Cell Morphology. Butyrate had a profound effect on the morphology of 9-1C cells in culture. 9-1C cells normally grow as a disorganized array exhibiting no apparent affinity for each other (Fig. 1A). Within 24 h after the addition of butyrate, the cells associated into clusters and exhibited a strong tendency to orient end-to-end, forming parallel columns or tracks through the monolayer (Fig. 1B). Butyrate-treated cells were also more uniform in shape and occupied larger areas on the dish surface than did control cells. This latter observation caused butyrate-treated cells to appear larger than control cells. This is particularly evident in areas of Fig. 1, A and B, where individual cells are isolated from surrounding cells. It also became apparent to us that butyrate-treated cells were in fact significantly larger than untreated cells when dissociated cells were observed.
during the course of making cell counts for the growth-comparison studies presented above (Charts 1 and 2). In order to obtain a comparison of the relative increase in cell size as a result of butyrate treatment, light scatter measurements were made on ethanol-fixed control and butyrate-treated cell populations. An approximately linear relationship exists between the cross-sectional area of cells in suspension and the amount of light scattered at small angles (21). We used this technique previously to show that retinoic acid (10 \( \mu \text{M} \)) caused an increase in the size of 9-1C cells (18). Cells cultured in the presence of 1 and 2 mM butyrate exhibited no apparent increase in light scatter properties over that of control cells. In the presence of 3 mM butyrate, however, cells were observed which had greater light-scattering properties than that of control cells (Chart 5). With increasing butyrate concentrations, there was a progressive increase in the frequency of cells possessing greater light-scatter properties, thus larger cross-sectional areas, and therefore greater volumes. It is also evident that the cell populations became more heterogeneous with respect to cell size as the butyrate concentration was increased. This made an estimation of the magnitude of increase in cell size difficult. Nevertheless, the data in Chart 5 show that only a small percentage of the cells cultured in the presence of 5 mM butyrate were as small as the majority of cells in the control cell population.

The data in Chart 5 also show that the frequency of objects with very small light-scatter properties increased with increasing butyrate concentrations. This phenomenon has been observed previously (18) in association with the action of retinoid on 9-1C cells and may be the result of increased cell fragility.

**DISCUSSION**

Retinoic acid has been shown to be a potent inducer of AP activity in a variety of cell types (17-20) including the 9-1C cell used in the present study. We have shown here that butyrate, a naturally occurring short-chain fatty acid, also induced AP activity in 9-1C cells, although the fatty acid was 2 to 3 orders of magnitude less effective than the retinoid. This difference may not be quite as large as it appears, however. A 4- to 5-fold increase in cell protein was observed after 72 h of exposure to butyrate, and although the comparison between AP induction by retinoid and butyrate was made during a much shorter time period (24 h), it is possible that increased protein synthesis during this time period could reduce, albeit slightly, the specific activity of AP in butyrate-treated cells. Also, although we know that all cells in the 9-1C population are responsive to retinoid (18), we do not know yet if this is the case with butyrate. Thus, on a per-cell basis, the magnitude of the difference in specific activities of AP in butyrate- and retinoid-treated cells may not be quite as great as indicated above. Nevertheless, the effective concentration ranges for the 2 inducers probably differ by at least 2 orders of magnitude. This is consistent with published values showing that, in general, butyrate is an effector of various cellular functions in the mM concentration range (15, 16), while retinoids are active at \( \mu \text{M} \) concentrations or lower (11). The 9-1C cell does appear to be less responsive to butyrate than other cell types. In some cells (2, 7, 9, 14), AP is induced at butyrate concentrations (<2 mM) which are well below that shown to be necessary for induction in 9-1C cells. Whether the reduced responsiveness of 9-1C cells to butyrate, relative to other cells, is due to an actual difference in cell-type response or to a media effect (4) is not clear. One seemingly unrelated action of butyrate, its rapid growth-inhibitory activity, may actually be a factor in the relatively low inducibility of AP by the fatty acid. The inducibility of the enzyme in 9-1C cells by retinoic acid has been shown to decrease sharply as confluence is reached (18). This suggests, among other possibilities, that induction of AP in 9-1C cells may be dependent upon continued cell cycling. This has been shown to be the case in HeLa cells, which are only inducible for the enzyme by glucocorticoids during S phase of the cell cycle (3). If this is also true for AP induction by butyrate and retinoic acid in 9-1C cells, then only those cells in S phase at the time of butyrate addition will be induced for the enzyme, while retinoic acid-treated cells, because they continue to proliferate, will remain inducible until confluence is reached. Experiments are in progress to determine if AP inducibility is limited to one phase of the cell cycle. We are also attempting to determine if butyrate and retinoic acid induce the same AP isozyme. Preliminary evidence suggests that they may, inasmuch as both the native (which is a \( M, 144,000 \) dimer) and monomeric (\( M, 72,000 \)) form, of the

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**Chart 5.** Effect of butyrate on cell volume. The experimental protocol is the same as that given in Chart 1. At the end of the 3-day exposure period to butyrate, replicate trypsin-dispersed cell populations were fixed in 70% ethanol and analyzed for forward-angle light-scatter flow cytometry as described previously (18). An approximately linear proportionality exists between the amount of light scattered at small angles and the cellular cross-sectional area (21). Ten thousand cells were analyzed per run. a, control; b, 3 mM butyrate; c, 4 mM butyrate; d, 5 mM butyrate.

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\[a\] D. H. Reese, manuscript in preparation.
EFFECT OF BUTYRATE ON PROSTATIC ADENOCARCINOMA CELLS

APs induced by both effectors appear to have the same mobilities on sodium dodecyl sulfate-polyacrylamide gels.

There are numerous reports in the literature showing that butyrate can reversibly or irreversibly suppress the growth of cells in culture (see Refs. 15 and 16 for reviews). In some tumor cells, primarily of neural origin, growth suppression is associated with lethality (15). This is not the mechanism of growth suppression in 9-1C cells, however. Even at extremely high concentrations (10 mM), there was no evidence of cytotoxicity. Furthermore, in cells cultured in the presence of 3 mM butyrate, which caused a greater than 90% inhibition of growth, inhibition was rapidly reversed, with a normal rate of growth being detected within 24 h after butyrate removal. The reason for the observed decrease in growth rate during the second 24-h period after butyrate removal is not clear. However, inasmuch as butyrate did cause a significant increase in cell size, it is possible that the cultures were becoming confluent prematurely during this period. This implies that the butyrate-induced increase in cell size was not reversed as rapidly as the growth inhibition, but no direct evidence for this is available at this point. It would appear that the underlying mechanisms by which butyrate and retinoic acid affect growth are quite different. Butyrate suppressed the rate at which 9-1C cells grew, while we have shown previously (18) that retinoic acid does not affect the growth rate appreciably but that it does cause cells to saturate at densities considerably lower (40%) than that of untreated cells. Studies are in progress to determine where in the cell cycle butyrate blocks growth.

Although butyric acid was the most effective fatty acid as an inducer of AP and in suppressing growth, the 3-, 5-, and 6-carbon acids were also active. These results are very similar, at least with respect to AP induction, to previous studies on the induction of AP activity by fatty acids in other cell types (2, 7, 9). In these studies and the present one, the general order of chain length effectiveness, with some exception (9), was 4 > 5 > 3 > 6. In the present study, this same order also holds for effectiveness in growth suppression. In contrast to this and the aforementioned studies, erythroid differentiation in erythroleukemic cells has been shown to be specific for the 4-carbon acid (10), and in view of this specificity, it was suggested that a receptor-ligand interaction may be involved. This seems a less likely possibility in the present study, although we did observe an apparent, albeit inexplicable, specific requirement for the 4-carbon acid in the stimulation of increased cell protein. Interestingly, a butyrate-specific binding protein has been demonstrated recently in mouse and rat livers (13).

The observed butyrate-induced increase in cell protein correlated with morphological observations and light-scatter measurements showing a concentration-related increase in cell size. The stimulation of increased cell size has also been observed following exposure of 9-1C cells to retinoic acid (18), and it is a frequently observed effect of butyrate treatment (15, 16, 22) but is not understood. A correlation has frequently been shown, however, between increased cell volume and increased AP activity (see Ref. 18 for a discussion), and it has been suggested (18) that the various transport functions attributed to AP (12) may, in part, contribute to the observed increase in cell size by affecting water movement. Morphologically, butyrate had a profound effect on the 9-1C cell. Although the cells gave the appearance of being larger, an observation which correlated with the light-scatter data showing increased cell size, we cannot rule out the possibility that the cells also became more flattened and spread out because of greater affinity for the culture surface. A change in affinity of the cells for the culture dish surface and for each other is certainly suggested by the strong contact-inhibited and organized pattern of the butyrate-treated cells. It is unclear at this point what factors contributed to the striking pattern of organized growth induced by the fatty acid. It is interesting that, although butyric acid and retinoic acid share many of the same effects on 9-1C cells, only butyrate caused the highly organized pattern of growth.

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Fig. 1. Effect of butyrate on cell morphology. Cells were grown on glass microscope slides in 100-mm culture dishes. Cultures were terminated by fixation in 10% neutral-buffered formalin. A, control; B, cultured 24 h in the presence of 5 mM butyrate. H & E, × 58.
Control of Growth, Morphology, and Alkaline Phosphatase Activity by Butyrate and Related Short-Chain Fatty Acids in the Retinoid-responsive 9-1C Rat Prostatic Adenocarcinoma Cell

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