Depletion of Sodium Butyrate from the Culture Medium of Friend Erythroleukemia Cells Undergoing Differentiation

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ABSTRACT

Friend erythroleukemia cells can be induced to undergo erythroid differentiation by a variety of unrelated compounds. The fact that sodium butyrate causes reversible alterations in growth, morphology, and biochemistry in many cell systems prompted us to reexamine its pattern of induction of differentiation and to compare it to that of dimethyl sulfoxide (DMSO) and hexamethylbisacetamide (HMBA). By the fourth day of induction, a peak in hemoglobin accumulation was reached in the cultures treated with each of these potent inducers. Differences, however, were noted in cultures in which there had been no change of medium for 7 days. Whereas DMSO or HMBA induced cultures reached a stationary stage of growth and maintained a high percentage of benzidine positive cells, butyrate treated cultures resumed active growth and showed a marked decrease in the percentage of benzidine positive cells. However, the actual number of terminally differentiated cells remained relatively constant. The addition of fresh butyrate to 4-day treated cultures prevented the decrease in the percentage of benzidine positive cells. Measurement of [14C]butyrate uptake into the cells showed a decrease in the incorporation of the inducer with time coincident with the decrease in the percentage of benzidine positive cells and of the butyrate in the medium. Incorporation of [3H]thymidine into cells undergoing differentiation for 4 days indicated that butyrate treated cells, but not cells treated with DMSO or HMBA were capable of active DNA synthesis and growth after removal of the inducers. These data suggest that butyrate, a natural fatty acid, is metabolized by the cells and with time its concentration is reduced to a level below that required to stimulate differentiation. Additional evidence to support this notion are the results obtained with conditioned medium (CM) from induced cultures. CM-DMSO and CM-HMBA retained the capacity to induce differentiation whereas CM-butyrate lost its potency with time.

INTRODUCTION

Murine erythroleukemia cell lines (FLC) originating from Friend leukemia virus transformed erythroid precursors have provided a model system for studying differentiation. FLC can be stimulated to mature along the erythroid pathway when cultured in the presence of DMSO or a variety of other compounds which have a similar capacity to modulate gene expression. These inducers cause morphological and biochemical alterations that parallel many of those that occur during normal erythropoiesis, including changes in globin synthesis, heme synthesis, enzymes involved in nucleotide metabolism, and membrane associated proteins (for review, see Refs. 1 and 2). Although the induced FLC acquire the ability to mimic the series of events that lead to the initiation of the program for terminal differentiation, yet cease to multiply. In addition, the molecular basis of the commitment process of the FLC is not well understood possibly because so many structurally and functionally unrelated compounds can trigger the complex series of events that lead to the initiation of the program for differentiation. However, the inducers can be divided into 2 classes based on ability to induce differentiation in variant clones (4) and to stimulate ODC (5). ODC, the rate limiting enzyme in the synthesis of polyamines, is regulated by many agents that stimulate cell growth and differentiation. DMSO and HMBA, inducers belonging to class 1, appear to trigger differentiation via a mechanism involving polyamine biosynthesis enzymes. Sodium butyrate, an inducer belonging to class 2, has little or no effect on ODC and is not inhibited by dexamethasone or 12-O-tetradecanoylphorbol-1-acetate (5) both of which inhibit class 1 inducers. In addition, it causes histone hyperacetylation in FLC (6) and at lower concentrations is not cytostatic and inhibits differentiation by HMBA (7). The effects of butyrate on FLC and other cell systems have been reviewed (8).

Whereas exposure to DMSO or HMBA for 12–18 h is sufficient for the cells to become irreversibly committed to the erythroid pathway (9), there are a number of studies (10, 11) which indicate that the cells exposed to butyrate for a similar period of time are not. If the treated cells were transferred to medium with or without butyrate, the percentage of benzidine positive cells in medium without the inducer was lower than that in the butyrate supplemented medium. We have reexamined the response of FLC to butyrate in order to gain a better understanding of the nature of the regulatory cascade that leads to terminal differentiation. Our results suggest that butyrate is metabolized by the cells and as a result the concentration of the compound in the culture medium is decreased. This decrease may account for the reversibility of the changes in growth, morphology, and biochemistry that butyrate has been reported to induce in a variety of human and murine cell lines (8, 12–14). We have found that if the concentration of butyrate is maintained by the addition of the compound to the cultures after the fourth day, the cells terminally differentiated by the seventh day. The possibility that there may be a component(s) which regulate differentiation and whose accumulation depends on the optimal concentration of butyrate is under investigation.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. The establishment, growth conditions, and characterization of FLC cell lines have been reviewed (2). Briefly, the cells of line 5-86, a subclone of line 745A were maintained in Glasgow minimum essential medium (Gibco) supplemented with 10% fetal calf serum (Rehies), penicillin (100 units/ml), and streptomycin (100 µg/ml). Cultures were incubated at 37 °C in 5% CO2/95% air. At designated intervals, the number of cells in each culture was

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The abbreviations used are: FLC, Friend erythroleukemia cells; FL, Friend leukemia; DMSO, dimethyl sulfoxide; ODC, ornithine decarboxylase; HMBA, hexamethylbisacetamide; CM, conditioned medium.

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BUTYRATE REQUIREMENT FOR STIMULATION OF FLC INDUCTION

determined by counting in a hemocytometer. Viability was determined by the trypan blue exclusion assay.

Conditioned Medium. CM from untreated cultures or cultures treated for 4 days with either DMSO, HMBA, or sodium butyrate was centrifuged and filtered through a Swinnex-HA, 0.45 mm, and then assayed for inducer activity. CMs were seeded with 1 × 10^6 fresh cells/ml and scored for benzidine positivity 4 and 7 days later.

Reverse Transcriptase Assay. The amount of virus released into the culture fluid was measured by incorporation of [3H]deoxythymidine monophosphate, using polyriboadenylc acid hybridized to multiple lengths of deoxythymidylicate, 10 residues long as template-primer under the conditions described previously (15).

Induction of Differentiation. Three compounds known to be potent inducers of the erythroleukemia cells were used: DMSO, Fischer Scientific Company (1.5% vol/vol); HMBA, kindly provided by R. Rubin (4 mm); and sodium butyrate, Baker Chemical Co. (1.5 mm).

Each was added directly to the culture at the time of seeding 1–2 × 10^6 cells/ml and where indicated fresh butyrate was added on day 4. The number of benzidine positive cells in cultures containing inducers was determined at the times indicated in the text by the method previously described (16, 17).

Labeling of Cells. To assay for DNA replication, the cells were pulse labeled with 1 μCi/ml [methyl-3H]thymidine (specific activity, 20 μCi/ mmol) for 1 h at 37°C at the intervals indicated in the text. The cultures were then centrifuged and the supernatant fluid aspirated. After washing the cells with phosphate buffered saline, the pellets were resuspended in 10% cold trichloroacetic acid and kept on ice for 20–30 min. The precipitates were collected on Millipore filters and washed several times with cold 10% trichloroacetic acid, dried, and the radioactivity was determined by means of a scintillation counter.

To determine butyrate uptake, n-butyric acid, sodium salt, 1-14C, (specific activity, 39 nCi/mmole; ICN Radiochemical) was added at 1 μCi/ml together with cold butyrate to give a final concentration of 1.5 mm sodium butyrate at the time the cultures were seeded. At designated intervals, 2-ml aliquots were centrifuged, and the cells were washed in phosphate buffered saline and collected on Millipore filters. Radioactivity was determined by scintillation counting.

The radioactivity remaining in the tissue culture medium, after centrifugation of the cells, was also determined using Biofluor (New England Nuclear) as scintillant. Quenching by the tissue culture medium components was determined by appropriate controls.

RESULTS

Patterns of Induction of Differentiation. The effect of exposure to the 3 potent inducers was compared in the first series of experiments. Because a more prolonged arrest in G1 is observed with DMSO and butyrate than with HMBA, cultures treated with these compounds were generally initiated with twice the number of cells (2 × 10^6 cells/ml) to provide a sufficient amount of cells for the assays. Butyrate at the concentration used (1.5 mm) was the most cytoplastic of the 3 inducers. Although the cell counts of the treated cultures were not always reproducible, the pattern of induction each followed was. All experiments were performed a minimum of 3 times. The data from representative experiments are shown. The response of the cells to each of the inducers was not affected by the difference in the number of cells used to seed the cultures. In the experiments shown in Table 1, there was no change of medium during the 7-day observation period. For some unexplained reason HMBA was more cytotoxic than usual in this experiment. However, each inducer caused substantial accumulation of hemoglobin by the fourth day as detected by the benzidine positive reaction. After longer periods of exposure, however, striking differences were observed. In cultures treated with butyrate, an acceleration of growth accompanied by a decrease in the percentage of benzidine positive cells was observed by the sixth and seventh day of exposure. This was in contrast to the behavior of the cells treated with DMSO or HMBA for the same period. These cells were no longer proliferating and were committed along the erythroid pathway as the increase in the number of benzidine positive cells with time indicated.

We also compared the replicative capacity of the virus in the chronically infected FL cells undergoing induction of differentiation. The reverse transcriptase activity released into the medium of each culture was measured daily for 4 days. As shown in Fig. 1, virus replication parallels cell growth. Although the values varied from experiment to experiment, in general 1.5 mm sodium butyrate inhibited both cell and virus replication.

In addition, we have examined the properties of the Friend leukemia virus genome and its expression during differentiation. The integration patterns and the number of proviral copies remain unchanged during induction by each of the inducers (18).

Comparison of Ability of Induced Cells to Synthesize DNA. To ascertain whether the treated cells were terminally differentiated and DNA synthesis was at a minimal level, we compared the extent of DNA synthesis in the cultures for 4 days to those transferred to fresh medium in the presence or absence of inducer. The incorporation [3H]thymidine into the acid insoluble fraction of cells pulse labeled for 1 h was measured (Table 2). No significant differences in the incorporation between the control and treated cells was observed on the fourth day, when all cultures generally reach a stationary stage. Differences were apparent, however, when the cells transferred to fresh medium with or without the inducer were assayed. After 2 further days of growth in the presence of the inducers (day 6), there was a 2- to 3-fold increase in incorporation of thymidine in all cultures. Growth was limited as shown in Table 1 and accordingly the capacity for DNA synthesis. The capacity for DNA synthesis remained at approximately the same level in DMSO or HMBA treated cells which had been transferred into fresh medium without inducer. In contrast, butyrate treated cells transferred into fresh medium without inducer showed a 5-fold increase in incorporation comparable to that which occurred in the control untreated cells transferred to fresh medium at the same time.

Requirement for Butyrate. Could the cellular effects we were observing be due to the depletion of butyrate from the medium? The question was raised since fatty acids, such as butyrate are natural products that can be metabolized by the cells. To determine whether this was occurring, the cells treated with each of the inducers for 4 days were adjusted to 2 × 10^6/ml and transferred to fresh medium with or without inducer. Assays were carried out again on the sixth day after the initiation of the experiments (Table 3). The cells transferred to medium containing the inducers proceeded along the pathway to terminal differentiation. Cultures maintained in butyrate were growth inhibited. Those maintained in the presence of medium containing either DMSO or HMBA were also inhibited but to a lesser extent. While all the cells from treated cultures transferred to medium without inducers grew well, the pattern of differentiation was similar to that shown in Table 1, i.e., the cells that had been treated with DMSO or HMBA remained committed whereas there was a sharp decrease in the percentage of benzidine positive cells in the butyrate treated cultures.

To determine whether butyrate was being depleted from the medium, the incorporation of [14C]butyrate was followed for a 7-day period with no change of medium in cultures seeded with 2 × 10^6 cells/ml. Two sets of experiments were performed in parallel to assay daily the incorporation of the labeled butyrate and the percentage of benzidine positive cells (Fig. 2). One set.
BUTYRATE REQUIREMENT FOR STIMULATION OF FLC INDUCTION

Table 1  Effect of inducers on cell growth and hemoglobin synthesis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fourth day</th>
<th>Sixth day</th>
<th>Seventh day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cells x 10^5/ml</td>
<td>% benzidine positive</td>
<td>Cells x 10^5/ml</td>
</tr>
<tr>
<td>None</td>
<td>25</td>
<td>0</td>
<td>20.5</td>
</tr>
<tr>
<td>DMSO (1.5%, v/v)</td>
<td>28</td>
<td>70</td>
<td>24.0</td>
</tr>
<tr>
<td>HMBA (4 mM)</td>
<td>15.5</td>
<td>81</td>
<td>19.2</td>
</tr>
<tr>
<td>Sodium butyrate (1.5 mM)</td>
<td>12.5</td>
<td>73</td>
<td>37.0</td>
</tr>
</tbody>
</table>

Table 2  Thymidine incorporation into cells induced for 4 and 6 days

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fourth day</th>
<th>Sixth day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[H]Thymidine incorporated (cpm/10^6 cells)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>With inducer</td>
</tr>
<tr>
<td>None</td>
<td>2,000</td>
<td>13,400</td>
</tr>
<tr>
<td>DMSO</td>
<td>3,700</td>
<td>5,700</td>
</tr>
<tr>
<td>HMBA</td>
<td>2,500</td>
<td>8,500</td>
</tr>
<tr>
<td>Sodium butyrate</td>
<td>3,000</td>
<td>6,400</td>
</tr>
</tbody>
</table>

received no supplemental butyrate whereas 1.5 mM cold butyrate was added to the second set on the fourth day when uptake of the labeled inducers had reached a peak. If no butyrate was added at this point, there was a decrease in the percentage of benzidine positive cells whereas those cultures which had received a supplement of cold butyrate maintained a high level of benzidine positive cells. The incorporation of labeled inducer decreased with time. When chased by cold butyrate, the label remained at the same level for 24 h and then decreased possibly because of degradation. Determination of the radioactivity in the culture medium (Table 4) showed that the level of [3H]butyrate decreased 50% after 4 days and 90% after 7 days. These data suggested that butyrate was being metabolized by the cells, thus reducing the concentration to a level below that required to stimulate differentiation.

Effect of Conditioned Medium from Induced Cultures. Additional evidence to support the notion that butyrate was being depleted from the medium was obtained from experiments in which CM from untreated cultures or cultures treated for 4 days with either DMSO, HMBA, or sodium butyrate was used. There were insufficient nutrients remaining in CMs from 4-day control cultures to support cell growth. CMs from the induced cultures (Table 5), however, permitted cell proliferation, the most active of which occurred in CM-butyrate. CM-DMSO and CM-HMBA had not lost induction potency and retained optimal concentrations of the respective inducer. By day 7, there were 68 and 84% benzidine positive cells in CM-DMSO and CM-HMBA, respectively. In marked contrast, there were only 8% benzidine positive cells in CM-butyrate cultures by day 7 (Table 5).

Similar results were obtained with CM from cultures treated for 7 days and scored for benzidine positivity 7 days later. Little growth occurred in cultures initiated with 7 day CMs. The cell counts in the CM-DMSO and CM-butyrate cultures were 2.3 \times 10^5/ml and 2.0 \times 10^5/ml, respectively, and 4.3 \times 10^5/ml in control cultures maintained for the same period in CM from untreated cells. However, cultures in CM-DMSO contained 63% benzidine positive cells in contrast to 16% benzidine positive cells in the cultures maintained in CM-butyrate.

In order to determine whether the butyrate was being inactivated during incubation, medium containing 1.5 mM butyrate was incubated at 37°C for 7 days before the cells were added. There was no loss of either induction or cytostatic activities of the preincubated butyrate (data not shown).

Determination of Actual Number of Benzidine Positive Cells. Taken together, our data were puzzling for they suggested that differentiation induced in FLC by butyrate was reversible as were the effects it induced in a number of other systems. This was a possibility in view of the fact that hemoglobin accumulation does not necessarily lead to terminal differentiation (19–21). The notion that the induced cells were capable of "dedifferentiation" was eliminated after we plotted the actual number rather than the percentage of benzidine positive cells in the cultures against time. These data clearly revealed that the

Table 3  Effect on cell growth and differentiation of removing inducer from cultures

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fourth day</th>
<th>Sixth day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cells x 10^5/ml</td>
<td>% benzidine positive</td>
</tr>
<tr>
<td>None</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>DMSO</td>
<td>20</td>
<td>45</td>
</tr>
<tr>
<td>HMBA</td>
<td>29</td>
<td>65</td>
</tr>
<tr>
<td>Sodium butyrate</td>
<td>10</td>
<td>75</td>
</tr>
</tbody>
</table>
O, sodium butyrate uptake; •, with additional cold butyrate; A, percentage of benzidine positive cells; A, with additional cold butyrate.

indicated, samples were collected and processed to determine radioactivity and benzidine positive cells as described in "Materials and Methods." After 4 days (arrow) one set of cultures received supplemental 1.5 nM cold sodium butyrate. O, sodium butyrate uptake; •, with additional cold butyrate; A, percentage of benzidine positive cells; A, with additional cold butyrate.

Table 4 Determination of [14C]butyrate in culture medium

<table>
<thead>
<tr>
<th>Days in culture</th>
<th>CPM</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>281,000</td>
<td>100.0</td>
</tr>
<tr>
<td>1</td>
<td>262,000</td>
<td>93.0</td>
</tr>
<tr>
<td>2</td>
<td>245,000</td>
<td>87.0</td>
</tr>
<tr>
<td>3</td>
<td>219,000</td>
<td>78.0</td>
</tr>
<tr>
<td>4</td>
<td>150,000</td>
<td>53.5</td>
</tr>
<tr>
<td>5</td>
<td>66,000</td>
<td>23.5</td>
</tr>
<tr>
<td>6</td>
<td>40,000</td>
<td>14.0</td>
</tr>
<tr>
<td>7</td>
<td>31,000</td>
<td>11.0</td>
</tr>
</tbody>
</table>

number of benzidine positive cells remained relatively constant from the fourth to seventh day of treatment with each of the inducers (Fig. 3), i.e., those cells that had differentiated had reached a point of no return. During this period, there was an acceleration of growth in butyrate treated cultures of the cells not yet committed that was correlated with the decrease in cytostatic and inducer activities. In contrast, growth in the untreated cultures and in the cultures treated with DMSO remained stationary and in the treated cultures, virtually all the cells were benzidine positive.

**DISCUSSION**

We have reinvestigated the kinetics of growth and hemoglobin accumulation in FL cells induced to differentiate with 3 well known potent inducers. The study was undertaken to address a number of questions concerning the mechanism of induction by butyrate since it induces reversible alterations in growth and morphology in many cell systems (8, 12–14) whereas the changes induced by DMSO and HMBA to our knowledge are apparently irreversible.

It is known that histones H3 and H4 from the erythroleukemia cells as well as from HeLa cells become hyperacetylated when incubated in the presence of sodium butyrate (6). This hyperacetylation is reversible 24 h after removal of butyrate from the medium. Using molecular hybridization techniques with RNA from the erythroleukemia cells treated with butyrate or control cells and nonrepetitive mouse DNA, about 38% new transcripts were found (22). Many of the new species of protein were not detected in control or DMSO treated cells. This effect was also reversible. The butyrate treated cells soon after transfer to normal medium showed the uninduced pattern of RNA and protein synthesis.

Our results provide further evidence that the mechanism of action of sodium butyrate differs from that of the other inducers. As compared to DMSO and HMBA, butyrate, at the concentration used in this study, is cytostatic for the first 3 days of culture. This is coincidental with the peak of butyrate uptake and hemoglobin accumulation by the cells (Fig. 2). After the fourth day of treatment, as the butyrate concentration is diminishing in the media (Table 4), the cells generally resume active growth and a decrease in the relative number of benzidine positive cells occurs. However, this cannot be attributed to reversibility. If the data are calculated on the basis of the actual number rather than on the percentage, the benzidine positive cells in butyrate treated cultures remain at approximately the same level through day 7, indicating that they are terminally differentiated (Fig. 3).

Studies on the incorporation of [3H]thymidine demonstrated that after 4 days of exposure, butyrate treated cells but not cells treated with DMSO or HMBA were capable of active DNA synthesis and division following the removal of the inducer. Further, measurement of the uptake of [14C]butyrate by the cells and in the culture medium showed a decrease in the incorporation of the inducer with time coincident with the decrease in the percentage of benzidine positive cells. These data support the notion that butyrate is readily metabolized, thereby reducing the concentration required to maintain differentiation.

Although we have pointed out some of the effects of butyrate that differ from those of the other inducers, there are other parameters that they affect in common. For instance, during induction by each of the inducers, the number of proviral copies and the integration patterns remain unchanged (18). A study of the DNA-binding proteins from nuclei of control and treated
cells. These alternative pathways are being brought to light by the various compounds which have the ability to repress the erythroleukemia cells as well as to other types of neoplastic effects of the inducers with ability to modulate gene expression. Partial remission.

In conclusion, the phenomenon we are observing cannot be attributed to a reversibility of the butyrate effects since the actual number of cells accumulating hemoglobin in the treated cultures remain committed. Instead our results suggest that butyrate is unique among the inducers in that it is a natural fatty acid that is rapidly metabolized. It remains to be determined whether our observations can be attributed to a process of degradation which might release a regulatory factor required to maintain differentiation. This factor may be unstable and active only in the presence of the inducer. Perhaps direct measurement of butyrate by chromatographic methods may yield further information. Of particular interest in this respect is the study of Novogrodsky et al. (24) who followed the serum level in dogs given an infusion of 3% butyrate (29 mg/kg/24 h). After the plasma was infused, the half-life was found to be extremely rapid, less than 5 min. Nevertheless, butyrate treatment of a child with acute myelogenous leukemia induced a partial remission.

The molecular basis for the differences which distinguish the effects of the inducers with ability to modulate gene expression remains unclear. As mentioned above the regulatory mechanisms in FL cells differ from those controlling erythropoiesis in normal RBC (2, 3). This suggests that alternative pathways to differentiation and cessation of growth may be available to the erythroleukemia cells as well as to other types of neoplastic cells. These alternative pathways are being brought to light by the various compounds which have the ability to repress the malignant phenotype.

REFERENCES

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