INDUCTION OF GLYCOLIPID BIOSYNTHESIS BY SODIUM BUTYRATE IN HELA CELLS

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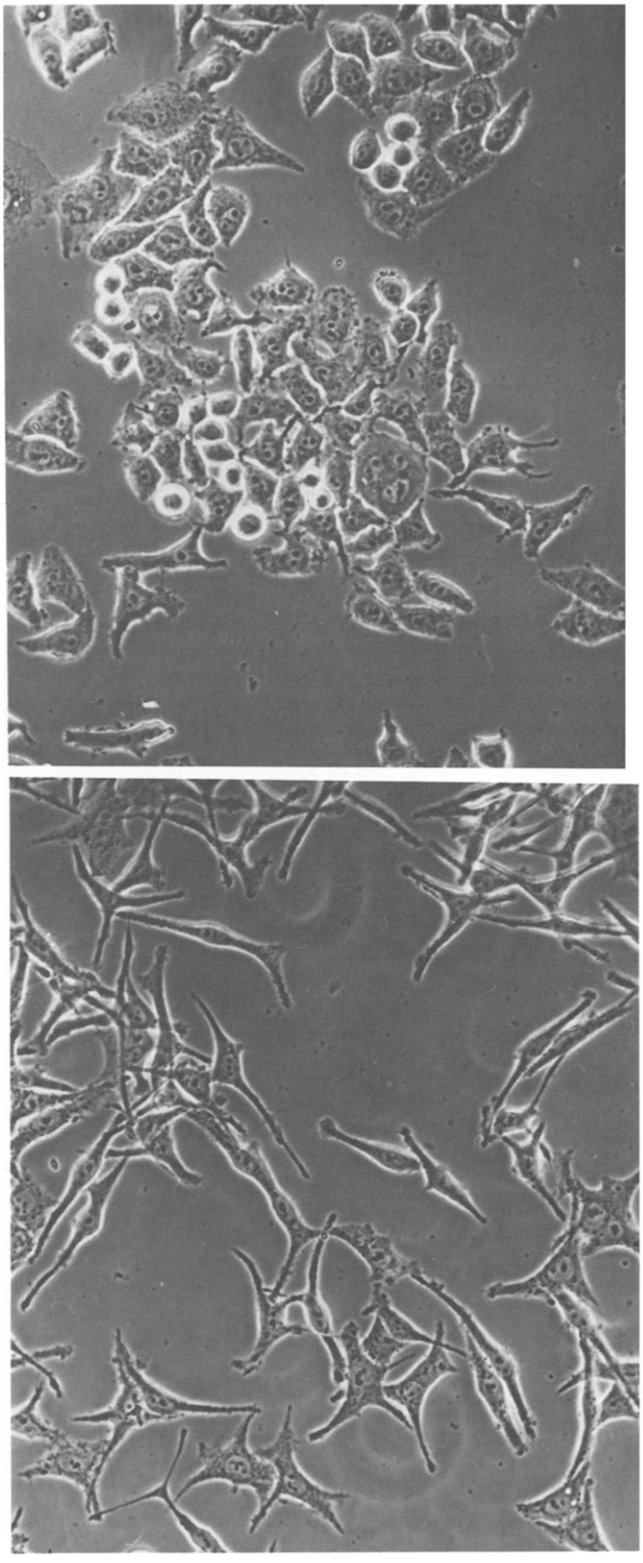
SUMMARY

Butyrate inhibits the growth of HeLa cells and markedly alters their morphology as the cells acquire a more fibroblast-like shape by greatly extending cellular processes. Butyrate simultaneously causes an increase in cellular sialylactosylceramide content by elevating CMP-sialic acid: lactosylceramide sialyltransferase activity (7-24 fold); whereas other glycolipid glycosyltransferase activities do not increase. Induction of this specific sialyltransferase is blocked by cycloheximide or actinomycin D. This first report on the induced synthesis of glycolipid components suggests that these complex carbohydrates have a role in cell growth and morphology.

Reductions in the biosynthesis of certain complex glycosphingolipids have been observed in cultured cells of various species following transformation by oncogenic viruses (1-4), chemical carcinogens (5,6), or X-irradiation (6). Transformation is often associated with characteristic changes in cell morphology and patterns of growth. Extensive alterations of HeLa cell morphology can be caused by small fatty acids such as butyrate (7). Following butyrate addition, the cells stop growth and develop extended processes (Fig. 1) which cause them to resemble fibroblasts or serum-deprived neuroblastoma cells (8). Since these shape changes resemble the reverse of cell transformation, we undertook an investigation of glycolipid biosynthesis in HeLa cells.

EXPERIMENTAL PRODEDURE

Cells and Cell Culture: HeLa cells, strain R, were obtained from Grand



Island Biological Company (GIBCO). The cells were cultured at 37° under 5% CO_2 in air in monolayer in 150 mm plastic Petri dishes (Falcon Plastics) containing Eagle's minimal essential medium supplemented with 10% calf serum and non-essential amino acids (9). In a typical experiment, cells were inoculated at a density of 20,000/cm^2 and incubated for 24 to 48 hours before the exposure to sodium butyrate. Subsequently, the culture medium was replaced by either normal medium or medium containing 5 mM sodium butyrate (pH 7.2). After exposure to butyrate for 12 to 24 hours, the cell monolayers were washed with Dulbecco's phosphate-buffered saline (10) without Ca and Mg. In this buffer the cells were detached by 10 min exposure to 0.05% trypsin (Nutritional Biochemicals Corporation) at 37°, collected by centrifugation at 4°, and washed twice more with cold buffer.

Glycolipid Analysis: The washed cell pellets were homogenized in three volumes of H_2O, an aliquot removed for protein determination (11), and the homogenate was lyophilized. The dried material was resuspended in 5 ml of chloroform-methanol (2:1, v/v) and extracted for 30 min at 50°. The residue was extracted with chloroform-methanol (1:2, v/v). Both extracts were filtered, combined, and taken to dryness under N_2. The lipids were dissolved in 5 ml chloroform-methanol (2:1, v/v), and 1 ml of H_2O was added. Following complete mixing and centrifugation, the upper phase was removed and the lower phase was washed four times with 2 ml of chloroform-methanol-water (3:48:47, v/v/v). The combined upper phases were taken to dryness and the gangliosides were separated by thin-layer chromatography, detected and quantitated as previously described (4).

Glycosyltransferase Assays: The washed cells were disrupted by freeze-

Fig. 1 Effect of butyrate on HeLa cells. HeLa cells were grown in Eagle's supplemented medium for 24 hours. The medium was replaced by fresh medium without (a) and with (b) 5 mM sodium butyrate. The pictures (160X) were taken 12 hours later.

thawing as previously described (12). The assay for CMP-NAN:CDH[1] sialyltransferase activity was as follows: the total volume of 25 µl contained 6 nmol CDH, 20 nmol CMP-[^{14}C]NAN (6.97 µCi/µmol from New England Nuclear), 50 µg cardiolipin (Supelco, Inc.), 375 µg cutscum (Fisher), 1.25 µmol Na-cacodylate (pH 6.8) and 10 µl of cell homogenate (200-300 µg of protein). After incubating at 37^{o} for two hours, the reactions were terminated by addition of 1.5 ml chloroform-methanol (2:1, v/v), 0.3 ml 2% $CaCl_2$, and 0.15 ml 1 N HCOOH. After thorough mixing the samples were centrifuged and the upper phases discarded. The lower phases were washed once with upper phase solvent and dried in counting vials for determination of radioactivity by liquid scintillation counting. Assays for UDP-Gal:CMH galactosyltransferase activity (3) and CMP-NAN:G_{M1} sialyltransferase activity (12) have been described elsewhere. Activities have been corrected for incorporation of label into endogenous acceptors and are expressed as picomoles of product formed per mg protein per hour.

RESULTS AND DISCUSSION

Ganglioside Content of Normal and Butyrate-Treated HeLa Cells: 24 hours after addition of 5 mM butyrate to HeLa cultures, the amount of ganglioside G_{M3} per mg cell protein increased significantly (Fig. 2). G_{D3}, the only other ganglioside present in these cells and the neutral glycosphingolipids were unaffected. When the cells were grown in a medium containing N-acetyl-[^{3}H]-mannosamine, a sialic acid precursor, the incorporation of radioactivity into G_{M3} increased correspondingly more in the butyrate-treated cells.

Glycosyltransferase Activities in Treated and Untreated HeLa Cells: Since the increase in G_{M3} content and the incorporation of N-acetyl[^{3}H]-mannosa-

[1] Abbreviations used are: NAN, N-acetylneuraminic acid; CDH, lactosylceramide; CMH, glucosylceramide; G_{M3}, N-acetylneuraminylgalactosylglucosylceramide; G_{D3}, N-acetylneuraminyl-N-acetylneuraminylgalactosylglucosylceramide; G_{M1}, galactosyl-N-acetylgalactosaminyl-[N-acetylneuraminyl]-galactosylglucosylceramide; G_{D1a}, N-acetylneuraminylgalactosyl-N-acetylgalactosaminyl-[N-acetylneuraminyl]-galactosylglucosylceramide

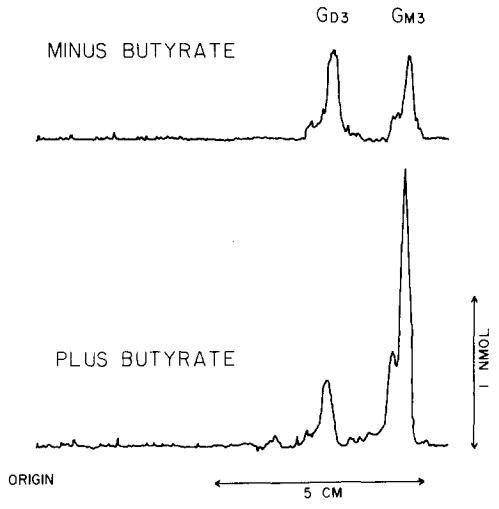


Fig. 2 Densitometric scans of thin-layer chromatogram of gangliosides extracted from HeLa cells grown with and without sodium butyrate. Gangliosides were extracted from HeLa cells (representing 5 mg protein) grown for 24 hours in Eagle's supplemented medium (top scan) and medium containing 5 mM sodium butyrate (bottom scan). The gangliosides were separated by thin-layer chromatography on silica gel G in the solvent system chloroform-methanol-0.25% aqueous $CaCl_2$ (60:35:8, v/v/v) and detected with resorcinol spray (4). The chromatograms were then scanned with a Zeiss chromatogram spectrophotometer. The distance along the ordinate is directly proportional to absorbance (up to 3 nmol). The vertical arrow represents the peak height of 1 nmol of G_{M3} standard which was chromatographed on the same plate as the ganglioside extracts.

mine into it indicated that butyrate treatment stimulated G_{M3} biosynthesis, the activity of CMP-NAN:CDH sialyltransferase (sialyltransferase I) was measured. After butyrate addition to the cultures, the specific activity of this glycosyltransferase increased greatly within 12 hours (Table I). Although the sialyltransferase I activity of different preparations varied (in controls: 2-9 pmol/mg protein/hr and in butyrate-treated cells: 42-159 pmol/mg protein/hr), butyrate always caused a significant increase (7-24 fold) when cells of the same experiment were compared. The assay remained linear for up to three hours and up to 300 µg of cellular protein.

The specific activities of two other glycosyltransferases involved in glycolipid biosynthesis were not significantly affected by sodium butyrate (Table 1). Galactosyltransferase I is the enzyme preceding sialyltransfer-

TABLE I

Effect of Butyrate on the Specific Activities of Glycosyltransferases in HeLa Cells

Glycosyltransferase	Reaction	Control	+ Butyrate
		pmol/mg protein/hr	
Galactosyltransferase I	CMH + UDP-Gal ⟶ CDH + UDP	233	156
Sialyltransferase I	CDH + CMP-NAN ⟶ G_{M3} + CMP	7	87
Sialyltransferase II	G_{M1} + CMP-NAN ⟶ G_{D1a} + CMP	203	177

Glycosyltransferase activities were measured 12 hours after addition of 5 mM sodium butyrate to the culture medium, as described under "EXPERIMENTAL PROCEDURES." The values represent the mean of three separate determinations.

ase I in the ganglioside biosynthetic pathway (13); its constant level indicates that the increase of sialyltransferase I activity is neither due to a general stimulation of glycolipid biosynthesis nor caused by a secondary increase of substrate (CDH) synthesis. Since sialyltransferase II activity is not increased, the possibility of a nonspecific stimulation of sialyltransferases is excluded.

Effects of Cycloheximide and Actinomycin D on Enzyme Increase: To determine whether the increase in sialyltransferase I activity in butyrate-treated cells required RNA and protein synthesis, HeLa cells were grown in the presence of cycloheximide (50 µg/ml) or actinomycin D (2 µg/ml) with or without the addition of butyrate (5 mM). Both of these inhibitors blocked the increase of sialyltransferase I activity in the presence of butyrate

TABLE II

Effect of Cycloheximide and Actinomycin D on Stimulation of Sialyltransferase I by Sodium Butyrate

Addition to the Medium	Control	+ Butyrate
	pmol/mg protein/hr	
None	8	67
Cycloheximide	9	12
Actinomycin D	6	7

HeLa cells were grown for 16 hours without and with 5 mM sodium butyrate in the presence of cycloheximide (50 µg/ml), actinomycin D (2 µg/ml), or no further addition. Sialyltransferase I activity was assayed as described under "EXPERIMENTAL PROCEDURES."

(Table II) as well as the previously described morphological changes (7).

The results of these experiments show a correlation between the morphology of HeLa cells in culture and their glycolipid content. In the presence of butyrate, there is an increase in ganglioside G_{M3} and the specific sialyltransferase involved in its biosynthesis. In addition, the effect of cycloheximide and actinomycin D indicates that the phenomenon of butyrate stimulated glycolipid biosynthesis is due to enzyme induction. These observations suggest a possible role of glycolipids in the control of cell growth and structure. They promise to provide a clearer understanding of the mechanisms of action of other small molecules such as dibutryl cyclic AMP and other nucleotides in altering those parameters. Finally, this relationship between morphology and glycolipids may provide insight into regulatory malfunctions associated with altered glycolipid biosynthesis in tumor cells.

REFERENCES

1. Cumar, F. A., Brady, R. O., Kolodny, E. H., McFarland, V. W. and Mora, P. T. (1970) Proc. Nat. Acad. Sci. USA 67, 757-764.
2. Den, H., Schultz, A. M., Basu, M. and Roseman, S. (1971) J. Biol. Chem. 246, 2721-2723.
3. Kijimoto, S. and Hakomori, S. (1971) Biochem. Biophys. Res. Commun. 44, 557-563.
4. Fishman, P. H., Brady, R. O., Bradley, R. M., Aaronson, S. A. and Todaro, G. J. (1974) Proc. Nat. Acad. Sci. USA 71, 298-301.
5. Den, H., Sela, B. A., Roseman, S. and Sachs, L. (1974) J. Biol. Chem. 249, 659-661.
6. Coleman, P. L., Fishman, P. H., Brady, R. O. and Todaro, G. J. J. Biol. Chem., manuscript submitted.
7. Ginsburg, E., Salomon, D., Sreevalsan, T. and Freese, E. (1973) Proc. Nat. Acad. Sci. USA 70, 2457-2461.
8. Seeds, N. W., Gilman, A. G., Amano, T., and Nirenberg, M. W. (1970) Proc. Nat. Acad. Sci. USA 66, 160-167.
9. Eagle, H. (1959) Science 130, 432-437.
10. Dulbecco, R. (1954) J. Exper. Med. 99, 167-182.
11. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275.
12. Fishman, P. H., McFarland, V. W., Mora, P. T. and Brady, R. O. (1972) Biochem. Biophys. Res. Commun. 48, 48-57.
13. Roseman, S. (1970) Chem. Phys. Lipids 5, 270-297.