

Sodium Butyrate Induction of Milk-related Antigens in Human MCF-7 Breast Carcinoma Cells¹

Miyako Abe and Donald W. Kufe²

Division of Medicine, Dana-Farber Cancer Institute, and Harvard Medical School, Boston, Massachusetts 02115

ABSTRACT

We have monitored the effects of sodium butyrate on expression of a human breast tumor-associated antigen defined by a murine monoclonal antibody designated DF3. This cell surface antigen has a molecular weight of approximately 300,000 and is related to a higher-molecular-weight antigen detectable in human milk. The results demonstrate that butyrate increases the membrane expression of the DF3 tumor-associated antigen. Further, butyrate treatment specifically enhances production of the DF3-related milk antigen. These effects occur at butyrate concentrations lower than that required for maximal expression of carcinoembryonic antigen and occur in the absence of changes in the expression of nonspecific cross-reacting antigen. The results suggest that butyrate induces a more differentiated MCF-7 phenotype and that this model may be useful in studying maturation of human breast cancer cells.

INTRODUCTION

Sodium butyrate is a potent inducer of erythroid differentiation in murine erythroleukemia cells (12) and of myeloid differentiation in human promyelocytic leukemia cells (6). Butyrate treatment increases sialyltransferase activity (9) and modulates the activity of other membrane-associated enzymes in human colon carcinoma cells (8). Butyrate-treated colon cancer cells also had increased levels of CEA³ (17, 18). Similarly, we have recently demonstrated that butyrate slows proliferation of MCF-7 breast carcinoma cells (1). This effect is associated with morphological changes and increased production of CEA. These findings have suggested that butyrate can enhance expression of differentiated phenotypes in carcinomas as well as in leukemias.

Another approach to assess differentiation of breast carcinomas involves monitoring for the synthesis of milk proteins. The synthesis of milk-related proteins has been used as a specific biochemical marker of differentiated function in mammary gland tissue and has been reported previously in human breast cancers (2, 15). We have recently developed a MAb reactive with an antigen present on the apical borders of more differentiated secretory mammary epithelial cells and in the cytosol of less differentiated cells (10). This MAb also reacts with an antigen present in human milk and thus provides an opportunity to monitor for a specific biochemical marker of differentiation.

The results of the present study demonstrate that butyrate enhances production of both the DF3 antigen in MCF-7 cells and

the cross-reacting higher-molecular-weight antigen detectable in human milk. The results also demonstrate that the enhanced expression of each of these antigens occurs on the cell membrane. Further, the increase in DF3 antigen expression occurs at butyrate concentrations distinct from that required for the maximal expression of CEA. In contrast, butyrate treatment had no detectable effect on the expression of NCA (19). These findings should provide a useful model for the study of human breast cancer cell maturation.

MATERIALS AND METHODS

Cell Culture. The human breast carcinoma MCF-7 cell line was obtained from the Michigan Cancer Foundation, Detroit, MI. Cells were grown as a monolayer in Dulbecco's modified Eagle's medium with the addition of 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 units penicillin/ml, 100 µg streptomycin/ml, and 0.25 IU insulin/ml.

MCF-7 cells in logarithmic growth phase were plated in 60- x 15-mm tissue culture dishes (Falcon Plastics, Oxnard, CA). Sodium butyrate was added 1 day after plating at concentrations of 1, 2, 3, and 4 mM. The medium was changed every other day. At the appropriate time, cells were harvested with 0.5% trypsin-0.2% EDTA, pelleted, and then resuspended in medium. Cell counts were performed by hemocytometer, and viability was monitored using trypan blue exclusion. Cell suspensions for antigen and protein determination were prepared by sonication in distilled water.

Solid-Phase Radioimmunoassay. The solid-phase radioimmunoassays using MAbs and sonicated cell suspensions were performed as described previously (5). The purified CEA and NCA antigens used in the solid-phase radioimmunoassay were kindly provided by Dr. Peter Thomas, Mallory Institute of Pathology, Boston, MA.

Cell Surface Binding Assay. MCF-7 cells in logarithmic growth phase were harvested with 0.5% trypsin-0.2% EDTA, pelleted, and resuspended in medium. This procedure had no detectable effect on DF3 antigenicity. The MCF-7 cells were then reacted with MAb DF3 and an isotype-identical control antibody for analysis by fluorescence-activated flow cytometry (11). Live cell radioimmunoassays were performed as described previously (5).

Molecular Weight Determination of MAb DF3-reactive Antigens. Antigen preparations from MCF-7 cells, a human breast carcinoma metastatic to liver (5) and human milk (4), were analyzed on a 4% polyacrylamide gel. After electrophoresis, the proteins from the polyacrylamide gel were transferred to nitrocellulose paper (Schleicher and Schuell, Inc., Keene, NH) using a Bio-Rad TransBlot apparatus (Bio-Rad, Rockville, MD) with a 25 mM Tris-192 mM glycine-20% methanol transfer buffer (pH 8.3). The blot was then incubated in 5% bovine serum albumin and washed 3 times. The washed nitrocellulose sheet was incubated with MAb DF3 for 120 min at room temperature, washed, incubated with rabbit anti-mouse immunoglobulin for 60 min, and then reincubated for an additional 120 min with ¹²⁵I-Protein A (specific activity, 2 × 10⁷ cpm/µg). The binding of radioactivity was determined by exposure of a Kodak X-AR film for 12 hr at -70°.

Preparation of MCF-7 Plasma Membrane, Mitochondrial-Endoplasmic Reticulum, and Supernatant Fractions. MCF-7 cells were harvested with trypsin-EDTA, washed in medium, and treated according

¹ This investigation was supported by the Dokton-Friends Special Fund at the Dana-Farber Cancer Institute.

² Supported by an American Cancer Society Faculty Research Award. To whom requests for reprints should be addressed, at Dana-Farber Cancer Institute, 44 Binney St., Boston, MA 02115.

³ The abbreviations used are: CEA: carcinoembryonic antigen; MAb: monoclonal antibody; NCA: nonspecific cross-reacting antigen.

Received April 8, 1984; accepted June 12, 1984.

to the method of Perdue (13). The isolation of the plasma membrane fraction was confirmed by assaying for CTPase activity (14).

RESULTS

The effects of varying concentrations of sodium butyrate on MCF-7 cellular proliferation are illustrated in Chart 1. Although 1 mM butyrate partially slowed MCF-7 growth, 2 mM butyrate inhibited proliferation by nearly 90% after 7 days of exposure. Cytostasis was achieved with 3 mM butyrate, and no loss of cell viability was observed following a 7-day exposure to either 3 or 4 mM butyrate. This inhibition of MCF-7 proliferation was associated with morphological changes that included an increase in cell size and development of an epithelioid configuration with long processes projecting from the cytoplasm (1).

The effect of butyrate on the expression of DF3 antigen in MCF-7 cells is illustrated in Chart 2A. DF3 antigen was detectable in untreated MCF-7 cells, and the level remained relatively constant during 7 days of culture. In contrast, 1 and 2 mM butyrate treatment resulted in up to 5-fold increases in total cellular DF3 antigen content. Although treatment with 3 and 4 mM butyrate also resulted in significant increases in DF3 antigen during the first 3 days of exposure, the subsequent increases were not as pronounced as those obtained with the lower concentrations of butyrate.

The increases in DF3 antigen following butyrate exposure could be related to increases in cell size and not due to a selective enhancement of DF3 antigen production. We have thus monitored total cellular protein following 7 days of butyrate treatment (Chart 2B). Total protein content increased with increasing butyrate concentration and duration of exposure. These findings were consistent with the observed changes in morphology. The DF3/protein ratio remained elevated in cells treated with 1 and 2 mM butyrate, while exposure to 3 or 4 mM butyrate had little if any effect on the DF3 antigen expression (Chart 2C). These findings are in contrast to the CEA/protein ratio which was increased in MCF-7 cells treated with 3 and 4 mM butyrate (1).

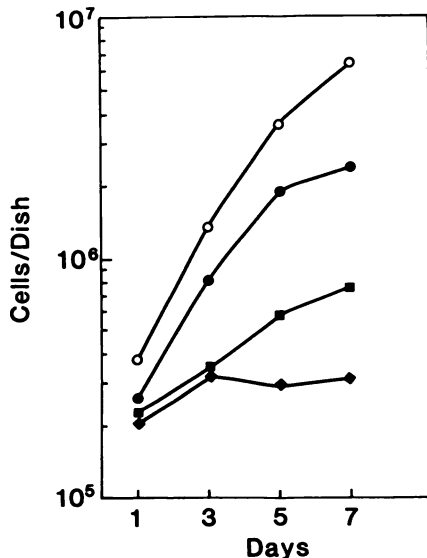


Chart 1. Effect of butyrate on MCF-7 proliferation. MCF-7 cells were seeded in 60- x 15-mm dishes for 24 hr prior to the addition of 1 mM (●), 2 mM (■), or 3 mM (▲) sodium butyrate. Cell counts were performed at 1, 3, 5, and 7 days of butyrate exposure. ○, control untreated cells. The results are expressed as the mean count for 5 separate dishes. Viability remained over 90% in all determinations.

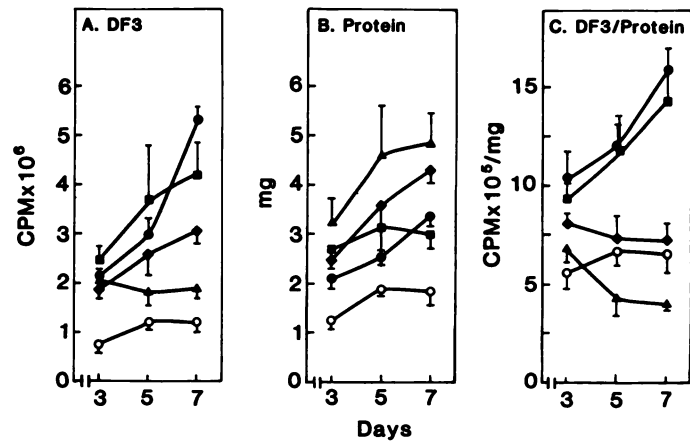


Chart 2. Effects of butyrate on DF3 antigen production and total protein content. MCF-7 cells were seeded for 24 hr and then treated with 1 mM (●), 2 mM (■), 3 mM (▲), or 4 mM (▲) sodium butyrate for 3, 5, and 7 days. ○, control untreated cells. DF3 content (A) was monitored using MAb DF3 in a solid-phase radioimmunoassay, and total protein (B) was determined by the Bio-Rad assay. Ratio (C) of ^{125}I cpm $\times 10^6$ /mg total protein. Results are expressed as the means of 5 separate determinations; bars, S.D.

Table 1
Effect of butyrate on expression of DF3, CEA, and NCA in MCF-7 cells

MAb	^{125}I cpm bound in solid-phase radioimmunoassay			
	Purified antigens		MCF-7 cells ^a (1×10^6)	
	CEA (10 ng)	NCA (10 ng)	Control	Butyrate
DF3	330	89	44,748 \pm 8,429 ^b	205,912 \pm 29,867
DF2	13,767	114	10,197 \pm 1,191	16,938 \pm 1,473
DF11	646	6,602	4,438 \pm 1,158	5,227 \pm 1,359

^a MCF-7 cells obtained after 7 days from control and 2 mM butyrate-treated cultures.

^b Mean \pm S.D. of 4 determinations.

The increase in DF3 antigen expression following a 7-day exposure to 2 mM butyrate was compared to that obtained with CEA and NCA production. The results obtained are listed in Table 1. MAb DF3 was unreactive with purified preparations of CEA and NCA. In contrast, MAb DF2 reacted specifically with CEA, while MAb DF11 reacted with NCA. As listed in Table 1, DF3 antigen expression increased by over 4-fold in MCF-7 cells treated with 2 mM butyrate for 7 days. A less pronounced increase in CEA production was obtained in the butyrate-treated cells, while there was no significant increase in the expression of NCA. These findings are consistent with the differential expression of certain genes following butyrate exposure and confirm the specific increase in DF3 antigen expression.

It was of further interest to determine whether the increase in DF3 antigen content was associated with cell surface expression. MCF-7 cells were treated with 2 mM butyrate for 1, 3, and 7 days, and the cell surface reactivity of MAb DF3 was determined by indirect immunofluorescence. The fluorescence histogram pattern of reactivity is illustrated in Chart 3. A nonreactive MAb 287 of the same IgG1 isotype showed no binding. In contrast, MAb DF3 was reactive with the cell surface of untreated and butyrate-treated cells. The pattern of reactivity was similar on Day 1, while membrane expression of DF3 antigen was significantly increased in butyrate-treated cells on Day 3 (Chart 3B). Further increases were observed in the butyrate-treated cells on Day 7 (Chart 3C). These findings have been confirmed using a live cell radioimmunoassay (Table 2). Using this method

Chart 3. Effect of butyrate on DF3 cell surface antigen expression using indirect immunofluorescence. MCF-7 cells were seeded for 24 hr and then treated with 2 mM butyrate for 1, 3, and 7 days. Immunofluorescence profiles represent reactivity of MAb DF3 with untreated cells (—) and butyrate-treated cells (---). - - - - -, reactivity of an isotope-identical control MAb 287.

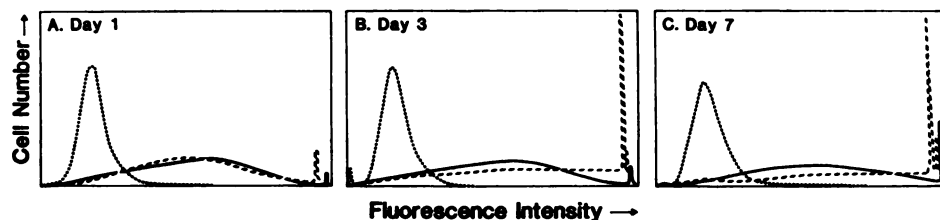


Table 2
Live-cell radioimmunoassay of DF3 cell surface antigen expression

Days of exposure	cpm bound/10 ⁵ cells ^a	
	Control	Butyrate (2 mM)
1	5,321	7,409
3	5,447	51,805
7	11,717	100,250

^a Mean of 2 determinations.

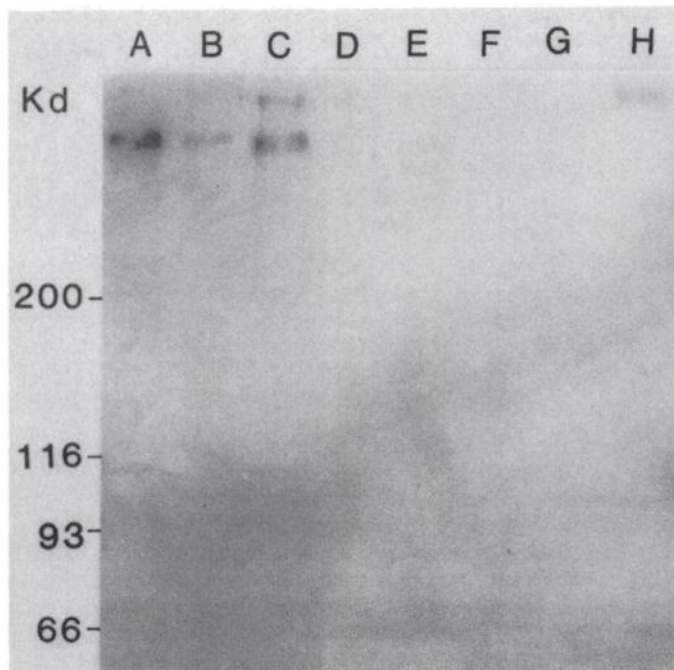


Fig. 1. Reactivity of MAb DF3 with metastatic breast carcinoma, butyrate-treated MCF-7 cells, and human milk. Lane A, membrane-enriched fraction of a breast carcinoma metastatic to liver (0.66 μ g); Lane B, MCF-7 cells (Day 1, 50 μ g); Lane C, MCF-7 cells treated with 2 mM butyrate (Day 1, 50 μ g); Lane D, MCF-7 cells (Day 3, 50 μ g); Lane E, MCF-7 cells treated with 2 mM butyrate (Day 3, 50 μ g); Lane F, MCF-7 cells (Day 7, 50 μ g); Lane G, MCF-7 cells treated with 2 mM butyrate (Day 7, 50 μ g); Lane H, human milk protein (50 μ g). The antigen preparations were subjected to electrophoresis on a 4% polyacrylamide gel, transferred to nitrocellulose paper, and analyzed for reactivity with MAb DF3.

of analysis, cell surface expression of DF3 antigen was increased by nearly 10-fold after 3 and 7 days of 2 mM butyrate exposure.

Since the immunogen used to prepare MAb DF3 was a membrane-enriched fraction of the human breast carcinoma metastatic to liver, we compared reactivity of MAb DF3 with the immunogen and MCF-7 cells (Fig. 1). The analysis revealed a reactive antigen having a molecular weight of approximately 300,000 in both the breast carcinoma extract and the MCF-7 cells (Fig. 1, Lanes A and B). The treatment of MCF-7 cells with 2 mM butyrate for 1, 3, and 7 days resulted in increased expression of the *M*, 300,000 antigen (Fig. 1, Lanes C, E, and G). Butyrate treatment also resulted in enhanced expression of a

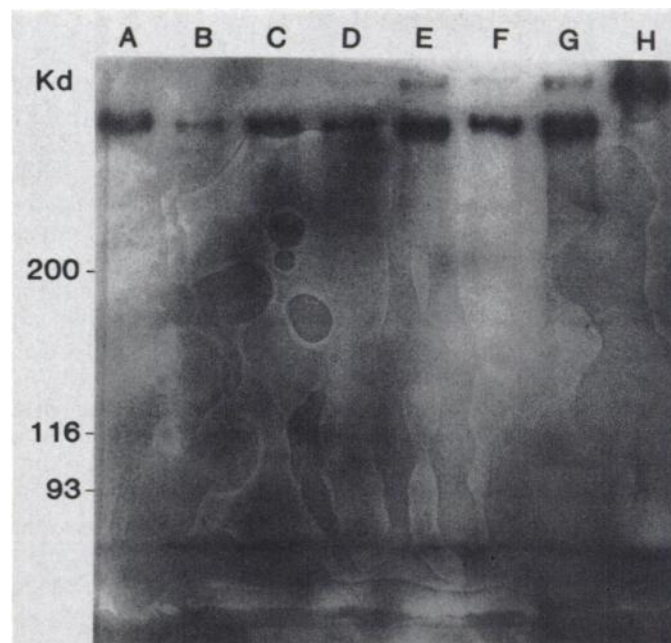


Fig. 2. Reactivity of MAb DF3 with cytoplasmic membrane preparations from Day 7 untreated and butyrate-treated MCF-7 cells. Lane A, membrane-enriched fraction of a breast carcinoma metastatic to liver (1 μ g); Lane B, cytoplasmic membrane fraction from untreated MCF-7 cells (1 μ g); Lane C, cytoplasmic membrane fraction from 2 mM butyrate-treated MCF-7 cells (1 μ g); Lane D, mitochondrial-endoplasmic reticulum fraction from untreated MCF-7 cells (1 μ g); Lane E, mitochondrial-endoplasmic reticulum fraction from 2 mM butyrate-treated cells (1 μ g); Lane F, supernatant fraction from untreated MCF-7 cells (1 μ g); Lane G, supernatant fraction from 2 mM butyrate-treated cells (1 μ g); Lane H, human milk protein (10 μ g). The antigen preparations were subjected to electrophoresis on a 4% polyacrylamide gel, transferred to nitrocellulose paper, and analyzed for reactivity with MAb DF3. CTPase activity (μ mol phosphate released/mg protein) for the butyrate-treated cells was as follows: cytoplasmic membrane fraction, 25.8; mitochondrial-endoplasmic reticulum fraction, 1.5; and supernatant fraction, 1.0.

higher-molecular-weight MAb DF3-reactive antigen (Lanes E and G). The higher-molecular-weight antigen was undetectable in the metastatic breast carcinoma extract, while an antigen of similar molecular weight was detectable in human milk (Fig. 1, Lane H). Finally, these increases in the expression of the MAb DF3 cross-reactive antigens in butyrate-treated cells were detectable at relatively higher levels in the plasma membrane fraction, as compared to the mitochondrial-endoplasmic reticulum or supernatant cellular fractions (Fig. 2).

DISCUSSION

MAb DF3 was generated using a membrane-enriched fraction of a human breast carcinoma metastatic to liver (10). This MAb has been shown to react with 87% of 52 primary breast carcinomas and with 100% of breast tumors metastatic to axillary lymph nodes or distal sites. The cross-reacting antigen has a molecular weight of approximately 300,000. The membrane lo-

cation of the DF3 antigen appears to be related to the functional activity of mammary cells as well as to the degree of differentiation of the tumor cells. Benign breast lesions showed only apical location of this antigen. In contrast, a cytoplasmic antigen location was always observed with infiltrating breast carcinomas.

Our previous results thus suggest that the DF3 antigen is present in high levels on apical borders of differentiated secretory mammary epithelial cells and in the cytosol of less differentiated malignant cells. Since milk fat globules are extruded from mammary gland cells by reverse pinocytosis, these structures are surrounded by apical cell membranes (4). The presence of DF3 antigen in human milk and on the apical borders of more differentiated cells suggests that the synthesis of this antigen represents a differentiated function of mammary epithelium.

Although there are several models for the study of leukemic cell differentiation (6, 12), there is presently no well-characterized model for the study of differentiation of malignant human epithelial cells. Several previous studies have monitored the effects of butyrate on colon carcinoma cells (8, 9, 17, 18), and the enhanced production of CEA has suggested maturation to a more differentiated phenotype. In this regard, CEA production in human breast cancer cells has reflected biological behavior (20), and an association has been made between CEA production and low histological grades (16). However, the production of milk antigens would clearly provide a more precise biochemical marker of differentiated function in malignant cells derived from mammary epithelium.

The results of the present study demonstrate that butyrate enhances production of DF3 antigen in MCF-7 cells. This antigen has a molecular weight comparable to that detectable in primary and metastatic breast carcinomas. More importantly, butyrate enhances production of a higher-molecular-weight MAb DF3-reactive antigen in MCF-7 cells which is also detectable in human milk. Further, butyrate treatment results in an increase in the expression of both the carcinoma-related and the milk-related antigens on the MCF-7 plasma membrane. These findings would be consistent with the appearance of MAb DF3 reactivity on the apical membrane borders of more differentiated mammary epithelium.

The present results also demonstrate a selective enhancement in DF3 antigen expression following exposure to 1 and 2 mM butyrate. Of particular interest is the finding that higher butyrate concentrations (3 and 4 mM) were not as effective in increasing DF3 antigen production. Although 2 mM butyrate exposure also increases CEA production, we have demonstrated previously that 3 and 4 mM butyrate treatment of MCF-7 cells results in a maximal enhancement of CEA content (1). Furthermore, the present study demonstrates that the selective increases in both DF3 antigen and CEA production are not accompanied by increases in the expression of NCA, an antigen not previously associated with a differentiated phenotype (19).

Although the mechanism by which butyrate induces differentiation remains unclear, the studies should provide a model for studying maturation of malignant human mammary epithelial cells. Butyrate alters histone acetylation patterns by reducing

nuclear histone acetyltransferase and inhibiting histone deacetylase (7). Butyrate also inhibits phosphorylation of histones and can interfere with methylation of nuclear protein fractions (3). These effects may be related to the induction of leukemic cell differentiation and the selective increase in MCF-7 milk-related antigen production. It will now be of interest to determine whether other inducers of differentiation will similarly affect DF3 antigen production and provide a model for the study of specific gene expression in human mammary epithelium.

REFERENCES

1. Abe, M., and Kufe, D. Effect of sodium butyrate on human breast carcinoma (MCF-7) cellular proliferation, morphology and CEA production. *Breast Cancer Res. Treat.*, in press, 1984.
2. Arklie, J., Taylor-Papadimitriou, J., Bodmer, W., Egan, M., and Mills, R. Differentiation antigens expressed by epithelial cells in the lactating breast are also detectable in breast cancers. *Int. J. Cancer*, 28: 23-29, 1981.
3. Boffa, L., Gruss, R., and Alfrey, V. Manifold effects of sodium butyrate on nuclear function. *J. Biol. Chem.*, 256: 9612-9621, 1981.
4. Ceriani, R., Thompson, K., Peterson, J., and Abraham, S. Surface differentiation antigens of human mammary epithelial cells carried on the human milk fat globule. *Proc. Natl. Acad. Sci. USA*, 74: 582-586, 1977.
5. Colcher, D., Horan Hand, P., Nuti, M., and Schlom, J. A spectrum of monoclonal antibodies reactive with human mammary tumor cells. *Proc. Natl. Acad. Sci. USA*, 78: 3199-3203, 1981.
6. Collins, S., Ruscetti, F., Gallagher, R., and Gallo, R. Terminal differentiation of human promyelocytic leukemia cells induced by dimethyl sulfoxide and other polar compounds. *Proc. Natl. Acad. Sci. USA*, 75: 2458-2462, 1978.
7. Covault, J., Sealy, L., Schnell, R., Shires, A., and Chalkley, R. Histone hypoacetylation following release of HTC cells from butyrate. *J. Biol. Chem.*, 257: 5809-5815, 1982.
8. Dexter, D. L., Crabtree, G. W., Stoeckler, J. D., Savarese, T. M., Ghoda, L. Y., Rogler-Brown, T. L., Parks, R. E., Jr., and Calabresi, P. *N,N*-Dimethylformamide and sodium butyrate modulation of the activities of purine-metabolizing enzymes in cultured human colon carcinoma cells. *Cancer Res.*, 41: 808-812, 1981.
9. Kim, Y., Tsao, D., Siddiqui, B., Whitehead, J., Arnstein, P., Bennet, J., and Hicks, J. Effects of sodium butyrate and dimethylsulfoxide on biochemical properties of human colon cancer cells. *Cancer (Phila.)*, 45: 1185-1192, 1980.
10. Kufe, D., Inghirami, G., Abe, M., Hayes, D., Justi-Wheeler, H., and Schlom, J. Differential reactivity of a novel monoclonal antibody (DF3) with human malignant versus benign breast tumors. *Hybridoma*, in press, 1984.
11. Kufe, D., Nadler, L., Sargent, L., Shapiro, H., Hand, P., Austin, F., Colcher, D., and Schlom, J. Biologic behavior of human breast carcinoma-associated antigens expressed during cellular proliferation. *Cancer Res.*, 43: 851-857, 1983.
12. Leder, A., and Leder, P. Burytric acid, a potent inducer of erythroid differentiation in cultured erythroleukemia cells. *Cell*, 5: 319-322, 1975.
13. Perdue, J. The isolation and characterization of plasma membrane from cultured chicken embryo fibroblast. *Methods Enzymol.*, 31: 162-168, 1974.
14. Perdue, J., and Sneider, J. The isolation and characterization of the plasma membrane from chick embryo fibroblasts. *Biochim. Biophys. Acta*, 196: 125-140, 1970.
15. Pich, A., Bussolati, G., and Carbanara, A. Immunocytochemical detection of casein and casein-like proteins in human tissues. *J. Histochem. Cytochem.*, 24: 940-947, 1976.
16. Shousha, S., Lyssiotis, T., Godfrey, V., and Scheuer, P. Carcinoembryonic antigen in breast-cancer tissue: a useful prognostic indicator. *Br. Med. J.*, 77: 777-779, 1979.
17. Tsao, D., Morita, A., Bella, A., Jr., Luu, P., and Kim, Y. S. Differential effects of sodium butyrate, dimethyl sulfoxide, and retinoic acid on membrane-associated antigen, enzymes, and glycoproteins of human rectal adenocarcinoma cells. *Cancer Res.*, 42: 1052-1058, 1982.
18. Tsao, D., Shi, Z., Wong, A., and Kim, Y. Effect of sodium butyrate on carcinoembryonic antigen production of human colonic adenocarcinoma cells in culture. *Cancer Res.*, 43: 1217-1222, 1983.
19. von Kleist, S., Chavanel, G., and Burtin, P. Identification of an antigen from normal tissue that cross-reacts with the carcinoembryonic antigen. *Proc. Natl. Acad. Sci. USA*, 69: 2492-2494, 1972.
20. Walker, R. Demonstration of CEA in human breast carcinomas by immunoperoxidase technique. *J. Clin. Pathol.*, 33: 356-360, 1980.