CHANGES IN GLYCOLIPID GLYCOSYLTRANSFERASES AND GLUTAMATE DECARBOXYLASE AND THEIR RELATIONSHIP TO DIFFERENTIATION IN NEUROBLASTOMA CELLS

Joseph R. Moskal,[+] David A. Gardner[*] and Subhash Basu

Department of Chemistry, Biochemistry and Biophysics Program, Univ. of Notre Dame, Notre Dame, Indiana 46556; and[*]Research Division, Miles Laboratories, Inc., Elkhart, Indiana 46514.

Received October 15,1974

SUMMARY - Glycolipid glycosyltransferase activities involved in the biosynthesis in vitro of neutral and acidic glycosphingolipids were measured in C-1300 tumors and cloned cells derived therefrom. An adrenergic clone (N1E-115) was grown in tissue culture in the presence of dibutyryl cyclic AMP and the levels of glycosyltransferases were measured before and after differentiation. Increased activities of galactosyltransferases and sialyltransferases with a concomitant increase in glutamate decarboxylase activity (the enzyme that catalyzes the synthesis of an inhibitory neurotransmitter, γ-aminobutyric acid) were observed.

The changes in glycosphingolipid and glycoprotein of eucaryotic cell membranes after viral transformation (1-6) and chemically induced differentiation (7-9), or during density dependent growth inhibition (10-11) and cell division (12), have formed an important area of investigation in recent years.

Clones of neuroblastoma cell lines derived from C-1300 tumors (13) have been utilized by many investigators for neurobiochemical research. These cells have been reported to generate action potentials (14) and accumulate enzymes involved in neurotransmitter synthesis (15-17) when morphogenic differentiation is stimulated either by the addition of dibutyryl cyclic AMP [$(But)_2$cAMP] or by reduction of serum from incubation medium.

A mouse neuroblastoma C-1300 clonal line, NB41A, was found to contain GM2, GD2, GM1, GD1a, and very little GM3 ganglioside by Dawson et al. (18). Marked variations in ganglioside patterns of various neuroblastoma clones having different electrical properties were reported by Yogeeswaran et al. (19). Stoolmiller et al. (20) reported that incubation of neuroblastoma clones (NB41) with norepinephrine (10 μM) caused a 6-fold increase of the GM1 + GM2/GM3 ratio in these cells.

Using an adrenergic clone (N1E-115) and a cholinergic clone (NS-20) derived from C-1300 tumors (21), we report here on the biosynthetic patterns in vitro of neutral and acidic glycosphingolipids in these clones. The changes

This work was supported by NIH Research Grant NS-09541-04 and a grant-in-aid from Miles Laboratories, Inc., Elkhart, Indiana (to S. B). [+]Predoctoral fellow in the Chemistry Department of the Univ. of Notre Dame, supported in part by the grant NS-09541 from the NIH.

in glutamate decarboxylase and glycolipid glycosyltransferase activities after adminstration of $(But)_2$cAMP in N1E-115 adrenergic cell culture were also observed.

MATERIALS AND METHODS

Cell culture: Neuroblastoma clones N1E-115 and NS-20 (21) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Gibco). Cultures were grown in 250-ml Falcon flasks containing 15 ml of medium in humidified atmosphere of 90% air-10% CO_2. Suspension cultures were grown in 500-ml spinner flasks containing Joklick's modified Eagle's medium supplemented with 10% fetal calf serum (Gibco).The medium was changed three times a week, and cells were subcultured when monolayers reached confluency, using 0.2% trypsin or 0.25% Viokase in phosphate buffered saline. A/J mice bearing C-1300 tumors were obtained from The Jackson Laboratory, (Bar Harbor, Me.). Both N1E-115 and NS-20 clones gave rise to malignant tumors when injected into A/J mice.

Donors: Unlabeled CMP-NAN was prepared according to Kean and Roseman (22). Unlabeled UDP-galactose was purchased from Calbiochem. CMP-[4, 5, 6, 7, 8, 9-^{14}C]NAN (229 mCi/ mmole) and UDP-[^{14}C]galactose (298 mCi/ mmole) were purchased from New England Nuclear.

Acceptors: Lactosylceramide (Galβ1-4Glc-cer) and GM3(NANAα2-3Galβ1-4Glc-

ACCEPTORS	[^{14}C]Galactose incorporated (pmoles/mg protein/hr)				
	C-1300	N1E-T	N1E-S	NS20-T	N1E-SB1
Endogenous	65	82	45	59	84
2-OH Ceramide	105	78	46	91	149
Glucosylceramide	227	224	496	262	ND
Lactosylceramide	81	593	301	258	477
LacTri-cer	2109	8055	5770	5342	5512
Lac-nTet-cer	106	634	324	453	ND
GanglioTri-cer	69	24	20	89	ND
GM2	45	98	75	121	ND

Table I. Glycolipid: galactosyltransferase activities. The complete incubation mixtures contained the following components (in micromoles) in a final volume of 0.05 ml: acceptor lipids, 0.05; Triton X-100, 195 μg; cacodylate-HCl buffer, pH 6.97, 10; $MnCl_2$, 0.25; UDP-[^{14}C]galactose (1.92×10^6 dpm/μmole), 0.02; and enzyme fraction, 0.25 to 0.6 mg of protein. The mixtures were incubated for 60 min. at 37°, and the reactions were stopped by adding 0.6 μmoles of EDTA (pH 7.0). The incorporation of radioactive glycose units into acceptor glycosphingolipids was assayed using the double chromatographic technique described previously (23-25). Under these conditions the rate of reaction remained constant up to one hour and was proportional to protein concentration between 0.1-0.5 mg/incubation volume. ND: not determined.

cer)were isolated from bovine spleen. GanglioTri-cer(GalNAcβ1-4Galβ1-4Glc-cer) and GloboTri-cer(Galα1-4Galβ1-4Glc-cer) were isolated from guinea pig and porcine erythrocytes, respectively(23, 24). Lac-nTet-cer(Galβ1-4GlcNAc-β1-3Galβ1-4Glc-cer) and LacTri-cer(GlcNAcβ1-3Galβ1-4Glc-cer) were prepared from rabbit erythrocyte B-active pentaglycosylceramide by sequential degradation of galactose units with purified fig α-galactosidase (25, 26), and papaya β-galactosidase(8). The purified glycosphingolipids were analyzed by gas chromatography (23, 25) and GC-mass spectrometry (27).

Enzyme source: A/J mice bearing C-1300 tumors were obtained from The Jackson Laboratory, and tumors were excised when they attained a diameter of 1-2 cm and stored at -18°. Before the experiment, tumors were thawed and homogenized in 3 volumes of 0.32 M sucrose with a Polytron 10-ST at 4-5°, and this homogenate was designated enzyme fraction C-1300.

N1E-115 clones grown in 250-ml Falcon T-flasks (containing 15 ml of medium) and in 500-ml spinner flasks (containing 250 ml of medium) were harvested (0.2 to 0.5 ml packed volume), homogenized in 1 volume of 0.32 M sucrose, and designated enzyme fractions N1E-T and N1E-S, respectively.

The cholinergic clone, NS-20, was grown in 250-ml Falcon T-flasks (containing 15 ml of medium), harvested (0.6 ml packed volume), homogenized in 1 volume of 0.32 M sucrose, and designated enzyme fraction NS20-T.

N1E-115 clone was grown in suspension culture for 5 days and then transferred to roller bottles (2.6×10^6 cells per ml of medium) containing 1.0 mM $(But)_2$cAMP, allowed to incubate for 24 hours, harvested (0.5 ml packed volume), homogenized in one volume of 0.32 M sucrose, and designated as enzyme fraction N1E-SB1.

The cells grown in T-flasks were harvested at confluency (5 days after subculture). The cells grown in suspension culture were harvested on the fifth day after inoculation (lag phase of growth curve). The cells were harvested without treatment with Viokase or trypsin and homogenized with a Polytron 10-ST (3 x 5 sec).

Incorporation of [^{14}C] galactose into glycolipids of N1E-115 cultures.

[^{14}C]Galactose (2.1×10^6 dpm ; 53 mCi/ mmole) was added to confluent N1E-115 cell cultures (1.7×10^7 cells / 75 cm^2 in 250-ml Falcon flasks) and incubated upto 3 days in the presence of 1.0 mM $(But)_2$cAMP (in 10 ml of medium). Cells were harvested with 3.0 ml of 0.2% trypsin in phosphate buffered saline (PBS), and 8.5 ml of chloroform-methanol (1:4) were added to the mixture to extract [^{14}C]glycosphingolipids. The mixture was refluxed for 30 min at 57°, filtered, and dialyzed against distilled water at 4° to remove free [^{14}C]galactose. The retentate was dried, dissolved in chloroform-methanol (2:1), and subjected to thin-layer chromatography(Fig. 1). Appropri-

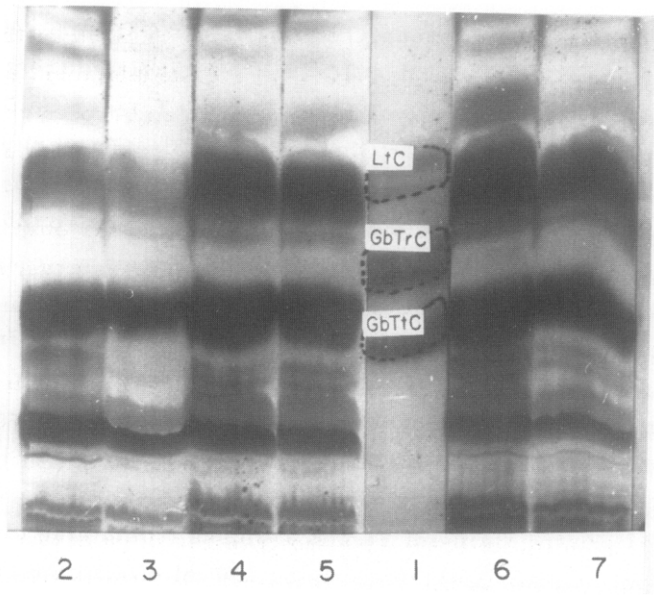

Figure 1. Effect of $(But)_2$cAMP on [^{14}C]galactose incorporation into N1E-115 cell glycosphingolipids. Lane 1. LtC (lactosylceramide), GbTrC (GloboTri-cer), and GbTtC(GloboTetra-ceramide). Lanes 2, 4, and 6 are ^{14}C-glycolipids obtained from N1E-115 cells grown in T-flasks after incubation in the presence of 1.0 mM $(But)_2$cAMP for 1, 2, and 3 days respectively. Lanes 3, 5, and 7 are ^{14}C-glycolipids obtained from control flasks incubated for 1, 2, and3 days respectively without the addition of $(But)_2$cAMP. 5-7000 dpm/sample was applied to silica gel G plates (0.5mm thick) and developed with chloroform-methanol-water (60:30:6). X-ray plate was developed after exposure for 80 days.

---

ate areas were scraped and radioactivity measured in a liquid scintillation system. In a separate experiment the radioactive areas were scraped and [^{14}C]glycolipids were eluted from the silica gel with chloroform-methanol-water(60:35:8). The three major radioactive bands (Fig.1), lactosylceramide (LtC), globoside(GbTtC) and GM3 ganglioside, were identified by their ability to be cleaved (83 to 95%) by purified papaya β-galactosidase, jack bean β-hexosaminidase and Cl. perfringens α-sialidase, respectively. Each radioactive product also co-chromatographed with authentic glycosphingolipids on silica gel G thin layer plate in two different solvent systems(chloroform-methanol-water, 60:30:6; n-propanol-water, 70:30).

RESULTS AND DISCUSSION

The glycosphingolipids of neuronal membrane (sulfatides and gangliosides or their precursors) are predominantly acidic in nature. Although the function of these acidic glycosphingolipids is not clearly understood at the present time, their stepwise biosynthesis in vitro has been proposed by several

laboratories (29-32). The mechanism of biosynthesis de novo of these glycosphingolipids and their relationship to neuronal cell development and differentiation are unknown. Basu et al. (33) reported that in embryonic chicken brain the UDP-galactose : 2-hydroxyceramide β-galactosyltransferase appeared after the 17th day of development, whereas UDP-galactose:GM2 β-galactosyltransferase appeared at earlier stages of development. This observation led us to study the appearance of these glycolipid glycosyltransferases in a simpler biological system such as specific clonal lines (N1E-115 and NS-20) derived from C-1300 neuroblastoma tumors.

A high level of UDP-galactose:LacTri-cer β-galactosyltransferase (34) was found in N1E-115 cells grown in T-flasks or suspension culture. The activity of this enzyme was not stimulated in the presence of $(But)_2$cAMP (Table I, last column). However, considerable stimulation of the two other galactosyltransferases (those catalyzing the transfer of galactose to 2-hydroxyceramide and lactosylceramide) was observed. The former lipid is a precursor for cerebroside synthesis, which is an important component of myelin membrane. The latter reaction yields the precursor (GloboTri-cer) of cell surface antigens such as globoside (5, 23). The appearance of mono- and diglycosylceramide was also observed in adrenergic clone N1E-115 grown in monolayer cultures in the presence of 1.0 mM $(But)_2$cAMP after 24 hours (Fig. 1). As shown in Table I, the galactosyltransferase (GalT) activities with glucosylceramide, lactosylceramide, LacTri-cer, and Lac-nTet-cer as

ACCEPTORS	[^{14}C]NAN incorporated (nmoles/mg protein/hr)				
	C-1300	N1E-T	N1E-S	NS20-T	N1E-SB1
Endogenous	0.44	2.07	2.18	0.65	2.35
Lactosylceramide	1.33	5.65	4.85	2.74	4.16
GM3	1.46	10.75	8.84	4.58	9.44
GanglioTet-cer	1.33	5.75	4.57	3.49	ND
Lac-nTet-cer	0.67	0.99	1.26	1.37	ND
GM1	0.26	1.36	2.52	0.85	4.38
GD1a	0.04	nd	0.59	nd	0.53
GD1b	0.04	0.33	1.45	0.15	1.64

Table II. Glycolipid: sialyltransferase activities. The complete incubation mixtures contained the following components (in micromoles) in a final volume of 0.05 ml: glycolipid acceptors, 0.05; Triton CF-54: Tween 80 (2:1 w/w), 200 μg; cacodylate-HCl buffer, pH 6.5, 10; $MgCl_2$, 0.1; CMP-NAN (1.27×10^6 dpm/μmole), 0.05; and 0.25 to 0.6 mg of protein. The mixtures were incubated for 60 min. at 37°. Reactions were terminated by addition of 0.6 μmoles of EDTA (pH 7.0) and assayed by the double chromatographic procedure described previously (23-25). Under these conditions the rate of reaction remained constant up to one hour and was proportional to protein concentration between 0.1 and 0.5 mg per incubation volume. ND: not determined, nd: not detectable under present assay conditions.

substrates, were 2- to 7-fold higher in the neuroblastoma clones than in the parent C-1300 tumors. These results in vitro suggest that there may be a blood group B-type glycosphingolipid actively synthesized de novo in neuroblastoma cells before differentiation and that the following pathways may exist in these clones (25, 29-36):

1. Glc-cer $\xrightarrow{[A]}$ Gal-Glc-cer $\xrightarrow{[B]}$ Gal-Gal-Glc-cer
2. GlcNAc-Gal-Glc-cer $\xrightarrow{[C]}$ Gal-GlcNAc-Gal-Glc-cer $\longrightarrow$ [D] Gal-Gal-GlcNAc-Gal-Glc-cer
3. Gal-Glc-cer $\xrightarrow{[E]}$ NAN-Gal-Glc-cer $\xrightarrow{[F]}$ $(NAN)_2$-Gal-Glc-cer

The [^{14}C]products of reactions [C], [D], and [F] were isolated, purified, and treated with specific glycosidases. The papaya β-galactosidase (8) cleaved 93% of Reaction-C product, the fig α-galactosidase (25, 26) cleaved 72% of Reaction-D product, and Cl. perfringens (30, 31) cleaved 90% of Reaction-F product. The [^{14}C]globoside obtained from N1E-115 culture-experiment (Fig. 1) migrated just behind the porcine erythrocyte globoside (GalNAcβ1-3Galα1-4-Galβ1-4Glc-cer) when mixed together. It is possible that the structure of this [^{14}C]globoside is related to Cytolipin R (GalNAcβ1-3Galα1-3Galβ1-4Glc-cer) found in rat lymphosarcoma (37).

Murray and his co-workers (19) detected an appreciable change in the pattern of the different gangliosides but not of the neutral glycosphingolipids in various clonal lines (N2A, NA, NB41A) of the mouse C-1300 neuroblastoma cells. Using adrenergic (N1E-115) and cholinergic (NS-20) clones, we tested various glycolipid:sialyltransferase (SialylT) activities (Table II). High levels of activity were observed with lactosylceramide, GM3, and GanglioTet-cer as acceptors. However, the enzyme activities catalyzing the synthesis in vitro of higher gangliosides were low. Except for the SialylT catalyzing the synthesis of GDIa from GMl, the other SialylTs remained unaffected in the presence of 1.0 mM $(But)_2$cAMP. From 5- to 10-fold elevations in activities were

	$^{14}CO_2$ Released (nmoles/mg protein/hr)				
	C-1300	N1E-T	N1E-S	NS20-T	N1E-SB1
-PLP	0.3	4.0	3.6	7.3	3.8
+PLP	2.1	4.9	5.7	12.5	9.5

Table III. Glutamate decarboxylase activity. The complete incubation mixtures contained the following components (in micromoles) in final volumes of 0.085 ml: HEPES buffer, pH 7.2, 10; Triton X-100, 75 μg; Pyridoxal 5'-monophosphate (PLP), 0.05; L-[1-^{14}C]glutamate (0.43 x 10^6 dpm/μmole), 0.15; and enzyme fraction, 0.25 to 0.6 mg of protein. The incubation mixture was placed in a dual microtube system and $^{14}CO_2$ released from this incubation mixture was absorbed onto Hyamine hydroxide-soaked Whatman 3MM paper and quantitatively determined in a toluene scintillation system as described recently (28).

observed with GM3-SialylT in the neuroblastoma clones grown in culture compared to the C-1300 tumors. Moreover, the product, GD3, is probably the predominant ganglioside in these clonal lines, instead of GM2, as shown by Yogeeswaran et al. (19) using N2A, NA, and NB41A neuroblastoma clones. The activity of GM3-SialylT in the cholinergic clone (NS20-T) was relatively low compared to the adrenergic cells (N1E-T and N1E-S).

A significant difference was observed between these two cell lines (N1E-T and NS20-T) when tested for inhibitory neurotransmitter (γ-amino-butyric acid) synthesis (Table III), with the cholinergic clone(NS20-T) having the higher content of this enzyme. An enhanced activity of glutamate decarboxylase was observed in extracts of the adrenergic cells (N1E-SB1) grown in the presence of 1.0 mM $(But)_2$cAMP for 24 hours. Further studies on the possible induction of this enzyme and the glycosphingolipid glycosyltransferases in the cholinergic clonal lines and their relation to neuroblastoma cell differentiation are under way.

ACKNOWLEDGMENTS: We would like to thank Dr. Shraga Makover of Hoffmann La Roche, Inc., for neuroblastoma clonal cell lines N1E-115 and NS-20, and Dr. Y.-T. Li of Tulane University for pure fig α-galactosidase as gift sample.

REFERENCES

1. Hakomori, S., Teather, C., and Andrews, H. (1968) Biochem. Biophys. Res. Commun. 33, 563-567.
2. More, P. T., Brady, R. O., Bradley, R. M., and McFarland, V. W. (1969) Biochemistry 63, 1290-1296
3. Makita, A., and Seyama, Y. (1971) Biochim. Biophys. Acta, 241, 403-411.
4. Yogeeswaran, G., Sheinin, R., Wherrett, J. R., and Murray, R. K. (1972) J. Biol. Chem. 247, 5146
5. Ghamburg C. G., and Hakomori, S. (1973) Proc. Nat. Acad. Sci. USA 70, 3329-3333.
6. Glick, M. C. (1974) Biology and Chemistry of Eucaryotic Cell Surfaces, pp. 213-240, Academic Press, New York.
7. Sakiyama, H., and Robbins, P. W. (1973) Arch. Biochem. Biophys. 154, 407-414.
8. Yeung, K-K., Moskal, J. R., Chien, J-L., Gardner, D. A., and Basu, S. (1974) Biochem. Biophys. Res. Commun. 59, 252-260.
9. Fishman, P. H., Simmons, J. L., Brady, R. O., and Freese, E. (1974) Biochem Biophys. Res. Commun. 59, 292-299.
10. Hakomori, S. (1970) Proc. Nat. Acad. Sci. USA 67, 1741-1747.
11. Robbins, P. W., and Macpherson, I. A. (1971) Proc. R. Soc. Lond. Ser. B Biol. Sci. 177, 49-58.
12. Chatterjee, S., Sweeley, C. C., and Velicer, L. F. (1973) Biochem. Biophys. Res. Commun. 54, 585-592.
13. Augusti-Tocco, G., and Sato, G. (1969) Proc. Nat. Acad. Sci. USA 64, 311-315.
14. Nelson, P., Ruffner, W., and Nirenberg, M. (1969) Proc. Nat. Acad. Sci. USA 64, 1004-1010.
15. Kates, J. R., Winterton, R., and Schlessinger, K. (1971) Nature (London) 229, 345.
16. Simantov, R., and Sachs, L. (1972) Eur. J. Biochem. 30, 123-129.
17. Waymire, J. C., Weiner, N., and Prasad, K. N. (1972) Proc. Nat. Acad. Sci. USA 69, 2241-2245.

18. Dawson, G., Kemp, S. F., Stoolmiller, A. C., and Dorfman, A. (1971) Biochem. Biophys. Res. Commun. 44, 687-694.
19. Yogeeswaran, G., Murray, R. K., Pearson, M. L., Sanwal, B. D., McMorris, F. A., and Ruddle, F. H. (1973) J. Biol. Chem. 248, 1231-1239.
20. Stoolmiller, A. C., Dawson, G., and Kemp, S. F. (1974) Fed. Proc. 33, 126.
21. Amano, T., Richelson, E., and Nirenberg, M. (1972) Proc. Nat. Acad. Sci. USA 69, 258-263.
22. Kean, E. L., and Roseman, S. (1966) J. Biol. Chem. 241, 5643-5650.
23. Chien, J-L., Williams, T., and Basu, S. (1973) J. Biol. Chem. 248, 1778-1785.
24. Basu, M., Chien, J-L., and Basu, S. (1974) Biochem. Biophys. Res. Commun. in press.
25. Basu, M., and Basu, S. (1973) J. Biol. Chem. 248, 1700-1706.
26. Li, Y-T., and Li, S-C. (1973) Methods Enzymol., 28, 714.
27. Sung, S-J., Esselman, W. J., and Sweeley, C. C. (1973) J. Biol. Chem. 248, 6528-6533.
28. Moskal, J. R., and Basu, S. (1974) Anal. Biochem. in press.
29. Roseman, S. (1974) Biology and Chemistry of Eucaryotic Cell Surfaces. pp. 317-354, Academic Press, New York.
30. Basu, S. (1966) Ph.D thesis, University of Michigan, Ann Arbor.
31. Kaufman, B., Basu, S., and Roseman, S. (1968) J. Biol. Chem. 243, 5804-5806.
32. Diceasare. J. L., and Dain, J. A. (1972) J. Neurochem. 19, 403-410.
33. Basu, S., Schultz, A. M., Basu, M., and Roseman, S. (1971) J. Biol. Chem. 246, 4272-4279.
34. Basu, M., and Basu, S. (1972) J. Biol. Chem. 247, 1489-1495.
35. Basu, S., Kaufman, B., and Roseman, S. (1968) J. Biol. Chem. 243, 5802-5804.
36. Stoffyn, P., Stoffyn, A., and Hauser, G. (1973) J. Biol. Chem. 248, 1920-1923.
37. Laine, R., Sweeley, C. C., Li, Y-T., Kiscic, A., and Rapport, M. M. (1972) J. Lipid Res. 13, 519.