BIOSYNTHESIS OF GLYCOSPHINGOLIPIDS BY MOUSE NEUROBLASTOMA (NB41A), RAT GLIA (RGC-6) AND HUMAN GLIA (CHB-4) IN CELL CULTURE[+]

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Summary. A mouse neuroblastoma cell line was found to synthesize at least four of the gangliosides characteristic of nervous tissue, G_{M2}, G_{M1}, G_{D2} and G_{D1a}. Since hematoside (G_{M3}) is virtually absent from the neuroblastoma cell line, the data suggest that asialo-G_{M2} is an intermediate in the synthesis and catabolism of gangliosides. Glial tumor cell lines of both rat and human origin contained mainly G_{M3} (90% of the total glycolipid content) and negligible amounts of hexosamine-containing glycosphingolipids.

Glycosphingolipids are membrane-bound constituents of all eukaryotic cells. Specific changes in their composition occur when cells are transformed either chemically or virally. Most established cell lines examined contain hematoside (G_{M3}) as the major glycosphingolipid (1) and do not synthesize the hexosamine-containing gangliosides associated with nervous tissue. Human skin fibroblasts (2) synthesize all the glycosphingolipids found in parenchymal organs, GL-1a, GL-2a, GL-3a, GL-4a, G_{M3}, and G_{D3}, but little or no gangliosides[1]. Recently, Cumar et al. (3) found that some established cell lines, such as AL/N and Swiss mouse 3T3, synthesized G_{M2}, G_{M1}, and G_{D1a} gangliosides. Further, the synthesis of these compounds was greatly reduced following transformation of the cells with a DNA virus and there was an associated loss of contact inhibition. This finding has been attributed to a loss of G_{M3}-N-acetylgalactosaminyltransferase activity (3).

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[1]Dawson, G., Hof, L., and Matalon, R., unpublished observations

Benda et al. (4) have established cell lines from N-nitrosomethylurea induced rat glial tumors which synthesize both a protein characteristic of the nervous system, the "S-100 protein", and acid mucopolysaccharides (5). Further, cloned cell lines established from mouse neuroblastoma (6) exhibit a number of properties of neurons including electrical excitability. The availability of these cells prompted us to examine their ganglioside content since these glycosphingolipids are limited almost exclusively to neurons. It was discovered that the neuroblastoma cells synthesized some of the gangliosides characteristic of neurons but that the glial cells did not. Chemical evidence is presented which suggests that the synthesis of gangliosides proceeds via asialo-G_{M2} rather than G_{M3}.

Materials and Methods.

A mouse neuroblastoma C 1300 clonal strain NB41A (7), a clonal strain (C-6) of rat glial cells (4), and human glial cells (CHB-4) were the gifts of Dr. Gordon Sato. Cells were cultured in Eagle's medium supplemented with 10% fetal calf serum (North American Biologicals) as modified by Matalon and Dorfman (8) in Falcon tissue culture dishes (100 x 20 mm) at 37° in an atmosphere of 10% CO_2: 90% air, 100% humidity. Trypsinized suspensions of neuroblastoma cells were plated at 2-4 x 10^6 cells per dish and grown for 8 days with feedings at two-day intervals. The growth medium was aspirated from 100 plates and cells were removed by a rubber policeman after several rinses with buffered saline. An average of 2.5 x 10^7 cells (7 mg dry wt) was obtained per dish. Trypsinized suspensions of glial cells were plated at 1.5 x 10^6 cells per dish and grown for 5 days. Cell cultures were fed at 24-hour intervals for 3 days, then 12-hour intervals for 2 days. Cells were collected as described above; an average of 1.02 x 10^8 cells per dish (20 mg dry wt) was obtained from 30 dishes.

Lyophilized cells (300 mg dry wt) were extracted with chloroform-methanol (2:1) and water (0.2 vol) was added to the filtrate and washings. The upper phase was evaporated to dryness, subjected to a modified alkaline methanolysis (9) after which the entire reaction mixture was exhaustively dialyzed, and subjected to thin-layer chromatography (TLC) on silica gel. The gangliosides were resolved by two sequential developments on TLC with chloroform-methanol 2.5 N NH_4OH (60:40:9) (10).

The lower phase was concentrated and the glycosphingolipid fraction

isolated by silicic acid chromatography (11). After alkaline methanolysis (9) and exhaustive dialysis, the individual glycosphingolipids were separated by TLC on precoated silica gel plates (Quantum Industries) with chloroform-methanol-water (110:40:6).

Individual glycosphingolipids were visualized with iodine, scraped from the plates, and eluted with chloroform-methanol-water (100:42:6). After the addition of mannitol (0.02-0.1 μ-moles) the glycosphingolipids were subjected to methanolysis in 1.0 N HCl in dry methanol (11, 12). Following neutralization with solid silver carbonate (12) re-N-acetylation with acetic anhydride (12) and removal of fatty acid methyl esters with hexane (11), the methyl glycosides were evaporated to dryness and converted to their trimethylsilyl ethers (11, 12). An HP402 Gas Chromatograph equipped with a glass column (6 ft x 1/8 in i.d.) packed with 3% OV-1 on 100/120 mesh Supelcoport was used as described previously (11, 12). The instrument was programmed from 160° to 240° at 2° per min, enabling the identification and quantitation of galactose, glucose, N-acetylgalactosamine, and N-acetylneuraminic acid during a single analysis (Fig 1).

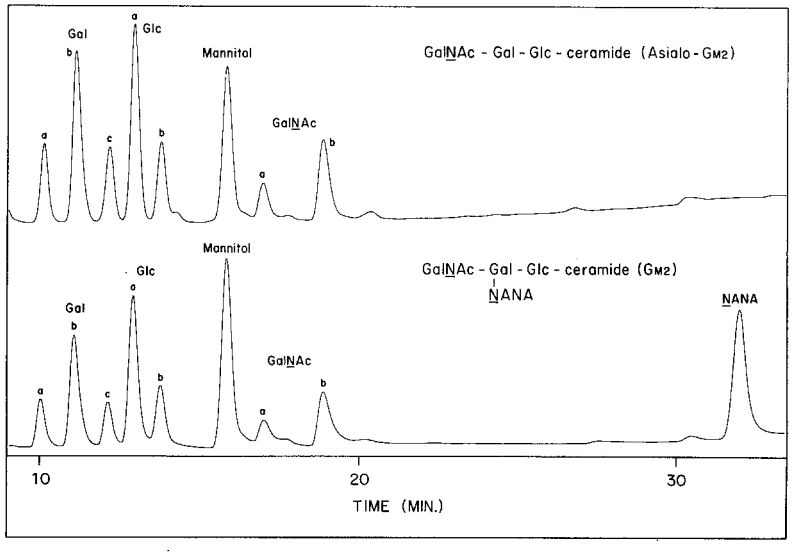

Figure 1. Gas-liquid chromatographic analysis of asialo-G_{M2} and G_{M2} as the trimethylsilyl ethers of the O-methyl glycosides. The column used was 3% OV-1 and the HP402 Gas Chromatograph was temperature-programmed at 2°/min from 160° to 240°. The peaks are identified as the ring forms of galactose, α/β-furanoside (a), α-pyranoside (b) and β-pyranoside (c); glucose, α-pyranoside (a) and β-pyranoside (b); N-acetylgalactosamine, β-pyranoside (a) and α-pyranoside (b); and N-acetylneuraminic acid. D-Mannitol was the internal standard.

Results and Discussion.

Thin-layer chromatography of the NB41A neutral glycosphingolipids showed four major fractions (Fig 2). These were identified by R_f value and gas-chromatography as GL-1a, GL-2a, asialo-G_{M2}, and an aminoglycosphingolipid, which on the basis of analyses could be either GL-4a or asialo-G_{M1}. Neither G_{M3}, which is found in both the neutral glycosphingolipid and ganglioside fractions, or the galactosylgalactosylglucosylceramide (GL-3a), characteristic of human skin fibroblasts (2), were detected in significant amounts (Table 1). The glycosphingolipid composition of the rat and human glial cells was much simpler; only GL-1a, GL-2a, and G_{M3} were found (Table 1) the latter constituting 90% of the total glycosphingolipid content (Fig 2).

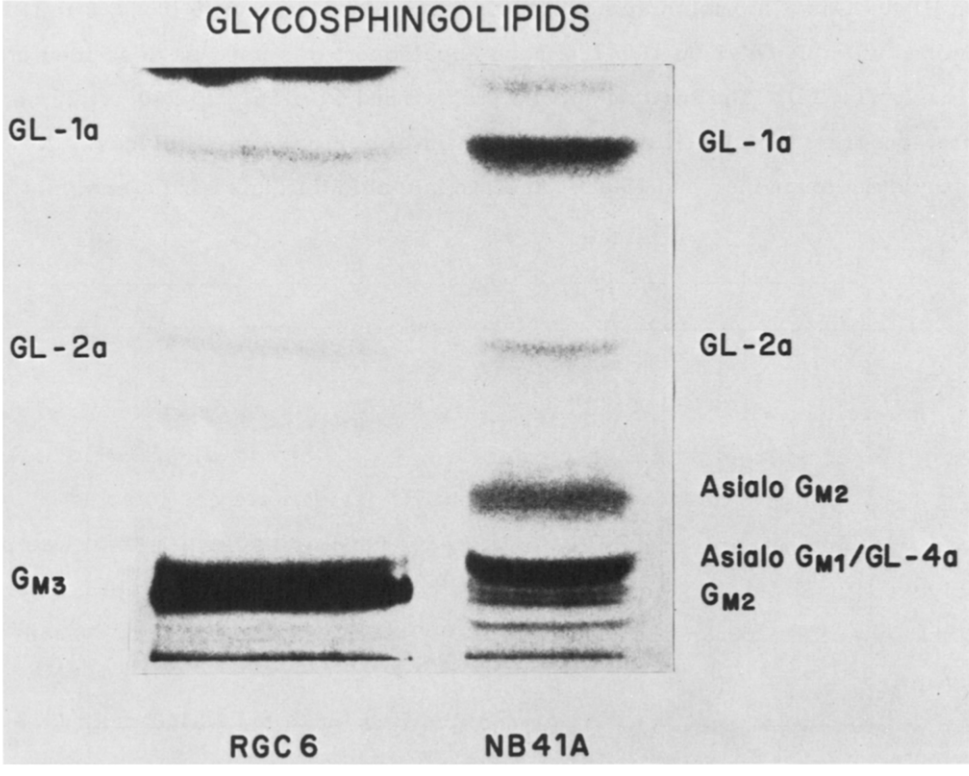

Figure 2. Thin-layer chromatographic separation of neutral glycosphingolipids from RGC-6 and mouse NB41A cell lines on pre-coated silica gel plates with chloroform-methanol-water (110:40:6).

Further marked differences between these cell lines were discovered upon examination of the ganglioside fraction. Human skin fibroblasts[1] and rat glial cells (Fig 3) contained only G_{M3} and a lesser amount of G_{D3}; neither G_{M3} nor

TABLE 1

Glycosphingolipid composition of mouse neuroblastoma (NB41A), rat glial (RGC-6) cell lines and normal human skin fibroblasts (NHSF).

Glycosphingolipid	μ-moles/g. dry wt. NB41A	RGC-6	NHSF
GL-1a	0.48	0.40	0.45
GL-2a	0.54	0.20	0.20
GL-3a	----	----	0.65
GL-4a	----	----	0.35
Asialo-G_{M2}	0.74	----	----
Asialo-G_{M1}	1.08	----	----
G_{M3}	----	2.15	1.09
G_{M2}	1.11	----	----
G_{M1}	0.28	----	----
G_{D3}	----	0.21	0.20
G_{D1a}/G_{D1b}	1.00	----	----
G_{D2}	0.11	----	----

G_{D3} were found in the NB41A cells (Table 1). The NB41A cells contained four major glycosphingolipids identified as G_{M2} (Fig 1) and its di-sialo form, G_{D2}; G_{M1}, G_{D1a} and probably G_{D1b}; trisialogangliosides were not detected. Material appearing in this region on thin-layer chromatography (Fig 3) was not glycosphingolipid in nature. These more complex gangliosides were not detected in either human skin fibroblasts or glial cells of both rat and human origin.

The total amount of glycosphingolipid present in the NB41A cells (5.3 μ-moles/g dry wt) (Table 1) was comparable to that found in rat and human neurons (13). Glial-rich fractions have been reported (13) to contain significant levels of gangliosides and neutral glycosphingolipids but such fractions probably remain contaminated with neurons, and it is not certain that normal glial cells synthesize gangliosides. The glial tumor cell lines studied here only synthesized G_{M3} (Table 1) and did not contain any of the glycosphingolipids associated with myelin.

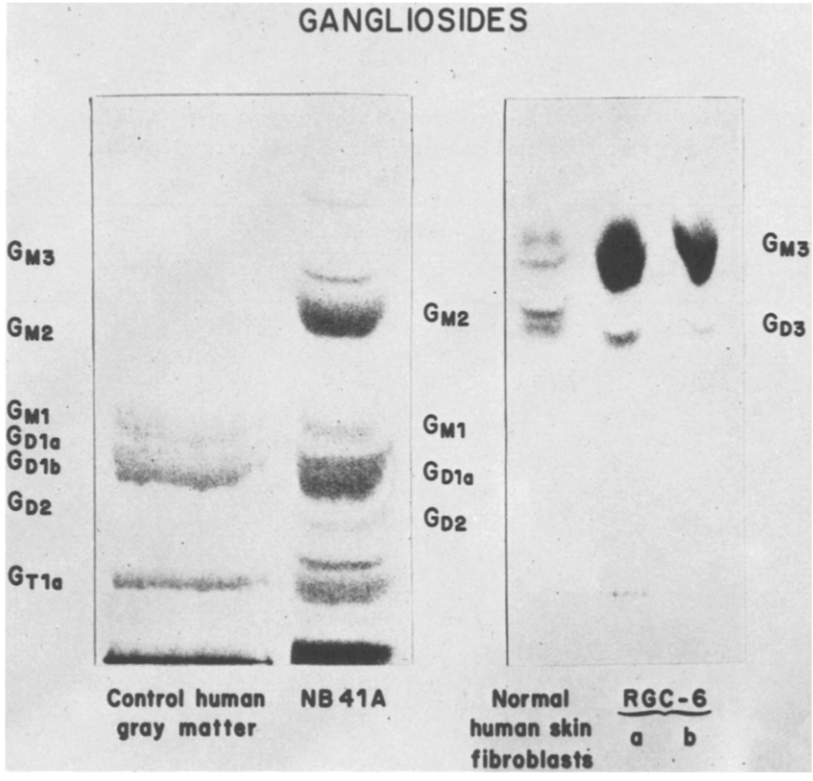

Figure 3. Thin-layer chromatographic separation of gangliosides isolated from RGC-6 (a, high cell density; b, low cell density) and mouse NB41A cell lines on pre-coated silica gel plates; two sequential developments in chloroform-methanol-2.5 N NH_4OH (60:40:9).

The pathway proposed by Kaufman et al. (14) for the biosynthesis of gangliosides in embryonic chick brain involves the conversion of GL-2a → G_{M3} → G_{M2} → G_{M1} → G_{D1a}; this has been confirmed in developing rat brain by Caputto et al. (15) who refuted proposals by Handa and Burton (16) that the actual pathway in rats was GL-2a → asialo-G_{M2} → G_{M2} → G_{M1} → G_{D1a}. A second pathway was proposed by Roseman (17) to account for the gangliosides containing a disialo unit; G_{M3} → G_{D3} → G_{D2} → G_{D1b} → G_{T1a}. Experimental evidence exists only for the first step (G_{M3} → G_{D3}) and Caputto et al. (15) have proposed recently that G_{M1} → G_{D1b} → G_{T1a}. Our analytical results indicate that the major ganglioside metabolic pathway in NB41A involves asialo-G_{M2} (Fig 4). Since there is no GL-3a in these cells, we believe that the "GL-4" is asialo-G_{M1}; work is in progress to confirm this structure by mass spectrometry (18) and possibly by other methods. The asialo-G_{M1} presumably

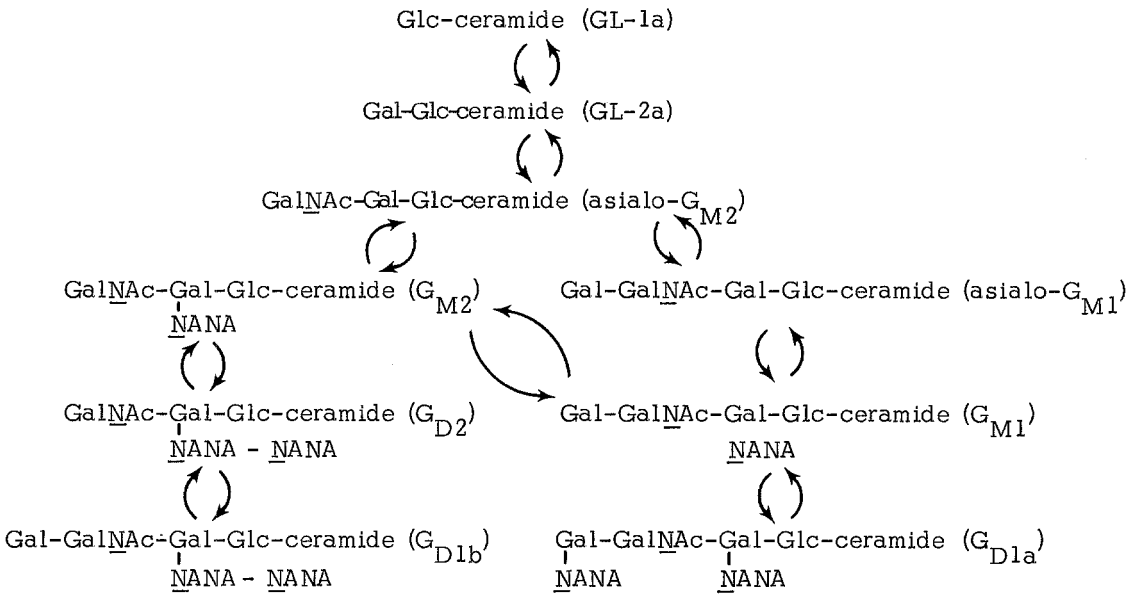

Figure 4. Proposed metabolic pathway to explain the glycosphingolipids found in mouse NB41A cells.

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arises by galactosylation of asialo-G_{M2}, a reaction demonstrated by Yip and Dain (19) in tadpole brain. G_{D1a} and G_{M1} can be synthesized from either asialo-G_{M1} or G_{M2}.

Because of the relative simplicity of the glycosphingolipid composition of NB41A cells it has been possible to demonstrate an interesting catabolic pathway involving $G_{D2} \rightarrow G_{M2} \rightarrow$ asialo-$G_{M2} \rightarrow$ GL-2a, all of which are trace constituents of normal brain and possibly $G_{D1a} \rightarrow G_{M1} \rightarrow$ asialo-$G_{M1} \rightarrow$ asialo-$G_{M2} \rightarrow$ GL-2a; G_{D1a} and G_{M1} are the major gangliosides in most vertebrate brains. For many years such a pathway has not been thought possible (20, 21) since commercial (and most brain) neuraminidase preparations exhibit no reactivity with respect to G_{M1} or G_{M2}; it has been assumed that the lack of reactivity resulted from steric hindrance by the N-acetylgalactosaminyl moiety. However, Kolodny et al. (21) have recently shown that a neuraminidase preparation from a number of mammalian tissues can hydrolyze sialic acid from G_{M2}. Our analytical data suggests that neuraminic acid can be cleaved from both G_{M1} and G_{M2} in this manner. The synthesis of gangliosides containing N-acetylgalactosamine, a characteristic of nervous tissue, is presumably of functional significance and clearly distinguishes the neuroblastoma cells

from the glial tumor cell lines, where the only gangliosides (G_{M3} and G_{D3}) are those associated with parenchymal cells. A detailed study of the biosynthetic and catabolic enzyme systems is now in progress since the absence of G_{M3} from these NB41A cells does not necessarily mean that it is not a rapidly metabolized biosynthetic or catabolic intermediate.

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References

1. Hakomori, S.-I., Teather, C., and Andrews, H., Biochem. Biophys. Res. Commun., 33, 563 (1968).
2. Matalon, R., Dorfman, A., Dawson, G., and Sweeley, C.C., Science, 164, 1522 (1969).
3. Cumar, F.A., Brady, R.O., Kolodny, E.H., McFarland, V.W., and Mora, P.T., Proc. Natl. Acad. Sci., U.S.A., 67, 757 (1970).
4. Benda, P., Lightbody, J., Sato, G., Levine, L., and Sweet, W., Science, 161, 370 (1968).
5. Dorfman, A., and Ho, P.-L., Proc. Natl. Acad. Sci., U.S.A., 66, 495 (1970).
6. Nelson, P., Ruffner, W., and Nirenberg, M., Proc. Natl. Acad. Sci., U.S.A., 64, 1004 (1969).
7. Augusti-Tocco, G., and Sato, G., Proc. Natl. Acad. Sci., U.S.A., 64, 311 (1969).
8. Matalon, R., and Dorfman, A., Proc. Natl. Acad. Sci., U.S.A., 56, 1310 (1966).
9. Dawson, R.M.C., Biochim. Biophys. Acta, 14, 374 (1954).
10. Ledeen, R., and Salsman, K., Biochemistry, 4, 2225 (1965).
11. Vance, D.E., and Sweeley, C.C., J. Lipid Res., 8, 621 (1967).
12. Clamp, J.R., Dawson, G., and Hough, L., Biochim. Biophys. Acta, 148, 342 (1967).
13. Norton, W.T., and Poduslo, S.E., J. Lipid Res., 12, 84 (1971).
14. Kaufman, B., Basu, S., and Roseman, S., In, Inborn Disorders of Sphingolipid Metabolism, Aronson, S.M., and Volk, B.W., Eds., Pergamon Press, Oxford, 1967, pp. 187-213.
15. Arce, A., Maccioni, H.J., and Caputto, R., Biochem. J., 121, 483 (1971).
16. Handa, S., and Burton, R.M., Lipids, 4, 589 (1969).
17. Roseman, S., Chem. Phys. Lipids, 5, 270 (1970).
18. Dawson, G., and Sweeley, C.C., J. Lipid Res., 12, 56 (1971).
19. Yip, M.C.M., and Dain, J.A., Lipids, 4, 270 (1969).
20. Svennerholm, L., J. Lipid Res., 5, 145 (1964).
21. Ledeen, R., J. Amer. Oil Chem. Soc., 43, 58 (1965).
22. Kolodny, E.H., Kanfer, J.N., Quirk, J.M., and Brady, R.O., J. Biol. Chem., 246, 14 (1971).