Biosynthesis and Localization of Gangliosides in Cultured Cells†

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ABSTRACT: Mouse neuroblastoma N18 cells contain a homologous series of gangliosides (G_{M3}, G_{M2}, G_{M1}, and G_{D1a}) which constitute a biosynthetic pathway. When added to the culture medium, tritium-labeled palmitate, galactose, and *N*-acetylmannosamine were incorporated into these gangliosides. Incorporation of [^{3}H]galactose into all four gangliosides was detected by 5 min and continued at essentially linear rates for several hours. When the cells were treated with *Vibrio cholerae* neuraminidase, the amounts of G_{M3} and G_{D1a} were reduced from 72% to 85%; there was a severalfold increase in G_{M1} and no change in G_{M2}. In spite of these large alterations in cellular ganglioside composition, there was no change in the rate of [^{3}H]galactose incorporation into the gangliosides. A large proportion of G_{M3} and G_{D1a} also was accessible to neuraminidase in neuroblastoma NB41A, Friend erythroleukemic, and rat glioma C6 cells. N18, NB41A, and Friend cells bound large amounts of ^{125}I-labeled cholera toxin with high affinity. At saturation, the ratio of G_{M1} content to toxin bound for the three cell lines was between 5.5 and 7. When treated with neuraminidase, the cells bound more toxin in correspondence to the increase in G_{M1} content. As each toxin molecule has five binding sites, these results suggest that most of the G_{M1} in these cells is on the surface. Our results indicate that the sequential glycosylation of one ganglioside to form the next higher homologue involves a very small pool of intermediates and that the bulk of the gangliosides are on the cell surface.

It is generally accepted that glycosylation of gangliosides involves the sequential transfer of monosaccharides from sugar nucleotides to the growing acceptor (Roseman, 1970; Fishman, 1974; Caputto et al., 1976). Each step appears to be catalyzed by a specific glycosyltransferase which exists as a multienzyme complex in the Golgi apparatus of the cell (Keenan et al., 1974; Pacuszka et al., 1978; Eppler et al., 1980). Based on the acceptor specificity of these glycosyltransferases, the major pathways for ganglioside biosynthesis have been elucidated [see Fishman & Brady (1976) and Caputto et al. (1976)]. When in vivo incorporation of labeled precursors into rat brain gangliosides was examined, Caputto et al. (1976) did not find the expected precursor/product relationships between different gangliosides. In fact, more complex gangliosides became labeled more rapidly than the simpler ones. In order to rationalize their data with the accepted biosynthetic pathways, they proposed that gangliosides were synthesized from a very small pool of intermediates which was separate from the main pool of cellular gangliosides and that each of the different types of gangliosides comprising the main pool was synthesized by its own multienzyme complex. Since brain consists of numerous cell types, each with its own pattern of gangliosides [see Duffard et al. (1977)] and possibly different biosynthetic rates, interpretation of in vivo studies may be very difficult. Mouse neuroblastoma N18 cells contain a homologous series of gangliosides corresponding to the major gangliosides of brain and possess the specific glycosyltransferases that represent this biosynthetic pathway (Duffard et al., 1977). We decided to examine the in vivo model proposed by Caputto et al. (1976) by using this cell line. We also decided to determine the localization of gangliosides in cultured cells. Although it is generally accepted that gangliosides are plasma membrane components (Fishman & Brady, 1976), several studies have indicated that gangliosides are also present intracellularly (Klenk & Choppin, 1970; Weinstein et al., 1970; Yogeeswaran et al., 1972; Keenan et al., 1972; Kanfer et al., 1976).

Experimental Procedures

Materials. D-[1-^{3}H]Galactose (2.8 Ci/mmol) was purchased from ICN (Irvine, CA). *N*-[G-^{3}H]Acetyl-D-mannosamine (4.65 Ci/mmol), NaB^3H_4 (5–7.7 Ci/mmol), and EN^{3}HANCE spray were obtained from New England Nuclear. [9,10(N)-$^3H_2$]Palmitic acid (590 mCi/mmol) was from Amersham Corp. Choleragen from Schwarz/Mann was iodinated as described by Cuatrecasas (1973). *Vibrio cholerae* neuraminidase (EC 3.2.1.18) was obtained from Calbiochem Behring Corp., galactose oxidase (EC 1.1.3.9) from Worthington Biochemical Corp., and Ready-Solv HP from Beckman.

Cells and Cell Culture. Mouse neuroblastoma N18 and NB41A cells were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum and 50 μg/mL gentamicin (Duffard et al., 1977). Rat glioma C6 and Friend erythroleukemic cells were cultured as described previously (Fishman & Atikkan, 1979). Cells routinely were used 5 days after subculturing and 2 days after a medium change. Cells were incubated with neuraminidase in either Dulbecco's phosphate-buffered saline containing Ca^{2+} and Mg^{2+} or serum-free Eagle's medium buffered with 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid at 37 °C.

Isolation and Analysis of Gangliosides. Cells were washed 3 times with ice-cold PBS,[1] detached from the culture vessels with a rubber policeman in PBS (except Friend cells), and collected by centrifugation. The washed cell pellet was dispersed in 2 mL of chloroform/methanol (1:2 v/v) by sonification and extracted at 37 °C for 30 min. After centrifugation, the residue was extracted as above and washed with 1 mL of the solvent. The combined extracts were taken to dryness under a stream of nitrogen. Gangliosides were purified from

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[1] Abbreviations: ganglioside nomenclature according to Svennerholm (1964); PBS, Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+} (Dulbecco & Vogt, 1954); $NaDodSO_4$, sodium dodecyl sulfate; Gal, galactose; ManNAc, *N*-acetylmannosamine; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

Table I: Incorporation of Labeled Precursors into Neuroblastoma N18 Cells[a]

precursor	^{3}H added (μCi)	^{3}H incorporated (μCi)	
		total	gangliosides
palmitate	219	177	12.0
Gal	103	10.0	2.12
ManNAc	145	1.68	0.674

[a] Confluent 75-cm^2 flasks of N18 cells were incubated with 10 mL of complete medium containing the indicated labeled precursor for 48 h at 37 °C. After the cells were washed and harvested, gangliosides were isolated as described under Experimental Procedures. Each flask contained 5 mg of cell protein (13.4 × 10^6 cells).

Table II: Incorporation of Labeled Precursors into Gangliosides of Neuroblastoma N18 Cells[a]

precursor	sp radioact. of gangliosides [(dpm/nmol) × 10^{-3}]			
	G_{M3}	G_{M2}	G_{M1}	G_{D1a}
palmitate	166	149	136	154
Gal	82	53	109	129
ManNAc	22	22	15	33

[a] Gangliosides were isolated from N18 cells labeled as described in Table I and separated by thin-layer chromatography. The labeled gangliosides were either quantified for sialic acid or detected by fluorography, scraped from the chromatograms, and counted as described under Experimental Procedures. Each preparation was analyzed in triplicate, and standard deviations (SD) of the means were less than 10%.

the crude lipid fraction by Sephadex G25, DEAE-Sephadex, and Unisil column chromatography and alkaline hydrolysis (Fishman et al., 1979b). The purified gangliosides were separated by thin-layer chromatography on silica gel 60, visualized by resorcinol spray, and quantified by scanning spectrodensitometry (Fishman et al., 1979b). Tritium-labeled gangliosides were detected by fluorography; the chromatograms were sprayed with EN^{3}HANCE and exposed to Kodak X-Omat AR film at −70 °C. The areas of the chromatograms corresponding to labeled gangliosides were scraped and transferred to scintillation vials. After 1 mL of water was added and the mixture was allowed to stand overnight, 10 mL of Ready-Solv HP was added to each vial, which was then counted on a Searle Mark III liquid scintillation counter.

Binding of Choleragen. Assay of choleragen receptors on intact cells was essentially as described previously (Fishman & Atikkan, 1979, 1980). Cells in suspension were incubated with 10 nM ^{125}I-labeled choleragen in 0.2 mL of medium containing 25 mM Tris-HCl (pH 7.4), 128 mM NaCl, 1 mM EDTA, 3 mM NaN_3, and 0.1% bovine serum albumin at 25 °C for 45 min. The samples were then filtered and the filters counted in a Beckman γ 4000. Nonspecific binding was determined by including 0.2 μM unlabeled toxin in the incubation. For each experiment, binding was determined in triplicate at two different concentrations of cells. In addition, the proportion of ^{125}I-labeled choleragen that bound to an excess of cells also was determined. This value which varied from 65 to 80% was used to calculate the specific radioactivity of the toxin in the assay. The concentration of stock solutions of choleragen was determined from the published extinction coefficient, $E^{1\%,1cm}_{280nm}$, of 11.41 (Finkelstein, 1973).

Other Methods. Cells were surface labeled by using galactose oxidase or $NaIO_4$ followed by reduction with NaB^3H_4 essentially as described by Moss et al. (1977). Choleragen/receptor complexes were extracted with NP-40 and immunoadsorbed with anticholeragen antiserum and fixed *Staphylococcus aureus* as described by Critchley et al. (1981). Protein was determined by the method of Lowry et al. (1951). Distribution of radioactivity into sugar residues of gangliosides was determined by acid hydrolysis and thin-layer chromatography (Fishman et al., 1979a) except Ready-Solv HP was used for counting and the counting efficiency was 29%.

Results

Biosynthesis of Gangliosides in N18 Cells. When N18 cells were incubated for 48 h in medium containing labeled precursors, the cells took up the label and incorporated it into gangliosides (Table I). Whereas most of the added palmitate was taken up by the cells, only a small fraction of Gal and even less of ManNAc were taken up. The same order of incorporation into the ganglioside fraction was observed, but the differences were less. Distribution of radioactivity into sugars and lipids also was determined. Following neuraminidase treatment of the labeled gangliosides and thin-layer chromatography, new bands corresponding to sialic acid and lactosylceramide were detected. With ManNAC as precursor, 35% of the label appeared as sialic acid compared to 37% that theoretically could be released from G_{D1a} and G_{M3}; no label was detected in lactosylceramide. With the other two precursors, less than 1% of the total radioactivity appeared as sialic acid, and the amount of labeled lactosylceramide exactly corresponded to the amount of labeled G_{M3}. Following acid hydrolysis, 4% of the total ^{3}H from gangliosides labeled with ManNAc appeared as galactosamine; with Gal as precursor, 75% was recovered as Gal, 20% as glucose (Glc), and 4.5% as galactosamine; with palmitate as precursor, no labeled sugars were detected.

The specific labeling of different gangliosides was determined (Table II). As expected, the specific radioactivity of the four gangliosides from palmitate-labeled cells was similar. With ManNAc as precursor, the monosialogangliosides were labeled to a similar extent whereas G_{D1a} had a higher specific activity. Incorporation of Gal into G_{M1} and G_{D1a}, which have two Gal residues, was greater than into G_{M2} and G_{M3}. A fluorograph of the labeled gangliosides separated by thin-layer chromatography showed that only gangliosides were labeled by Gal and ManNAc; with palmitate, however, several additional minor labeled components also were present in the ganglioside fraction.

The kinetics of ganglioside biosynthesis were determined with Gal as precursor. Incorporation into all of the gangliosides was detected within 5 min of exposing the cells to the labeled medium (not shown). Incorporation was essentially linear with time for up to 4 h (Figure 1A). During the first hour, specific labeling of different gangliosides occurred at a constant rate, and there was no evidence of precursor/product relationships (Figure 1B). Similar experiments were attempted with the other two precursors. There was insufficient incorporation of ManNAc at the early times in order to obtain significant results. With palmitate, G_{M2} had the highest specific radioactivity at the early time points. This suggested the possibility of the presence of labeled contaminants in the G_{M2} fractions (see above).

Binding of Choleragen to Intact Cells. When N18 cells were incubated with increasing concentrations of ^{125}I-labeled choleragen, saturation was reached by 2 nM and half-saturation by 0.3 nM (Figure 2). Similar results were obtained with Friend erythroleukemic cells and neuroblastoma NB41A cells (Figure 2). We also determined the amount of G_{M1} in these cells and compared it to the amount of toxin bound at saturation (Table III). Although the absolute values varied among the cell lines,[2] the ratio of G_{M1} to toxin only ranged

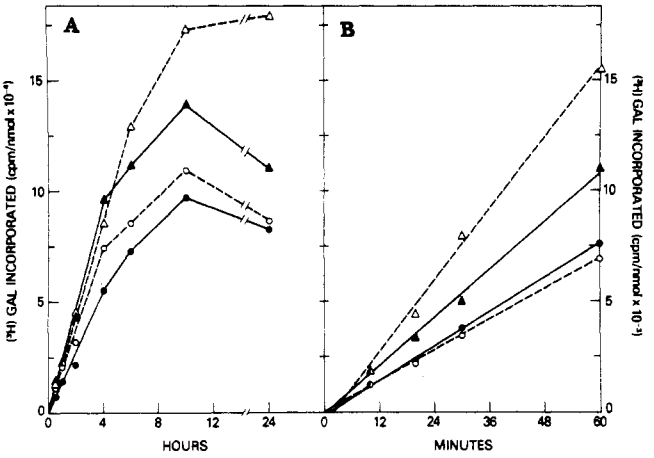

FIGURE 1: Kinetics of [^{3}H]Gal incorporation into gangliosides of neuroblastoma N18 cells. Cells were incubated in medium containing 150 (A) and 63.5 (B) μCi/mL [^{3}H]Gal for the indicated times. Gangliosides were isolated, quantified, and assayed for ^{3}H as described under Experimental Procedures. G_{D1a} (△); G_{M1} (▲); G_{M2} (○); G_{M3} (●).

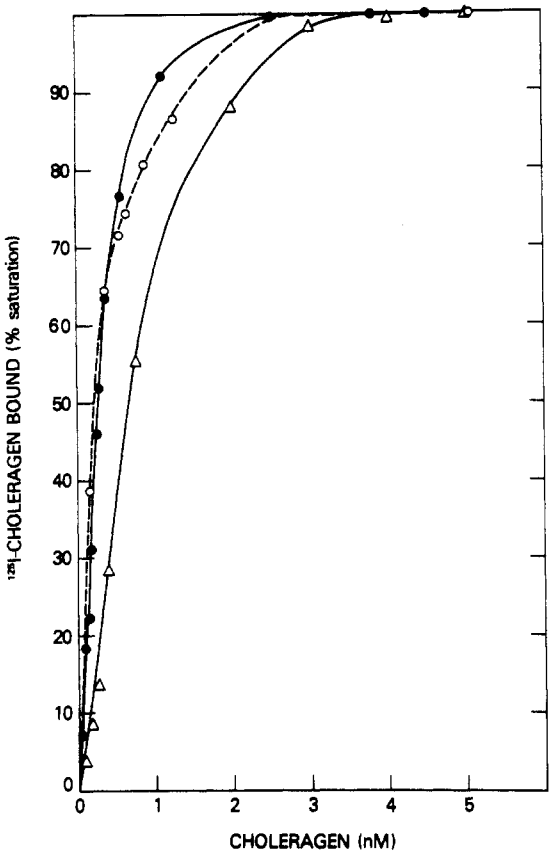

FIGURE 2: Binding of ^{125}I-labeled choleragen to intact cells as a function of choleragen concentration. Neuroblastoma N18 (●) and NB41A (△) cells and Friend erythroleukemic cells (○) were incubated with increasing concentrations of ^{125}I-labeled choleragen in the presence and absence of 0.2 μM unlabeled toxin and assayed for specifically bound choleragen as described under Experimental Procedures.

between 5.5 and 7.

In order to confirm that choleragen was binding to G_{M1} on the cell surface, we surface labeled N18 cells in the presence

[2] The ganglioside composition of different cultures of N18 and NB41A cells varied considerably. Effects of culture conditions, serum, and passage number on ganglioside composition have been observed previously (Hollenberg et al., 1974; Kemp & Stoolmiller, 1976). We found that the longer the cells were serially cultured the greater were the changes and that NB41A cells varied more than N18 cells.

Table III: Choleragen Receptors and G_{M1} Content of Different Cell Lines[a]

cell line	pmol/10^6 cells: G_{M1} content	pmol/10^6 cells: toxin receptors	ratio
neuroblastoma N18	89.2	13.5	6.6
	106	15.9	6.7
	138	23.5	5.9
	137	21.0	6.5
			mean: 6.44 ± 0.36
neuroblastoma NB41A	16.1	2.49	6.4
	11.0	2.05	5.4
	3.38	0.62	5.4
	3.84	0.55	7.0
			mean: 6.05 ± 0.79
Friend erythroleukemic	28.5 ± 1.29	4.96 ± 0.13	5.75 ± 0.30

[a] Cells were assayed for specific ^{125}I-labeled choleragen binding and G_{M1} content as described under Experimental Procedures. For neuroblastoma cells, each value represents results from a separate culture. For Friend cells, three separate batches of cells from the same culture were analyzed.

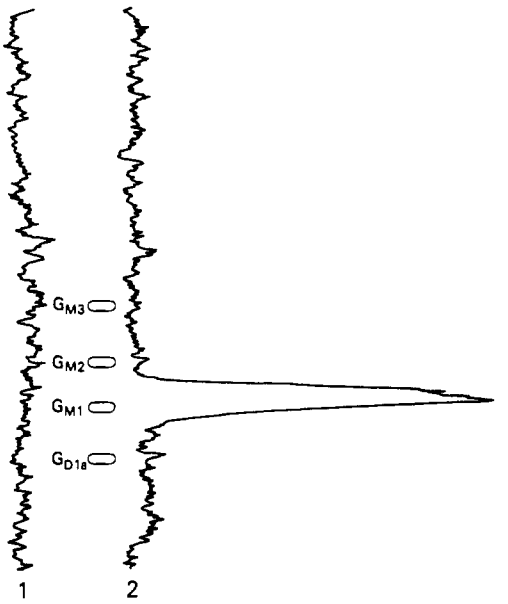

FIGURE 3: Effect of choleragen on surface labeling of neuroblastoma N18 gangliosides by galactose oxidase and NaB^3H_4. Cells were incubated in 2 mL of serum-free medium for 30 min at 37 °C in the presence (scan 1) and absence (scan 2) of 50 nM choleragen and then 60 min in the presence of 100 units of galactose oxidase. The cells were washed and incubated for 30 min in 2 mL of PBS containing 2 mCi of NaB^3H_4 (5 Ci/mmol). After the cells were washed, gangliosides were isolated, separated by thin-layer chromatography, and detected by radioscanning. In the absence of galactose oxidase, the radioscan was the same as scan 1.

and absence of choleragen (Figure 3). When intact cells were treated with galactose oxidase followed by NaB^3H_4, the only ganglioside labeled was G_{M1}; labeling of G_{M1} was completely prevented by first exposing the cells to the toxin.

We also used immunoadsorption to analyze choleragen receptors in these cells (Critchley et al., 1979, 1981). N18 cells were labeled with [^{3}H]Gal, homogenized, and incubated with and without excess choleragen. After extraction with NP-40, the soluble extracts were incubated with anticholeragen antiserum followed by fixed *S. aureus*. The immune precipitates then were analyzed by thin-layer chromatography (Figure 4A) or $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 4B). Although 95% of the radioactivity was extracted by the detergent, only a few percent was recovered in the

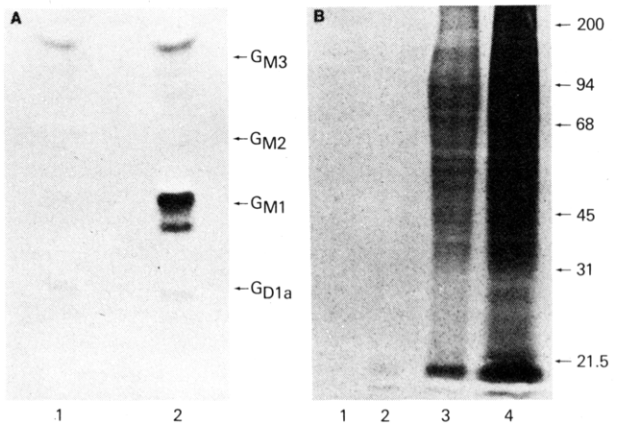

FIGURE 4: Immunoadsorption of choleragen receptors from neuroblastoma N18 cells labeled with [^{3}H]Gal. Cells were labeled with [^{3}H]Gal as described in Table I and homogenized. Portions (470 μg of protein) containing 1 μCi of ^{3}H were incubated with and without 7 μg of choleragen for 1 h at 0 °C. After NP-40 was added to 1%, the samples were sonicated, extracted for 4 h at 0 °C, and centrifuged at 10000*g* for 5 min. The extracts then were incubated with anticholeragen antiserum followed by fixed *S. aureus* (Critchley et al., 1981). The immunoprecipitates were analyzed by thin-layer chromatography (A) (lane 1, minus toxin; lane 2, plus toxin) or Na-DodSO$_4$–polyacrylamide gel electrophoresis (B) (lane 1, minus toxin; lane 2, plus toxin; lanes 3 and 4, 38 and 150 μg of cell protein).

Table IV: Effect of Neuraminidase Treatment on Cellular Gangliosides[a]

cell line	neuraminidase	pmol/mg of protein G_{M3}	G_{M2}	G_{M1}	G_{D1a}
neuroblastoma N18	−	829	1080	683	2200
	+	225	1050	2400	511
neuroblastoma NB41A	−	1040	1140	42	714
	+	127	1090	432	165
Friend erythroleukemic[b]	−			93	570
	+			454	135
rat glioma C6[c]	−	6590			
	+	1405			

[a] Cells were incubated in the presence and absence of neuraminidase (10 units/mL) for 2 h and analyzed for gangliosides as described under Experimental Procedures. [b] No G_{M3} or G_{M2} was detected chemically, but small amounts were detected by the $NaIO_4/NaB^3H_4$ method. [c] No G_{M2}, G_{M1}, or G_{D1a} was detected chemically or by the $NaIO_4/NaB^3H_4$ method.

immune precipitates. Of the numerous glycoproteins labeled by [^{3}H]Gal, none were immunoadsorbed. In contrast, labeled G_{M1} was specifically absorbed from the toxin-treated cells; in addition, there were small and similar amounts of other labeled gangliosides in both the control and choleragen-treated samples. Of the total label incorporated by the cells, 2.61% was associated with G_{M1}. With the use of this value and the recovery of known amounts of [^{3}H]G_{M1} carried through the immunoadsorption procedure (75%), all of the label specifically adsorbed could be accounted for as G_{M1}. We repeated the experiment except the intact cells were incubated with and without choleragen and washed before detergent extraction. Identical results were obtained. In addition, no labeled G_{M1} or glycoproteins were recovered from toxin-treated cells incubated with *S. aureus* in the absence of anticholeragen antibodies.

Effect of Neuraminidase on Gangliosides. When intact cells were incubated with neuraminidase, there was a substantial change in the ganglioside composition (Table IV). The amount of G_{M3} in N18, NB41A, and rat glioma C6 cells was reduced by 73–88%, and the amount of G_{D1a} in NI8, NB41A, and Friend cells was reduced by similar amounts. In addition,

Table V: Choleragen Receptors and G_{M1} Content of Cells Treated with Neuraminidase[a]

cell line	pmol/10^6 cells G_{M1} content	choleragen receptors	ratio
N18	565	96.1	5.9
NB41A	74.2	13.5	5.5
Friend	122	19.4	6.3

[a] Cells were incubated with neuraminidase for 2 h and assayed for specific ^{125}I-labeled choleragen binding and G_{M1} content as described under Experimental Procedures.

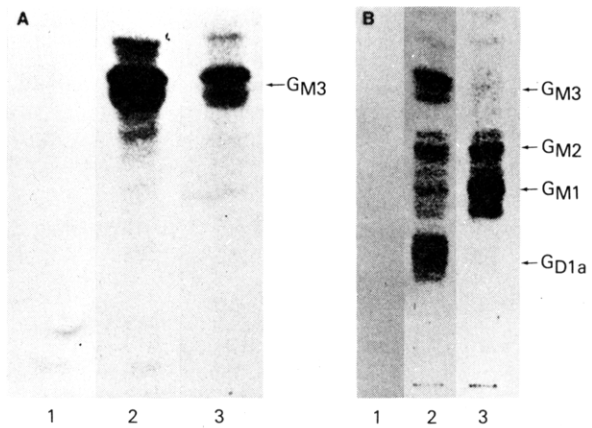

FIGURE 5: Surface labeling of the cellular gangliosides by $NaIO_4$ and NaB^3H_4. Cells were incubated for 2 h in the absence and presence of neuraminidase, washed, and incubated for 30 min at 0 °C in PBS with and without 2 mM $NaIO_4$. The cells were washed and incubated in PBS containing 1 mCi of NaB^3H_4 (7.7 Ci/mmol) for 30 min at 25 °C. After the cells were washed, gangliosides were isolated, separated by thin-layer chromatography, and detected by fluorography as described under Experimental Procedures. (A) Rat glioma C6 cells; (B) neuroblastoma N18 cells. Lanes 1, control cells minus $NaIO_4$; lanes 2, control cells plus $NaIO_4$; lanes 3, neuraminidase-treated cells plus $NaIO_4$.

the amount of G_{M1} increased in the latter three cell lines. When we assayed the neuraminidase-treated cells for choleragen binding, there was a corresponding increase (Table V). Maximum effects appeared to occur under the conditions chosen (2 h with 10 units/mL) as similar changes in gangliosides and toxin binding were observed at shorter times or different concentrations of enzyme. Thus, when NB cells were incubated with 0–15 units/mL for up to 2 h, the maximum increase in toxin binding was observed at 15 min and was the same as that observed with 2.5 units/mL at 2 h. At 1 h, degradation of G_{D1a} was maximum with 10 units/mL neuraminidase. Similar results were obtained with N18, C6, and Friend cells. Ganglioside degradation reached a maximum limit at 2 h with 10 units/mL enzyme, and no further hydrolysis was observed even with 50 units/mL neuraminidase.

When intact cells were oxidized with $NaIO_4$ and reduced with NaB^3H_4, all of the gangliosides became labeled (Figure 5, lanes 2). In the absence of $NaIO_4$, there was no incorporation of ^{3}H into gangliosides (Figure 5, lanes 1). Following exposure of the cells to neuraminidase, there was a significant reduction in the labeling of G_{D1a} and G_{M3} and an increase in that of G_{M1} (Figure 5, lanes 3).[3]

[3] Following neuraminidase treatment, labeling of G_{D1a} in N18 cells by the $NaIO_4/NaB^3H_4$ procedure was reduced 96% compared to a 77% reduction in G_{D1a} content (Table IV). For G_{M3} in C6 cells, the corresponding reductions were 86 and 79%. Thus, a small amount of G_{M3} may remain on the cell surface after enzyme treatment.

Table VI: Effect of Neuraminidase Treatment on Ganglioside Biosynthesis in Neuroblastoma N18 Cells[a]

ganglioside	incorporation of ^3H (cpm/mg of protein)	
	control cells	neuraminidase-treated cells
G_{M3}	882	795
G_{M2}	956	864
G_{M1}	974	1170
G_{D1a}	1670	1770

[a] Cells were incubated in the presence and absence of neuraminidase for 2 h, washed, and incubated for 1 h in complete medium containing 10 μCi/mL [^{3}H]Gal. Gangliosides were isolated and analyzed for ^3H as described under Experimental Procedures.

We then determined the effect of neuraminidase treatment on the incorporation of [^{3}H]Gal into gangliosides of N18 cells (Table VI). Although the ganglioside composition was drastically altered by neuraminidase, incorporation of label into the four gangliosides during a 1-h pulse was not changed. Similar results were obtained during 2- and 4-h labeling times (data not shown).

Discussion

Consistent with an earlier study (Duffard et al., 1977), mouse neuroblastoma N18 cells contain large amounts of gangliosides G_{M3}, G_{M2}, G_{M1}, and G_{D1a}, which they are able to synthesize de novo from radioactive precursors added to the culture medium. Of those examined, galactose appeared to be the best in terms of rate of uptake and lack of contamination of the purified gangliosides. Based on the kinetics of [^{3}H]Gal incorporation into the different gangliosides, there was no evidence of precursor/product relationships. All of the gangliosides became labeled at essentially linear rates and at specific radioactivities consistent with their carbohydrate structure. Thus, G_{M1} and G_{D1a} with two Gal residues became more highly labeled than G_{M3} and G_{M2}. These results indicate that both Gal residues are being incorporated essentially at the same time. Our results, therefore, are consistent with the model proposed by Caputto et al. (1976). Gangliosides are synthesized from a very small pool of intermediates which does not mix with the major pool of cellular gangliosides.

Kemp & Stoolmiller (1976) examined the biosynthesis of monosialogangliosides in NB41A cells. Using [^{14}C]Gal as a precursor, their results were similar to ours. With [^{3}H]-ManNAc, however, they observed some precursor/product relationships between G_{M3} and G_{M2} and G_{M2} and G_{M1}. Thus, G_{M3} was labeled at essentially a linear rate whereas there was a 10-min delay before G_{M2} and G_{M1} became labeled. Although we are unable to explain these different results, we did find that ManNAc was not an effective precursor. In this regard, the specific radioactivities reported by Kemp & Stoolmiller (1976) were less than 40 cpm/nmol at 10 min. In addition, if there was a 10-min lag between the G_{M3} and G_{M2} synthesis, there should be a corresponding delay between the appearance of the two newly synthesized gangliosides on the cell surface. We found, however, that the transport time from site of synthesis to plasma membrane was very similar for different gangliosides (Miller-Podraza & Fishman, 1982). The linear rates of synthesis and appearance on the cell surface of different gangliosides indicate that once glycosylation is initiated it is completed very rapidly (relative to our sampling time).

Our results indicate that the major pool of cellular gangliosides is on the cell surface. When the cells were incubated with neuraminidase, the levels of G_{M3} and G_{D1a} decreased substantially. Others have also observed similar effects, but no quantitative data were provided (Yogeeswaran et al., 1972; Critchley & Vickers, 1977). We found that between 12 and 27% of the G_{M3} and 23% of the G_{D1a} were resistant to enzyme attack. In addition, the G_{D1a} and G_{M3} that were resistant to the enzyme were less accessible to surface labeling by $NaIO_4$ and NaB^3H_4.

Choleragen also was used as a probe for surface G_{M1}, the toxin receptor (Fishman, 1980). We were able to show by means of surface labeling and immunoadsorption techniques that choleragen binds specifically to G_{M1} on N18 cells. At saturation, the mean ratios of G_{M1} content to toxin bound for these cells as well as NB41A and Friend erythroleukemic cells were between 5.75 and 6.44. After treatment with neuraminidase, the content of G_{M1} and toxin receptors increased proportionally, and the ratios remained in the same range. It is known that the binding component of choleragen is composed of five identical polypeptide chains (Gill, 1976; Kurosky et al., 1977; Lai, 1977) and that each toxin can bind to five G_{M1} oligosaccharide chains (Fishman et al., 1978). Thus, if each choleragen molecule has bound to five G_{M1} molecules on the plasma membrane, between 13 and 22% of the G_{M1} was inaccessible to the toxin. It is unlikely that steric hindrance is a factor as each NB41A cell bound as few as 330000 toxin molecules and each neuraminidase-treated N18 cell as much as 58 million.

In summary, our results indicate that the major pool of cellular gangliosides is localized in the plasma membrane with their oligosaccharide chains exposed to the external environment. The remaining gangliosides presumably are located within the cell being degraded by lysosomes, being synthesized by the Golgi apparatus, or being transported to the plasma membrane.

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Translocation of Newly Synthesized Gangliosides to the Cell Surface†

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Appendix: Computer Analysis of Rates of Incorporation of [^{3}H]Galactose into Total and Cell-Surface Pools of Gangliosides

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ABSTRACT: A new method was developed to follow the translocation of gangliosides from their site of synthesis within the cell to the plasma membrane. Cultured mouse neuroblastoma N18 and rat glioma C6 cells were labeled for increasing times with D-[1-^{3}H]galactose and then subjected to mild oxidation with $NaIO_4$. Under the conditions chosen, oxidation was essentially restricted to cell-surface sialic acid residues, which were converted to derivatives with an aldehyde function. The labeled gangliosides were isolated from the cells and reacted with dinitrophenylhydrazine to form dinitrophenyl (DNP) derivatives of the oxidized gangliosides. The DNP-gangliosides then were separated from their unmodified counterparts by thin-layer chromatography. Thus, the rate of labeling of surface gangliosides was distinguished from the rate of labeling of total gangliosides. Our results indicated that the transfer of gangliosides from the site of synthesis to the cell surface required approximately 20 min and that newly synthesized gangliosides appeared to be transported to the plasma membrane at a constant rate. No essential differences were found in the rates of translocation of different ganglioside species by N18 cells or between gangliosides of N18 and C6 cells.

The biosynthetic pathways of many gangliosides have been described (Fishman & Brady, 1976). It is generally accepted that the carbohydrate chains of gangliosides are formed by membrane-bound multienzyme complexes which catalyze the stepwise addition of activated monosaccharides to glycolipid acceptors (Roseman, 1970; Fishman, 1974; Caputto et al., 1976). It is also believed that glycosylation of gangliosides takes place in the Golgi apparatus and/or in the endoplasmic reticulum of cells (Keenan et al., 1974; Fleischer, 1977; Pacuszka et al., 1978; Eppler et al., 1980) from where the newly synthesized compounds move to their final destination, the plasma membrane. Very little is known at present about the intracellular transport of gangliosides. In the preceding paper (Miller-Podraza et al., 1982), we described the biosynthesis of gangliosides in cultured cells and demonstrated that they are predominantly located in the plasma membrane. We now describe a new method for distinguishing between surface and intracellular gangliosides and examine the kinetics of ganglioside transport from an intracellular site of synthesis to the plasma membrane.

Experimental Procedures

Materials. $NaIO_4$ was obtained from Sigma and 2,4-dinitrophenylhydrazine from Aldrich Chemical Co. D-[1-^{3}H]-Galactose (2.8 Ci/mmol) was obtained from ICN (Irvine, CA). NaB^3H_4 (7.8 Ci/mmol) and EN^{3}HANCE spray were from New England Nuclear. Silica gel coated glass plates (E. Merck, no. 5763) were obtained through EM Laboratories (Elmsford, NY).

Cell Culture. Mouse neuroblastoma N18 and rat glioma C6 cell lines were grown in Dulbecco's modified Eagle's medium containing 10% and 5% fetal calf serum, respectively,

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