Uptake and metabolism of exogenous gangliosides by cultured cells: effect of choleragen on the turnover of G_{M1}

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Abstract When added to the culture medium, ^{3}H-labeled G_{M1} (tritiated predominantly in the terminal galactose residue) was taken up by murine NCTC 2071 and rat glioma C6 cells, both of which are G_{M1}-deficient. Upon incubating the labeled cells in fresh medium, the cell-associated G_{M1} was metabolized by the cells with a half-life of 1 to 2 days. Some of the G_{M1} was converted to G_{D1a} but the bulk of the label appeared in the medium as degradation products. When G_{M1} labeled in the sialic acid or lipid portion of the molecule was utilized, G_{M2} also was detected with time in the cells and only a small fraction of the radioactivity was detected in the medium. The rat glioma C6 cells appeared unable to degrade the G_{M2} that they accumulated; this was demonstrated directly by incubating the cells with labeled G_{M2}. The uptake and subsequent metabolism of G_{M1} was observed over a wide range of G_{M1} concentrations (10^{-8} to 10^{-4} M). The G_{M1}-treated cells initially bound more iodinated choleragen than did untreated cells; but with time, binding capacity decreased. When G_{M1}-treated cells were transferred to fresh medium in the presence of excess choleragen, the amount of cell-associated G_{M1} remained relatively constant for several days; the conversion of G_{M1} to G_{D1a} also was blocked. Although labeled G_{M3} and G_{D1b} also were taken up by the cells, choleragen had no effect on their subsequent metabolism. Choleragenoid, the binding subunit of choleragen, also inhibited G_{M1} metabolism without activating adenylate cyclase. These results indicate that exogenous gangliosides taken up by cultured cells are metabolized and that choleragen, which binds with high affinity to G_{M1}, specifically prevents the metabolism of this ganglioside.—**Fishman, P. H., R. M. Bradley, B. E. Hom, and J. Moss.** Uptake and metabolism of exogenous gangliosides by cultured cells: effect of choleragen on the turnover of G_{M1}. *J. Lipid Res.* 1983. **24:** 1002–1011.

Supplementary key words adenylate cyclase • membranes • glycosphingolipids

In previous publications from our laboratories, we have reported that G_{M1}-deficient cells can take up exogenous G_{M1} from the culture medium (1–6) or from serum (4). The exogenous G_{M1} became functionally incorporated into the cells as choleragen was able to bind to the cells and to activate adenylate cyclase (1, 2, 4–6). In addition, the toxin protected the newly incorporated G_{M1} from oxidation when cells were incubated with galactose oxidation or sodium periodate (3). Other gangliosides also were taken up by these cells but they did not sensitize the cells to choleragen (2, 5). These results are consistent with reports of other investigators that G_{M1} is the receptor for choleragen (7–10). We also observed that the incorporated exogenous G_{M1}, in addition to being functionally active, was also metabolized (2, 6). Other investigators, however, have reported that exogenous gangliosides are not metabolized by cultured cells (11, 12). The present studies describe the fate of exogenous G_{M1} that has been taken up by cultured cells and demonstrate that choleragen specifically protects the ganglioside from being metabolized.

EXPERIMENTAL PROCEDURES

Materials

G_{M1} was purified from bovine brain as previously described (13) and labeled by galactose oxidase and NaB^3H_4 (1); this procedure labeled the ganglioside predominantly in the terminal galactose residue (3). G_{M1} also was labeled in the sialic acid residue by oxidation with $NaIO_4$ and reduction with NaB^3H_4 (6, 14) and in the ceramide moiety by $PdCl_2$-catalyzed reduction (15).

Abbreviations: G_{M1}, II^{3}NeuAc-GgOse$_4$Cer, Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1′Cer; G_{M2}, II^{3}NeuAc-GgOse$_3$Cer, GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1′Cer; G_{M3}, II^{3}NeuAc-LacCer, NeuAcα2-3Galβ1-4Glcβ1-1′Cer; G_{D1a}, IV^{3}NeuAc, II^{3}NeuAc-GgOse$_4$Cer, NeuAcα2-3Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1′Cer; G_{D1b}, II^3(NeuAc)$_2$-GgOse$_4$Cer, Galβ1-3GalNAcβ1-4(NeuAcα2-8NeuAcα2-3)Galβ1-4Glcβ1-1′Cer; NeuAc, N-acetylneuraminic acid.

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[^{14}C]G_{M3} labeled in the sialic acid was synthesized as described previously (16). [^{3}H]G_{D1b} and G_{M2} were prepared by the galactose oxidase/$NaB^{3}H_4$ procedure (1). $NaB^{3}H_4$ and $Na^{125}I$ were obtained from Amersham Corp. Choleragen and choleragenoid were purchased from Schwarz/Mann. ^{125}I-Labeled choleragen was prepared using the chloramine-T procedure essentially as described by Cuatrecasas (7).

Cells and cell culture

NCTC 2071 cells and rat glioma C6 cells, obtained from the American Type Culture Collection, were grown as described elsewhere (1, 17) usually for 5 days before studies were initiated. The NCTC 2071 cells in 100-mm dishes were incubated for 18 hr in NCTC 135 medium with gangliosides added from a stock solution prepared in sterile distilled water (6). The C6 cells in 75-cm^2 flasks or 35-mm wells of cluster dishes were incubated for 24 hr in Dulbecco's modified Eagle's medium with 0.45% glucose but no serum, with and without gangliosides. After incubation, the medium was removed and the cells were washed three times with phosphate-buffered saline. The cells were then either incubated further in fresh medium or analyzed for radioactive gangliosides or ^{125}I-labeled choleragen binding as described below.

Analysis of gangliosides

Cells were scraped from the culture vessels in ice-cold phosphate-buffered saline and collected by centrifugation. Gangliosides were isolated from the cells as described previously (1, 6). After separating the gangliosides by thin-layer chromatography, they were detected by radioscanning and quantified as described previously (3). Radioactivity released into the culture medium was analyzed by several procedures. Portions were counted before and after lyophilization. Samples of the freeze-dried residue were dissolved in chloroform–methanol–water 60:30:4.5 (v/v), desalted on a small Sephadex G-25 column (18) and analyzed by thin-layer chromatography for labeled gangliosides as described above or dissolved in 50 mM ammonium bicarbonate (pH 7.8) and analyzed by gel filtration column chromatography.

Other methods

Binding of ^{125}I-labeled choleragen to cells attached to culture dishes (6) or in suspension (19) was determined in triplicate and corrected for nonspecific bind-

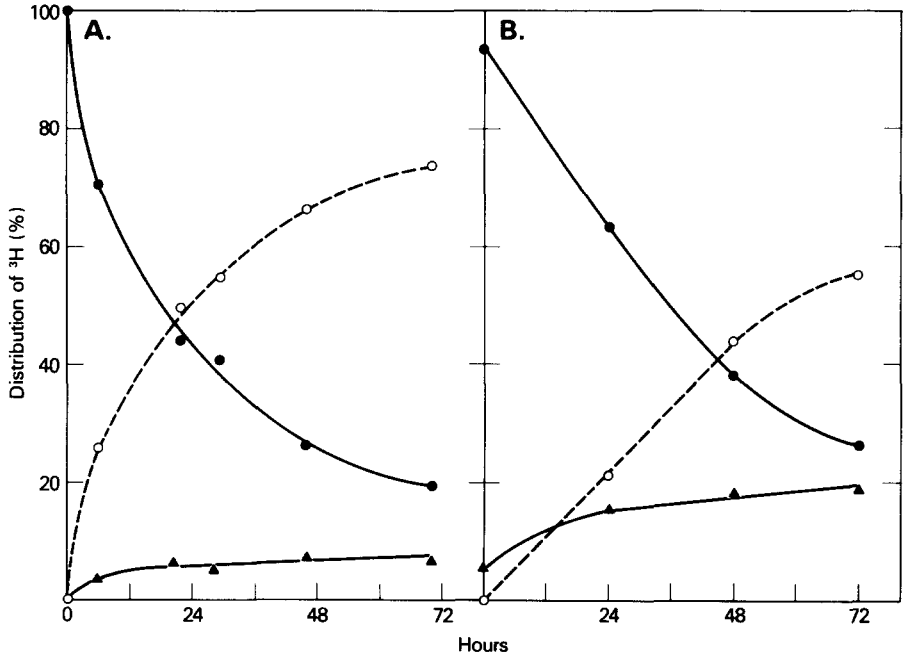

Fig. 1. Degradation of exogenous [^{3}H]G_{M1} incorporated into G_{M1}-deficient cells. A, Each 10-cm dish of NCTC 2071 cells was incubated with 10 nM [^{3}H]G_{M1} (200,000 cpm in 7 ml of medium) for 18 hr. The cells were washed extensively and cultured in fresh medium for the indicated times. The cells from three dishes were harvested, pooled, and analyzed for [^{3}H]G_{M1} (●) and [^{3}H]G_{D1a} (▲) as described under Experimental Procedures. Portions of the culture medium were counted (○). The total mean radioactivity (cells plus medium) for all of the samples was 28,200 ± 2300 cpm. The radioactivity not extracted from the cells was 2850 ± 98 cpm and was not included in the percent distribution. B, Same as in A except each 75-cm^2 flask of rat glioma C6 cells was incubated with 0.5 μM [^{3}H]G_{M1} (200,000 cpm in 4 ml of medium) for 24 hr. The total mean radioactivity for all of the samples was 40,000 ± 910 cpm. Radioactivity not extracted from the cells was 846 ± 112 cpm.

TABLE 1. Analysis of radioactivity in culture medium from cells treated with [^{3}H]G_{M1}

Time	^{3}H in Culture Medium	
	Nonvolatile	Lipid-extractable[a]
hr	%	
24	45.9	12.3
48	34.3	11.3
72	27.7	10.1

Rat glioma C6 cells were incubated for 24 hr in medium containing 0.5 μM [^{3}H]G_{M1}, washed, and incubated in fresh medium for the indicated times. Portions (1 ml) of the culture medium were counted for ^{3}H before and after lyophilization. In addition, the freeze-dried medium was dissolved in 0.1 ml of water, diluted with 2 ml of chloroform–methanol 2:1 (v/v) and applied to a column of Sephadex G-25 superfine equilibrated in chloroform–methanol–water 120:60:9 (v/v) which then eluted with 8 ml of the same solvent (13). The entire eluate was collected, evaporated, and counted for ^{3}H.

[a] When a known amount of [^{14}C]Gal was applied to the column, 1.9% was recovered in the eluate. Recovery of a known amount of [^{3}H]G_{M1} was 98%.

ing as measured in the presence of 2×10^{-7} M unlabeled toxin. Intracellular cyclic AMP was assayed as described previously (5, 6). Protein was determined by the method of Lowry et al. (20) with bovine serum albumin as the standard.

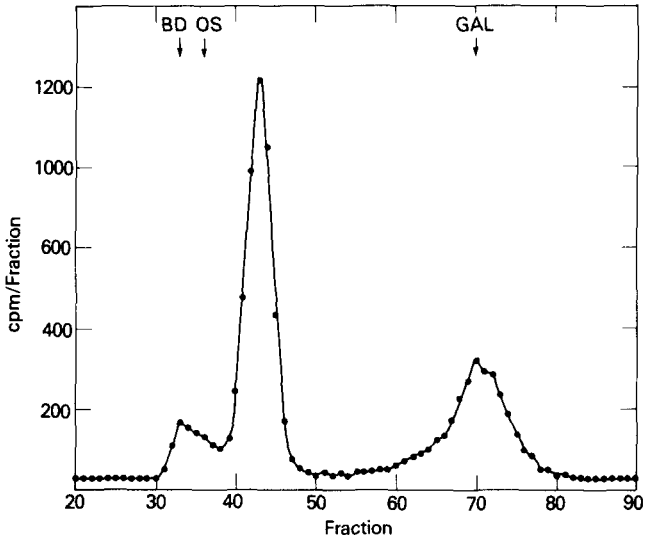

Fig. 2. Analysis of radioactivity in the medium from cultures of [^{3}H]G_{M1}-treated rat glioma C6 cells. Cells in 35-mm dishes were incubated in medium containing 0.5 μM [^{3}H]G_{M1} (300,000 cpm/ml) for 24 hr. The cells were extensively washed and cultured in fresh medium (1.5 ml/dish) for 24 hr. The medium was removed and portions were counted before (14,800 cpm/ml) and after (8590 cpm/ml) lyophilization. The freeze-dried material was dissolved in 50 mM ammonium bicarbonate (pH 7.8) and applied to a 0.9 × 60 cm Bio-Gel P-2 (100–200 mesh) column that was eluted with the same buffer. Fractions of 0.5 ml were collected and counted for ^{3}H. The column was calibrated with blue dextran (BD), [^{3}H]G_{M1}-oligosaccharide (OS) and [^{14}C]galactose (GAL) as indicated by the arrows. Similar results were obtained with medium from 48-hr cultures.

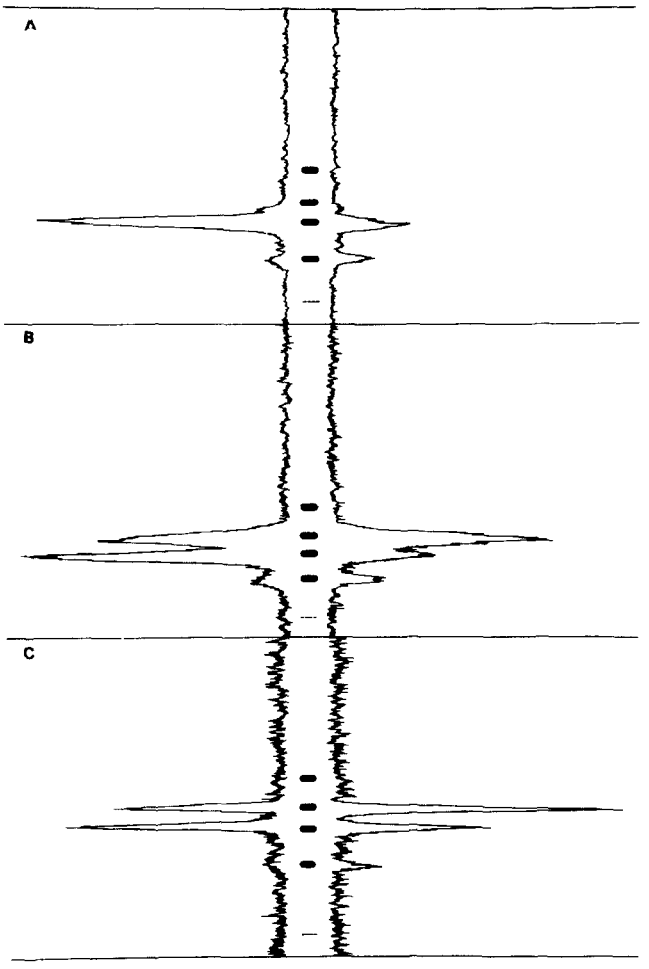

Fig. 3. Radioscans of thin-layer chromatograms of labeled gangliosides isolated from [^{3}H]G_{M1}-treated rat glioma C6 cells. Rat glioma C6 cells in 35-mm dishes were incubated for 24 hr in medium containing 0.5 μM [^{3}H]G_{M1} labeled in the galactose (A), sialic acid (B), or ceramide (C) portion of the molecule. The cells were either harvested (left scans) or cultured in fresh medium for an additional 48 hr and then harvested (right scans). Lipids were extracted from the cells, separated by thin-layer chromatography, and detected by radioscanning as described under Experimental Procedures. Positions of ganglioside standards applied to the same chromatograms are indicated: from top to bottom, G_{M3}, G_{M2} G_{M1}, and G_{D1a}. (-----), origin.

RESULTS

Uptake and metabolism of G_{M1} by cultured cells

NCTC 2071 and rat glioma C6 cells were incubated overnight in medium containing [^{3}H]G_{M1}, washed extensively, and incubated in fresh medium for different times (**Fig. 1**). Cell-associated G_{M1} decreased with time with a half-life of 21 hr in NCTC 2071 cells (Fig. 1A) and 37 hr in C6 cells (Fig. 1B). With both types of cells, there was a corresponding increase with time in radioactivity appearing in the culture medium. In addition, some of the cell-associated radioactivity was identified as G_{D1a} and its amount increased with time. By 3 days,

TABLE 2. Distribution of radioactivity in cultures of rat glioma C6 cells treated with [^{3}H]G_{M1}

Location of Label	Cell-Associated ^{3}H[a]		Distribution of ^{3}H in Cultures after 48 hr[b]		
	0 hr	48 hr	Cell Residue	Cell Lipid	Total Medium
	cpm × 10^{-3}			%	
Gal	17.8 ± 1.00	12.6 ± 0.76	2.35 ± 0.18	57.9 ± 1.1	39.8 ± 1.3 (61.2)
NeuAc	15.9 ± 1.26	14.8 ± 1.47	0.47 ± 0.16	94.7 ± 0.9	4.9 ± 0.8 (90.0)
Ceramide	16.1 ± 1.98	13.4 ± 1.13	0.89 ± 0.27	90.7 ± 1.1	8.4 ± 1.2 (73.4)

C6 cells cultured in 35-mm dishes were incubated for 24 hr in medium containing 1 μM [^{3}H]G_{M1} labeled in different portions of the molecule and washed extensively. Some of the cultures were harvested and the remainder were incubated for 48 hr in fresh medium and harvested. Data are the mean ± S.D. of values from triplicate cultures.

[a] In a separate experiment, the cell-associated ^{3}H had decreased to 51.2 (Gal), 100 (NeuAc), and 95.7% (ceramide) after 48 hr

[b] Values in parentheses represent the % of nonvolatile radioactivity in the medium.

G_{D1a} represented a significant percentage of the remaining cell-associated radioactivity in both cell lines (Fig. 1). Although the rate of G_{M1} metabolism varied somewhat from experiment to experiment, the same pattern was observed. Only ~10% of the cell-associated radioactivity was not extracted by chloroform–methanol and ~90% of the lipid-extractable radioactivity was recovered as gangliosides.

Analysis of gangliosides and metabolic products

As indicated in Fig. 1, there was an increase in radioactivity in the culture medium with time. When the medium was analyzed, a large fraction of the radioactivity was found to be volatile and the proportion increased with the time in culture (**Table 1**). About 10% of the total radioactivity in the medium was recovered in the lipid fraction; all of it migrated as G_{M1} on thin-layer chromatograms (data not shown). When the nonvolatile radioactivity in the medium was analyzed by gel filtration column chromatography, three labeled peaks were detected (**Fig. 2**). A minor peak representing 9% of the radioactivity applied to the column eluted in the void volume; this peak presumably was G_{M1} which behaves as a large molecular weight micelle under these conditions (21, 22).[2] The next peak (46%) eluted from the column after G_{M1}-oligosaccharide and before D-galactose. The third peak (30%) co-eluted with galactose.

To analyze further the metabolic products of G_{M1}, C6 cells were incubated with G_{M1} labeled in the sialyl or lipid moieties for 24 hr, washed, and incubated in fresh medium for 48 hr. The cells contained, in addition to G_{M1} and G_{D1a}, a labeled ganglioside that migrated on the thin-layer chromatograms as G_{M2}; the proportion of this ganglioside increased with time (**Fig. 3**). In contrast to what we had observed with G_{M1} labeled in the terminal galactose, the total amount of cell-associated radioactivity remained constant after 48 hr when G_{M1} labeled either in the sialic acid or lipid was used (**Table 2**). In addition, much less of the cell-associated radioactivity was resistant to lipid extraction. Finally, much less radioactivity was recovered in the spent culture medium and a larger proportion of it was nonvolatile.

When C6 cells treated with [^{3}H]G_{M1} labeled in the lipid or sialic acid were cultured for up to 5 days, no additional labeled lipids were detected in the cells. This finding suggested that the C6 cells were unable to degrade G_{M2}. To directly test this possibility, the cells were incubated with [^{3}H]G_{M2}, washed, and incubated for up to 3 days (**Table 3**). There was no evidence of any degradation of the G_{M2}. In contrast, mouse neuroblastoma cells incubated for 24 hr with G_{M1} labeled in the lipid or sialic acid accumulated labeled gangliosides corresponding to G_{D1a}, G_{M1}, G_{M2}, and G_{M3}; the proportion of the latter two gangliosides increased 24 hr after the medium was changed (data not shown).

TABLE 3. Effect of time on metabolism of [^{3}H]G_{M2} taken up by rat glioma C6 cells

Time	^{3}H in Lipid Fraction[a]
hr	*cpm*
0	4560 ± 244
30	4470 ± 551
73	4130 ± 152

C6 cells in 35-mm dishes were incubated for 19 hr in medium containing 130 pmol/ml of [^{3}H]G_{M2} (24,000 cpm), washed extensively, and cultured in fresh medium for the indicated times. The cells were harvested, extracted, and analyzed for lipid-associated ^{3}H as described under Experimental Procedures. Values are the mean ± S.D. of triplicate determinations.

[a] When analyzed by thin-layer chromatography, all of the radioactivity in the lipid fractions migrated as G_{M2}.

[2] When [^{3}H]G_{M1} was applied to the column, a single peak was recovered in the void volume; some of the ganglioside, however, was absorbed to the column. This may explain why the recovery of radioactivity from the medium in the void volume was less than would be expected from the data in Table 1.

Effect of G_{M1} concentration and cell growth on G_{M1} metabolism

The amount of GM1 taken up by rat glioma C6 cells was proportional to its concentration in the medium over the range of 10 nM to 3 μM as described previously (5, 6). After the labeled cells were incubated in fresh medium for 48 hr, the decrease in cell-associated G_{M1} represented a relatively constant percentage (62 to 67%) of the amount initially incorporated. In addition, [^{3}H]G_{D1a} represented between 46 and 51% of labeled gangliosides in the cells at 48 hr. Thus, the rate of degradation of G_{M1} and its conversion to G_{D1a} appeared to be independent of the amount of G_{M1} taken up by the cells over a 300-fold range.

Rat glioma C6 cells also were exposed to 100 μM unlabeled G_{M1} for 24 hr, then were shifted to fresh medium and analyzed for gangliosides after different times (**Table 4**). The predominant endogenous ganglioside in these cells is G_{M3} with only trace amounts of other gangliosides (23). During the initial incubation, the amount of G_{M1} taken up by the cells exceeded their content of G_{M3}; in addition, some of the G_{M1} became converted to G_{M2} and G_{D1a}. With time in fresh medium, the amount of cell-associated G_{M1} decreased, the amount of G_{M2} increased, and the amount of G_{D1a} initially increased and then decreased. Although the amount of endogenous G_{M3} increased with time, this also was observed in cells not treated with G_{M1} (Table 4). Thus, the cells were able to take up large amounts of G_{M1} from the medium and convert it to G_{D1a} and G_{M2}. As indicated above, the cells were unable to degrade the G_{M2}.

TABLE 4. Effect of high G_{M1} concentration on its uptake and metabolism by rat glioma C6 cells

G_{M1} Treatment	Time of Metabolism	Cell-Associated Gangliosides			
		G_{M3}	G_{M2}	G_{M1}	G_{D1a}
hr	*days*	*pmol/mg protein*			
0	0	6590	ND[a]	ND	ND
0	4	9730	ND	ND	ND
1	2	6480	2200	1110	422
24	0	6320	3180	11900	548
24	1	6840	6590	5750	817
24	2	6910	8280	2850	717
24	4	8760	8040	1710	617

Rat glioma C6 cells cultured in small flasks were incubated with 2.5 ml of serum-free medium containing 100 μM G_{M1} for the indicated times, washed extensively, and cultured in fresh serum-free medium for the indicated number of days. Gangliosides were isolated from the cells, purified, separated by thin-layer chromatography, detected with resorcinol reagent, and quantified by scanning spectrodensitometry as described previously (23).

[a] ND, not detected; the major ganglioside in C6 cells is G_{M3} (23).

TABLE 5. Effect of cell density and growth on metabolism of G_{M1}

Density	Serum	Time	Protein	[^{3}H]G_{M1}
		hr	*μg/dish*	*cpm/dish*
Low	−	0	442	15,800
	−	48	506	6,330
	+	48	844	7,480
High	−	0	686	17,000
	−	48	855	7,900
	+	48	1210	6.980

Rat glioma C6 cells plated at 12,500 (low) and 25,000 (high) cells/cm^2 in 35-mm diameter dishes were grown for 4 days, then incubated with 0.5 μM [^{3}H]G_{M1} for 24 hr and washed extensively. The cells either were harvested immediately or after a further incubation for 48 hr in 2 ml of fresh medium with or without 5% fetal calf serum. The cells were analyzed for [^{3}H]G_{M1} as described under Experimental Procedures. Each value represents the mean of three dishes; the variation in cpm/μg protein was less than 10% among each set of three dishes. There are 5 million cells per mg protein.

We also incubated the C6 cells with 1 μM [^{3}H]G_{M1} for 1 hr. The amount of G_{M1} initially present was 20% of that detected in cells treated for 24 hr with the ganglioside. After 48 hr in fresh medium, the cell-associated G_{M1} content had been reduced to 47% and [^{3}H]G_{D1a} was present. Similar results were obtained when the cells were exposed to 100 μM unlabeled G_{M1} for 1 hr. In addition to G_{D1a}, G_{M2} also was detected in the cells after 48 hr in fresh medium (Table 4).

We next explored the effects of cell density and cell growth on G_{M1} metabolism. C6 cells at different densities were incubated with [^{3}H]G_{M1}, washed, and incubated with fresh medium with or without 5% fetal calf serum for an additional 48 hr. Neither condition had a dramatic effect on the rate of G_{M1} metabolism even though the serum-fed cells doubled their protein content during the process (**Table 5**).[3] When the effect of serum and cell growth was examined daily over a 3-day period, there was again no substantial difference in the rate of G_{M1} metabolism (data not shown).

Binding of choleragen to G_{M1}-treated cells

We had shown previously that rat glioma C6 cells incubated with exogenous G_{M1} exhibited an increase in choleragen binding that depended on time and the concentration of G_{M1} (5, 6). When C6 cells treated with G_{M1} were incubated in fresh medium, their increased capacity to bind ^{125}I-labeled choleragen decreased with time (**Fig. 4**). Toxin binding appeared to decrease more rapidly when cells were cultured in medium containing serum and therefore growing more rapidly.[3]

[3] From the data in Table 5 and Fig. 4B, it appears that C6 cells cultured at lower densities incorporate more exogenous G_{M1} per mg protein than cells cultured at higher densities.

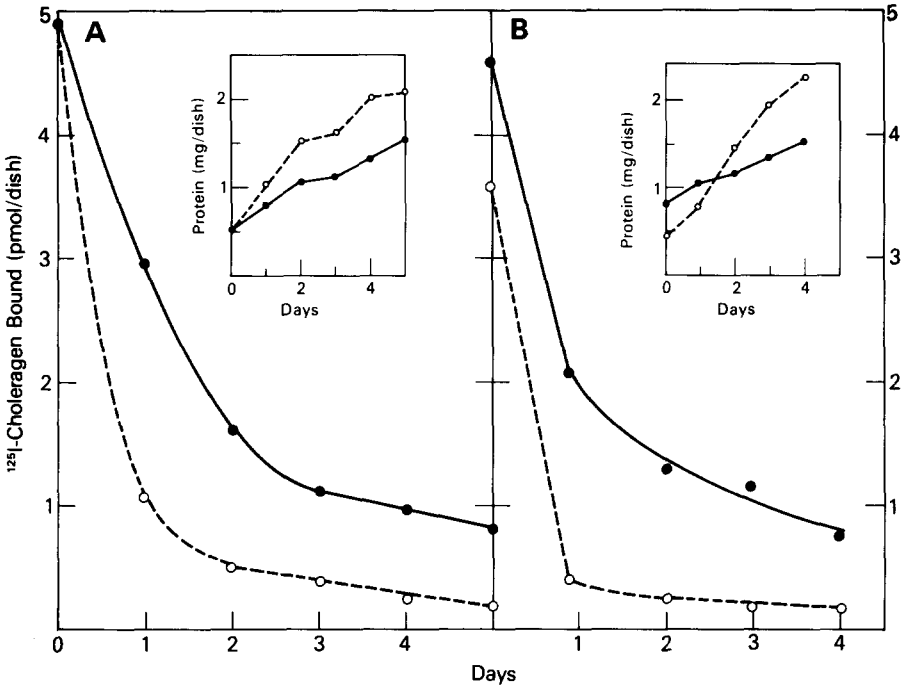

Fig. 4. Effect of cell growth and density on decrease in binding of choleragen by G_{M1}-treated rat glioma C6 cells with time. Cells in 35-mm dishes were incubated in medium containing 0.5 μM Gm_1 for 1 hr, washed extensively, and cultured in fresh medium without (●) or with (○) 5% fetal calf serum for the indicated times. The cells then were washed and assayed for specific ^{125}I-labeled choleragen binding and protein (insert) as described under Experimental Procedures. In panel A, the cells were plated at 12,500 cells/cm^2; in panel B, the cells were plated at 12,500 (○) and 25,000 (●) cells/cm^2.

When loss of toxin binding and cell-associated [^{3}H]G_{M1} were determined in the same experiment (**Fig. 5**), it was clear that the ability to bind choleragen decreased more rapidly ($t_{1/2}$ of 18 hr) than did the content of G_{M1} from the cells ($t_{1/2}$ of 35 hr). Initially, the ratio of G_{M1} molecules to toxin bound was 7.04 ± 0.17 (n = 3); after 4 hr, this ratio increased to over 10 (Fig. 5).[4]

Effect of choleragen on metabolism of G_{M1}

When the G_{M1}-treated NCTC 2071 and C6 cells were incubated in fresh medium in the presence of excess choleragen, the loss of cell-associated G_{M1} was inhibited (**Fig. 6**). In addition, choleragen inhibited the conversion of G_{M1} to G_{D1a} by the cells. After 48 hr, toxin-treated NCTC 2071 cells contained only 31% as much [^{3}H]G_{D1a} as did untreated cells. The inhibition was less with C6 cells (62% as much G_{D1a} after 48 hr) but this might be due to the increased conversion of G_{M1} to G_{D1a} observed in these cells (see Fig. 1). Similar results were obtained in two additional experiments with each cell line. In some of these experiments, there was no loss of G_{M1} in the toxin-treated cells even after 6 days (data not shown). The effect of choleragen on G_{M1} metabolism appeared to be specific. C6 cells treated with unlabeled G_{M1} and labeled G_{M3} or G_{D1b} were able to metabolize these latter gangliosides but choleragen (which was able to bind to the unlabeled G_{M1} taken up by the cells) had no effect on their degradation (**Table 6**). As shown in **Table 7**, choleragenoid, unlike choleragen, did not activate adenylate cyclase but, like the toxin, did block G_{M1} metabolism.

DISCUSSION

G_{M1}-deficient NCTC 2071 and C6 cells incorporated G_{M1} from the culture medium; when the cells were then cultured in fresh medium, the amount of cell-associated G_{M1} decreased with time and appeared to be converted to other gangliosides. In our initial studies, we used G_{M1} labeled in the terminal galactose, which is identical to native G_{M1} except for a tritium instead of a hydrogen. With time, some of the cell-associated G_{M1} was converted to labeled G_{D1a} but much of the radioactivity appeared in the culture medium as galactose and un-

[4] If each toxin molecule binds to five G_{M1} molecules (24, 25), then most of the cell-associated G_{M1} is still on the surface after a 24-hr exposure to medium containing the ganglioside. After 4 hr in G_{M1}-free medium, over 50% of the cell-associated G_{M1} appears not to be accessible to choleragen as the ratio increased to 10.1:1.

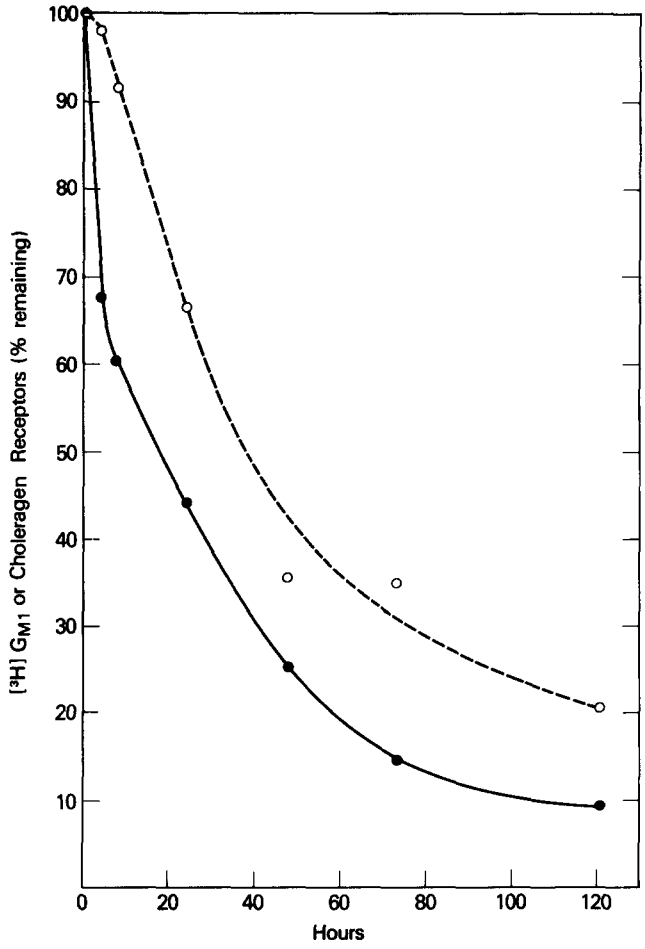

Fig. 5. Loss of choleragen binding capacity and [^{3}H]G_{M1} from rat C6 cells with time. Cells cultured in small flasks were incubated in medium containing 0.5 μM [^{3}H]G_{M1} for 22.5 hr, washed extensively, and cultured in fresh medium for the indicated times. The cells then were washed, harvested, and assayed for [^{3}H]G_{M1} (○) or specific ^{125}I-labeled choleragen binding (●) as described under Experimental Procedures.

identified nonvolatile and volatile products. We also exposed the cells to G_{M1} labeled in the sialic acid and lipid moieties by procedures that altered the structure of the ganglioside. Using these derivatives, labeled G_{M2} in addition to G_{D1a} was detected in the cells and very little of the cell-associated radioactivity appeared in the medium. We were able to confirm that the cells converted the G_{M1} to G_{M2} and G_{D1a} by incubating them with large amounts of unlabeled G_{M1} and chemically quantifying the cell-associated gangliosides.

G_{D1a} is synthesized from G_{M1} by addition of sialic acid from CMP-sialic acid, a reaction catalyzed by a specific sialyltransferase that is present in NCTC 2071 cells (2).[5]

[5] Rat glioma C6 cells contain 1.2 nmol/hr per mg protein of CMP-NeuAc:G_{M1} sialyltransferase activity, which is similar to the level found in NCTC 2071 cells (2).

Other studies have indicated that this sialyltransferase is localized in the Golgi apparatus of the cell (13, 26). G_{M2} is formed from G_{M1} by removal of the terminal galactose via a beta-galactosidase that is also present in NCTC 2071 cells (2) and is believed to be a lysosomal enzyme (27). Further degradation of G_{M2} to G_{M3} requires a second lysosomal enzyme, beta-hexosaminidase (28). The rat glioma C6 cells were unable to metabolize either the G_{M2} that was formed from exogenous G_{M1} or that was directly taken up by the cells. Thus, they appear to be deficient in this enzyme.

The rat glioma C6 cells were able to take up and degrade G_{M1} over a wide range of ganglioside concentration (10^{-8} to 10^{-4} M). The latter concentration is above the reported critical micellar concentration (29, 30); gangliosides, however, appear to self-associate even at 10^{-8} M (21, 22). At present, it is not known in what state the gangliosides are incorporated into the cells. When exposed to G_{M1} for only 1 hr, the cells took up less ganglioside than during a 24-hr treatment; but they were able to metabolize the incorporated G_{M1}. We also observed that metabolism of the incorporated G_{M1} was not substantially affected by the growth phase of the cells. Both growing and nongrowing cells were able to metabolize G_{M1} to a similar extent.

The cells, which are G_{M1}-deficient (1, 5, 23), were able to bind more choleragen after being exposed to the ganglioside. When transferred to G_{M1}-free medium, choleragen binding per culture decreased with time. The toxin binding capacity of the cells diminished more rapidly than did the G_{M1} content. Thus, the exogenous G_{M1} is initially incorporated into the outer surface of the plasma membrane and is internalized prior to being metabolized.

When the G_{M1}-treated cells were cultured in medium containing choleragen, metabolism of the ganglioside was inhibited. Although other labeled gangliosides were taken up by the cells, their metabolism was not blocked by the toxin. In addition, the B component of choleragen (choleragenoid) prevented the metabolism of G_{M1}. We conclude from these latter observations that the ability of the toxin to inhibit G_{M1} turnover is related directly to its binding to the ganglioside and not to some secondary process such as activation of adenylate cyclase. We have recently reported that cell-bound choleragen is internalized and metabolized by cultured cells, that the B component is metabolized more slowly than the A component, and that some of the toxin remains on the cell surface for several days (31, 32). These latter two observations may be related to the ability of the B component to inhibit G_{M1} metabolism. It is known that the B component binds tightly to G_{M1} through the terminal galactosyl and sialyl residues of the ganglioside

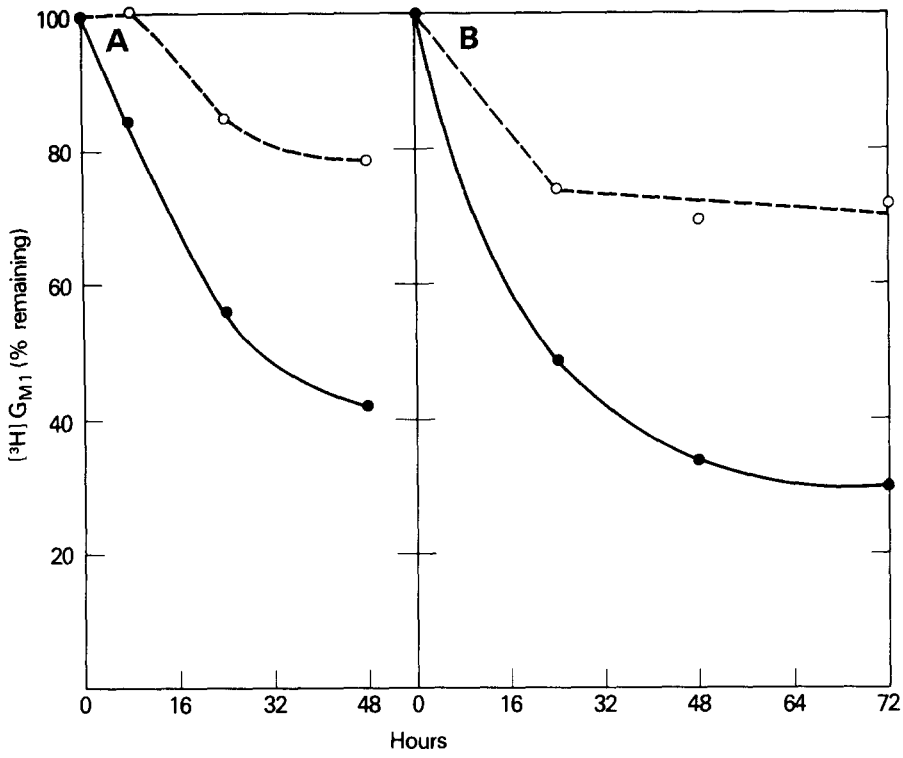

Fig. 6. Effect of choleragen on metabolism of $[^3H]G_{M1}$. The NCTC 2071 cells (A) initially contained 5.4 pmol of $[^3H]G_{M1}$ per dish and were incubated with 10 ml of medium without (●) or with (○) 12 nM choleragen; three dishes were pooled for each sample. The rat glioma C6 cells (B) initially contained 24 pmol of $[^3H]G_{M1}$ per 35-mm dish and were incubated with 2 ml of medium without (●) or with (○) 30 nM toxin; two dishes were pooled for each sample.

(3, 33). If G_{M1} is complexed to the B subunit and this complex is slowly internalized, the sugars of the gangliosides may be inaccessible to enzymes such as sialyltransferase and beta-galactosidase.

TABLE 6. Effect of choleragen on metabolism of exogenous gangliosides taken up by rat glioma C6 cells

Experiment	Time	Choleragen	Cell-Associated Labeled Gangliosides[a]		
			G_{M3}	G_{M1}	G_{D1b}
	hr	*nM*	*% remaining*		
1	29	0	76	74	63
		110	79	94	69
2	44	0	52	33	38
		45	56	85	45

Rat glioma C6 cells were incubated for 24 hr in medium containing only $[^3H]G_{M1}$ or unlabeled G_{M1} plus $[^{14}C]G_{M3}$ or $[^3H]G_{D1b}$ (1 μM of each ganglioside in experiment 1 and 0.5 μM in experiment 2). The cells were washed extensively and either harvested or incubated in fresh medium in the absence and presence of excess choleragen for the indicated times and then harvested. The cells were analyzed for labeled gangliosides as described under Experimental Procedures.

[a] As previously described (5), uptake of the individual gangliosides by the cells was similar.

Our results appear to differ from those reported by other investigators. O'Keefe and Cuatrecasas (11), using RNA virus-transformed Balb 3T3 cells which are G_{M1}-

TABLE 7. Effect of choleragen and choleragenoid on activation of adenylate cyclase and metabolism of exogenous G_{M1} taken up by rat glioma C6 cells

Treatment	Cyclic AMP[a]	$[^3H]G_{M1}$[b]
	pmol/dish	
None	25.9 ± 3.8	10.3 ± 0.3
Choleragen	805 ± 28	22.4 ± 0.4
Choleragenoid	30.5 ± 2.5	18.8 ± 0.4

Rat glioma C6 cells cultured in 35-mm dishes were incubated in 1 ml of medium containing 0.25 μM $[^3H]G_{M1}$ for 24 hr and washed extensively. Some cells were incubated in 2 ml of medium containing 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 1 mM isobutylmethylxanthine, and 12.5 nM choleragen or choleragenoid as indicated for 1 hr and analyzed for cyclic AMP content (5, 6). Other cells were analyzed for $[^3H]G_{M1}$ without further incubation or after incubation for 48 hr in 2 ml of fresh medium with 12.5 nM toxin or choleragenoid as indicated.

[a] Values are the mean ± S.D. of cyclic AMP levels from three separate dishes, each assayed in duplicate.

[b] Values represent amount of G_{M1} that remained after 48 hr and were determined on duplicate dishes (mean ± range). The amount of G_{M1} initially present was determined on three separate dishes and was 20.1 ± 0.96 pmol/dish.

deficient (34), found that cells treated with G_{M1} bound increased amounts of choleragen. When the cells were washed and maintained in a nondividing state, the number of toxin receptors per cell remained unchanged for up to 4 days. When the G_{M1}-treated cell cultures were split daily and assayed for toxin binding the next day, the number of receptors per cell decreased in inverse proportion to the increase in cell number. Thus, their results suggest that exogenous G_{M1} is not internalized and/or metabolized by these cells (11).

Wiegandt and coworkers (12, 35–37) have reported that exogenous gangliosides are taken up by cells but that they are incorporated in the plasma membrane in a different way than endogenous gangliosides and they are not degraded. Thus, exogenous gangliosides were more susceptible to neuraminidase than endogenous gangliosides on the same cells and 70–85% of the cell-associated gangliosides were removed by treating the cells with trypsin. They also observed that most of the gangliosides were removed by adding serum or serum albumin to the culture medium (37). We found, however, that little if any exogenous G_{M1} was released from the cells by trypsin (6). In the present study, the addition of 5% serum to the culture medium had no effect on the loss of cell-associated G_{M1}. In most of their studies, Wiegandt et al. (12, 35–37) exposed the cells to high concentrations of ganglioside for short times, whereas in most of our experiments we treated the cells to low concentrations for 24 hr. When our cells were exposed to 100 μM G_{M1} for 1 hr, they did incorporate and metabolize the ganglioside.

Differences in types of cells used might account for some of these contrasting results. Although we were able to demonstrate that exogenous G_{M1} was metabolized with a half-life of less than 2 days in two different cell lines, one of rat and one of murine origin, it is possible that other cell lines may not metabolize G_{M1}. In this regard, the rat glioma C6 cells appeared unable to metabolize G_{M2}. Although the cells used in our studies are G_{M1}-deficient (1, 5, 23), we do not believe that their ability to incorporate and degrade exogenous G_{M1} is related to its not being a normal constituent of these cells. The C6 cells contain endogenous G_{M3} and were able to take up and degrade exogenous G_{M3}. We also observed that neuroblastoma cells that contain G_{M1} were able to metabolize exogenous G_{M1}. Thus, our present results are consistent with our previous concept that exogenous gangliosides can be incorporated into the plasma membrane in a manner analogous to endogenous gangliosides under appropriate conditions (1–6).

Although it is generally accepted that the bulk of the gangliosides are in the plasma membrane and degradation occurs in the lysosomes, little is known about the intracellular processing of gangliosides. Our present studies demonstrate that exogenous G_{M1}, which has been incorporated into the outer surface of the plasma membrane, becomes internalized. Once inside the cell, some of the G_{M1} must enter the Golgi apparatus where it is converted to G_{D1a} and some of it must be taken up by lysosomes where it is degraded to G_{M2}. Thus, the uptake and metabolism of exogenous gangliosides by cultured cells may be useful for delineating the normal pathway for ganglioside turnover.

We thank Brian Adornato for culturing the rat glioma C6 cells.

Manuscript received 3 September 1982 and in revised form 14 March 1983.

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