Effects of human brain cell culture conditions on [^{14}C]glucosamine radioactivity incorporation into gangliosides

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Abstract Human glioma cells (*12-18*) and fetal neural cells (CH II) in culture were exposed for 20 hr to [^{14}C]glucosamine to determine the level and distribution of radiolabel incorporated into gangliosides. Cells of identical passage levels at two stages of growth, preconfluent and confluent, were preincubated for 0 to 60 hr in serum-free medium (SFM). Both higher cell densities and longer incubations in SFM caused a change in the amounts and patterns of radiolabeled gangliosides. Preincubation for 60 hr in SFM caused an increase ($P < 0.05$) in the percent of total recovered ganglioside radiolabel in GM_1 of CH II cells, from 10.5 to 16.7% in preconfluent cells and from 14.1 to 31.9% in confluent cells. Conversely, the proportion of radiolabel in GM_3 and GM_2 decreased with longer preincubations in SFM. A similar preincubation of glioma cells caused an increase in the proportion of label into GD_{1a} of both preconfluent and confluent cells ($P < 0.02$) from 4 to 11% of the total ganglioside radioactivity. Higher cell densities also resulted in consistently higher percent (of total ganglioside) incorporation into GD_{1a} of *12-18* cells ($P < 0.05$) and GM_1 of CH II ($P < 0.01$). These results show that there is a shift in the incorporation of precursor label into more complex gangliosides under conditions associated with the arrest of cell division. These phenomena may represent a regulatory response of the ganglioside biosynthetic apparatus to changes in extracellular environment and cell contact.—**Liepkalns, V. A., C. Icard-Liepkalns, A. J. Yates, S. Mattison, and R. E. Stephens.** Effects of human brain cell culture conditions on [^{14}C]glucosamine radioactivity incorporation into gangliosides. *J. Lipid Res.* 1983. **24:** 533–540.

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Gangliosides are sialic acid-containing glycosphingolipids present in the plasma membranes of mammalian cells. These amphiphilic compounds have been implicated in mechanisms of immunosuppression (1–3), cell growth regulation (4, 5), and cellular differentiation (6, 7). Some gangliosides can modulate biological effects on cells through their role as receptors for cholera toxin (8) and interferon (9, 10). Specific gangliosides have been determined to be tumor markers for human melanoma (11) and human colon carcinoma (12) for which monoclonal antibodies have been prepared.

We utilized two human neural cell lines for this study. One was derived from a spontaneous brain tumor (glioblastoma multiforme, *12-18*) (13), which has been described previously, and the other was cultured from the cerebral hemispheres of a normal fetus at 16 weeks gestation (CH II). We recently reported the biochemical characteristics of the glioma and two fetal brain cell lines in order to substantiate their usefulness as model systems for the study of growth control (14–19). Potter (20) has proposed that undifferentiated fetal tissue may have characteristics which correlate with and may help to elucidate the biochemistry of neoplasms.

We have been able to detect quantitative changes in the ganglioside and neutral glycolipid composition of glioma cells in culture with increasing cell contact (15, 16). This was in contrast to previous reports that indicated that transformed cell lines had lost this response to cell density changes (21). One purpose of this study is to show further and independently that the formation of complex gangliosides accompanies controlled increases in brain tumor cell contact. In order to delineate the effects of cell division from cell contact we incubated our cell lines at preconfluent and confluent cell densities in serum-free medium for periods of up to 60 hr. We then employed the method of isotopic incorporation into specific ganglioside species from a radiolabeled neuraminic acid precursor, [^{14}C]glucosamine. The results indicated that the formation of specific complex gangliosides is associated with a high degree of cell contact *and* with the arrest of brain tumor cell division. To our knowledge this is the first study to control cell den-

Abbreviations: SFM, serum-free medium; *12-18*, human glioma cells. This paper follows the ganglioside nomenclature of Svennerholm (42).

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sity and cell division for the purpose of elucidating relationships of ganglioside formation to human brain cell contact and proliferation.

MATERIALS AND METHODS

Cell biology

Our media and method of growing human glioma and fetal brain cells from a 14-week-old fetus in culture has been reported elsewhere (14). Cell biological characterization of human cells from 14- or 16-week-old fetuses has been described (14–19, 22) and these fetal cell lines are similar in all aspects studied to date.

The glioma cells (*12-18*) passage 10, and the fetal neural cells (CH II), also passage 10, were seeded at 5000 cells/cm^2 and grown to similar cell densities in 150-cm^2 flasks. At specified densities up to confluency, serum-containing medium was removed from some of the flasks and the cell surfaces were gently washed with serum-free medium (SFM). The cells were further preincubated in SFM at 37°C for periods of 20, 40, or 60 hr before addition of isotope. Seven μCi of D-[1-^{14}C]glucosamine (60 mCi/μmol) (New England Nuclear, Boston, MA) was added in 16 ml of fresh medium to each of at least two flasks per preincubation time point, each of which was analyzed separately (see Biochemistry, below). Flasks with isotope were incubated for an additional 20 hr, then washed three times with ice-cold Dulbecco's phosphate-buffered saline (Gibco, Grand Island, NY) and cells were harvested by scraping with a rubber policeman. Cell pellets were obtained by centrifuging at 100 *g* for 20 min. Pellets were frozen at −35°C until ganglioside extraction. Three 75-cm^2 flasks (Corning, Corning, NY) were seeded, incubated, and washed in parallel to all experimental flasks for cell density determinations as described previously (14).

Biochemistry

All pellets were transferred to Thomas tissue grinders (size BB) and 150 μg of ganglioside prepared from normal human cerebral cortex was added as a carrier to avoid losses during dialysis. Cells were first homogenized in 5 ml of chloroform–methanol 2:1 and then 2.5 ml of methanol was added. Following low speed centrifugation, the supernatant was removed and this step was repeated once with chloroform–methanol 2:1 and once with chloroform–methanol–water 1:2:5, pooling all the supernatants. This total lipid extract was taken to dryness, reconstituted in chloroform–methanol 2:1, and partitioned by the method of Suzuki (23) with three additional washes of theoretical upper phase containing water. The pooled upper phases were dried, treated with alkaline phosphatase and phosphodiesterase (24), subjected to alkaline methanolysis, neutralized (25), and dialyzed against distilled water with several changes over 12 hr. The ganglioside fraction was then purified by silicic acid column chromatography and the radioactivity in this fraction was determined by counting an aliquot in Aquasol II on a Beckman LS-8000 liquid scintillation counter (93% efficiency). Another aliquot containing about 5000 cpm was applied to a silica gel thin-layer plate and chromatographed in chloroform–methanol–water 60:40:10 with 0.02% $CaCl_2 \cdot 2H_2O$. The plates were dried, Kodak XR-2 film was placed over the gel and allowed to develop in the dark for several weeks. The developed spots were identified by comparing their mobilities with known standards run on the same plate visualized with resorcinol spray. Radiolabeled ganglioside standards were prepared by injecting 15 μCi of D-[1-^{14}C]glucosamine intracerebrally into 13-day-old rat brains. One day later animals were killed by decapitation and gangliosides were prepared as described above except that they were not subjected to either the enzyme treatment or alkaline methanolysis. Normal brain cerebral cortex gangliosides were prepared from normal postmortem brain. Gels containing the visualized spots, as well as areas between the spots, were scraped into separate scintillation vials and radioactivity was determined as above. Areas scraped for counting were small and similar (~1 cm^2). It was determined that differential quenching was far too insufficient to account for the consistent differences in label in the bands. Brunngruber, Tettamani, and Berra (26) have evaluated ganglioside extraction procedures and concluded that these extracts contain very little, if any, glycoprotein. There is a report of an amphipathic membrane protein associated with gangliosides in erythrocyte membrane. However, the purified protein has not been shown to contain sialic acid (27). Absolute purity of our ganglioside extract cannot be guaranteed, but to our knowledge, this is the purest human brain ganglioside extract available (12). All critical samples were counted to give a counting error of 5% or less; most had radioactivity at levels of ten times background and none less than twice background.

Statistics

The data in Table 1 were represented by a model in which cell type and cell density were treated as class variables and time of incubation in SFM was considered a continuous variant affecting the total incorporation of radioactivity. The model is called analysis of covariance since there is a continuous variable and a classification variable in the same model (28).

The percentages of incorporation into species of gangliosides (Tables 2 and 3) were treated by multiple regression analysis within each cell type (29). Multiple

regression analysis entailed models with all continuous variables, i.e., time of incubation and incorporation of radioactivity into particular gangliosides. We used a series of F-tests to determine the fitness of the model (30). The values were regressed upon time of incubation in SFM for all cell densities of a cell line and also for preconfluent versus confluent cell densities. We used a series of other F-tests to determine whether values of the parameters were close to zero. The parameters are slopes in the multiple regression models.

All of these analyses were made under the usual assumptions of analysis of variance (28). We chose P values approaching or less than 0.05 as evidence of correlation.

RESULTS

Table 1 shows the amounts of radioactivity on a per cell basis incorporation from D-[1-^{14}C]glucosamine, a neuraminic acid precursor, into total gangliosides of both cell types. Statistical analyses including all data for both cell types indicated an overall trend; higher cell density correlated with less radioactivity in gangliosides per cell ($P < 0.05$). This was less of a phenomenon for glioma ($P < 0.09$) with two exceptions at 60 hr, than the fetal neural ($P < 0.001$) cells (one exception at 40 hr SFM preincubation). However, glioma cells always incorporated more isotope than fetal neural cells ($P < 0.001$). With longer SFM exposure there was a general increase in label incorporation into ganglioside extracts, but this was not considered significant ($P > 0.15$).

Table 2 shows the percentage distribution of total recovered radioactivity among different gangliosides of the glioma cells. Cell preparations at higher cell densities had a lower proportion of ganglioside radioactivity in GM_2 ($P < 0.015$) and GM_1 ($P < 0.015$) and proportionately more radiolabel in GD_3 ($P < 0.01$) and GD_{1a} ($P < 0.05$). An analysis of data for both preconfluent and confluent glioma cells showed that longer durations

TABLE 1. Incorporation of radioactivity into total ganglioside extracts of human glioma (*12-18*) and fetal brain (CH II) cells from D-[1-^{14}C]glucosamine

Preincubation Time in Serum-free Medium	Glioma (*12-18*)		Fetal Neural (CH II)	
	Cell Density[a]	Radioactivity in Ganglioside	Cell Density[a]	Radioactivity in Ganglioside
hr	*cells/cm²*	*cpm/10⁶ cells*	*cells/cm²*	*cpm/10⁶ cells*
0[b]	15,126 ± 2000	2207	18,600 ± 2378	1566
		4119		1437
	54,133 ± 1245	1304	52,112 ± 7299	891
		1198		889
0	14,014 ± 2400	3238	14,533 ± 2133	1096
		1881		1413
	56,100 ± 4318	2071	54,136 ± 3782	809
		1857		650
20	38,780 ± 1100	2273	17,733 ± 2800	1139
		1500		1365
	59,444 ± 952	1350	57,066 ± 8266	928
		1095		817
40[c]	26,926 ± 2900	5754 ± 3374	28,453 ± 2453	1014
				588
	58,700 ± 3113	2237 ± 275	37,715 ± 4505	975
				1327
60[c]	23,000 ± 2100	2522 ± 604	21,040 ± 2026	1660 ± 453
	48,100 ± 3040	3346	43,008 ± 4370	1457 ± 38
		3230		

[a] Cell counts are means ± SE (n = 3). Each of three 75-cm² flasks was trypsinized and counted at the same time that 150-cm² flasks were scraped and extracted. Flasks for cell counts must be trypsinized, but cells for lipid extraction were not exposed to proteases.

[b] Cells incubated with isotope in medium plus serum.

[c] Three individual 150-cm² flasks available for analysis (n = 3).

Cells were preincubated in SFM for the times shown, prior to addition of isotope. After addition of isotope to serum-free medium, each flask was incubated for 20 hr. The radioactive medium was then removed and the cells were washed gently three times with ice-cold Dulbecco's phosphate-buffered saline. The cells were harvested by scraping and centrifugation at 100 *g* for 20 min. Cell pellets were extracted for ganglioside (23), and radioactivity in the sample was determined by counting an aliquot. Each cpm number represents an individual experiment in which a 150-cm² flask was seeded and incubated, and cell gangliosides were extracted as described in Methods.

TABLE 2. Glioma cells: percent of recovered ganglioside radioactivity

Preincubation Time in Serum-free Medium	PD[a]/20 hr	Cell Density[b]	GM_3[c]	GM_2	G_x	GM_1	GD_3	GD_{1a}
		cells/cm²						
0[d]	0.2	15,126 ± 200	11.8	39.8	22.1	7.8	2.7	4.1
	0.2	54,133 ± 2245	12.7	38.0	25.8	5.2	4.7	3.7
0	0.1	14,019 ± 2400	11.5	44.9	25.9	8.4	1.3	2.4
	0.3	56,100 ± 4318	16.5	35.0	23.2	5.3	3.5	5.3
20	1.5	38,780 ± 1100	10.1	44.0	24.0	7.2	1.6	6.3
	0.1	59,444 ± 952	7.4	25.5	24.8	5.6	5.2	12.3
40[b]	0	26,926 ± 2900	9.6 ± 0.5	38.6 ± 3.0	25.1 ± 2.0	8.6 ± 0.8	2.4 ± 0.2	9.9 ± 1.7
	0	58,700 ± 3113	10.7 ± 0.6	29.2 ± 1.1	24.0 ± 1.8	5.3 ± 0.9	6.7 ± 0.6	13.4 ± 2.4
60[b]	0	23,100 ± 2100	8.2 ± 0.4	37.6 ± 1.7	24.5 ± 0.4	9.0 ± 0.2	2.9 ± 0.2	10.9 ± 1.0
	0	48,100 ± 3040	10.5	29.7	24.6	3.9	5.4	11.2

[a] PD, cell population doublings for duration of isotope exposure.
[b] Results are means ± SE (n = 3). Single numbers are means of duplicates from separate flasks.
[c] Ganglioside nomenclature is according to Svennerholm.
[d] Cells incubated with isotope in medium plus serum.

Preconfluent cells had undergone 1.4 population doublings prior to SFM exposure; confluent cells had undergone 3.8 population doublings.

Percent distribution of recovered radioactivity in specific gangliosides of glioma cells after incubation with D-[1-^{14}C]glucosamine in fresh serum-free medium (SFM). Total ganglioside extracts were separated on silica gel thin-layer plates. Kodak film (XR-2) was placed over the plates and allowed to develop. Specific gangliosides were identified by comparing mobilities to standards, the appropriate areas of gel were scraped into scintillation vials, and radioactivity was determined.

than 0 or 20 hr in serum-free medium were correlated with a greater proportion of the total ganglioside radioactivity in GD_{1a} ($P < 0.02$) and possibly in GD_3 ($P < 0.09$) with a "fall-off" at 60 hr preincubation. The proportion incorporated into G_x was constant regardless of cell density or length of time in serum-free medium.

In the fetal neural cells (**Table 3**), a lower proportion of radiolabel into the monosialogangliosides GM_3 ($P < 0.018$), GM_2 ($P < 0.01$), and G_x ($P < 0.001$) correlated with higher cell densities. However, the increased proportion of radioactivity in GM_1 at higher cell densities regardless of preincubation conditions approached sig-

TABLE 3. Fetal neural cells: percent of recovered ganglioside radioactivity

Preincubation Time in Serum-free Medium	PD[a]/20 hr	Cell Density[b]	GM_3	GM_2	G_x	GM_1	GD_3	GD_{1a}
		cells/cm²						
0[c]	0.3	18,600 ± 2378	21.8	17.8	19.5	10.5	12.6	4.8
	0.4	52,112 ± 7299	26.7	12.2	15.6	14.1	12.0	4.0
0	0	14,533 ± 2133	31.8	17.8	19.5	10.5	12.6	4.8
	0.4	54,136 ± 3782	19.7	10.2	13.6	17.3	15.9	5.7
20	0.3	17,733 ± 2800	19.8	17.9	18.3	13.5	17.5	5.6
	0.1	57,066 ± 8266	15.2	9.4	12.2	18.4	17.5	5.8
40	0.7	28,453 ± 2453	20.5	12.3	14.5	16.8	19.9	5.9
	0	37,715 ± 4505	13.6	6.0	12.4	28.5	20.4	3.7
60[b]	0	21,040 ± 2026	19.2 ± 1.7	11.6 ± 3.3	14.3 ± 0.6	16.7 ± 1.1	19.2 ± 1.3	6.4 ± 0.3
	0.2	43,008 ± 4370	11.6 ± 0.6	4.6 ± 0.3	12.5 ± 0.5	31.9 ± 2.2	22.2 ± 0.9	4.0 ± 0.3

[a] PD, cell population doublings for duration of isotope exposure.
[b] Results are means ± SE (n = 3). Single numbers are means of duplicates.
[c] Cells incubated with isotope in medium plus serum.

Preconfluent cells had undergone 1.6 population doublings prior to exposure to SFM; confluent cells had undergone 3.0 population doublings prior to SFM exposure.

Percent distribution of radioactivity in ganglioside species of fetal neural cells after incubation with D-[1-^{14}C]glucosamine in fresh SFM. Methods are the same as in Table 2.

nificance ($P < 0.09$). Cell density had no significant relationship with the proportion of total ganglioside radioactivity incorporated into either GD_3 or GD_{1a} of the fetal neural cells. Longer preincubation time in SFM produced less of the total ganglioside radioactivity in GM_3 ($P < 0.018$), GM_2 ($P < 0.016$), and G_x ($P < 0.004$), and more in GM_1 ($P < 0.05$) and GD_3 ($P < 0.003$) Table 3). In the case of GM_3, this trend plateaued for cell preparations at preconfluent fetal brain cell densities. Unlike the glioma cells, fetal neural cultures incorporated radioactivity from glucosamine into GD_{1a} at a (proportionately) relatively low rate that was unaffected by the length of preincubation in SFM.

Fig. 1 shows a representative radioautogram of a thin-layer plate of separated gangliosides from several confluent glioma samples. All scraped bands were well separated and identifiable in relation to ganglioside standards run on the same plate. The recovery of radioactivity scraped from plates was usually about 90% and the lowest was 76%. The only major band of radioactivity of uncertain identity (called G_x) ran between GM_1 and GM_2, usually as a doublet.

In Tables 2 and 3, the observed cell population doublings obtained from growth curves are shown for 20-hr periods during incubation time of isotope. We observed an arrest of cell division in cultured glioma cells after 40 hr incubation in SFM (Table 2). Similarly, fetal neural cell populations were quiescent after 40 hr in SFM, with a slight recovery at 60 hr.

DISCUSSION

We have previously reported the growth characteristics of the cell lines employed in this study, which were obtained from a glioblastoma multiforme biopsy (*12-18*) and the fetal brain (14, 15, 18, 19). The glioma cells have higher chromosome numbers, twice as much DNA per cell, more rapid [^{3}H]thymidine incorporation per cell, and longer DNA synthesis phase than fetal brain

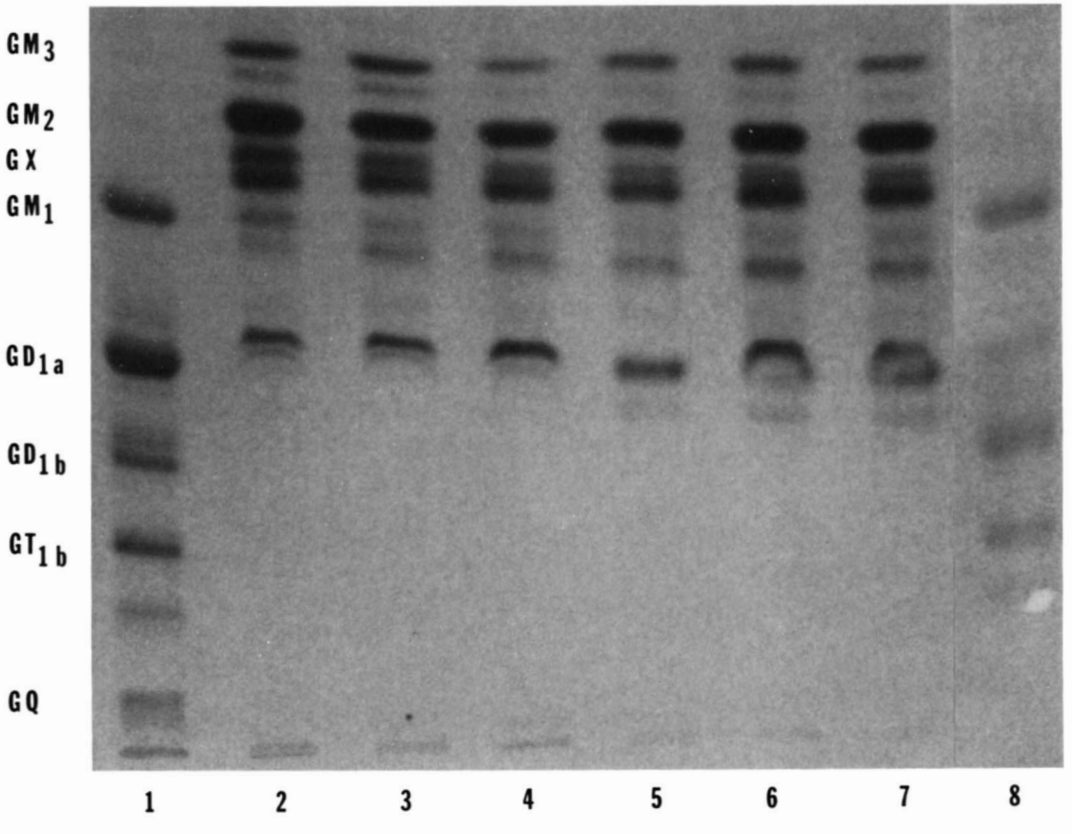

Fig. 1. Combined radioautogram of radioactive gangliosides (4600 cpm) and sprayed lane of ganglioside standards. After developing the X-ray film, the part of the film covering the ganglioside standards was excised. The part of the thin-layer plate containing standards was sprayed with resorcinol reagent and heated at 140°C for 6 min. The developed film was then placed on the plate for photography and identification of gel areas containing ganglioside bands to be scraped. Lane 1: radiolabeled 14-day-old rat brain gangliosides. Lane 2: confluent glioma exposed to radiolabeled glucosamine for 20 hr in the presence of fetal calf serum. Lanes 3 to 7: confluent glioma cells exposed to radiolabeled glucosamine for different time periods in fetal calf serum-free medium (lane 3, 20 hr; lane 4, 40 hr; lanes 5 and 6, 60 hr; lane 7, 80 hr). Lane 8: normal human cerebral cortex gangliosides, 15 μg NeuAc.

cells. Thus they display cell characteristics in culture that make them useful as models for neoplastic neural cells in vivo. The value of studies on neoplastic tissue in conjunction with fetal tissue has been discussed by Potter (20) and one purpose of previous reports from our laboratory was to establish CH cells as a useful in vitro model of fetal human glial cells.

As a function of human neural cell density, we observed that protein per cell decreased, DNA per cell remained relatively constant, and choline and ethanolamine phosphoglyceride per cell were relatively constant, but ganglioside sialic acid per cell increased. However, these determinations were made along the growth curve in presence of serum with proliferating cells (14, 15).

Both of these cell lines, *12-18* and CH, are able to survive and metabolize in our serum-free medium as measured by [^{3}H]thymidine incorporation (19, 22). This property of the cells proved advantageous in the present study which required up to 60 hr incubation in serum-free medium in order to obtain growth arrest. Since fresh medium was added with isotope, this helped to counter the conditioning of medium and stimulation of cell division by the cells' own secreted growth factors. However, it is possible that with sufficiently extended exposure to nutrient serum-free medium these cells would respond to secreted growth factors by re-initiation of cell division and its associated mechanisms.

The significance of our findings resides first in the ability to demonstrate that human neural cells (not of neuronal origin) are able to incorporate a sialic acid precursor into cell gangliosides under serum-free culture conditions. Previous reports have suggested that cells of glial origin do not have the capability to synthesize complex gangliosides (31, 32). Secondly, our previous quantitative data indicated that at higher cell densities, glioma cells (*12-18*) accumulated complex gangliosides (15). Glioma cells and fetal brain cells accumulated neutral glycolipids with increasing cell density (16). This study demonstrates independently that these cells exhibit a patterned response to cell contact, as assayed by radioactivity incorporation into ganglioside extracts.

Since the glioma cells were obtained from a biopsy of a "spontaneous" human brain tumor we have also demonstrated here that, in contrast to a previous report (21), not all neoplastic cells lose the ability to respond to cell contact (via formation of complex glycolipid) after oncogenic insult. Thirdly, this study represents the first comparison of ganglioside formation in neural cell lines that controls for the effects of both cell density and cell proliferation.

Previous investigators have shown that glucosamine is a precursor to the polar moieties (sialyl, galactosyl) of gangliosides (33). The polar portion of a ganglioside is synthesized by the sequential addition of monosaccharide residues from a nucleotide sugar donor to a specific acceptor (34). Our data could, to some extent, reflect differences in glucosamine uptake, conversion to glucose, and conversion to sugar nucleotide, as well as ganglioside synthesis and catabolism. However, in view of our previous quantitative findings (15), we conclude that the net formation of complex gangliosides, in particular those in the pathway for GD_{1a}, was a response to glioma cell contact and associated with the arrest of glioma cell growth. As determined from carefully monitored cell populations, growth of the fetal neural cell population ceased in 40–60 hr in serum-free medium under our culture conditions; growth of the glioma cell population ceased similarly after 40–60 hr. Therefore, after a 40–60 hr preincubation in our serum-free medium, we obtained cell populations that were quiescent and at two different categories of density, preconfluent and confluent. Thus the incorporation of isotope into GD_{1a}, GD_3, and GM_1 was correlated with the arrest of cell proliferation under culture conditions of controlled cell density.

The remarkable constancy of G_x incorporation with respect to other gangliosides in the glioma cells, despite the variety of experimental conditions imposed, might follow if G_x were in a separate pathway from GD_{1a} synthesis. Such would be the case if it were structurally related to GM_{1b}, a ganglioside with which G_x co-migrates, reportedly isolated from rat hepatoma cells (35), but the exact structure of G_x remains to be elucidated. At this point we must conclude further only that G_x is a compound which co-purifies with human brain gangliosides, which is resorcinol-positive, and incorporates radioactivity from [^{14}C]glucosamine. We have observed this band previously (13, 15) in the chromatography of human brain ganglioside preparations, the same kinds of extracts that were used for "carrier" in these studies.

We have based our conclusions on thorough statistical analysis, and all together the results reported here suggest that mechanisms for the synthesis of specific complex gangliosides are closely associated with the control of fetal brain and brain tumor cell growth in vitro.

It has been reported by other investigators that the ganglioside GD_{1a} or GD_3 may also be associated with muscle cell differentiation (36, 37) and cell-to-cell contact in mouse fibroblasts (38). We have further observed that physiological concentrations of gangliosides (extracted from human brain) added to the culture medium can inhibit division of fetal neural cells (19, 22) and extend the cell cycle of astrocytoma cells in culture (39).

Future studies should focus on the cell function-structure relationships of cell surface glycolipids. Such investigations may reveal specifically how particular cell surface lipids are involved in brain cell differentiation and de-differentiation. Critchely (40) had suggested that the appearance of complex glycolipids at the cell surface presents receptors for growth inhibitory molecules. Subsequently we reported the inhibition of human neural cell division by a ganglioside extract (19, 22) and Kinders, Rintoul, and Johnson (41) observed that GM_1 sensitizes mouse fibrosarcoma cells to growth inhibitory peptides.

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