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Glycolipid Synthesis in Normal and Virus-Transformed Hamster Cell Lines

(hamster sarcoma virus/polyoma virus/saturation density/Forssman antigen)

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ABSTRACT **Studies have been continued on the synthesis of glycolipids by the NIL 2 line of hamster cells. Several clones were isolated from this line. These clones vary in morphology, saturation density, and glycolipid composition. Contrary to expectation there was no correlation between saturation density and complexity of the glycolipid pattern. In fact, the clone with the highest saturation density was the only one to show the complete set of glycolipids found previously in NIL 2 cells.**

All untransformed NIL clones show an increase in the level of "higher" (more than two saccharides/ceramide) glycolipids as the cells approach confluence. In the line containing all three "higher" neutral glycolipids, all three increased as cells approached saturation density. In the line containing only hematoside, this compound increased at confluence. Cells transformed by hamster sarcoma virus or polyoma virus showed no density-dependent glycolipid synthesis.

The glycolipids of cultured mammalian fibroblasts have been studied intensively during the last few years. The fibroblasts of murine origin as well as those from many other species have principally gangliosides, glycolipids containing sialic acid. In contrast, fibroblasts from the established hamster lines, BHK 21 and NIL 2 cells, have a series of neutral glycolipids and only a single glycolipid containing sialic acid, hematoside (G_{M3}; see Table 1). The structures of the neutral hamster glycolipids have been determined by Hakomori *et al.* (1) and, as shown below, our work has confirmed these structures. Table 1 presents the structures of the hamster fibroblast glycolipids and the designations used for them in this paper.

Experiments done by Robbins and Macpherson (2) and also by Hakomori and coworkers (3) have shown that viral transformation of NIL or BHK cells causes the disappearance of GL-3, GL-4, and GL-5. GL-1, GL-2, and G_{M3} are present in substantial amounts in virus-transformed cells and share no fixed pattern of variation after transformation. Of exceptional interest is the finding that GL-3, GL-4, and GL-5 in untransformed cells are all present in larger amounts in confluent than in sparsely growing cultures. This observation suggests that, in these cells, cell-to-cell contact serves as a positive control for the synthesis of these glycolipids and that absence of contact or loss of contact inhibition (e.g., by transformation) could bring about the lowering of the concentrations of these three glycolipids.

In order to extend these findings we isolated several clones from the NIL cell line. These clones had differing morphologies, saturation densities, and glycolipid patterns. One clone was transformed by polyoma virus and hamster sarcoma virus. This paper reports studies on the properties of these clones and on the density-dependent synthesis of glycolipids that is shown by all of the untransformed cell lines.

MATERIALS AND METHODS

Cell Lines. All cell lines were derived from the NIL 2 line isolated from embryonic hamster tissue by Diamond (4). NIL B1 cells were obtained from Dr. I. Macpherson by way of Dr. W. Eckhart. NIL 2e cells were obtained directly from Dr. Macpherson's laboratory. One clone from NIL B1 cells (NIL 1c1) and three clones from NIL 2e cells (NIL 2c1, NIL 2c2, and NIL 2c3) were selected by Puck's method (5). Saturation densities of these cell lines are shown in Table 3. The cells were cultivated in Eagle's minimal essential medium containing four times the normal concentration of amino acids and vitamins, 10% fetal calf serum, 50 μg streptomycin/ml, and 75 units penicillin/ml. Cells were grown in Falcon plastic plates, in plastic bottles, or in roller bottles.

Transformation. B-34 Cells, which produce hamster sarcoma virus, were the gift of Dr. Macpherson. Medium was collected from B-34 cells and was used as a virus source. NIL 1c1 cells were infected with hamster sarcoma virus by the method described by Zavada and Macpherson (6). Wild-type polyoma virus was provided by Dr. Eckhart. Cells were infected with polyoma virus in suspension (7). Cells transformed by either hamster sarcoma virus or polyoma virus were selected in soft agar by standard methods (8).

TABLE 1. *Structures of glycolipids*

Abbreviation	Structure	Name
GL-1	Glucosyl–ceramide	Monohexosyl ceramide
GL-2	Galactosyl–glucosyl–ceramide	Dihexosyl ceramide
GL-3	Galactosyl–galactosyl–glucosyl–ceramide	Trihexosyl ceramide
GL-4	*N*-Acetylgalactosaminyl–galactosyl–galactosyl–glucosyl–ceramide	Globoside
GL-5	*N*-Acetylgalactosaminyl–*N*-Acetylgalactosaminyl–galactosyl–galactosyl–glucosyl–ceramide	Forssman antigen
G_{M3}	*N*-Acetylneuraminyl–galactosyl–glucosyl–ceramide	Hematoside

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Labeling of Cells with [1-^{14}C]Palmitate. Labeled palmitate was prepared for use according to the method described earlier (9). For the labeling studies, cells were grown on 5-cm Falcon plastic petri dishes. To obtain "growing" cultures, we plated 2×10^5 cells and to obtain "confluent" cultures, we plated 2×10^6 cells. Growing cultures were labeled the day after inoculation and confluent cultures were labeled after cells reached saturation density. Cells were counted both at the beginning and at the end of labeling to assure that proper growth occurred or that the stationary density was maintained during the labeling interval. Cells were labeled with 5 μCi of [1-^{14}C] palmitate for 24 hr, and lipids were extracted by the method described (9). A small amount of extract was counted for determination of uptake of palmitate. The protein content of the residue was determined by the method of Lowry *et al.* (10).

Analysis of Lipids by Thin-Layer Chromatography. The chloroform–methanol extract was dried under a stream of nitrogen, and two-dimensional thin-layer chromatography and subsequent autoradiography were performed by the method of Gray (11), as described by Robbins and Macpherson (9).

Extended Labeling. NIL 2c1 cells were used for this experiment. For growing cultures 3×10^4 cells and for confluent cultures 3×10^5 cells were inoculated per plate and incubated overnight to allow attachment. The plate medium was labeled with [^{14}C]palmitate. Growing cultures were harvested 3 days later, and confluent cultures were harvested at 3, 4, and 6 days after labeling. The number of cells was determined at these times.

Thymidine Uptake. For growing cultures 2×10^5 cells and for confluent cultures 2×10^6 cells were inoculated per plate. [^{3}H]Thymidine (4 μCi per plate) was used for labeling. At the end of a labeling period of 24 hr plates were washed with warm phosphate-buffered saline (12), and the cells were dissolved in 2 ml 1 N NaOH. Trichloroacetic acid-insoluble radioactivity was determined in a liquid scintillation counter. The number of cells was determined at the time of harvest.

Quantitative Determination of Lipids (Sphingosine). Cells were grown in roller bottles. Bottles containing growing cells and those containing confluent cultures were both labeled with [^{14}C]palmitate for 24 hr.

The isolation of the lipids was done as described earlier. The extracted lipid was hydrolyzed by the method of Sweeley and Moscatelli (13). After hydrolysis, the acid solution was extracted with petroleum ether and the fatty acids thus extracted were counted. The solution was made alkaline, and sphingosine was extracted with ethyl ether. An aliquot was counted. The remaining ether phase was dried, and sphingosine was determined by the method of Robins *et al.* (14).

RESULTS

Lipid patterns of NIL clones

We first observed that the lipid patterns of NIL B1 and NIL 2e cells, which were derived originally from the same clone, differed considerably. Specifically, NIL B1 cells contain neither globoside (GL-4) nor trihexosyl ceramide (GL-3). However, they do have hematoside (G_{M3}) and the Forssman glycolipid (GL-5). Both G_{M3} and GL-5 are present in lesser amounts in growing cells than in confluent cells of NIL B1. NIL 2e cells contain all of the glycolipids shown in Table 1. GL-4 and GL-5 increase on confluency, but G_{M3} does not show this density-dependent change in NIL 2e cells (Table 3). It thus became important to examine whether these differences would become accentuated in individual clones derived from these cell lines.

One clone was isolated from NIL B1 cells, and three clones were isolated from NIL 2e cells. The morphological appearances of the clones are quite different. Cells of NIL 1c1 look flatter than all other clones. NIL 2c1 cells are smaller than all others and show a parallel arrangement after they reach confluency. Cells of NIL 2c2 and NIL 2c3 look similar to each other. In the medium from confluent cultures of both of these lines, there are many floating cells. Growing cultures of these lines contain fewer floating cells. These floating cells exclude colloid dyes such as Erythrosine B and may amount to 40% of the total number of cells. Table 2 shows the uptake of [^{3}H]thymidine into growing and confluent cells. The rate of DNA synthesis of confluent NIL 2c1 and polyoma-transformed NIL 1c1 cells decreased to 7.3 and 6%, respectively, of those shown by growing cells. Since we found many floating cells in the medium of NIL 2c2 cultures, uptake into floating cells and into cells attached to the plate was examined separately. Thymidine uptake into floating cells was 39.5% and into attached cells was 29.4% of that observed in growing cells. These data suggest that NIL 2c2 cells are growing even after they reach confluency and that they continuously release cells into the medium. We do not know at what stage in the cell cycle cells detach from the plate.

We found that palmitate uptake and incorporation into glycolipids and phospholipids is about three times higher, as measured in cpm lipid/μg of protein, in growing cells than it is in confluent cells during a labeling period of 24 hr. When we look at the pattern of incorporation of [^{14}C]palmitate into the individual glycolipids relative to the incorporation into all lipids, we find that NIL 1c1 cells, which have the lowest saturation density, lack higher glycolipids such as GL-3, GL-4, and GL-5. However, they do contain hematoside (G_{M3}), which increases considerably on confluency. Cells of NIL 2c1 contain GL-3, GL-4, and GL-5. These three glycolipids increased sharply on confluency, and hematoside remained about constant during the transition from the growing state to confluency. NIL 2c2 and NIL 2c3 cells, which never stop growing, lack GL-3 and GL-4, but they have GL-5 and G_{M3}. Furthermore, GL-5 increased significantly on confluency in spite of the release of floating cells and the continued growth observed in this clone. Most of the phospholipids remained more or less constant at different cell densities. However, more phosphatidyl inositol was found in confluent cells (Table 3).

The only clone that was observed to contain all three

TABLE 2. [^{3}H]*Thymidine incorporation into growing and confluent cells*

Cell line	Growing cells (cpm/cell)	Confluent cells (cpm/cell)
NIL 2c1	0.144	0.0105
NIL 2c2	0.121	0.0355
floating cells		0.0478
Polyoma-transformed NIL 1c1	0.229	0.0136

TABLE 3. *Synthesis of lipids by NIL cells at high and low density*

NIL cell line	B1		2e		1c1		2c1		2c2		2c3	
Saturation density*	4.8×10^6		4.2×10^6		2.5×10^6		7.0×10^6		3.5×10^6		3.5×10^6	
Growth phase	G	C	G	C	G	C	G	C	G	C	G	C
Incorporation of [^{14}C]palmitate† (cpm/μg protein)					1.3×10^4	5.6×10^3	1.2×10^4	3.4×10^3	1.5×10^4	2.9×10^3	1.5×10^4	3.8×10^3
Incorporation of [^{14}C]palmitate‡												
GL-5	5.1	10.6	4.8	6.4	0	0	12.3	33	2.0	8.5	0.8	2.7
GL-4	0	0	3.8	10.0	0	0	3.6	16.5	0	0	0	0
GL-3	0	0	0	0	0	0	0	2.6	0	0	0	0
GL-2	3.5	1.1	0	0.5	11.4	10.5	0.5	1.3	0.6	0.5	0.4	0.4
GL-1	6.7	5.8	3.2	1.6	5.9	8.1	5.6	5.1	1.2	3.2	3.2	1.8
G_{M3}	17.4	31.4	7.9	4.1	27.5	53.2	19.3	18	9.9	10.4	16.6	6.5
PC + SM	790	765	800	725	706	694	695	660	734	706	755	761
PE	131	116	132	113	117	127	158	154	146	147	137	90.5
PI	11.6	30.1	14	38.5	31.6	36	38.7	44.8	35.9	54.5	31	48.3

* Number of cells per 5-cm plastic plate.

† The cells were labeled with [^{14}C]palmitate as described in *Methods*. Incorporation into all lipids.

‡ Incorporation into individual lipids relative to incorporation into all lipids. The quantities represent cpm × 1000 divided by total cpm incorporated into phospholipids and glycolipids.

G, growing culture; C, confluent culture; SM, sphingomyelin; PC, phosphatidyl choline; PE, phosphatidyl ethanolamine; PI, phosphatidyl inositol.

"density-dependent" glycolipids was NIL 2c1. We observed the same density dependence as reported earlier. It became interesting to examine at what cell density these glycolipids started to increase. Cells of NIL 2c1 were inoculated at low and high density and labeled with [^{14}C]palmitate for long periods. It was found that GL-5 increased before confluency was reached, whereas GL-4 started to increase only when cells had almost attained confluency. In growing cells GL-3 was not observed at all, but it was found after cells reached confluency. Again, G_{M3} was not density dependent (Table 4). As stated in *Methods*, these cells were labeled for a long period. However, relative amounts of each lipid was the same as in the 24-hr labeling experiment. Thus, real (i.e., nonturnover) differences in the lipid patterns between growing and confluent cells are probably being observed when a labeling period of 24 hr is used. The chemical determination of glycolipid concentrations is described below.

TABLE 4. *Density dependence of glycolipids of NIL 2c1*

No. days of labeling	3	3	4	6
Cell number per plate at the time of harvest	2.8×10^5	2.4×10^6	5.9×10^6	6.7×10^6
Incorporation of [^{14}C]palmitate*				
GL-5	6.2	24.8	31.9	38
GL-4	1.5	3.2	11.6	11.6
GL-3	0	0	1.8	2.1
GL-2	1.4	1.6	1.5	1.7
GL-1	10.0	2.5	6.5	4.7
G_{M3}	37.5	21.1	28.7	25.8
PC + SM	690	555	550	572
PE	159	222	205	183
PI	29.4	40.6	48	50

* The quantities represent cpm × 1000 divided by total cpm incorporated into phospholipids and glycolipids. (See Table 3 for abbreviations.)

Glycolipid pattern of transformed NIL 1c1

NIL 1c1 lacks GL-3, GL-4, and GL-5, the glycolipids that show the typical density-dependent response described above. In this clone, however, hematoside shows a dramatic increase as the cells become confluent (Table 3). This clone was transformed with hamster sarcoma virus and polyoma virus. One clone of cells transformed by polyoma virus and three clones of cells transformed by hamster sarcoma virus were selected in soft agar. The saturation density of the transformed clone by polyoma virus increased to 1.4×10^7 cells per plate. However, the saturation densities of three clones transformed by the hamster sarcoma virus did not increase dramatically (Table 5), probably because live cells were constantly being released into the medium. The morphology and the orientation of cells transformed by hamster sarcoma virus are very different from those of untransformed cells. NIL 1c1 cells appear quite square and cells do not overlap. However, the cells transformed by hamster sarcoma virus look rather triangular and have long processes. Cells also tend to overlap one another even in rather sparse cultures. Glycolipid patterns were examined in these transformed cells (Table 5). All of the transformed clones showed less hematoside than the parent line both in the growing state and on confluency. The density-dependent effect was not seen in any of the clones, whether they were transformed by an RNA virus (hamster sarcoma virus) or a DNA virus (polyoma). The three clones of NIL 1c1 transformed by hamster sarcoma virus had decreased GL-1 and GL-2. These decreases did not occur in the transformed clone by polyoma virus.

TABLE 5. *Effect of transformation by oncogenic virus on lipid pattern of NIL 1c1 cells*

Cell line	NIL 1c1		Polyoma-transformed Nil 1c1		HSV-1-1		HSV-1-2		HSV-1-3	
Saturation density*	2.5×10^6		1.4×10^7		3.5×10^6		3.5×10^6		3.5×10^6	
Growth phase	G	C	G	C	G	C	G	C	G	C
Incorporation of [^{14}C]palmitate* (cpm/μg protein)	1.3×10^4	5.6×10^3	1.5×10^4	3.7×10^3	6.5×10^3	3.8×10^3	6.5×10^3	4.2×10^3	7.1×10^3	5.3×10^3
Incorporation of [^{14}C]palmitate*										
GL-5	0	0	0	0	0	0	0	0	0	0
GL-4	0	0	0	0	0	0	0	0	0	0
GL-3	0	0	0	0	0	0	0	0	0	0
GL-2	11.4	10.5	14.6	12.8	1.3	0.6	0.9	0.8	2.4	0.6
GL-1	5.9	8.1	3.5	3.4	2.0	2.5	0.9	1.0	1.1	1.3
G_{M3}	27.6	53.4	20	19.3	16.0	15.1	19.6	13.9	13.1	9.7
PC + SM	706	694	650	695	700	680	675	750	680	714
PE	177	127	202	163	172	102	186	96	180	101
PI	31.6	36	36	37	39	63	47	58	38.9	61

* See footnotes of Table 3.

Chemical measurements of glycolipid concentrations

The increase in GL-3, GL-4, and GL-5 that occurs as cells become confluent was confirmed by direct chemical measurement. An assay of sphingosine was chosen since this moiety serves as the backbone of all glycolipids. The moles of lipid per milligram of protein in growing and confluent NIL 2c1 cells varied in the same direction as that shown by the radioactive assays (Table 6).

Biochemical results

Distribution of Palmitate Label in Glycolipids. Since it is known that palmitic acid can serve as a precursor of the alkyl chain of the sphingosine molecule (15), it seemed of interest to determine the distribution of label between sphingosine and fatty acid in the labeled glycolipids. The labeled glycolipids, GL-1 and GL-2, were extracted from a thin-layer plate and the individual lipids were hydrolyzed in methanolic HCl. The fatty acids were extracted from the acidic solution with petroleum ether, and sphingosine was extracted with ethyl ether after the solution was made alkaline. When the radioactivity of these two components was measured, it was observed that about 50% of the radioactivity went into each of those two building blocks of the glycolipids.

Structure of GL-5. Hakomori *et al.* (1) have assigned the structure shown in Table 1 to GL-5. In confirmation of this structure we have made the following observations: (*a*) It is known that glucosamine is incorporated by fibroblasts into only the glucosamine, galactosamine, and sialic acid moieties of glycolipids and glycoproteins (16). Cells were labeled simultaneously with [^{14}C]palmitate and [^{3}H]glucosamine. Hematoside and GL-5 were then isolated after thin-layer chromatography. If one assumes equal specific activities for the amino sugars and sialic acid, the ^{3}H-to-^{14}C ratio should be a reflection of the number of these sugar moieties per glycolipid molecule. This ratio was about twice as great in GL-5 as in G_{M3}, clearly indicating two amino sugar residues in GL-5. (*b*) Cells were uniformly labeled with [^{14}C]glucose, and the glycolipids were isolated as described above. After appropriate hydrolysis and electrophoresis it was clear that GL-5 did not contain sialic acid. After more vigorous hydrolysis and paper chromatography it was found that GL-4 gave galactosamine, galactose, and glucose in a ratio of 1:2:1 and that GL-5 gave the same sugars in a ratio of 2:2:1.

DISCUSSION

Complex glycolipids are important components of the cell plasma membrane. It is, therefore, of interest to look for correlations between the glycolipid patterns of cell lines and biological properties such as saturation density and state of transformation. In the original work with the present system it was shown that untransformed NIL cells contained G_{M3}, GL-3, GL-4, and GL-5 and that the concentrations of all of these except G_{M3} increased as cells became confluent. Virus-transformed lines derived from NIL contained only G_{M3} and its concentration did not vary with the state of confluence. Of the clones isolated in the present study, one (NIL 1c1) contained only G_{M3}, two (NIL 2c2 and 3) contained G_{M3} and GL-5, and one contained all four glycolipids. There is no apparent correlation between the presence or absence of any of these glycolipids and the saturation density or other physiological properties of the cells. Of special interest, however, is the observation that all of the untransformed lines showed the "density-dependent" increase in glycolipid concentrations as cells approached confluence. In the line containing only G_{M3}, this glycolipid responded to cell density as shown in Table 3. In lines containing GL-5, or GL-3,

TABLE 6. *Quantitative assay of sphingosine in NIL 2c1 cells*

Lipid	Growing cells (nmol sphingosine/mg protein)	Confluent cells (nmol sphingosine/mg protein)
G_{M3}	2.03	1.03
GL-5	1.87	2.72
GL-4	0.51	1.5
GL-1	0.81	0.95

GL-4, and GL-5, however, the density-dependent response was limited to these neutral glycolipids and no increase in G_{M3} was seen. In general, it could be stated that the density-dependent response leads to the increased synthesis of the larger neutral glycolipids in NIL cells and that in the absence of these components the excess glycolipid is "shunted" into G_{M3}. This explanation, however, is obviously an oversimplification of the situation. A more detailed model, if it could be developed, should account for the quantitative findings and should also give a clear indication of the point(s) at which enzymatic reactions are controlled. The proposal that the density-dependent increase in synthesis resulted primarily from an increase in the formation of GL-3 must certainly be revised in the light of the finding that the G_{M3} concentration in NIL 1c1 rises considerably as cells become confluent.

The finding that cells transformed by virus, which have the same glycolipid pattern as NIL 1c1, do not share the density-dependent response is important. This lack of density-dependent glycolipid synthesis appeared in the cell lines transformed by both the polyoma and hamster sarcoma viruses. When the growth of NIL 1c1 and NIL 2c1 cells transformed by hamster sarcoma virus was stopped by treatment with dibutyryl cyclic AMP, there was not an increase in the incorporation of [^{14}C]palmitate into glycolipids (Sakiyama and Robbins, in preparation). These results suggest that loss of the density-dependent effect may be closely associated with a primary effect of viral transformation. Mora *et al.* (17) have suggested that the viral genome may play an important role in the control of glycolipid synthesis by transformed mouse lines.

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