Alterations in Glycosphingolipid Patterns in a Line of African Green Monkey Kidney Cells Infected with Herpesvirus

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The major glycosphingolipids (GSLs) of a line of African green monkey kidney cells (BGM) were characterized as glucosylceramide, lactosylceramide, galactosyl-galactosyl-glucosylceramide, and *N*-acetylgalactosaminyl-galactosyl-galactosyl-glucosylceramide. Neutral GSLs accounted for approximately 80% of the total GSLs isolated. The predominant gangliosides were *N*-acetylneuraminyl-galactosyl-glucosylceramide, *N*-acetylgalactosaminyl-*N*-acetylneuraminyl-galactosyl-glucosylceramide, and galactosyl-*N*-acetylgalactosaminyl-*N*-acetylneuraminyl-galactosyl-glucosylceramide. The incorporation of labeled galactose into GSLs was compared in mock-infected and herpes simplex virus type 1-infected BGM cells. Herpes simplex virus type 1 infection resulted in a three- to four-fold increase in galactose incorporation into glucosylceramide and a decrease in galactose incorporation into galactosyl-galactosyl-glucosylceramide and *N*-acetyl-galactosaminyl-galactosyl-galactosyl-glucosylceramide. The virus-induced alteration in the GSL labeling pattern occurred early in infection, before the release of infectious virus, and was not prevented by the presence of cytosine arabinoside. Treatment of uninfected BGM cells with cycloheximide resulted in alterations in the GSL pattern which were similar to those observed in herpes simplex virus type 1-infected cells. These observations suggest that an early virus function such as inhibition of host cell protein synthesis is responsible for the observed alterations of GSL metabolism. Experiments with a syncytium-producing strain of herpes simplex virus type 1, herpes simplex virus type 2, and pseudorabies virus indicated that other herpes viruses altered GSL metabolism in a manner similar to herpes simplex virus type 1.

It is now well documented that herpesvirus infection of cells results in extensive modification of cellular membranes (3, 8, 12, 13, 18, 23). Numerous virus-determined glycoproteins appear on the cell surface early in infection (3, 8, 13, 18), imparting altered antigenic reactivity (12, 23) and affecting cell-to-cell interaction (13). Although there is ample evidence for the participation of glycosphingolipids (GSLs) in cell receptor (7, 9, 11, 27), antigenic (20), and virus-induced cell fusion (10) phenomena, there are few reports on the effects of herpesvirus infection on cellular GSL metabolism.

Brennan et al. reported that cells infected with herpes simplex virus (HSV) had enhanced incorporation of [¹⁴C]galactose into galactose of glycoproteins and into glucose and galactose of glucosyl- and lactosylceramides (5). Similarly, Ruhlig and Person (21) observed that infection of human embryonic lung cells by several strains of HSV type 1 (HSV-1) resulted in increased

incorporation of [¹⁴C]galactose into glucosyl- and lactosylceramides relative to more complex GSLs. These effects were diminished or delayed when cells were infected with syncytium-producing mutants of HSV-1. HSV-1 infection of BHK-21 hamster cells resulted in increased synthesis of ceramide, glucosylceramide, and lactosylceramide, suggesting a simplification of the GSL pattern (17).

We have investigated the effect of herpesvirus infection on GSL metabolism in a line of African green monkey kidney cells. Our studies have also indicated that HSV infection results in enhanced incorporation of [¹⁴C]galactose into glucosylceramide. However, we have observed a more pronounced block in the synthesis of the more complex neutral GSLs and gangliosides than has been reported previously. Furthermore, evidence is presented which suggests that HSV-induced alterations may be the result of virus-mediated suppression of host cell protein synthesis.

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MATERIALS AND METHODS

Cells and viruses. BGM cells are a continuous line derived from African green monkey kidney cells (2). BGM cells were cultured in Eagle minimal essential medium in a base of Earle saline (EMEM; GIBCO Laboratories, Grand Island, N.Y.) supplemented with 1.2 g of sodium bicarbonate per liter, 200 U of penicillin per ml, 200 μ g of streptomycin per ml, and 10% by volume of fetal calf serum. After 3 to 4 days in culture, the medium was replaced with EMEM containing 5% fetal calf serum. Cells used in these experiments were from 6- to 7-day confluent cultures.

Virus strains used include HSV-1 strain VR3 (HSV-VR3; obtained from M. Ito, State University of New York at Buffalo, Buffalo), HSV-2 strain 333 (HSV-333; obtained from K. C. Chada, Roswell Park Memorial Institute, Buffalo, N.Y.), pseudorabies virus (obtained from H. R. Thacore, State University of New York at Buffalo), and a syncytium-producing strain of HSV-1 (HSV-syn) which arose spontaneously in a stock of HSV-VR3 during passage in our laboratory.

Virus infection and cell labeling. Confluent monolayers of BGM cells grown in 25-cm² plastic flasks (Falcon Plastics, Oxnard, Calif.) were infected with 10 to 50 PFU of the various virus strains per cell or mock infected with growth medium. After a 1-h adsorption period at room temperature, the excess inoculum was removed, and the medium was replaced with 2.5 ml of EMEM and 5% fetal calf serum. Infected or mock-infected cells were incubated at 36°C for 4 h and washed four times with warm phosphate-buffered saline containing 0.3 mM calcium chloride and 1.0 mM magnesium chloride, pH 7.4 (PBS), and the medium was replaced with 2.5 ml of EMEM containing 5% fetal calf serum and [¹⁴C]galactose (1 μ Ci/ml, 50.4 mCi/mol; New England Nuclear Corp., Boston, Mass.). Metabolic labeling of the cells was carried out for 2 to 16 h, as described in the text.

Inhibitors. Cytosine arabinoside (ara-C; 20 μ g/ml; Sigma Chemical Co., St. Louis, Mo.) was added after the virus adsorption step. Cycloheximide (20 μ g/ml; Sigma Chemical Co.) was added when parallel cultures were infected. The concentration of ara-C utilized had been found in preliminary experiments to inhibit the production of infectious virus in HSV-1-infected cells by over 99%. The concentration of cycloheximide utilized inhibited the incorporation of labeled leucine into trichloroacetic acid-precipitable material by over 95%.

Isolation and identification of GSLs. BGM monolayers in 29-ounce (870-ml) glass prescription bottles were washed three times with 0.02% EDTA in PBS and incubated for 10 min at 36°C with a volume of the same reagent sufficient to cover the monolayer. Cells were suspended by scraping with a rubber policeman, washed three times with PBS, and collected by low-speed centrifugation. Extractions of lipids from cell pellets, partitioning of lipid fractions, and isolations of neutral GSL fractions were performed as described by Saito and Hakomori (22).

Neutral GSL fractions were dried under vacuum in a rotary evaporator, dissolved in a small volume of chloroform-methanol (2:1), and separated by thin-layer chromatography (TLC) on Q4 Silica Gel G plates (Quantum Industries, Fairfield, N.J.) with a chloroform-methanol-water (65:25:4) solvent mixture. Individual components were detected by spraying heavily with orcinol (24).

Ganglioside-containing aqueous-phase fractions were evaporated to a small volume in a rotary evaporator, dialyzed at 4°C against distilled water, and lyophilized. The dried residues were redissolved in chloroform-methanol-water (1:1:0.3), and the individual gangliosides were resolved by TLC on Q4 Silica Gel G plates with a chloroform-methanol-ammonium hydroxide-water (60:35:1:7) solvent mixture. Gangliosides were detected by light spraying with resorcinol (25).

Neutral GSLs and gangliosides were isolated from labeled cells as described above and were detected on TLC plates by autoradiography, using Kodak single-coated medical X-ray film SB-54. In these studies, cells from each labeled culture were mixed with unlabeled cells obtained from one 29-ounce prescription bottle confluent monolayer. For quantitation of label, the developed autoradiograms were realigned with the chromatogram, the labeled areas were marked, and the silica gel was scraped into scintillation vials. The silica gel was suspended in 0.5 ml of distilled water mixed with 9.5 ml of Bray scintillation fluid per vial (4) and counted in a Mark II liquid scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill.). Data were corrected for quenching by the channels ratio method.

GSL reference standards. Neutral GSL standards were obtained from human kidney and brain cells and were provided by R. K. Murray (University of Toronto, Toronto, Ontario, Canada). *N*-Acetylneuraminyl-galactosyl-glucosylceramide (G_{M3}) was provided by S. C. Basu (University of Notre Dame, South Bend, Ind.). A ganglioside fraction containing *N*-acetyl-galactosaminyl-*N*-acetylneuraminyl-galactosyl-glucosylceramide (G_{M2}) was prepared from human brain cells obtained from a patient with Tay-Sach's disease and was provided by P. J. Carmody (State University of New York at Buffalo).

Analytical procedures. Neutral GSL and ganglioside fractions were separated by preparative TLC as described above, and the individual components were detected by spraying heavily with methanol-water (1:1). Lipid-containing zones were marked, the plates were redried, and the individual zones were scraped from the plates. Neutral GSLs were eluted from the silica gel with chloroform-methanol-water (65:25:4); gangliosides were eluted with chloroform-methanol-ammonium hydroxide-water (60:35:1:7). After evaporation to dryness, the GSLs were redissolved in a known volume.

Individual neutral GSLs were analyzed for sphingosine content by the spectrophotometric procedure of Lauter and Trams (14), using octadecylamine hydrochloride (Eastman Kodak Co., Rochester, N.Y.) as a standard. Gangliosides were analyzed for sialic acid content by the resorcinol procedure of Svennerholm (25) as modified by Miettinen and Takki-Luukkainen (16). *N*-Acetylneuraminic acid (Sigma Chemical Co.)

was used as a standard.

Protein determinations were performed on cell pellets by the procedure of Lowry et al. (15). Bovine serum albumin (Sigma Chemical Co.) was used as a standard.

RESULTS

Characterization of GSL components. Figure 1 shows the TLC pattern of the neutral GSL fraction of BGM cells. Based on mobility values of known standards, these components were identified (in order of decreasing mobility) as follows: glucosylceramide (GL-1), lactosylceramide (GL-2), galactosyl-galactosyl-glucosylceramide (GL-3), and *N*-acetylgalactosaminyl-galactosyl-galactosyl-glucosylceramide (GL-4). The glycolipids were purified by preparative TLC, and the sugar composition was confirmed by gas-liquid chromatographic analysis of the trimethylsilyl ethers of the *O*-methyl glycosides as described by Vance and Sweeley (26) and Clamp et al. (6) (unpublished observations).

The corresponding ganglioside fraction from BGM cells was chromatographed with known standards (Fig. 2), and the major ganglioside

FIG. 1. Thin-layer chromatogram of neutral GSL standards and the neutral GSL fraction of BGM cells. Neutral GSLs of human kidney cells (lane 1), BGM cells (lane 2), and human brain cells (lane 3) were chromatographed on 0.25-mm Silica Gel G plates, using the chloroform-methanol-water (65:25:4) solvent mixture.

FIG. 2. Thin-layer chromatogram of ganglioside standards and the ganglioside fraction of BGM cells. GM_3 (lane 1), gangliosides of human brain cells (lane 2), gangliosides of BGM cells (lane 3), and a ganglioside fraction containing GM_2 (lane 4) were chromatographed on 0.25-mm Silica Gel G plates, using the chloroform-methanol-ammonium hydroxide-water (60:35:1:7) solvent mixture.

components were tentatively identified (in order of decreasing mobility) as follows: GM_3 , GM_2 , and galactosyl-*N*-acetylgalactosaminyl-*N*-acetylneuraminyl-galactosyl-glucosylceramide (GM_1). Lesser quantities of *N*-acetylneuraminyl-galactosyl-*N*-acetylgalactosaminyl-*N*-acetylneuraminyl-galactosyl-glucosylceramide (GD_{1a}), galactosyl-*N*-acetylgalactosaminyl-(*N*-acetylneuraminyl)₂-galactosyl-glucosylceramide (GD_{1b}), and (*N*-acetylneuraminyl)₂-galactosyl-*N*-acetylgalactosaminyl-*N*-acetylneuraminyl-galactosyl-glucosylceramide (GT) were also detected. Further confirmation of the identity of the gangliosides was obtained by examination of the products of partial acid hydrolysis of individual gangliosides purified by preparative TLC (28; unpublished observations).

The relative quantities of neutral GSLs from BGM cells were determined by analysis of chromatographically separated GSLs for sphingosine content (Table 1). GL-1, GL-2, GL-3, and GL-4 accounted for 13, 9, 16, and 25%, respectively, of the total sphingosine content of the fraction. A non-glycosylated component (presumably ceramide) which moved ahead of GL-1 in the chromatogram accounted for 16% of the sphingosine content of the fraction. Residual gangliosides represented 9% of the sphingosine content of the fraction.

Determinations of the total amount of lipid-bound sialic acid in the corresponding ganglioside fraction yielded 1.1 nmol of sialic acid per mg of protein. Assuming a 1:1 molar ratio of

TABLE 1. *Distribution of sphingosine in a neutral GSL fraction from BGM cells^a*

GSL component(s)	Sphingosine ^b (nmol/mg of protein ^c)	Distribution (%)
Total	10.5	100
Non-glycosylated	1.7	16
GL-1	1.4	13
GL-2	0.9	9
GL-3	1.7	16
GL-4	2.7	25
Gangliosides ^d	1.0	9
Recovery	9.3	88

^a Total cells extracted, 5.1×10^7 .^b Mean of two determinations.^c Total protein, 293.5 mg.^d Residual gangliosides not removed from the neutral GSL fraction.

sialic acid to sphingosine for the principal gangliosides of BGM cells, the total ganglioside content would represent approximately 20% of BGM GSLs. Thus, the neutral GSLs are the principal GSL components of BGM cells.

Effect of HSV-1 infection on the incorporation of labeled galactose into the GSLs of BGM cells. The effect of HSV-1 infection on the incorporation of labeled galactose into the GSLs of BGM cells was examined. Figures 3 and 4 show autoradiograms of TLC patterns of the neutral and acidic GSLs extracted from virus-infected and mock-infected cells. [¹⁴C]galactose was readily incorporated into all of the major GSLs in mock-infected cells. However, viral infection resulted in a marked reduction of the label incorporated into neutral GSLs more complex than GL-1 and into the gangliosides more complex than G_{M3}. Increased incorporation of label into GL-1 and its ceramide precursor was apparent.

The quantitative distribution of label into the various GSLs is shown in Table 2. Incorporation of label into GL-1 was 3.5 times greater in HSV-1-infected cells than in mock-infected cells. On the other hand, there was a marked decrease in incorporation of label into GL-3 and GL-4 (8- and 16-fold, respectively). GL-2, a relatively minor component of BGM neutral GSLs, appeared little affected by the infection. [¹⁴C]galactose labeling of all BGM cell gangliosides was reduced two- to sevenfold by HSV-1 infection. Qualitatively similar patterns of labeling of infected versus mock-infected BGM GSLs were observed when [¹⁴C]glucosamine or [¹⁴C]palmitic acid was used as the labeled precursor (unpublished data).

FIG. 3. Autoradiogram of neutral GSLs from [¹⁴C]galactose-labeled BGM cells. Neutral GSLs from mock-infected BGM cells (lane 1) and from HSV-1-infected BGM cells (lane 2) were labeled for 7 h, beginning at 4 h after infection or mock infection. Chromatography was carried out as described in the legend to Fig. 2. The arrow indicates the origin of the chromatogram. CER, Ceramide.

FIG. 4. Autoradiogram of gangliosides from [¹⁴C]galactose-labeled BGM cells. Gangliosides from mock-infected BGM cells (lane 1) and from HSV-1-infected BGM cells (lane 2) were labeled for 7 h, beginning at 4 h after infection or mock infection. Chromatography was carried out as described in the legend to Fig. 2. The arrow indicates the origin of the chromatogram.

Kinetic studies. The incorporation of [14 C]-galactose into GSLs of BGM cells was studied as a function of time after viral infection. As shown in Fig. 5, decreased labeling of the more complex GSLs and increased labeling of GL-1 were established 7 h after infection and before the first 2-log₁₀ increase in infectious virus in the culture medium. Similarly, decreased labeling of the individual gangliosides (G_{M3}, G_{M2}, and G_{M1}) was also noted 7 h after infection.

Effect of metabolic inhibitors. To determine whether HSV-1-induced alterations of GSL metabolism occurred before viral replication, we carried out experiments similar to those described above in the presence of ara-C. Table 3 shows that ara-C did not prevent HSV-1 induced alterations of GSL metabolism (Table 2).

The above results indicated that an early event in the HSV-1 lytic cycle was responsible for altered GSL metabolism. Viral infection is known to cause a pronounced inhibition of host cell-specific macromolecular synthesis (19). Therefore, it was possible that in the absence of protein synthesis, differential lability of preexisting glycosyltransferases could have produced the observed alterations of GSL metabolism. Thus, we examined the effect of cycloheximide, an inhibitor of translation, on the incorporation

FIG. 5. Kinetics of [14 C]galactose incorporation into neutral GSLs of HSV-1-infected BGM cells. Labeled galactose was added 4 h after infection or mock infection, and labeling was continued until 7, 11, or 21 h after infection. Data are expressed as a ratio of infected to uninfected disintegrations per minute per milligram of protein and are shown relative to the release of infectious virus.

TABLE 2. Distribution of label from [14 C]galactose in GSLs of mock-infected versus HSV-1-infected BGM cells^a

GSL component(s)	dpm $\times 10^{-4}$ /mg of protein		Ratio of I/M ^b
	Mock infected	HSV-1 infected	
Neutral GSLs			
Total ^c	31.6	27.0	0.85
Ceramide	0.9	1.4	1.48
GL-1	5.8	20.1	3.46
GL-2	0.8	0.9	1.05
GL-3	8.4	1.1	0.13
GL-4	10.7	0.6	0.06
Gangliosides ^d	1.9	0.6	0.34
Gangliosides			
Total ^c	8.9	3.1	0.35
G _{M3}	1.4	0.6	0.41
G _{M2}	1.4	0.2	0.14
G _{M1}	1.3	0.4	0.34
Others ^e	1.6	0.9	0.56

^a Cells were labeled for 12 h, beginning at 4 h after infection or mock infection.

^b Ratio of infected to mock infected.

^c Total neutral GSL or ganglioside fractions before TLC.

^d Residual gangliosides not removed from the neutral GSL fraction.

^e Remaining gangliosides with chromatographic mobility less than G_{M1}. Origin material is included.

of [14 C]galactose into GSLs of BGM cells. Table 3 shows that in the presence of cycloheximide, altered patterns of GSL metabolism that were similar to the virus-induced patterns were obtained. These data, therefore, strongly suggest that HSV-induced alterations of GSL metabolism are a reflection of virus-mediated inhibition of host cell-specific protein synthesis.

Effects of other herpesviruses. Three different herpesviruses were tested for their effects on [14 C]galactose incorporation into GSLs of BGM cells (Table 4). HSV-syn, HSV-2, and pseudorabies virus all caused substantial increases in the labeling of GL-1 and marked decreases in the labeling of GL-3 and GL-4. HSV-2 and pseudorabies virus induced two- and threefold increases in GL-2 labeling, respectively. All three virus strains caused two- to threefold decreases in labeling of ganglioside fractions. The pseudorabies virus strain tested caused a somewhat less pronounced alteration of GSL metabolism than did other HSV strains. Thus, of the herpesviruses tested, all appeared to alter GSL metabolism in a similar manner.

DISCUSSION

We, as well as others (17, 21) have observed that the overall effect of HSV infection is a

TABLE 3. *Effects of ara-C and cycloheximide on the distribution of label from [¹⁴C]galactose in GSLs of mock-infected versus HSV-1-infected BGM cells^a*

GSL component(s)	dpm × 10 ⁻⁴ /mg of protein					
	ara-C (20 µg/ml)			Cycloheximide (20 µg/ml)		
	Mock infected	HSV-1 infected	Ratio of I/M ^b	Mock infected	HSV-1 infected	Ratio of I/M ^b
Neutral GSLs						
Total	23.2	26.0	1.12	26.5	24.3	0.92
Ceramide	0.8	1.3	1.68	5.6	2.3	0.41
GL-1	4.8	20.5	4.27	18.1	18.6	1.03
GL-2	0.8	1.3	1.70	0.4	0.7	1.75
GL-3	6.4	1.3	0.21	1.7	0.8	0.47
GL-4	8.7	0.8	0.09	0.3	0.3	1.00
Gangliosides ^c	1.3	0.5	0.39	0.4	0.5	1.25
Gangliosides						
Total	7.1	4.2	0.59	1.1	0.9	0.82

^a Cells were labeled for 12 h, beginning at 4 h after infection or mock infection.^b Ratio of infected to mock infected.^c Residual gangliosides not removed from the neutral GSL fraction.

reduction in the synthesis of more complex glycolipids with a concomitant increase in the synthesis of GL-1. Increased synthesis of GL-2 has also been reported in HEL cells by Ruhlig and Person (21) and in BHK-21 cells by Ray and Blough (17). Ruhlig and Person (21) have proposed that HSV-induced alterations of GSL metabolism may be due to an HSV gene product that acts to decrease glycosyltransferase activity. The failure of ara-C to prevent HSV-1-induced alterations of GSL metabolism indicates that the underlying mechanism is related to an early virus function. The striking similarity between the patterns of [¹⁴C]galactose incorporation after HSV infection and after treatment with cycloheximide suggests that altered GSL metabolism is due to virus-induced inhibition of host cell macromolecular synthesis (19). Anderson and Dales (1) have reached a similar conclusion as a result of their studies on the effect of vaccinia virus infection on GSL metabolism. They also found similar alterations of GSLs in virus-infected cells and in uninfected cells treated with protein synthesis inhibitors.

Changes in GSL synthesis may thus reflect differential turnover rates of the glycosyltransferases. UDP-Glucose-ceramide glycosyltransferase may have a longer half-life than the other transferases in the sequence. As a result, GL-1 may accumulate because it cannot be further glycosylated. In addition, in the absence of other glycosyltransferase activity, there may be increased availability of nucleotide sugars. Alternatively, Anderson and Dales (1) have proposed that regulatory mechanisms controlling the rates of synthesis of the individual GSLs may be disrupted as a result of viral infection, or by inhib-

TABLE 4. *Effects of different herpesvirus strains on the distribution of label from [¹⁴C]galactose in GSLs of BGM cells^a*

GSL component(s)	dpm × 10 ⁻⁴ /mg of protein			
	Mock infected	Virus-infected BGM cells		
		HSV-syn	HSV-2	Pseudo-rabies virus
Neutral GSLs				
Total ^b	28.5	29.5	38.1	32.1
GL-1	5.8	25.1	30.4	18.6
GL-2	0.8	0.6	1.7	2.5
GL-3	8.4	1.1	2.1	4.9
GL-4	10.7	0.5	1.4	2.6
Gangliosides				
Total ^b	6.3	2.3	3.4	3.4

^a Cells were labeled for 12 h, beginning at 4 h after infection or mock infection.^b Total neutral GSL and ganglioside fractions before TLC.

itors. Thus, removal of the UDP-glucose-ceramide glycosyltransferase regulatory block could result in increased synthesis of GL-1.

The role of altered patterns of GSLs in the herpesvirus lytic cycle remains uncertain. However, it is likely that the character of cellular membranes, when altered by herpesvirus infection, facilitates the assembly or release of virus from infected cells or both (17, 18). The combined effects of herpesvirus infection on the glycoprotein and glycolipid profiles of the membranes of infected cells may be necessary for optimal expression of viral functions.

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