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Alterations of Neutral Glycolipids in Cells Infected with Syncytium-Producing Mutants of Herpes Simplex Virus Type 1

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The isolation of syncytium-producing mutants of herpes simplex virus type 1 (KOS strain), which cause extensive cell fusion during otherwise normal infections, has been reported previously (S. Person, R. W. Knowles, G. S. Read, S. C. Warner, and V. C. Bond, J. Virol. 17:183-190, 1976). Seven of these mutants, plus two syncytial strains obtained elsewhere, were used to compare the incorporation of labeled galactose into neutral glycolipids of mock-infected, wild-type-infected, and syncytially infected human embryonic lung cells. Five predominant cellular glycolipid species were observed, denoted GL-1 through GL-5 in order of increasing oligosaccharide chain length; for example, GL-1 and GL-2 correspond to glycolipids that contain mono- and disaccharide units, respectively. Wild-type virus infection caused an increase in galactose incorporation into GL-1 and GL-2 relative to GL-3 through GL-5. For a single labeling interval from 4 to 10 h after adsorption, syncytial infections generally resulted in a relatively greater incorporation into more complex glycolipids than did wild-type infections. One mutant, *syn* 20, was compared with wild-type virus throughout infection by using a series of shorter labeling pulses and appeared to delay by at least 2 h the alterations observed during wild-type infections. These alterations are apparently due to defects in synthesis, since prelabeled cellular glycolipids were not differentially degraded during mock or virus infection.

Oligosaccharide chains of glycolipids and glycoproteins may be important determinants of specificity of interactions at the cell surface. Differences have been observed in the neutral glycolipid and ganglioside profiles of normal and transformed cells, sparse and confluent cells, and cells in various stages of the cell cycle. In addition, changes in ganglioside patterns accompany differentiation in the brain and changes in cell morphology. Specific gangliosides serve as surface membrane receptors for cholera toxin and thyroid-stimulating hormone (for reviews see 1, 6, 9). Infection of cells in culture with herpes simplex virus (HSV) caused an increase in neutral glycolipids with mono- and disaccharide units (2; E. W. Schroeder and J. M. Merrick, Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, S140, p. 227).

We used syncytial (*syn*) mutants of HSV type 1 to investigate alterations specifically related to cell fusion induced during productive infections. A comparison of the properties of cells infected with *syn* mutants and cells infected with wild-type virus, which causes very little syncytia formation, should provide a distinction between changes specifically related to fusion and those generally related to the infection process. Since fusion occurs over a period of several hours, temporal correlations between biochemical events and fusion are possible. Glycoproteins have been implicated in studies of cell fusion induced by paramyxoviruses (see, for example, 4), pox viruses (19), and HSV (11-15, 18), but comparable investigations concerning glycolipids do not exist. Therefore, we compared the neutral glycolipids synthesized in wild-type and *syn* mutant infections.

MATERIALS AND METHODS

Cell culture and virus growth. Human embryonic lung (HEL) cells were grown in 16-ounce (ca. 0.47-liter) prescription bottles, using a growth medium containing F12, supplements, and fetal bovine serum as described previously (17). The preparation and storage of virus stocks have also been described previously (17). HSV type 1, strain KOS, and syncytial mutant 804 were kindly provided by Priscilla Schaffer (Baylor College of Medicine, Houston, Tex.). The syncytial mutant was originally isolated by Schaffer. Syncytial strain MP was obtained from Bernard Roizman (University of Chicago, Chicago, Ill.). The isola-

tion of *syn* mutants of KOS is described elsewhere (16; G. S. Read, S. Person, and P. M. Keller, manuscript in preparation).

Before infection, cells were grown to a subconfluent density, 7×10^4 to 9×10^4 cells per cm^2, and washed twice with Tricine-buffered saline (17). Cell layers were mock-infected or exposed to 5 to 10 PFU per cell in a final volume of 0.8 ml of Tricine-buffered saline per 16-ounce bottle. After 1-h adsorption interval, cells were washed with Tricine-buffered saline and incubated at 34°C in 6 to 10 ml of growth medium per bottle. Under these conditions, 75% of the added virus are adsorbed (17).

Radioactivity. D-[1-^{14}C]galactose (50 mCi/mmol) and D-[*U*-^{3}H]galactose (1.6 Ci/mmol) were obtained from New England Nuclear Corp., Boston, Mass., and added to the growth media at final activities of 0.15 to 3 and 20 to 50 μCi/ml, respectively, as required for particular experiments.

Neutral glycolipid extraction, separation, and analysis. An extraction procedure similar to that of Mora et al. (16) was followed. After various labeling periods, cells were scraped from the bottles, pelleted, and washed twice with Tricine-buffered saline. The cells were suspended and extracted with 2.0 ml of chloroform–methanol (2:1, vol/vol) at 50°C for 15 min, pelleted, and extracted again with 1.0 ml of chloroform–methanol (1:2). The polar components, including most of the gangliosides, were removed by adding 1.0 ml of chloroform and 1.0 ml of water to the combined extracts and following the procedure of Folch et al. (7). The lower nonpolar phase containing the neutral glycolipids was dried under nitrogen and applied to Silica Gel G thin-layer chromatography (TLC) plates (Analabs Inc., Newark, Del.) in less than 0.05 ml of chloroform–methanol (2:1). Plates were developed in chloroform–methanol–water containing 0.25% $CaCl_2$ (60:35:8). Glycolipid spots were detected by autoradiography, and their R_f values were correlated with those of standards (glucosyl ceramide, tetrahexosyl ceramide, and Forssman hapten) (Supelco, Inc., Bellefonte, Pa.), which were visualized by α-naphthol staining. The Silica Gel containing the radioactive glycolipid spots was then scraped into scintillation vials, saturated with 0.4 ml of water, and shaken with 4.0 ml of Aquasol-2 (New England Nuclear Corp., Boston, Mass.). The radioactivity was determined with a Beckman liquid scintillation counter. The percentage of radioactivity in each glycolipid species relative to that in the total neutral glycolipids was determined. Double-label analysis was done by labeling two cell preparations, one with [^{14}C]galactose and the other with [^{3}H]galactose, and combining them before extraction.

RESULTS

Incorporation of labeled galactose into the neutral glycolipids of mock-infected, wild-type-infected, and *syn* mutant-infected HEL cells. Figure 1 shows an autoradiogram of the TLC patterns of neutral glycolipids extracted from mock-infected and virus-infected cells labeled with [^{14}C]galactose from 4 to 10 h after adsorption. The mock-infected cells contained five predominant lower-phase components referred to as GL-1 through GL-5 in order of decreasing mobility. Identification of the five components based on mobility values of standard glycolipids and previous reports from the literature (3, 5, 8) was as follows: GL-1, glucosylceramide (Previous studies have shown that [^{14}C]galactose is incorporated into both glucose and galactose residues of glycolipids [2, 3, 8]. Galactosylceramide is also observed, but the amount is small relative to that of glucosylceramide [3, 8].); GL-2, lactosylceramide; GL-3, galactosyl-galactosyl-glucosylceramide; GL-4, *N*-acetyl-galactosaminyl-galactosyl-galactosyl-glucosylceramide; GL-5, probably a mixture of Forssman hapten (*N*-acetylgalactosaminyl-*N*-acetylgalactosaminyl-galactosyl-galactosyl-glu-

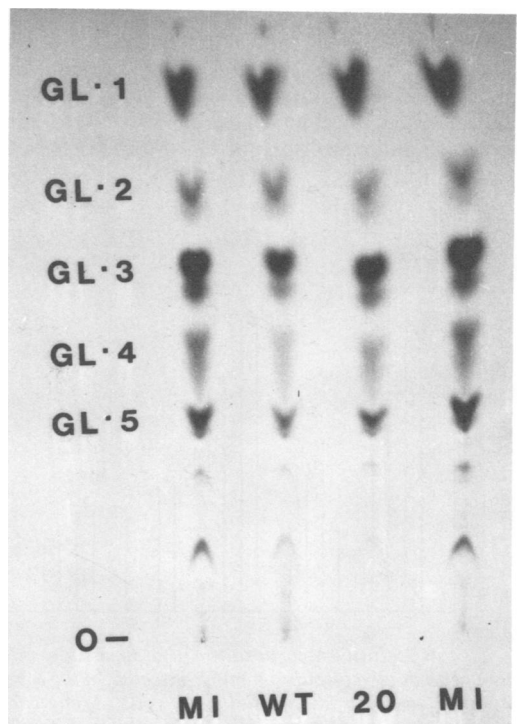

FIG. 1. *Radioautogram of neutral glycolipids of mock-infected (MI), wild-type (WT)-infected, and syn 20 (20)-infected HEL cells. Cells, mock-infected or virus-infected at a multiplicity of infection of 10 PFU/cell, were labeled with [^{14}C]galactose at 0.9 μCi/ml from 4 to 10 h after adsorption. The neutral glycolipids were extracted, chromatographed, and detected by radioautography as described in the text. The predominant glycolipid species are labeled GL-1 through GL-5 in order of decreasing mobilities and were identified as described in the text. The origin (O) is at the bottom of the figure.*

cosylceramide) and hematoside (*N*-acetylneuraminyl-galactosyl-glucosylceramide) (G_{M3}), since it has been observed that about 50% of hematoside partitions into the lower nonpolar phase of the Folch extract (5, 16), and Forssman hapten and hematoside have the same mobilities in the solvent system used. It is evident from Fig. 1 that wild-type and *syn* mutant infections caused a relative decrease in the more complex glycolipids.

For a quantitative comparison of glycolipids, the five major species detected by autoradiography were scraped from the TLC plates, and the radioactivity was determined for each component. Figure 2 shows a comparison of the fraction of the total radioactivity incorporated into each glycolipid species for mock-infected, wild-type-infected, and *syn* 20-infected cells. Whereas GL-3 was the predominant species in mock-infected cells, it was notably decreased in virus-infected cells, and GL-1 became the predominant species. In each of nine separate experiments, HSV-infected cells were found to have larger fractions of radioactive GL-1 and GL-2, smaller fractions of GL-3 and GL-4, and generally smaller fractions of GL-5 than did the mock-infected cells. The average values for each glycolipid species for *syn* 20-infected cells were intermediate to those for mock-infected and wild-type-infected cells.

The glycolipids synthesized during infection with a number of syncytial strains were compared with those synthesized during wild-type infection, using a double-label analysis. Wild-type- and *syn*-infected cultures, one labeled with [^{14}C]galactose and the other labeled with [^{3}H]-galactose, were harvested and combined before extraction and separation of glycolipids. The ratios in Table 1 indicate the amounts of radioactivity in each glycolipid species relative to wild-type virus for seven *syn* mutants isolated in our laboratory, as well as for the syncytial strains 804 and MP. All of the syncytial infections, except *syn* 1, resulted in significantly greater amounts of the more complex glycolipids GL-3 and GL-4 than did wild-type infections. *syn* 1, which fuses cells very slowly does not show this effect for any glycolipid species. The relative amounts of radioactivity for two cell

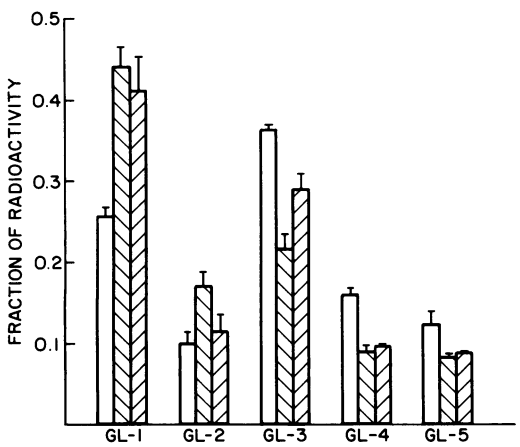

FIG. 2. *Relative incorporation of radioactivity into the neutral glycolipids of mock-infected, wild-type-infected, and syn 20-infected HEL cells. Areas containing glycolipid species GL-1 through GL-5 as visualized by autoradiography (Fig. 1) were scraped from the thin-layer plates and analyzed for radioactivity. The fraction of radioactivity for each species is the counts per minute for each area divided by the total counts per minute for all five areas. For the single experiment shown, the labeling interval was from 4 to 10 h after adsorption. Data are shown for mock-infected (□), wild-type-infected (▨), and syn 20-infected (▩) cells. The error bars represent one standard deviation and were calculated by averaging data from separate bottles: 6 for mock-infected cells, 7 for wild-type-infected cells, and 2 for syn 20-infected cells.*

TABLE 1. *Ratios of [^{14}C]- to [^{3}H]galactose incorporated into neutral glycolipids from combined pairs of mock-infected (MI), wild-type (WT)-infected, and syn mutant-infected cell preparations*[a]

Determination[b]	^{14}C/^{3}H cpm ratio				
	GL-1[c]	GL-2	GL-3	GL-4	GL-5
MI/MI	1.00	0.83	1.08	1.08	1.00
WT/WT	1.00	0.83	1.10	1.10	0.83
WT/MI	1.00	0.77	0.30	0.23	0.33
20/MI	1.00	0.78	0.58	0.47	0.49
1/WT	1.00	0.98	0.95	0.92	0.63
6/WT	1.00	1.57	1.75	1.54	0.90
8/WT	1.00	1.61	2.37	2.43	1.96
20/WT	1.00	1.23	1.38	1.26	1.16
30/WT	1.00	1.10	2.25	2.25	2.14
31/WT	1.00	1.00	1.64	1.62	1.30
33/WT	1.00	1.23	1.64	1.52	1.02
804/WT	1.00	1.32	2.78	2.71	2.78
MP/WT	1.00	0.75	1.59	1.84	1.57
Avg mutant/WT	1.00	1.16	1.72	1.69	1.43

[a] Cells were infected (multiplicity of infection, 5 to 10) or mock-infected and labeled with either [^{14}C]galactose (0.2 to 3.0 μCi/ml) or [^{3}H]galactose (20 to 50 μCi/ml) beginning 4 h after adsorption. After 10 to 13 h of infection, cell preparations were harvested and combined in ^{14}C^{3}H-labeled pairs. The neutral glycolipids were extracted, separated, and quantitated with a liquid scintillation counter. The ^{14}C/^{3}H counts per minute ratios were normalized, using a value of 1.00 for GL-1. In some experiments, cell preparations labeled with [^{3}H] galactose and [^{14}C]galactose were reversed. In these cases, the isotope ratios were also found to be reversed

[b] 1, 6, 8, 20, 30, 31, and 33 refer to cells infected by mutants *syn* 1, *syn* 6, etc. MP and 804 are other syncytial strains.

[c] GL-1 through GL-5 refer to glycolipid species described in the text.

preparations of the same type, e.g., [^{14}C]galactose-labeled, mock-infected cells combined with [^{3}H]galactose-labeled, mock-infected cells, are also shown to provide an estimate of the experimental error (lines 1 and 2).

Kinetics of synthesis of neutral glycolipids in wild-type- and *syn* 20-infected HEL cells. The results of a pulse-labeling experiment, comparing the time dependence of glycolipid synthesis during wild-type and *syn* 20 infections, are shown in Fig. 3 and 4. HEL cells were infected with wild-type or *syn* 20 virus, or were mock-infected, and labeled for 3-h intervals from 3 to 18 h after virus adsorption. Autoradiograms of TLC patterns of [^{14}C]galactose incorporation into glycolipids of infected-cell extracts are shown in Fig. 3. The intensities of complex glycolipid spots decrease with time in profiles of infected-cell extracts, with the effect being more evident for extracts from wild-type-infected cells.

A quantitative analysis of the fractional compositions of glycolipids is shown in Fig. 4, where the relative amount of each species is plotted as a function of time after infection. The amount of each glycolipid for mock-infected cells did not vary substantially over the course of the experiments, the composition being similar to the 3-h labeling data for infected cells and to the data shown in Fig. 2 (Fig. 4, legend). For wild-type infections, the shift to the less complex glycolipids began early and continued throughout most of the experiment. The alterations in the amounts of GL-1, GL-2, and GL-3 were delayed by at least 2 h in *syn* 20-infected cells with respect to the same alterations in wild-type-infected cells. Early and late in infection, differences in the respective fractional compositions for *syn* 20- and wild-type-infected cells are less evident. The above results were also supported by double-label analyses (M. A. Ruhlig, unpublished data).

Fate of prelabeled cellular neutral glycolipids in mock-infected, wild-type-infected or *syn* 20-infected HEL cells. To determine whether the neutral glycolipid alterations were due to the synthesis of new glycolipids or to the breakdown of preexisting glycolipids, the fate of prelabeled cellular glycolipids was examined 0 to 18 h after virus adsorption. After labeling, cells were infected or mock-infected and incubated in nonradioactive medium for 0, 6, 12, or 18 h before the neutral glycolipids were analyzed (Table 2). No significant changes in the fractional amount of GL-2, GL-3, or GL-4 were observed over the time interval. All cell preparations showed an initial decrease in the fractional amount of GL-1 within 6 h after removal of the radioactive medium, which may reflect its conversion to the more complex spe-

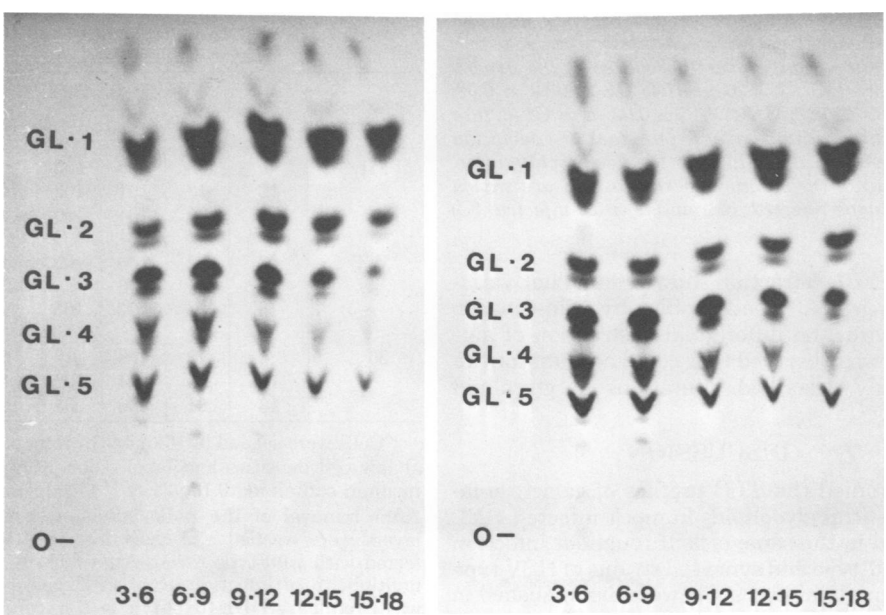

FIG. 3. *Radioautograms illustrating the kinetics of neutral glycolipid synthesis in wild-type- and syn 20-infected HEL cells. Cells were infected at a multiplicity of infection of 10 PFU/cell and labeled with [^{14}C]-galactose (2 μCi/ml) for 3-h intervals as indicated in the figure. The neutral glycolipids were extracted, separated by TLC, and detected by autoradiography. Profiles are shown for wild-type-infected (left) and syn 20-infected (right) cells. The origins (O) are at the bottom of the autoradiograms.*

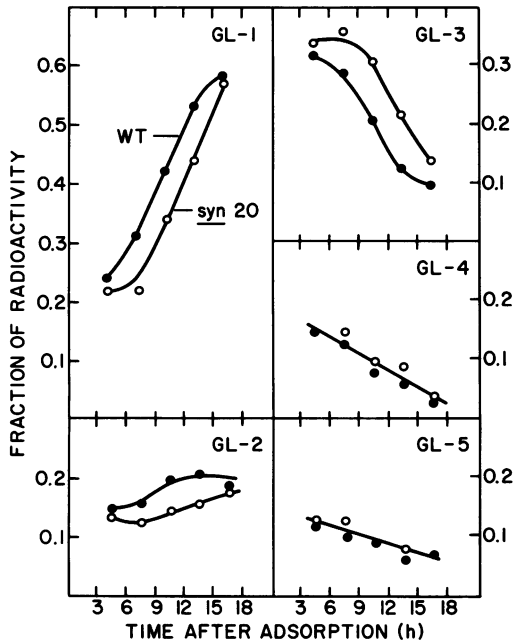

FIG. 4. *Quantitative analysis of the kinetics of synthesis of neutral glycolipids in wild-type and syn 20-infected cells. Areas corresponding to GL-1 through GL-5 of Fig. 3 were scraped from the chromatogram and analyzed for radioactivity. The radioactivity for each glycolipid species is plotted as a fraction of the total radioactivity, as in Fig. 2. Data points are plotted at the midpoints of each labeling interval. The average values for mock-infected cells, which showed little change over the course of the experiments, were: GL-1, 0.26 ± 0.04; GL-2, 0.12 ± 0.02; GL-3, 0.32 ± 0.02; GL-4, 0.16 ± 0.03; and GL-5, 0.14 ± 0.02. The variations refer to one standard deviation calculated from the values for the five labeling intervals. Data are from one experiment and are shown for wild-type-infected* (●) *and syn 20-infected* (○) *cells.*

cies. The GL-5 fraction showed small but variable changes. After adsorption by wild-type or *syn* 20 virus, no differential destruction of glycolipids was observed that could account for the previously described alterations in glycolipid profiles.

DISCUSSION

We studied the TLC profiles of galactose-labeled neutral glycolipids in mock-infected HEL cells and in the same cells throughout infection with wild-type and syncytial strains of HSV type 1. Five predominant spots were distinguished in the mock-infected cell extracts. These were found to have mobilities similar to those previously observed for neutral glycolipids. GL-5 probably contains the ganglioside G_{M3} as well as Forssman hapten. The profile and the fractional composition of neutral glycolipids observed here are similar to those reported by others for cultured mammalian cells (3, 5, 8).

The overall result of wild-type infection is to shorten the average oligosaccharide chain length. GL-3, GL-4, and GL-5 decreased in amounts relative to GL-1 and GL-2. GL-1 became the predominant spot and comprised about 65% of the total neutral glycolipids synthesized late in infection (discounting GL-5). The shortening of glycolipid chains is associated with a synthetic, as opposed to a degradative, process, since prelabeled cellular glycolipids do not turn over substantially during viral infection. In a recent study of herpes-infected cells, neutral glycolipids were not separated from gangliosides. Of the neutral glycolipids, GL-1 and GL-2 were distinguished, and these were noted to qualitatively increase during HSV type 1 and type 2 infections. GL-1 and GL-2 have also been noted to appear in HSV type 1 virions (2). A significant increase in [^{14}C]galactose incorporation into less complex glycolipids during HSV type 1 and 2 infections has also been reported (Schroeder and Merrick, Abstr. Annu. Meet. Am. Soc. Microbiol. 1976).

Since the overall incorporation of radioactive galactose is at least as great in HSV-infected as in mock-infected cells (data not shown), it is unlikely that the shorter chain lengths are due

TABLE 2. *Fate of neutral glycolipids in HEL cells labeled before infection*[a]

Determination	Time (h)	% Control fractional radioactivity				
		GL-1	GL-2	GL-3	GL-4	GL-5
MI	6	53	90	103	100	141
	12	43	101	102	105	128
	18	37	104	105	100	100
WT	6	52	103	105	102	112
	12	51	96	95	109	158
	18	54	103	103	91	145
20	6	73	109	117	96	91
	12	63	94	109	110	119
	18	61	106	104	109	129

[a] Cells were seeded in 16-ounce prescription bottles at low cell densities and labeled for 30 h in growth medium containing 0.15 μCi of [^{14}C]galactose per ml. After removal of the radioactive medium, the cell layers were washed and mock-infected (MI) or infected with wild-type (WI) or *syn* 20 (20) virus at a multiplicity of infection of 10 PFU/cell. One bottle was then harvested to obtain a 0-h control value. Other bottles were incubated for 6, 12, and 18 h in growth medium, and the glycolipids were extracted and analyzed. Values given are expressed as percentages of control fractional radioactivity obtained at 0-h post-infection for each glycolipid species.

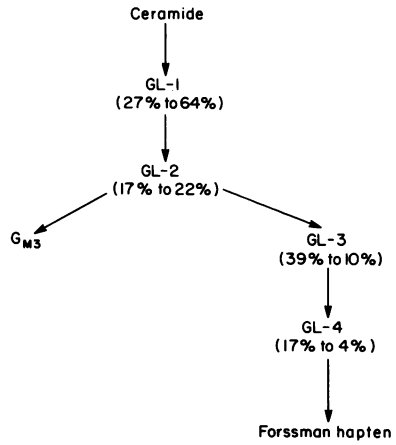

FIG. 5. *Biosynthesis of the predominant glycolipids in HEL cells.*

to a decrease in galactose caused by virus infection. In addition, Brennan et al. (2) have found that the absolute amount of UDP-galactose increases and the ratio of UDP-galactose to UDP-glucose remains nearly constant during HSV infection. Therefore, it is likely that the decrease in oligosaccharide chain lengths is due to a block at a specific step in the glycolipid biosynthesis pathway.

A scheme for the biosynthesis (see for example 1, 6, 9) of the predominant glycolipids found in HEL cells is given in Fig. 5. TLC of Folch extracts revealed that gangliosides more complex than G_{M3} were present only in small quantities in HEL cells (M. A. Ruhlig, personal observation; S. M. Steiner, personal communication). The first and second numbers listed in parentheses refer to the percent compositions synthesized early and late in wild-type infection, respectively. GL-5 was omitted from the percentage calculations because it is a mixture of Forssman hapten and G_{M3}.

It is clear from the diagram that the marked decrease in GL-3 and GL-4 is accompanied by an accumulation of GL-1 and, to a lesser extent, GL-2. These data could be explained either by a block between GL-1 and GL-2 or between GL-2 and GL-3. If the block is between GL-1 and GL-2, the lack of a large decrease in GL-2 could be due to the presence of a separate enzyme that converts GL-1 to GL-2 for the subsequent production of G_{M3} (GL-2 consists of two separate pools). If the block is between GL-2 and GL-3, the lack of a large buildup of GL-2 could be due to the conversion of some GL-2 to GL-3. Of course, it is possible that the TLC spots contain more than one glycolipid species. Regardless of the particular site of the block, it seems likely that an HSV gene product acts to decrease the overall cellular glycosyl transferase activity. We cannot propose an unique reason for the existence of such a virus function.

Other explanations for the altered patterns of glycolipid synthesis in infected cells are also possible. For example, virus infection is known to turn off host cell protein synthesis, and differences in the lability of preexisting cellular glycosyl transferases could have produced the observed patterns. The *syn* mutation would then be hypothesized to result in a difference in the shut-off of host cell protein synthesis. However, no differences have yet been observed in the sodium dodecyl sulfate-polyacrylamide gel electrophoresis profiles of host or viral proteins synthesized early in infection (R. W. Knowles and S. Person, to be published). Therefore, although possible, this explanation is considered to be less likely than the direct alteration of a glycosyl transferase activity by an HSV gene product.

The average labeled glycolipid composition 6 to 12 h after infection is different in *syn* mutant- and wild-type-infected cells. However, if the kinetic data for *syn* 20-infected cells were shifted to earlier times in infection by about 2.5 h, then the data for *syn* 20 and wild-type infections would be essentially indistinguishable. This may indicate that syncytial strains cause a delayed inhibition of glycosylation.

Since MP-infected cell plasma membranes lack a specific viral glycoprotein, it has been proposed previously that fusion in HSV involves a fusion inhibitor activity (18). However, we note that MP is altered in glycolipid synthesis as well. We propose that wild-type infections are characterized by the presence of a fusion factor and a fusion inhibitor, the combination resulting in a small amount of fusion. The fusion inhibitor hypothesis is based on three lines of evidence. (i) *syn* mutant-infected cells fuse efficiently with each other and with mock-infected cells but fuse poorly with wild-type-infected cells (10, Read et al., in preparation). (ii) A small but reproducible amount of fusion occurs for a brief period during wild-type infection and then stops. A sharp decrease or termination in the rate of fusion may also occur during *syn* 20 infection, but it occurs about 4 h later than during wild-type infection (17). (iii) Using the [^{125}I]lactoperoxidase surface labeling technique, at least one virus-specific glycoprotein is labeled 2.5 h later in *syn* mutant infections than in wild-type infections (R. W. Knowles, S. Person, G. S. Read, T. C. Holland, P. M. Keller, and S. C. Warner, manuscript in preparation).

Perhaps the delay in the appearance of surface glycoproteins, the delay in the shortening of the average glycolipid chain length, and the delay in the decrease in the rate of fusion found in

syn-infected cells are all due to an altered glycosyl transferase activity. It is possible that the *syn* mutation is a defect in the gene responsible for the inhibition of glycosyl transferase activity in wild-type-infected cells, the defect resulting in a decreased inhibitor activity. The above results warrant the continued investigation of glycosylation with respect to fusion production and fusion inhibition by syncytial strains of HSV type 1. Studies of specific glycosyl transferase activities would be of special interest.

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