The effects of *N*-hexyl-*O*-glucosyl sphingosine on normal cultured human fibroblasts: a chemical model for Gaucher's disease

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Abstract Normal human skin fibroblasts were grown in the presence of *N*-hexyl-*O*-glucosyl sphingosine (HGS), an inhibitor of aryl glucosidase and glucocerebrosidase. Tests of the cells with aryl glycosides showed that β-glucosidase activity in the cells was drastically reduced while other enzyme activities (α-glucosidase, β-galactosidase, and *N*-acetyl-β-hexosaminidase) were normal or elevated. Exposure of cells to HGS for 28 days resulted in increased values for cell weight per plate, glucocerebroside concentration, and galactosylgalactosylglucosyl ceramide concentration. The concentrations of total lipid, cholesterol, and protein were unchanged, as was the fatty acid distribution within the glycolipids. Chemically, the inhibitor-treated cells exhibited a model form of Gaucher's disease. Although many membranous cytoplasmic inclusions were induced by HGS, they were unlike the characteristic inclusions seen in individuals with the genetic disorder. Skin fibroblasts from a Gaucher patient showed no abnormalities in composition or appearance.

Gaucher's disease is a recessively inherited disease characterized chemically by a deficiency of the enzyme, glucocerebrosidase, which normally releases the β-linked glucose of glucocerebroside, or GL-1a (1). As a result of this deficiency, GL-1a accumulates in the tissues of affected individuals. The accumulated lipid is associated with morphological lesions in tissue histiocytes, consisting of inclusions which appear by electron microscopy as membrane-bound secondary lysosomes filled with tubular structures (2).

Clinical manifestations in the patients vary greatly, with respect to onset of symptoms, organs most severely affected, progress of the disorder, and types of complaints. The relationship between the severity of the symptoms and the degree of glucosidase deficiency is not simple (3). It would therefore be useful in understanding the disorder to have an in vitro model which can be manipulated in several dimensions, such as degree of glucosidase deficiency, rate of GL-1a synthesis, and concentration of glucocerebroside in the environment (medium).

Cultured human fibroblasts synthesize and degrade GL-1a (4) while Gaucher fibroblasts derived from adult patients with the disease are deficient in glucosidase and are said to store the glycolipid (5). However the distinctive "Gaucher inclusions" have not been demonstrated in the Gaucher fibroblasts (6) and the degree of GL-1a accumulation is somewhat less than that seen in organs from patients. Nevertheless, the accessibility of cultured human cells for experimental manipulation may offer compensation for these deficiencies.

This report gives data from an attempt to develop such a model with the use of a synthetic inhibitor of glucocerebrosidase, *N*-hexyl-*O*-glucosyl sphingosine, previously demonstrated to be a strong inhibitor of the corresponding enzyme in rat spleen (7) and cultured mouse and rat tumor cells (8).

MATERIALS AND METHODS

The culture system used in this study involves initiation of cell cultures from explants of surgical skin specimens obtained from a variety of sites (9). Cells were grown on 100 × 20 mm Falcon plastic culture plates (Falcon Plastics, Oxnard, Cal.) in 5% CO_2–95% air at 37°C in 10 ml of Eagle's MEM containing 10% fetal calf serum. The medium also included 0.1 mM serine, 1 mM pyruvate, and 10,000 units of penicillin G and 0.5 mg of streptomycin sulfate per 100 ml. Pronase B (Calbiochem, San Diego, Cal.), 25 mg/100 ml of Ca^{2+} + Mg^{2+}-free balanced salt solution, was used to remove cells from the plate (10). The cells were replicated at a concentration of

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Abbreviations: HGS, *N*-hexyl-*O*-glucosyl sphingosine; GL-1a, glucocerebroside or glucosyl ceramide; GL-3a, digalactosyl glucosyl ceramide; TLC, thin-layer chromatography; GLC, gas–liquid chromatography.

TABLE 1. Conditions used for assaying fibroblast hydrolases

	β-Glucosidase	α-Glucosidase	Phosphatase	Galactosidase	Hexosaminidase
Buffer	citrate/PO_4	Na acetate	Na acetate	Na acetate	Na acetate
Buffer conc.	50 mM	50 mM	50 mM	50 mM	33 mM
Substrate conc.	1 mM	1 mM	6 mM	1 mM	5 mM
Buffer pH	4.0 and 5.3	4.0	5.0	4.0	4.0
Homogenate vol.	50 μl	100 μl	10 μl	10 μl	10 μl
Total vol.	0.25 ml	0.3 ml	0.21 ml	0.16 ml	0.16 ml
Incubation time	1 hr	2 hr	1 hr	0.5 hr	0.5 hr

7.5×10^5 cells per plate. Five days later ascorbic acid was added (50 μg/ml) and half the plates received medium containing 0.5 μg/ml of HGS. The cultures were maintained an additional 11 or 28 days with medium changed 3 times per week. Fresh HGS was added with each medium change at a concentration of 0.5 μg/ml for the first 5 feedings and 1 μg/ml for the remaining feedings. The HGS was evaporated from solution in organic solvent in a sterile vessel, neutralized with sterile HCl solution, and added to the medium.

At the end of the culture period, the cells in each plate were washed with Ca^{2+} + Mg^{2+}-free solution, removed with Pronase, centrifuged, washed twice with saline, and homogenized immediately in water for enzyme assays, or frozen and stored at −60°C for lipid analysis.

For electron microscopy the cells were fixed on the plate with 1% glutaraldehyde in Millonig's buffer, pH 7.3, for 60 min at 4°C. The cells were then scraped into test tubes, centrifuged, washed with the same buffer for 30 min, and postfixed in 1% OsO_4 in the same buffer for 2 hr at 4°C. Dehydration with graded acetone solutions was followed by embedding in Araldite, curing for 1–3 weeks at 50°C, and cutting sections with glass knives on a Porter-Blum ultramicrotome. The sections were stained with lead citrate (11) and uranyl acetate (12) and examined with an RCA EMU 3G electron microscope.

Aqueous homogenates of the fresh cells (0.6–1 mg protein/ml) were prepared in a Dounce glass hand grinder and assayed for β-D-glucosidase, β-D-galactosidase, and *N*-acetyl-β-D-glucosaminidase with the 4-methylumbelliferyl glycosides (Pierce Chemical Co., Rockford, Ill.) as described in **Table 1.** The reactions were stopped by adding 3 ml of 0.17 M glycine carbonate, pH 10.7. Acid phosphatase and α-D-glucosidase were assayed with the corresponding *p*-nitrophenyl derivatives (13). Protein was determined by a modification of the method of Lowry et al. (14) in which the copper was stabilized with EDTA[4].

β-Glucosidase was found to have a pH optimum between 4.0 and 4.5, an apparent K_m of 0.14 mM, and a constant rate of hydrolysis for over 2 hr. Glucocerebrosidase was measured in Gaucher, normal, and HGS-treated normal cells with a natural substrate, [^{14}C]stearoyl glucosyl sphingosine.[5]

For the chemical analyses, the frozen cells were lyophilized to obtain the dry weight, then suspended in water (4 ml/g) and homogenized with chloroform–methanol 1:1 (100 ml/g) containing an internal standard, galactocerebroside made from nonadecanoic acid (0.67 mg/g). The insoluble residue thus produced was washed, dried, and analyzed for protein content by the procedure of Hess and Lewin (15). The lipid extract was washed by partitioning with chloroform, methanol, and saline, then evaporated to dryness and weighed.

Some of the lipid samples were examined by TLC on silica gel with hexane–ether–acetic acid 70:30:4 or chloroform–methanol–water 90:35:6. The lipid bands were located with bromothymol blue followed by a charring spray.

For quantitative analysis, the dried lipids were exposed to NaOH–chloroform–methanol to degrade the ester-type lipids, then chromatographed on silica gel with acetone–methanol 9:1 to elute the simple glycolipids, using 100 ml/g of silica gel (16). The eluted lipids were applied to 0.5 mm thick silica TLC plates in bands 1 cm long, then separated with chloroform–methanol–water 111:40:6 and visualized with bromothymol blue. Bands corresponding to GL-1a and GL-3a were scraped from the plates into small columns and eluted with chloroform–methanol–ammonium hydroxide 7:7:1, 25 ml/g. Blanks derived from lipid-free zones of the corresponding R_f's on the same plate were also eluted. Prior to scraping the GL-3a zone, internal standard 19:0 galactocerebroside was applied to the TLC powder.

After washing the eluted glycolipids by solvent partitioning as above, we determined the fatty acids in the samples by GLC with a DEGS column (17). Small corrections were made for the peaks in the blanks, mainly 16:0, 16:1, 18:0, and 18:1. In order to keep the blanks below 1 μg, we found it necessary to use redistilled solvents, methanolic-HCl prepared in the laboratory (not bought), freshly made TLC plates which were washed with the same solvent prior to activation (60 min at 112°C), and silica gel kept from contact with plastic or storage in wooden boxes. The sample's content of glycolipid was calculated by adding up the number of moles of each fatty acid found (based on comparison with the GLC response to the added 19:0 standard).

HGS was synthesized by an improved method (7), using equimolar amounts of bromohexane and glucosyl sphingosine, and heating for 5 hr at 100°C. The analogous compound, *N*-hexyl-*O*-galactosyl sphingosine, was made similarly for comparison. Cholesterol was determined colorimetrically on the chloroform eluate from the first silica gel column.

[4] Robertson, W. V. B., personal communication.
[5] Hyun, J. C., and N. S. Radin, unpublished work.

TABLE 2. Enzyme activities in fibroblasts cultured with HGS

	β-Glucosidase		α-Glucosidase	Phosphatase	Galactosidase	Hexosaminidase
	pH 4	*pH 5.3*				
Normal cells	104 ± 41	55 ± 19	98 ± 28	2430 ± 620	934 ± 340	5380 ± 1200
Gaucher cells	13 ± 5	10 ± 5	83 ± 40	2050 ± 550	657 ± 240	4670 ± 1500
Experiment 1—11 days						
Control CA	93	40	74	1480	935	3580
+HGS 200 μg	2	0	93	1290	860	3270
Experiment 2—28 days						
Control PM	38	27	91	2390	338	4370
+HGS 95 μg	4	1	165	3680	1380	5850
Experiment 3—28 days						
Control ST	77	49	107	2030	1030	6760
+ HGS 95 μg	2	0.5	115	2490	1270	8110
Experiment 4—28 days						
Control ST	79	44	48	1450	713	4710
+ HGS 95 μg	1	0.3	90	2690	1060	7420
+ HGalS 95 μg	83	38	24	1450	678	5890

Enzyme activities are nmoles of substrate hydrolyzed per hr per mg of protein. The top line gives average values (and SD) from 10 normal cell lines; the second line gives averages from 4 Gaucher cell lines. One plate of cells was used in each experiment, except in Expt. 2, where two plates were pooled. The weight of HGS shown is the total amount added during the entire span of growth. HGalS is the galactose analog of HGS. The medium used in Expt. 1 had no added ascorbic acid.

TABLE 3. Effect of HGS on fibroblasts grown 28 days with a total of 95 μg/plate of HGS (20 plates total)

	Normal Cell ST		Normal Cells PM		Gaucher Cells
	−HGS	+HGS	−HGS	+HGS	−HGS
Cell yield[a]	6.21	9.42	4.91	5.59	3.90
Cerebroside[b]	0.39	0.80	0.29	0.85	0.44
GL-3a[b]	1.68	1.96	1.83	2.68	1.64
Cholesterol[b]	24	22	20	21	21
Total lipid[b]	166	167	152	166	163
Total protein[b]	570	570	590	570	440
Cerebroside[c]	2.42	7.54	1.42	4.75	1.72

[a] mg dry wt/plate.
[b] mg/g cells.
[c] mg/plate.

RESULTS

Enzyme changes

The top part of **Table 2** shows the enzyme activities of normal and Gaucher fibroblasts after 19 days in culture, assayed with aryl substrates. The data for the latter cells were derived from two patients with the adult form and two with the infantile form. As previously reported (18), only β-glucosidase activity was significantly low in Gaucher fibroblasts and there was proportionately less activity at pH 4 than at 5.3. The major part of Table 2 shows that the activity of β-glucosidase in cells cultured with HGS was decreased to levels somewhat below those found in the mutant cells. A point of difference is that the decrease was greater at pH 5.3, rather than 4.0.

The effect of HGS on the other hydrolases studied was a distinct enhancement, in most cases, analogous to the increases reported in patients with specific hydrolase deficiencies (19). The galactosyl analog of HGS (last line, Table 2) produced no effect on β-glucosidase; possibly its only effect was a decrease in α-glucosidase.

HGS produced the same effect on glucosidase activity when added to homogenates of normal cells. The amine acted as an effective competitive inhibitor with respect to methylumbelliferone glucoside, with K_i about 2.5 μM. Similar kinetics have been observed with rat spleen and glucocerebroside as substrate (7). The effect of 7 μM HGS on GL-1a hydrolase in normal fibroblast homogenates was found to be a 97% reduction in activity. A culture of Gaucher cells showed 5% of the normal activity with the natural substrate.

Morphologic changes in the cells could be seen with media containing HGS concentrations above 4.5 μM and the cells died within a week. In the lower concentrations used in this study, toxic effects could not be seen by light microscopy and the cultures could be maintained at least 4 weeks.

Lipid changes

Visual examination of the total lipids on TLC plates showed that the treated cells had a normal level of cholesterol and ceramides (the precursor of GL-1a). Two blue bands could be detected in the extracts of HGS-treated cells when the plate was sprayed with bromothymol blue; this color is characteristic of amines. One band corresponded to HGS while the faster band corresponded to *N*-hexyl sphingosine. Evidently the fibroblasts absorbed HGS from the medium and gradually hydrolyzed the glucoside linkage. Experiments with [^{14}C]HGS in rats showed a similar ability to degrade HGS to hexyl sphingosine (20).

Spraying the plates with a charring spray showed that the amounts of the amines were relatively very small and that glucocerebroside could be seen only in the treated cells. Lactosyl ceramide could not be seen but GL-3a was quite visible. No concentration differences could be seen in the major phospholipids of the HGS-treated cells.

TABLE 4. Fatty acid distribution in cerebroside and GL-3a from normal fibroblasts grown 28 days

Fatty acid	Cell Line ST				Cell Line PM			
	Cerebroside		Trihexoside		Cerebroside		Trihexoside	
	−HGS	+HGS	−HGS	+HGS	−HGS	+HGS	−HGS	+HGS
	% of total fatty acids							
16:0	14.9	19.4	14.7	15.5	17.9	14.9	13.7	13.2
17:0	4.2	4.6	1.8	1.7	5.9	4.0	1.9	1.3
18:0	4.2	5.6	2.7	2.0	6.7	8.1	2.2	2.4
18:1	0	0.4	1.1	0.1	0.7	2.0	0.1	0.1
20:0	2.9	1.4	1.3	1.6	3.1	2.3	1.8	1.9
22:0	15.5	16.3	14.4	14.4	13.7	15.0	12.8	12.6
22:1	0	0	0.2	0.9	0	0	0.2	0.4
23:0	10.4	5.0	3.1	3.0	4.6	4.4	3.6	3.5
23:1	0	0	0.5	1.2	0	0	0.8	0.9
C_{24}[a]	48.0	47.3	56.7	57.5	47.9	49.3	61.2	61.6

[a] The last line gives the percent of 24:0 + 24:1 since the two acids were poorly resolved.

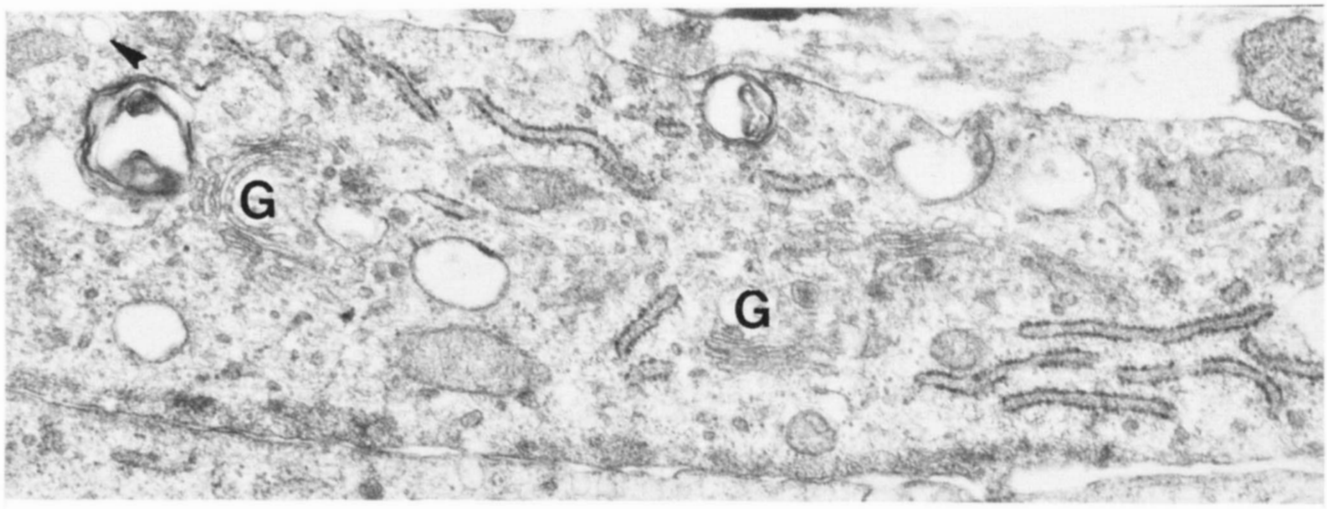

Fig. 1. Electron micrograph of normal fibroblast, showing prominent Golgi zones (G), abundant rough endoplasmic reticulum cisternae, and poorly formed myelin figures. Pinocytotic vacuoles are indicated by arrow head. Mag × 35,420.

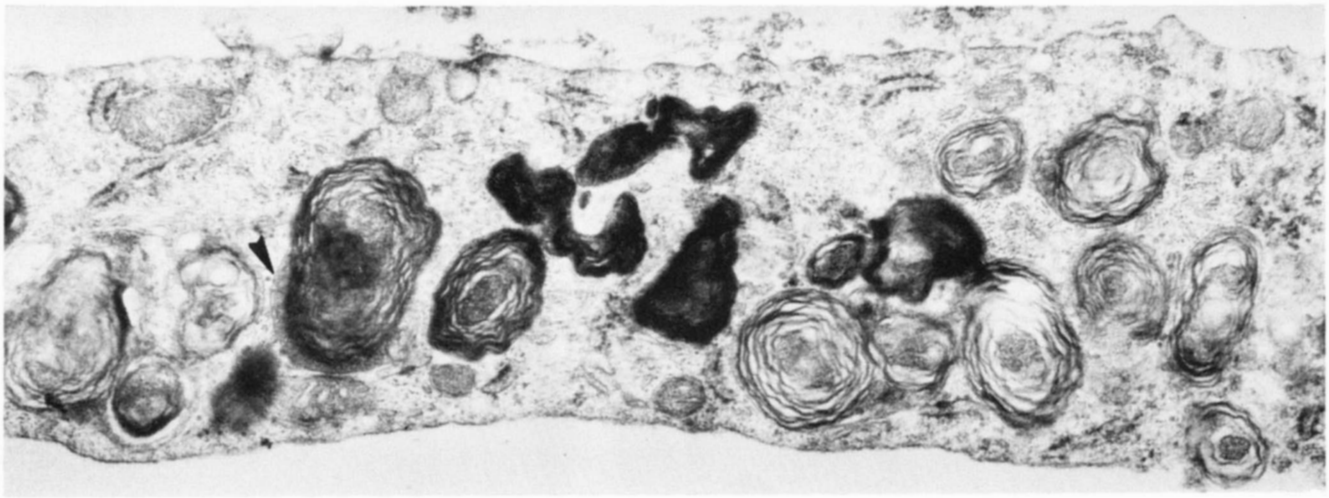

Fig. 2. Electron micrograph of fibroblast treated with HGS. Note numerous membranous cytoplasmic inclusions, some consisting of loose lamellae and others of dense osmiophilic material. A few are bounded by a single membrane (arrow head). Mag × 35,420.

Quantitative data on the cells treated for 28 days with HGS (**Table 3**) show that HGS induced three changes: glucocerebroside concentration increased 2- or 3-fold, GL-3a concentration increased somewhat less, and the dry weight of cells per tissue culture plate increased. In terms of total GL-1a per plate, the increase was 3-fold in both series of experiments. A set of Gaucher cells, grown without HGS, yielded values much like those of the normal cells.

The distribution patterns for the fatty acids in GL-1a and in GL-3a showed no effect of HGS exposure (**Table 4**). Fatty acid data for the Gaucher cells, not shown here, showed an elevated 16:0 and 18:0 and diminished 20:0 and 22:0 in GL-1a. The Gaucher GL-3a distribution was similar to that shown in Table 4.

Morphological changes

Untreated fibroblasts, Gaucher fibroblasts, and cells grown in the presence of hexyl galactosyl sphingosine showed normal morphology, with prominent Golgi zones, focally dilated rough endoplasmic reticulum, rare myelin figures, lipid bodies, normal nuclei, abundant cytoplasmic filaments, pinocytotic vacuoles, and collagen along the cell surfaces (**Fig. 1**). Cells grown in the presence of HGS contained many membranous cytoplasmic inclusions 0.5–1.5 μm in diameter. Most inclusions consisted of concentric layers of smooth membranes arranged loosely around granular material in the center (**Fig. 2**). Other membranes were very compactly arranged into irregular osmiophilic bodies. The inclusions appeared to be in close proximity to the rough endoplasmic reticulum, in relation to the Golgi apparatus, or in direct continuity with cytoplasmic filaments. Many were bounded by a single membrane or formed part of the contents of autophagic vacuoles. The rough endoplasmic reticulum, Golgi zones, mitochondria, nucleus, cytoplasmic filaments, pinocytotic vacuoles, and collagen in the HGS-treated cells appeared normal.

DISCUSSION

These experiments show that HGS competitively inhibits the activity in human fibroblasts of the enzymes that hydrolyze methylumbelliferone glucoside and GL-1a. Studies with human placental glucocerebrosidase have also demonstrated the strong inhibitory power of HGS (21). These observations indicate that the human glucosidases resemble rat and mouse glucosidases, at least in the region of the active site.

Our experiments also show that human fibroblasts can absorb HGS from the medium and that the inhibitor reaches the glucosidase in the cells, producing primarily a blockade of the catabolic metabolism of GL-1a. While we did not determine the amount of HGS accumulated in the cells, it is evident from the considerable dilution used in assaying the β-glucosidase activity (Table 1) that the amount was more than ample for blocking all the enzyme within the cells. Only secondary effects were produced on the other hydrolases assayed.

The extent of GL-1a accumulation in the treated cells was not as great as is observed in some Gaucher tissues of intact patients. However the incubation period used in this study cannot be expected to yield accumulations corresponding to those seen over many years in the adult patients. Moreover, skin (the source of our cells) is not noted for its high accumulation of GL-1a in the disease. The normal levels of GL-1a in our untreated Gaucher cells suggest that glucocerebroside turnover is not rapid in these cells or that the hydrolase activity, even in mutant cells, is relatively high compared with other tissues. There is the possibility that excess GL-1a is lost from cultured cells when the medium is replaced with fresh medium (the calf serum may equilibrate with GL-1a on the cell surface).

The inhibitor also produced some accumulation of GL-3a, which may be a characteristic of the disease. Two studies of Gaucher spleen (22, 23) reported no increase in GL-3a concentration, but one laboratory reported almost double the normal value in four spleens (24) and another found a doubling and a 20-fold increase in two other spleens (25). Cultured Gaucher fibroblasts were reported to have markedly elevated GL-3a levels (5); indeed, the elevation was greater than that seen in GL-1a. An increased GL-3a concentration might arise because of increased availability of GL-1a, its precursor. Another possibility is that GL-1a is an inhibitor of α-galactosidase, which degrades GL-3a. A third possibility is that GL-3a is a major constituent of the cytoplasmic particles that accumulate in Gaucher organs and in our HGS-treated fibroblasts. Lysosomes of liver are relatively enriched in neutral glycolipids, particularly GL-3a (26), and it is possible that the accumulating Gaucher deposits are derived from lysosomes. We are inclined to reject a fourth hypothesis, that there is a "damming up" of all precursors (via hydrolysis) of GL-1a. If this were so, one would expect to see an accumulation of GL-2a, but this has not been observed in patients or in our cells.

The values we observed for GL-1a in normal cells are similar to those reported in similar cells (4). However the latter study found GL-3a concentrations of 0.56–0.78 nmoles/mg while ours were 1.52 and 1.65 nmoles/g. Dawson, Matalon, and Dorfman (4) showed that the concentration in fibroblasts of GL-3a, but not of GL-1a, was somewhat affected by the nature of the culture medium. Perhaps GL-3a content in all cells is particularly sensitive to environmental conditions.

Our findings on fatty acid distribution are somewhat different from those reported before (4) and are more typical of mammalian glycosphingolipids in that we found very little oleate and much more of the very long chain acids.

Although the chemical and enzymatic changes induced in normal cells by HGS closely mimicked the chemical phenotype found in Gaucher tissues, the cellular pathology demonstrated by electron microscopy was distinctly atypical. A variety of patterns may be seen in the inclusions that accumulate in fibroblasts cultured from patients with various lipidoses and other storage diseases (6, 27). Inclusions with morphology similar to that produced by HGS have been observed in fibroblasts from patients with Niemann-Pick disease, types A (27) and C (6). In addition, chloroquine and chlorpromazine, which are lipoidal amines and thus resemble HGS, produce similar cytoplasmic inclusions in vitro (28, 29) and in vivo

(30). Therefore the possibility exists that the inclusions in our treated fibroblasts are primarily deposits of the inhibitor complexed with acidic macromolecules or β-glucosidase. Glew and Lee (31) and Glew et al. (32) have shown that the particles accumulating in Gaucher spleen are not only rich in GL-1a but also contain appreciable amounts of glucocerebrosidase, as well as other substances. Perhaps the particles in our cells were similar but could not form the typical inclusions because the cells did not synthesize a compound necessary to orient the stored glycolipid into the tubular structure.

The activity of β-glucosidase can now be regulated in normal fibroblasts by controlling the concentration of HGS added to the culture medium. This model may permit the molecular analysis of the storage process and clarify further the sequence of chemical reactions that produces cellular pathology in Gaucher's disease. A detailed understanding of this process may be necessary since proposed therapies, such as enzyme supplementation, may require correction of several factors in order to be effective.

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