Glucosylceramide and the Level of the Glucosidase-Stimulating Proteins

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The concentration of β-glucosidase-stimulating proteins (called cohydrolase here) was measured in mouse liver and brain by immunoassay. Factors that might influence the levels of cohydrolase were examined. Injecting mice with an inactivator of glucosidase (conduritol B epoxide) rapidly produced elevations in liver glucosylceramide (the enzyme's substrate) and in liver and brain cohydrolase. Injection of glucosylceramide emulsified with Myrj 52 produced the same two effects in liver but not in brain. The increases in cohydrolase level induced by the enzyme inhibitor persisted in both organs for at least seven days, reaching 61–70% above the normal level. Injection of emulsified galactocerebroside, sphingomyelin and mixed glucosphingolipids but not of ceramide also produced rises in cohydrolase level. An increase in cohydrolase level resulted from injection of phenylhydrazine, which produces hemolysis and consequently an increased workload for the glucosidase of liver. When the enzyme inhibitor and/or larger amounts of glucosylceramide emulsion were injected (750 mg/kg body weight), increases in liver weight of 13 to 37% appeared within one day. The increased weight was characterized by increases in the weights of protein, total lipid and DNA and a very high increase in glucosylceramide level. These procedures have produced a rapidly developing model version of Gaucher disease in mice. Injected glucocerebroside also induced an elevated level of glucosidase activity.
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Gaucher disease is a heritable human disorder characterized by a deficiency in the activity of glucosylceramide (GlcCer) glucohydrolase (EC 3.2.1.45). A heat-stable glycoprotein preparation capable of stimulating this enzyme was discovered in the spleen of a patient with the disorder (1,2). The GlcCer glucosidase activator, variously called Factor P (1), heat-stable factor (3), sphingolipid activator protein 2 (4) and cohydrolase sphingolipid I (5), will be referred to here as cohydrolase (CH). The activator, a mixture of similar low molecular weight proteins, occurs in a variety of normal tissues and also activates two other sphingolipid hydrolases, galactosylceramide (GalCer) galactosidase and sphingomyelinase (4,6).

Considerable accumulations of both cohydrolase sphingolipid-I (CH) and GlcCer are found in the spleen of patients with Gaucher disease. In addition, there is a pathologically large increase in the size of the reticuloendothelial system, represented by a generalized increase in functioning tissue rather than the simple accumulation of CH and GlcCer. While accumulation of the enzyme's substrate is readily explained by the inadequate hydrolase activity, the mechanism causing CH accumulation and organ hypertrophy is still obscure.

The study reported here tests the hypothesis that the concentration of GlcCer in a reticuloendothelial tissue is an important controlling factor for the tissue level of cohydrolase and organ size. If this is correct, interventions that increase the level of GlcCer ought to increase the level of CH and the organ size. We report here on the results of three such approaches with mice.

MATERIALS AND METHODS

Materials. Most of the materials have been described (5,7). Conduritol B epoxide, which specifically inactivates glucocerebrosidase in mice (8), was prepared chemically from *myo*-inositol (9,10) and injected intraperitoneally in saline solution at a dose level of 0.1 mg/g body weight. Sphingomyelin and GalCer were isolated from bovine brain, ceramide (stearoyl sphingosine) was prepared chemically and GlcCer was isolated from a human Gaucher spleen. Calf thymus DNA, Type V, was from Sigma Chemical Co. (St. Louis, Missouri).

A mixture of glycosphingolipids was prepared from outdated human red cells by conventional procedures: extraction with hexane-isopropanol, alkaline methanolysis of the ester lipids and silica gel chromatography with chloroform/methanol. The mixture consisted primarily of di-, tri- and tetraglycosylceramides, with a small amount of an unidentified phospholipid migrating on TLC plates between lactosylceramide and hydroxy GalCer. We are calling these lipids "glucolipids" because they are catabolized to GlcCer.

Each sphingolipid was coevaporated from solution with half its weight of a low-toxicity nonionic detergent, Myrj 52 (polyoxyethylene 40 stearate, ICI America Inc., Wilmington, Delaware) and emulsified in 0.9% saline by slight warming in an ultrasonic bath for 15 min. The concentration of sphingolipid was 9 or 45 mg/ml. The slightly milky emulsion was injected intraperitoneally at a dosage of 0.15 or 0.75 mg/g.

Animals. Hsd CFl mice (Harlan Industries), nominally 16 days old, were matched by computer into groups of five, each group having a similar mean and distribution of weights; two groups were chosen at random from a computer-generated table for each control or treatment (11). Each nursing mother was assigned two groups of five. The brains or livers from each group of five were pooled and each homogenate was assayed in duplicate, so that the average values cited in this paper (and standard deviations, where shown) were obtained from four values derived from 10 mice. The purpose of choosing this unusual approach was to reduce the importance of animal variability as well as the analytical workload. The analytical data from the two five-mouse groups in each treatment group typically differed by 5% or less.

Assay methods. CH was measured by a peroxidase-linked immunoassay after extraction, heat treatment and purification with a size exclusion column (5). This method of measurement is much more sensitive than the original assay method (stimulation of glucosidase) and more accurate because it avoids interference by endogenous enzyme and activation inhibitors (12,13).

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Glucosidase was assayed with [^{3}H]GlcCer (14). This assay does not detect the cytosolic glucosidase of unknown function (15), nor is it dependent on the tissue content of CH since an exogenous activator (taurocholate) is included in the incubation medium. In some of the liver samples (see Results), a considerable accumulation of GlcCer was seen and the possibility existed that this lipid might interfere with the assay. However, comparisons with 1, 2 and 4 mg of liver per incubation tube yielded similar specific activities with control and experimental groups. Evidently the radioactive micellar substrate did not equilibrate with the suspension of GlcCer in the homogenate during the 1-hr incubation.

Protein was determined with the Folin-Ciocalteu reagent with bovine serum albumin as standard (16); DNA was determined by the diaminobenzoic acid method (17).

The liver homogenates were analyzed for lipids after extraction with hexane/isopropyl alcohol (18). Total lipids were determined by weighing the extract nonvolatile contents. TLC was performed with precoated plates of silica gel 60, 10 × 10 cm (EM Laboratories), which were heated with a charring reagent. Some plates were quantitated with a computer video camera (19). Chloroform/methanol/aqueous 0.02% KCl (60:35:8, v/v/v) was used for the polar lipids; chloroform/methanol/HOAc (90:2:8, v/v/v) was used for ceramide.

RESULTS

Inactivation of glucosidase with conduritol B epoxide (CBE). In experiment 1, mice (4.9–7.8 g) were injected at time zero with either saline (control mice) or a 0.9% solution of CBE in saline. Liver and brain homogenates were made in 0.25 M sucrose and compared with the membranous and cytosolic fractions (140,000 × g for 30 min). Virtually all of the glucosidase activity (>95%) in the control mice was found to be in membrane-bound form. As noted before (20), CBE injection produced almost a complete loss of glucosidase activity within 1 hr. There was no noticeable recovery of activity within 5 hr.

The CH concentrations in control brain and liver (Table 1) were similar to those found before (5). CBE injection produced increases in liver total CH levels, 28% above the control value by 3 hr and 45% by 5 hr. An increase of 14% was visible even within 1 hr. While this is a relatively small increase, it is likely to be significant in view of the monotonic nature of the concentration/time relationship.

Brain CH rose in response to CBE too, but only after a delay. By 5 hr after injection the concentration had risen 11% compared to zero-time animals and 24% compared to 5-hr controls.

The cytosol from brain contained no detectable CH, but that from liver contained about 10% of the total activator protein (Table 1). The changes in CH noted above were mirrored in the membrane-bound CH, but the amount of cytosolic CH in liver did not change after CBE injection.

Summing the values for the CH in the cytosolic and membranous fractions yielded recoveries that were 81–93% (87% average) of the values in the unfractionated homogenate. This suggests that CH was relatively stable to enzymatic degradation during the subcellular separation step. Only the total CH was measured in the subsequent experiments.

Longer-term effects of CBE. In experiment 2, the injection of saline or epoxide was repeated as above and the mice were killed after 1, 4 or 7 days. All animals, control and experimental, were killed at the same time of day to eliminate the possibility of diurnal changes. Homogenates were prepared in extraction buffer (5 mM phosphate pH 7, 1 M NaCl) (5) instead of sucrose solution.

Both organs showed some restoration of glucosidase activity within 1 day (left side of Table 2), and a substantial restoration several days after inactivation of the enzyme. Similar data were found elsewhere (8,14). Glucosidase activities in control mice peaked in the livers of the four-day group (at weaning time) but showed decreasing values in brain with age. A similar drop in rat brain glucosidase activity with the approach of weaning has been found (21), probably a reflection of a decreasing turnover rate for gangliosides.

In the CBE-treated mice, the CH concentrations in both organs (right side of Table 2) were markedly elevated over the control values at each time point. The degree of elevation was greater than that seen after 5 hr (Table 1), and it is evident that the effect of CBE continued well after all of the inhibitor had been excreted (22).

While the CH level in liver was quite constant with increasing age over the seven-day period studied, brain showed a gradual decrease. This may reflect the known slowdown in ganglioside turnover that occurs at this age.

A repetition of the first two experiments (experiment 3) again showed that CBE produced increases in liver total CH (19% after 1 hr, 54% after 4 days). As before, brain showed an increase only after an initial delay—65% above normal by 4 days.

Effects of glucosphingolipid injection. A second mode of manipulating GlcCer levels in tissues was tested. In experiment 4, mice (6.2–7.8 g) were injected with either detergent solution in saline (control mice) or with the emulsion of red cell ceramide oligosaccharides (dosage about 0.15 mg/g body weight). One set of 20 mice (10 control, 10 experimental) was killed after one day. A second set was injected three times—at time zero, after one day and after four days; they were then killed one day later, five days after starting.

TABLE 1

Changes in Cohydrolase Following Injection of Mice with Conduritol B Epoxide

	Cohydrolase concentration (ng/mg wet tissue) in liver in mice killed at				Cohydrolase concentration (ng/mg wet tissue) in brain of mice killed at			
	0 hr	1 hr	3 hr	5 hr	0 hr	1 hr	3 hr	5 hr
Controls								
Homogenate	3.34			3.26	1.67			1.50
Cytosol	0.30			0.31	—			—
Membranes	2.62			2.63	1.52			1.46
CBE-treated								
Homogenate		3.81	4.28	4.72		1.66	1.68	1.86
Cytosol		0.31	0.32	0.30		—	—	—
Membranes		2.90	3.43	3.81		1.48	1.62	1.78

TABLE 2

Longer-Term Changes in Glucosidase and Cohydrolase Levels in Liver and Brain after Injection of Glucosidase Inactivator, Conduritol B Epoxide

Group	Glucosidase activity (nmol/hr/mg)	% of control	Cohydrolase concentration (ng/mg)	% of control
Liver				
Controls, 1 day later	3.12 ± .15		3.17 ± .18	
+ CBE 1 day later	0.24 ± .11	8	5.10 ± .08	161
Controls, 4 days later	3.63 ± .07		3.05 ± .13	
+ CBE 4 days later	1.58 ± .06	44	4.90 ± .08	161
Controls, 7 days later	2.94 ± .03		3.10 ± .08	
+ CBE 7 days later	1.24 ± .03	42	4.58 ± .06	156
Brain				
Controls, 1 day later	1.23 ± .03		1.83 ± .13	
+ CBE 1 day later	0.14 ± .06	11	3.08 ± .09	168
Controls, 4 days later	1.07 ± .08		1.68 ± .06	
+ CBE 4 days later	0.27 ± .03	25	2.87 ± .05	171
Controls, 7 days later	1.06 ± .03		1.57 ± .05	
+ CBE 7 days later	0.48 ± .04	45	2.37 ± .05	151

Experimental design as in Table 1. Groups of five mice each were injected at time 0 with saline or CBE. Data, based on wet weight of tissue, are calculated from two groups killed at each time point.

The levels of brain CH and glucosidase did not change in either set. This may mean that entry of the lipid emulsion was blocked by the blood-brain barrier. However, both proteins responded positively in liver. The increase in liver glucosidase activity was 14% by one day and 23% by five days (Fig. 1). The CH concentration responded more vigorously to the glycolipid load: a 33% increase within one day and a 60% increase by five days (Fig. 2). Except for the glucosidase activities after one day, the observed increases are clearly significant. These results can be interpreted to mean that the injected glucosphingolipids had reached the liver and, after catabolic degradation to GlcCer, had induced an elevated level of CH and, to a smaller extent, of glucosidase. An elevation in liver glucosidase activity following injection of red cell sphingolipids has also been observed in rats (23).

The injection of erythrocyte glucosphingolipids was repeated (experiment 5) and again there was no change in the level of brain CH while the level of liver CH rose 34% in one day.

Effect of injecting simple sphingolipids. Since CH also activates GalCer and sphingomyelin hydrolases (4,6), we thought that elevations in the tissue levels of these lipids might act like the hypothesized elevation in GlcCer in experiments 4 and 5. A similar injection schedule and dosage (three injections) was followed in experiment 6 with individual sphingolipids—ceramide, GalCer and sphingomyelin—and the animals were analyzed only after five days. As expected from the previous experiment, there were no significant changes in brain, presumably because of the blood-brain barrier. In liver, only small changes were seen in the glucosidase activity, except for an 11% rise after sphingomyelin injection (left column of Table 3). Cohydrolase levels in liver (right column of Table 3) showed distinct increases after injection of sphingomyelin (25%) and GalCer (23%). Ceramide injection produced no clear effect.

The injection of the three sphingolipids was repeated (experiment 7), but with only one injection of lipid and animals being killed on the fifth day. Even with this lower lipid load, the glucosidase activity in liver was again slightly elevated (7%) by sphingomyelin injection. The CH concentration in liver again showed a distinct positive response to loading the animals with sphingomyelin and GalCer (+21% with both) but not to ceramide.

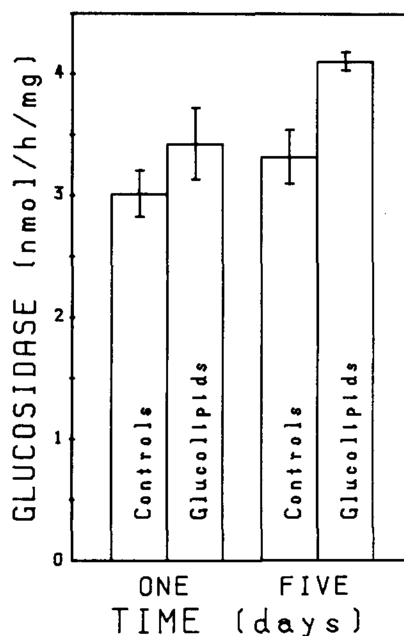

FIG. 1. Changes in liver glucosidase activity produced by injecting emulsified glucolipids from red cells (experiment 4). Liver glucocerebrosidase was assayed as described in the text. The left bar in each pair is from the control mice killed after 1 or 5 days. Error bars are calculated from the four analytical values obtained from the two five-mouse pools used for each bar (while the standard deviation formula used was used for the calculation, this is not strictly the same as the standard deviation obtained from single analyses of four different mice.)

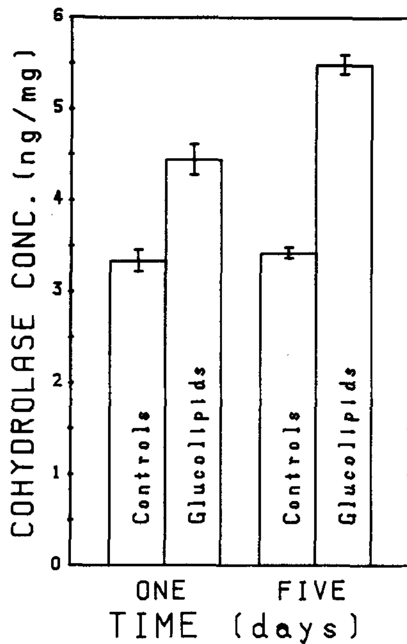

FIG. 2. Changes in liver cohydrolase concentration produced by injecting glucosphingolipids from red cells (experiment 4). Animals are the same ones indicated in Fig. 1.

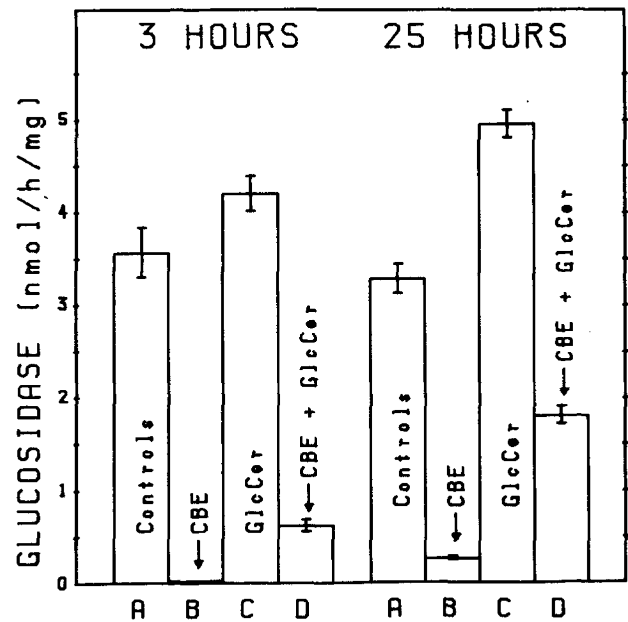

FIG. 3. Changes in glucosylceramide glucosidase of liver on injecting emulsified ceramide (A), emulsified ceramide with CBE (B), emulsified GlcCer (C) or emulsified GlcCer with CBE (D). Data are shown for mice killed 3 hr and 25 hr after injection (experiment 8).

TABLE 3

Effects on Liver of Injecting Sphingolipid Emulsions

	Glucosidase activity (nmol/hr/mg tissue)	Cohydrolase concentration (ng/mg tissue)
Myrj controls	2.78 ± .14	3.64 ± .15
Ceramide/Myrj	2.51 ± .22	3.46 ± .11
Galactosylceramide/Myrj	2.92 ± .11	4.46 ± .18
Sphingomyelin/Myrj	3.09 ± .25	4.54 ± .14

Each mouse was given three injections of detergent solution or lipid emulsion at time 0, after 1 day and after 4 days and then was killed after 5 days. Data reported are the averages of four analyses derived from two sets of five mice each.

The above loading experiments are similar to those of Kampine et al. (23), who showed that injecting rats with red cell sphingolipids (including sphingomyelin) induced a rise in liver sphingomyelinase. It appears likely that injected sphingomyelin and GalCer reach the liver.

In experiment 8, loading was carried out with emulsified GlcCer. The dosage of lipid in this experiment was fivefold higher, 0.75 mg/g body weight. Because of the difference in molecular weights between GlcCer and red cell glycolipids, the molar dosage of GlcCer was actually somewhat greater than fivefold higher. The control mice were injected with emulsified ceramide (at the same molar dosage as the cerebroside) instead of with detergent alone. In addition, since inactivation of tissue glucosidase should protect the GlcCer load against catabolic loss, half of the control and cerebroside-injected mice were also injected with CBE as in experiment 1. The animals were killed after 3 and 25 hr; only the livers were analyzed.

As seen before, the glucosidase levels in CBE-injected mice were virtually zero at the 3-hr time point, and some reappearance of active enzyme molecules was visible at the 25-hr point (Fig. 3, bars B). GlcCer injected into normal mice (bar C vs bar A) produced distinct increases in enzyme activity: 18% and 51% above normal after 3 and 25 hr, respectively. A similar augmentation in glucosidase activity was seen in the mice injected with both CBE and lipid (bar D vs B). The reappearance of enzyme activity was remarkably fast in the latter animals: 16% of normal at 3 hr and 55% of normal at 25 hr. It appears likely that the GlcCer absorbed by the liver induced more rapid synthesis of the glucosidase or protected it against normal catabolic loss.

Cohydrolase responded similarly (Fig. 4), with the rise in concentration being greatest 25 hr after injecting the combination of inhibitor and glucolipid (bar D is 70% higher than bar A). Even in a short interval (3 hr) after CBE or GlcCer injection, a small but distinct increase in CH was visible. As with the previous experiment with CBE (Table 1), CH synthesis or level seemed to respond quickly to an increased glucosidase workload. Calculation of the total CH in liver, taking into account the liver growth (see next section), shows that the amount of CH in the CBE/GlcCer animals was 96% above normal within 25 hr.

Liver enlargement. The liver weight (Fig. 5) was markedly increased by all three treatments of experiment 8, especially with the combination of CBE and GlcCer. The maximal effect was a 37% increase at 25 hr.

The control livers after 25 hr contained, on the average, 49.5 mg protein (18.1% of total weight), and the CBE/GlcCer livers each contained 77 mg protein (20.5% of total weight). Thus the gains in liver weight were accompanied by an even greater gain in total organ protein,

56%. This shows that the increase in liver size could not be the result of hydration or fat storage. This sudden enlargement was not a reflection of a generalized increase in body weight.

Since experiment 8 was done with controls injected with ceramide/Myrj, the possibility had to be considered that this emulsion had a toxic effect that made the livers smaller than those of the animals injected with GlcCer/Myrj. This question was tested in experiment 9, in which two five-membered groups of control mice were given Myrj alone (no ceramide) and two groups were given Myrj + GlcCer + CBE at the same dosages used in experiment 8. After 25 hr, the average body weights were 5.79 g and 5.50 g for the control and experimental mice (−5%). The average liver weights were 243 mg for control mice and 304 mg for experimentals (+25%). The effect of this treatment on CH concentration, as observed in the previous experiments, was to raise the level considerably (+77%). Injected GlcCer again speeded the restoration of glucosidase activities (to 49% of normal). Thus the effects on liver weight, CH level and glucosidase activity found in experiment 8 were repeatable and not artifacts due to the use of ceramide in the control animals.

In addition we tested the possibility that the Myrj in the control animals might have had some effect (experiment 10). Groups of 10 mice were injected with either *a*, saline; *b*, saline + CBE; *c*, saline + CBE + Myrj + ceramide; *d*, saline + CBE + Myrj; or *e*, saline + Myrj, and were killed 25 hr later. The individual liver weights were measured to permit use of analysis of variance with the conservative Bonferroni Multiple Comparison procedures (Table 4). The body weights were not significantly affected by the treatments. The two controls, *a* and *e*, also did not differ significantly with respect to liver weight (i.e., Myrj injection did not affect the liver weight). However, the two control groups differed from the three CBE groups with a high degree of significance. The largest effect, an increase in liver weight of 22% ($p < 0.0001$), was seen on comparing the CBE/ceramide/Myrj group with the Myrj control mice.

When the groups were compared on the basis of percentage of liver (Table 4, column 3), highly significant differences ($p < 0.0001$) were seen between the CBE groups and Myrj controls. The differences were significant with regard to the saline control mice also, but only when the ordinary t-test was used, evidently because of the greater statistical noise due to variability in body weights.

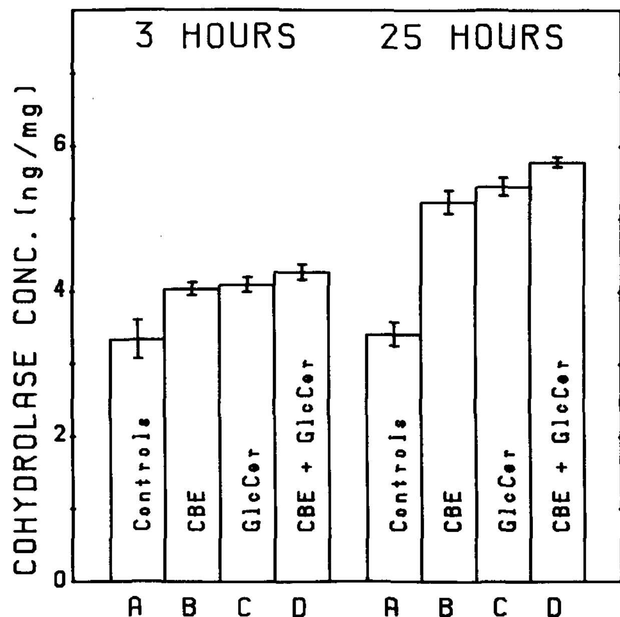

FIG. 4. Changes in cohydrolase concentration on injecting emulsified ceramide (A), emulsified ceramide with CBE (B), emulsified glucosylceramide (C) or glucosylceramide with CBE (D). See legend to Fig. 3 (experiment 8).

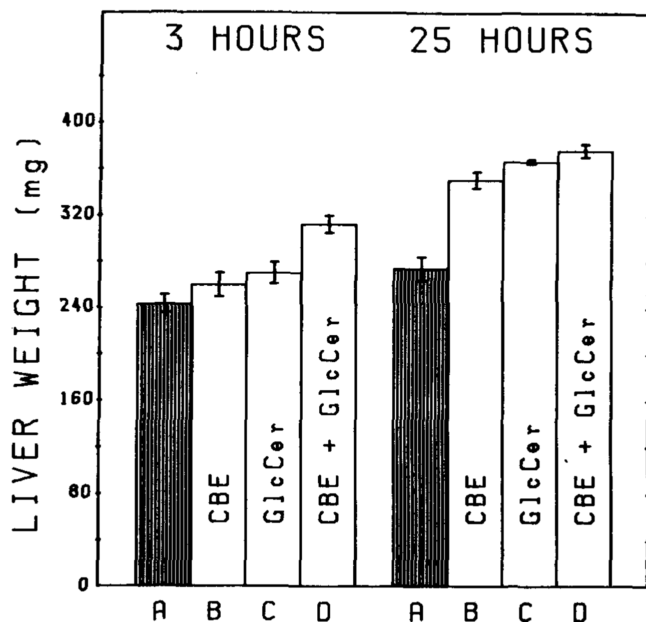

FIG. 5. Changes in liver weight on injecting ceramide (A), ceramide + CBE (B), glucosylceramide (C) or glucosylceramide + CBE (D) (experiment 8). Error bars show deviation from the mean of the two groups of mice (five mice/group).

TABLE 4

Effect of CBE Injection, With or Without Detergent and Ceramide, on Liver and Body Weight

Treatment	Body weight (g)	Liver weight (mg)	Liver/body (%)
a: Saline controls	6.43 ± .43	256 ± 16	4.00 ± .31
b: + CBE	6.51 ± .60	289 ± 17	4.47 ± .42
c: + CBE + Ceramide/Myrj	6.78 ± .59	304 ± 3	4.52 ± .41
d: + CBE + Myrj	6.31 ± .60	282 ± 9	4.52 ± .50
e: + Myrj	6.89 ± .51	249 ± 20	3.63 ± .43

Significant differences (p values): liver weight, 0.0001 (*a* vs *b* and *c*; *e* vs *b*, *c* and *d*); 0.002 (*a* vs *d*); 0.015 (*c* vs *d*); liver/body, 0.0001 (*e* vs *b*, *c* and *d*). Mice were injected with conduritol B epoxide (0.1 mg/g body weight) in saline, with added Myrj (0.27 mg/g) or with added stearoyl sphingosine (0.54 mg/g) in Myrj. They were killed 25 hr later and each animal's liver was weighed separately. The averages and S.D.s are listed. See experiment 10 for details.

The inclusion of ceramide with CBE and Myrj (*c* vs *d*) significantly enhanced the CBE stimulation of liver growth (Table 4). Thus it would appear that part of the growth effect seen in experiment 8, which included ceramide/Myrj in the CBE, was due to a synergistic interaction. This may arise from the fact that ceramide is the lipoidal precursor of GlcCer; the injected lipid may increase the rate of GlcCer synthesis and thus enhance the CBE effect on liver growth.

In none of the groups in this last experiment was there an effect on brain weight. We had previously reported a statistically significant increase of 13% in brain weight (and 9% in liver weight) of mice injected with eight daily doses of CBE, compared to saline control mice (20). Evidently a 25-hr period of glucosidase depletion is too short to produce this intriguing increase in brain weight.

Determination of the protein concentrations in the various livers from experiment 10 showed slightly elevated values in the mice with the heaviest livers: 11.3 and 11.6% in the two groups of saline control mice, *a* and *e*, and 12.3% in the CBE/Myrj/ceramide mice. This confirms the similar observation made in experiment 6.

In experiment 11, a comparison was made of mice injected with GalCer and GlcCer, both emulsified in Myrj and injected at the same dosage (0.75 mg/g). Twenty-five hours later the two groups did not differ significantly in body weight, but the GlcCer animals had livers that were 16% heavier ($p < 0.001$). Thus the weight enhancement, unlike the enhancement in CH concentration by both lipids (Table 3), was quite specific with respect to the sugar moiety of the cerebroside.

The DNA concentrations in the livers of experiment 10 were very similar in all groups (2.44 to 2.49 mg/g), and the weight of total DNA was 20% higher in the larger livers (*c* vs *e*). In experiment 11, the total DNA was 0.611 mg/liver in the animals injected with GalCer and 0.715 mg/liver in animals injected with GlcCer (+17%).

Changes in liver lipid composition. The concentrations of total lipids in the 25-hr livers in experiment 8 were 66, 77, 77 and 102 mg/g wet wt for controls, CBE mice, GlcCer mice and CBE/GlcCer mice, respectively. The maximum increase was $(102 - 66)/66 = 55\%$, a striking change. Even at the 3-hr time point there was a noticeable increase (12%) in liver lipids of the CBE/GlcCer group. The increases in the 25-hr mice were even more marked when compared on a total liver basis: the weights of lipid were 18, 27, 28 and 38 mg per liver for the same four groups (a maximal increase of 111%).

Examination of the total lipids from this experiment by semiquantitative thin layer chromatography (TLC) showed that the animals treated with CBE, GlcCer or the two together had accumulated a striking amount of GlcCer. At the sample level applied to the plate, equivalent to 0.13 mg of liver, the GlcCer spot from the control mice was barely visible while the experimental groups contained GlcCer at a level similar to those of the major lipids.

The GlcCer concentrations were 0.04, 6.5, 7.7 and 29 mg/g liver for the four groups. This lipid accounted for a substantial portion of the increases seen in the total lipids.

Ceramide typically shows a dual spot on TLC plates in which the upper one (very long chain acids) is heavier than the lower one (mainly stearoyl sphingosine) in nonneural organs. The mice injected with emulsified stearoyl sphingosine, compared with Myrj-injected mice, showed a small but definite increase in the intensity of the lower spot, suggesting that a small portion of the injected ceramide had reached the liver. The mice treated with GalCer and GlcCer emulsions showed a slight elevation in the concentrations of both ceramide spots, especially the ones with shorter fatty acids. This kind of fatty acid distribution is characteristic of the GlcCer that accumulates in Gaucher spleen (24) and illustrates how closely our Gaucher mice mimic the natural form.

Quantitative analysis (25) of the liver extracts from experiment 10 revealed ceramide concentrations of about 110 μg/g in the control mice and about double this in the mice given CBE (with or without ceramide).

Responses to internal hemolysis. We next tested a third method of raising the GlcCer concentration in liver. Injection of phenylhydrazine induces hemolysis and an increased requirement on the part of the reticuloendothelial system to destroy the damaged red cells. This was found to induce elevated activities of glucocerebrosidase, galactocerebrosidase and sphingomyelinase in rat spleen (23) and glucosidase in mouse spleen and liver (14). In experiment 12, 5.1-6.8 g mice were given a single injection of phenylhydrazine.HCl (0.04 mg/g body weight) in saline. As noted before (14), the glucosidase activity in liver rose in response (Table 5). Analysis after 1, 2 or 4 days showed activities that were 9, 15 and 54% above each pair of corresponding control groups, respectively. The CH concentrations also rose (Table 5), but with a longer lag period: 0, 11 and 30%, respectively.

DISCUSSION

Our data show that mouse liver, and brain in some cases, can respond to various treatments by developing an elevated concentration of cohydrolase. Three types of treatments produced this effect: inactivation of β-glucosidase by an inhibitor (experiments 1, 2, 3 and 8), injection of certain sphingolipids (experiments 4, 5, 6, 7 and 8) and induction of hemolysis (experiment 12). All three

TABLE 5

Effects of Phenylhydrazine Injection on Glucosidase and Cohydrolase Levels in Liver

	Glucosylceramide hydrolase (nmol/hr/mg tissue)	Cohydrolase (ng/mg tissue)
Controls		
0 time	3.36 ± .05	3.04 ± .05
1 day	3.39 ± .16	3.01 ± .08
2 days	3.17 ± .10	3.00 ± .07
4 days	2.42 ± .04	3.04 ± .05
Phenylhydrazine		
1 day	3.70 ± .17	2.98 ± .05
2 days	3.64 ± .29	3.34 ± .05
4 days	3.72 ± .22	3.96 ± .11

Groups of five mice each received a single injection of phenylhydrazine.HCl or saline and were sacrificed after 1, 2 or 4 days. Details in experiment 12. The data are calculated from two groups sacrificed at each time point.

treatments have in common the effect of increasing the tissue concentration of substrate for one or more of the three enzymes known to respond to cohydrolase (4,6). In the case of the first approach, increased levels of GlcCer are derived from the normal catabolic degradation of tissue glucolipids (gangliosides and ceramide hexosides); with liver there is the additional load of hydrolyzing blood elements as their life spans come to a conclusion. In the case of the hemolytic approach, the increased substrate levels are derived from the hydrolytic breakdown of red cells. Direct demonstration of an increase in GlcCer was shown by TLC in mice injected with CBE and with GlcCer (experiment 8). The same effect has been reported for CBE by Kanfer et al. (26).

Our observed accumulation of CH corresponds to that seen in a human genetic disorder, Gaucher disease. Our findings show that the increase in CH appears rapidly and cannot be attributed to some secondary, late-developing phenomenon that might occur in the natural disorder.

It is apparent from our TLC results that the tissue concentration of GlcCer was many-fold higher than normal in some animals, yet the increase in CH (and glucosidase) was much smaller. It may be that only a portion of the accumulated GlcCer was localized in a region that could influence the formation of CH. Another explanation is that the mechanism by which the CH accumulation was produced has a limited velocity, and longer-term experiments would show increases comparable to those seen in the human disorder. The latter develop over a much longer time.

Cohydrolase was not the only substance found to accumulate in liver as the result of injecting GlcCer. Total and specific activity of glucosidase also increased (Fig. 3) and, in the case of mice that were also given CBE (experiments 8 and 9), the restoration toward normal activity of the enzyme was hastened by the GlcCer. Injection of GlcCer also increased total protein and protein concentration (experiments 8 and 10) and total DNA weight per liver (experiments 6, 7, and 8). These findings may mean that GlcCer causes an actual increase in the concentration of tissue glucosidase or the proliferation of certain cell types that are rich in content of the enzyme. We have recently shown that GlcCer is absorbed by neuroblastoma cells and increases their utilization of [^{3}H]thymidine for the synthesis of DNA (Datta, S.C., Snider, R.M., and Radin, N.S., unpublished data).

The unexpectedly rapid liver growth produced in our mice by accumulating GlcCer (experiments 8, 9, 10 and 11) is consistent with the great enlargements in spleen, liver and bone marrow that develop over time in Gaucher disease. Because of the link sometimes observed between Gaucher disease and proliferation of B-cells, the proposal has been made that GlcCer could stimulate their formation (27). GlcCer stimulates the formation of fruiting bodies in a fungus and wheat (28). The possibility should be considered that this lipid plays a specific role in determining the size of certain organs or in controlling the rate of proliferation of certain cells.

Our finding that GlcCer, but not GalCer or ceramide, reached and entered the liver from the peritoneum rather efficiently finds a parallel in an in vitro uptake study with cultured macrophage (29).

While speculation over a mechanism for the effects of GlcCer may be inappropriate here, it should be mentioned that GlcCer is the lipoidal precursor of the gangliosides. That cells loaded with GlcCer send some of their GlcCer "upstream" (by addition of sugars) has been demonstrated for cultured Gaucher fibroblasts (30). Gangliosides have been reported to stimulate nerve growth in cultures (cf. 31). Ganglioside GM_1 is involved in thymocyte proliferation (32).

Other researchers have reported large, rapid increases in liver and DNA weight as the result of single injections: insulin in diabetic rats (33), trioiodothyronine in rats (34) and lead nitrate in rats (35). Further studies (to be published separately) in this laboratory of the growth phenomenon have confirmed the effect described in this paper.

Our unconventional research design has been used before (36) and has recently been described in more detail (11). It was found in preliminary studies that the primary source of variability in results arose in individual mice, rather than in the analytical procedures. The use of a "multimouse"—a pool of five mice—was adopted to reduce this source of experimental noise. Every experiment was run twice since two multimice were used for each treatment group. Each multimouse was independently assayed in duplicate, so that four values were averaged to yield many of the values reported here. If one mouse in a multimouse group exhibited a highly aberrant value, the effect on the pool value should be greatly reduced by the other four mice, and the chance of the second multimouse in the same experimental group having a similar aberrant value in the same direction would be very small. Since the duplicate enzyme, CH, and protein assays typically agreed within a few percent; since the time studies generally showed monotonic progression; since the two multimice at each point yielded similar values; and since all the experiments involving the same treatment, such as CBE injection, showed identical kinds of responses (smaller or larger increases above the control values), our observed relationships can be considered secure. The values for CH in the various multiple control groups were similar to each other within each experiment (see, for instance, the four control groups—8 × 5 mice—listed in Table 5).

In the case of CH, when 46 control multimice were compared with 56 multimice treated with CBE, sphingolipids or phenylhydrazine, an increased concentration was seen in the livers of every experimental multimouse. A total of 230 individual control mice and 280 experimental mice were used in this study. In one experiment, individual livers were weighed and all weight increases proved highly significant by a standard statistical test.

The idea of selecting animals to produce matched groups, with similar distributions around the mean of a selected variable, has been recommended by several biostatisticians (37-39).

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