Stimulation of Liver Growth and DNA Synthesis by Glucosylceramide

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The nature of the growth-stimulating effect of glucosylceramide was studied. Mice were injected intraperitoneally with emulsified glucosylceramide and conduritol B epoxide, an inhibitor of cerebroside glucosidase. Within one or two days, the liver grew 18–24%, as reported. Two enzymes involved in DNA synthesis also increased more than the weight. The total liver activity of thymidine kinase increased 46–73%, and the total activity of ornithine decarboxylase increased as much as 101%. It is suggested that elevated liver levels of glucocerebroside stimulate cell proliferation through a relatively direct mechanism.
Lipids 23, 508–510 (1988).

It is evident that the growth of liver and spleen in Gaucher patients, who lack a specific glucohydrolase (EC 3.2.1.45), is far more than can be accounted for by accommodation to the weight of stored glucosylceramide (GlcCer), the enzyme's substrate. The chemical nature of the enlarged organs and the mechanisms of the causational sequence still are obscure. In a study in which we injected mice intraperitoneally over an eight-day period with conduritol B epoxide (CBE), an inhibitor of GlcCer glucosidase, we found significant increases in liver and brain size (9% and 13%, respectively) (1). Presumably, the CBE caused an accumulation of the enzyme's substrate (2) and, therefore, induced a temporary model form of Gaucher disease. In a later study (3), we injected emulsified GlcCer once and found that much of the lipid reached the liver and that a rapid enlargement of the liver ensued, 10–37% within 25 hr. Co-injection of CBE with GlcCer enhanced the growth phenomenon. The enlargement was accompanied by an increase in total DNA, lipid and protein (as well as the glucosidase-activator proteins that are known to accumulate in Gaucher spleen); it was evident that the increase in liver size could not be attributed to edema. The detergent alone did not produce a change in liver weight. We have postulated that GlcCer can induce cell division and growth. The study reported here was directed at clarifying the mechanism by which this occurs.

METHODS AND MATERIALS

Materials. Most of the materials, the mice used and the method for selecting them for each experimental group have been described (3,4). The GlcCer, isolated from Gaucher spleen, was emulsified with 3/4 of its weight of Myrj 52, a low-toxicity nonionic detergent (polyoxyethylene stearate, ICI America Inc.), and injected i.p. into 16-day-old mice (3). Labeled thymidine was from ICN and labeled ornithine was from DuPont NEN.

Assays. Thymidine kinase was measured by following the phosphorylation of [^{3}H]thymidine, 182 cpm/nmol, in a tube containing Tris-Cl$^-$ pH 7.5, ethylenediamine tetraacetic acid (EDTA), mercaptoethanol, adenosine triphosphate (ATP), $MgCl_2$, NaF, bovine serum albumin, creatine phosphate and creatine kinase (5,6). The incubation with liver cytosol was performed for 30 min. The resultant thymidine phosphate was isolated with ion exchange paper.

Ornithine decarboxylase was measured by following the release of [^{14}C]O_2 from L-[1-^{14}C]ornithine (22.9 cpm/pmol) in EDTA, Tris-Cl$^-$ pH 7.4, DTT, pyridoxal phosphate (7, 8), and 2-methylornithine (in half of the tubes). Each assay tube contained the supernatant suspension from centrifugation of liver homogenates at 30,000 g for 25 min.

Protein and DNA were determined as described (3).

RESULTS

In one experiment, thymidine kinase specific activity was determined in the livers of mice injected with GlcCer/Myrj in saline, then (24 hr later) with CBE in saline. A day later, the mice were killed, and the livers were analyzed (Table 1). Control mice received two injections of saline alone. The previous observations, increases in protein and DNA that paralleled liver growth, were confirmed. In addition, a distinct rise in thymidine kinase total activity (46%) was seen.

In another experiment, GlcCer/Myrj and CBE were injected as a mixture once, and the animals were killed 24 or 48 hr later. Thymidine kinase total activity rose 73% in 24 hr and subsided to 43% in 48 hr.

A trial with a single injection of GlcCer/Myrj without CBE produced smaller effects, with a 30% increase in total enzyme activity after 24 hr. No significant rise was seen at seven hr.

In vitro tests were run to determine whether the addition of GlcCer could directly stimulate thymidine kinase. Various amounts of GlcCer emulsion (5, 10, 25 and 50 μg/ml) in Tris buffer were added to the incubation mixture just before the addition of liver enzyme. Incubation for 30 or 60 min, with or without GlcCer, produced similar observed activities. In an experiment with 50 μg/ml of GlcCer, with and without 5 μg/ml of phosphatidylserine (from bovine brain, Sigma Chemical Co., St. Louis, MO), with and without a two-hr preincubation, there was also no significant effect of either lipid. However the phosphatidylserine + GlcCer exerted a small inhibitory effect (about 12%). The phospholipid was tested because it is an activator of GlcCer glucosidase.

Another enzyme noted for its responsiveness to various growth promoters, ornithine decarboxylase (9), showed larger responses in activity to GlcCer and CBE (Table 2). This was done as part of the second experiment, above. Again, the liver increase in weight was seen, similar at the two time points. The total activity of ornithine decarboxylase in the stimulated mouse livers was 101% above controls after 24 hr, 64% above after 48 hr.

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Abbreviations: CBE, conduritol B epoxide; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; GlcCer, glucosylceramide.

TABLE 1

Thymidine Kinase Activity in Mice Injected with Glucosylceramide

Measurement	Controls	Treated	Increase (%)
Body wt. (g/mouse)	6.70 (0.38)	6.65 (0.27)	−0.7
Liver wt. (mg/mouse)	277 (20)	327 (27)	18
Protein in liver (mg)	27.6 (1.6)	31.6 (1.1)	14
DNA in liver (μg)	644 (50)	758 (30)	18
Thymidine kinase (μmol/hr)	1.30 (0.09)	1.90 (0.12)	46

Nine Hsd CF1 mice were injected with GlcCer (250 mg/kg emulsified with Myrj, 188 mg/kg) and, a day later, with CBE (80 mg/kg). Saline was injected into nine controls, and all mice were killed 24 hr later. The thymidine kinase activity shown is μmol/hr of thymidine converted to the monophosphate by the cytosol from one mouse liver, less the blank activity from boiled liver. The numbers in parentheses are standard deviations derived from six values (duplicates from three groups of three pooled livers) or nine individual values in the case of the weights. All increases were statistically significant by Student's t-test ($p < 0.005$).

TABLE 2

Ornithine Decarboxylase Activity in Mice Injected with GlcCer and CBE

Measurement	Controls	Treated	Increase (%)
Body wt. 24 hr later (g)	6.71 (0.77)	6.78 (0.46)	0
Body wt. 48 hr later (g)	6.57 (0.66)	6.95 (0.96)	6
Liver wt. 24 hr (mg)	278 (25)	342 (27)	23*
Liver wt. 48 hr (mg)	268 (34)	333 (55)	24*
Ornithine decarboxylase			
24 hr later (nmol/hr)	400 (21)	805 (55)	101*
48 hr later (nmol/hr)	387 (39)	636 (75)	64*

*$p < 0.005$.

The protocol was similar to that shown in Table 1, but both drugs were injected together at time zero, and the animals were killed 24 hr or 48 hr later. Standard deviations are shown in parentheses. Decarboxylase activity shown is the amount of ornithine decarboxylated by one liver in one hr, corrected for the blank activity in the presence of decarboxylase inhibitor.

DISCUSSION

We have shown that an emulsion of GlcCer acts on the liver to stimulate or increase the amount of thymidine kinase, a major enzyme of DNA synthesis (10). The mechanism for the kinase stimulation may not be through a direct action on the enzyme, as indicated by the lack of effect in vitro. However, the activation phenomenon may require other factors that are needed for in vitro stimulation.

The increase in ornithine decarboxylase activity that we observed is consistent with the reported two-fold increase produced by incubating cells with ganglioside, a glycolipid derived from GlcCer (11). The incubated ganglioside probably was hydrolyzed in part to GlcCer, and the GlcCer injected into our mice presumably was converted in part to ganglioside. It remains to be seen which glycolipid or intermediate between them was the actual stimulator. Ornithine decarboxylase formation is induced, generally, by phorbol diester, which apparently acts also to stimulate phosphorylations by a protein kinase C (12) as well as by a second pathway (9). Both pathways apparently activate the formation of ornithine decarboxylase (13). Gangliosides were reported to stimulate a protein kinase C (14) and to exert a proliferative action on cells such as neuroblastoma (15).

Phorbol diester may act through its stimulating effect on glycolipids, exemplified in the finding that it increased the levels of cerebroside, lactosylceramide and ganglioside GM3 in cultured leukemia cells (16). Both GlcCer and phorbol diester increased interleukin formation by macrophage cells (17).

It has been reported that lysosphingolipids are strong inhibitors of protein kinase C and of phorbol diester binding, and the speculation was offered that the lysolipids are responsible for the pathological features of sphingolipidoses such as Gaucher disease (18). Glucosylsphingosine does accumulate in this disorder (albeit to a low concentration) and might be formed to a certain extent in our GlcCer-loaded mice, but one generally cannot conclude that the inhibition of protein kinase C should lead to cell proliferation and increases in enzymes involved in DNA synthesis. Stimulation of protein kinase C leads to increased levels of ornithine decarboxylase (12,13), and we temporarily could conclude that GlcCer injection produces an increase in the kinase, not an inhibition.

In one experiment, we weighed the mouse spleens and found no significant change in weight after injecting GlcCer. The lack of effect can be attributed to our finding of rapid uptake of the exogenous GlcCer by liver (3). This is consistent with an observation made recently with labeled L-glucosylceramide, an unnatural enantiomer that apparently is unmetabolizable (19).

Omitted in this preliminary study was a control group of mice that had been injected only with detergent, instead of saline. Myrj alone had been found to have no effect on liver weight (3), and it has been found to be replaceable by lecithin for production of GlcCer's growth effect (Datta and Radin, unpublished data). Moreover, our experiment with GlcCer/Myrj without CBE produced a smaller increase in thymidine kinase, a result that is consistent with the assignment of the effect to the lipid rather than to the detergent. GlcCer has been found to enhance the proliferation of Ehrlich ascites carcinoma cells in vivo without the inclusion of detergent (20). While it seems highly unlikely that the detergent is responsible for the interesting increases in enzyme activity reported here, future studies should include a detergent or lecithin control group.

We suggest that GlcCer has mitogenic properties, acting through a relatively direct mechanism. It may function not only as a precursor for nearly all the glycolipids but also to control organ growth in normal animals. The possible roles of GlcCer and/or related glycolipids in cell proliferation and cancer have been discussed more fully (21).

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