Inhibition of cerebroside synthesis in the brains of mice treated with L-cycloserine

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Abstract Subcutaneous injection of L-cycloserine resulted in a 28% reduction in cerebroside levels in mouse brain but had no effect on the levels of gangliosides. In contrast, intraperitoneal injection results in a reduction of ganglioside as well as cerebroside + sulfatide levels. The route of injection influenced the degree of 3-ketodihydrosphingosine synthase inhibition. Intraperitoneal injection caused a rapid decrease in synthase activity followed by recovery over 48 hr, whereas subcutaneous injection resulted in no inhibition over this time; only after daily injection for a week was synthase activity reduced 35%. One week following cessation of L-cycloserine administration, enzyme activity had recovered, whereas the cerebroside level continued to fall. All lipids and enzymes showed normal levels 3 weeks post-cycloserine administration. L-[^{3}H]serine incorporation into glycolipids showed that cerebroside synthesis was most affected, whereas sulfatide synthesis was less affected. One week after cessation of cycloserine treatment, cerebroside synthesis was still severely inhibited, whereas sulfatide levels were near normal. Two weeks after cessation of L-cycloserine administration, synthesis of these glycolipids was similar to that of controls. —**Sundaram, K. S., and M. Lev.** Inhibition of cerebroside synthesis in the brains of mice treated with L-cycloserine. *J. Lipid Res.* 1985. **26:** 473–477.

Supplementary key words 3-ketodihydrosphingosine • gangliosides

Cerebroside metabolism in animals has been studied from two aspects. Starvation of young animals has been shown to reduce cerebroside accumulation in brain (1) and vitamin B_6-deficient rats have shown a reduced sphingolipid synthesizing capability (2). On the other hand, a number of inhibitors of catabolic enzymes of cerebrosides has been studied (3, 4). These result in accumulation of specific cerebrosides and hence mimic lipid storage diseases. We have recently shown that D- and L-cycloserine (4-amino-3-isoxazolidinone) are potent inhibitors of the first enzyme of the sphingolipid pathway, 3-ketodihydrosphingosine (3KDS) synthase derived from *Bacteroides levii* or brain microsomes, and also that intraperitoneal administration of L-cycloserine to mice results in significantly reduced levels of gangliosides and cerebrosides plus sulfatides in the brains of these animals (5). We have further shown that L-cycloserine selectively inhibits synthesis of phosphosphingolipids in growing cultures of *B. levii* whereas other phospholipids are inhibited to a much lesser degree (6). Of a number of potential inhibitors of 3KDS synthase examined, L-cycloserine was by far the most potent (7).

Intraperitoneal injection of L-cycloserine at 150 mg/kg body weight for 1 week results in an inability of the mice to gain weight during this period (5). This regimen also gives rise to transient effects which have been ascribed to inhibition of GABA-T and ALA-T (8). In order to avoid these untoward effects of L-cycloserine, a different route of administration was examined. The results of subcutaneous administration are described in this report. L-cycloserine administered by this route caused a significant reduction of cerebroside content with no significant effect on the level of gangliosides in mouse brain. In contrast to intraperitoneally injected mice, the mice injected subcutaneously for 1 week gained weight, although the gain was less than that of the control group.

MATERIALS AND METHODS

Male Swiss mice weighing 8–12 g, obtained from our vivarium, were used at 16 days of age (postweaned) for all experiments. Food and water were given ad libitum. They were injected either subcutaneously or intraperitoneally with L-cycloserine dissolved in 0.05 ml of saline (prepared immediately before use). After L-cycloserine treatment, the animals were decapitated and the brains were removed and homogenized with 20 volumes (20 ml/g wet tissue) of chloroform–methanol 2:1. The lipids were extracted and partitioned by the procedure of Folch, Lees, and Sloane Stanley (9).

The upper phase was dialyzed, lyophilized, and used for total ganglioside estimation by the resorcinol method (10), as modified (11) employing free N-acetyl neuraminic acid as the standard. From the lower phase, cerebrosides and sulfatides were separated by TLC (non-borate plates)

Abbreviations: 3KDS, 3-ketodihydrosphingosine; TLC, thin-layer chromatography.

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using chloroform–methanol–water 65:25:4 (v/v/v) (12). The bands co-chromatographing with standard cerebroside and sulfatide were visualized by iodine vapors, removed separately, and used for galactose estimation (13). Glucocerebroside and galactocerebroside were estimated together. In these experiments 70% recovery of the lipids was obtained following extraction from TLC plates.

Microsomes were prepared from the brains essentially according to the procedure of Morell and Radin (14), with the modification that the suspending medium contained 0.25 M sucrose, 1 mM pyridoxal phosphate, and 1 mM dithiothreitol in 50 mM phosphate buffer (pH 7.4). 3KDS synthase activity was assayed as described previously (15).

Labeling of brain cerebrosides and sulfatides was performed by intracranial administration of L-[^{3}H]serine (16). Uniformly labeled L-[^{3}H]serine (4 Ci/mmol) was used without dilution. Mice after 1 week's treatment with L-cycloserine, or 1 or 2 weeks after recovery, were injected intracerebrally with 1 μCi/g body weight of L-[^{3}H]serine in 10 μl of 0.9% NaCl. After 6 hr the animals were killed, their brains were removed and pooled, and lipids were extracted (9). The lower phase was subjected to TLC and the bands co-chromatographing with standard cerebrosides and sulfatides were removed and counted in a scintillation counter.

L-cycloserine was a generous gift from Dr. P. Sorter, Hoffman-LaRoche, Rahway, NJ. E. Merck (Dramstadt, Germany) was the source of silica gel G plates. Cerebrosides and sulfatide standards were purchased from PL Biochemicals, (Milwaukee, WI). Other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). All the chemicals were of reagent grade or of the best quality available. Statistical analyses were performed using the Student's *t*-test.

RESULTS

Ganglioside and cerebroside levels

The daily administration of 100 mg/kg body weight of L-cycloserine subcutaneously for 1 week resulted in a 28% reduction in cerebroside level, but had no effect on the content of gangliosides (**Table 1**, group A). This result was confirmed in repeat experiments (data not shown).

Influence of the route of injection on ganglioside content

When L-cycloserine was injected intraperitoneally, a significant reduction was found in the level of gangliosides (average 26%). In contrast, subcutaneous administration of L-cycloserine resulted in no significant change in the level of gangliosides in the brains of the mice (**Table 2**).

Effect of subcutaneous and intraperitoneal administration on brain 3KDS synthase activity

The difference in glycolipid type inhibited following intraperitoneal or subcutaneous administration could be related to a differential effect on synthase activity. Mice were therefore given one injection of 100 mg/kg body weight L-cycloserine by each route and the brains were assayed for enzyme activity 2, 4, 6, 8, 16, 24, and 48 hr later. As shown in **Fig. 1**, 2 hr following intraperitoneal injection, an 80% inhibition of synthase activity was found. This activity recovered to 84% of the control over the 16–48-hr period. In contrast, a subcutaneous injection of L-cycloserine produced no discernible inhibition of the synthase over the 2–48-hr period.

Mice that had been injected intraperitoneally with 100 mg/kg body weight L-cycloserine daily for 1 week showed a 15% inhibition of synthase activity in brain microsomes (data not shown).

Recovery of glycolipid and 3KDS-synthase levels following cessation of L-cycloserine administration

It was of interest to determine the recovery of both synthase and glycolipid levels following a course of subcutaneous cycloserine treatment. Experiments were performed in which L-cycloserine was administered for 1 week; some mice were killed and their brains were assayed for enzyme activity, cerebroside, sulfatide, and ganglioside content. Others were not treated further with cycloserine; these were examined 1 and 3 weeks later for enzyme activity and glycolipid content. The results are shown in **Fig. 2**. After administration of cycloserine for 1 week, the cerebroside content was diminished by 28% whereas synthase activity was 36% inhibited. One week after cessation of treatment, the enzyme activity had recovered reaching that of the control group; the cerebroside content, however, continued to decline reaching 34% inhibition ($P < 0.001$). Three weeks following cessation of treatment, enzyme and cerebroside levels were normal. Sulfatide accumulation showed a small (10%) inhibition after treatment for 1 week and no effect in subsequent samples (Table 1). Although no significant inhibition of ganglioside levels was observed, there was a 15% reduction in ganglioside levels 1 week after L-cycloserine treatment was stopped.

L-[^{3}H]serine incorporation into brain cerebrosides and sulfatides

To study the synthesis of brain glycolipids, L-[^{3}H]serine was injected intracerebrally into mice that had been treated with daily subcutaneous injections of L-cycloserine for 1 week. Six hours following L-[^{3}H]serine administration, the animals were killed and incorporation into the glycolipids was determined. Similar incorporation studies

TABLE 1. Inhibition and recovery of cerebrosides, sulfatides, gangliosides, and 3KDS synthase activity following subcutaneous administration of L-cycloserine

	Group A		Group B		Group C	
	Control	Experimental	Control	Experimental	Control	Experimental
1. Glycolipids (mg/g wet tissue)						
a. Cerebrosides	2.878 ± 0.344	2.068 ± 0.174[a]	2.730 ± 0.156	1.796 ± 0.119[b]	2.466 ± 0.115	2.466 ± 0.332
b. Sulfatides	1.752 ± 0.165	1.619 ± 0.198	1.332 ± 0.17	1.384 ± 0.12	2.053 ± 0.48	2.075 ± 0.18
c. Gangliosides	0.320	0.326	0.393	0.334	0.333	0.361
2. 3KDS synthase activity (nmol 3KDS formed)	2.899	1.843	2.630	2.663	2.064	1.945
3. Body weight (g)	12.50 ± 1.64	10.42 ± 1.11	20.26 ± 0.80	15.92 ± 1.13	24.70 ± 0.56	26.78 ± 0.77

Mice were injected daily with L-cycloserine for 1 week. Group A mice were killed immediately after cessation of treatment. Group B and C mice were killed 1 and 3 weeks, respectively, after cessation of cycloserine treatment. The brains were divided for glycolipid estimation and determination of 3KDS synthase activity. Values are means ± SEM of five animals per group. Ganglioside levels and 3KDS synthase activities were obtained from pooled brain samples.

[a]Significant at the 0.05 level.

[b]Significant at the 0.001 level.

were performed on mice treated with L-cycloserine as described above and maintained with no further treatment for 1 and 2 weeks. As can be seen in **Table 3**, incorporation of serine into cerebrosides and sulfatides was inhibited 78% and 60%, respectively, following cycloserine administration for 1 week. After cycloserine treatment had been stopped for 1 week, inhibition of serine incorporation was 69% for cerebrosides and 10% for sulfatides. Two weeks following cessation of cycloserine administration, no inhibition of incorporation into cerebrosides was found; in contrast incorporation into cerebrosides showed a slight increase.

DISCUSSION

Our results show that the route of administration of L-cycloserine profoundly influences the class of glycolipid inhibited. The subcutaneous injection of L-cycloserine for 1 week resulted in a 28% inhibition of cerebroside level with no effect on ganglioside content. The subcutaneous route was less stressful than intraperitoneal administration inasmuch as the mice did gain weight when subcutaneously injected whereas mice with intraperitoneally administered L-cycloserine did not (5). Three weeks post-cycloserine administration, the weights of the control and experimental groups were similar.

The inhibition of cerebroside but not ganglioside levels in mouse brain following subcutaneous injection of L-cycloserine was unexpected since both classes of glycolipids share the same initial biosynthetic pathway that L-cycloserine inhibits. Hypotheses to explain this result would include accessibility of the enzyme to the inhibitor: that the 3KDS synthase responsible for cerebroside synthesis is inhibited whereas the enzyme responsible for ganglioside synthesis is less accessible. Accessibility could be a factor even though no significant inhibition of ganglioside levels occurred, if, for example, the 3KDS synthases responsible for synthesis of gangliosides and cerebrosides were spatially separated in the cell. However, a 15% fall in ganglioside levels was seen 1 week following cessation of cycloserine treatment.

A second hypothesis is that ganglioside synthesis is susceptible only to very severe inhibition of synthase activity, whereas cerebroside synthesis is susceptible to a much smaller degree of synthase inhibition. We have shown that L-cycloserine injected intraperitoneally in a single dose results in a dramatic inhibition of synthase activity 2 hr following dosage and recovery is 80% complete 16 hr later, whereas the same level administered subcutaneously did not produce inhibition of this enzyme over a 48-hr period. Only after daily subcutaneous injections for 1 week was an effect seen—a 36% drop in activity.

This differential effect on the activity of the enzyme obtained by the different routes of inoculation obviously

TABLE 2. Effect of the route of injection on ganglioside accumulation in L-cycloserine-treated mice

Route	Control	Experimental
	mg/g brain	
Intraperitoneal	0.320	0.100
	0.428	0.336
Subcutaneous	0.256	0.272
Subcutaneous[a]	0.368	0.376

Mice (fiver per group) were injected with 150 mg/kg body weight L-cycloserine daily for 1 week. Controls received saline. The animals were killed and the brains were removed and pooled. Ganglioside accumulation was determined as described in the Materials and Methods.

[a]Mice in this group received 100 mg/kg body weight of L-cycloserine.

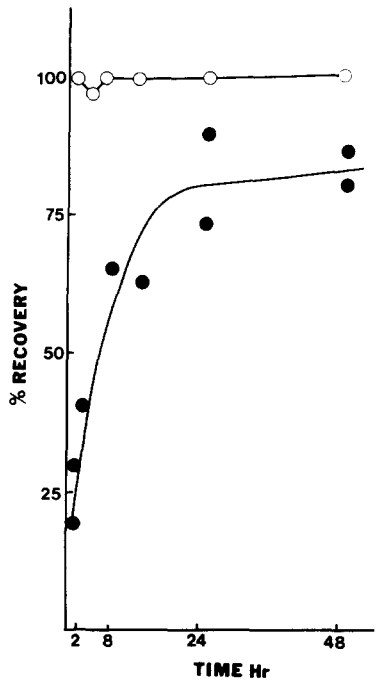

Fig. 1. Activity of brain 3KDS synthase following L-cycloserine administration. L-cycloserine at 100 mg/kg body weight was injected ● intraperitoneally and ○ subcutaneously into six groups of 16-day-old male mice (five animals/group). Controls received saline. After various time intervals, the animals were killed, the brains from each group were pooled, and brain microsomes were isolated and assayed for 3KDS synthase activity. Twenty-four and 48-hr post-injection experiments were repeated.

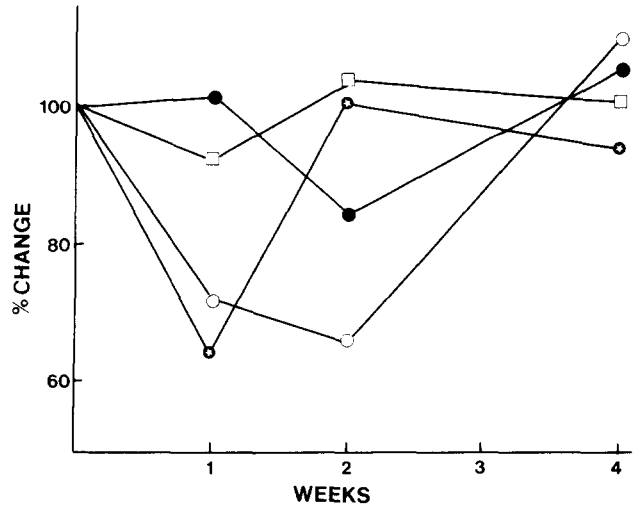

Fig. 2. Inhibition and recovery of glycolipid and 3KDS synthase activity following subcutaneous L-cycloserine administration. The experimental animals (five per group) were injected with L-cycloserine (prepared fresh in saline) 100 mg/kg body weight daily for the first week and allowed to recover for the following 3 weeks. Controls were injected with saline. At the end of 1, 2, and 4 weeks the animals were killed and the brains were assayed for 3KDS synthase ⊛, cerebrosides ○, sulfatides □, and gangliosides ● as described in Materials and Methods. The decreases in cerebroside level at the end of the 1st and 2nd weeks are significant at $P < 0.05$ and $P < 0.001$ levels, respectively.

reflects the rate of diffusion of the compound from the site of injection. Thus, intraperitoneal injection is characterized by a severe, immediate inhibition of the brain microsomal synthase activity and recovery, whereas subcutaneous administration has a gradual, cumulative effect on this enzyme. The very low intermittent levels of synthase activity observed during intraperitoneal administration could, therefore, be responsible for the inhibition of ganglioside accumulation.

A second unexpected result was the continued drop in cerebroside levels following withdrawal of L-cycloserine, which occurred when synthase activity had returned to normal. This result shows that there was a definite lag in recovery of cerebroside accumulation following inhibition of the first enzyme of the pathway. This lag may be related to the diversion of the biosynthetic system to ganglioside and sulfatide synthesis. Full recovery of cerebroside levels had occurred by the third week after L-cycloserine withdrawal. Of interest, too, is the small (15%) degree of inhibition of ganglioside content seen in brains 1 week following cessation of cycloserine administration and at a time when cerebroside inhibition is at its greatest. This leads to the speculation that continued administration of L-cycloserine by this route could eventually affect ganglioside accumulation. Alternatively, if specific inhibition of cerebrosides were desired, intermittent subcutaneous

TABLE 3. Effect of L-cycloserine on L-[^{3}H]serine incorporation in brain cerebrosides and sulfatides

	Group A		Group B		Group C	
	Control	Experimental	Control	Experimental	Control	Experimental
			cpm/g brain			
Cerebrosides	45,110	10,110	42,320	13,180	43,060	47,480
Sulfatides	8,220	3,320	6,900	6,240		

Five mice per group were injected subcutaneously with L-cycloserine (100 mg/kg body weight) daily for 1 week. Control animals were injected with saline. The mice were then injected intracerebrally with L-[^{3}H]serine (1 μCi/g body weight). The mice in Group A were killed 6 hr later and brains were removed to determine L-[^{3}H]serine incorporation into glycolipids as described in the Materials and Methods. Mice in Groups B and C were treated with cycloserine in the same manner as Group A mice except that serine injections and lipid analyses were performed 1 and 2 weeks, respectively, after L-cycloserine treatment was terminated. Measurements shown are cpm/g brain wet wt.

cycloserine administration would be indicated. One week after cessation of cycloserine administration, when the level of cerebrosides was markedly reduced (69%), sulfatide levels had largely recovered in the experimental group.

Cerebrosides are known to be intermediates in the synthesis of sulfatide and gangliosides (17). It therefore appears that synthesis of these end products is given priority as shown by the continued severe inhibition of cerebroside accumulation and synthesis together with the recovery of sulfatide and ganglioside levels 1 week following cessation of cycloserine treatment. A control mechanism may exist which would signal when sufficient sulfatides and gangliosides are made and when cerebrosides would accumulate. It is of interest to note that inhibition of long chain base formation by D, L-α-fluoropalmitic acid resulted in inhibition of incorporation of [^{14}C]palmitate into ceramide and gangliosides, whereas incorporation into sulfatides was increased (18).

A third explanation for the differential effects of L-cycloserine on cerebroside and ganglioside synthesis following subcutaneous administration is that an enzyme specific for cerebroside synthesis is affected in addition to 3KDS synthase. The inhibition of this hypothetical enzyme could account for the continued drop in cerebroside levels observed during recovery following cycloserine administration when 3KDS synthase activity had reached control levels.

The ability to select the type of sphingolipid inhibited may have implications for those sphingolipidoses where cerebrosides accumulate or where disturbances of cerebroside metabolism occur, e.g., in Gaucher's disease where glucocerebroside accumulates or in Krabbe's disease, where psychosine, presumably derived from galactocerebroside, accumulates in human brain (19).

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