ANNALS OF CLINICAL AND LABORATORY SCIENCE, Vol. 5, No. 1

Plasma Cerebrosides in Stroke and Multiple Sclerosis*

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ABSTRACT

This investigation was conducted to determine whether or not plasma galactosyl ceramides were elevated in patients with stroke and multiple sclerosis, and to determine glycosyl ceramide concentrations in older, normal subjects. It was hypothesized that central nervous system destruction, like that which occurs in stroke or in the demyelination characteristic of multiple sclerosis, might be reflected by changes in plasma glycosyl ceramides, specifically by an increased percentage of galactosyl ceramide.

Glycosyl ceramides were analyzed in duplicate 10 ml aliquots of plasma from each of seven patients with stroke, five patients with multiple sclerosis and five control subjects age-matched with stroke patients. Mean percentages of galactosyl ceramide for both controls (11.06 percent) and multiple sclerosis (11.40 percent) were strikingly similar. The percent of galactosyl ceramides for stroke was slightly elevated (14.32 percent) but there were no significant differences at the $p = >0.05$ level.

Introduction

Glycosphingolipids have been isolated from many mammalian tissues and are known for their importance as structural components, especially in the nervous system. Recent research[2,4] has shown them to figure prominently in a group of genetically-determined diseases, the lipidoses, which are characterized by specific enzyme deficiencies and various syndromes involving demyelination, lipid storage, mental retardation, organ and tissue malfunction and shortened life span.[22] All classes of glycosphingolipids have been implicated in the lipidoses,[2,4,24] and the occurrence of increased quantities of cerebrosides (monohexosylceramides) or other glycosphingolipids in plasma,[1,18,21,23,29,30] urine,[9] cerebrospinal fluid[7,23,27,28] and other tissues[8,10,13,15,16,20,25,26] depends on the metabolic error. Glycosphingolipid alterations have not been studied so extensively in other central nervous system disorders.

For example, multiple sclerosis, a disease of the central nervous system of unknown etiology, is also accompanied by demyelination and elevated cerebrospinal fluid galactosyl ceramide. Often, the demyelination found in the periventricular white

* This investigation was supported in part by the National Multiple Sclerosis Society, Grant #ORD 9871.

matter is in contact with circulating cerebrospinal fluid. Since galactosyl ceramide is the cerebroside which is characteristic of myelin, the presence of the lesion is apparently responsible for the elevation of this specific cerebroside in the cerebrospinal fluid of patients with multiple sclerosis.[6] A single study on pooled plasma of patients with multiple sclerosis[21] showed no significant differences in cerebroside composition or levels from normal controls.

While patterns of tissue destruction in stroke vary according to the specific lesion, both gross and microscopic changes usually include interruption and disintegration of myelin.[22] However, the extent to which the destruction of myelin and associated pathology in stroke causes changes in cerebrospinal fluid and plasma cerebrosides has not been documented.

In this investigation, plasma from patients with stroke and multiple sclerosis was examined in order to determine whether or not changes in the composition or concentration of glycosyl ceramides accompany these disorders. Since reported control values of plasma cerebrosides were restricted to normal healthy individuals age 35 and under,[1,21,25,29,30,31] it was also necessary to establish the cerebroside composition and concentrations in older controls age-matched with the stroke patients.

Materials and Methods

Blood samples were obtained from hospitalized patients. In most instances, specimens from stroke patients were obtained within two weeks following the onset of symptoms.

From patients and apparently healthy individuals age-matched with stroke patients, fifty ml of blood were drawn into heparin, the samples centrifuged for 30 minutes at 2,500 rpm, the plasma separated from the cells and either frozen or analyzed immediately. No hemolyzed samples were used. Duplicate 10 ml samples of plasma (measured to the nearest 0.05 ml) were analyzed.

Redistilled solvents were used throughout. Materials included were Unisil (200–325 mesh),* the silylating reagent, Regisil,† and Supelcoport column packing (3 percent SE 30).‡ Commercial thin layer plates coated with silica gel G§ were prerun in diethyl ether to remove contaminants. Mannitol 2 μmole per ml in 0.5 N methanolic HCl was used as an internal standard.

Total lipids were extracted from each 10 ml aliquot of plasma by the addition of 200 ml chloroform-methanol (2:1) using the biphasic system of Folch et al[12] and the modifications of Vance and Sweeley.[30] The lower phase containing the total lipids was resuspended in chloroform and applied to a 2 g silicic acid column suspended in $CHCl_3$. Glycosphingolipids were eluted with 100 ml of acetone:methanol (9:1) following elution of the neutral lipid fraction with 50 ml of chloroform. All eluates were reduced to dryness before proceeding to the next analytical step in a rotary evaporator or under a nitrogen stream. Residues were reconstituted in a small quantity of solvent.

Contaminating phospholipids were removed by mild alkali-catalyzed methanolysis[11,30] and the glycosphingolipids were applied to thin layer plates with authentic standards and chromatographed in a solvent system of chloroform-methanol-water (100:42:6). Cerebrosides were visualized by exposure to iodine vapor[7,21,31] and blueprints made for permanent reference (figure 1). Cerebrosides were eluted from the silica gel with chloroform-methanol-water (100:50:10) and evaporated to dryness.

Thirty μl of the mannitol standard and 3 ml of 0.5 N methanolic HCl were added

* Clarkson Chemical Co., Williamsport, PA.
† Regis Chemical Co., Chicago, IL.
‡ Supelco Inc., Bellefonte, PA.
§ Quantum Industries, Fairfield, NJ.

to the samples which were then incubated at 80° for 20 to 24 hours. Methyl esters of fatty acids were removed by three extractions of equal volumes of hexane. The methyl glycoside fraction was neutralized with granular, reagent grade silver carbonate.

Following trimethylsilylation, the trimethylsilyl derivative of methyl glucosides, methyl galactosides, and mannitol were separated on a 3 percent SE 30 column gas chromatograph at 160° (figure 2). Calculations were made from the gas-chromatographic tracings according to the method of Vance and Sweeley[30] and converted to nanomoles per ml.

Results

The values given in table I indicate the plasma levels of total monohexosyl ceramides (glycosyl ceramides or cerebrosides), galactosyl ceramides and glucosyl ceramides of the individuals constituting the control group. The mean total cerebroside concentration was 28.19 nanomoles per ml; the mean galactosyl ceramide concentration was 3.07 nanomoles per ml; and the percentage of galactosyl ceramide was 11.05.

In table II are shown the average of paired samples of the group of multiple sclerosis patients. Total cerebrosides (28.34 nanomoles per ml), galactosyl ceramides

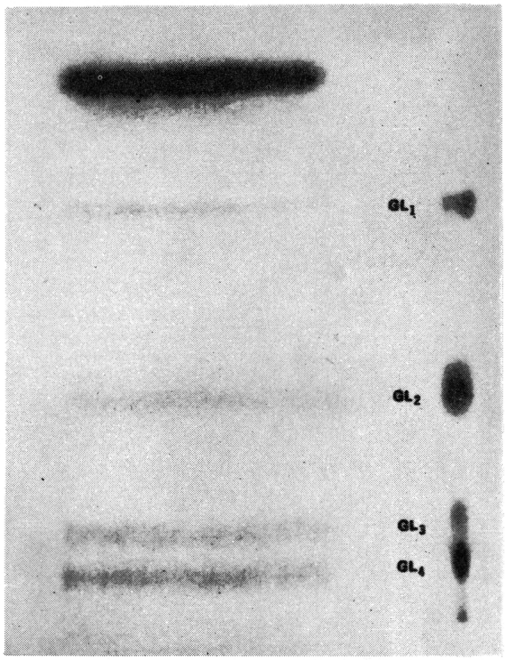

FIGURE 1. Thin-layer chromatogram of glycosyl ceramides from human plasma. The bands which were visualized after exposure to iodine vapor are monohexosyl ceramide (GL_1), dihexosyl ceramide (GL_2), trihexosyl ceramide (GL_3), and globoside (GL_4).

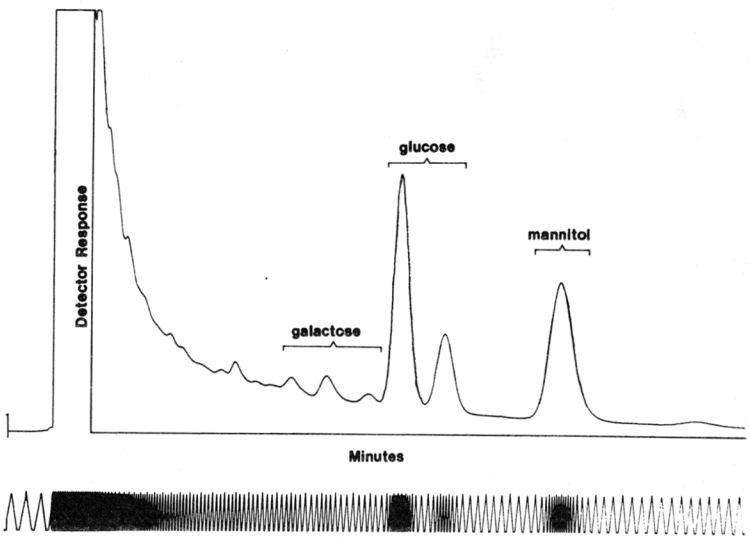

FIGURE 2. Gas-liquid chromatogram of TMSi methyl glycosides of D-glucose and D-galactose with mannitol added as an internal standard. Run on a 2mm × 3mm column of 3 percent SE-30 on 100/120 Supelcoport at 150°. A Hewlett-Packard F & M model 402 gas chromatogram was used with a flash heater at 250° and a nitrogen carrier gas flow rate of 35 ml per minute.

TABLE I

Concentration of Plasma Glycosyl Ceramides from Controls*

Patient	Age Sex	Gal	Controls GLC	Total GL_1	Percent Gal
V.W.	50m	5.11	38.62	43.74	11.76
J.D.	61m	2.89	28.87	31.76	9.08
B.G.	47f	2.79	24.92	27.71	10.14
F.D.	50m	2.10	17.51	19.61	10.71
C.H.	66m	2.46	15.16	18.11	13.55
X		3.07	25.02	28.19	11.05

*Average of paired samples (nanomoles per ml).

(3.19 nanomoles per ml) and glucosyl ceramides (25.20 nanomoles per ml) show a striking similarity to those values determined for the control group. The galactosyl ceramide of the control group ranged from 9 to 14 percent while that of the multiple sclerosis group ranged from 8 to 15 percent.

The concentration of cerebrosides in plasma of individuals suffering from stroke is presented in table III. Total cerebrosides (21.99 nanomoles per ml), glycosyl ceramides (17.75 nanomoles per ml), and galactosyl ceramide (2.80 nanomoles per ml) were decidedly lower than those values of the control group, while the percentage of galactosyl ceramides (14.32) was higher.

In one stroke patient E.S., an 84-year-old female with a 50-year history of diabetes, the plasma cerebrosides contained 25.66 percent galactosyl ceramide. The range of galactosyl ceramide from other patients in the group ranged from 8 to 14 percent.

TABLE II

Concentration of Plasma Glycosyl Ceramides from Multiple Sclerosis Patients*

Patient	Age Sex	Gal	Multiple Sclerosis GLC	Total GL_1	Percent Gal
D.P.	24f	1.94	11.35	13.34	14.95
D.W.	44f	2.88	31.64	34.52	8.34
E.M.	46m	2.79	22.00	24.80	10.51
C.V.	28m	4.81	35.77	40.53	11.86
L.V.	37f	3.54	25.25	28.49	11.37
X		3.19	25.20	28.34	11.40

*Average of paired samples (nanomoles per ml).

TABLE III

Concentration of Plasma Glycosyl Ceramides from Stroke Patients*

Patient	Age Sex	Gal	Stroke GLC	Total GL_1	Percent Gal
A.K.	65m	1.56	11.20	12.76	12.21
E.S.	84f	4.57	14.90	19.47	25.66
B.M.	76m	4.96	25.57	30.36	14.52
G.M.	58m	2.28	22.65	24.93	9.26
P.S.	55m	1.20	8.55	9.75	12.32
C.D.	56m	1.96	14.49	16.45	11.94
D.S.	76m	3.36	36.89	40.23	8.36
$\bar{X}$		2.80	17.75	21.99	14.32†
X'		2.51	19.89	22.41	11.44

*Average of paired samples as nanomoles per ml.
†P = >0.05
X' shows mean values when patient E.S. is not included.

Analysis of the glycosyl ceramide concentration and percentage of galactosyl ceramides, excluding patient E.S., showed the total cerebrosides to be 22.41 nanomoles per ml and galactosyl ceramides (11.44 percent) to be even more closely related to the control group (table III). The range of galactosyl ceramides expressed as percent of total glycosyl ceramides between the individuals constituting the stroke group is the same as for the multiple sclerosis group. Galactosyl ceramides are decreased to 2.51 nanomoles per ml. There was no significant difference ($p = >0.05$) between these two groups.

In table IV is shown a comparison of concentration and percentage of glycosyl, galactosyl and glucosyl ceramides of the

TABLE IV

Comparison of Concentration and Percentage of the Glycosyl, Galactosyl and Glucosyl Ceramides*

	GL_1	Gal	Percent Gal	GLC
Controls	28.19	3.07	11.05	25.02
Multiple sclerosis	28.34	3.19	11.40	25.20
Stroke	21.99	2.80	14.32	17.75

*All groups given as nanomoles per ml.
P = >0.05

three groups. Using analysis of variance, there was no significant difference ($p = >0.05$).

Recalculation of data from the stroke group following elimination of the patient who appeared to be afflicted with more than one disease entity and who showed an approximate two-fold elevation of galactosyl ceramide above the other patients in the stroke group gave the following results: the total cerebrosides were slightly elevated; galactosyl ceramides decreased considerably, but not to a significant degree; and the percentage of galactosyl ceramides was in very close agreement with the other two groups.

Discussion

Multiple sclerosis has been studied extensively from many perspectives. Plasma cerebroside composition, however, has been determined only on pooled samples from patients with this disorder.[21] In the present study which utilized duplicate samples from individual patients, the range of galactosyl ceramide percentages found in patients with multiple sclerosis was not significantly different from the figure established by Rathke and Jones.[21] The mean percentage of galactosyl ceramides of individual patients compared very closely with that of controls in this and other studies.

The increase in cerebrospinal fluid galactosyl ceramide reported in multiple sclerosis patients need not be reflected by changes in the plasma cerebroside composition for a variety of reasons. For example, a several-fold dilution would occur even if the relatively minute amounts of cerebrospinal fluid cerebroside did enter the plasma freely. The rather wide normal range of percentage of plasma galactosyl ceramide could obscure small but significant increases from the cerebrospinal fluid. Also, assuming that the blood-brain barrier is damaged in the formation of central nervous system lesions, no direct evidence exists that galactosyl ceramide enters the plasma unchanged. Again, the relatively small increases, which theoretically could be added to the plasma pool from this route, might be obscured by the normal range.

In patients with multiple sclerosis and retrobulbar neuritis, Tourtellotte and Haerer[28] were able to demonstrate that cerebrospinal fluid cerebroside levels correlated well with age of patient and extent of lesion. Thus, the greater amounts of galactosyl ceramides in the plasma of patients C.V. and L.V. in our study may be indicative of more extensive demyelination. Possible relationships between plasma cerebroside composition and the presence or extent of demyelinating lesions in the central nervous system could be explored by serial determinations of plasma cerebrosides following relapses. Millar[19] has postulated that multiple sclerosis could involve a defect of the blood-brain barrier and has outlined a sequence of events which possibly accompany demyelination. This sequence is similar to that described for vascular disease.

Massive destruction of the central nervous system which occurs in the various kinds of stroke is known to disrupt the blood-brain barrier,[17] in fact, antibodies to various central nervous system constituents including cerebrosides have been demonstrated after destructive or demyelinating disorders, indicating the possibility of plasma contamination.[3] It was predicted that the galactosyl ceramide, which is characteristic of myelin, might appear in the plasma from this source. This hypothesis would seem to be disproved by the data obtained in this study. However, qualifications about conclusions are necessary and further studies need to be undertaken before the hypothesis is completely nullified.

For example, the time of sampling following onset of symptoms could be very

significant, and the range of normals is sufficiently broad so that analysis of serial samples over a period of time might be necessary to document changes in plasma cerebroside composition. The extent of the lesion almost certainly would affect the possible quantity of cerebrosides reaching the plasma. Also, the dilutional effects of the plasma could be compensated in some way by analyses of galactosyl ceramide more specifically characteristic of the central nervous system. Differences exist in the fatty acid composition of the monohexosyl ceramides of brain and plasma. Possibly a combination of cerebroside hexose and fatty acid analyses could establish with certainty the contribution of myelin cerebroside to the plasma pool in the presence of massive destruction.

Patient E.S. had 25.66 percent of plasma cerebrosides present as galactosyl ceramides. This patient was an 84-year-old female and a known diabetic for 50 years. Her history gave no indication of the presence of diabetic neuropathy. The diagnosis in this patient was cerebral thrombosis. However, three obvious categories were present in this patient: aging, stroke and atherosclerosis. Studies on aging have brought to light few biochemical parameters which accompany the aging process in normal subjects. No information is available to describe if, or at what point, the structural molecules of aging organs begin to appear in the plasma. Possibly the increased plasma cerebrosides in the controls in this study reflect a normal concomitant of aging.

Acknowledgments

The authors acknowledge the technical assistance of Mr. Warren Taylor and the secretarial assistance of Ms. Sandra Randall. Gratitude is expressed to the Pathology Departments of the Detroit Osteopathic and E. W. Sparrow Hospitals, to Dr. Louis Rentz, Dr. Paul Jakubiac, Dr. Eileen Rathke and Dr. Charles Sweeley.

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