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Demonstration of a Deficiency of Glucocerebroside-cleaving Enzyme in Gaucher's Disease*

ROSCOE O. BRADY,† JULIAN N. KANFER, ROY M. BRADLEY, AND DAVID SHAPIRO

(From the Laboratory of Neurochemistry, National Institute of Neurological Diseases and Blindness, Bethesda, Md., and the Department of Organic Chemistry, Weizmann Institute of Science, Rehovoth, Israel)

The accumulation of abnormal quantities of glucocerebroside in the reticuloendothelial cells of patients with Gaucher's disease is well documented (2–7). Previous studies in this laboratory indicated no abnormality in cerebroside formation in spleen tissue obtained from patients with Gaucher's disease (8). These observations suggested that the biochemical lesion in these patients might be on the pathway of cerebroside catabolism. To pursue such investigations, we chemically synthesized glucocerebroside-^{14}C labeled in carbon atom 1 of the D-glucose portion of the cerebroside molecule (9). With this material, it was possible to demonstrate and partially purify an enzyme in human spleen tissue that catalyzes the hydrolysis of the glycosidic bond of the cerebroside molecule. The products of the reaction catalyzed by the most highly purified enzyme preparation were shown to be glucose and ceramide (*N*-acylsphingosine) (9).

When this information became available, studies were undertaken to determine the level of the glucocerebroside-cleaving enzyme in human spleen tissue obtained from patients with various conditions. The present report describes the results obtained in these experiments and presents evidence of a marked attenuation of glucocerebroside-cleaving activity in the spleen of patients with Gaucher's disease.

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† Address requests for reprints to Dr. Roscoe O. Brady, National Institutes of Health, Bethesda, Md. 20014.

Methods

Clinical material. All of the spleen tissue used in this series was obtained at operation. In the control group, splenomegaly was present only in the three patients with hemolytic anemia. The largest spleen was obtained from patient WR and weighed 2,300 g. A varying degree of splenomegaly was present in all of the patients with Gaucher's disease. The largest spleen, weighing 1,100 g, was obtained from patient AK. The diagnosis of Gaucher's disease was established by Jena-Giemsa staining of the tissue specimens. In all instances, bone marrow aspiration also revealed the presence of Gaucher's cells. Only patient SZ, with the infantile form of Gaucher's disease, showed any neurological abnormality.

Assay procedure. Each of the specimens of spleen tissue was packed in dry ice within 5 minutes after excision. The samples were thawed and assayed for glucocerebroside-cleaving activity within 24 hours. Control studies indicated that this enzymatic activity did not diminish appreciably under these conditions. However, there was a gradual decrease in activity of about 5% per day on storing the frozen human tissue at −15° C (9).

Cell-free extracts of the thawed tissue were prepared by mincing the tissues and homogenizing them in 2 vol of 0.1 M potassium phosphate buffer, pH 7.0, with an all glass TenBroeck homogenizer. The suspensions were filtered through two layers of cheesecloth and centrifuged at $600 \times g$ for 12 minutes. The $600 \times g$ supernatant suspensions were then centrifuged at $100{,}000 \times g$ for 1 hour. The activity of the glucocerebroside-cleaving enzyme was assayed on samples of the high speed supernatant solutions. The incubation mixtures contained 50 μmoles of potassium phosphate buffer, pH 7.0, 0.140 μmole of glucose-1-^{14}C cerebroside (440,000 cpm per μmole), 0.8 mg of Cutscum (isooctylphenoxypolyoxethanol [1]), and enzyme (13 to 30 mg of protein) in a final volume of 0.65 ml. The mixtures were incubated for 1 hour at 37° C in air. The reaction was stopped by the addition of 0.17 ml of 7% perchloric acid and worked up as described previously (9). The hydrolysis of glucocerebroside-^{14}C was determined by measuring the formation of water-soluble radioactive products. Glucocerebroside itself is completely insoluble in water under these conditions. The glucocerebroside-cleaving cata-

[1] Fisher Scientific Co., Silver Spring, Md.

lytic activity was found to be proportional to the amount of enzyme solution employed.

Because of the relatively large amount of glucocerebrosides that accumulates in the spleens of patients with Gaucher's disease, two techniques were employed to correct for dilution of labeled substrate. In the first procedure, the amount of endogenous glucocerebroside was quantitated by the isotope dilution technique (10). A known amount of radioactive glucocerebroside was added to samples of the enzyme preparations, and the mixtures were extracted three times with 19 vol of warm (55° C) chloroform-methanol, 2:1 (vol/vol) (11). The suspensions were filtered through glass wool to remove denatured protein. One-fifth vol of water was added to the extract, and the mixtures were clarified by centrifugation. The upper phases were decanted and the lower phases equilibrated with 0.2 vol of chloroform-methanol-water, 3:48:47 (vol/vol/vol). The upper phases were removed and the lower phases taken to dryness. The residue was saponified and partitioned as described by Kishimoto, Davies, and Radin (12). The chloroform phase was dried over anhydrous Na_2SO_4 and the solvent subsequently removed *in vacuo*. The residue was dissolved in a small volume of chloroform-methanol 50:1 and applied to a 1- × 10-cm column of silicic acid. The column was eluted with 30-ml portions of chloroform-methanol, 50:1, 30:1, and 9:1. The major portion (more than 90%) of the radioactivity was recovered in the 9:1 fraction. The amount of glucocerebroside was estimated colorimetrically by the phenol-sulfuric acid procedure (13) and the specific activity determined. This value, which indicated the extent of dilution of the added glucocerebroside-^{14}C, was used to calculate the quantity of endogenous glucocerebroside.

TABLE I

Determination of dilution of labeled substrate by endogenous cerebroside

Patient	Age	Sex	Method: Rat spleen enzyme (1)	Method: Isotope dilution technique
	years		Dilution factor	
GM	33	F	1.3	1.2
AK	3	F	1.4	1.3
SZ	1	M	1.1	1.1
SG	39	M	1.1	1.2
SL	3	M	1.5	2.0
PA	4	M	1.2	1.3

The dilution of labeled substrate was also determined by another method. This procedure consisted of measuring the amount of enzymatic hydrolysis of glucocerebroside-^{14}C catalyzed by an enzyme preparation from rat spleen tissue, alone, and in the presence of an equal amount of the Gaucher spleen preparations (1). The decrement in the amount of radioactivity released from glucocerebroside-^{14}C catalyzed by the combination of the two preparations was contrasted with that expected from incubating each preparation alone. The endogenous dilution was calculated from these values. The two methods gave quite good agreement in the initial six spleen samples from patients with Gaucher's disease (Table I).

TABLE II

Level of glucocerebroside-cleaving enzyme in human spleen preparations

Condition	Patient	Age	Sex	Enzymatic activity
		years		*mμmoles cleaved/mg protein/hr*
Sickle cell anemia	MS	12	F	0.11
Congenital hemolytic anemia	WR	76	F	0.13
Hemolytic anemia	DS	15	F	0.10
Aplastic anemia	FB	68	F	0.13
Tetralogy of Fallot	CD	11	F	0.08
Mitral stenosis	JD	40	M	0.09
Congenital spherocytosis	FA	4	M	0.15
Idiopathic thrombocytopenic purpura	SM	37	F	0.11
Congenital hemolytic anemia	CF	14	F	0.10
Chronic nephritis	RP	21	M	0.12
				Mean 0.11 ± 0.001 SE
Gaucher's disease, adult form	GM	33	F	0.008
Gaucher's disease, adult form	AK	3	F	0.006
Gaucher's disease, infantile form	SZ	1	M	0.001
Gaucher's disease, adult form	SG	39	M	0.022
Gaucher's disease, adult form	SL	3	M	0.010
Gaucher's disease, adult form	PA	4	M	0.020
Gaucher's disease, adult form	JR	13	M	0.005
Gaucher's disease, adult form	TM	5	M	0.015
Gaucher's disease, adult form	MW	12	F	0.019
Gaucher's disease, adult form	GK	14	F	0.021
Gaucher's disease, adult form	SK	15	F	0.032
				Mean 0.015 ± 0.002 SE

Therefore, only the latter procedure was employed in the assays performed on the five subsequent spleen specimens. The use of the mixed rat and human enzyme preparations has the additional advantage of possibly permitting recognition of any inhibitors in the Gaucher spleen preparations.

Results

The level of glucocerebroside-cleaving enzyme in human spleen tissue was originally determined in four control and three Gaucher spleen samples (1). As additional material became available, the number of specimens in the control group was expanded to ten and those in the Gaucher's to eleven. The previous incubation time of 14 hours was reduced to 1 hour in the current study to insure that the assays were performed during a period when the rate of reaction was linear with time (9). In the previous study, the amount of enzymatic activity was expressed in terms of counts per minute of radioactive water-soluble products obtained. However, to obtain a more precise quantitative assay in the present experiment, we determined the level of endogenous glucocerebroside by two separate procedures. This knowledge permitted an accurate expression of enzymatic activity in terms of millimicromoles of substrate cleaved per milligram of protein per hour. The mean glucocerebroside-cleaving activity in preparations of spleen tissue from the ten control patients was 0.11 mμmole per mg of protein per hour (Table II). In enzyme preparations from eleven spleens of patients with Gaucher's disease, the mean value was 0.015 mμmole per mg of protein per hour. The dilution of labeled substrate by endogenous glucocerebroside in the non-Gaucher's enzyme preparations was from 2 to 5%. The figures for enzymatic activity in these preparations were not corrected for dilution of labeled material. However, in the Gaucher spleen enzyme preparations, the dilution of labeled substrate varied from 10 to 100%. Therefore, all of the figures reported for the assays with these preparations have been corrected for endogenous dilution with the value obtained by the mixed rat and human enzyme procedure.

Discussion

The present experiments comprise a comprehensive documentation of our preliminary finding (1) of an attenuation of glucocerebroside-cleaving enzyme in spleen tissue of patients with Gaucher's disease. Recent studies with fresh, unfrozen spleen tissue homogenized in isotonic sucrose solution indicate that a considerable portion (40 to 65%) of the glucocerebroside-cleaving enzyme may be initially bound to subcellular particles (14). However, the activity of the enzyme in the present study was determined in the 100,000 × g supernatant solutions for the following reasons. Initial experiments (9) indicated that most of the total enzymatic activity in the tissue homogenates was released in soluble form under the conditions described in these studies. The necessity for freezing the samples of spleen tissue for transportation from various parts of the country dictated the use of this procedure. The subcellular distribution of the cerebroside-cleaving enzyme was examined and found to be the same as in the non-Gaucher spleen preparations.

The results reported in a recent communication by Patrick (15) seem to substantiate our finding of a deficiency of glucocerebroside-cleaving enzyme in the Gaucher spleens. There are a number of discrepancies between the observations in the two laboratories that will have to be resolved by further experimentation. In particular, in contrast with Patrick's report of a complete absence of glucocerebroside-cleaving activity in the Gaucher's spleen preparations, in the present study there was evidence of some, albeit markedly diminished, glucocerebrosidase activity in all of the spleens from the ten examples of the adult form of Gaucher's disease. In view of the variation of time of onset of pathological signs and symptoms in patients with Gaucher's disease, it seems reasonable that varying degrees of residual enzymatic activity might be present in the adult form of this disease. No consistent proportionality could be shown in human spleen preparations between hydrolysis of glucocerebroside and artificial substrates such as p-nitrophenyl-β-D-glucopyranoside (9). Therefore, it would appear unlikely that the use of the latter material can give a reliable indication of the level of glucocerebroside-cleaving enzyme as proposed by Patrick. The assay employed by Patrick was based upon the determination of free glucose liberated from glucocerebroside with glucose oxidase. In the present studies, none of the radioactive products obtained from glucose-1-^{14}C cere-

broside after incubation with the 100,000 × *g* supernatant enzyme preparations appeared to be free glucose (9). Almost all (95%) of the radioactivity was retained by an anion exchange resin, suggesting conversion of the labeled reaction products to compounds such as phosphate esters or even further metabolism to various acidic products of glycolysis. The appearance of such products would probably be undetected by Patrick's procedure. In unfractionated Gaucher spleen tissue homogenates, the metabolic conversion of free glucose-1-^{14}C to $^{14}CO_2$ was completely within normal limits.

The present observations, coupled with the earlier demonstration that cerebroside synthesis appears normal in Gaucher spleen (8), make it appear quite likely that a deficiency of activity of the glycosidic enzyme is somehow responsible for the accumulation of glucocerebroside in the reticuloendothelial cells of these patients. In view of the number of clinical differences among the infantile, adult, and possibly juvenile (16) forms of Gaucher's disease, it seems likely that there may be more than a single genetic mutation involved. Further experimentation is required to determine the extent and nature of enzymatic abnormalities in these various categories.

Summary

The level of glucocerebroside-cleaving enzyme has been determined in human spleen tissue specimens with the use of glucose-1-^{14}C cerebroside. The level of enzymatic activity was found to be markedly attenuated in eleven preparations of Gaucher spleen tissue compared with the values observed in ten non-Gaucher spleen samples. This metabolic defect may account for the accumulation of excessive quantities of glucocerebrosides in Gaucher's disease.

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