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Immunological and Catalytic Quantitation of Splenic Glucocerebrosidase from the Three Clinical Forms of Gaucher Disease

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SUMMARY

The enzymatic activity of glucocerebrosidase in splenic extracts of the adult nonneurological form of Gaucher disease (type I) was 15% ± 7% of normal, and the titer of enzyme cross-reacting material (ECRM) in these spleens was 54% ± 9% of normal. The titer of ECRM in splenic extracts of tissues obtained from patients with the neurological forms of Gaucher disease (types II and III) was essentially the same as in type I Gaucher spleens (59% ± 10% of normal), but the measurable catalytic activity of glucocerebrosidase in these spleens was substantially lower than that found in type I Gaucher spleens (2.3% ± 0.6% of normal). Thus, the attentuated glucocerebrosidase activity in spleens from all three forms of Gaucher disease appears to stem from a structurally mutated enzyme that is altered in its catalytic efficiency and possibly in its antigenic expression.

INTRODUCTION

An attenuation in the measurable catalytic activity of glucocerebrosidase has been a well-documented and salient biochemical feature underlying the molecular pathogenesis of Gaucher disease [1]. The finding of partially deficient glucocerebrosidase levels in obligate heterozygote carriers strongly suggests that the primary genetic lesion of Gaucher disease affects in some direct fashion the catalytic expression of glucocerebrosidase [2]. Specific mutations involving the

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structure of the enzyme, the synthesis or turnover of the enzyme, or of enzyme activators have all been considered. Direct and conclusive evidence for a structurally altered and catalytically compromised enzyme was provided by isolation of residual glucocerebrosidase from an adult (type I) Gaucher spleen [3]. This latter study was limited by the low yield of purified enzyme (< 2%) and the analysis of a single affected spleen. The question of glucocerebrosidase characterization in the other clinical forms of Gaucher disease known as types II and III, respectively, was not addressed.

Our study was designed to develop a practical immunological means for characterizing residual glucocerebrosidase in crude tissue homogenates. This method was applied to a study of 11 Gaucher spleens and has led to the conclusion that a structural mutation of glucocerebrosidase is a common feature in all of the clinical subtypes of Gaucher disease.

MATERIALS AND METHODS

Immunization and Antibody Preparation

A rabbit was immunized by intradermal injection of purified human placental glucocerebrosidase with a specific activity of 1×10^6 nmol of glycolipid hydrolyzed/hr per mg protein [4]. The enzyme in phosphate-buffered saline was mixed with an equal volume of Freund's complete adjuvant and administered to the rabbit at a dose of 1 mg/kg body weight. Three similar booster doses of the enzyme in incomplete Freund's adjuvant were injected at 2-week intervals. Raised lesions at the site of injection were observed after each dose. Serum was obtained from the rabbit before immunization and 2 weeks after the final booster dose. Goat antibody to rabbit γ-globulin was obtained from Calbiochem-Behring. IgG fractions of immunized serum, preimmunized serum for control, and of goat antirabbit γ-globulin serum were prepared by ammonium sulfate precipitation [5].

Quantitative Immunoassay of Glucocerebrosidase

The quantitative immunoassay method developed by Carson et al. [6] was adapted for use in our study. Purified human placental glucocerebrosidase was serially diluted with 50 mM citrate-phosphate buffer, pH 6.0, containing 0.2% Cutscum and 1.0% sodium taurocholate. Enzyme dilutions of 5–400 U in 0.2 ml of the above buffer were used. Immunized rabbit IgG (53 μg protein) was added to the enzyme dilutions in 0.025 ml aliquots in the above buffer. The solutions were adjusted to 2.5% human serum albumin with Cutter's 25% albumin (0.025 ml). These primary incubations were for 1 hr at room temperature followed by 24 hrs at 4°C. Pre-immunized rabbit IgG was used for the control tubes. Goat antirabbit γ-globulin (1 mg) was added, the tubes mixed, and the incubation procedure repeated. All tubes were subsequently centrifuged at 49,000 *g* for 1 hr to sediment the floculant material formed. The supernatants were assayed for unprecipitated glucocerebrosidase activity.

Enzymatic Assays

Glucocerebrosidase was routinely assayed by incubation with D-[1-^{14}C]glucocerebroside as described [7]. Sphingomyelinase was determined with [choline-ME-^{14}C]sphingomyelin [8]. The activities of the other lysosomal hydrolases were determined fluorimetrically using 2.5-mM solutions of various glycosides of 4-methylumbelliferone in 50 mM citrate-phosphate buffer, pH 5.0, with 0.13% albumin. To determine the specific activities of the enzymes studied, frozen spleen samples were allowed to partially thaw and were homogenized in 3 vol (w/v) of 50 mM citrate-phosphate buffer, pH 6.0, containing 0.2% Cutscum and 1.0% sodium taurocholate in a Waring blender. Following centrifugation at

49,000 *g* for 1 hr, the clear supernatants were collected and assayed for enzymatic activity. This same supernatant was also used to measure and compare the glucocerebrosidase cross-reacting material (CRM) titers of normal and affected tissues since it was shown that in both Gaucher and normal homogenates, greater than 90% of the total enzymatic activity could be solubilized with the two detergents (data not shown). Protein was determined by the method of Lowry et al. [9]. One U of catalytic activity is defined as the hydrolysis of 1 nmol of substrate per hr at 37°C.

Tissue Storage

Spleens from Gaucher patients or from normal autopsy cases (< 6 hrs) were obtained over a 2-year period and kept frozen at −40°C. The spleens from the adult nonneuropathic (type I) form of the disease came from male and female patients ranging in age from 15 to 45 years. The two spleens from the juvenile form (type III, Norbottian variety) of the disorder came from two boys aged 6 and 14. The single spleen representing the infantile (type II) form of Gaucher disease came from a 9-month-old female patient at autopsy.

RESULTS

Enzymatic Assays of Normal and Gaucher Spleen Extracts

While all the splenic extracts contained a rather uniform level of cholesterol or phosphatidyl ethanolamine for the most part, the glucocerebroside levels in Gaucher spleens were, as expected, 11–200 times higher than in normal tissues (table 1). The specific catalytic activity of glucocerebrosidase was dramatically lower in Gaucher spleen extracts, ranging from 2.3% ± 0.6% in spleens of Gaucher patients with the type II and III forms of the disorder to 15% ± 7% of normal in type I spleens (table 1). Several other lysosomal hydrolases, while generally elevated in the affected spleens, showed no statistically significant differences between the nonneurological and neurological forms of the disorder (table 1).

Standardization of Glucocerebrosidase Immunoprecipitation Curve and Immunotitration of Catalytic Activity in Normal and Gaucher Spleen Extracts

When increasing quantities of purified human placental glucocerebrosidase were added to a constant quantity of rabbit antihuman glucocerebrosidase γ-globulin, a decreasing percentage of the standard added enzyme was precipitated. An experimentally determined standard curve was generated from this series of enzyme dilutions (fig. 1). From this curve, the absolute amount of immunoreactive glucocerebrosidase protein in normal and Gaucher spleen extracts could be compared to the immunoprecipitation of purified standard enzyme by measuring the percent of catalytic activity precipitated from a given aliquot of extract. A unit of antigenic activity is defined as the amount of CRM that will precipitate to the same extent as 1 catalytic U of purified standard glucocerebrosidase. The immunoprecipitation of crude glucocerebrosidase was shown to be in the dose response range since differing aliquots of spleen extracts gave the same CRM titer (table 2). This titer was lower in Gaucher spleens. The Gaucher tissue extracts themselves did not interfere in the interaction of enzyme with antibody since a mixture of normal and affected spleen extracts gave an antigenic titer that was additive (table 2). Partial purification of glucocerebrosidase from the crude splenic extracts did

TABLE 1

GLUCOCEREBROSIDE LEVELS AND LYSOSOMAL HYDROLASE ACTIVITIES IN NORMAL AND GAUCHER SPLEEN EXTRACTS

Type	Glucocerebroside (mg/g wet tissue)	Glucocerebrosidase	Sphingomyelinase	Acid phosphatase (U/mg protein)	α-Galactosidase	β-Hexosaminidase	β-Galactosidase	β-Glucuronidase	α-Mannosidase
Normal:									
1	0.14	24	6.6	4,645	11	105	55	58	18
2	0.21	23	5.7	3,333	31	118	67	94	46
3	0.08	24	2.3	2,727	6	85	30	36	18
4	0.13	19	5.4	3,484	21	110	53	95	1
5	0.05	24	2.9	4,000	48	194	65	127	27
6	0.10	18	2.2	2,625	9	113	56	80	1
Mean:	0.12 ± 0.06	22 ± 3	4.2 ± 2.0	3,469 ± 768	21 ± 16	121 ± 38	54 ± 13	82 ± 32	18 ± 17
Gaucher (type I):									
1	24	2.6	5.0	4,365	20	394	100	199	58
2	12	6.0	8.3	5,419	21	347	110	126	42
3	19	2.0	4.4	3,243	11	304	51	116	35
4	14	5.0	7.3	4,571	16	128	81	57	37
5	23	1.6	3.5	3,429	10	309	48	105	27
6	16	3.0	6.1	6,000	19	352	83	129	42
7	22	2.4	5.0	4,941	22	354	71	157	38
8	21	3.1	3.4	3,789	12	271	59	125	37
Mean	17 ± 7	3.2 ± 1.5	5.4 ± 1.7	4,470 ± 1,000	16.4 ± 4.8	307 ± 82	75 ± 22	127 ± 41	39.5 ± 9
Gaucher (type III):									
1	26	0.57	2.9	4,114	11	317	61	· · ·	33
2	26	0.50	4.6	3,600	15	444	47	· · ·	50
Gaucher (type II):									
1	22	0.36	4.7	3,111	30	474	111	· · ·	56
Mean (types II and III)	24 ± 7	0.48 ± 0.11	4.1 ± 1	3,610 ± 500	19 ± 10	412 ± 83	73 ± 33	· · ·	46 ± 12

NOTE: Enzyme assays were carried out as described in the text with crude extracts obtained from frozen tissue samples. A unit is defined as the hydrolysis of 1 nmol substrate/hr at 37°C. Glucocerebroside levels were measured by extraction of lipids from the crude tissue extract with chloroform-methanol (2:1, v/v) and a subsequent separation and quantitation of the lipids by thin-layer chromatography as described [10].

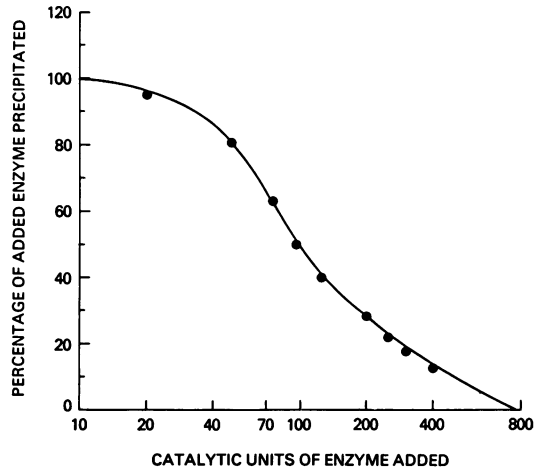

FIG. 1.—Standard immunoprecipitation curve for glucocerebrosidase. To triplicate tubes containing dilutions of highly purified human placental glucocerebrosidase, a constant quantity of rabbit antihuman glucocerebrosidase antibody was added, followed by goat antirabbit globulin antibody. The percentage of glucocerebrosidase immunoprecipitated was plotted against the initial enzyme U added in order to generate the experimentally determined standard curve.

not affect its immunoprecipitation properties (data not shown). The enzymatic and antigenic specific activities of glucocerebrosidase in normal spleen extracts were equivalent (table 3), showing that the construction of the standard immunoprecipitation curve with purified placental glucocerebrosidase is valid for the immunological quantitation of splenic enzyme. Additional confirmation of the lower CRM titer in Gaucher tissues is seen by comparing the complete antibody dose response curves of the normal and Gaucher spleen enzymes (fig. 2). Equal protein concentrations of each extract were incubated with a fixed serial dilution of antibody. Lower concentrations of γ-globulin were needed throughout the

TABLE 2

ANTIGEN DOSE RESPONSE FOR THE IMMUNOPRECIPITATION OF GLUCOCEREBROSIDASE IN NORMAL AND GAUCHER SPLEEN EXTRACTS

Extracts	% of activity precipitated	Antigenic U/mg protein
Normal spleens (6):		
0.1 ml	62 ± 10	23 ± 3
0.2 ml	34 ± 4	22 ± 2
Gaucher spleens (11):		
0.1 ml	85 ± 5	12 ± 2
0.2 ml	60 ± 4	11 ± 2
Mixture		
.1 ml normal + .1 ml Gaucher	50	17

NOTE: Normal and Gaucher spleen extracts were prepared and assayed for glucocerebrosidase CRM as described in text. From the percent of catalytic activity precipitated, the antigenic activity equivalent to that of standard purified enzyme (antigenic U) could be obtained by using the standard curve defined in figure 1. Two different aliquots of each spleen extract was tested. The values are the statistical summation of the six normal and 11 affected spleens listed individually in table 3.

TABLE 3

CATALYTIC AND ANTIGENIC QUANTITATION OF GLUCOCEREBROSIDASE IN NORMAL AND GAUCHER SPLEENS

Type	Enzymatic activity (catalytic U/mg protein)	Antigenic activity (antigenic U/mg protein)	Absolute specific activity (enzymatic activity/antigenic activity)
Normal:			
1	24	22	1.09
2	23	21	1.10
3	24	25	0.96
4	19	21	0.90
5	24	25	0.96
6	18	20	0.90
Mean	22 ± 3	22 ± 3	0.99 ± 0.09
Gaucher (type I):			
1	2.6	13.0	0.20
2	6.0	15.0	0.40
3	2.0	10.0	0.20
4	5.0	14.0	0.36
5	1.6	10.0	0.16
6	3.0	12.0	0.25
7	2.4	11.0	0.22
8	3.1	10.0	0.31
Mean	3.2 ± 1.5	12 ± 2	0.26 ± 0.09
% of normal	15% ± 7%	54% ± 9%	26% ± 1%
Gaucher (type III):			
1	0.57	11	0.05
2	0.50	13	0.04
Gaucher (type II):			
1	0.36	15	0.02
Mean (type II and III)	0.48 ± 0.11	13 ± 2	0.04 ± 0.02
% of normal	2.3% ± 0.6%	59% ± 10%	4% ± 2%

NOTE: Enzymatic and immunological assays were carried out as described in text. Catalytic U is defined as hydrolysis of 1 nmol substrate/hr at 37°C. Antigenic U is amount of CRM that will precipitate to the same extent as a catalytic U of purified standard glucocerebrosidase.

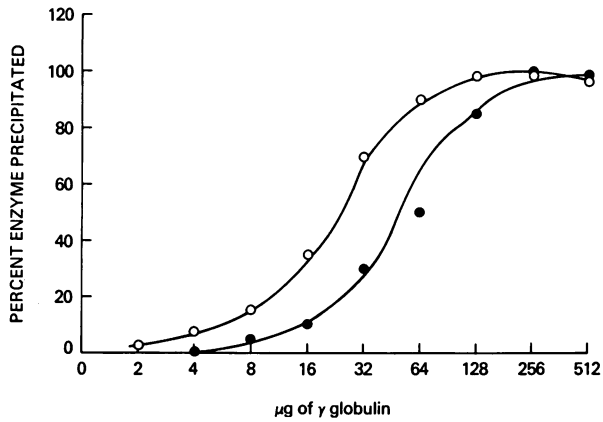

FIG. 2.—Antibody dose response curves for precipitation of glucocerebrosidase from normal and Gaucher spleen extracts. Splenic extracts were prepared as described in text. Aliquots of normal (spleen no. 4) and Gaucher (type I, spleen no. 4) (table 3) containing 1 mg of protein were assayed for glucocerebrosidase CRM as noted with increasing concentrations of rabbit antiglucocerebrosidase γ-globulin. ○—○, Gaucher; ●—●, normal.

curve for the precipitation of glucocerebrosidase from Gaucher extract compared to normal.

DISCUSSION

No significant differences in the elevated glucocerebroside levels were noted among the three clinical forms of Gaucher disease. An unusually low quantity of glycolipid was noted in one of the nonneurological type I Gaucher patients (spleen no. 4, female, age 40). The catalytic activity of glucocerebrosidase in splenic extracts of type I Gaucher patients was reduced to 15% ± 7% of normal. The spleens from the three Gaucher patients afflicted with the neurological forms of Gaucher disease showed a statistically significant lower level of glucocerebrosidase activity (2.3% ± 0.1% of normal) than from the nonneurologically involved (type I) patients. This difference in catalytic activity between the nonneurological and neurological forms of Gaucher disease was not noted with seven other hydrolases. It will be of interest in the future to study the relative levels of glucocerebrosidase activity in a larger and more varied group of Gaucher tissues in order to determine if this difference in enzyme activity reflects a consistent biochemical parameter that distinguishes the neurological and nonneurological forms of this disorder.

The absolute specific activity of glucocerebrosidase (catalytic/antigenic) [11] in all the Gaucher spleens was significantly lower than that of the normal tissues (table 3), indicating that all three clinical forms of Gaucher disease have as a common molecular feature a structurally mutated and catalytically deficient glucocerebrosidase molecule. The measurable glucocerebrosidase CRM titer in the Gaucher spleen extracts was substantially lower than those found in normal spleens. This lower CRM titer was consistently found irrespective of the clinical form of the disorder, the tissue environment in which the immunoassay was carried out,

or the degree of purity of the enzyme. Thus, the attenuated levels of measurable catalytic glucocerebrosidase activity in crude tissue extracts appear to reflect a structurally mutated enzyme compromised both in its catalytic efficiency and possibly in its antigenic expression. Further comparative studies of the turnover and antigenic equivalency of normal and mutated glucocerebrosidase will have to be carried out in order to answer these questions more fully.

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