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Quantitation of the Enzymically Deficient Cross Reacting Material in G_{M1} Gangliosidoses

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INTRODUCTION

G_{M1} gangliosidoses are glycolipid storage diseases characterized by an accumulation of G_{M1} ganglioside and a deficiency of β-galactosidase activity towards this substrate [1]. These disorders have been classified into two major types which are inherited as autosomal recessive traits [2]. G_{M1} gangliosidosis type 1 is distinguished by congenital retardation of psychomotor development, rapid degeneration of the central nervous system, prominent skeletal abnormalities and visceromegaly, and death before 2 years of age [3]. In type 2, neurological deterioration progresses slowly without significant bony abnormalities or visceromegaly, and survival may be as long as 10 years [4]. In addition to these two major types, a number of adult variants of G_{M1} gangliosidosis have recently been reported [5–7]. Several of the adult patients have exhibited a mild neurodegenerative course [5, 6], while others have had normal intelligence with severe bony abnormalities [7].

The β-galactosidase that hydrolyzes G_{M1} ganglioside and is deficient in G_{M1} gangliosidoses is also known to hydrolyze galactose from aryl-β-D-galactosides [8], asialofetuin [9], and lactosylceramide under specified assay conditions [10]. In G_{M1} gangliosidosis type 2 and in the adult variants, residual β-galactosidase activities towards these substrates are higher in liver and skin fibroblasts than in type 1 [11]. These higher residual activities may account for the clinical differences among the various types. Material cross reacting with antiserum to β-galactosidase has been demonstrated in liver of patients with G_{M1} gangliosidoses by double gel immunodiffusion and by immunotitration of the residual enzymic activity. The approximate amount of cross reacting material (CRM) has been estimated to be 15%–30% of normal in one patient [12] and about normal in several others [11, 13]. We present here a sensitive immunoassay for the precise quantitation of G_{M1} β-galactosidase CRM.

MATERIALS AND METHODS

Clostridium perfringens neuraminidase and galactose oxidase were obtained from Worthington Biochemical (Freehold, N.J.), Sepharose 4B from Pharmacia (Piscataway, N.J.), agarose

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from Aldrich Chemical (Milwaukee, Wis.), and Eagle's minimal essential medium (MEM) from Gibco (Grand Island, N.Y.). Bovine brain gangliosides and lactosyl-*N*-stearoyl-dihydrosphingosine were from Miles Laboratories (Elkhart, Ind.), 4-methylumbelliferyl-β-D-galactoside, naphthol AS-LC-β-D-galactoside and bovine serum albumin from Sigma (St. Louis, Mo.), and sodium taurocholate, sodium taurodeoxycholate, and oleic acid from Calbiochem (La Jolla, Calif.). Sodium boro[^{3}H]hydride 270 mCi/mmol was from New England Nuclear (Boston, Mass.). Goat antiserum to rabbit immunoglobulins was purchased from Behring Diagnostics (Somerville, N.J.). Immunoglobulin G (IgG) fractions of rabbit antisera to human liver β-galactosidases A_1 and A_2 were prepared as previously described [14]. β-Galactosidases A_1 and A_2 were purified from human placenta as previously described for liver [14].

Tissue Preparation

Fibroblasts were derived from skin biopsies of normal controls and of patients with Krabbe disease, I-cell disease, and G_{M1} gangliosidosis types 1 and 2. One G_{M1} gangliosidosis type 1 cell line (GM 806) and one I-cell strain (GM 1586) were obtained from the Human Mutant Cell Repository, Camden, N.J. Fibroblast cultures from two patients with the adult type of G_{M1} gangliosidosis (B. J. and R. N.) were obtained from Dr. J. S. O'Brien. The cells were cultivated in MEM supplemented with 15% fetal calf serum. At confluency, the medium was decanted, the cells were washed twice with isotonic saline and harvested using 0.25% trypsin in Hank's solution. The cell pellets were washed three times in isotonic saline, resuspended in distilled water, and following five cycles of freezing and thawing, the insoluble fraction was removed by centrifugation for 15 min at 12,500 *g*. Preparation of liver and placenta homogenates was carried out as previously described [15]. The Folin method [16] was used for protein assay with bovine serum albumin as a standard.

Enzyme Assays

4-Methylumbelliferyl-β-galactosidase activity was determined by an Aminco-Bowman spectrofluorometer as previously described [15], and naphthol-β-galactosidase activity was demonstrated in precipitin lines [14]. Lactosylceramide was labeled with tritium by the galactose oxidase-sodium boro[^{3}H]hydride method [17]. Lactosylceramidase I activity was assayed according to Wenger et al. [18], and lactosylceramidase II activity was assayed in the presence of sodium taurodeoxycholate [10]. G_{M1} ganglioside prepared from bovine brain gangliosides [19] was tritiated by modifying [20] the procedure of Radin et al. [17]. G_{M1} β-galactosidase activity was assayed according to Norden and O'Brien [9]. Radioactivity was measured in a liquid scintillation spectrometer.

Immunochemical Procedures

Double immunodiffusion was carried out in 1% agarose gel in phosphate buffered saline (PBS), 0.01 M phosphate, 0.14 M NaCl, pH 7.0. Immunonoadsorbants were prepared by coupling IgG fractions of antisera against β-galactosidases A_1 and A_2 to cyanogen bromide-activated Sepharose 4B [21]. Tissue preparations (20 ml, 4 mg protein/ml) were passed through the immunoadsorbant conjugates (5 ml, 28 mg IgG/ml), and after washing, the original sample and the unadsorbed fraction were assayed for the various enzymic activities.

Single radial immunodiffusion was performed in 1% agarose gel in PBS according to Mancini et al. [22], modified as follows: the rabbit antisera to β-galactosidase isozymes were incorporated in the gels, and 15 ml of antibody-containing agarose were poured onto a petri dish (9 cm diameter). Holes with 3 mm diameter were punched at 10 mm intervals and 10 μl of enzyme-containing solutions were applied to the wells using a microliter syringe (Hamilton, 701N). Diffusion was allowed to proceed for 48 hr at 4°C; the dishes were then washed with PBS over the next 48 hr at 4°C. The gels were then covered by goat antiserum to rabbit immunoglobulins and after 24 hr diffusion and 48 hr wash, stained for protein with 1% Naphthol Blue-Black in acetic acid:methanol:water 1:5:5 (v/v/v). The diameter of the stained rings was measured and the area calculated.

TABLE 1

IMMUNOADSORPTION OF G_{M1} β-GALACTOSIDASE ACTIVITY BY SEPHAROSE-IgG CONJUGATES OF ANTISERA TO HUMAN LIVER β-GALACTOSIDASES A_1 AND A_2

Tissue Preparations	Original Activity	Unadsorbed with Anti-A_1	Unadsorbed with Anti-A_2
Liver homogenate	247 (100)	18.0 (7.3)	28.9 (10.5)
Placenta homogenate	66 (100)	4.2 (6.4)	5.2 (7.9)
Fibroblast lysates	214 (100)	16.0 (7.5)	21.0 (9.8)

NOTE. — Activity = nmol/hr/mg protein (%).

RESULTS

Precipitin lines of identity were demonstrated by double immunodiffusion between purified placenta isozymes and crude preparations of normal fibroblasts, liver, and placenta. Identity was also found between skin fibroblast lysates of normal controls and patients with Krabbe disease, I-cell disease, and G_{M1} gangliosidosis types 1 and 2 and the adult type. Enzymic activity was demonstrated after 1 hr incubation at 37°C with naphthol-AS-LC-β-D-galactoside in the precipitin bands of normal liver, placenta, and normal and Krabbe fibroblasts.

Results obtained following immunoadsorption of G_{M1} β-galactosidase activity from preparations of normal liver, placenta, and skin fibroblasts using antibody-Sepharose conjugates are shown in table 1. Similar adsorption was demonstrated with the IgG fraction of either of the two anti-β-galactosidase antisera. Adsorption of more than 85% of the original activity was also found for 4-methylumbelliferyl-β-galactosidase and lactosylceramidase II, but no significant adsorption of lactosylceramidase I occurred.

Calibration of the single radial immunodiffusion assay for the detection of both β-galactosidases A_1 and A_2 was carried out with the purified placenta isozymes in agarose gel containing either of the two anti-liver β-galactosidase antisera. As seen in figure 1, there is a linear relationship between ring area and enzyme concentration with identical sized rings for both isozymes in each of the antibody plates.

Fibroblast lysates of the various cell types and concentrated medium of an I-cell culture were assayed for β-galactosidase activity towards G_{M1} ganglioside. The CRM was quantitated in these preparations by the single radial immunodiffusion assay, and the ratio of G_{M1} β-galactosidase activity to CRM was calculated. The results are shown in table 2. Both CRM and G_{M1} β-galactosidase activity were higher than normal in Krabbe cells and lower in I-cell preparations, resulting in CRM specific activity similar to controls. In G_{M1} gangliosidosis type 1, normal amounts of CRM were found with about 1/500 of control CRM specific activity. Elevated quantities of CRM were found in G_{M1} gangliosidosis type 2, but the CRM specific activity was only 1/100 of control cells. In the two adult variants, normal amounts of CRM were present so that CRM specific activity was only reduced to 1/30 of normal.

DISCUSSION

In this paper, we report a sensitive and reliable immunoassay for quantitative determination of G_{M1} β-galactosidase CRM, independent of its catalytic activity.

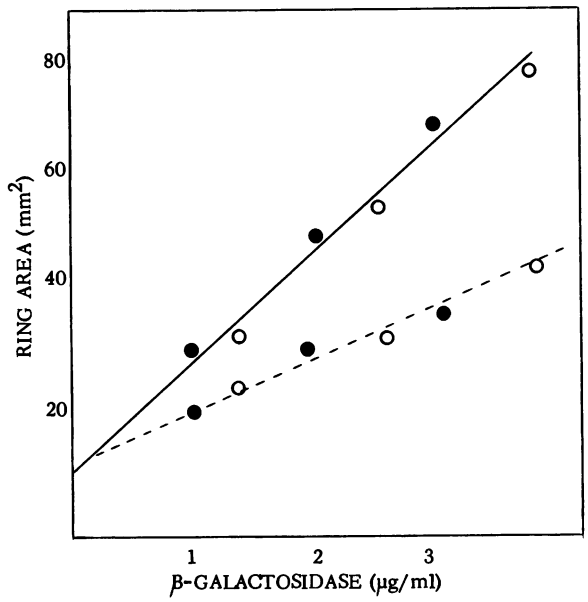

FIG. 1.—Single radial immunodiffusion of human placenta β-galactosidases A_1 (•) and A_2 (○) in 1% agarose gels in PBS, containing IgG fractions of antisera to human liver β-galactosidases A_1, 0.21 mg IgG/ml (—) and A_2, 0.85 mg IgG/ml (---).

Introduction of a second antibody to the single radial immunodiffusion assay, namely, goat antiserum to rabbit immunoglobulins, results in high sensitivity [23] and allows the quantitation of CRM in tissue preparations with a concentration of 1–2 μg/ml. The identity found among the enzymes of the various tissues as well as between the normal and the patients' CRM excludes the possibility of tissue specificity of the enzyme and/or antigenic dissimilarity between the normal enzyme and the CRM in the various diseases studied.

The levels of CRM specific activity demonstrated in our study of patients with G_{M1} gangliosidoses are comparable to those derived from the data of O'Brien and Norden in one patient with type 2 [20] and three with the adult type [13]. Using this assay, additional differences were demonstrated between G_{M1} gangliosidosis types 1 and 2, regarding quantity and specific activity of G_{M1} β-galactosidase CRM. Although increased quantities of CRM were observed in type 2, this does not solely account for the difference in residual activity between types 1 and 2, since CRM specific activity was also significantly higher in type 2. The CRM specific activity was even higher in the two adult variants studied, each of which exhibited normal levels of CRM. This method may serve as a tool for the study of the molecular defect in related β-galactosidase disorders.

SUMMARY

Normal quantities of G_{M1} β-galactosidase cross reacting material (CRM) (0.31–0.47 μg/mg protein) were detected by a sensitive radial immunodiffusion assay in skin fibroblasts from patients with G_{M1} gangliosidosis type 1 and adult variants, whereas

TABLE 2

COMPARISON OF G_{M1} β-GALACTOSIDASE ACTIVITY AND ANTIGENICALLY CROSS REACTING MATERIAL (CRM) IN SKIN FIBROBLASTS

Cell Strain	No.	Activity*	CRM†	Activity/CRM‡
Normal	15	110–289	0.28–0.45	436–611
Krabbe	8	276–398	0.47–0.61	522–595
I-cell	3	34–44	0.06–0.08	558–567
Medium	1	6.8	0.015	453
G_{M1} type 1	7	0.2–1.2	0.31–0.47	0.6–2.6
G_{M1} type 2	6	2.2–5.3	0.41–0.72	3.9–8.9
G_{M1} adults	2	4.8,7.0	0.29,0.36	17,19

* G_{M1} β-galactosidase activity: nmol/hr/mg protein.
† CRM: μg/mg protein.
‡ CRM specific activity: nmol G_{M1}/hr/μg CRM.

elevated levels were found in G_{M1} gangliosidosis type 2 (0.41–0.72 μg/mg protein). The specific activity of the immunologically CRM towards G_{M1} ganglioside of normal fibroblasts was about 500 times that of type 1, 100 times that of type 2, and 30 times that of the adult variants.

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