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Nature of the Mutation in Adult β-Galactosidase Deficient Patients

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INTRODUCTION

Evidence suggests that the mutation in G_{M1} gangliosidosis is structural. When liver samples from 11 patients with infantile (type 1) and juvenile (type 2) G_{M1} gangliosidosis were analyzed by double immunodiffusion, crossreacting materials to anti-β-galactosidase A antibody were found in each [1]. Analysis by Meisler and Rattazzi [2] of a patient with type 1 G_{M1} gangliosidosis gave the same result. In another patient (C. E.) with a phenotype similar to type 2, the quantity of structurally altered β-galactosidase was within the normal range, but the catalytic activity was 1% of normal [3]. The mutant enzyme from C. E. had a more positive electophoretic charge than normal both before and after treatment with neuraminidase, and K_m for G_{M1} was five times normal. Thus, all 13 patients with G_{M1} gangliosidosis studied thusfar can be classified as crossreacting positive (CRM+) mutants. Patient C.E. is a proven kinetic mutant and the others are likely so.

Several unusual β-galactosidase deficient adults who are chronically affected but with less severe involvement than patients with types 1 and 2, have recently been described [4–6]. In a patient from Holland, cell hybridization of his fibroblasts and those from patients with types 1 or 2 gave complementary β-galactosidase activity in the fused heterokaryons [7]. It was suggested that a regulator mutation in the Holland patient might account for the complementation.

We describe here three unusual β-galactosidase deficient adults, including the Holland patient, who can be classified as CRM+ mutants, synthesizing nearly normal quantities of structurally altered β-galactosidase.

MATERIALS AND METHODS

Clinical descriptions of the three adults studied here have been reported previously. Patient Holl is the Holland patient reported by Loonen et al. [4]; patient B. J. is the younger of the two siblings reported by Wenger et al. [5]; and patient R. N. is the girl with an unusual type of spondyloepiphyseal dysplasia reported by O'Brien et al. [6].

Cultured fibroblasts from these three individuals, from controls, and from nine patients with

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G_{M1} gangliosidosis types 1 and 2 were grown as described previously [8]. The media was washed twice with isotonic saline 2–3 weeks after subculture, and the cells were harvested by scraping. Five volumes of 20 mM sodium acetate buffer, pH 4.3, containing 4 mM sodium chloride were added, and the sample was frozen and thawed three times in acetone-dry ice and centrifuged at 11,000 *g* for 15 min to obtain the supernatant. In all instances, greater than 90% of the β-galactosidase activity was in the supernatant.

The activities of G_{M1} β-galactosidase, 4-methylumbelliferyl β-D-galactosidase, and asialofetuin β-galactosidase were assayed as previously described [9, 10]. *N*-Acetyl-β-D-glucosaminidase activity was measured as described [8]. Immunoglobulin G (IgG) was prepared from control serum and from antisera against human liver β-galactosidase A [9] by the method of Fahey [11]. The IgG was concentrated to 40 mg of protein per milliliter by ultrafiltration. IgG solutions were then heated at 55°C for 30 min and centrifuged at 50,000 *g* for 30 min to inactivate rabbit serum β-galactosidase. Supernatants were used for immunotitrations which were performed as follows: 50 μl of β-galactosidase solution was added to 50 μl of IgG which was diluted in 10 mM sodium phosphate buffer containing 10 mM sodium chloride (pH 7.0). Tubes were left at 4°C for 16 hr and centrifuged at 50,000 *g* for 30 min. The supernatants were then assayed. Addition of anti-β-galactosidase IgG to control supernatants without centrifugation did not inhibit activity of the enzyme.

Precise quantitation of β-galactosidase activity was not possible in the immunotitration experiments. This was due to the small volume of tissue available and the low activity present in the mutant lines, necessitating long incubation times where linearity of enzyme activity was not maintained. For the same reason, immunotitration experiments were not possible in any of the patients with G_{M1} gangliosidosis type 1. Precise quantitation, however, was possible in supernates, and the values in table 1 were obtained under conditions where activity was linear.

RESULTS

The 4-methylumbelliferyl and the G_{M1} β-galactosidase assays were sensitive enough to measure activity in the mutant supernates at 0.05% of the control values; with

TABLE 1

ENZYME ACTIVITY IN CULTURED SKIN FIBROBLAST SUPERNATANTS

Subjects	4MU β-Gal	ASF β-Gal	GM_1 β-Gal	4MU GluNac
Controls (No. = 15)	24.5 (15.7–46.6)	0.61 (0.18–2.0)	5.31 (2.4–13.8)	170 (88–228)
GM_1 Type 1:				
K. W.	0.48	0.027	0.032	190
C. M.	0.32	0.023	0.021	150
J. K.	0.62	0.080	0.020	182
K. D.	0.47	0.033	0.040	235
GM_1 Type 2:				
D. J.	0.63	0.072	0.12	170
C. J.	0.60	0.040	0.21	...
W. J.	7.40	0.028	0.60	212
C. E.	2.70	...	0.21	269
M. J.	0.68	...	0.48	129
Adult Type:				
Holl	1.29	0.050	0.28	190
B. J.	1.18	0.100	0.32	202
R. N.	0.85	0.008	0.37	160

NOTE. — Activities are expressed as nmol/mg protein per min using fibroblast supernatants. β-Gal = β-galactosidase; β-GluNac = *N*-acetyl-β-D-glucosaminidase; 4 MU = 4-methylumbelliferyl; ASF = asialofetuin. Descriptions of all patients, except K. W. (unpublished) and W. J. [13], can be located by consulting table 1 in reference [3].

asialofetuin the value was 0.5%. Remarkable deficiencies of G_{M1} β-galactosidase, 4-methylumbelliferyl β-galactosidase, and asialofetuin β-galactosidase were present in the cultured fibroblasts from the adult variants (table 1). G_{M1} β-galactosidase activities in the adult cell lines were 10 times higher than in patients with type 1 G_{M1} gangliosidosis and similar to those with type 2.

Immunotitration curves for 4-methylumbelliferyl β-D-galactosidase, G_{M1} β-galactosidase and asialofetuin β-galactosidase activities give nearly identical curves in control fibroblasts (fig. 1). The results indicate that these activities are the property of the same enzyme, a fact that has been previously established in normal human liver [9] but not in cultured fibroblasts.

When β-galactosidase was precipitated with anti-β-galactosidase A IgG from mutant supernates at the same cell protein concentration as controls, the shape of the immunoprecipitation curves was similar to those of controls. However, the activity of β-galactosidase was much lower in the supernates from the mutant lines. In patients B. J. and R. N., using the 4-methylumbelliferyl substrate (fig. 2) as well as G_{M1} (not shown), activity was less than 10% of control values. In patient Holl, about 25% residual activity at each dilution of antibody was found using the 4-methylumbelliferyl substrate (fig. 3*a*), and 10%–14% activity was found when G_{M1} was used (fig. 3*b*). On a cell protein basis, each cell line from the adult patients had a concentration of

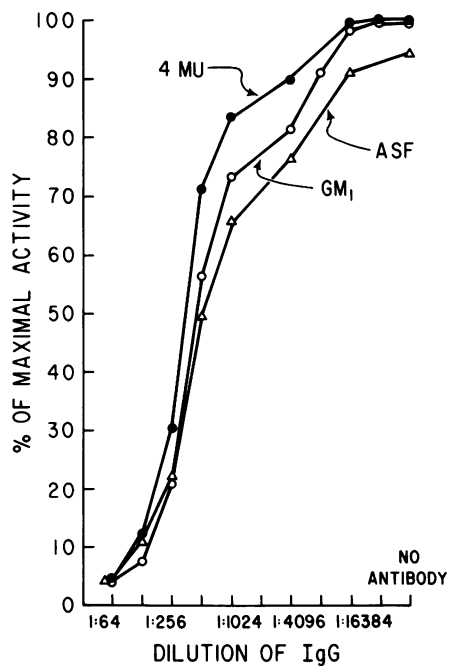

FIG. 1.—Immunotitration of control fibroblast acid β-galactosidase using anti-β-galactosidase immunoglobulin G (IgG). Activity is expressed as percent of maximal activity for 4-methylumbelliferyl β-galactosidase (●—●, 4MU), G_{M1} β-galactosidase (○—○, G_{M1}) and asialofetuin β-galactosidase (△—△, ASF).

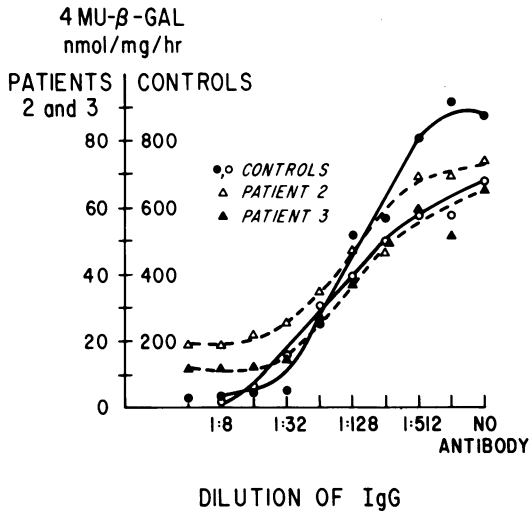

FIG. 2.—Immunotitration of 4-methylumbelliferyl β-galactosidase (4MU-β-Gal) from controls (•, ○) and patients 2, B. J., (△) and 3, R. N., (▲). Note the different activity scales for controls and patients.

immunologically reactive β-galactosidase which fell within, or exceeded, the normal range.

When anti-β-galactosidase A IgG was added to supernatants from controls or the adults' cells and activity was measured (prior to the centrifugation step) after 1 hr incubation at 37°C, no inhibition or stimulation of activity was found. Measurement of the activity of 4-methylumbelliferyl β-D-*N*-acetylglucosaminidase in supernatants after immunoprecipitation with anti-β-galactosidase A IgG revealed no change in activity. IgG prepared from nonimmunized rabbits failed to precipitate β-galactosidase activity in controls or patients cells.

Kinetic studies using 4-methylumbelliferyl β-D-galactoside gave a K_m in all three adult patients close to normal (200 μM). It was not possible to obtain substrate-saturation curves using G_{M1} or asialofetuin as substrates due to the low residual activity. For the same reason it was not possible to visualize 4-methylumbelliferyl β-D-galactosidase on starch gels after electrophoresis.

DISCUSSION

Cells from the three adults appear to be synthesizing nearly normal quantities of catalytically altered β-galactosidase. The mutant enzyme in each patient was shown to possess G_{M1} β-galactosidase activity as well as 4-methylumbelliferyl β-D-galactosidase activity. Unfortunately, due to the low activity present it was not possible to determine whether the enzyme in the patients had the same molecular weight, electrophoretic mobility, and substrate saturation kinetics with G_{M1} and asialofetuin as the normal enzyme. Failure to demonstrate an altered K_m using the 4-methylumbelliferyl substrate does not rule out the possibility that they are kinetic mutants. Our previous study of β-galactosidase isolated from the liver from patient C. E. revealed a K_m for the 4-methylumbelliferyl substrate which was twice normal; with ganglioside G_{M1} as

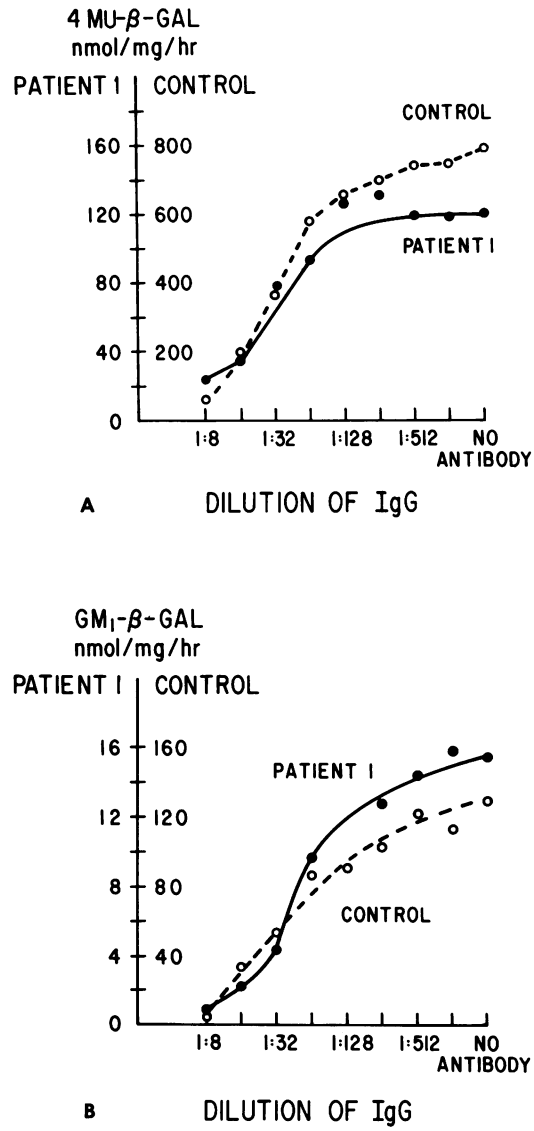

FIG. 3.—*A*, Immunotitration of 4-methylumbelliferyl-β-galactosidase (4MU-β-Gal) from control (○) and patient 1, Holl, (●); *B*, immunotitration of G_{M1} β-galactosidase (G_{M1}) from control (○) and patient 1 (●).

substrate, a K_m five times normal was observed [3]. In order to carry out this study in the adult patients, partial purification of the enzyme from a large volume of fibroblasts must be made.

The immunological titration data suggest that all three adults are CRM+ mutants. If one assumes antigenic equivalence with normal liver β-galactosidase A, the adult enzyme has about 10% of normal catalytic activity at the same level of immunological activity as controls. Admittedly, this quantitative assessment is crude and subject to

error due to the semi-quantitative nature of the immunoprecipitation assay. Nonetheless, the results indicate that approximately normal quantities of structurally altered β-galactosidase is biosynthesized in the mutant cells.

Somatic cell hybridization of cells from patients with type 1 or type 2 G_{M1} gangliosidosis and from one of the adults in this study (Holl) have yielded complementary β-galactosidase in the mixed heterokaryons [7]. A regulator mutation was suggested in the Holl cells to account for this result. Subunit rearrangements within the mixed heterokaryons was considered unlikely as a basis for the complementation since evidence suggests that the native enzyme has a single subunit [9]. The present studies indicate that the mutation in patient Holl is structural, not regulatory, and the nature of the complementation in the mixed heterokaryons remains unexplained. It has been suggested [1] that complementation could occur even with two allelic mutants if the structural alteration in one (or both) of the parent lines led to an alteration in the tertiary structure of the enzyme which would impair catalysis (folding) but would be corrected in the mixed heteropolymer due to favorable protein-protein interactions between the mutant monomers (unfolding). This situation is similar to that in E. coli mutants [12] in which restoration of β-galactosidase activity occurs when anti-β-galactosidase antibodies are introduced. Complementation could also occur after the fusion of two allelic mutants of a monomeric protein that are individually unstable, if a mixture of the two forms stabilized each other. In this instance, at least one of the original mutants would have reduced or absent CRM. This possibility has been ruled out for the G_{M1} mutants studied here. Another possibility is that β-galactosidase B, which has a molecular weight about 10 times that of β-galactosidase A and crossreacts with A [9], contains a polypeptide not shared with A. If the unique polypeptide is affected by a second mutation, it might explain some of the complementation seen in patient Holl. Purification and analysis of β-galactosidase B (now underway) should be illuminating. Further studies on the nature of the mutation in patient Holl are clearly necessary before conclusions can be drawn concerning the molecular basis of the complementation.

The present results add three patients with β-galactosidase deficiency to the previous 13 who have been determined to be CRM+ mutants. It appears that mutations leading to the synthesis of unstable, rapidly degraded enzyme, to enzyme leakiness from cells or tissue, or to depressed synthesis secondary to a structural or regulatory mutation are uncommon in humans with β-galactosidase deficiency.

SUMMARY

Fibroblasts from three chronically affected, β-galactosidase deficient adults were shown to synthesize nearly normal quantities of immunologically reactive catalytically deficient β-galactosidase, indicating that they are CRM+ structural mutants.

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