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Genetics

Mutations of glucocerebrosidase: Discrimination of neurologic and non-neurologic phenotypes of Gaucher disease

(alleles/processing/multiple molecular forms/electroblotting/crossreacting material)

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ABSTRACT **Multiple molecular forms of β-glucocerebrosidase that permit discrimination between neurologic and non-neurologic phenotypes of Gaucher disease have been identified radioimmunologically in fibroblasts and human brain tissue. In normal human fibroblasts these forms have been shown by NaDodSO$_4$/polyacrylamide gel electrophoresis to have apparent M_r of 63,000 (form A_1), 61,000 (form A_2), and 56,000 (form B). The M_r 63,000 form may be a precursor of the M_r 56,000 form. Non-neurologic Gaucher disease (type 1) fibroblasts and normal brain tissue are characteristic in that they contain only one major immunoreactive protein, the M_r 56,000 form. In contrast, fibroblast extracts and brain tissue from neurologic Gaucher disease phenotypes contain only the higher molecular weight forms A_1 and A_2. These data and the low residual activity of the enzyme in all the variants of Gaucher disease suggest that the mutations of β-glucocerebrosidase are allelic and involve the active site.**

Gaucher disease is one of several inherited lipidoses that have a wide spectrum of clinical presentations. All of the Gaucher disease phenotypes are characterized by a deficiency of glucocerebrosidase with resultant tissue accumulation of the lipid glucocerebroside (1). Correlations between Gaucher disease subtype or clinical severity and residual enzyme activity or tissue glucocerebroside content (or both) have been inadequate (2–5).

Biochemical characterizations of β-glucosidase from normal and Gaucher disease tissues have suggested that the clinical subtypes of Gaucher disease contain different altered forms of β-glucosidase (6–14). However, consistent differences in properties of the enzyme have not been uniformly demonstrated, so that no adequate differentiation of subtype or severity within subtype has been provided (15–19). Recently, multiple forms of normal and Gaucher disease tissue β-glucocerebrosidase have been described by isoelectric focusing techniques, but this still has not permitted differentiation of Gaucher disease phenotypes (20–26).

Here we report the identification of multiple molecular forms of β-glucocerebrosidase in human brain tissue and fibroblasts that differ in NaDodSO$_4$/polyacrylamide gel electrophoresis determination of molecular weight and that permit discrimination between the neurologic and non-neurologic Gaucher disease phenotypes. A hypothesis for the biochemical and genetic bases for the clinical heterogeneity of neurologic and non-neurologic Gaucher disease is presented.

METHODS

Enzyme Purification and Assay. Human placental β-glucocerebrosidase isolated according to Furbish *et al.* (27) was further purified to homogeneity by either HPLC (unpublished data) or NaDodSO$_4$/polyacrylamide gel electrophoresis. β-Glucocerebrosidase activity was measured as described (27) by using 175 μl of 100 mM potassium phosphate buffer (pH 5.8) containing 0.15% Triton X-100 and 5 μl of D-β-[1-^{14}C]glucocerebroside (7.5 mg/ml in sodium taurocholate at 50 mg/ml).

Antiserum Production. The band located at M_r 65,000 on NaDodSO$_4$/polyacrylamide gel electrophoresis from a preparation of human placental β-glucocerebrosidase was removed from the gel. An aliquot containing 180 μg of protein was placed in complete Freund's adjuvant and injected subcutaneously into a 6-kg rabbit. Booster doses of β-glucocerebrosidase (180 and 200 μg) in incomplete Freund's adjuvant were given at 20 and 28 days. Antiserum obtained from this rabbit 10 wk after the initial inoculation was used in these experiments. Pre-immune serum was from the same rabbit. Immune and pre-immune sera were stored at −20°C.

Fibroblast Culture and Preparation. Skin fibroblasts from normals and Gaucher disease patients were obtained from patient biopsies and from the Human Genetic Mutant Cell Repository. All fibroblasts were cultured to confluency in McCoy's 5A medium supplemented with 10% fetal calf serum and antibiotics. Cells were harvested by trypsinization and washed with culture medium and phosphate-buffered saline (P_i/NaCl) at pH 7.2. Fibroblasts stored in a 50% (vol/vol) mixture of Cryoprotective media (M.A. Bioproducts, Walkersville, MD) and McCoy's 5A with 40% fetal calf serum were rapidly thawed at 37°C and washed with P_i/NaCl at pH 7.2.

Fibroblast pellets were suspended in 60 mM potassium phosphate (pH 6.6) containing 0.1% Triton X-100 and were then sonicated at 40 W for 5 sec at 4°C. The sonicates were centrifuged at 4°C at 48,000 × *g* for 20 min and the supernatants were stored at −20°C.

Tissue Extraction. Normal and type 2 Gaucher brain tissues were homogenized (20% wt/vol) in 60 mM potassium phosphate (pH 6.6) containing 0.1% Triton X-100. After sonication at 40 W for 30 sec at 4°C the homogenates were centrifuged at 4°C at 48,000 × *g* for 20 min. The supernatants were stored at −20°C. Protein was determined by the method of Bradford (28).

Polyacrylamide Gel Electrophoresis and Electroblotting. Samples to be electrophoresed were brought to a total volume of 200 μl of 125 mM Tris·HCl (pH 6.5) containing 10 mM dithiothreitol, 10% glycerol, and 1% NaDodSO$_4$. Prior to loading, the fibroblast and white cell samples were heated at 95°C for 2 min. Brain tissue samples were heated at 37°C for 2 min.

Electrophoresis was performed on vertical slab gels cooled

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Abbreviations: P_i/NaCl, phosphate-buffered saline; CRM, crossreacting material.

to 8–12°C in LKB model 2001 apparatus as described by Hasilik and Neufeld (29). The ratio of acrylamide to *N,N'*-methylenebisacrylamide was 30:0.312. Total monomer concentrations were 12.8% and 5.8% in the separating and stacking gels, respectively. The cathode buffer contained 6 g of Tris, 28.9 g of glycine, and 1 g of $NaDodSO_4$ per liter. $NaDodSO_4$ was omitted from the anode buffer. Electrophoresis was stopped 1½–2 hr after the bromphenol blue dye marker ran off the gel.

After electrophoresis, the gel was overlaid with nitrocellulose paper (Bio-Rad nitrocellulose or Schleicher & Schuell BA85 nitrocellulose) and transferred to a horizontal electrophoretic blotting apparatus. Electroblotting was performed for 2½ hr at 23°C at 200–300 mA in 25 mM Tris·HCl (pH 8.3) containing 192 mM glycine and 20% (vol/vol) methanol (30). After electroblot transfer, the lanes of nitrocellulose paper that contained the protein standards were stained with 0.1% Coomassie blue. The remaining nitrocellulose electroblot was processed for antigen detection by using antibody ^{125}I-labeled protein A (^{125}I-protein A) radiography.

Antibody ^{125}I-Protein A Radiography. After the electroblotting procedure, the nitrocellulose sheets were air dried and then soaked at 37°C for 60 min in P_i/NaCl (pH 7.2) containing 0.1% bovine serum albumin and 0.05% Tween 20. The nitrocellulose blots were transferred to 50-ml polypropylene centrifuge tubes (31) and washed twice with 10 ml of P_i/NaCl (pH 7.2) containing 0.05% Tween 20. Twenty microliters of antisera in 10 ml of P_i/NaCl (pH 7.2) containing 0.05% Tween 20 was added and the tubes rotated at 37°C for 60 min. The blot sheets were then washed three times for 5 min by rotation with 10 ml of P_i/NaCl (pH 7.2) containing 0.05% Tween 20. Ten milliliters of P_i/NaCl (pH 7.2) containing 0.05% Tween 20 and 25 μl of ^{125}I-protein A (New England Nuclear; 60–80 μCi/μg; 1 Ci = 3.7×10^{10} becquerels) was added and rotation was continued at 23°C for 1 hr. The nitrocellulose blots were then washed six times by rotation with 10 ml of P_i/NaCl (pH 7.2) containing 0.05% Tween 20 and were dried (Bio-Rad slab dryer) between two sheets of cellophane (noncoated 150 PD cellophane; Du Pont). Radiography was performed on XAR-5 film (Kodak) at 23°C for 1–5 days.

RESULTS

Prior to HPLC purification, $NaDodSO_4$/polyacrylamide gel electrophoresis demonstrated that placental β-glucocerebrosidase contained protein bands with apparent M_r of ≈65,000 and ≈46,000. Immunoradiochemical nitrocellulose-blot radiography of this preparation of placental β-glucocerebrosidase with antibody made against homogeneous glucocerebrosidase showed only one band of M_r 65,000. The M_r 46,000 polypeptide showed neither glucocerebrosidase activity (unpublished data) nor reactivity with the antibodies against the M_r 65,000 component. The immunoradiochemical nitrocellulose blots processed without immune sera or with pre-immune sera were blank. The radiograph bands detected with the antiserum thus represent polypeptides that are antigenically similar to homogeneous M_r 65,000 human placental β-glucocerebrosidase.

Immunoradiochemical nitrocellulose blots demonstrating crossreacting material (CRM) to human placental homogeneous β-glucocerebrosidase found in normal and types 1, 2, and 3 Gaucher-disease confluent fibroblast extracts are shown in Fig. 1. Normal fibroblasts have major forms with apparent M_r of ≈63,000 (A_1) and 56,000 (B) and a minor form having an apparent M_r of ≈61,000 (A_2). Non-neurologic Gaucher disease (type 1) fibroblasts contain a single major CRM band at M_r 56,000 and comparatively minor CRM bands at M_r 63,000 and 61,000. Non-confluent type 1 Gaucher disease fibroblast extract contained variable proportions of CRM band at M_r 63,000 and

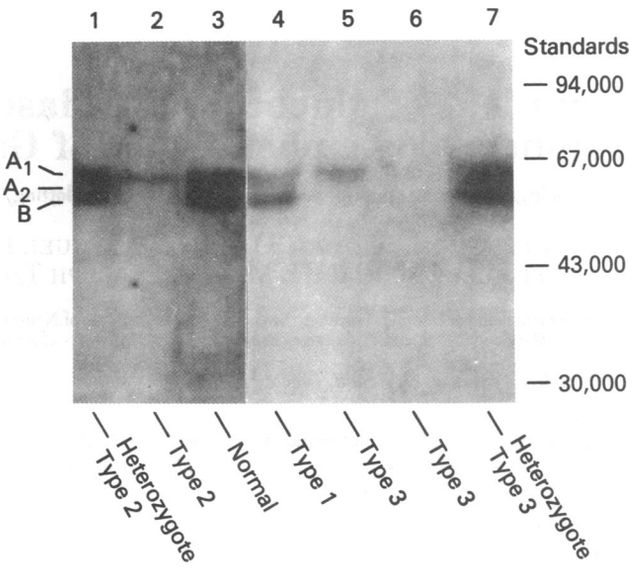

FIG. 1. Immunoradiochemical nitrocellulose blot radiograph of $NaDodSO_4$/polyacrylamide gel electrophoresis of multiple molecular forms of glucocerebrosidase in fibroblasts. Fibroblast extracts were prepared as described. Total protein in each sample was: lane 1, 79 μg; lane 2, 96 μg; lane 3, 33 μg; lane 4, 79 μg; lane 5, 38 μg; lane 6, 33 μg; lane 7, 37 μg.

56,000 depending on the state of confluency. In contrast, $NaDodSO_4$/polyacrylamide gel electrophoresis of both confluent and non-confluent fibroblasts from neurologic Gaucher disease phenotypes demonstrates only a major CRM band at M_r 63,000. Boiling normal fibroblast extracts for 5 min in the sample solubilizer for $NaDodSO_4$/polyacrylamide gel electrophoresis did not alter the immunoradiochemical nitrocellulose blot. $NaDodSO_4$/polyacrylamide gel electrophoresis of confluent fibroblast extracts thus permits separation of multiple forms of β-glucocerebrosidase and discrimination between neurologic and non-neurologic Gaucher disease phenotypes.

The predominant form of glucocerebrosidase in normal brain tissue extracts was identified by antibody to have an apparent M_r of 56,000 (Fig. 2). An antigenic species with a M_r >100,000 is also present. This M_r 100,000 form may result from aggregation of the smaller species because the amount of this larger form increases when samples are heated at higher temperatures. Type 2 Gaucher disease brain tissue completely lacks a CRM band at M_r 56,000. The specific activity of β-glucocerebrosidase from these tissues was <5% of that of control brain tissue. These data suggest that the deficiency of the M_r 56,000 form results in the neurologic Gaucher disease phenotype.

In contrast to CRM of confluent fibroblast and brain tissue extracts, CRM from normal and types 1 and 3 Gaucher disease circulating mononuclear white blood cell extracts show only one major band corresponding to M_r ≈63,000 (unpublished data). Minor amounts of CRM at M_r 61,000 and 56,000 are seen. Differentiation of Gaucher disease phenotypes by using circulating mononuclear white blood cells is not possible with this radiographic technique.

DISCUSSION

The data presented in this paper demonstrate mutations of glucocerebrosidase that are characteristic of the neurologic or non-neurologic subtypes. The most reasonable and simplest interpretation of these observations is that there are multiple mutations of glucocerebrosidase at the same locus. This is consistent with preliminary results of studies in polyethylene

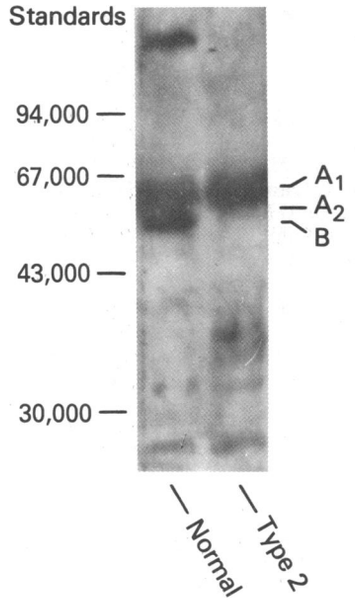

FIG. 2. Immunoradiochemical nitrocellulose blot radiograph of $NaDodSO_4$/polyacrylamide gel electrophoresis of multiple molecular forms of glucocerebrosidase in brain. Brain extracts were prepared as described. Total protein in each lane was 39 μg.

glycol-fused fibroblasts (unpublished data) that showed the three phenotypes to be noncomplementary. The low residual enzymatic activities in the three phenotypes of the disease also suggest that the multiple allelic mutations each affect the active site. Moreover, the very low residual β-glucocerebrosidase activity in type 2 brain suggests that the mutated M_r 63,000 and 61,000 forms do not express normal enzyme activity. Alternative explanations involving multiple genes at different loci have not been eliminated but are neither consistent with complementation studies nor supported by our experience with over 300 families in which Gaucher disease subtype is unique to the kindred. The methodology reported in this paper should allow characterization of the genotype in the kindred described by Wenger *et al.* in which markedly different phenotypes occurred (32).

An hypothesis encompassing our data and explaining the occurrence of neurologic and non-neurologic Gaucher disease phenotypes is shown in Fig. 3. We postulate that form B results from processing [e.g., polypeptide cleavage or glycosylation (or both)] of a larger precursor that could be form A. A change in type 1 Gaucher disease fibroblast CRM bands from variable proportions of forms A and B to predominantly form B as the cultures become confluent supports this processing scheme. This is also consistent with data on pulse-chase processing studies of glucocerebrosidase in porcine kidney cells (A. Erickson, personal communication). Assuming this is correct, one would expect to find some proportion of forms A and B in any tissue at all times. The amount of each form present would depend upon tissue characteristics (such as maturation) and might be expected to vary. This is further suggested by the predominance of form A in circulating mononuclear cells but a predominance of form B in type 1 Gaucher disease pulmonary tissue macrophages (unpublished results). Factors that stabilize, activate, or compartmentalize the precursor or processed forms may play a role in these observations. We suggest that the mutation that occurs in type 1 Gaucher disease in some way alters the active site but still allows processing of the inactive enzyme to a M_r 56,000 form albeit at a slower than normal rate. The mutations in the neurologic forms of Gaucher disease affect the active site but do not allow processing of the precursor to form B, the predominant form in brain tissue. Different extents of partial precursor processing (e.g., glycosylation, etc.) might occur in types 2 and 3 phenotypes, and the use of monoclonal antibodies to detect CRM might permit discrimination between chronic and acute neurologic subtypes.

Recently, evidence has been presented that the two β-subunits of hexosaminidase are not identical although they are coded for by the same gene (33). This implies a significant degree of processing of the subunits of hexosaminidase. Similar mechanisms may pertain in the assembly of other lysosomal enzymes and may form a rational basis for the classification of

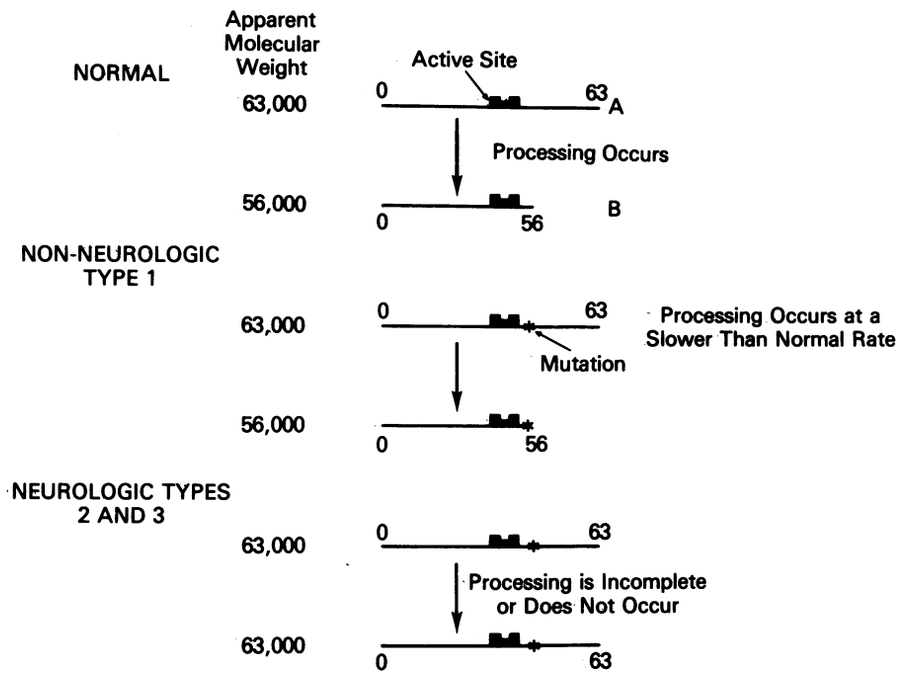

FIG. 3. Hypothesis for occurrence of Gaucher disease phenotypes.

multiple forms of these glycoproteins (34, 35). Functional characteristics of the enzyme especially at membrane interfaces may depend on these structural differences and thus may account for differences in clinical presentation of a mutation.

It is also possible that in some Gaucher disease patients a variable amount of a β-glucosidase is present that has some glucocerebrosidase activity. The existence of a β-glucosidase with activity toward glucocerebroside and having a pI of 4.5–4.8 has been reported for white blood cells and spleen (21, 26). The presence of such an isozyme could help explain the clinical diversity within subtype. In addition, a β-glucosidase without glucocerebrosidase activity has also been reported (24, 25, 36). The relationship between these β-glucosidases and glucocerebrosidase is unclear and further studies are needed to ascertain whether the β-glucosidases have a role in the phenotypic expression of Gaucher disease.

The more frequent occurrence of type 1 Gaucher disease and the widespread occurrence of type 1 disease in Ashkenazi Jews is consistent with an allelic mutation of glucocerebrosidase different from the mutations in types 2 and 3. The discovery of the multiple molecular forms (A_1, A_2, and B) of β-glucocerebrosidase now permits the discrimination of the neurologic and nonneurologic phenotypes of Gaucher disease. Preliminary work suggests that these forms also occur in spleen and liver. Purification and biochemical characterization of specific forms will result in a more rational basis for the description and understanding of the clinical heterogeneity in Gaucher disease and other inherited sphingolipidoses.

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