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Gaucher disease: Physical, kinetic and immunologic investigations of human and canine acid β-glucosidase

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GAUCHER DISEASE: PHYSICAL, KINETIC AND IMMUNOLOGIC INVESTIGATIONS OF HUMAN AND CANINE ACID β-GLUCOSIDASE

by

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A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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Abstract

GAUCHER DISEASE: PHYSICAL, KINETIC AND IMMUNOLOGIC INVESTIGATIONS OF HUMAN AND CANINE ACID β-GLUCOSIDASE

by

DIANE ELIZABETH FABBRO

Thesis Advisor: Dr. Gregory A. Grabowski

Kinetic and immunologic techniques were developed to investigate the nature of the acid β-glucosidase (β-Glc) defects which result in human and canine Gaucher disease (GD). Two new affinity columns, using the potent inhibitors of β-Glc (N-alkyl-deoxynojirimycins) as affinity ligands, were synthesized and methods were developed to obtain homogeneous β-Glc from normal human placenta. Polyclonal and monoclonal (representing 14 different epitopes from 18 clones) antibodies were produced to the pure normal β-Glc. Monospecific polyclonal IgG and tritiated-bromo-conduritol B epoxide ([^{3}H]Br-CBE), a specific covalent active site directed inhibitor of β-Glc, were used to quantitate the functional catalytic sites in normal and Type 1 Ashkenazi Jewish GD (AJGD) enzyme preparations: The k_{cat} values for several new substrates with the mutant enzymes from spleen were about 1.5-fold less than the respective normal enzyme, indicating a nearly normal catalytic capacity of the mutant enzymes. Immunoblotting studies with polyclonal or several monoclonal antibodies indicated three molecular forms of β-Glc (M_r = 67,000, 62,000 to 65,000 and 58,000) in fibroblast extracts from normals and Type 1 AJGD patients. In comparison, only one form of cross-reacting immunologic material (CRIM) was detected in fibroblast

extracts from Types 2 and 3 or several non-Jewish Type 1 GD patients. These single CRIM forms were of variable M_r in the different patients. Deglycosylation of the β-Glc in these fibroblast extracts resulted in a single CRIM form (M_r = 56,000) in all cases, indicating that the core β-Glc polypeptide from all GD patients had a normal M_r as assessed by SDS-PAGE. The CRIM molecular forms in the normal and GD sources were shown to bind [^{3}H]Br-CBE, i.e., they were β-Glc. Four monoclonal antibodies were shown to inhibit β-Glc activity by three different mechanisms: 1) blocking of the active site, 2) induction of a conformational change at the active site, or 3) both of these mechanisms. One of these monoclonal antibodies, MCAb61, proved useful for the delineation of the variants of GD based on the degree of its inhibition of the residual enzymatic activity. Studies of the canine GD indicated that it was an analogue of the neuronopathic human GD with normal levels of CRIM and thermolabile β-Glc activity. These studies indicate that human GD is highly heterogeneous at the phenotypic as well as the biochemical levels. The results also support the concept that all subtypes and variants of GD result from single base substitutions in the β-Glc structural gene which lead to a variety of processing abnormalities as well as a uniquely altered active site in Type 1 AJGD. Based on these studies, it is proposed that the allelic defects in the subtypes and variants of GD result from a dislocalization and/or an <u>in vivo</u> instability of β-Glc.

FORWARD

Portions of this thesis have been presented in the following publications:

I Farrow, B.R.H., Hartley, W.J., Pollard, A.C., Fabbro, D., Grabowski, G.A., and Desnick, R.J.: Gaucher disease in the dog. In: Gaucher Disease: A Century of Delineation and Research, Desnick, R.J., ed., Alan R. Liss, Inc., New York, pp. 645-653, 1982.[1]

II Desnick, R.J., Grabowski, G.A., Dinur, T., Fabbro, D., Goldblatt, J., and Gatt, S.: Gaucher disease: A membranous enzymopathy. In: Membranes and Genetic Disease, Sheppard, J.R., Anderson, V.G., and Eaton, J.V., eds., Alan R. Liss, Inc., New York, pp. 193-215, 1982. [1]

III Fabbro, D., Desnick, R.J., and Gatt, S.: Lysosomal β-glucosidase of rat liver. Enzyme 31:122-127, 1984.

IV Dinur, T., Osiecki-Newman, K., Fabbro, D., Legler, G., Gatt, S., Desnick, R.J., and Grabowski, G.A.: Human acid β-glucosidase: Affinity purification on N-alkyl-deoxynojirimycin sepharose and the amino acid sequences of the N-terminus and a peptide containing the active site. In: Enzymes of Lipid Metabolism-2, Freysz, L., Dreyfuss, H., Masarel, R., and Gatt, S., eds., Plenum Press, New York, in press.[2]

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VIII Osiecki-Newman, K., Fabbro, D., Dinur, T., Legler, G., Gatt, S., Desnick, R.J., and Grabowski, G.A.: Human acid β-glucosidase: Use of inhibitors and alternate substrates to investigate the properties of the normal and Gaucher disease active sites. Biochem., in review.

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LIST OF ABBREVIATIONS

β-Glc	acid β-glucosidase (E.C.3.2.1.45)
GD	Gaucher disease
dNM	deoxynojirimycin
C_{10}-dNM	N-(10-carboxydecyl)dNM
C_{12}-dNM	N-(12-carboxydodecyl)dNM
CBE	Conduritol B epoxide
CRIM	cross-reactive immunologic material
AJGD	Type 1 Ashkenazi Jewish Gaucher disease
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
GC	glucosyl ceramide
4MU-Glc	4-methyl-umbelliferyl-β-D-glucoside
C_n-U-Glc	4-alkyl-umbelliferyl-β-D-glucoside (where n = number of carbons in the alkyl chain
NBD-C_{12}-GC	12-[N-methyl-N-(7-nitrobenzo-2-oxa-1,3,-diazol-4-yl)] aminododecanoyl-(2 -N-sphingosyl-1-O-β-D-glucoside)
NBD-C_6-GC	[6-(7-nitrobenzo-2-oxa-1,3,-diazol-4-yl)aminohexanoyl]-(2-N-sphingosyl-1-O-β-D-glucoside)
MCAbs	monoclonal antibodies
GS	glucose sphingosine
TC	sodium taurocholate
PS	phosphatidylserine
PBS	phosphate buffered saline
HSA	human serum albumin
Buffer A	0.04 M citrate/0.05 M phosphate, pH 5.5, 4 mM β-mercaptoethanol, 1 mM EDTA
Buffer B	0.125 M Tris-HCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10% glycerol
Buffer C	0.04 M citrate/0.05 M phosphate, pH 5.5, 1 mM β-mercaptoethanol, 5 mM EDTA

I. OBJECTIVES

Gaucher disease (GD), the most prevalent lysosomal storage disease, is of particular interest to geneticists and biochemists since the molecular basis of the phenotypic heterogeneity in this disease remains unknown. This family of inherited glycosphingolipid disorders is characterized by the defective activity of the lysosomal hydrolase, acid β-glucosidase (β-Glc; EC 3.2.1.45). The resultant accumulation of this enzyme's natural substrate, glucosylceramide (GC), in cells of the reticuloendothelial system leads to various clinical manifestations. Three major phenotypes of GD have been differentiated by the absence (Type 1) or presence and severity (Types 2 and 3) of neuronopathic involvement.

The aim of these studies is to elucidate the nature of the allelic mutations which result in these phenotypes. The presence of residual β-Glc activity in all GD tissues has permitted comparative physical, kinetic and immunologic studies of the GD phenotypic variants. Furthermore, the availability of a naturally occurring canine analogue provided a unique opportunity for investigations of GD and normal canine β-Glc for comparison to the human enzymes.

The specific aims of this research included:

1. Purification of the normal human and canine β-Glc by two newly synthesized affinity supports, N-(10-carboxy-decyl)- and N-(12-carboxy-dodecyl)-deoxynojirimycin bound to Sepharose, for comparative physical, kinetic and immunologic studies, as well as the production of mono-specific poly- and mono-clonal antibodies.

2. Determination and comparison of the turnover number (k_{cat}) for the normal β-Glc and the residual β-Glc activity in two Type 1 AJGD patients.

3. Use of immunoblotting to evaluate the number and origin of the molecular forms of β-Glc and to estimate the amount of CRIM in the subtypes and variants of human GD.

4. Production of a panel of anti-β-Glc monoclonal antibodies (MCAbs) to investigate the structure and functions of normal and GD β-Glc.

5. Investigations of the canine model of GD by 1) quantitation of the glycosphingolipid substrate (GC) and substrate analogue, glucose sphingosine, in normal and GD canine tissues, 2) comparative characterization of two β-Glc activities in normal and GD canine tissues, and 3) evaluation of the canine GD mutation using a variety of inhibitor and activator probes of β-Glc activity.

II. BACKGROUND AND RATIONALE

A. Gaucher Disease: Clinical Heterogeneity:

GD was first described in 1882 by Philippe Gaucher in a 32 year old woman with splenomegaly and peculiar large cells in the spleen (1). Twenty years later, GD was recognized as an inherited disorder (2), and subsequent family studies established an autosomal recessive mode of inheritance (3,4). Subsequently, three major phenotypes of GD have been delineated by the absence, or presence and severity of neuronopathic involvement. Type 1 GD, the most common phenotype, has a high prevalence in the Ashkenazi Jewish population (gene frequency = 0.02) (5). However, this form of GD has been reported in Black, Hispanic, Afrikaner and other ethnic groups (6,7). The clinical manifestations are characterized by the absence of neuronopathic involvement, as well as hepatosplenomegaly and bony deterioration. The onset of these clinical manifestations in affected patients is highly variable within and among families and ethnic groups. Type 2 GD is a rare, panethnic, acute neuronopathic disease of infancy which has a stereotyped clinical course leading to death by two years. Type 3 GD, a juvenile onset, subacute neuronopathic disease, was first described in 1959 in Norrbotten, Sweden (8). This phenotype of GD is characterized by variable neuronopathic manifestations, splenomegaly and severe skeletal deterioration during early childhood. These major phenotypes of GD all result from the defective activity of the same lysosomal hydrolase, β-Glc (9). However, the differential nature of the mutations which result in these phenotypes has not been elucidated.

B. Acid β-Glucosidase: Enzyme Purification and Characterization:

Knowledge of the structure and function of normal β-Glc is requisite to understanding the nature of the heterogeneity in the GD phenotypes. Early attempts to purify β-Glc were hampered, since it is highly hydrophobic and is membrane associated (10). Pentchev et al. (11), using conventional methods, purified the placental enzyme about 4,000-fold with a 5% yield. Subsequently, Furbish et al. (12) exploited the enzyme's hydrophobic nature for large scale partial purification of β-Glc with 30% recovery. Pentchev et al. (14) used this technology to demonstrate similar purification properties of the splenic β-Glc from a normal individual and a Type 1 GD patient. Similarly, sequential chromatography on Concanavalin A (Con A) and phosphatidylserine (PS)-Sepharose has provided high yields of partially purified β-Glc from placenta (13). Each of these methods resulted in partially purified enzyme with varying degrees of contamination by human serum albumin (15) and other proteins.

More recently, apparently homogeneous β-Glc has been obtained by newly developed affinity chromatographic methods. Strasberg et al. (16) used epoxy linked glucosyl sphingosine (GS) to obtain homogeneous β-Glc. However, this column appeared to undergo degradation after one or two applications of enzyme and was not useful for large scale purification (15). The use of two substrate analogue ligands, GS and GC bound to Sepharose, has resulted in apparently homogeneous β-Glc with a 22,000-fold purification from human placenta. These affinity supports were not degraded in over 1 year of use. Recently, we have synthesized two new, stable affinity ligands with great capacity and specificity for active

β-Glc. These supports use the potent inhibitors, N-(10-carboxy-decyl)- and N-(12-carboxy-dodceyl)-deoxynojirimycin (C_{10}-dNM and C_{12}-dNM), as the affinity ligands. The use of these columns provided sufficient homogeneous β-Glc for the studies presented here (Section V.A.).

The subunit structure and post-translational modification of purified β-Glc have been evaluated by electrophoretic and isoelectric focusing studies. Analytical polyacrylamide gel electrophoretic (PAGE) studies of the denatured purified β-Glc from normal placenta have demonstrated the enzyme to be a homomer with a subunit molecular weight of about 67,000 (11,12,15). Although native PAGE analyses of β-Glc have been hindered by the inability of the non-denatured enzyme to migrate into gels, recent HPLC studies [in this laboratory and others (17)] have suggested that the native normal enzyme purified from placenta is a monomer.

Glycosylation of placental β-Glc was suggested by its tight binding to Con A-Sepharose, however, the hydrophobic interactions between β-Glc and the Con A support have precluded examination of the type of oligosaccharide modifications (e.g., complex) by differential elution (18). Several molecular forms of β-Glc have been identified using granular bed gels. These studies have demonstrated that the β-Glc from several tissues has multiple forms with pI values between pH 3.2 to 6.5 (19-21). Isoelectric focusing of the purified placental enzyme resolved several molecular forms with pI values of 4.2 to 6.2 (15,21). Sequential digestion of some of these preparations with neuraminidase, β-galactosidase and β-N-acetyl-glucosaminidase resulted in a single major enzyme form with a pI value of 7.8 (22). Five potential sites for N-linked carbohydrate addition have been predicted by the cDNA sequence of β-Glc

(23,24). The demonstration of a mixture of high mannose and complex oligosaccharide side chains on purified placental β-Glc (25) has suggested that the various isoelectric forms are due to differential glycosylation (see Section V.C.3.).

The low level of the defective β-Glc activity in GD patients has hindered purification of the mutant gene products for comparative kinetic and structural studies. Recently, immunologic detection of β-Glc in crude tissue homogenates after electroblotting sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) has permitted the determination of the molecular weight of β-Glc in GD patients which obviated the need for enzyme purification. However, the presence of several molecular weight forms of β-Glc and the differences between the forms of normal and GD CRIM which were detected by Ginns et al. (26-28) has not been verified by other investigators (29; see Section V.C.).

C. Kinetic, Physical and Immunologic Characterization of Normal and GD β-Glc: Discrimination of Gaucher Disease Phenotypes:

To date, few investigations are available which detail the kinetic properties of human β-Glc. In crude or pure preparations, β-Glc has been shown to require detergents, negatively-charged lipids and/or a "co-glucosidase" protein for optimal hydrolysis of the water soluble substrate, 4-methylumbelliferyl-β-D-glucoside (4MU-β-Glc), or the natural lipid substrate, GC (30,31,49,50). Hyun et al. (32) used partially purified placental β-Glc and a variety of synthetic inhibitors to demonstrate the specificity of two binding sites on β-Glc: 1) a catalytic site and 2) an allosteric site presumed for "cationic"

inhibitors. More recently, Grabowski et al. (33) conducted extensive modifier studies with the pure β-Glc to evaluate the binding specificity of three domains near or in the active site. These domains have specificities for the polar, sphingosyl or fatty acid acyl groups of the natural substrate and participate in substrate binding and hydrolysis. These conclusions were based on extensive interaction studies of enzyme modifiers and provided a model to evaluate potential site-specific mutations in GD phenotypes. Recently, using [^{3}H]Br-CBE as a covalent affinity lable, a β-Glc amino acid critical for substrate hydrolysis has been identified at Asp^{444} of the 497 amino acid sequence of β-Glc (34). This residue is thought to reside in an α-helical structure flanked by two hydrophobic regions which may be components of the three sites described by the above domain model.

The occurrence of clinically distinct phenotypes of GD and the fact that residual β-Glc activity can be detected in each phenotype (35-39), suggests the presence of different allelic mutations which retain partial activity. Indeed, many investigators have shown the residual enzyme in GD to be more thermolabile than the normal enzyme (37-39). Using the method of Turner et al. (37), this laboratory has demonstrated that the β-Glc from AJGD Type 1 GD fibroblasts was more heat labile than the normal enzyme. Karazeh recently confirmed this finding (34). In addition, the residual β-Glc from several non-Jewish Type 1 GD patients had either normal, increased or decreased thermostability (40), suggesting that there were several different mutant gene products which resulted in Type 1 GD.

A unique feature of the mutant gene products in all GD phenotypes is the normal K_m value of the residual β-Glc for natural and artificial substrates (35-40). Thus, the different mutations of β-Glc in the GD variants were thought to affect the catalytic capacity (velocity) of the enzymes (see Section V.B.). In an effort to account for this decrease in hydrolytic capacity, investigators have directed efforts to quantitate the amount of residual activity and β-Glc protein, and to examine the qualitative properties of β-Glc in the different phenotypes. Several investigators have reported that the levels of residual activity correlated with the clinical subtype (41,42), e.g., less residual activity results in the more rapid accumulation of the substrate (GC) and a more severe clinical course of the disease. However, using natural or artificial substrates, other investigators have not corroborated this finding (43,44). Pentchev et al. (45) suggested that the neuronopathic and non-neuronopathic GD phenotypes could be distinguished by the level of residual activity based on the amount of CRIM. However, these studies assumed antigenic and catalytic equivalence of the normal and mutant gene products (see Section V.B.). More recently, estimation of CRIM specific activity using MCAbs demonstrated similar levels in Type 1 and Type 2 GD patients and provided no discrimination of GD phenotypes (46).

Qualitative differences between the residual β-Glc in the neuronopathic and non-neuronopathic forms of GD have been demonstrated by the degree and magnitude of enzymatic responses to lipoidal modifiers. Using PS or gangliosides (G_{M1} and G_{M2}), Mueller et al. (47) demonstrated that the residual enzyme in Type 1 GD fibroblasts had a 2-fold greater activation than either the normal or the Type 2 or 3 enzymes. Wenger et

al. (48) achieved similar results in leukocyte or fibroblast extracts using PS and a natural protein effector molecule. Glew et al. (49,50) have developed an assay, based on differential PS activation of the residual β-Glc, which discriminated Type 1 and Type 2 GD by the level of β-Glc specific activity in splenic or brain extracts. However, the significance of these observations to the understanding of the enzymatic defects in the neuronopathic and non-neuronopathic forms of GD is limited since the residual β-Glc from only a very few patients were examined. More recently, Grabowski et al. (40,51) delineated the kinetic properties of the residual enzyme in a variety of GD patients from diverse ethnic backgrounds. These studies indicated substantial genetic heterogeneity within and among the three GD phenotypes and subclassified the residual β-Glc activity in the major phenotypes and variants of GD into three groups. Group A residual activities had normal interactions with the inhibitors, glucose sphingosine (GS), N-hexyl-GS, conduritol B epoxide (CBE), and the negatively charged lipids, taurocholate (TC) and phosphatidylserine (PS). Group A residual activity was found in all Type 2 and Type 3 patients as well as several non-Jewish Type 1 patients. Group B residual β-Glc activities had 5- to 7-fold increased K_i values for the above inhibitors (40), 3- to 5-fold increased I_{50} or K_i values for CBE or Br-CBE (51), and abnormal interactions with TC and PS as well as with several glycon and alkyl-glycon derivatives. Most but not all Type 1 GD patients, both Ashkenazi Jewish and non-Jewish patients, were characterized as Group B. Group C included a single Afrikaner Type 1 GD patient whose residual activity

had intermediate results with these enzyme effectors (40,51). Based on pedigree studies, this patient likely represents a heteroallelic compound for Group A and Group B mutations.

Immunologically detectable differences in the residual β-Glc from the Type 1 and Types 2 and 3 phenotypes have been reported by Ginns et al. (26-28) using immunoblotting. Three forms of β-Glc in normal and GD Type 1 fibroblasts (M_r = 63,000, 61,000 and 56,000) were detected. In contrast, only the 63,000 molecular weight form was observed in the neuronopathic (Type 2 or 3) phenotypes of GD. Based on these findings, these investigators postulated an arrested maturation of β-Glc in Type 2 or 3 GD and normal maturation of β-Glc in the Type 1 patients. However, other investigators have detected only one CRIM form $M_r \cong 63,000$ in both normal and GD fibroblasts and have failed to verify the findings of Ginns et al. (26-28; see Section V.C.).

To examine the <u>in vivo</u> residual enzyme activity in the GD phenotypes, investigators have quantitated the levels of accumulated substrates in a variety of tissues. In GD liver and spleen, GC was present in 300- to 400-fold increase over normal levels, while in plasma and brain, 2- to 80-fold increased levels were found, respectively. The level of accumulated GC did not provide discrimination between the neuronopathic and non-neuronopathic phenotypes (45). However, the levels of GS (52,53), a putative neurotoxin, did distinguish these GD phenotypes but only in brain extracts. In cerebellar or cerebral cortex, GS was present at 30- to 50-fold elevated levels in the Types 2 and 3 GD compared to the minimally increased levels in Type 1 GD. These results indicated that the mutations of β-Glc in the Type 2 and 3

patients may alter the catabolism of both GS and GC, while only the latter is altered in Type 1 GD or that there may be a critical level of β-Glc activity required for GS catabolism.

The lack of sufficient GD tissues and the rarity of Type 2 and 3 GD have limited investigations of the pathophysiology of the accumulating substrates. The recently described canine model of GD may be invaluable for these studies, as well as for the determination of the age dependent lipid accumulation and investigations of neuronopathic involvement.

D. The Canine Model of Gaucher Disease:

An animal analogue of human GD has been described in the Australian Silky Terrier (54,55). Of the subtypes of the human disease, the phenotype of the GD dog, with neurologic symptoms and progressive development of tremors and seizures, most closely resembles that of the neuronopathic forms, Types 2 and 3 GD (54). Pathologic studies of an affected dog demonstrated the presence of "Gaucher cells" in hepatic sinusoids, lymphoreticular tissue and cerebellum (54) and, to a lesser extent, in the spleen (54). Ultrastructural studies of these tissues revealed that the accumulated material was in "tubular" deposits similar to those observed in human GD (54). Identification and quantitation of the accumulated lipid has not been demonstrated until the present studies (Section V). Furthermore, a marked deficiency of β-Glc activity at acidic pH values was demonstrated in the canine Gaucher tissues and leukocytes using an artificial substrate (55). However, at neutral pH values normal levels of β-glucosidase activity were measured. Further

investigations of these findings and characterization of two β-glucosidase activities in canine tissues using the substrate, 4-methylumbelliferyl-β-D-glucoside (4MU-Glc) are presented here (Section V.E.). Use of the natural substrate, GC, demonstrated the deficiency of one of these enzymes, β-Glc, in canine GD and the membrane association, selected physical, kinetic and immunologic properties of the normal canine β-Glc are compared to those of the residual mutant enzyme (Section V.E.).

III. MATERIALS

Chemicals and Reagents: Chemicals and reagents were from the following commercial sources: 4-methylumbelliferyl-β-D-glucopyranoside (4MU-Glc) and other 4-alkyl-umbelliferyl-1-O-β-D-glucosides, 4-C_n-U-Glc (where n = alkyl chain length and varied from n=7 to n=11) (Research Products International, Mount Prospect, IL); pure sodium taurocholate (TC), dodecyl-β-D-maltoside, and castanospermine (1,6,7,8-tetrahydroxyoctahydroindoline) (Calbiochem-Behring Corp., La Jolla, CA); Triton X-100, L-α-phosphatidyl-L-serine (PS), human serum albumin, Nonidet P-40, Tween 20, decyl agarose, 1-O-methyl-α-D-mannopyranoside and δ-gluconolactone (Sigma Chemical Co., St. Louis, MO); 12-[N-methyl-N-(7-nitrobenzo-2-oxa-1,3-diazol-4-yl)]aminododecanoic acid [NBD-dodecanoic acid, C_{12}-NBD] and NBD-aminohexanoic acid [C_6-NBD] (Molecular Probes, Junction City, OR); fluorescamine (Hoffmann-LaRoche Inc., Nutley, NJ); octyl Sepharose, CH-Sepharose, Concanavalin A-Sepharose 4B, aminohexyl-Sepharose 4B-CL (Pharmacia Fine Chemicals, Piscataway, NJ); protein C-4 column (4.6 x 250mm/1) (The Nest Group, South Boro, MA); cellulose acetate gels (17 x 17 cm x 0.35 cm) (Kalex Scientific Co., Manhasset, NY); Ultrodex™ and ampholines (LKB Products, Rockville, MD); nitrocellulose (0.45 μm, Schleicher and Schuell, Keene, NH); Vectastain ABC Kit (Vector Lab, Burlingame, CA); 4-Chloro-1-naphthol and sodium docecyl sulfate (SDS), (Bio-Rad, Richmond, CA). Staph A cells, prestained protein standards (BRL, Gaithersburg, MD); Zetachrom 200 cartridge (AMF Lab, Meriden, CT), V-8 protease (Miles, Naperville, IL); N-Glycanase™ (Genzyme, Boston MA); Silica Gel G Thin Layer Chromatography (TLC) plates (250 μm) with or without 5% boric acid (Analtech, Newark, DE);

Sylon BTZ-Silyating Reagent, SE-30 gas crhomatogrpahy (GC) column, (100-120 mesh, Supelcoport) (Supelco, Bellefonte, PA); silicic acid (UNISIL) (Clarkson Chem., Williamsport, PA); Mouse Immunoglobulin Subtype Identification Kit (Boehringer-Mannheim, Indianapolis, IN). All other reagents were of the highest grade available.

The following tissue culture supplies were obtained from commercial sources: Dulbecco's Modified Eagle's Medium (DMEM), glutamine, penicillin, streptomycin, and non-essential amino acids (Whittaker MA BioProd, Walkersville, MA); fetal calf serum, prescreened for hybridoma growth (Gibco, Gaithersburg, MD); thymocyte conditioned media and media supplement (Hana Biologicals, Berkeley, CA).

GC and glucose sphingosine (GS) and galactose sphingosine were isolated from human GD spleen or brain as described (56,57). The fluorescent GC substrates, C_6-NBD-GC and C_{12}-NBD-GC, were synthesized from GS and the respective NBD-hexanoic or dodecanoic acids (10).

Conduritol B-epoxide (CBE) and [^{3}H]Br-CBE, a specific covalent inhibitor of β-Glc (58), nojirimycin (NM), deoxynojirimycin (dNM) and N-dodecyl-dNM were gifts from Dr. Gunter Legler and N-hexyl-sphingosyl-1-O-β-D-glucoside (N-hexyl-GS) was a gift from Dr. Shimon Gatt.

The N-alkyl-dNM supports were synthesized from N-(10-carboxydecyl)dNM (C_{10}-dNM) or N-(12-carboxydodecyl)dNM (C_{12}-dNM) by carbodiimide dehydration with aminohexyl-Sepharose (59). The ligand concentrations were 4 or 7 μmol/ml of settle gel for C_{10}-dNM or C_{12}-dNM, respectively (59). GS-Sepharose was synthesized as described (15) using CH-Sepharose 4B. GS ligand concentration was 6 mg/ml of settle gel.

IV. METHODS

The methods section is constructed for readibility of the Results and Discussion sections. As a result, there is some redundancy between the methods sections described below.

A. Affinity Purification of the Normal Placental and Gaucher Disease Splenic β-Glc on N-Alkyl-Deoxynojirimycin-Sepharose:

1. Enzyme Preparation: Normal β-Glc from human placentae was partially purified by hydrophobic chromatography (12) as modified by Grabowski and Dagan (15). Briefly, human placentas which had been frozen at -20°C within 30 minutes of delivery, were partially thawed in 0.9% saline, containing 0.2% sodium azide and 0.005 mM phenylmethylsulfonyl fluoride (PMSF). The tissues were homogenized in 25 mM phosphate, pH 6.0, and centrifuged (5000 x g, 45 min). The pellets were then extracted with 25 mM citrate, pH 7.0, containing 1% cholate. The cholate extract was further purified using 35% ammonium sulfate fractionation, citric acid precipitation (pH 5.35) and butanol extraction using the methods described by Furbish (12). Butanol extraction was required for further purification on affinity or hydrophobic supports. After dialysis of the butanol extract, the sample was purified by chromatography on decylagarose and octyl-Sepharose, used sequentially. β-Glc was eluted off either column in a linear ethylene glycol gradient (0 to 90% ethylene glycol) in citrate buffer, pH 5.0. Highly purified fractions were pooled and diluted to less than 5% ethylene glycol concentrations prior to application to N-alkyl-dNM supports.

The Type 1 Ashkenazi Jewish Gaucher disease (AJGD) β-Glc was purified from spleen which had been obtained at splenectomy and was frozen at -20°C until use. The residual enzyme in fibroblasts and splenic extracts from this patient had the physical and kinetic properties characteristic of this form of Gaucher disease (40,51). For enzyme purification from the Type 1 AJGD spleen, the procedure of Grabowski and Dagan (15) was modified as follows: 1) the citric acid precipitation was eliminated to avoid major losses (> 85%) of the mutant enzymatic activity and 2) the supernatant from the ammonium sulfate step was delipidated with 20% butanol. The dialyzed butanol extract of the Type 1 AJGD spleen and the normal enzyme obtained from the ethylene glycol eluant of the octyl-Sepharose column was used for subsequent affinity purification procedures.

2. Analytical Procedures: Enzyme activities were determined fluorometrically with 4 mM 4MU-Glc containing 4.0 mM Triton X-100, 4.65 mM TC and 0.05 M phosphate/0.04 M citrate, pH 5.5. Assays were terminated using 0.1 M ethylene diamine (33). Protein concentrations were estimated by the Lowry procedure (60). Reverse phase HPLC and sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were as described (34,78). Capacities of the affinity columns were estimated as the amount of β-Glc activity eluted off the column after 10% of activity being applied appeared in column flow through (15). Amino acid composition and sequence analyses were accomplished by Dr. Kenneth Williams of the Protein Chemistry Facility in the Department of Biophysics and Biochemistry at Yale University.

B. Use of Conduritol B Epoxide Derivatives to Investigate the Catalytically Active Normal and Gaucher Disease Enzymes:

1. Enzyme Sources: Normal β-Glc was purified from human placentae by affinity chromatography on N-alkyl-deoxynojirimycin-Sepharose (see Sect. IV.A.). Homogeneity of the preparation was documented by the presence of a single protein species on SDS-polyacrylamide electrophoresis (64,78) or reverse phase HPLC (34) and by a single N-terminal amino acid sequence (Sect. V.A.). Normal spleen was obtained at surgery from a patient with idiopathic thrombocytopenic purpura and stored at -20°C for six months. The β-Glc from this spleen was purified through the butanol delipidation step (15) and was about 60-fold enriched. The Type 1 AJGD spleens were obtained at splenectomy from 25 and 54 yr. old patients and stored at -20°C until used (6-12 months). The mutant splenic β-Glc were purified either by hydrophobic (12) or N-alkyl-deoxynojirimycin-Sepharose chromatography (Sect. IV.A.). These final preparations were about 500- and 7500-fold enriched and several protein bands were observed on SDS-polyacrylamide gel electrophoresis. The 500-fold enriched Type 1 AJGD enzyme also was delipidated with 40% of a 1:1 (v/v) mixture of butanol: isopropyl ether to ensure that intrinsic lipids were removed (61). All enzyme preparations were stable for several months when stored at 4°C in 0.04 M citrate/0.05 phosphate, pH 5.5, 4 mM β-mercaptoethanol, and 1 mM EDTA (buffer A) containing 60-80% ethylene glycol. These four different enzyme preparations of varying purity were used in comparative studies to control for the effect of contaminants on the kinetic measurements since homogeneous Type 1 AJGD β-Glc could not be obtained in an active form.

Using the pure normal placental β-Glc and the partially purified normal or Type 1 AJGD splenic enzymes [which had been deglycosylated with N-glycanase (Genzyme, Boston, MA)], identical immunoblot molecular weights (56,000) were obtained with monospecific polyclonal or monoclonal antibodies to the normal placental β-Glc (Sect. V.D.). In addition, immunoblots demonstrated identical profiles of cross-reacting immunologic material (CRIM) from the glycosylated enzymes in splenic or fibroblast extracts from several normal individuals or other Type 1 AJGD patients although the profiles in each tissue source were different. These studies indicated that the partially purified mutant enzymes examined had essentially identical molecular weight to the normal placental or splenic enzymes and they were similar to those from several other Type 1 AJGD patients.

2. Kinetic Studies: Hydrolysis of NBD-GC derivatives (62), 4MU-Glc, or 4-C_n-U-Glc (where n was the alkyl chain length) (33,40) was determined fluorometrically. The typical reaction mixture (0.2 ml) contained 0.05 M phosphate/0.04 M citrate, pH 5.5, Triton X-100 (4 mM), 4.65 mM taurocholate, 4 mM β-mercaptoethanol, 1 mM EDTA, substrate, < 1% ethylene glycol, and enzyme. Assays with the highly purified Type 1 AJGD enzyme contained 0.6% human serum albumin in the incubation mixtures to maintain enzyme stability. Under these conditions, human serum albumin did not alter the k_{cat} or V_{max} values of the more stable pure normal placental or delipidated splenic β-Glc, respectively. The lipoidal substrates in chloroform/methanol (2:1; v/v) were added to dry tubes, the solvents evaporated under nitrogen and then with high vacuum for 2 to 4 h. The residue was resuspended in buffer containing Triton X-100

and the reactions were initiated by the addition of enzyme. The amount of enzyme was adjusted to ensure that less than 5% of the substrate was hydrolyzed. Reactions at 37°C were terminated after 0.5 to 2 h. Protein concentrations were estimated by the method of Lowry et al. (60).

3. Determination of k_{cat} Values: The k_{cat} values for alternate substrates with the normal and Type 1 AJGD β-Glc were calculated from $V_{max} = [E]_t\ k_{cat}$ assuming that only those active sites which retained their respective full catalytic activity were labelled by [^{3}H]Br-CBE (see below). V_{max} values for the 4MU-Glc and C_7-U-Glc and NBD-C_6- or NBD-C_{12}-GC substrates were obtained from linear Lineweaver-Burk plots which had been evaluated by the least squares method. The graphic method of Cleland (63) was used to determine K_m and V_{max} for the 4-C_9- and -C_{11}-U-Glc, since substrate inhibition was observed. The k_{cat} values were based on six separate experiments performed in duplicate for each substrate and enzyme source. For studies which determined k_{cat} from the relationship of the enzyme activity to the number of catalytic sites (i.e., Fig. 3), enzyme activities were determined with subsaturating amounts of substrate (4 mM 4MU-Glc or 0.2 mM NBD-C_{12}-GC) to avoid the problem of substrate insolubility at high concentrations: The V_{max} was calculated from the substrate concentrations and respective K_m values.

$[E]_t$ was estimated by quantitating the number of catalytic sites in each enzyme preparation using [^{3}H]Br-CBE (8000 cpm/pmole) as follows: various amounts of enzymatic activity or protein from the different enzyme preparations in buffer A were incubated with a large excess of [^{3}H]Br-CBE (2-8 µl; 10 µM final concentration) in 0.6% human serum albumin. Complete inactivation of each enzyme was achieved with this

concentration of [^{3}H]Br-CBE by 2 h at 22°C. To ensure that all [^{3}H]Br-CBE binding sites were saturated, the mixtures were incubated at 22°C for 24 h. Human serum albumin was required to maintain enzyme stability under these conditions for up to 24 h.

To separate [^{3}H]Br-CBE from that bound specifically to β-Glc, the enzyme-[^{3}H]Br-CBE complexes were immunoprecipitated quantitatively with monospecific rabbit anti-human β-Glc IgG and Staphylococcus aureus Protein A (40). The resultant supernatants were reprecipitated successively with additional IgG and Protein A until no additional increase in precipitated radioactivity was observed; immunoprecipitation usually was quantitative after a single cycle. The resultant pellets were washed by resuspension and centrifugation (10,000 x g; 40 min) twice in phosphate buffered saline, containing 1% human serum albumin, 0.5 M NaCl and 0.05% Tween 20 and then twice in phosphate buffered saline, containing 0.05% Tween 20. The washed pellets were dissolved (24 h, 22°C) with 100 µl Protosol™ (New England Nuclear, Boston, MA) in 900 µl of water and the radioactivity determined. With a fixed amount of the pure placental or crude splenic enzymes (< 2 nmol 4MU-Glc hydrolyzed/min) from normal sources, the number of catalytic sites was constant when the concentration of [^{3}H]Br-CBE was varied between 2 and 20 µM. With similar amounts of enzymatic activity from the Type 1 AJGD enzyme preparations, the number of catalytic sites remained constant when the concentration of [^{3}H]Br-CBE was varied between from 5 and 20 µM. Based on these results, a final concentration of 10 µM [^{3}H]Br-CBE was used in these experiments.

The concentration of catalytic sites/mg protein of original enzyme solution was determined from the radioactivity in the precipitates. The total enzyme concentration, $[E_t]$, was based on a molecular weight of 56,000 for the pure unglycosylated normal enzyme which was calculated from amino acid composition (see Sect. V.A.). This protocol provided a 1:1 mol/mol stoichiometry of $[^3H]$Br-CBE in the precipitates and pure normal placental enzymatic protein (34).

C. Type 1, 2 and 3 Gaucher Disease: Genetic Heterogeneity Within and Among Subtypes Delineated by Immunoblotting:

1. Preparation of Fibroblast Lysates and Isolation of β-Glc from Media: Most of the skin fibroblast cultures were established from forearm explants by standard procedures and additional fibroblast cell lines (GM2627, GM1260, GM877) were obtained from the Human Genetic Mutant Cell Repository (Camden, NJ).

The Norrbottnian Type 3 GD fibroblast cell lines were provided by Dr. Lars Svennerholm.

Skin fibroblasts from normal and GD patients were cultured to confluency in RPMI 1640 supplemented with 10% fetal calf serum containing 1% glutamine and 1% penicillin/streptomycin. Fibroblasts were harvested into 0.9% saline with a rubber policeman and then washed three times with 0.9% saline by resuspension and centrifugation (1000 x g; 10 min) prior to storage at -20°C. Pilot studies demonstrated that storage at -20°C for up to three months had no effect on immunoblotting pattern.

β-Glc activity was solubilized either by 1) homogenization (Dounce) of cells in 0.25 M sucrose (50 to 100 µl) or 2) sonication of cells suspended in 60 mM phosphate, pH 6.6, containing 0.1% Triton X-100 (Tx) or 0.1% Tx and 0.5% sodium cholate. For comparative studies, sonication was conducted at 4°C using a cup sonicator (Branson Cell Disrupter 200) at 40 watts, 5 sec, or with bursts of 30 sec, 10 sec, 10 sec, or using a probe sonicator (Heat System Co.), 40 watts, 5 sec. Aliquots of the sonicates were removed and the remaining samples were centrifuged at 48,000 x g for 20 min (4°C). The supernatants (S) were removed and the resultant pellets, resuspended to original volume with corresponding lysis buffer, were assayed immediately for β-Glc activity.

CRIM in cell-free media from fibroblast cultures was concentrated by adsorption to rabbit anti-human placental β-Glc IgG immobilized on CNBr-Sepharose (5mg IgG/ml of settled gel). Fifty to one hundred ml of fibroblast culture media were decanted, filtered (0.45 µm; Corning Glass, Corning, NY) and mixed with 50 µl of Sepharose-bound anti-β-Glc IgG (16 h, 4°C). The Sepharose beads then were washed three times by resuspension and centrifugation (100 x g; 10 min) in phosphate buffered saline (PBS) containing 0.05% Tween-20. The pelleted IgG Sepharose beads with bound β-Glc were, then, resuspended in 50 µl of Buffer B, (0.125 M Tris-HCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10% glycerol), and heated at 100°C for three min. After centrifugation (5000 x g, 30 min), the resultant supernatants were subjected to SDS-polyacrylamide gel electrophoreses (SDS-PAGE) and immunoblotted using monoclonal anti-β-Glc antibody, MCAb 61, for detection (see below). A control sample consisting of 50 ml of RPMI 1640 media containing 10% fetal calf serum

was processed exactly as described above and no immunoblotting signal was detected.

2. Immunoblots: Fibroblast homogenates or sonicates (3.5 to 150 µg of protein) were subjected to slab gel (1.5 mm) SDS-PAGE (7.5%) (64). After SDS-PAGE, the gels were equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, pH 8.3, containing 10% methanol) for 30 min and proteins were then electroblotted onto nitrocellulose membranes in transfer buffer (70 volts, 2 h) (65). After transfer, the membranes were rinsed for 15 min with PBS and blocked with 5% non-fat dry milk in PBS (PBS-BLOTTO) (66) for 20 min. The blocked membranes were then agitated (16 h at 4°C) in PBS-BLOTTO containing polyclonal (5µg IgG/ml) or monoclonal antibodies (1:1000 dilution of ascites fluid) to β-Glc. The membranes then were washed for 10 min with PBS, rinsed with PBS-BLOTTO containing 0.05% NP-40 and washed for an additional 10 min with PBS. Bound antibody was detected using Vectastain ABC Kit according to the manufacture's instruction but washes were conducted as described above.

Anti-human placental β-Glc IgG was purified from polyclonal rabbit antisera by chromatography on a Zetachrom 200 cartridge according to the manufacturers instructions. Monoclonal antibodies in ascites fluid from hybridoma clones -1, -37, -61 or -9 were obtained as described (67) (Sect. IV.D.). The human placental β-Glc prepartions used for immunization were purified by affinity chromatography and contained a single N-terminal amino acid sequence (Sect. V.A.). This sequence of twenty-two amino acids was co-linear with that encoded by two different β-Glc cDNAs (23,24).

3. N-Glycanase™ Digestion: Reduced and denatured proteins in fibroblast sonicates were deglycosylated with N-Glycanase™ as described (68). Briefly, 20 to 100 µg of fibroblast protein in 0.5% SDS and 0.1 mM β-mercaptoethanol were boiled for 3 min. The samples were brought to 0.2 M sodium phosphate, pH 8.6, and 30 mM EDTA using concentrated buffer solutions. NP-40 was added to ensure a seven to one concentration ratio of NP-40 to SDS. N-Glycanase™ (0.3 units per 20 µg of lysate) was added and the resultant mixtures were incubated for 12 to 24 h at 30°C. The samples were then diluted in sufficient three-fold concentrated Buffer B to achieve the final concentrations of Buffer B and subjected to SDS-PAGE and immunoblotting. Increasing the amount of N-Glycanase™ or time of digestion produced no additional change of β-Glc migration.

4. [^{3}H]Br-CBE Labeling of Fibroblast β-Glc: The β-Glc in fibroblast sonicates were labeled with [^{3}H]Br-CBE, a covalent active site directed inhibitor of β-Glc, as follows: fibroblasts were solubilized at 4°C in 60 mM phosphate, pH 6.6, containing 0.1% Triton X-100 and 0.5% cholate using the cup sonication method. After centifugation (48,000 x g; 20 min) 40-50 µg of supernatant protein were incubated (22°C) with [^{3}H]Br-CBE for 72 h. Based on the total enzymatic activity in these samples and the known k_{cat} values for the 4MU-Glc substrate (see Sect. V.B.), [^{3}H]Br-CBE was estimated to be in at least 10,000-fold molar excess. After 72 h, the samples were diluted in 3-fold concentrated buffer B and subjected to SDS-PAGE and immunoblotting (see above). After visualization of CRIM bands on the immunoblots, the nitrocellulose membranes were cut into strips which were then dissolved in 10 ml of ACS II (Amersham, Arlington Heights, IL) and the radioactiv-

ity associated with each strip was determined.

5. Assay Conditions: β-Glc activity was assayed in aliquots of diluted (20:1, v/v) fibroblast lysates. The incubation mixtures contained 4.65 mM TC, 4.0 mM Triton X-100, 4.0 mM 4-methylumbelliferyl-β-D-glucoside, 0.05 M phosphate/0.04 M citrate, pH 5.5. Reactions were terminated with 0.1 M ethylenediamine and fluorescence intensity determined (33,40).

Proteins were estimated using the method of Lowry (60).

D. Characterization and Use of Monoclonal Antibodies to Investigate the Structure and Function of Normal and GD β-Glc:

1. Production of Hybridomas:

a) In Vivo Immunization: Purified β-Glc (40 µg) was emulsified in complete Freund's adjuvant and injected into the foot pad and inguinal and axillary regions of female Balb/C mice (6-16 wk of age). On days 7 and 14, booster injections (40 µg in Freund's incomplete adjuvant) were given, and after 3-8 weeks, a final boost (40 µg in PBS) was injected intravenously. Three days after the final boost, splenic cells were harvested by disaggregation and fused with P3-X-63-Ag 8.653, a mouse myeloma cell line, which had been maintained in log growth. Hybridoma fusions were conducted by the method of Kennet (67), fusing cells in 1:5 ratio of myeloma cells:spleen cells with polyethylene glycol (PEG) 1000, pH 8.6, or with modifications of Fazekas De St. Groth (70) using PEG 4000. Fused cells were diluted to 2.5 to 5 x 10^5 myeloma cells/ml and plated into microtitre wells. Hybridomas were selected by growth in HAT media. Mass cultures were screened for

anti-β-Glc antibodies by ELISA (see below) and the positive cultures were cloned into microtiter plates by limiting dilution in fluid phase as described by Mao (69).

b) In Vitro Sensitization (IVS): IVS involves sensitization of mouse splenic cells to antigen in cell culture as described by Luben (71) and modified by Hana Biologicals. IVS avoids the requirement for the long term immunization regimen required by the in vivo method and requires as little as 5 µg of antigen as compared to 100-250 µg for in vivo immunization.

For IVS, five unimmunized mice were sacrificed, the spleens aseptically removed, and the splenic cells were disaggregated by perfusion with DMEM. After lysis of erythrocytes and resuspension in media, cells were pelleted by centrifugation at 400 x g for 5 min. Viable spleen cells (1.6×10^8) were resuspended in 16 ml of media containing 50% thymocyte conditioned media (Hana Biologicals), 0.05 mM β-mercaptoethanol, 20% fetal calf serum and 25 µg of purified β-Glc. The cells were then incubated in T-75 flasks for four days. A parallel experiment using immunization media without 20% fetal calf serum resulted in almost complete cell death. After four days, sensitized spleen cells were fused with PX63 mouse myeloma cells using PEG 1000. Screening and cloning were conducted as described above.

2. Screening Assays for β-Glc Hybridomas: Homogeneous β-Glc was used in ELISA assays to select anti-β-Glc secreting hybridomas (67). The antigen (100 µl), diluted to 1-2 µg/ml with bicarbonate buffer, pH 9.6, or with PBS, pH 7.3, was coated onto wells of microtiter plates by incubation at 20°C for 2-4 h. The wells were washed with PBS, contain-

ing 0.05% Tween-20 (PBS/Tween) and unoccupied sites were blocked with 1% human serum albumin (HSA) in bicarbonate buffer or PBS for 1 h. The wells were washed several times with PBS/Tween and then, 100 µl aliquots of spent media, ascites fluid, or purified immunoglobulin from cloned hybridomas were placed in each well. After 2-3 h at room temperature, the wells were washed with PBS/Tween and alkaline phosphatase conjugated goat anti-mouse immunoglobulin was added (1:1000 dilution in PBS/Tween). After 4-8 h, the wells were washed with PBS/Tween and the alkaline phosphatase substrate, p-nitrophenyl phosphate, was added in 1% diethanolamine, pH 9.8, containing 0.3 mm $MgCl_2$. After incubation at 37°C for 30 min, the absorbance was quantitated spectrophotometrically at a wavelength of 405 nm.

3. Ascites Production of Monoclonal Antibodies: Positive clones were injected intraperitoneally into female Balb/c mice (10-12 weeks) which had been primed with 0.5 ml pristane, one week prior to and the day before injection of hybridoma cells (67). The ascites fluid was collected by paracentesis.

4. Characterization and Purification of Monoclonal Antibodies to β-Glc: Immunoglobulin subtypes were determined by the ELISA method with a commercial kit (Boehringer-Mannheim, Indianapolis, IN). IgM or IgG immunoglobulins were purified from ascites fluid by ammonium sulfate fractionation (20-45%) and by ion exchange chromatography or S-300 Sephacryl chromatography, respectively (72).

Epitope specificities were determined by the ELISA additivity index (AI) as described by Friguet et al. (73,77). For these studies, known amounts of pure β-Glc were bound (at pH 7.3 or 9.6) to each well of a

microtitre plate and the amount of a given MCAb was adjusted to reach maximum absorbance readings. This quantity of each possible pair of the different MCAbs were combined, incubated with bound β-Glc, and the change in the absorbance determined. The AI was calculated from:

$$\frac{2\,A_{1+2}}{A_1+A_2} - 1 \quad X\ 100 = AI \qquad \text{Eq 1}$$

where A_1, A_2 and A_{1+2} are absorbance readings for one MCAb, a different MCAb, and the two MCAbs combined, respectively. AI values less than 30 were chosen to be indicative of similar epitope specificities for the two MCAbs.

Several other criteria were used to determine epitope specificity as monitored by ELISA. The ratio of absorbance readings achieved with β-Glc immobilized at pH 7.3 (β-Glc, 7.3) to β-Glc immobilized at pH 9.6 (β-Glc, 9.6), the effect of temperature for MCAb interactions, and the percent recognition of partially purified canine β-Glc were used to distinguish the antigen binding properties of the MCAbs from the 18 stable anti-β-Glc producing hybridomas.

The sensitivity of detecting native or SDS denatured β-Glc on dot blots, the recognition pattern of immunoblotted partial V-8 protease digests of β-Glc, and the effect of the MCAbs on β-Glc activity further defined the epitope specificities (75-77).

5. Kinetic Studies using Inhibitory MCAbs: All 18 MCAb were evaluated for their effects on β-Glc activity as follows: normal or GD fibroblasts sonicated in PBS (Branson 200 cup sonicator, 30 s) or partially purified enzymes, were diluted in PBS, pH 7.3, containing 1% human serum albumin (HSA). MCAb, ascites fluid [heat inactivated (50°C,

2 h) and dialyzed (vs PBS)], or purified immunoglobulin (diluted in PBS with 1% HSA) was added to the enzyme source and incubated at 22°C. After 2 h, the test tubes were assayed for β-Glc activity using 4 mM 4MU-Glc, 0.05 M phosphate/0.04 M citrate, pH 5.5, 4.0 mM Triton X-100 (33,40). A control sample was similarly processed but contained no MCAb.

Four inhibitory MCAbs, two of which have been determined to recognize the same epitope, were further investigated. The rate of inhibition and the effect of various modifiers of β-Glc activity on inhibition and binding by these MCAb were examined in solution and by ELISA. The effects of various lipoidal modifiers were determined as follows: stock solutions of GS, N-hexyl-GS, TC or PS and other modifiers were prepared in chloroform:methanol (2:1, v/v). The desired amount of the modifier was aliquoted into test tubes and the solvent evaporated. More water soluble modifiers, e.g., dNM, N-C_{12}-dNM, or castanospermine were dissolved in water and appropriate amounts added to tubes. The incubations were initiated by addition of the enzyme source followed by the addition of the MCAb. Incubations were continued for 2 h at 22°C, except where noted. Immediately prior to enzyme assay, the incubation mixture was diluted (20- to 100-fold) in PBS and 50 µl was used for β-Glc assay as described above.

The ELISA assay contained β-Glc either directly immobilized to ELISA wells in PBS or via polyclonal anti-β-Glc IgG which had previously been bound to the plate. After incubation with the MCAbs, and washes with PBS/Tween, 120 µl of the β-Glc assay mixture, containing 4 mM 4MU-β-Glc, 0.05 M phosphate/0.04 M citrate, pH 5.5, and 0.5% Triton X-100 (and TC where noted) was added to each well (33,40). After incubation at 22°C

for 2-4 h, 100 µl of the assay mixture was removed into test tubes. The reactions were terminated with ethylene diamine and the fluorescence intensity determined (33).

E. Physical, Kinetic and Immunologic Investigations of the Canine Model of Gaucher Disease:

1. Identification and Quantitation of Glycosphingolipids: The glycosphingolipids of canine tissues were quantitated and characterized using the method of Vance et al. (79). Lipids were extracted from minced tissues with chloroform/methanol (2:1, v/v). A biphasic Folch separation to remove water soluble lipids was conducted and then the neutral sphingolipids were then purified by silicic acid chromatography, alkaline hydrolysis of contaminating phospholipids, and resolution of glycosphingolipids by thin layer chromatography (TLC) in chloroform: methanol:water (65:25:4; v/v/v). The region of the TLC plates for individual glycolipids were scraped into tubes and the glycolipids eluted using chloroform:methanol:water (10:5:1; v/v/v). The sugar moieties were hydrolyzed from the lipids by acidic methanol and derivitized to form volatile tri-methyl silane compounds for identification and quantitation by gas liquid chromatography (SE-30 glass column) using mannitol as an internal standard.

GS was isolated from tissues by the method of Raghavan et al. (80). The tissues were extracted with chloroform/methanol and repeatedly washed with acidic methanol and water to extract GS into the aqueous phase. The acidic upper phases were pooled, adjusted to pH 9.0 and extracted with chloroform/methanol/water (86:14:1, v/v/v). The dried

chloroform phase was applied to a silicic acid column. GS was eluted in 20% methanol and further purified on TLC using chloroform/methanol/water/ammonium hydroxide (65:35:2.5:2.5; v/v/v/v) as the solvent. The presence of GS was established with ninhydrin and anthrone sprays for the detection of amino and carbohydrate groups, respectively. GS was further identified and distinguished from galactose sphingosine by using TLC plates containing 5% boric acid developed in chloroform:methanol: ammonium hydroxide (14:5:1; v/v/v) (81) and by the detection of glucose peaks and absence of galactose peaks on gas chromatographic analysis.

2. Enzyme Preparation: Tissues for determination of β-Glc activity were prepared as follows: tissue samples were Potter-Elvejhem homogenized (4°C) in 5 vol 0.25 M sucrose and clarified by centrifugation at 120 x g. The supernatants containing > 95% of total β-Glc activity were used to determine the level of β-Glc activity in normal and GD sources. The remainder of the clarified supernatants was extracted at 4°C by the dropwise addition of n-butanol (1:1; v/v). After 30 min of stirring, the solution was centrifuged at 8,000 x g for 45 min (4°C). The resulting aqueous layers, containing between 75 and 90% of the β-Glc activity from either the normal or GD sources, were removed and dialyzed overnight against 0.05 M phosphate/0.04 M citrate, pH 5.5, containing 5 mM EDTA and 1 mM β-mercaptoethanol (Buffer C). These butanol-extracted enzyme sources were used for all kinetic and thermostability studies. The β-Glc activities in the butanol extracts were stable at -20°C for several months.

3. Determination of β-Glucosidase Activities: For determination of the hydrolytic rates of 4MU-Glc, the standard reaction mixture

(0.2 ml) contained 0.05 M phosphate/0.04 M citrate, pH 5.5, 4 mM 4MU-Glc, 4.65 mM TC, 4.0 mM Triton X-100 and appropriately diluted enzyme source (33,40). For the estimation of "nonspecific" β-Glc activity (30), TC and Triton X-100 were omitted. The reactions were terminated and the fluorescence intensity determined in 0.1 M ethylene diamine, pH 11.0 (33). Assays with NBD-C_{12}-GC were conducted as described (57). The NBD-C_{12}-GC substrate was a 1:20 (mole/mole) mixture of fluorescent NBD-C_{12}-GC and authentic GC; this mixture was designated as GC.

The assays were linear with time (0.25 to 4 h) and protein concentration (20 to 150 μg/assay). Other enzymatic activities were determined using the appropriate 4MU-glycosides (82). Protein concentration was estimated by a fluorescamine procedure (83).

4. Kinetic Studies: The effects of various lipoidal modifiers were determined as follows: stock solutions of GS, N-hexyl-GS, dodecyl-β-D-maltoside, TC or PS and other inhibitors, were prepared in chloroform:methanol (2:1; v/v). The desired amount of the modifier(s) was aliquoted into tubes and the solvents evaporated under nitrogen. Appropriate amounts of substrate in 4.0 mM Triton X-100 and 0.05 M phosphate/0.04 M citrate, pH 5.5, were added and the mixtures were vigorously agitated. Water soluble modifiers, dNM, C_{12}-dNM, CBE or δ-gluconolactone were dissolved in water and appropriate amounts aliquoted into tubes. The reactions were initiated by the addition of the enzyme source and assayed as described above. The K_m values were determined from Eadie-Hofstee (84) plots and represent the mean of three determinations: GC concentrations varied between 0.05 mM and 1.5 mM. GS

and N-hexyl-GS were shown to be noncompetitive inhibitors of human (33,40) and canine (normal or GD) β-Glc (data not shown). Thus, the K_i values were derived from Dixon plots and represent the mean of three determinations for each enzyme source. For CBE, the time required to inhibit enzymatic activity by 50%, $t_{1/2}$, was determined by adding CBE (0.2 mM final concentration) to the enzyme in buffer C, containing 2 mM TC and incubated at 23°C (51). At specified time points, aliquots (10 µl) were removed, added to 190 µl of substrate solution and the hydrolysis of GC was determined as described above (1 h, 37°C). CBE inhibitable activity (4MU-Glc) was determined using up to 2 mM CBE in the absence of Triton X-100 and TC.

Using the appropriate substrates, the levels of twelve other hepatic lysosomal enzyme activities were found to be normal, including: α-L-arabinosidase, arylsulfatase A and B, aspartylglucosaminidase, α-L-fucosidase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucuronidase, β-hexosaminidase, α-mannosidase and β-xylosidase (82).

5. Membrane Association of β-Glc: To determine the degree of membrane association of β-Glc, tissue samples were homogenized and clarified as described above. The clarified supernatants were then centrifuged at 100,000 x g for 45 min (4°C). The resultant pellets were resuspended to the original volume with 0.9% saline and then sonicated for 30 sec [80 output; 4°C (Branson Model 200)]. The high speed supernatants (S) and the dispersed pellets (P) were assayed immediately. Total recovery of β-Glc in these fractions was 80 to 100%.

6. Electrophoresis and Isoelectric Focusing: Cellulose acetate gel electrophoresis of crude tissue homogenates and determinations of GC

and 4MU-Glc activities in gel sections were performed exactly as described (85). Recovery of applied activities ranged between 30 and 50%.

Granular bed isoelectric focusing was conducted according to the manufacturer's instructions using ampholines, pH 4.0 to 8.0. The tissue samples (2 grams) were homogenized at 4°C with a Potter-Elvejhem homogenizer in 4 volumes of water. Sodium cholate (1% w/v) was added and the mixtures were stirred for 30 min at 4°C. After centrifugation, 14,000 x g for 30 min, the supernatants, containing 90% of β-Glc activity, were dialyzed overnight (4°C) against 1% glycine. Assays were conducted in gel sections eluted with 0.1 M citrate, pH 5.0, containing 70% ethylene glycol. Total recovery of various activities was from 35 to 60%.

7. Con A Chromatography: Con A-Sepharose chromatography was conducted as described (85). Briefly, the clarified supernatants from three grams of homogenized normal or GD liver tissue were mixed with Con A-Sepharose at 23°C for 2 h and then poured into columns. The flow-through and subsequent buffer washes were termed the "unbound fraction." After extensive washing with Con A buffer (0.05 M citrate, pH 6.0, containing 1 M NaCl, and 5 mM each of $MgCl_2$, $MnCl_2$ and $CaCl_2$), the "bound fraction" was eluted in the above buffer containing 0.5 M 1-O-methyl-α-D-mannoside and 50% ethylene glycol. The fractions were then dialyzed against buffer A and concentrated in an ultrafiltration cell (YM 10 membrane; Amicon, Danvers, MA). Total recovery of applied 4MU-Glc activity typically ranged between 80 and 90%.

8. Thermostability Studies: Thermostability studies were performed as described (37). The dialyzed butanol extracts of normal or GD liver, brain, kidney or spleen preparations were equalized for activity (GC substrate) by dilutions with water. These samples (100 µl) were mixed 1:1 (v/v) with phosphate/citrate buffer at various pH values (pH 5.0-7.0) containing 2 mM Triton X-100, 7.4 mM TC and 2 mg/ml HSA. For mixture experiments, equal activities of the GD and appropriately diluted normal β-Glc were mixed (1:1; v/v) and diluted with the above buffer. After the samples were heated at 50°C for the designated time, aliquots were removed, immediately cooled to 4°C and assayed using the GC substrate. In control experiments, the thermostabilty of the canine β-hexosaminidase activity was found to be identical in normal or GD sources.

9. Immunoblotting Studies: Tissue samples (0.1 g) from normal or GD Silky Haired Terriers were Potter-Elvejhem homogenized in 4 volumes of 0.06 M phosphate, pH 6.6, containing 0.5% cholate, 0.1% Triton X-100 and then sonicated for 30 seconds at 4°C (Branson 200 cup sonicator, 80 output). Aliquots (80 µg protein/well) were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 1.5 mm slab gels (64). After electrophoresis, proteins were electroblotted (70 volts, 90 min) onto a nitrocellulose membrane in transfer buffer, 0.025 M TRIS 0.19 M glycine, pH 8.3, containing 10% methanol (65). The nitrocellulose membrane was blocked with PBS-BLOTTO (66) and incubated for 16 h at 4°C with monospecific rabbit anti-human placental β-Glc IgG (40) (5 µg/ml PBS-BLOTTO) or a monoclonal antibody to human β-Glc (Section V.D.) to human β-Glc. The bound IgG was detected with Vectastain ABC kit.

Comparative immunotitration studies indicated that the canine β-Glc had about 50% cross-reactivity with the anti-human β-Glc polyclonal IgG (data not shown).

10. Purification of Canine Splenic β-Glc: Partially thawed canine splenic tissues were homogenized in 20 mM phosphate/10 mM citrate, pH 7.0, containing 0.02% sodium azide, 0.01 mM PMSF, and further extracted by mixing this solution with 1% cholate (w/v) at 4°C for 1 h. After centrifugation (5000 x g, 45 min), the supernatant was fractionated with 30% ammonium sulfate and centrifuged (5000 x g, 45 min) (12). The resultant supernatant was diluted by the addition of one-half the total volume of 3-fold concentrated Con A buffer (85). The diluted supernatant was gently stirred with 200 ml of Con A Sepharose for 16 h at 4°C. After extensive washing of the Con A Sepharose with Con A buffer, the beads were poured into a column, and β-Glc activity was eluted with Con A buffer containing 0.5 M α-methylmannoside and 50% ethylene glycol. The high specific activity fractions were pooled and extracted with 35% butanol. A greater concentration of butanol than used with the human enzyme (20%) was required to achieve a good biphasic separation due to the high salt concentration of the enzyme eluted from Con A.

After dialysis against Buffer C, the sample was applied to a previously equilibrated GS-Sepharose column at 3-5 ml/min. After extensive washing with buffer C, and washing with buffer C containing 20% ethylene glycol, β-Glc activity was eluted in a linear 20-90% ethylene glycol gradient in buffer C (15). Canine β-Glc was eluted at about a 65% ethylene glycol concentration. Samples with high specific

activity were pooled and immediately diluted in Buffer A (canine β-Glc was not stable when stored in high concentrations of ethylene glycol at 4°C) to less than 5% ethylene glycol concentration and applied to C_{12}-dNM Sepharose at a rate of 1 ml/min. The column was washed with buffer A, containing 4 mM β-mercaptoethanol, buffer A containing 1% TC and then buffer A alone. The canine enzyme was eluted from the C_{12}-dNM Sepharose with 50-75% ethylene glycol concentration in a 10-90% linear ethylene glycol gradient in buffer A.

V. RESULTS AND DISCUSSION

A. Affinity Purification of the Normal Placental and Gaucher Disease Splenic β-Glc on N-Alkyl-Deoxynojirimycin-Sepharose:

1. Affinity Purification of Normal β-Glc: Table I summarizes typical purifications of β-Glc from 10 kg of human placentae by C_{10}- and C_{12}-dNM chromatography. The enzyme was purified about 20,000-fold from the crude homogenate (9500-fold from the cholate extract) with an overall yield of 14 to 18%. The final enzyme preparations from either column had similar specific activities (1.4 to 1.7 x 10^6 nmol/h/mg protein using 4 mM 4MU-Glc as substrate) and were stable in 60% ethylene glycol at 4°C for four to six months. The homogeneity of these preparations was evidenced by a single protein species on SDS-PAGE gels (detected by the silver-staining technique (78); data not shown), and on reverse phase HPLC (Fig. 1). In addition, these enzyme preparations had a single N-terminal amino acid sequence. The first 22 residues are shown below with tentative assignments in parentheses and unidentifiable residues designated by X:

Ala-Arg-Pro-X-Ile-Pro-Lys-Ser-Phe-Gly-Tyr-
Ser-Ser-Val-Val-X-(Val)-X-X-Ala-Thr-Tyr....

This sequence was colinear with that predicted from the human β-Glc cDNA nucleotide sequence (23,24) and indicates the junction of the leader piece and the mature N-terminus. Amino acid composition analyses of β-Glc (Table II) revealed 35% hydrophobic amino acids (Ile + Leu + Met + Val + Aromatics) which was in good agreement with the composition predicted from the cDNA (23,24).

TABLE I

Purification of Acid β-Glucosidase from Normal and Type 1 AJGD Tissues

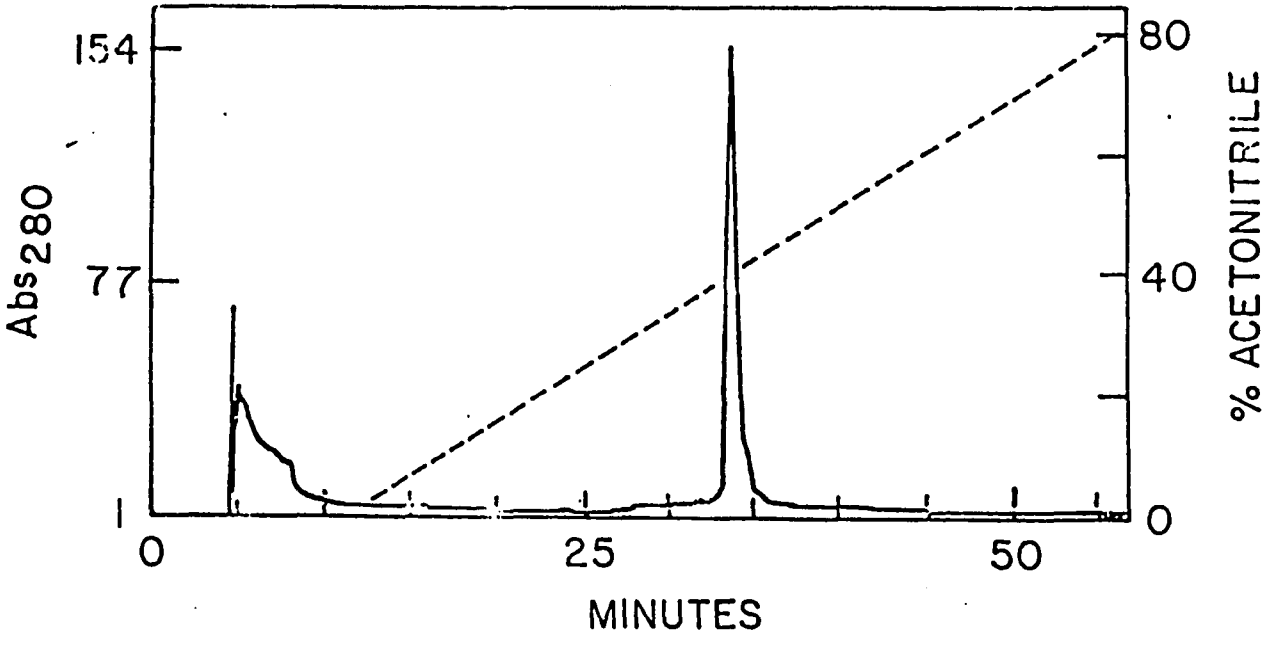
Purification Step	Normal		Type 1 AJGD	
	Specific Activity[a]	Yield	Specific Activity[a]	Yield
	(U/mg protein)	(%)	(U/mg protein)	(%)
Cholate extract	170	100	4.5	100
35% Ammonium sulfate	380	70	9.9	63
Acid precipitate/ Butanol extract	160	43	4.0[b]	51
Decyl agarose	137,000	45	—	—
Purification 1:				
Octyl Sepharose I	338,000	40	—	—
N-C_{10}-dNM Sepharose	1.6×10^6[b]	14	26,500	10.9
Purification 2:				
Octyl Sepharose II	306,000	45		
N-C_{12}-dNM Sepharose A	948,000	30		
N-C_{12}-dNM Sepharose B	1.7×10^6	18		

[a]nmol of 4MU-Glc (4 mM) hydrolyzed per h.

[b]Acid precipitation was omitted.

[c]Final specific activity ranged from 1.46 to 1.74×10^6 U/mg protein.

Figure 1: Reverse phase HPLC profile of normal placental β-Glc purified by C_{10}-dNM-Sepharose. Five nmoles (280 μg) of β-Glc were applied and then eluted at 41% acetonitrile in 0.05% trifluoroacetic acid. The peaks at 5 to 10 min were due to absorbing materials in the buffer.


154
77
1
Abs280
0
25
50
MINUTES
80
40
0
% ACETONITRILE

The placental β-Glc, partially purified by hydrophobic chromatography, was diluted with 0.05 mM citrate, pH 5.5, containing 4 mM β-mercaptoethanol and 1 mM EDTA (buffer A) to an ethylene glycol concentration of < 5% and applied (1 ml/min) to equilibrated columns of either C_{10}- or C_{12}-dNM-Sepharose. The columns were then subjected to sequential washes (100 column vol each) with buffer A, buffer A containing 1% taurocholate and then, buffer A alone. The enzyme then was eluted from either affinity support at about 40 to 60% ethylene glycol in a 10 to 90% linear gradient in buffer A (Fig. 2). The enzyme eluted from the C_{12}-dNM support typically contained variable amounts (5 to 15%) of a contaminant ($M_r \cong 47,000$) which could be resolved from β-Glc by reapplication followed by elution with a 40 to 90% ethylene glycol gradient in buffer A (Table I). In comparison, the enzyme obtained from C_{10}-dNM-Sepharose was homogeneous (Fig. 1). The capacities of the affinity supports were about 0.5 to 0.75 mg β-Glc/ml of settled gel and remained unchanged without regeneration through ten uses of either column.

Importantly, all solutions and buffers as well as the affinity columns must contain 4 mM β-mercaptoethanol to maintain enzyme stability. At β-mercaptoethanol concentrations between 0.5 and 3.0 mM, the enzyme activity had a $t_{1/2}$ of 0.1 to 0.5 h, whereas at 4 mM β-mercaptoethanol, the β-Glc activity was stable for 4 to 6 months. Interestingly, when the enzyme activity of a homogeneous preparation was allowed to decrease (in 2 mM β-mercaptoethanol) to a specific activity of 750,000 nmol/h/mg protein, increasing the β-mercaptoethanol concentration to 4 mM, prevented any further loss of activity. The original specific activity (1.5×10^6 nmol/h/mg protein) of homogeneous β-Glc

TABLE II

Amino Acid composition of β-Glc

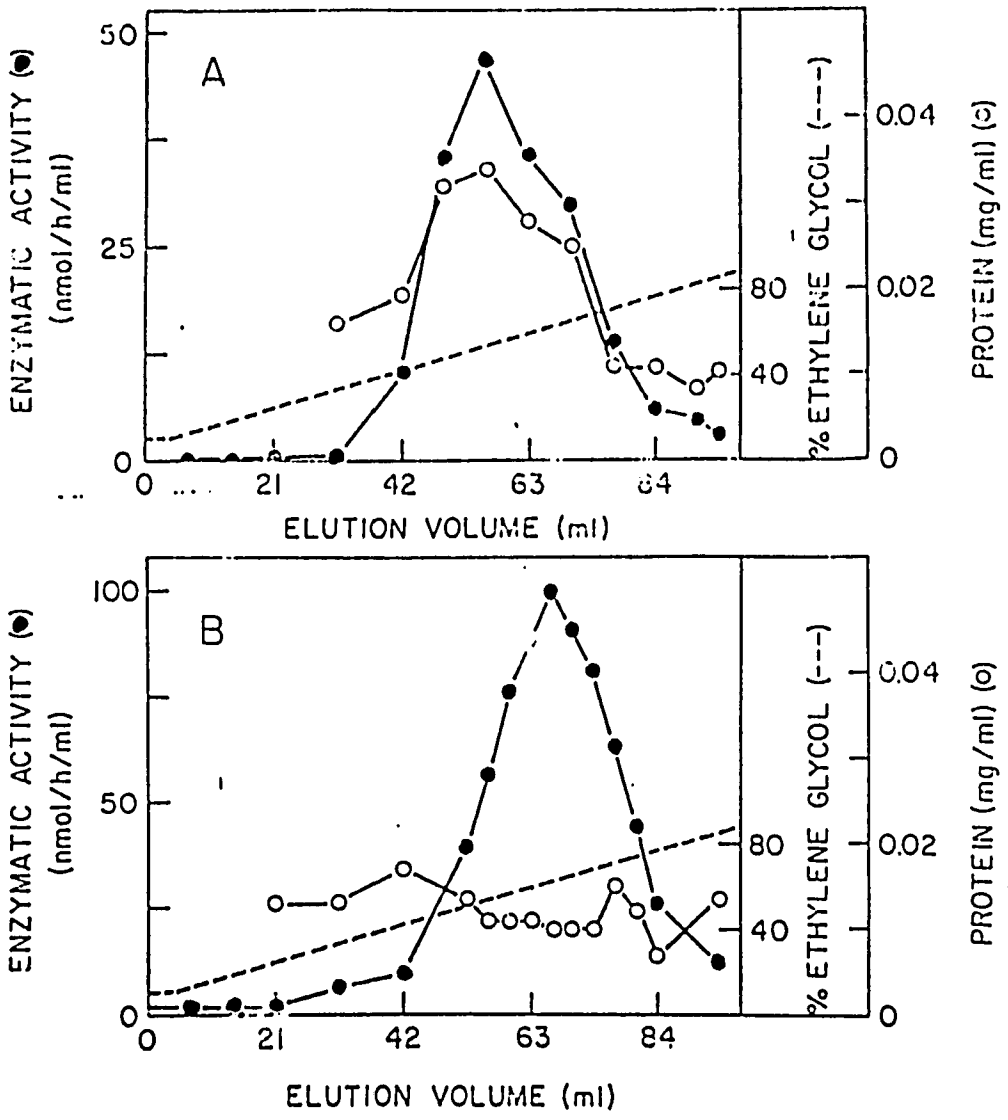
Amino Acid	Number of Residues[a]
Asx[b]	48
Thr	28
Ser	29
Glx[b]	43
Pro	34
Gly	39
Ala	43
Val	32
Met	8
Ile	21
Leu	58
Tyr	19
Phe	28
His	18
Lys	24
Arg	26
Cys	—
Trp	—

[a]Based on MW = 55,382

[b]Asx = Asp + Asn; Glx = Glu + Gln

Figure 2: Elution profile of normal (A) and Type 1 AJGD (B) β-Glc activity from C_{10}-dNM-Sepharose. Enzyme activity (●) and protein (O) eluted coincidentally in a 10 to 90% ethylene glycol gradient (---) after extensive washes (see text). The enzyme activities in A were multiplied by 10^{-3}.

Figure 2


A
ENZYMATIC ACTIVITY (●)
(nmol/h/ml)
50
25
0
0
21
42
63
84
ELUTION VOLUME (ml)
80
40
% ETHYLENE GLYCOL (---)
0.04
0.02
0
PROTEIN (mg/ml) (○)
B
ENZYMATIC ACTIVITY (●)
(nmol/h/ml)
100
50
0
0
21
42
63
84
ELUTION VOLUME (ml)
80
40
% ETHYLENE GLYCOL (---)
0.04
0.02
0
PROTEIN (mg/ml) (○)

could be fully restored by repurifying the above preparation on C_{10}-dNM. About 50% of the homogeneous protein was detected in the 1% taurocholate wash of the affinity column and was inactive. In contrast, about 50% of the homogeneous protein was eluted with the ethylene glycol gradient. This latter protein had a specific activity typical of pure β-Glc. These results suggest that only active enzyme bound to the affinity support and was eluted specifically with ethylene glycol. Thus, this procedure provided a method to obtain purified preparations which contain only active enzyme. This observation was supported by finding a 1:1 mol/mol stoichiometry of β-Glc protein and [^{3}H]Br-CBE (34). These findings also may account for the higher specific activities of these preparations than those obtained with other substrate affinity ligand methods (15,16).

2. Affinity Purification of Type 1 AJGD β-Glc: Table 1 also summarizes the purification of β-Glc from 4 kg of Type 1 AJGD spleen by C_{10}-dNM chromatography. The final preparation was about 7500-fold enriched over that in the crude homogenate (about 5900-fold over the cholate extract). This preparation was obtained in an 11% yield and several protein bands were detected on silver stained SDS-PAGE gels (78) (data not shown). Only a band at $M_r \cong 67,000$, which corresponded to the pure normal glycosylated placental β-Glc, was detected by immunoblotting using polyclonal or monoclonal antibodies to normal β-Glc. Reapplication and elution of the final preparation on the C_{10}-dNM column resulted in recoveries of 10 to 15% of the applied enzymatic activity

and insufficient protein was obtained for analysis. In buffer A containing 60% ethylene glycol and 4 mM β-mercaptoethanol, the enzyme activity was stable for at least five months.

To obtain yields greater than 1% of the original Type 1 AJGD enzymatic activity, the acid precipitation and hydrophobic chromatography steps (11,12,15) were eliminated. The cold (4°C) butanol extract, obtained after dialysis against 2% butanol and containing 4 mM β-mercaptoethanol, was applied directly to a 5 ml column of C_{10}-dNM (1 ml/min). The washes and elution were exactly the same as for normal placental β-Glc except that the mutant enzyme activity in the crude splenic extract was eluted with 50 to 70% ethylene glycol (Fig. 2). Extensive comparative kinetic studies of the normal and Type 1 AJGD β-Glc preparations indicated that the mutant β-Glc had a specific active site defect (40,51).

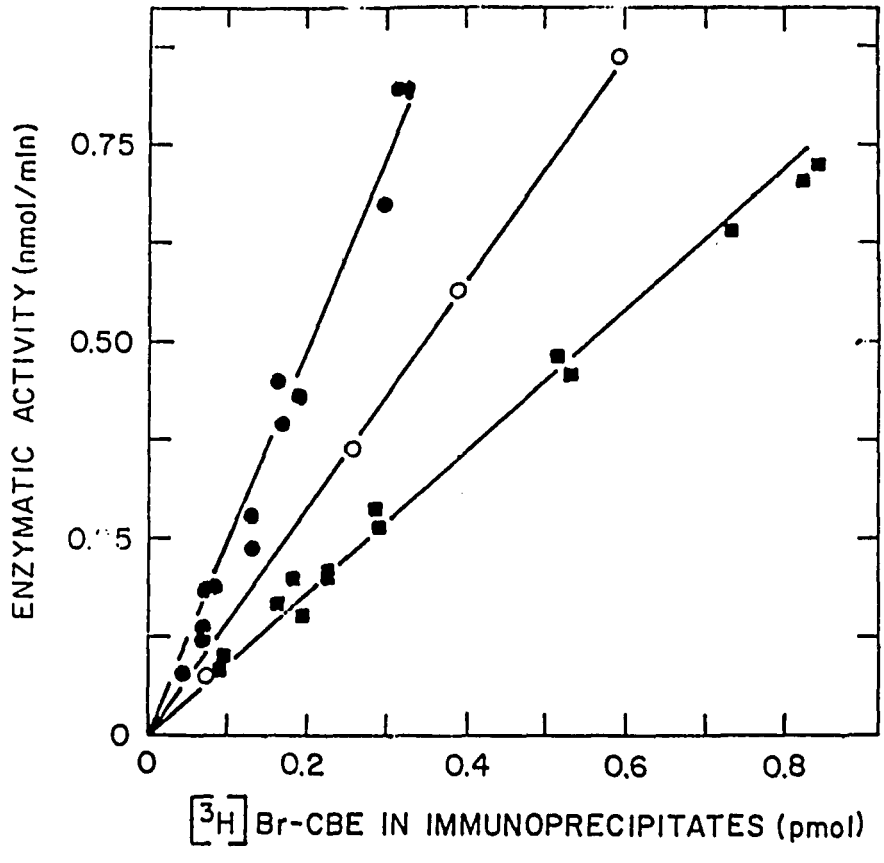
3. Conclusions: In summary, two new affinity columns employing potent [K_i = .050 to .100 μM (59)] β-Glc inhibitors have been shown to be useful for purification of normal and Type 1 AJGD β-Glc. Since pilot studies have indicated that the N-alkyl-dNM supports bind other glucosidases (e.g., neutral β-glucosidase and acid α-glucosidase isozyme 1 and 2), the affinity columns might be adapted to the sequential purification of these enzymes by differential elution with specific substrates or inhibitors. For β-Glc, these affinity supports provided a high yield of homogeneous normal enzyme which retained full catalytic activity and facilitated investigations of this enzyme's structure and

function. In addition, the C_{10}-dNM support provided a simple, rapid and higher yield alternative to the low yield, multi-procedural method (14) for purifying mutant β-Glc from Gaucher disease spleen.

B. Use of Conduritol B Epoxide Derivatives to Investigate the Catalytically Active Normal and Gaucher Disease Enzymes:

1. Determination of k_{cat} Values: To determine the k_{cat} values for the normal and Type 1 AJGD active enzymes, the concentration of catalytic sites in a homogeneous normal placental β-Glc preparation, a crude normal splenic preparation (60-fold enriched) and partially purified splenic preparations from two AJGD patients (500- and 7500-fold enriched) were determined using [^{3}H]Br-CBE and monospecific rabbit anti-human β-Glc IgG. As shown in Figure 3, the amount of enzymatic activity (4MU-Glc) was directly related to the number of catalytic sites which were specifically labeled by [^{3}H]Br-CBE in the respective enzyme preparations; similar results were obtained using NBD-C_{12}-GC [200 μM; K_m = 30 μM (Table III)] as substrate (data not shown). The k_{cat} values for the normal and Type 1 AJGD enzymes with 4MU-Glc were obtained directly from the slopes of the respective curves in Figure 3 and the corresponding data with NBD-C_{12}-GC as substrate. The k_{cat} values for the normal homogeneous placental enzyme, with the 4MU-Glc and NBD-C_{12}-GC substrates, were 2360 min^{-1} (range 2240 to 2480) and 2440 min^{-1} (range 2320 to 2560), respectively. Using the delipidated normal splenic extract, the k_{cat} values were 1380 min^{-1} (range 1370 to 1390) for the 4MU-Glc substrate (Fig. 3) and 1480 min^{-1} (range 1410 to 1510) with

Figure 3: Correlation of enzymatic activity (nmol 4MU-Glc hydrolyzed/min) and the number of catalytic sites in homogeneous normal placental β-Glc (●), delipidated normal splenic enzyme (O) and partially purified Type 1 AJGD splenic β-Glc preparations (■). [^{3}H]Br-CBE specifically bound to the respective β-Glc was determined by quantitative immunoprecipitation of the enzyme-[^{3}H]Br-CBE complex with rabbit anti-human placental β-Glc IgG and Staphylococcus aureus protein A. The k_{cat} values for each enzyme preparation were determined from the slopes of the unweighted linear regression curves (see text and Table III). The data points for the mutant enzyme were from two different enzyme preparations which differed in degree of purification by 15-fold.


ENZYMATIC ACTIVITY (nmol/min)
0.75
0.50
0.25
0
0
0.2
0.4
0.6
0.8
[3H] Br-CBE IN IMMUNOPRECIPITATES (pmol)

NBD-C_{12}-GC. The corresponding k_{cat} values for the Type 1 AJGD splenic enzymes were 920 min^{-1} (range 830 to 1000) with 4MU-Glc and 980 min^{-1} (range 880 to 1080) with NBD-C_{12}-GC.

In separate sets of experiments similar to those in Figure 3, the number of catalytic sites in the normal or Type 1 AJGD β-Glc sources was determined as a function of total protein concentration in the respective enzyme preparations. These data indicated that [^{3}H]Br-CBE bound to the homogeneous normal placental enzyme in a 1:1 mole ratio with enzymatic protein (34). The normal splenic β-Glc was estimated to represent about 0.4-0.6% of the total protein in the preparation. Similarly, the Type 1 AJGD active enzymatic protein was 0.7% and 10.5% of the total protein in the two different preparations. From these data, the total enzyme concentrations, [E_t], were calculated to be between 0.5 and 15 nM: i.e., about 20,000- to 700-fold less than the concentration of [^{3}H]Br-CBE (10 μM) used in these experiments.

The k_{cat} values (Table III) for the pure normal placental enzyme and the Type 1 AJGD splenic enzymes with each substrate were determined from $V_{max} = [E_t]k_{cat}$, assuming that only enzyme molecules which had retained full catalytic activity had been quantitated by the [^{3}H]Br-CBE method. For each substrate, the k_{cat} values were about 1.5- to 3-fold lower for the Type 1 AJGD enzymes than the respective values for pure normal placental β-Glc. Essentially identical k_{cat} values were obtained with either Type 1 AJGD splenic preparation. As indicated above, the k_{cat} values for 4MU-Glc and NBD-C_{12}-GC as substrates with the crude normal splenic preparation were about 1.3- to 1.7-fold greater than those for

TABLE III

Comparison of Kinetic Constants of the Normal and Type 1 AJGD β-Glc[a] with Alternate Substrates

Substrate Aglycon	K_m (mM)		kcat (min^{-1})				
			Normal Placental		Normal Splenic	AJGD Splenic	
	Normal	AJGD	Method 1[b]	Method 2[c]	Method 1	Method 1	Method 2
2-N-(NBD-C_{12}-)-sphingosyl	0.03±0.01	0.03±0.02	2440 (2320-2560)	2135 (2090-2178)	1480 (1410-1510)	980 (880-1080)	826 (749-893)
2-N-(NBD-C_6-)-sphingosyl	0.05±0.02	0.05±0.03		3200 (2790-3610)			1090 (963-1218)
4-C_{11}-U	0.05±0.02	0.05±0.02		773 (729-818)			306 (278-329)
4-C_9-U	0.17±0.03	0.15±0.02		2220 (1973-2460)			914 (850-989)
4-C_7-U	0.12±0.02	0.28±0.05		1972 (1820-2124)			713 (670-748)
4-C_1-U	1.9±0.3	2.9±0.5	2360 (2240-2480)	2235 (2038-2430)	1380 (1370-1390)	920 (830-1000)	893 (862-929)

[a]Homogeneous normal placental β-Glc and partially purified normal or Type 1 AJGD splenic enzyme were used for these studies.

[b]Method 1: k_{cat} was derived from the slopes of enzymatic activity as a function of [^{3}H]Br-CBE in the immunoprecipitates as determined by other investigators in this laboratory.

[c]Method 2: k_{cat} was derived from $V_{max} = [E_t]k_{cat}$ where $[E_t]$ was determined from the radioactivity of [^{3}H]Br-CBE in immunoprecipitates after complete inactivation of a fixed amount of enzyme activity by 10 μM of [^{3}H]Br-CBE (see text).

the Type 1 AJGD splenic enzymes. The respective K_m values for each substrate were similar in the Type 1 AJGD and normal enzyme preparations (Table III).

2. Discussion: This study has provided evidence for a defect of active site function of β-Glc from patients with Type 1 AJGD. Compared to the normal enzyme, the defect in the Type 1 AJGD enzyme resulted in a small decrease (1.5- to 3-fold) in the k_{cat} values for several substrates while a 5-fold decreased affinity for CBE, a covalent catalytic site inhibitor, has been demonstrated by other investigators in this laboratory (51). These results imply that the deficiency of enzymatic activity in Type 1 AJGD cannot be explained by a major alteration in the catalytic capacity of the active mutant enzyme. This explanation for the nature of the defect in Type 1 AJGD differs from that previously suggested by others (14) and by us (33,51) which proposed that the defect in the Type 1 AJGD enzyme resulted in a large decrease in V_{max} or k_{cat}. Based on comparative immunologic studies of normal and Type 1 Gaucher disease enzymes in highly purified splenic preparations Pentchev et al. (14) suggested a mutation in the β-Glc structural gene which resulted in a 10- to 20-fold decrease in V_{max} and, by inference, k_{cat} of the Type 1 GD enzyme. Using immunologic, inhibitor and inactivation (CBE) studies of the residual enzyme in fibroblast extracts, we proposed a specific active site defect which could account for this V_{max} abnormality in Type 1 AJGD (51).

Previous studies which determined Vmax have been limited by the lack of homogeneous Type 1 AJGD enzyme which retained full catalytic activity and by assumptions of antigenic identity of the normal and Type 1 AJGD enzymes (40,45,46). To overcome these limitations, we used the covalent inhibitor, [^{3}H]Br-CBE, to determine the k_{cat} values from V_{max} values and the concentration of β-Glc catalytic sites, [E_t], in pure normal placental β-Glc, delipidated crude normal splenic extracts, and the Type 1 AJGD enzyme preparations. This approach obviated the need for homogeneous mutant enzyme which retained full catalytic activity and only required that all enzyme which bound [^{3}H]Br-CBE could be completely immunoprecipitated. Thus, the results of these studies were dependent upon the degree of labeling obtained with the Type 1 AJGD enzyme. Complete labeling of the active Type 1 AJGD enzyme was supported by the following findings: 1) [^{3}H]Br-CBE was recovered from SDS-polyacrylamide electrophoretic gels of the pure normal enzyme and the corresponding protein band in the Type 1 AJGD enzyme preparation following immunoabsorption with monoclonal antibody to normal β-Glc. These results indicated covalent binding of [^{3}H]Br-CBE to either enzyme without large losses of label especially under the less stringent procedures used in the present experiments. 2) Labeling conditions were optimized to ensure that all [^{3}H]Br-CBE binding sites were saturated. 3) A 1:1 mole to mole stoichiometry was obtained with the pure normal enzymatic protein and [^{3}H]Br-CBE (34). 4) A linear relationship was found between the amount of incorporated radioactivity and enzymatic activity for the normal or Type 1 AJGD enzymes (Fig. 3) which had grossly similar primary structure. The k_{cat} calculated from these curves for the pure normal placental enzyme agreed well with the observed value based on protein

determinations (Fig. 3 and Table III). 5) The maximal rate of inactivation (k_{max}) of the Type 1 AJGD enzyme by CBE, determined by other investigators in this laboratory, was similar to that for the normal placental β-Glc. This result is inconsistent with a major alteration in k_{cat}, if the steps required for covalent binding of [^{3}H]Br-CBE and substrate hydrolysis are the same (58). In addition, we assumed that only active enzyme could bind [^{3}H]Br-CBE covalently and that these molecules had retained their respective full catalytic activities. The finding of the 1:1 stoichiometry of inhibitor binding to the normal enzyme based on protein (34) or activity determinations support this assumption. The minor decrease in k_{cat} values for the Type 1 AJGD splenic enzymes provide evidence for the binding of [^{3}H]Br-CBE only to catalytically active mutant β-Glc. If the first assumption was incorrect or the immunoprecipitation procedure quantitated non-specifically bound (i.e., non-β-Glc) [^{3}H]Br-CBE, the differences in k_{cat} values between the normal placental β-Glc and Type 1 AJGD active enzyme would be less than those obtained.

Although the present studies were confined to splenic enzymes from two Type 1 AJGD patients, these results most likely are representative of the mutation(s) in affected Ashkenazi Jewish patients since the mutant enzymes which were selected had kinetic and immunologic properties characteristic of β-Glc found in a large series of Type 1 AJGD patients (40,51). Also, the kinetic properties of delipidated normal splenic β-Glc and normal or Type 1 AJGD enzymes in crude fibroblast extracts or lymphocytes were essentially identical to those of the respective enzymes reported here (40,51). These results indicated that the respective kinetic properties of the normal and Type 1 AJGD enzymes

were consistent in different tissue sources (40,51). However, the slightly lower k_{cat} values obtained with the delipidated normal splenic enzyme, compared to the placental enzyme, suggest minor differences in kinetic properties of β-Glc (or its different molecular forms) from various tissues. Based on these considerations, the respective k_{cat} values for the normal β-Glc and the Type 1 AJGD preparations were similar with each of six different substrates (Table III) representing two different types of leaving groups.

These data provide insight into the nature of the molecular pathology of the deficient enzyme activity in Type 1 AJGD. Previous studies have indicated that the inhibitors, glucose sphingosine (40) and sphingosine (51), have altered affinities for the active site of the Type 1 AJGD enzyme. These data suggested that a particular domain or region within or near the mutant active site alters the function of the active site (33,40,51). Immunologic studies with polyclonal (40,45) and monoclonal (46) antibodies have indicated that the normally processed β-Glc protein in Type 1 AJGD tissues was present at about 20 to 55% of normal levels, but that the enzymatic activity per mg of CRIM was 8- to 20-fold decreased (14,40,45,46). The minor decrease in k_{cat} found in these studies implies that a percentage of detectable CRIM in previous studies was catalytically inactive enzyme protein. Thus, it appears likely that the defect within or near the Type 1 AJGD active site leads to <u>in vivo</u> lability of the enzymatic activity.

C. Type 1, 2 and 3 Gaucher Disease: Genetic Heterogeneity Within and Among Subtypes Delineated by Immunoblotting:

1. Solubilization of β-Glc Activity: Due to the membrane association of β-Glc, various extraction conditions were evaluated to ensure solubilization of β-Glc for immunoblotting studies. As shown in Table IV, the amount of β-Glc activity retained in the homogenate or sonicate supernatants after centrifugation was variable and 0.1% Triton X-100, 0.5% cholate provided the maximal solubilization of β-Glc activity. The type of sonicator (cup or probe) or length of sonication time had only minor effects on the specific activity or percentage of β-Glc activity solubilized (data not shown). No consistent difference in solubilization of normal and GD β-Glc activity was apparent.

In view of the fact that complete solubilization of the normal or mutant β-Glc activities could not be consistently achieved, whole fibroblasts were sonicated in 0.1% Triton X-100 and 0.5% cholate for immunoblotting studies. This method was also used since dissolution of whole fibroblasts in SDS-PAGE buffer B alone resulted in poorly resolved immunoblots.

2. Immunoblotting Studies: The molecular weight forms of CRIM were determined in fibroblast sonicates from seven normal individuals and thirty-three GD patients. Very similar CRIM patterns were observed using polyclonal IgG or three different monoclonal antibodies to human β-Glc. Figure 4 demonstrates the consistency and variability of the immunoblotting patterns using polyclonal IgG (Figs. 4A and B) or using monoclonal antibody 61 (Fig. 4C). Normal fibroblasts (Fig. 4A, lane 8; Figs. 4B and C, lane 7) consistently contained CRIM forms of $M_r \cong$

TABLE IV

Solubilization of β-Glc Activity in Normal, Type 1, and Type 2 GD Fibroblasts

Conditions	% of Total Activity Recovered in Supernatant		
	Normal	Type 1 GD	Type 2 GD
Homogenization			
0.25 M Sucrose	17.1% (2)[a] (17.1-17.6)	3.0% (2) (0-6.0)	6.7% (2) (0-13.6)
Sonication			
60 mM phosphate, pH 6.6	32.3% (6)	14.8% (3)	27.2% (4)
0.1% Triton X-100	(7.4-66)	(4.3-30.5)	(4.9-30.0)
60 mM phosphate, pH 6.6	67.4% (5)	80.1% (4)	74.6% (4)
0.1% Triton, 0.5% cholate	(24-95)	(30.5-98)	(48-93.2)

[a]( ) indicates number of experiments.

67,000 to 58,000. The $M_r \cong$ 67,000 and/or 58,000 forms were typically present as discrete bands, whereas the forms $M_r \cong$ 61,000 to 64,000 were diffuse and were usually not detected as distinct forms. As shown in Figure 4, the relative staining densities of these CRIM forms were somewhat variable. This variability was found with different normal individuals and with the same normal individual. No consistent variation was observed with passage number or cell density of the fibroblast cultures.

The CRIM forms (M_r = 67,000 to 58,000) in most Type 1 GD fibroblasts, including nine AJGD and ten non-Jewish GD patients, had normal patterns using polyclonal or monoclonal antibodies (Fig. 4, see legend). The amount of CRIM per mg cell protein always appeared less than normal (Fig. 4). These 19 Type 1 GD patients were previously classified as Group B by physical and kinetic studies (40,51). In contrast, fibroblasts from five different non-Jewish Type 1 GD patients had immunoblot CRIM patterns which differed from normal and only one or two of the normally present CRIM forms were detected (Fig. 4, see legend). Previous kinetic investigations had classified these Type 1 GD patients as Group A. Importantly, the polyclonal or monoclonal antibody preparations resulted in slightly different CRIM patterns in this group of patients. In Figures 4B and C (lane 4), the fibroblast extract from an African Black Type 1 GD patient had one CRIM form ($M_r \cong$ 63,000) detectable with polyclonal IgG and two CRIM forms ($M_r \cong$ 67,000 and 63,000) detected with monoclonal antibody 61. Also, as shown in Figure 4B and C (lane 5) a very faintly detected CRIM form with $M_r \cong$ 63,000 was found

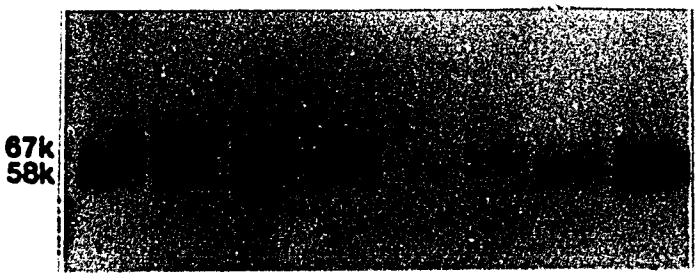
in fibroblast extracts from a Cape Colored Type 1 GD patient even though these lanes contained four times the amount of protein that had been applied from normal fibroblast extracts.

Intrafamilial variability of the CRIM patterns was observed with fibroblasts from several neuronopathic patients. Figure 5A (lane 3) shows a typical example of the CRIM pattern observed in seven of the Types 2 or 3 GD patients with a single discrete band detected at $M_r \cong$ 67,000. Two unrelated Type 2 GD patients had one CRIM form at $M_r \cong$ 62,000 in their fibroblast extracts (Fig. 5B, lane 1). Cell line GM2627, classified as an atypical Type 2 GD patient (46) and one Type 3 GD patient had two CRIM forms at $M_r \cong$ 67,000 and 63,000. Unlike all other fibroblast extracts from neuronopathic patients, which required more than 10 times the amount of cell protein to produce equally dense CRIM bands, only twice the amount of protein was required to obtain an intense CRIM signal from GM 2627 (data not shown). This observation confirms that of Beutler et al. (46) who found 50-82% of normal amounts of CRIM in this "atypical" Type 2 GD fibroblast line. Fibroblasts from one American Black Type 2 GD patient had no detectable CRIM with up to 200 µg of cell protein applied to the gels (data not shown).

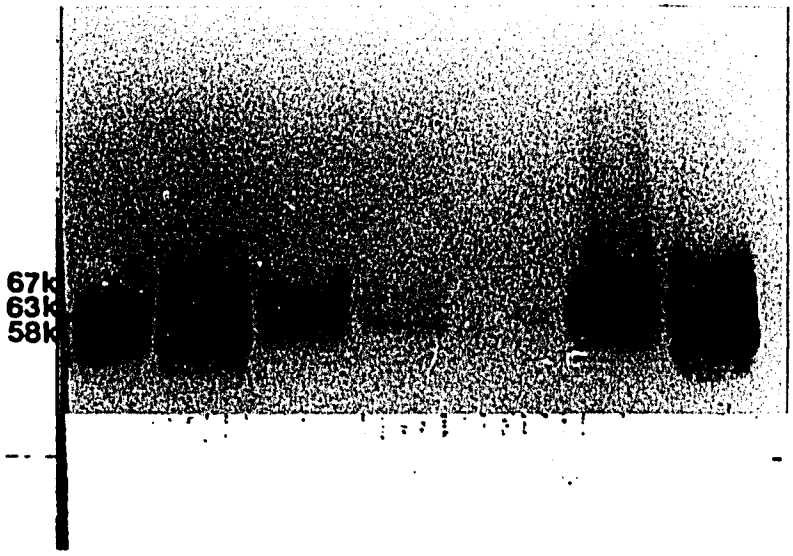
As shown in Figures 4 and 5, when equal amounts of normal and GD fibroblast protein were applied to SDS-PAGE, GD cell lines typically had a visually less intense immunoblotting signal. Densitometric scanning of these immunoblots, standardized to increasing amounts of normal fibroblast protein, provided an estimate of the relative amount of the CRIM in GD cells. Assuming antigenic identity of GD β-Glc and the normal enzyme, the amount of CRIM in two Group B Type 1 patients, a non-Jewish Group A Type 1 patient, and one Type 2 GD (GM 1260) patient were

Figure 4: Immunoblot of fibroblast sonicates from normal individuals and Type 1 GD patients using anti-β-Glc polyclonal (A and B) or MCAb 61 (C). Normals are in Lane 8 (A; 40 µg protein) and Lane 7 (B and C: 15 µg protein). Type 1 GD which were representative of Group B are in Lanes 1-4 (A) and Lanes 1, 2 and 6 (B and C). The classic Type 1 GD Group B is the Type 1 AJGD. Type 1 GD which were representative of Group A are in Lanes 5 and 7 (A) and Lanes 3-5 (B and C). Lane 6 (A) was from a Type 3 patient of AJ/Irish descent. All GD samples had 80 µg of fibroblast protein.

A



B



C

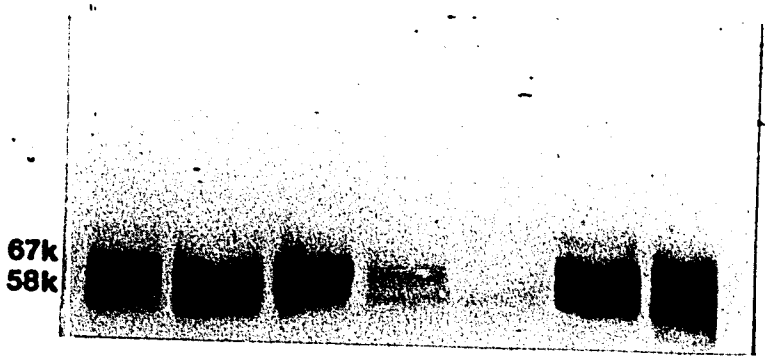
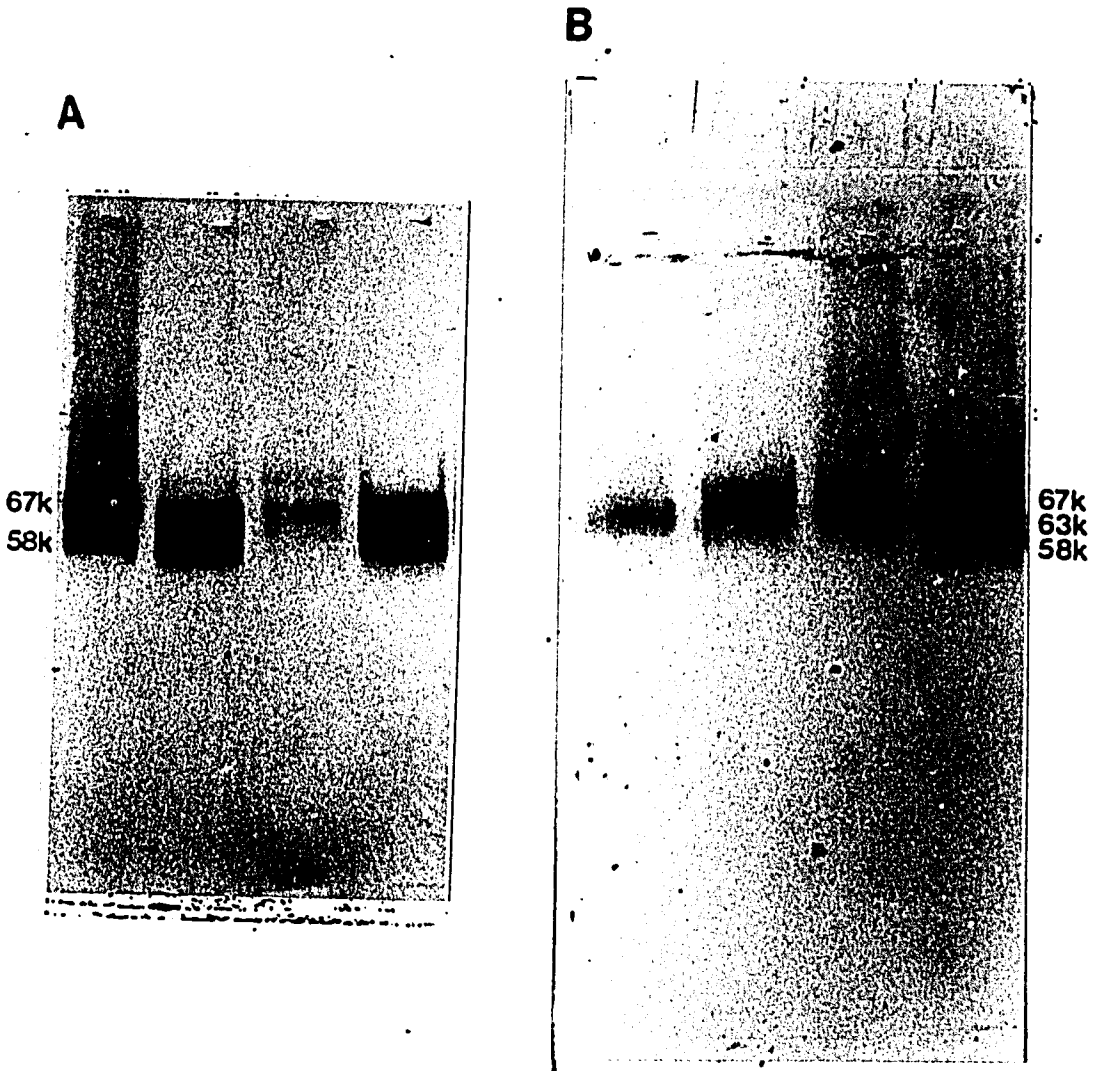


Figure 5: Immunoblots of normal and Group A Types 1 and 2 GD fibroblast sonicates using anti-β-Glc polyclonal antibody. Lane 1 and 2 in A contain 18 and 88 µg protein from normal fibroblasts, respectively. Lane 3 in A contains 83 µg protein from Type 2 GD fibroblasts (GM 877). Lane 4 in A contains 80 µg protein from a Group B Type 1 GD patient. In B, Lane 4 contains 15 µg protein from normal fibroblasts and Lanes 2 and 3 contain 132 µg and 108 µg protein from two different Group A Type 1 GD (non-Jewish) patients' fibroblasts. Lane 1 in B contains 110 µg protein from a Type 2 GD fibroblast line (GM 1260). It should be noted that the Type 2 cell extracts in A and B are from different patients.

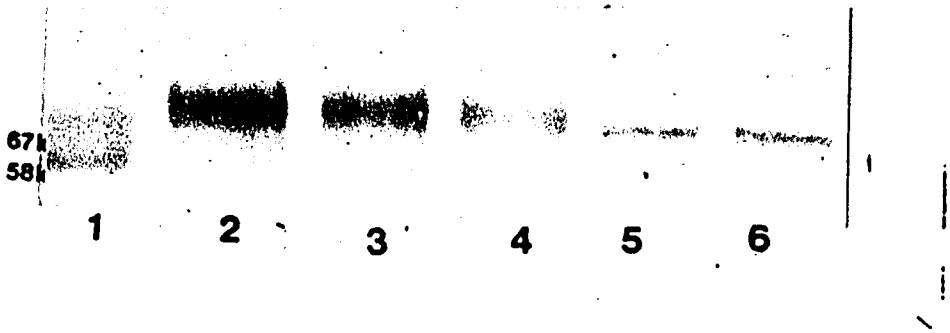

A
67k
58k
B
67k
63k
58k

estimated to be 10.5% (range 9-12%), 3% and 1.5% of the normal level, respectively. In general, for a fixed amount of fibroblast protein the neuronopathic forms of GD resulted in the weakest immunoblotting signals and thus had the lowest amounts of detectable CRIM.

The CRIM detected with monoclonal antibody 61 in the immune absorbed (polyclonal antibody) cell culture media from normal and AJGD fibroblasts had slightly greater M_r than that observed in the respective cell sonicates (Fig. 6). In the media from normal and Type 1 AJGD (Group B) cells, the predominant CRIM form had a M_r about 2000 greater than the highest M_r form (67,000) detected in fibroblast sonicates. The CRIM from the media of Type 2 GD (GM 1260) cells also had a greater $M_r \cong$ 67,000 as acompared to the CRIM form detected in the cells($M_r \cong$ 63,000, Fig. 6) when equal volumes of media were exhaustively immunoabsorbed on the polyclonal column and the eluted CRIM subjected to immunoblotting, the intensity of the CRIM signal was less than normal and greatly decreased in media from Type 1 AJGD cells and Type 2 GD (GM 1260) cells, respectively. These results are consistent with the relative amounts of CRIM detected in the respective cell sonicates.

The assumption that the various immunodetected forms of CRIM were actually β-Glc was supported by the production of similar immunoblot patterns using either polyclonal antisera or any of three monoclonal antibodies, which have been shown to recognize different epitopes of β-Glc and one of which inhibits β-Glc activity (Sect. V.D.). In addition, after labeling normal or Type 1 AJGD fibroblast sonicates with [^{3}H]Br-CBE, a covalent inhibitor specific for β-Glc, radioactivity was

Figure 6: Immunoblot of CRIM from fibroblast culture media which had been adsorbed to anti-β-Glc IgG-Sepharose and detected with MCAb 61. Lane 1 contains a normal fibroblast sonicate (40 µg protein). Lanes 2 and 3 were from 50 and 10 ml of culture media from normal fibroblasts, respectively. Lane 4 was from 50 ml of culture media from Type 1 AJGD fibroblasts. Lanes 5 and 6 were from 50 and 100 ml, respectively, of culture media from Type 2 GD fibroblasts (GM 1260).


67k
58k
1
2
3
4
5
6

recovered in the various CRIM forms when immunodetected bands were cut and counted (Figure 7). A similar experiment with Type 2 GD cells was performed but the recovered radioactivity was barely above background.

3. N-Glycanase™ Digestion: To investigate the origin of the molecular forms of CRIM and the size of the β-Glc protein backbone, N-Glycanase™ was used to remove N-linked carbohydrate moieties from the glycoproteins in normal and GD fibroblast lysates. N-Glycanase™ digestion of two normal, two Type 1 AJGD and two Type 2 GD fibroblast lysates (GM 2627, GM 877) reduced the various CRIM forms to one CRIM form of similar molecular weight: $M_r \cong 56,000$ (Fig. 8). The 72,000 molecular weight CRIM form, most easily detected in one of the two Type 2 GD cell lines investigated (GM 877), was resistant to N-Glycanase™ digestion and no change in electrophoretic mobility was apparent. N-Glycanase™ digestion of immunoabsorbed β-Glc from culture media of normal and Type 1 AJGD fibroblasts also resulted in a single $M_r \sim$ 56,000 CRIM form (data not shown).

4. Discussion: The immunoblotting studies reported here provide evidence for genetic heterogeneity within and between the subtypes and variants of GD and indicate the origin of the several molecular weight forms of β-Glc in the cultured fibroblasts. During the course of these studies, a controversy has developed as to the number of β-Glc molecular weight forms present in cultured fibroblasts (26,27,29). Consequently, several different approaches were used to provide support for ascribing the different molecular weight forms of CRIM to β-Glc. The enzyme used for the production of polyclonal antisera and the screening of the antibody producing hybridomas was

Figure 7: Incorporation of [^{3}H]Br-CBE in immunodetected CRIM forms of normal and Type 1 AJGD fibroblasts. 40-50 µg of labelled fibroblast protein were subjected to SDS-PAGE and immunoblotting. Immunodetected CRIM forms were cut out of the nitrocellulose and radioactivity was determined. Radioactivity is normalized per mg of fibroblast protein and the bars refer to the respective M_r areas cut from the nitrocellulose filter.

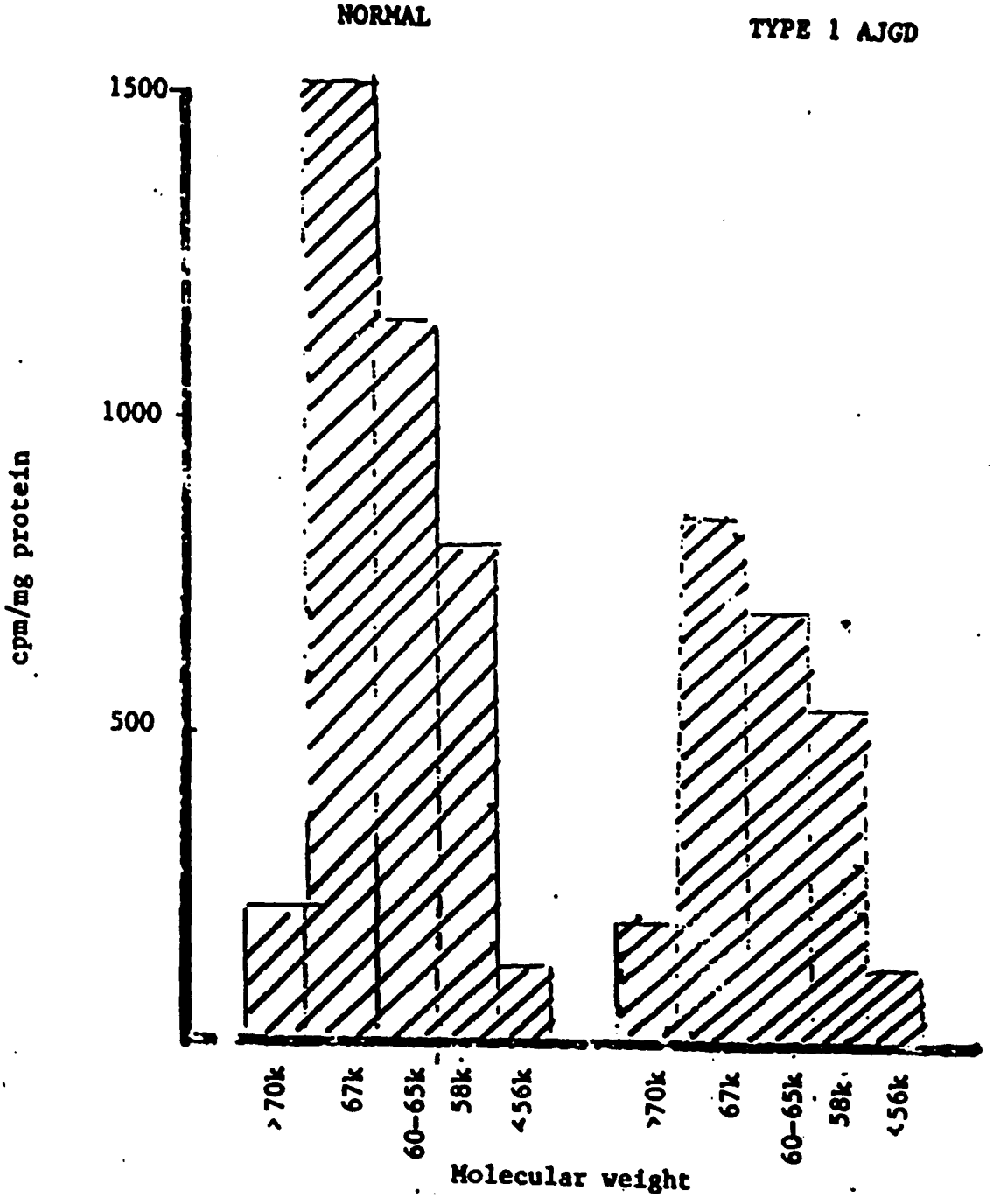
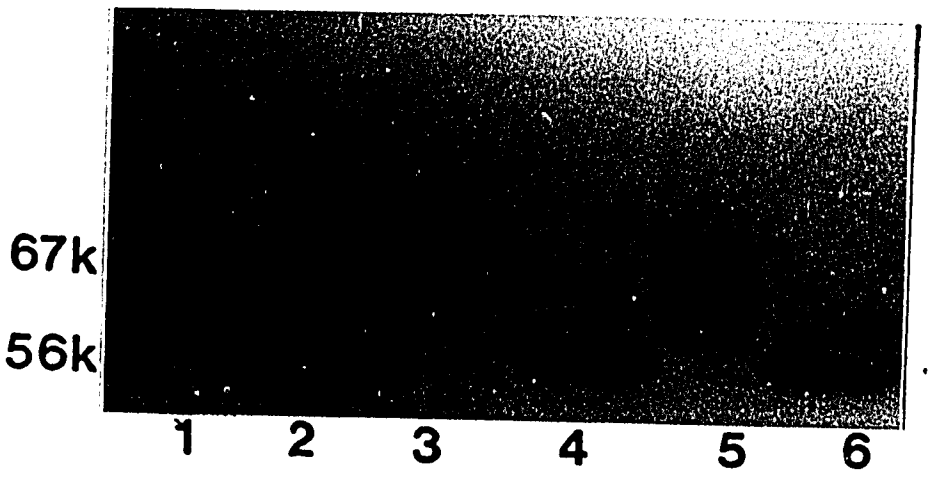

NORMAL
TYPE 1 AJGD
1500
1000
500
cpm/mg protein
>70k
67k
60-65k
58k
<56k
>70k
67k
60-65k
58k
<56k
Molecular weight

Figure 8: Immunoblots of N-glycanase-treated fibroblast sonicates from normal or GD variants. Prior to N-glycanase treatment of normal, Type 2 GD (GM 2627), Type 1 (Group B) GD, are shown in Lanes 1, 3 and 5, respectively, and after treatment are in Lanes 2, 4 and 6, respectively. Each lane contains 80 µg of protein.


67k
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homogeneous, as demonstrated by SDS-PAGE, HPLC (reverse phase) and the presence of a single N-terminal amino acid sequence (22 residues), with exact colinearity to the hepatic β-Glc cDNAs (23,24). The CRIM patterns detected with polyclonal antibody were reproducible with several, monoclonal antibodies to β-Glc, in particular, the monoclonal antibody 61 which has been shown to inhibit β-Glc activity (Section V.D.). The patterns of CRIM molecular weight forms varied with the subtype or variant of GD, indicating that non-specific (non β-Glc) constitutive proteins were not being detected. Also, the immunoblots from cells of a patient with no detectible enzymatic activity had no immunoblotting signal using either polyclonal or monoclonal antibodies, indicating that non-β-Glc protein even on overloaded gels was not being detected. Furthermore, all the CRIM forms detected on immunoblots were reduced to a single CRIM form $M_r \cong 56,000$ after complete deglycosylation with N-Glycanase™. No other bands were observed. Finally, [^{3}H]Br-CBE, a specific covalent inhibitor of β-Glc, was used to localize β-Glc in the immunoblots: only the parts of the nitrocellulose membrane corresponding to CRIM contained radioactivity. Based on these results, we conclude that the CRIM forms identified in these studies represent β-Glc.

The differences in the CRIM patterns within and between the subtypes and variants of GD suggested that the allelic variants of β-Glc resulted in differential effects on the post-translational processing and/or stability of the mutant enzymes. Ginns et. al. (26,27) previously proposed a unique processing defect in the neuronopathic subtypes of GD to account for the single CRIM form detected in Type 2 and 3 GD fibroblasts. Our studies confirm these findings using the same cell lines but indicate that the presence of a $M_r \cong 63,000$ CRIM form was not character-

istic of all Type 2 GD patients nor was it unique to the neuronopathic variants. With fibroblast cultures from different variants of non-Jewish Type 1 GD, single CRIM forms ($M_r \cong$ 59,000-67,000) were also observed. Since deglycosylation resulted in one CRIM form of $M_r \cong$ 56,000 which corresponded to the predicted molecular weight of the mature, normal β-Glc polypeptide chain, the CRIM forms in the GD subtypes and variants investigated in this report likely arose from differential posttranslational glycosylation. This conclusion and the lower level of CRIM (assuming antigenic identity with the normal enzyme) detected in GD fibroblasts and in the cell culture media suggest that several different amino acid substitutions (or deletions which do not dramatically alter the size of the protein backbone) give rise to β-Glcs which are unstable and/or are poor substrates for processing enzymes.

Although the effect on the trafficking of the abnormally glycosylated mutant enzymes in some Type 2 GD cell lines is uknown, it is possible in these variants of GD that β-Glc remains extra lysosomal. If this speculation is correct, in some variants of GD, the level of β-Glc in lysosomes may be negligible and the level of β-Glc determined by standard assays may not reflect the amount of enzymatic activity available in vivo for GC (or GS) hydrolysis. Furthermore, since the levels of other lysosomal enzymes are normal in GD, and GC (and GS) are the only accumulated compounds in GD tissues and the fact that β-Glc has nearly normal intracellular levels in I-cell disease (91), suggest that β-Glc may have a unique processing pathway and/or signals for trafficking to the lysosome. Indeed, the detection of an N-Glycanase™ resistant β-Glc form with $M_r \cong$ 72,000 in some other Type 2 GD fibroblast lines suggests the persistence of a precursor form of β-Glc containing a

signal (leader) peptide sequence, at least transiently, during processing of the normal enzyme (87). Such precursors have been detected recently in pulse chase studies in normal fibroblasts (87), although the M_r values for the β-Glc forms differ from those reported here and by Ginns et al. (26,27).

Several observations from these studies are of diagnostic importance. Among the Type 1 AJGD or other Type 1 GD patients classified as Group B, the normal CRIM forms were detected in all fibroblast extracts. No neuronopathic patient's fibroblasts were discovered to have this CRIM pattern. However, several non-Jewish Type 1 (Group A) cell lysates had one or two CRIM forms which could be confused with the pattern in the Type 2 cell line [GM 1260 (M_r = 63,000)]. Also, the fibroblasts from one neuronopathic patient of Ashkenazi Jewish/Irish ancestry had a CRIM profile which was essentially identical to that from a Type 1 (Group C) Afrikaner patient. Thus, the diagnostic usefulness of the immunoblotting technique may be limited to families where the phenotype of GD is well established. The heterogeneity of the CRIM patterns within and between the subtypes and variants of GD makes prediction of the GD phenotype on the basis of CRIM pattern uncertain.

D. Characterization and Use of MCAbs to Study the Structure and Kinetic Properties of Normal and GD β-Glc:

1. Characterization of Monoclonal Antibodies (MCAb): Three fusions were conducted which yielded eighteen stable anti-β-Glc producing hybridomas (of 112 total). The isotypes of these antibodies are shown in Table V, columns 2 and 3. Several criteria (Tables V,VI) were used to determine the epitope specificity of these anti-β-Glc MCAbs. Three groups of epitope specificities were initially identified by differences in the maximal absorbance (ELISA) of antibody bound to β-Glc which had been immobilized at pH 7.3 [β-Glc (7.3)] or 9.6 [β-Glc (9.6)] (Table V, col.4). With seven MCAbs, slightly greater binding was observed with β-Glc (7.3) compared to β-Glc (9.6) and were named Group 1, whereas six MCAbs had much greater binding to β-Glc (7.3) (Group 2) and five MCAbs bound preferentially to β-Glc (9.6) and/or were IgM (Group 3). The specificities of the MCAbs in these three groups were further defined by AI values (Table VI) for each possible pair of MCAbs. For these studies an arbitrary AI, $\geq$30, was used to indicate binding to different epitopes; inconsistent results were not used for defining the epitope specificities.

Within Group 1, three epitope specificities were defined by MCAb 4, MCAb 74, or MCAbs 9, 38, 61, 65 and 37. The latter five MCAbs gave consistently low AI values when cross-tested with each other. Within Group 2, MCAb pairs 32 and 42 or 77 and 122, defined two additional epitopes, whereas MCAbs 90 and 20 gave inconsistent results. In parallel studies, conducted with β-Glc (9.6), a similar segregation of epitopes

TABLE V

Antigen Binding Properties of Anti-β-Glc MCAbs

MCAb No.	Isotype		Abs[β-Glc(7.3)] / Abs[β-GLc(9.6)]	Effect of Temperature		Cross-activity with Canine β-GLc	Detection Limit[a]	Percent Inhibition[b]
	Light	Heavy		β-Glc(7.3)	β-GLc(9.6)			
Group 1[c]							(ng)	
4	K	G_1	1.8	22 > 37 > 4	nd[d]	56	0	16
74	K	M	1.6	37 = 22 > 4	nd	8	50	0
9	K	G_1	1.2	22 > 37 > 4	nd	0	0.5	0
38	K	G_1	1.3	22 > 37 = 4	37 > 22 > 4	8	0.1	48
61	K	G_1	1.0	22 = 4 = 37	37 > 22 > 4	10	0.1	64
65	K	G_1	1.4	22 > 37 = 4	4 = 22 > 37	24	10	0
37	λ	G_1	1.5	22 > 37 > 4	-	42	50[e]	-13
Group 2								
32	K	G_{2A}	6.0	22 = 4 > 37	4 = 22 > 37	72	0	29
42	K	G_1	7.5	22 = 4 > 37	4 > 22 = 37	75	0	19
90	K	G_1	17.0	4 > 22 > 37	4 > 22 = 37	74	10	19
20	K	G_1	13.0	22 > 4 > 37	4 > 37 = 22	78	50	24
77	λ	G_1	9.0	22 > 37 > 4	nd	45	50	3
122	λ	G_1	4.0	22 > 37 > 4	4 > 22 = 37	54	10	-12
Group 3								
1	K	G_2	1.0	22 > 4 = 37	nd	40	0.5	0
123	K	M	0.9	22 > 4 > 37	37 > 4 > 22	59	10	0
F-1	K	M	1.2	33 > 22 = 4	37 > 22 = 4	70	10	30
F-2	K	M	2.6	22 > 37 = 4	37 > 22 = 4	60	10	41
59	K	M	0.7	37 > 22 > 4	4 > 22 = 37	>50	Stained Background	0

[a]Limit of detection on dot blots with native β-Glc.

[b]Percent inhibition compared to control; negative number indicates activation.

[c]Group was assigned based on the ratios of absorbances for β-Glc (7.3) and β-Glc (9.6).

[e]MCAb 37 was the only antibody that had greater sensitivity for the detection of SDS-denatured β-Glc.

[d]nd indicates that no difference in absorbance readings were observed at different temperatures.

TABLE VI
Additivity Indices for Various MCAbs[a]

MCAb No.	4	74	9	38	61	65	37	32	42	77	122	90	20	1	123	F-1	F-2	59
4	-																	
74	90[b]	-																
9	39	59	-															
38	79	36	16	-			GROUP 1											
61	33	64	12	18	-													
65	35	65	3	7	13	-												
37	38	78	8	23	33	15	-											
32	23	48	66	64	78	93	80	-										
42	19	76	33	23	35	43	33	0	-									
77	40	71	8	23	37	34	01	70	26	-			GROUP 2					
122	42	95	29	42	50	26	09	68	29	08	-							
90	32	112	21	43	40	32	38	22	32	10	08	-						
20	35	162	69	78	86	79	87	10	06	33	55	07	-					
1	33	73	52	51	60	66	76	39	38	62	79	73	97	-				
123	67	113	63	61	51	42	57	87	41	41	85	24	0	63	-			
F-1	41	0	26	16	41	18	16	25	23	18	11	0	18	85	46	-		GROUP 3
F-2	48	6	52	11	48	14	26	70	20	07	10	24	19	79	135	35	-	
59	17	45	51	57	57	69	45	26	16	51	67	53	12	58	52	67	132	-

[a] β-Glc (7.3) was used; data for β-Glc (9.6) not shown.

[b] The numbers are the additivity indices: AI values < 30 were chosen to indicate similar specificity.

based on AI indices were obtained (data not shown). The inconsistent results observed with some MCAbs were interpreted as suggesting either steric hindrance or conformational interactions with the MCAbs (see below). Within Group 3, the five MCAbs appeared to recognize distinct epitopes. However, cross-checking the F-1 and F-2 (IgM) MCAbs with those in Groups 1 and 2 resulted in inconsistent assignment of epitope specificities. Using Groups 1, 2 and 3, defined above, we interpreted the results to indicate that: 1) MCAbs 1, 4, 74, 59, and 123 recognize five unique epitope specificities; 2) MCAbs 9, 38, 61, 65 and 37 bind either to a single epitope or overlapping epitopes and 3) three MCAb pairs, 32 and 42, 77 and 122, and F-1 and F-2, each recognized a single or contiguous epitope. Kinetic studies (see below) indicate that in solution, MCAbs F-1 and F-2 bind to different epitopes. The epitope specificity of MCAb 122 was distinguished from that of MCAb 77 by the marked decrease in thermostability of β-Glc activity induced by the binding of MCAb 122 to the enzyme (data not shown).

The effect of temperature (37°C, 22°C, and 4°C) on MCAb binding to β-Glc (7.3) and β-Glc (9.6) provided additional delineation of epitope specificity. A difference in binding was assigned only with reproducible ELISA absorbance changes of greater than 10% at each temperature. Since these studies were conducted with saturating amounts of antibodies, the differences in absorbance were likely due to conformational changes of β-Glc and not altered diffusion rates of the MCAb. This suggestion also was supported by the improved binding of several MCAbs to β-Glc at 4°C. For most MCAbs, binding to β-Glc (7.3) was best at 22°C. However, MCAbs 74, 59 and F-1 had increased binding at 37°C and the lowest binding at 4°C. These results provide support for the different epitope specific-

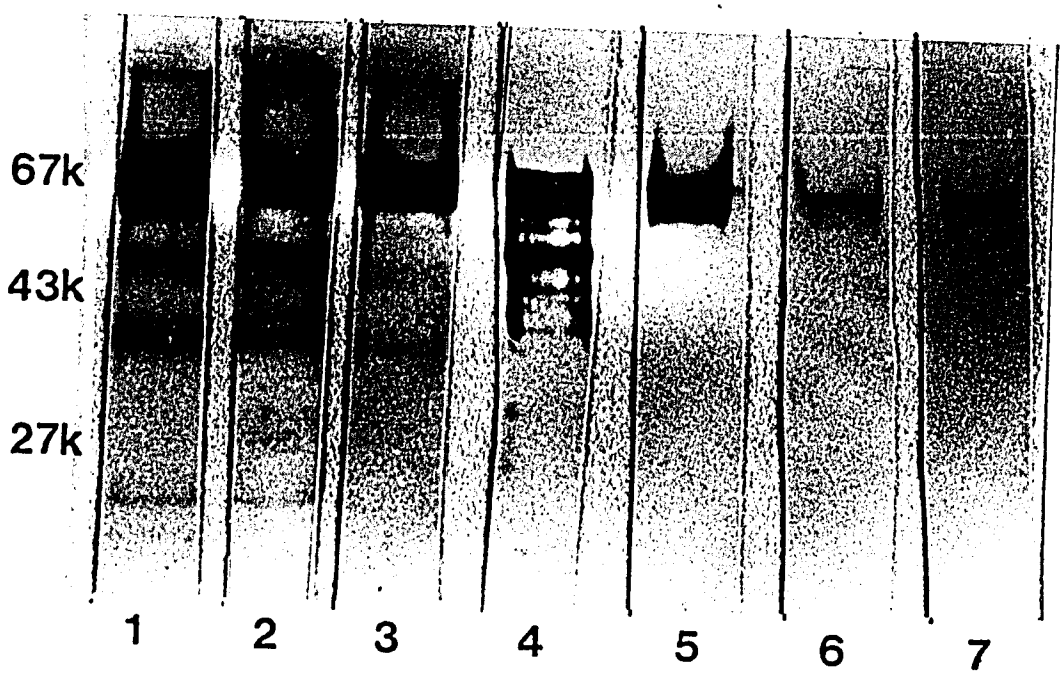
ities of MCAbs 74 and 59 and suggest that F-1 had a unique epitope specificity. Among the Group 1 MCAbs which were thought to have similar epitope specificity as determined by AI indicies (i.e., 9, 37, 38, 61, 65) only 61 and 38 had similar temperature dependence of binding to β-Glc. These results suggested at least two additional epitope specificities in Group 1. Using the temperature dependence criteria, Group 2 MCAbs, 32, 42 and 20, defined one epitope and MCAb 90 another.

The binding of MCAbs to partially purified canine splenic β-Glc immobilized to ELISA plates at pH 7.3 or 9.6 (Table V, col.7), and the detection sensitivity of native, or reduced and denatured human β-Glc on dot blots (Table V, col.8) suggested subtle distinctions between epitopes. For example, MCAbs 38 and 61 gave concordant results with these tests but the results with MCAbs 9, 65 and 37 were discordant suggesting different epitope specificities. The specificities of these five MCAbs also were distinct as determined by the CRIM patterns with electroblotted peptides from partial V-8 digests of β-Glc. MCAbs 38 and 61 detected identical CRIM patterns while MCAbs 37 and 65 detected a different pattern of peptides which were a subset of those recognized by MCAb 9 (Fig. 9).

The final criteria for epitope specificity was the ability of MCAbs to alter human β-Glc activity in solution. Four MCAbs (38, 61, F-1 and F-2) reproducibly inhibited β-Glc hydrolysis of 4MU-Glc or NBD-C12-GC. Importantly, 38 and 61 recognized the same epitope, whereas, F-1 and F-2 each defined a unique epitope specificity (see below).

Using all the criteria described above, a total of 14 different patterns of antigen binding, i.e., epitope specificities, were assigned from these eighteen MCAbs.

Figure 9: Recognition of peptides in the same protease digest of pure β-Glc using immunoblots with different MCAbs. MCAbs 38, 9 37, 1, 65, 90, and 122 were used for immunodetection in Lanes 1-7, respectively.


67k
43k
27k
1
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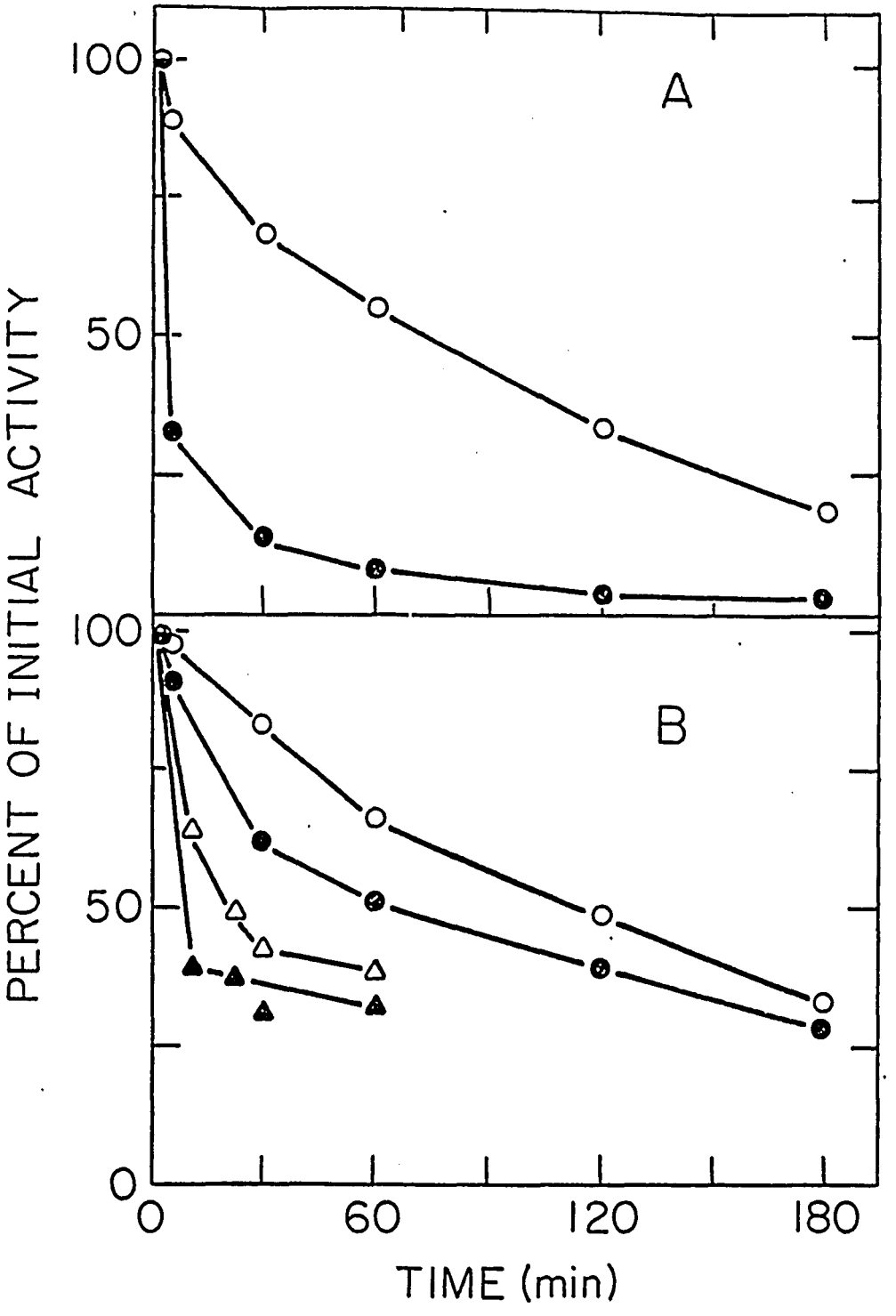
6
7

2. Interaction of β-Glc with Inhibitory MCAbs: The interactions of β-Glc, enzyme modifiers and the inhibitory MCAbs 61, 38, F-1 and F-2 were evaluated to gain insight into the structure and function of the normal β-Glc active site. MCAbs F-1 and F-2 completely inhibited β-Glc activity after 2 h of incubation, whereas, MCAbs 38 and 61 were only partially inhibitory (Fig. 10). Ten-fold increased concentrations of MCAbs 38 or 61 as well as prolonged incubation times did not increase the degree of inhibition (65% inhibition). To determine whether the inhibition by MCAbs 38 and 61 was due to a partially active MCAb/β-Glc complex or to free β-Glc which was fully active in the presence of completely inactive MCAb/β-Glc complexes, the MCAb/β-Glc mixtures were treated with Staph A cells, after maximum inhibition had been obtained. All β-Glc activity was precipitated by Staph A cells, indicating a partially active MCAb/β-Glc complex (data not shown). Since identical results were obtained with MCAbs 61 and 38, all remaining studies were conducted with MCAb 61.

The observed rates of inhibition were altered by the amount of β-Glc activity or the amount of MCAb present in the incubation mixtures (Fig. 10). The $t_{1/2}$ for maximal inhibition of β-Glc by F-1 (6.1 µg/assay) was increased about 18-fold by a 10-fold increase in the amount of β-Glc activity in the incubation mixtures. In comparison, only a two-fold increase of the respective $t_{1/2}$ values was observed for F-2 (5.4 µg/assay) or 61 (1.0 µg/assay) under the above conditions. To determine whether the rates of inhibition of β-Glc were due to the rate of MCAb binding to the enzyme, β-Glc was immobilized on ELISA plates at pH 7.3 [β-Glc (7.3)] or by binding the enzyme to immobilized rabbit

Figure 10: The rate of inhibition of normal β-Glc activity using MCAb F-1 (A) or MCAbs F-2 (circles) and 61 (triangles) (B). Open symbols indicate 3.6 nmol/h of β-Glc activity; closed symbols indicate a 10-fold decreased level of enzyme activity, i.e., 0.36 nmol/h.


100
50
A
100
50
B
0
0
60
120
180
PERCENT OF INITIAL ACTIVITY
TIME (min)

polyclonal anti-β-Glc IgG [β-Glc (IgG)], a noninhibitory antibody preparation. Both β-Glc (7.3) and β-Glc (IgG) were catalytically active. With MCAb F-1, a maximum of 10% inhibition of enzyme activity was observed with β-Glc (7.3), whereas a direct correlation between the amount of F-1 bound and the degree of inhibition was obtained with β-Glc (IgG). This result suggests that the induced inhibition of β-Glc, but not the binding by F-1, resulted from a conformational change in the enzyme. Similarly, with F-2 and 61, the degree of enzyme inhibition was related to the amount of MCAb bound to β-Glc (IgG) (Fig. 11). At any level of β-Glc inhibition by the MCAbs (F-1, F-2, or 61), no further increase in the degree of enzyme inhibition was observed after the excess unbound MCAb was removed from the ELISA wells. These findings suggest that the inhibitory effects were immediate and not the result of a slow inductive (conformational) process.

3. Effects of Substrates and Effectors on Inhibition of Normal β-Glc by MCAbs: The interactions between substrates or modifiers of β-Glc activity on the inhibitory effects and binding of MCAbs 61, F-1 and F-2 were evaluated in solution and on ELISA plates. For these studies, substrate was added to the enzyme solution simultaneously with the MCAbs. Incubations of enzyme and MCAb in solution and in the presence of 4 mM 4MU-Glc or 0.3 mM NBD-GC, demonstrated that the MCAb inhibition of β-Glc activity was unaffected (MCAb 61), partially prevented (MCAb F-1) or completely prevented (MCAb F-2) by these substrates (Table VII). For these studies, substrate was added to the enzyme solution simultaneously with the MCAbs. Studies using the ELISA system [with β-Glc (IgG)] demonstrated that with MCAb F-1, the decreased

TABLE VII

Effect of Substrate on Inhibition of β-Glc Activity By Monoclonal Antibodies F-1, F-2 and 61

Monoclonal Antibody	Incubation	
	Without Substrate	With Substrate
F-1 - MCAb	4.28	4.67
+ MCAb	1.25	3.15
% Inhibition	71%	33%
F-2 - MCAb	4.16	4.92
+ MCAb	2.78	4.99
% Inhibition	33%	0
61 - MCAb	4.26	4.74
+ MCAb	1.74	1.82
% Inhibition	59%	61%

degree of inhibition in the presence of 4MU-Glc was accompanied by decreased MCAb binding. In comparison, the presence of 4MU-Glc had no effect on the binding of MCAb-61 (Fig. 11) or F-2 to β-Glc (IgG). However, 4MU-Glc did prevent the inhibition of enzymatic activity by F-2, but not by MCAb 61 (Fig. 11).

A series of β-Glc inhibitors and activators were tested for their effects on the induced inhibition by MCAbs F-1 and F-2 (Table VIII). These studies were conducted with MCAb, enzyme and enzyme modifiers in a preincubation mixture (2 h; 22°C), followed by a 20- to 100-fold dilution prior to assay with 4MU-Glc substrate. Thus, the effect of the modifier on antibody induced inhibition was being tested and is expressed as the percent change in degree of inhibition, i.e., percent inhibition by MCAb alone; minus the percent inhibition by MCAb in the presence of the effector. No compound increased the percent inhibition by the MCAb F-1. Substrates and most of the potent inhibitors of β-Glc, such as deoxynojirimycin (mixed inhibition) or glucose sphingosine (non-competitive inhibition), protected the enzyme from inhibition by MCAb F-1. Importantly, the potent (mixed type) inhibitors of β-Glc, castanospermine and nojirimycin, which are structurally similar to deoxynojirimycin, had little or no effect on the induced inhibition by F-1. Galactosyl sphingosine, which does not inhibit β-Glc activity, also had no effect on the ability of MCAb F-1 to inhibit β-Glc.

TC and PS, activators of β-Glc activity, partially protected the enzyme from inhibition by MCAb F-1. This degree of protection was related to the potency of the activators since greater concentrations of TC, a less potent activator, were required to achieve the same degree of

Figure 11: The effect of 4MU-Glc substrate on the binding of β-Glc (circles) and on the inhibition of β-Glc activity (squares) by MCAbs F-1 (A), F-2 (B) and 61 (C) as detected on ELISA. Open symbols denote incubations of MCAb and enzyme done in the absence of substrate; closed symbols (dashed lines) indicate incubations done in the presence of 4 mM 4MU-Glc. MCAbs F-1 and 61 were used as purified immunoglobulins. MCAb F-2 was used as heat-inactivated (50°C, 2 h), dialyzed (versus PBS) ascites fluid.

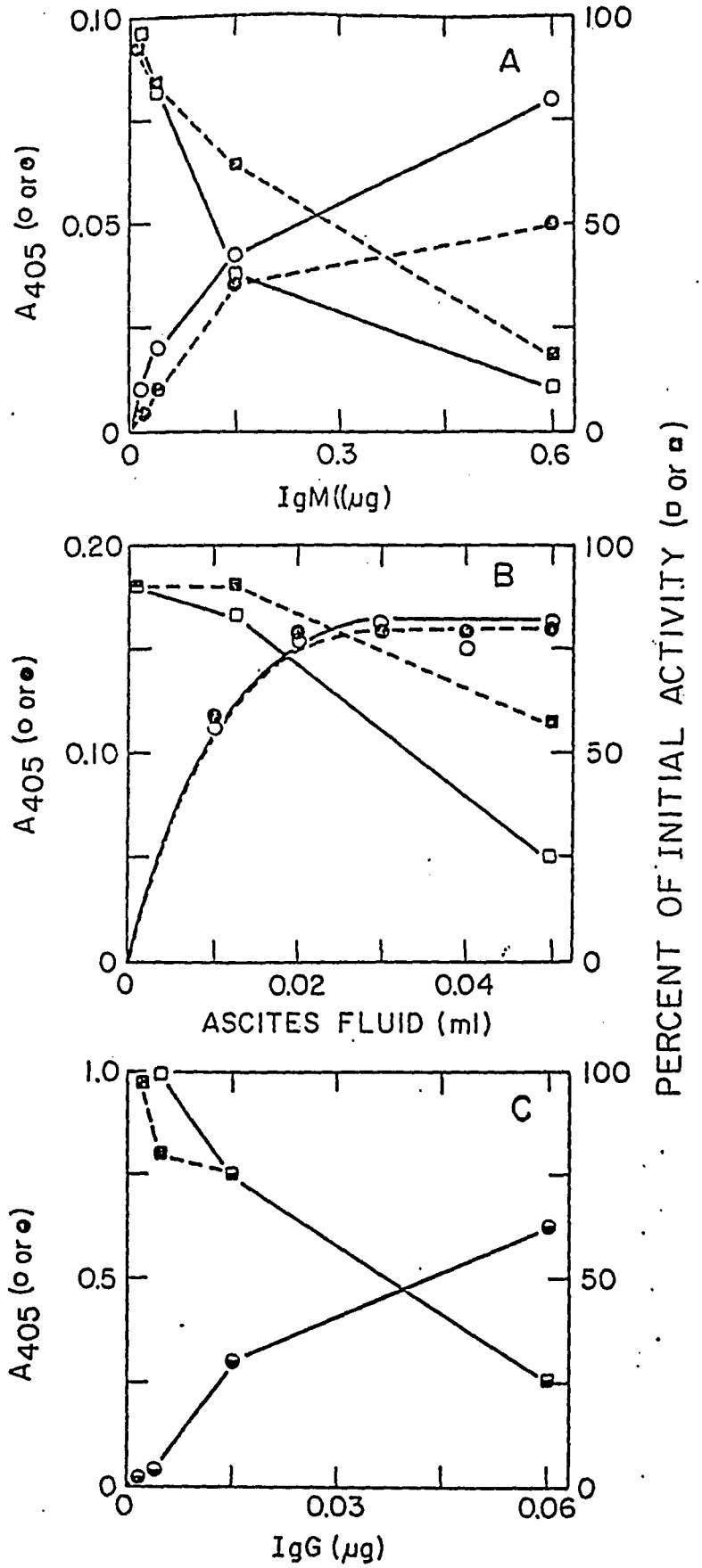

A
B
C
A405 (o or •)
PERCENT OF INITIAL ACTIVITY (o or □)
IgM((µg)
ASCITES FLUID (ml)
IgG (µg)
0.10
0.05
0
0.3
0.6
100
50
0.20
0.02
0.04
1.0
0.5
0.03
0.06

TABLE VIII

Effect of Inhibitors and Activators on the MCAb F-1 Induced Inhibition of Acid β-Glucosidase Activity

Compound (mM)	Effect on β-Glc	% Change in Inhibition by MCAb F-1[a]
4MU-Glc (4)[b]	Substrate	68
NBD-GC (0.3)	Substrate	62
Phosphatidylserine (1.6)	A[c]	25
Taurocholate (1.9)	A	5
" " " (4.65)	A	30
" " " (9.2)	A	40
Sphingosine (0.10)	C/NC[c]	4
" " " (0.53)	C/NC	39
Glucose Sphingosine (0.10)	NC[c]	67
N-Hexyl-Glucose Sphingosine (0.001)	NC	28
Galactose Sphingosine (0.10)	No Effect	0
Octyl β-Glucoside (1.0)	C/NC	0
Octyl α-Glucoside (1.0)	No Effect	0
1-Deoxynojirimycin (1.25)	C/NC	68
N-Dodecyl Deoxynojirimycin (0.00125)	C/NC	72
Nojirimycin (0.020)	C/NC	20
Castanospermine (0.040)	C/NC	-4
1-O-Methyl α-Glucoside (500)	C/NC	1
Triton X-100	N[c]	5
Human Serum Albumin	N	0
Ethylene Glycol	N	0

[a]All control samples were 65-78% inhibitory by MCAb F-1 in the absence of modifiers.

[b]All concentrations of β-Glc modifiers were equal to or slightly greater than I_{50} values (33,40,51).

[c]A, activator; C/NC, mixed competitive/non-competitive inhibitor; NC, noncompetitive; N, non-specific.

TABLE IX

Effect of MCAb 61 on the Kinetic Interactions of Normal and Type 1 AJGD β-Glc

	Normal β-Glc		AJGD Type 1 β-Glc	
	-MCAb61	+MCAb61	-MCAb61	+MCAb61
K_m (4MU-Glc)[a]	2.1 mM	2.0 mM	2.4 mM	2.4 mM
K_i GS	5 μM	10 μM	75 μM	230 μM
K_i N-Hexyl-GS	0.05 μM	0.12 μM	0.43 μM	0.60 μM
K_i Castanospermine	7.5 μM	10.9 μM	7.5 μM	6.9 μM
"Co-Glucosidase" Activation	3.8-fold	5.0-fold	2.0-fold	3.8-fold
TC Activation (.75%)	2.2-fold	4.8-fold	12.0-fold	14.0-fold
PS Activation (4 mM)	3.5-fold	5.0-fold	14.5-fold	7.5-fold

[a]All assays contained 0.5% Triton X-100. Binding of MCAb61 had similar effects on the K_i values for GS, N-Hexyl GS, and castanospermine when assays contained 0.5% Triton X-100 and 0.25%-0.5% TC.

protection as PS, a more potent activator. Triton X-100, human serum albumin and ethylene glycol, which interact with β-Glc nonspecifically, had no protective effects.

In contrast, although substrate protected normal β-Glc from inhibition by both F-1 and F-2, GS and, to a lesser extent, TC, which protected the enzyme from F-1 inhibition, had no effect or slightly enhanced the inhibition by MCAb F-2, respectively.

Due to the partial inhibition induced by MCAb-61 and the rapid rate at which this MCAb binds and inhibits β-Glc activity, kinetic analyses were conducted after maximal inhibition was obtained (Table IX). With the normal enzyme, the β-Glc activity in the MCAb-61/enzyme complex had unchanged K_m values for 4MU-Glc and K_i values for castanospermine. The K_i values for the noncompetitive inhibitors, GS and N-hexyl-GS, were slightly increased (1.25- to 2.5-fold) in the MCAb/enzyme complex. The normal enzyme when bound with MCAb-61 had a greater degree of activation by TC, PS, and "co-glucosidase", a naturally occuring activator protein. These results suggest that MCAb 61 induced a conformational change in β-Glc leading to a partially active enzyme with unchanged affinity for the substrate or competitive inhibitors. However, the effects of noncompetitive effectors, inhibitors or activators, were altered. Indeed, the effects of the negatively charged lipids or the protein activator partially restored the activity of the inhibited enzyme.

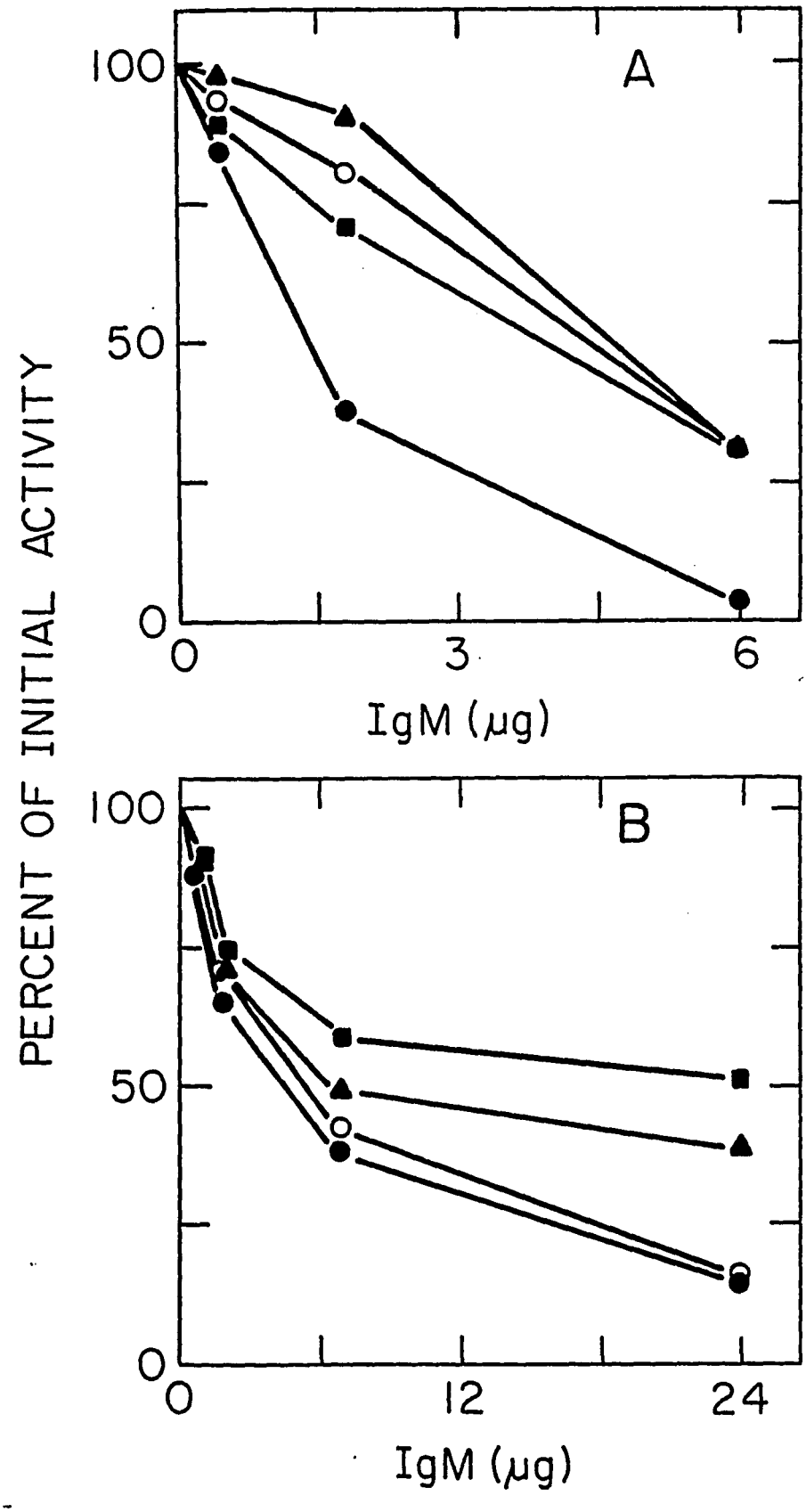
In order to ensure that the apparently increased sensitivity of the enzyme/MCAb complex to activation by TC, PS or the "co-glucosidase" was not due to the release of the antibody from β-Glc, enzyme and MCAb were incubated together until maximal inhibition was achieved. Then, 0.5% TC was added and incubated for 30 min at 37°C. The incubation mixtures were

then diluted 50-fold and assayed for β-Glc activity (with 0.5% Triton X-100). No change in the degree of inhibition was observed in the presence or absence of TC, as compared to a control sample containing the 50-fold diluted solution of MCAb 61 added directly to the β-Glc assay mixture. Similar results were found when the amount of MCAb bound to β-Glc was monitored by the ELISA method: no difference in the amount of MCAb 61 bound to β-Glc was observed in incubations of this MCAb in the presence or absence of 0.5% TC.

4. The effect of MCAbs F-1, F-2 and 61 on inhibition of GD β-Glc activity: When equal amounts of normal or GD β-Glc activity from fibroblasts were incubated with MCAbs F-1 or F-2, the rate of inhibition (data not shown) and the amount of MCAb required to inhibit the GD β-Glc activity by 50% (I_{50}) was increased 4-fold and 1.5- to 6-fold by F-1 and F-2, respectively, compared to that of the normal enzyme (Fig. 12). When the amount of normal β-Glc activity was increased by ten-fold, the I_{50} for F-2 was essentially unchanged. However, the I_{50} for F-1 inhibition of the normal enzyme was increased about 4-fold; this I_{50} value was comparable to that obtained with ten-fold lesser amounts of β-Glc activity from either the Type 1 or 2 GD fibroblast extracts.

The effects of enzyme modifiers on the inhibition of Type 1 AJGD β-Glc activity by F-1 were similar to the results obtained with the normal enzyme: i.e., substrates, GS, N-hexyl-GS and dNM protected the Type 1 AJGD enzyme from inhibition. Castanospermine and 1-O-methyl-α-D-glucoside, a more potent inhibitor of the Type 1 AJGD enzyme than the normal β-Glc activity had no effect on the ability of F-1 to inhibit the type 1 AJGD β-Glc. However, in comparison to the normal enzyme, TC had

Figure 12: Inhibition of equal amounts of β-Glc activity in normal (circles), Type 1 AJGD (■), and Type 2 (Δ) GD fibroblasts by MCAbs F-1 (A) and F-2 (B). Points designated by closed symbols represented an initial β-Glc activity of 2.3 nmol/h. The normal sample denoted by (0) had a 10-fold increased initial level of β-Glc activity, 23.0 nmol/h.

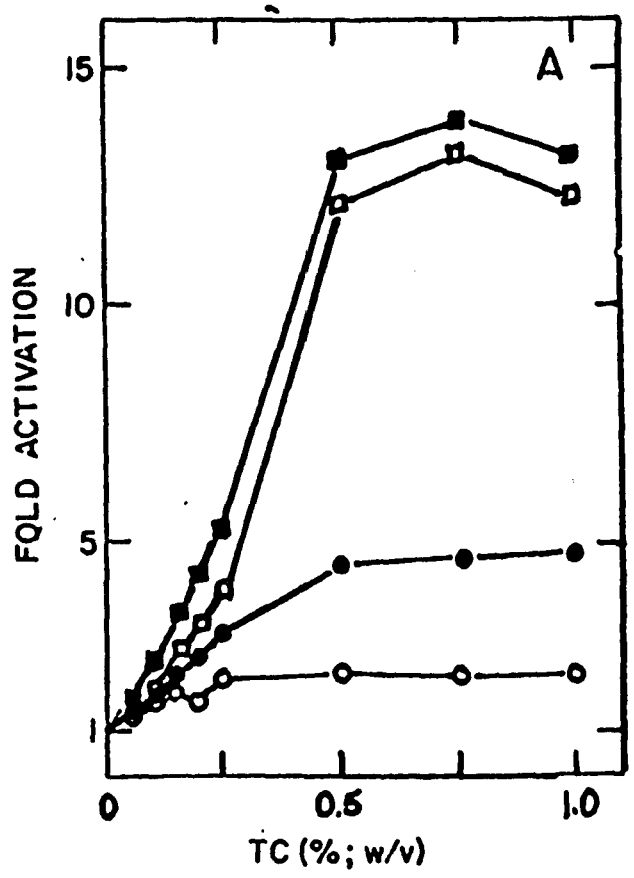

A
100
50
0
0
3
6
IgM (μg)
B
100
50
0
0
12
24
IgM (μg)
PERCENT OF INITIAL ACTIVITY

less protective effect on the Type 1 AJGD enzyme and 9.2 mM of TC protected the Type 1 AJGD β-Glc by only 20% as compared to 40% protection of equally inhibited normal β-Glc activity.

The effects of MCAb 61 on the properties of the Type 1 AJGD enzyme differed from those observed with the normal enzyme. Although the K_m for 4MU-Glc and the K_i for castanospermine were unchanged, the K_i values for GS and N-hexyl-GS were 1.5- to 4.5-fold increased in the MCAb 61/enzyme complex, compared to only 1.2- to 2.5-fold with the normal enzyme (Table IX). In addition, the degree of activation of the Type 1 AJGD enzyme in the antibody/enzyme complex was (Fig. 13) 1) unchanged by TC; 2) decreased (by 2-fold)by PS and 3) was increased (2-fold) by "co-glucosidase". These results suggest that the kinetic effects of TC, PS and "co-glucosidase" are mediated by different mechanisms.

5. Delineation of the Heterogeneity of GD Using MCAb 61: Since the normal β-Glc had increased activation by TC and the AJGD enzyme had unaltered activation by TC when bound to MCAb 61 (Fig. 13), the usefulness of this MCAb for discrimination of the subtypes and variants of GD was evaluated. For these studies, fibroblast extracts from five different normal individuals or various GD patients (21 patients) were incubated with or without MCAb 61 in the presence of 0.5% TC and then assayed for β-Glc activity in the presence of 0.25% Triton X-100 and 0.5% TC. Using 4MU-Glc or GC as substrates, the normal or Types 2 and 3 β-Glc (from seven different GD patients) were inhibited about 10-15% by MCAb 61 whereas the Type 1 AJGD enzyme (from ten different patients) was inhibited 60-70% by this antibody. This discrimination was apparent only under these experimental conditions and all enzymes were inhibited to

Figure 13: The effect of MCAb 61 on the TC (A) or PS (B) activation of normal (circles) and Type 1 AJGD (squares) β-Glc activity. Open symbols denote control assays done in the absence of the MCAb. Closed symbols indicate β-Glc which had been maximally inhibited by MCAb 61 prior to β-Glc assays in the presence of the indicated concentrations of TC or PS.


A
15
10
5
1
FOLD ACTIVATION
0
0.5
1.0
TC (%; w/v)

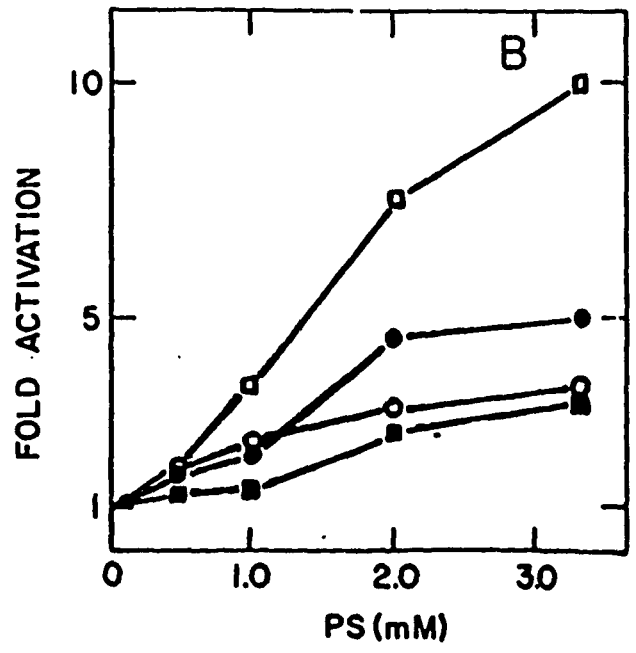
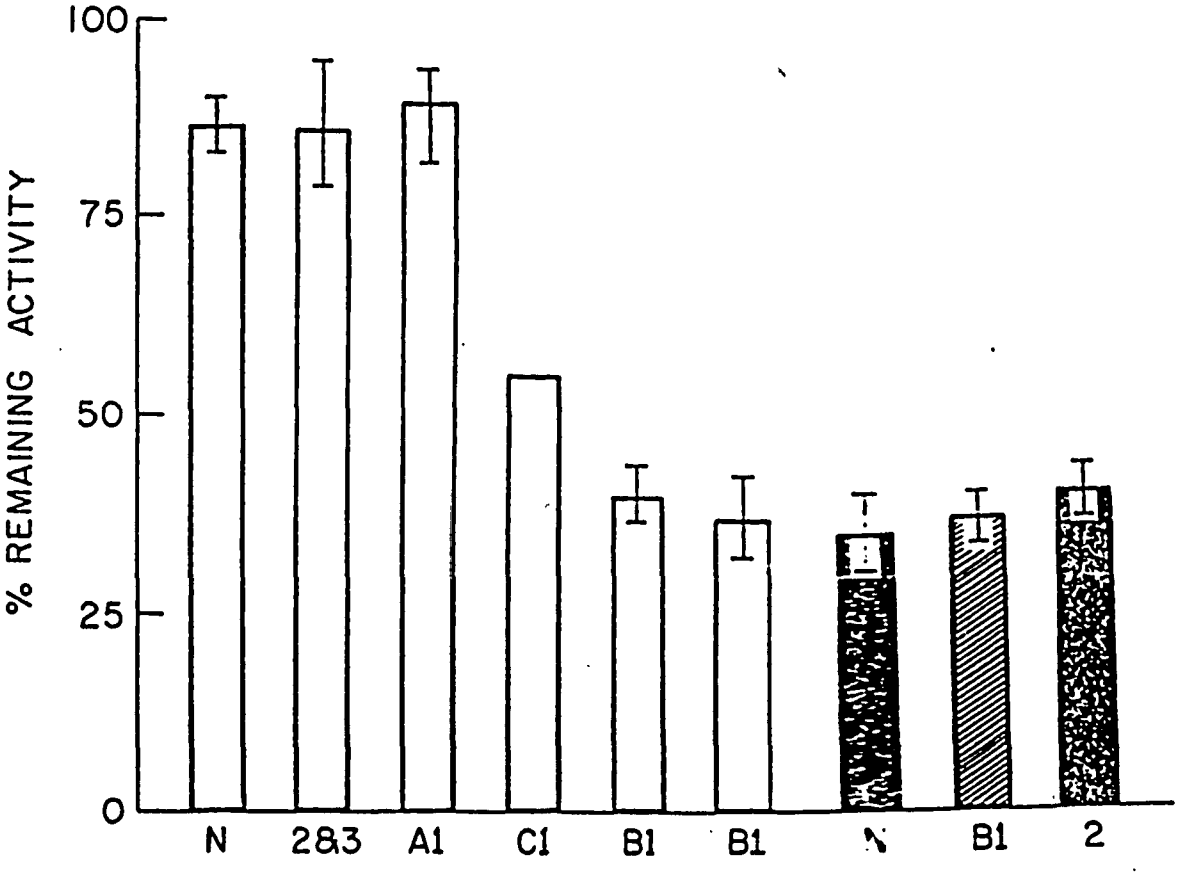

B
10
5
1
FOLD ACTIVATION
0
1.0
2.0
3.0
PS (mM)

Figure 14: The differential effects of MCAb 61 on the β-Glc activity from normal and GD variant fibroblasts. Fibroblast sonicates from normal individuals are designated as N and those from Type 2 or 3 GD patients, 2 or 3, respectively. β-Glc from Type 1 GD fibroblasts classified as Group A, B or C by physical and kinetic analyses are designated A1, B1 or C1, respectively. Shaded and cross-hatched bars indicate assays done with MCAb 61 in the absence of 0.5% TC.

DIFFERENTIAL EFFECTS OF F-61 ON ACID β-GLUCOSIDASE ACTIVITY FROM NORMAL AND GD VARIANT FIBROBLASTS



the same degree (60-70%) in the absence of TC. With increasing concentrations of TC from 0-0.5%, the differences in the degree of inhibition between the normal and the Type 1 AJGD β-Glc became increasingly greater (Fig. 13) while with concentrations of TC between 0.5-1.0%, the difference decreased. Similarily, Type 1 GD enzymes from ten non-Jewish patients, who had been shown previously to have either normal (Group A) or abnormal (Group B) responses to TC (and other modifiers of β-Glc activity) segregated with the normal and Types 2 and 3 enzymes (Group A) or the Type 1 AJGD (Group B) enzymes, respectively, using the inhibitory response to MCAb 61 (Fig. 14).

6. Discussion: In this communication, studies with MCAbs to human β-Glc have provided insight into the structure and function of the normal and GD β-Glc. In addition, one of these MCAbs (MCAb 61) was useful for the discrimination of allelic mutations of β-Glc which result in the subtypes and variants of GD. Several criteria were used to characterize eighteen MCAbs and to identify fourteen different antigen binding sites on the normal enzyme. Of these fourteen epitopes, three were associated with enzymatic inhibition. Although the number of identified epitopes was greater than classically predicted for a 56,000 kd protein (one epitope per 5000 Kd), recent studies have indicated that overlapping epitopes may cover the entire surface of proteins (96) and that many epitopes, interior to the protein, may be accessible to antibodies only if the protein is unfolded (77). The finding that MCAbs 37 and 59 preferred binding to denatured or inactive enzyme and MCAbs 61, 37, 9 and 65 could be distinguished by their recognition of different peptides in V-8 protease digests of β-Glc

indicates such inaccessibility of some epitopes on β-Glc. Furthermore, the identification of epitopes based on differential reactivity of MCAbs to β-Glc immobilized at pH 7.3 or 9.6, temperature dependence of binding, or binding of native vs SDS-denatured enzyme indicated the conformational dependence of certain epitopes. The conformational dynamics of epitope specificity were also evident from the differential rates of inactivation of β-Glc by equal concentrations of MCAbs F-1, F-2 and 61, suggesting that the slower binding MCAbs may recognize "buried" epitopes (77,96).

The functional consequences of the MCAb-enzyme interactions yielded information on the mechanism of β-Glc action and the structure of the β-Glc active site. The MCAbs F-1, F-2 and 61 inhibited β-Glc activity without precipitation and therefore must exert their inhibitory effects by direct binding to the enzyme and not via formation of extended immune complexes. This was further supported by the finding that MCAb 61, an IgG MCAb, retained its partial inhibition of enzyme activity in the Staph A precipitated complexes whereas F-1 and F-2 completely inhibited β-Glc activity in solution. Furthermore, the mechanisms by which these MCAbs inhibit β-Glc activity appear to be different (see below). This would not be expected if inhibition resulted only from immune-complex formation.

Three mechanisms could account for the inhibitory effects of the MCAbs: 1) the antibodies could bind to residues within or near the active site and block access of substrates to this site; 2) the antibodies could induce a conformational change or "freeze" the enzyme in a conformation, thereby preventing hydrolysis or 3) a combination of these two mechanisms. These three mechanisms were evaluated indirectly by

assessing the influence of substrates, inhibitors and activators on the extent of binding and the degree of enzyme inhibition by the MCAbs. With MCAb F-1, the 4MU-Glc or GC substrates prevented the MCAb binding as well as the resultant inhibition of the enzymatic activity. This apparent competition of F-1 and substrate for β-Glc may be due either to their binding to the same (or overlapping) site or to a substrate induced conformational change which renders the MCAb site inaccessible. Such substrate induced changes in protein conformation have been demonstrated with several protein kinases (95). Other modifiers of β-Glc activity, i.e., sphingosine and glucose or galactose sphingosine, had more limited effects on F-1 binding, apparently concealing or exposing F-1 binding sites, respectively. The presence of substrate had no affect on the binding of MCAbs F-2 and 61 to β-Glc, indicating binding sites different than that of F-1: i.e., ones which were accessible to the MCAbs when substrate was bound to the enzyme.

When the interaction of F-1 and enzyme was assessed by the ELISA system using β-Glc (7.3), which was partially active, MCAb F-1 had no inhibitory effect but binding of MCAb F-1 did occur. In comparison, when fully active β-Glc (IgG) was used, both the binding and the inhibition of the enzyme by F-1 were observed. These results indicate that a conformational change of the enzyme induced by the binding of F-1 was necessary for the resultant inhibition and immobilization of β-Glc (7.3) directly to the ELISA plate appeared to constrain the enzyme's ability to change conformation. However, the conformational change induced by F-1 must be different than that required for substrate hydrolysis, since β-Glc (7.3) and β-Glc (IgG) were enzymatically active.

The fact that GS, the deacylated analogue of GC, had equivocal effects on F-1 binding yet prevented MCAb inhibition, further supports the necessity of a conformational change for inhibition of β-Glc activity. Moreover, galactosyl sphingosine, which promoted the binding of F-1 to β-Glc as monitored by the ELISA system, had no influence on the inhibitory effects of F-1 in the solution system. This result supports the suggestion that GS exerted its protective effect by inducing a conformational change only upon binding β-Glc rather than by a nonspecific hydrophobic mechanism. Taken together, these results suggest that F-1 induces inhibition of β-Glc by mechanism three above, with competition between substrate and F-1 for binding and the induction of a conformational change which results in enzyme inhibition. This mechanism could be conceptualized by proposing that the recognition of only part of an epitope (linear sequence) is required for binding but that inhibition would result only by a conformational change induced by binding to the complete epitope which spans the active site cleft and blocks the active site. Binding of substrate or inhibitors or immobilization of β-Glc directly to ELISA plates would induce conformational effects which prevent recognition of the full epitope by the MCAb leading to MCAb binding but not active site blockade.

In comparison, using MCAb F-2, the inhibition of β-Glc but not the binding was prevented by substrates. In contrast to results with F-1, GS did not affect and TC slightly enhanced F-2 inhibition of β-Glc activity. Because of the different effects of GS or TC on the F-2 inhibition of β-Glc, these effectors were considered to induce conformational effects which differed from that obtained with substrate. The finding that F-2 bound more efficiently to enzyme immobilized in the

native state or through an IgG (polyclonal) bridge and recognized only undigested β-Glc after partial proteolysis by V-8 suggested that the epitope for recognition by this antibody was conformation dependent.

The partial inhibition of and the binding to the enzyme by MCAb 61 were unaffected by substrates or GS. These results suggest that the inhibition induced by this MCAbs was mediated primarily through conformational changes in the enzyme which stabilized the enzyme in a suboptimal conformation for catalytic activity. The fact that MCAb 61 bound similarly to denatured or native β-Glc immobilized by a variety of methods at pH 7.3 or pH 9.6, suggested that the epitope for this MCAb was in a contiguous sequence and that a particular conformation was not required for recognition. The recognition of low molecular weight peptides in V-8 protease digests of β-Glc also indicates the sequence dependency of this epitope. The rapid rate of MCAb 61 binding and inhibition suggest that this epitope is exposed on the surface of β-Glc (77).

Previously, a three domain model of the β-Glc active site was proposed to include: 1) the glycon binding domain for recognition of the polar head group of substrates and inhibitors, 2) the aglycon binding domain with specificity for the alkyl or acyl chains of inhibitors or substrates and 3) the third domain which recognized sphingosyl moieties and negatively charged lipids. These domains were proposed to function in the orientation and binding and hydrolysis of substrates. The present MCAb studies support and extend this model of the β-Glc active site.

The concordant effects of GS and, to a lesser extent, the negatively charged lipids (TC and PS) on the inhibition by MCAb F-1 suggested that these compounds may bind to the same domain in the active site and/or

induce a similar conformational change in β-Glc as has been previously proposed (33). The discordant effects of these compounds on the inhibition of the enzyme by F-2, i.e., GS had no effect and TC increased the degree of inhibition, indicated that these effectors may bind to different regions or overlapping areas on the enzyme. This conclusion was supported by the differential effects of MCAb 61 resulting in a decrease of the enzyme's affinity for GS and an increased activation by (perhaps increasing the affinity for) TC and PS.

Since Triton X-100 and human serum albumin, nonspecific activators of β-Glc, had no effect on the inhibition by any of these MCAbs, the results cannot be ascribed to "detergent enhancement" or nonspecific effects.

Using MCAb 61, the activation of the Type 1 AJGD β-Glc by the "co-glucosidase" was increased while the activation by TC or PS was not affected or was decreased, respectively. These results indicate that this naturally occurring protein effector bound to a different region or induced a different conformational change in the enzyme than the negatively charged lipids, TC or PS. This latter finding was implicit in the studies of Glew and co-workers (49,50) and Berent and Radin (61). Thus, it appears that β-Glc has several domains for the recognition of effector molecules and that GS, TC and PS seem to interact with separate or overlapping sites and not the same third active site domain as originally proposed (33). Due to the effect of substrate and GS in preventing F-1 from inhibiting β-Glc activity, substrate and GS bind the enzyme at the active site whereas TC and PS mediate their effect via two different domains. THese domains appear to induce an optimal conformation at the active site for substrate hydrolysis and the binding of

particular inhibitors (33). The same conclusion can be reached with "co-glucosidase" which has been previously proposed to "create" the active site of β-Glc (61).

Although substrate, GS, dNM, and C_{12}-dNM, seem to cause conformational changes which prohibit F-1 inhibition of β-Glc activity, an unexpected finding was the discordant effects of dNM and nojirimycin and, particularly, castanospermine, on the inhibition of β-Glc by F-1. This result was unexpected since these compounds are very close structural analogues and exert their potent inhibitory effect at the active site of the enzyme. However, these results are consistent with recent kinetic studies of the normal and Type 1 AJGD enzymes which indicated that the interaction of dNM with the β-Glc active site requires the electrostatic interaction of two residues (possibly histidyl) whereas the latter compounds require only one major residue (98, Forward VIII). Furthermore, the same kinetic studies suggested that one of the histidyl groups for dNM interaction participated in the stabilization of the binding of GS and sphingosine derivatives, via the NH_2 groups, but that this group was not primarily involved in the binding of castanospermine and nojirimycin. The concordant effects of dNM or GS on the inhibition of β-Glc by F-1 provide additional support for this conclusion. These results also suggest that a conformational change at the active site is required for the optimal alignment of critical residues for the binding of GS and dNM, but that this may not be required for the binding of castanospermine or nojirimycin.

In addition to providing insight into the organization of effector sites on β-Glc, the studies with MCAb 61 provided a unique method for the determination of allelic variants of GD. Under optimal conditions

the degree of MCAb 61 inhibition of β-Glc activity from normal and Type 1 AJGD β-Glc fibroblasts could be used to distinguish these enzymes. This discrimination was dependent on the concentration of TC or PS in the assay incubations and was primarily determined by the ability of MCAb 61 to increase the activation of the normal enzyme by these compounds and its lack of effect on TC activation or the prevention of activation by PS with the Type 1 AJGD enzymes. Interestingly, the Type 1 GD enzymes previously determined to belong to Group A, i.e., they had normal response to TC or PS and normal K_i values for GS, had normal inhibition by MCAb 61. Those non-Jewish Type 1 enzymes which belonged to Group B, i.e., with abnormal response to TC or PS and increased K_i values for GS, consistently had the 60-70% inhibition characteristic of the Type 1 AJGD enzyme. Importantly, the Types 2 and 3 enzymes (Group A) could be distinguished only from the Group B Type 1 GD enzymes. Thus, the residual enzymes cannot be unequivocally assigned by this or any current method to the neuronopathic (Type 2 and 3) or nonneuronopathic (Type 1 GD) in the absence of clinical information.

E. Physical, Kinetic and Immunologic Investigations of the Canine Analogue of Gaucher Disease:

1. Quantitation of Glycosphingolipids: As shown in Table X, the kidney and liver of the GD dog had 35- and 80-fold elevated levels of glucose ceramide (GC), respectively. However, GD brain had only a 3-fold increase in the level of GC and the amount of GC accumulation in the GD spleen was minimal. The levels of lactosyl ceramide (GL-2) were also elevated from 2- to 10-fold in all GD dog tissues with the excep-

tion of spleen. The other glycosphingolipids, GL-3 (globotriosyl ceramide) and GL-4 (globotetraosylceramide) were increased slightly, about 2-fold above the normal levels, as determined by staining intensity on TLC and quantitation by gas chromatography. However, until further structural studies and identification of canine glycosphingolipids are conducted, no definitive conclusions can be drawn about GL-3 and GL-4 accumulation.

GS, a poor substrate of β-Glc and a putative neurotoxin, was detected in GD dog spleen, liver and brain, but not in kidney samples. GS was not present in detectable levels in normal canine tissues similar to the lack of GS in normal human tissues (Table X).

2. Residual Activities: Table XI compares the β-glucosidase specific activities in the normal and GD Silky Haired Terrier liver, brain, spleen, and kidney. In all GD tissues a marked deficiency (< 4% of normal) of GC activity was observed. In contrast, deficient 4MU-Glc activity in liver, brain and kidney from the GD dog was apparent only in assays containing TC. Only in the GD splenic homogenates was a marked deficiency of 4MU-Glc activity evident in the presence or absence of detergents (Table XI). These observations suggested the presence of a "nonspecific" β-glucosidase(s) (except in spleen) similar to that observed in human tissues (88). A 4-fold variation in the level of hepatic "nonspecific" β-glucosidase activity was found in mongrel dogs in the absence of detergents. In the GD dogs, this enzymatic activity was within the normal range.

TABLE X

Neutral Glycosphingolipids and Glucose Sphingosine in Tissues of the Canine Gaucher Disease and Normal Dogs

Source	Glucosyl Ceramide	Lactosyl Ceramide	Glucose Sphingosine
	(nanomoles/gram wet weight)		
Spleen			
Normal 1	24.8	5.1	
Normal 2	41.6	18.4	ND[a]
Gaucher	56.1	8.5	+
Liver			
Normal 1	7.4	6.5	ND
Gaucher	554.0	70.0	+
Kidney			
Normal 2	20.0	30.5	ND
Gaucher	699.0	54.8	ND
Brain			
Normal 1	89.2	23.7	ND
Gaucher	280.4	64.5	++

[a]Indicates that the presence of GS was not detected

TABLE XI

"Specific" and "Nonspecific" β-Glucosidase Activities in Tissues from Normal and GD Australian Silky Haired Terriers

	Specific Activity (nmol/h/mg)					
	Glucosyl Ceramide		4MU-Glc with Detergents[a]		4MU-Glc without Detergents	
Source	Normal	Gaucher	Normal	Gaucher	Normal	Gaucher
Liver	145.30	1.54	71.91	6.91	15.90	19.51
Brain	105.21	3.10	42.78	3.04	7.84	8.12
Spleen	62.44	0.07	16.95	0.03	2.72	0.10
Kidney	30.86	1.29	11.97	0.98	8.41	2.64

[a]Detergents were 4.0 mM Triton X-100 and 4.65 mM TC.

TABLE XII

Membrane Association of β-Glc Activity in Normal and Gaucher Dog Tissues[a]

	Normal[b]		Gaucher[c]	
	nmol/h/g wet wt	% of total	nmol/h/g wet wt	% of total
Brain:				
Crude Supernatant[d]	1559.04	100	36.98	100
S[e]	130.07	8.3	12.64	34.1
Liver:				
Crude Supernatant	4990.35	100	47.46	100
S	1738.90	34.8	20.50	43.2
Kidney:				
Crude Supernatant	966.91	100	27.29	100
S	219.57	22.7	15.08	55.3
Spleen:				
Crude Supernatant	3299.07	100	3.17	100
S	519.85	15.8	1.69	53.2

[a] GC hydrolytic rates. Total recovery of activity varied between 80-110%.

[b] Average of two normal dogs.

[c] Average of two experiments in same Gaucher dog tissues.

[d] Clarified supernatant, see text.

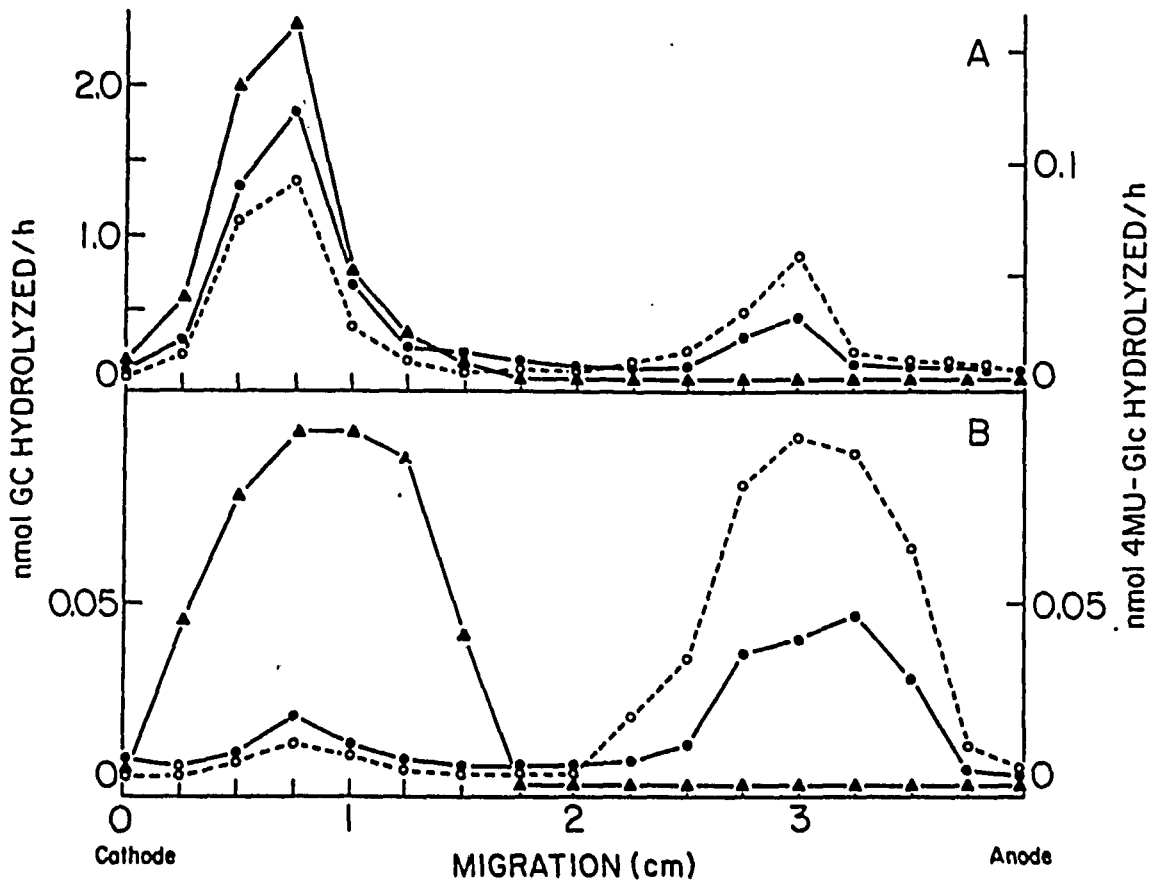
[e] 100,000 x g supernatant, see text.

3. Membrane Association of β-Glucosidases: As shown in Table XII, the β-Glc activity (GC substrate) was partially membrane-associated in all normal and GD tissues. In the normal tissues, the degree of membrane-association was highest in brain (> 90%) while in liver, kidney and spleen about 15 to 35% of the β-Glc activity was recovered in the high speed supernatants (S). In contrast, in the GD tissues greater solubility of the GC activity was observed (Table XII).

The 4MU-Glc activity in the S and P fractions was characterized further using CBE. In normal dog liver, the 4MU-Glc activity in the P fraction was completely inhibited by 2 mM CBE (> 96%) while the activity in the S fraction was only 80% inhibited. Similarly, in the GD dog liver preparations this concentration of CBE inhibited over 85% and about 10% of the 4MU-Glc activity in the P and S fractions, respectively. The presence of CBE resistant β-glucosidase in the S fraction from the normal and GD livers indicated that the "non-specific" β-glucosidase was soluble. In the GD dog, the β-Glc deficiency leads to the apparent preponderance of "nonspecific" glucosidase activity in the S fraction.

4. Cellulose Acetate Electrophoresis and Con A Chromatography: Further demonstration of the presence of two 4MU-Glc activities and the deficiency of β-Glc was obtained by cellulose acetate gel electrophoresis of liver extracts from the normal and GD dogs. As shown in Figure 16, either enzyme source had two peaks of 4MU-Glc activity when cut strips from cellulose acetate gels were assayed in the absence of detergents. The faster migrating activities from the normal and GD liver extracts comigrated, were inhibited by detergents (Triton X-100 and TC), hydrolyzed only the 4MU-Glc substrate and were not inhibited by 2 mM

Figure 15: Cellulose acetate gel electrophoresis of β-glucosidase activities in liver extracts from normal (A) and Gaucher (B) dogs. 4MU-Glc hydrolysis was determined in the presence (●) and absence (O) of 4.0 mM Triton X-100 and 4.65 mM TC. (Δ) denotes GC hydrolysis (β-Glc activity).


A
B
2.0
1.0
0
0.1
0
0.05
0
0.05
0
0
1
2
3
Cathode
MIGRATION (cm)
Anode
nmol GC HYDROLYZED/h
nmol 4MU-Glc HYDROLYZED/h

CBE. The amount of this "nonspecific" β-glucosidase activity varied by over 5-fold in three mongrel dog livers and was highest in the normal Silky Haired Terrier liver. In contrast, the slower migrating activities from normal and GD livers comigrated, hydrolyzed GC as well as 4MU-Glc, were activated by detergents, and were completely inhibited by 2 mM CBE. Using this electrophoretic system, only this activity was deficient in the GD dog liver extracts (Fig. 15) as well as extracts from brain, kidney and spleen (data not shown).

Con A-Sepharose chromatography provided separation of β-Glc and "nonspecific" β-glucosidase activities. Table XIII summarizes the properties of the β-glucosidase activities in the "bound" and "unbound" Con A fractions from normal and GD liver extracts. In normal canine liver, approximately 50% of 4MU-Glc activity (assayed in the absence of detergents) and 70% of the GC activity was bound to Con A. The "bound" 4MU-Glc activity was activated 10-fold by 4.65 mM TC and 4.0 mM Triton X-100 compared to the level in the absence of detergents and was completely inhibitable by CBE (Table XIII). The pH optima for 4MU-Glc activity of the "bound" and "unbound" fractions were essentially identical (pH 5.25). The "unbound" 4MU-Glc activity was activated about 3-fold by the detergents and only partially inhibitable by CBE (Table XIII). When the "unbound" fraction was reapplied to Con A, only an additional 10% of the 4MU-Glc and GC activities bound to the lectin. The presence of β-Glc and "nonspecific" β-glucosidase in the "unbound" fraction was confirmed by cellulose acetate electrophoresis (Table XIII). The faster migrating activity in the "unbound" fraction

TABLE XIII

Properties of "Unbound" and "Bound" β-Glucosidases in Canine Normal and GD Liver Following Con A Chromatography

Source	Property					
	pH Optimum	GC[a] Hydrolysis	CBE[b] Inhibition	Detergent[c] Effect	Km (mM)[d] 4MU-Glc	Electrophoretic Forms[e]
Normal:						
Unbound	5.25	30%	60%	3-Fold	ND	A,N
Bound	5.25	70%	96%	10-Fold	1.9	A
Gaucher:						
Unbound	5.0-8.0	50%	0	Inhibition	ND	A,N
Bound	5.25	50%	>90%	2-Fold	1.4	A

[a]Percent of total GC activity in each fraction.

[b]CBE (2 mM) inhibition of 4MU-Glc activity in the absence of detergents.

[c]Fold stimulation of 4MU-Glc activity by 4.65 mM taurocholate and 4.0 mM Triton X-100 compared to the activity in the absence of these detergents.

[d]Determined in the absence of detergents.

[e]A = acid β-glucosidase (e.g., β-Glc); N = "non-specific" β-glucosidase.

comigrated with the "nonspecific" β-glucosidase found in crude homogenates, whereas the slower migrating activity co-migrated with β-Glc from crude homogenates.

The "unbound" fraction from the canine GD liver contained about 80% of the total 4MU-Glc activity (assayed in the absence of detergents) and about 50% of the total GC activity. The "unbound" 4MU-Glc activity had a broad pH optimum (pH 5 to 8) and was partially inhibited (60%) by the addition of 4.0 mM Triton X-100 and 4.65 mM TC. Furthermore, this "unbound" 4MU-Glc activity was not detectably inhibited by 2 mM CBE. In contrast, this activity in the "bound" fraction had a pH optimum of 5.25, was stimulated by detergents and was inhibited more than 90% by 2 mM CBE (Table XIII). After reapplication of the "unbound" fraction to Con A, about 90 to 94% of the GC activity was recovered in the flow-through. Cellulose acetate electrophoresis of each fraction demonstrated identical results to those obtained in the normal liver except for the very large amount of "nonspecific" β-glucosidase relative to β-Glc.

5. Isoelectric Focusing: Figure 17 demonstrates the isoelectric focusing patterns of β-glucosidase activities in liver homogenates from normal (Fig. 16) and the GD dog (Fig. 16). A β-glucosidase activity with a pI value of 4.6 to 4.8 was found in the normal and GD liver homogenates. In the GD dog, this activity hydrolyzed only the 4MU-Glc substrate and was not detectably inhibited by 2.0 mM CBE. Five molecular forms of β-Glc with pI values of about 4.8, 5.2, 6.0, 6.4 to 6.6, 7.6 were found in the normal and GD enzyme sources.

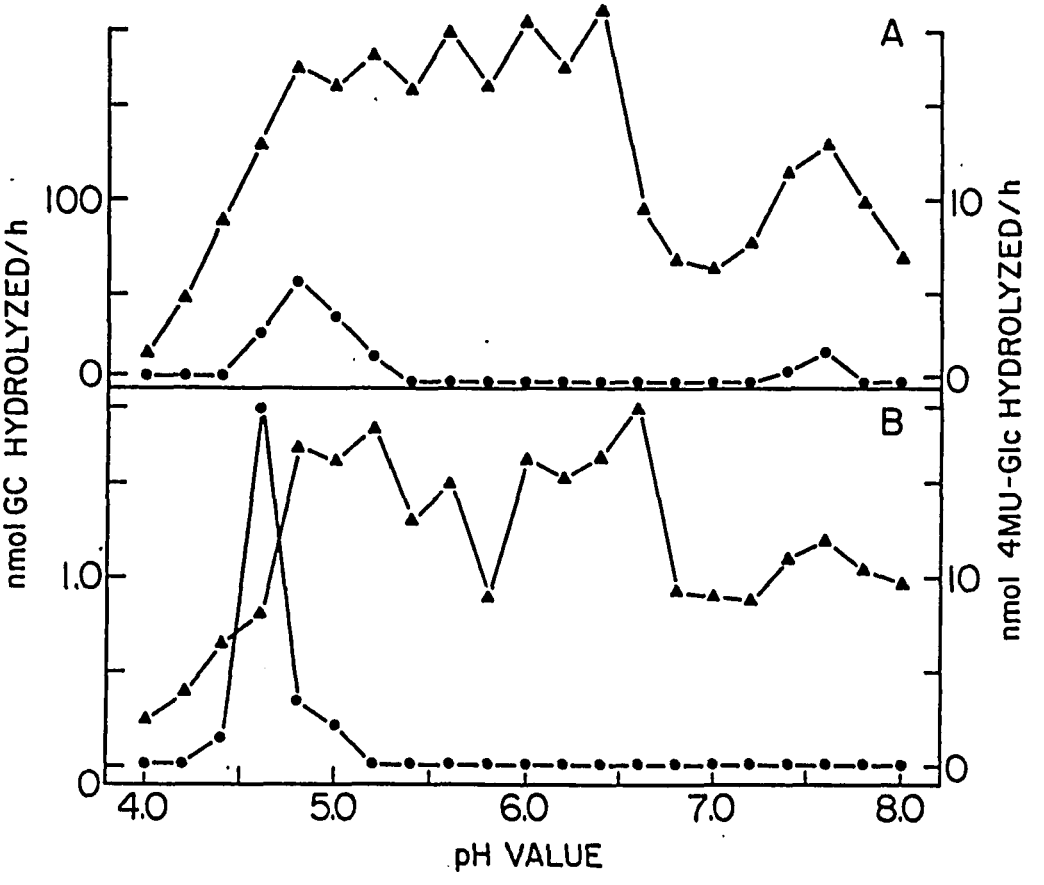
TABLE XIV

Effects of Modifiers on GC Hydrolysis in Butanol Extracts of Liver from Normal and GD Dogs

Property	Source	
	Normal	Gaucher
	(μM)	
K_m	150	180
K_i (Glucosylsphingosine)	7.0	5.0
K_i (N-hexyl-glucosylsphingosine)	0.28	0.38
I_{50} (Sphingosine)[a]	225	225
I_{50} (dNM)	90	80
I_{50} (N-dodecyl-dNM)	0.11	0.13
I_{50} (δ-Gluconolactone)	140	200
I_{50} (CBE)	25	27

[a]Amount of inhibitor which results in 50% inhibition of initial activity

Figure 16: Granular bed isoelectric focusing of β-glucosidase activities in normal (A) and GD (B) liver homogenates. β-Glc activity (Δ) (nmol/GC hydrolyzed/h). 4MU-Glc hydrolysis was determined in the absence (O) of detergents.


A
B
100
0
10
0
1.0
0
10
0
4.0
5.0
6.0
7.0
8.0
pH VALUE
nmol GC HYDROLYZED/h
nmol 4MU-Glc HYDROLYZED/h

6. Kinetic Studies of β-Glc Activity: Using the GC substrate, the pH optima (5.25) for the normal and GD β-Glc in liver extracts were identical. The kinetic parameters obtained at this pH for the normal and GD β-Glc are shown in Table XIV. In the presence of 4.0 mM Triton X-100 and 2.0 mM TC, the apparent K_m values (0.15 mM) were similar for the normal and GD enzymes. The K_i values for GS (7 μM) and N-hexyl-GS (0.28 μM), as well as the I_{50} values for sphingosine (225 μM), dNM (90 μM), N-dodecyl-dNM (0.11 μM), δ-gluconolactone (140 μM) and CBE (25 μM) were essentially identical for the normal and GD β-Glc. The $t_{1/2}$ value with CBE (0.2 mM) for either β-Glc was identical (50 min).

In contrast, differential effects of the normal and GD β-Glc were observed using the inhibitor, dodecyl-β-D-maltoside, and the modifiers, TC and PS, or the human activator protein. The I_{50} for the inhibitor, dodecyl-β-D-maltoside, was 12.8 mM for the GD enzyme and 4.0 mM for the normal β-Glc. Using the human splenic activator protein, the GD dog β-Glc was stimulated 2.8-fold by 40 μg of this protein while the normal enzyme had only a 1.5-fold increase in GC hydrolytic rate.

Figure 17 shows the typical curves for GC hydrolysis by butanol extracts of liver from normal and GD dogs in the presence of 4.0 mM Triton X-100 and increasing concentrations of TC or PS. Under these conditions, GC hydrolysis by extracts from normal liver was stimulated up to 1.4-fold by low concentrations of TC (< 0.5 mM) and low concentrations of PS (< 1 mM) had no effect. With higher levels of these negatively charged lipids, decreasing hydrolytic rates were observed. In contrast, GC hydrolysis by butanol extracts of the GD liver was stimu-

lated up to 1.8-fold with 1.5 mM TC or 1.3-fold with 0.75 mM PS and little inhibition was found at higher concentrations of either negatively charged lipid.

7. Thermostability Studies: Differential heat inactivations (50°C) were obtained with the normal and GD canine β-Glc in butanol extracts of liver. As shown in Figure 19, the normal GC activity had a $t_{1/2}$ of about 80 min at pH 6.0 while the GD β-Glc was markedly thermolabile ($t_{1/2}$ = 6 min). In mixture experiments, the expected biphasic inactivation curves for two enzymes with different thermostabilities was obtained (Fig. 18, broken curve). Similar results were obtained using butanol extracts of normal or GD spleen, kidney or brain (data not shown). For all tissue sources the $t_{1/2}$ values were pH dependent, i.e., at pH 5.0, the normal enzyme activity had a $t_{1/2}$ of 100 min whereas the GD β-Glc $t_{1/2}$ was 35 min. In comparison, at pH 7.0 the normal and GD β-Glc $t_{1/2}$ values were about 14 and 4 min, respectively. No differences in the stabilities of the normal and GD β-Glc activities were observed during each of seven cycles of freezing and thawing.

8. Immunoblotting Studies: Immunoblots using monospecific polyclonal or monoclonal anti-human β-Glc IgG, permitted the determination of the molecular weight forms of canine β-Glc cross-reacting immunologic material (CRIM). Two major forms of CRIM, $M_r \cong$ 69,000 and 94,000 were detected in normal and GD tissues (spleen, brain, kidney, and liver). Normal liver and kidney had additional forms of CRIM with $M_r \cong$ 77-72,000 and another CRIM form with $M_r \cong$ 48,000 was present only

Figure 17: Effect of taurocholate (Δ,▲) or phosphatidylserine (O,●) on GC hydrolysis by butanol extracted liver homogenates from normal (open symbols) or GD dog (closed symbols). GC hydrolysis was determined in presence of 4.0 mM Triton X-100 and various concentrations of TC or phosphatidylserine.

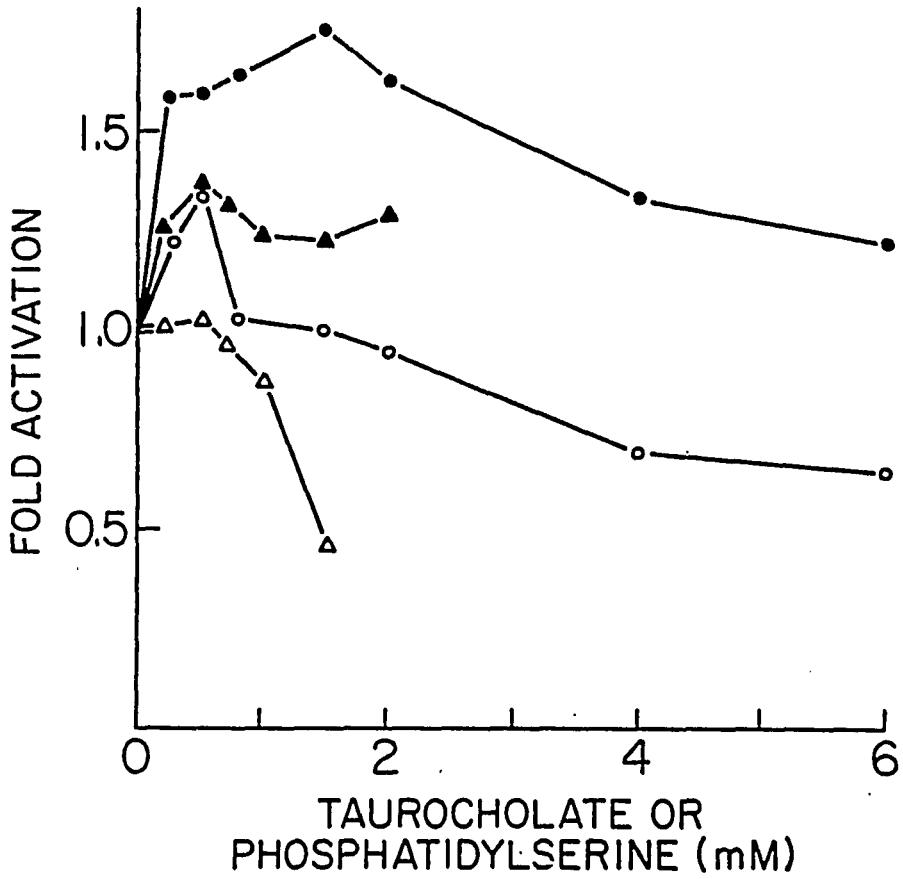
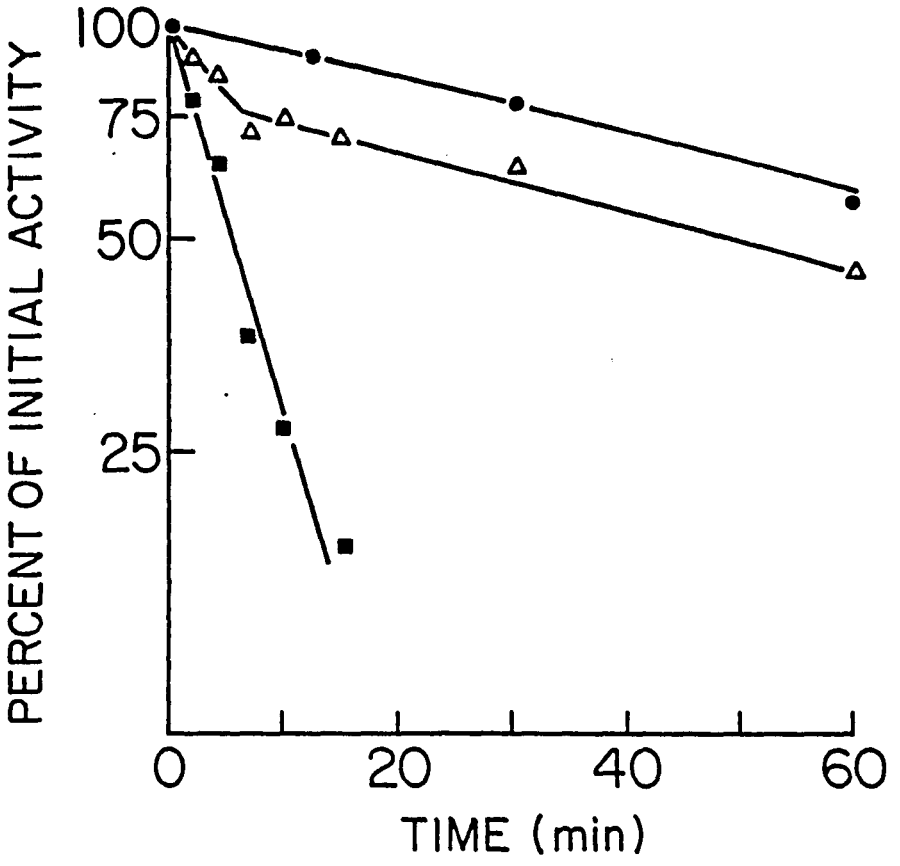

FOLD ACTIVATION
1.5
1.0
0.5
0
2
4
6
TAUROCHOLATE OR
PHOSPHATIDYLSERINE (mM)

Figure 18: Heat inactivation of GC activity in liver extracts from normal (●) and GD (■) dogs. Heat inactivations were conducted at 50°C in 0.1 M phosphate/0.08 M citrate, pH 6.0, containing 2 mM Triton X-100 and 7.4 mM taurocholate. In mixture experiments, equal amounts of GC activity from the normal and GD liver extracts were combined prior to heat inactivation (Δ).

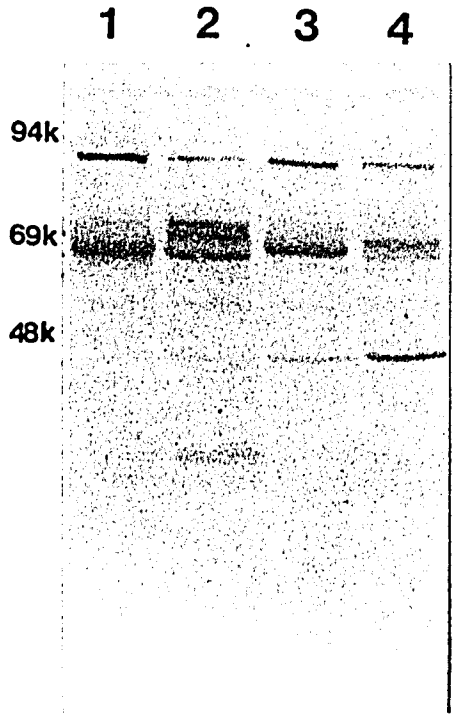

PERCENT OF INITIAL ACTIVITY
100
75
50
25
0
20
40
60
TIME (min)

in liver (Fig. 19). In the GD dog a marked deficiency of the 77-72,000 molecular weight CRIM forms was observed in the liver and kidney extracts.

CRIM-specific activity of β-Glc in GD liver extracts was estimated by the use of immunoblots. For these studies, the staining density of the CRIM associated with each molecular weight form of a fixed amount of GD β-Glc activity was visually compared to that obtained with 0-, 10-, 50-, 75- and 100-fold greater amounts of normal β-Glc activity. Based on these comparisons, about 75 times more normal β-Glc activity was required to equal the staining density observed in the GD dog extract. These results indicated that the CRIM-specific activity in the GD dog was approximately 1.5% (1/75th) of normal.

9. Purification of Normal Canine Splenic β-Glc: Initial purification of canine splenic β-Glc was attempted using the purification scheme developed for the human enzyme (Table XV). However, the canine enzyme was extremely unstable after acid precipitation and butanol extraction and, thus, the entire homogenate was used for purification of the canine enzyme. Ammonium sulfate fractionation of the homogenate was optimized at 30%, since the 35% cut used for the human enzyme resulted in 20% loss of canine β-Glc activity with no additional purification. Con A chromatography of these extracts provided 300- to 600-fold purification. β-Glc activity was eluted off of Con A by 0.5 M α-methylmannoside, 50% ethylene glycol in Con A buffer in a broad trailing peak of activity in approximately a 55% yield. This eluant was then extracted with butanol (35%) prior to further purification by affinity or hydrophobic chromatography. Chromatography on the

Figure 19: Immunoblotting patterns of CRIM in kidney (lanes 1 and 2) and liver (lanes 3 and 4) homogenates from normal (N) (lanes 2 and 4) and GD (G) (lanes 1 and 3) dogs using polyclonal anti-human β-Glc IgG. Each lane contained 80 μg of homogenate protein.


1
2
3
4
94k
69k
48k

GS-Sepharose affinity column was conducted as described (15) and provided an additional 10- to 30-fold purification. Less than 10% of the total β-Glc activity flowed through the column or eluted off non-specifically with the 20% ethylene glycol wash. However, storage of canine β-Glc at 4°C in high concentrations of ethylene glycol (60-75%), resulted in rapid loss of activity. Subsequent stability studies indicated that if stored at -20°C with high concentrations of ethylene glycol or at 4°C in less than 40% ethylene glycol, the partially purified canine β-Glc was stable.

Application to and elution of the partially purified canine β-Glc from C_{12}-dNM affinity column, as described for the human enzyme, resulted, in an additional 3.3-fold purification with a specific activity of of 615,000 nm/h/mg protein. Samples subjected to SDS-PAGE revealed a predominant $M_r \cong$ 72,000 molecular weight protein and low levels of smaller molecular weight contaminants.

10. Discussion: The present studies demonstrate the increased level of GC substrate and GS in canine GD tissues, confirm the specific deficiency of acid β-glucosidase (β-Glc) and provide the first characterizations of the residual activity in the GD dog. The GC levels were markedly increased in canine GD liver and kidney with lower accumulation evident in spleen and brain. These results correlate well with the pathologic findings of the GD dog which demonstrated Gaucher cells only occasionally evident in GD spleen compared to the heavily infiltrated liver and kidney (54). The low level of splenic GC accumulation is interesting in light of the absence of splenomegaly in the six GD dogs previously examined on autopsy (54,55). The GS accumulation in canine GD

TABLE XV

Comparative Purification of Canine Splenic β-Glc Using Purification Protocol for Human β-Glc or Using Con A and C_{12}-dNM-Sepharose

Purification Step	Human β-Glc Protocol		Con A/C_{12}-dNM-Sepharose	
	Specific Activity	Yield	Specific Activity	Yield
	(U/mg protein)	(%)	(U/mg protein)	(%)
Homogenization	22.4	100	12.5	100
30% Ammonium Sulfate	19.7	62	13.0	118
Acid Precipitation	256.7	50	___	___
Con A-Sepharose	___	___	6518	55
Butanol Extract/Dialysis	261.3	30[a]	5976	58
GS-Sepharose	10,091	1.5	188,259	25.2
C_{12}-dNM	___	___	618,600	12.7

[a]Fifty percent loss of β-Glc activity during dialysis

tissues also supports the clinical designation of the GD dog as a model of the neuronopathic Type 2 and 3 forms of human GD. GS, a putative neurotoxin, has been found in elevated levels in the brain samples of Type 2 and Type 3 GD patients and is only minimally elevated or absent in Type 1 GD and normal brain samples, respectively (52,53).

In support of the previous findings with the artificial 4MU-Glc substrate and assays at acidic pH values, low levels (< 4% of normal) of natural substrate activity β-Glc were detected in liver, spleen, brain and kidney of the affected dog. Twelve other lysosomal hydrolases were present at normal levels. Furthermore, as suggested by Van De Water et al. (55), a "nonspecific" β-glucosidase obscured the specific deficiency of β-Glc in the GD dog when determined with 4MU-Glc substrate.

Preliminary characterization of the "nonspecific" β-glucosidase in normal and GD dog livers demonstrated 1) lack of GC hydrolytic activity, 2) broad pH optimum for 4MU-Glc, 3) isoelectric point of pH 4.6-4.8, 4) more anodal migration than β-Glc on cellulose acetate gel electrophoresis, 5) lack of binding to Con A, 6) inhibition by Triton X-100 and sodium TC, and 7) absence of inhibition by CBE, a covalent inhibitor of several β-Glc activities (58). A similar "nonspecific" β-glucosidase has been described in human and bovine tissues (88,89).

Recent speculations, based on the study of human GD tissues, have suggested a relationship between the "non-specific" and "specific" β-glucosidases, particularly in the neuronopathic forms of the disease (88). In the present study no differences between the "non-specific" β-glucosidase found in normal and GD dog tissue extracts were apparent by electrophoresis, isoelectric focusing and Con A binding. Moreover, the absolute level of this activity, as determined with 4MU-Glc in the

absence of detergents, was normal in the GD dog tissue extracts although the level of activity varied over a 4- to 5-fold range in hepatic extracts from different normal dogs. The establishment of a breeding colony of inbred Australian Terriers with neuronopathic GD disease may permit closer examination of the possible relationships of these glucosidases in a more controlled genetic milieu.

Use of a fluorescent derivative of the natural substrate of β-Glc (GC) allowed the comparative characterization of normal β-Glc and the residual β-Glc activity in the GD dog. In liver, only minor differences in Con A binding were observed between normal and GD canine β-Glc. In addition, the number and pI values of active β-Glc molecular forms found on isoelectric focusing (Fig. 16), the pH optima, apparent K_m values, inhibitor interactions, and $t_{1/2}$ values (CBE inhibition) and cryostability of the normal and GD β-Glc were identical (Table XIV). However, differences between the normal and GD β-Glc were found in their membrane association, qualitative responses to selected modifiers of GC activity, variation in molecular weight forms and thermostabilities.

The β-Glc activities in normal and GD sources were only partially membrane-associated as determined in these once freeze/thawed tissues (Table XII). However, the activity in the GD dog tissues appeared to be less membrane-associated than the normal dog activity. Since the normal and GD tissues were stored and handled in a similar manner, the differences were most likely due to an abnormality of the GD β-Glc. However, confirmatory studies of membrane association in fresh, unfrozen tissues must await the availability of more GD dogs.

The velocity curves of GC hydrolysis with increasing amounts of TC or PS in the presence of 4 mM Triton X-100 demonstrated that the GD enzyme activity was enhanced more by low concentrations and was inhibited less by higher concentrations of these modifiers than the normal enzyme. The differential effects of the human activator protein and dodecyl-β-D-maltoside (elevated I_{50}) on the normal and GD enzymes indicated that the abnormal response of the GD enzyme was not restricted to ionic detergents but also included other hydrophobic reagents.

The marked decrease of estimated CRIM specific activity and the thermolability of the GD β-Glc suggested that the mutant enzyme activity was unstable. The severely decreased thermostability of the GD β-Glc at pH 6.0 suggests the possibility of a pH sensitive in vivo instability of the mutant enzymatic activity during transit from the Golgi to the lysosomes. Furthermore, the deficiency of the 72-77,000 molecular weight forms of β-Glc in GD liver and kidney (Fig. 19) suggested an abnormal processing of the mutant enzyme in particular tissues. These results and the altered interaction of the GD enzyme with hydrophobic agents suggest a mutation which results in complex effects on the membrane binding, stability, and certain kinetic properties of the mutant enzyme. However, the major effects of this mutation lead to a marked instability of the enzymatic activity.

The characterization studies presented here and the previous pathologic and biochemical reports indicate that the canine GD is an analogue of the human Type 2 or Type 3 GD. The normal active site function of the canine GD β-Glc, as indicated by inhibitor studies, is analogous to the residual β-Glc in the neuronopathic (Type 2/Type 3)

forms of human GD. However, the marked decrease in CRIM specific activity and the thermolability of the residual enzymatic activity seems to be unique to the canine mutation.

Purification of normal canine β-Glc has revealed similarities to the normal human enzyme as demonstrated by the elution and purification on PS-Sepharose, GS-Sepharose, GC-Sepharose and C_{12}-dNM Sepharose. SDS-PAGE has demonstrated the canine enzyme ($M_r \cong 72,000$) which is similar to the 67,000 molecular weight of human splenic β-Glc (14,15). However, the instability of canine β-Glc activity after acid precipitation and when stored under conditions which are optimal for the human enzyme suggest different enzymatic properties.

The canine β-Glc appeared to be less hydrophobic than the human enzyme as demonstrated by the easier solubilization of canine β-Glc activity into the supernatants of crude homogenates, the more complete recovery of canine activity off Con A Sepharose, and the elution off C_{12}-dNM and GS Sepharose columns at slightly lower ethylene glycol concentrations than that of the human enzyme. The 1- to 1.5-fold activation of canine β-Glc by TC and PS as compared to the 2- to 3-fold activation of the human enzyme suggests a different interaction of canine β-Glc with lipids and lipoidal modifieres. Furthermore, MCAb 61, a MCAb which inhibits human β-Glc activity (Sect. V.D.) and had no effect on canine β-Glc activity. In contrast, MCAbs F-1 and F-2 completely inhibit both human and canine β-Glc activity. Thus, future comparative studies of the human and canine β-Glc by kinetic, immunologic, and genetic techniques may provide insight into the evolutionary functional relationships of the β-Glc structural domains.

IV. CONCLUSIONS

Gaucher disease has provided biochemists and geneticists with a model system to investigate the biology of a lysosomal enzyme and the effects of allelic mutations in a structural gene on the phenotypic expression of a disease. However, the unique properties of β-Glc, among lysosomal enzymes, and the variety of β-Glc mutations resulting in GD might be exploited to understand the normal biology of β-Glc as well as other lysosomal enzymes. To approach these issues, two questions related to β-Glc and GD will be addressed: 1) Is β-Glc membrane-bound, i.e., an integral membrane protein, and, if so, does this property require unique mechanisms unrelated to other lysosomal enzymes. 2) What is the nature of the mutations in GD which account for the presence or absence of neuronopathic manifestations. Since neither of these questions have been resolved completely, the answers will be somewhat speculative in nature and will attempt to point toward directions for resolving these issues.

The evidence for the membrane binding of β-Glc is all circumstantial. Although β-Glc does sediment with membrane fractions upon subcellular fractionation, β-Glc has never been demonstrated to exist within a membrane by direct observation and has not been shown to be reconstituted into artificial membrane systems. However, it is clear that the enzymatic activity and the enzyme protein require detergents (Triton, bile acids or SDS) for solubilization. Indeed, as indicated in Section V.C. of this thesis, even high concentrations of detergents, together with sonication, failed to solubilize a large percentage of the β-Glc from fibroblasts. Detergents also were required to solubilize the enzyme for purification and all current purification methods for β-Glc

incorporate such extractions. The fact that delipidation is necessary for the specific interaction of this enzyme with hydrophobic supports or with "co-glucosidase" and that enzymatic activity is reconstituted only by the addition of specific lipids indicate the presence of intrinsic lipids on the enzyme. These lipids probably derive from the membrane and represent membrane components. Thus, at least operationally, β-Glc is a membrane protein. How much of the enzyme is buried in the membrane is completely unknown. Accepting the above argument, one can question whether variant GD enzymes are differentially membrane-bound. The data in Section V.C. of this dissertation suggests that all active GD enzymes are solubilized from fibroblast extracts by the same procedures and to about the same extent. If solubilization properties are an indication of membrane binding, certainly no major differences exists among the active normal and mutant enzymes. However, whether inactive forms of β-Glc with altered membrane binding are produced in GD has not been addressed.

This reasoning implies that the nature of the different mutations in the variants of GD does not create either a more or less membrane-bound catalytically active β-Glc. This is in contrast to the GD dog mutation which appears to render the GD β-Glc activity less membrane-bound and more easily solubilized (Sect. V.E.). Consequently, the differences in the human GD phenotypes cannot be explained on the basis of a dyslocalization of the enzyme due to abnormal membrane binding. This does not imply that abnormal localization to the lysosomal compartment cannot occur (see below).

The high molecular weight CRIM form detected in Group A (Type 2 and Type 3 and some Type 1 GD) fibroblasts and the apparent deficiency of the lower molecular weight forms, suggests the presence of an incompletely processed glycosylated precursor of β-Glc (87) or the instability of the lower molecular weight, more mature forms, of β-Glc in GD. Therefore, the Group A mutant β-Glc may be hindered in its efficient transport into the lysosomes or it may be rapidly degraded once in the lysosome.

The fact that β-Glc is one of two lysosomal enzymes present in normal levels in I-cell disease fibroblasts (91) suggests an alternative to the mannose-6-phosphate lysosomal targeting system used for other lysosomal enzymes. Indeed, mannose-6-phosphate has not been shown to be incorporated into the carbohydrate structure of β-Glc (25). Although this is not firmly established (J. Tager, personal communication), our recent pulse-chase studies in COS cells suggest that mannose-6-phosphate is not incorporated into the β-Glc carbohydrate chains. It is possible that the major lysosomal targeting of β-Glc occurs via the secretion and uptake at the plasma membrane as proposed by Von Figura and Weber (92) as an alternative mechanism for other lysosomal enzymes. The presence of higher M_r glycosylated forms of β-Glc in fibroblast culture media compared to intracellular forms (Sect. V.C.) (87), suggests the secretion of different β-Glc forms which possibly contain non-mannose-6-phosphate signals for attachment to the plasma membrane. Alternatively, normal and GD β-Glc may remain membrane-associated throughout the β-Glc maturation process as has been observed with other membrane-bound

proteins (93). Therefore, the dyslocalization of β-Glc from the lysosome in Type 2 or Type 3 GD might not be evidenced as an increased ease of solubilization of β-Glc activity.

The genetic heterogeneity of GD and the etiology of the neuronopathic involvement in Types 2 and 3 GD have been a central focus for many investigations of GD. The fact that the level of *in vitro* β-Glc activity does not correlate well with the severity or presence of neuronopathic involvement in GD suggests that these assay systems may not reflect the true lysosomal β-Glc activity. Such a difference in activity may be due to either defective targeting to the lysosome, abnormal membrane insertion and/or altered interactions with the "co-glucosidase". Until *in situ* assays for β-Glc activity are developed, the correlation of *in vitro* and *in vivo* enzyme levels will remain obscure. However, the findings of abnormal activation of the Type 1 AJGD enzyme by negatively charged lipids and/or "co-glucosidase" *in vitro* suggest that this mutant enzyme may not interact with membrane components *in vivo* in a normal manner (47,50,97). The finding of extra-lysosomal and non-lysosomal β-Glc (in ER) by immunoelectron microscopy (Tager, personal communication) in Type 2 GD supports the dyslocalization of the mutant enzyme in this form of GD. Thus, the deficiency of lysosomal β-Glc in some neuronopathic variants may be absolute whereas the lysosomal β-Glc in Type 1 AJGD may merely be defective. In the Group A Type 1 GD cells with abnormal M_r forms, the possibility must be considered that these forms derive from different processing defects which permit localization of β-Glc to the lysosome in sufficient amount to preclude neuronopathic manifestations.

GS, the deacylated analogue of GC, has been shown to accumulate in the brains of Types 2 and 3 GD patients and to be only minimally elevated or absent in Type 1 GD patients (52,53). Based on such results, and in analogy to Krabbe disease, it has been hypothesized that GS is the neurotoxin in Type 2 and 3 GD (80). However, unlike galactose sphingosine in Krabbe disease (β-galactocerebrodsidase deficiency) (90), glucose sphingosine has been shown to be a very poor substrate for β-Glc and kinetic analyses have shown GS to be a very potent inhibitor of the normal and Group A enzymes. Thus, it is interesting to speculate that GS may create a viscious cycle of inhibition of the low levels of β-Glc (lysosomal) in the Types 2 and 3 GD. In comparison, the greately decreased affinity but, presumably, normal hydrolysis of GS by the Type 1 AJGD enzyme may protect the brain from the toxic effects of GS. This speculation implies a threshold of β-Glc activity (lysosomal) for the prevention of neuronopathic effects.

The detection of GS accumulation in tissues from the neuronopathic canine model of GD and the absence of GS in normal canine tissues (Sect. V.E.) is supportive evidence of GS as being a neurotoxic factor in GD. Furthermore, the lack of detectable GC accumulation in GD canine kidney which had 4.1% of normal levels of β-Glc activity supports the hypothesis that a threshold level of β-Glc activity is required to prevent GS accumulation. This is particularly suggestive since GS did accumulate in canine liver, brain and spleen which had 1%, 2.9% and 0.1% of the respective normal β-Glc activities.

Finally, the demonstration of decreased levels of CRIM (Sect.V.C.) in GD patients suggests an altered synthesis or stability of mutant β-Glc in Type 1 and Types 2 and 3 GD patients. Since comparative studies

of synthesis and degradation of β-Glc in normal and GD fibroblasts have not been reported, the decreased levels of CRIM specific activity in GD and the normal k_{cat} values in Type 1 AJGD β-Glc (Sect.V.B.) suggested that only a portion of the β-Glc present in Type 1 AJGD appeared to be catalytically active. The mutant enzyme activity, therefore, must be unstable. This catalytic instability was also demonstrated in the canine model of GD (Sect.V.E.) which had normal levels of CRIM and low levels of CRIM specific activity. Thus, it could be hypothesized that all forms of GD result from unstable mutant enzymes which, because of their dyslocalization (Types 2 and 3) or abnormal kinetic properties (Type 1 GD) become more susceptible to proteolytic digestion.

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