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ALPHA-GALACTOSIDASE A REPLACEMENT THERAPY

FOR FABRY DISEASE

by

Ken M. Zeidner

A dissertation submitted to the Graduate Faculty in Biomedical Sciences

in partial fulfillment ofthe requirements for the degree of Doctor of Philosophy.

The City University of New York

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ABSTRACT

ALPHA-GALACTOSIDASE A REPLACEMENT THERAPY FOR FABRY DISEASE

by

Ken M. Zeidner

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Fabry disease is an X-linked glycosphingolipid storage disease resulting from a deficiency of lysosomal α-galactosidase A (α-Gal A; EC 3.2.1.21) and accumulation of its principle glycosphingolipid substrate, globotriaosylceramide (Galα1-4Galβ1-4βGlc1-4Cer; GL-3), primarily in the endothelium, leading to heart and kidney failure and death by the fourth decade of life. The accumulation is due to increased circulating plasma GL-3, carried by the low density lipoprotein particles and deposited in the vasculature. Presently, no treatment exists for this disease. These studies were undertaken to characterize a murine model of Fabry disease and to assess the effectiveness of enzyme replacement therapy (ERT) in these animals to provide the rationale for this treatment in Fabry patients.

Mice hemizygous or homozygous for a null α-Gal A allele appeared normal at birth and possessed normal lifespans and fertility. These mice were completely deficient of α-Gal A activity in all tissues examined, including liver, spleen, heart, lung, brain, kidney, white blood cells, fibroblasts, and plasma. Cultured fibroblasts from these mice also lacked enzymatic activity against GL-3 *in vivo*, but did retain partial *in vitro* activity due to the presence of a related lysosomal enzyme, α-galactosidase B.

In order to quantitate the accumulated glycosphingolipid in these mice and in future clinical ERT trials with Fabry patients, a rapid and sensitive ELISA was developed. The

assay was based on the specific affinity of an *Escherichia coli* toxin, verotoxin, for GL-3. Utilizing this ELISA, markedly increased levels of GL-3 were detected in the liver, spleen, heart, kidneys, skin, and plasma of α-Gal A-deficient mice and plasma of Fabry hemizygous males. Immunofluorescence microscopy of primary α-Gal A-deficient mouse fibroblasts using verotoxin detected endosomal/lysosomal storage of GL-3, though no cell membrane staining was observed in either wild-type or α-Gal A-deficient mice, in contrast to normal and Fabry human fibroblasts, which possessed high levels of GL-3 in their plasma membranes. This was supported by the fact that mouse fibroblasts were resistant to verotoxin cytotoxicity in culture, whereas human fibroblasts were sensitive.

Based on previous observations of the effects of various α-Gal A glycoforms on GL-3 depletion in Fabry patients, α-Gal A-deficient mice were administered four different glycoforms of recombinant human α-Gal A to determine their biodistributions and pharmacokinetics. These glycoforms differed in their content of sialic acid and mannose-6-phosphate residues. At a single dose of 1 mg enzyme/kg body weight (mg/kg), the tissue distribution of each glycoform was remarkably similar. The majority of enzyme activity was recovered from the liver, with a small amount present in the spleen and kidneys. No activity was detected in the lungs, heart, or, as expected, brain. All glycoforms were cleared rapidly from the circulation ($t_{1/2} < 5$ min), with the highly sialylated forms slightly more persistent, though these differences were not expected to be clinically significant. This rapid clearance of the injected enzymes was due to the reticuloendothelial mannose receptor, as injection of mannans prior to enzyme administration prolonged their circulation and led to a redistribution of activity to various tissues. As no significant differences existed between the various glycoforms, only the most sialylated, mannose-6-phosphorylated form of α-Gal A was used in further studies. In tissues, the enzyme exhibited a half-life of ~40 hr in liver and ~20 hr in spleen and kidney, suggesting that the enzyme was endocytosed and correctly targeted to lysosomes. Increasing the dose administered, this glycoform was distributed to other tissues, including detectable levels in

heart and kidney. Multiple administrations of α-Gal A completely restored enzyme levels to those of wild-type mice in all tissues examined, except brain.

The effect of this α-Gal A glycoform on GL-3 accumulation in α-Gal A-deficient mice was examined. Single administrations of α-Gal A at doses of 0.3-10 mg/kg hydrolyzed the accumulated GL-3 in liver, spleen, heart, kidney, and skin in a dose-dependent manner. Multiple administrations of 3 or 10 mg/kg cleared all the accumulated GL-3 from liver, spleen, heart, and kidney and greatly reduced the GL-3 accumulation in skin. In the liver, there was a >95% depletion of GL-3 at all doses tested. In the heart, there was a >75% reduction of GL-3 at the low doses and >95% reduction at the high dose. In the kidney, all doses tested significantly decreased GL-3 accumulation, with the high dose effecting the maximal decrease. In spleen, all doses tested significantly decreased GL-3, with the high dose completely depleting GL-3 accumulation. In skin, there was a dose-dependent depletion of GL-3, with maximal depletion of about 75% of controls. Administration of 0.3-10 mg/kg α-Gal A led to a decrease in circulating levels of GL-3 in a dose-dependent manner. Thirty days following a single 3 mg/kg dose, GL-3 levels in the liver were still below detection, whereas GL-3 levels in plasma, spleen, and heart were still below the predose values. In addition, GL-3 reaccumulation in plasma preceded reaccumulation in tissues, suggesting that plasma GL-3 levels may be an early indicator of GL-3 storage in tissues. Antibodies against exogenous α-Gal A were produced in mice administered the highest doses of enzyme, though they were not neutralizing and did not appear to affect the clinical outcome of the experiments.

These studies provide the rationale for ERT for Fabry disease. These results indicate that following infusion into α-Gal A-deficient mice, recombinant human α-Gal A can reach all tissues analyzed and can catabolize the accumulated GL-3 in a dose-dependent manner. Changes in plasma GL-3 levels appear to accurately reflect changes in organ storage, thereby allowing a rapid and simple method for evaluating ERT. Furthermore, these result suggest that infrequent administrations of enzyme can reverse GL-3

accumulation and potentially maintain the lipid at normal levels. It is therefore concluded that ERT is warranted for Fabry disease.

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To Wendy, for coming along at the perfect time.

And to myself, for finally finishing something.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

1. Background

Fabry disease was originally described in 1898 independently by two dermatologists, Anderson and Fabry, who noted proteinuria and abnormal vasculature, suggesting that the disease was a generalized disorder of blood vessels and kidney (Desnick et al., 1995). Thus, the disease was originally characterized as angiokeratoma corporis diffusum universale. Pompen et al. (1947) described postmortem findings in two brothers who died from renal failure, namely vacuolization in blood vessels throughout the body and correctly suggested that the disease was a storage disorder. Scriba and associates determined the storage was lipid in nature (Desnick et al., 1995), and Sweeley and Klionsky (1963) characterized two neutral glycosphingolipids from the kidney of a Fabry patient, which were identified as globotriaosylceramide and galabiosylceramide. Thus, Fabry disease was characterized as a glycosphingolipidosis. In 1965, Opitz et al., using pedigree analysis, determined that Fabry disease is inherited in an X-linked fashion. Two years later, Brady et al. (Brady et al., 1967) demonstrated a deficiency of lysosomal ceramide trihexosidase in individuals with Fabry disease, and soon thereafter, Kint (1970) (Kint, 1970) demonstrated that ceramide trihexosidase was an α-galactosidase. In 1971 it was determined that globotriaosylceramide possessed a terminal α-D-galactopyranosyl residue (Bensuade et al., 1971; Clarke et al., 1971; Hakomori et al., 1971; Handa et al., 1971; Li and Li, 1971); thus, Fabry disease was finally classified as an X-linked inborn error of glycosphingolipid metabolism, resulting from a deficiency of the lysosomal hydrolase, α-galactosidase A (Fig. 1).

2. Lysosomal Enzymes and Storage Diseases

Lysosomal Enzymes: Lysosomes have been described functionally as acidic cellular organelles which function as terminal degradative compartments (Kornfeld, 1987).

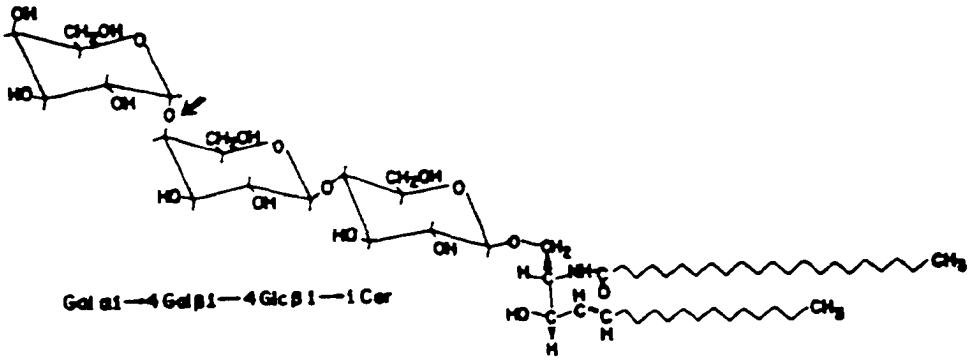

Figure 1. Chemical structure of globotriaosylcermide (GL-3), the principle accumulated substrate in Fabry disease. The arrow indicates the α-galactosyl bond, which is normally hydrolyzed by α-Gal A. Taken from Desnick et al., 1995.

They have also been described biochemically as mannose-6-phosphate receptor (M6PR) negative and lysosomal glycoprotein positive organelles (Kornfeld and Mellman, 1989) which maintain their low pH through the actions of a V-type proton ATPase (Mellman et al., 1986). They are a reservoir of approximately 40 acidic hydrolases which reside in the lumen of this organelle and catalyze the step-wise degradation of complex carbohydrate, protein, and lipid substrates (Storrie and Desjardins, 1996). The enzymes are synthesized on membrane-bound polysomes (Kornfeld, 1987) and translocated through the rough endoplasmic reticulum (ER) membrane (Walter et al., 1984). In the lumen of the ER, the enzymes are cotranslationally glycosylated at specific asparagine residues, with subsequent trimming of the oligosaccharides in the Golgi apparatus (Kornfeld and Kornfeld, 1985). Certain mannose residues on soluble lysosomal enzymes acquire phosphate moieties in a series of two reactions (Von Figura and Hasilik, 1986). First, UDP-*N*-acetylglucosamine-1-phosphotransferase catalyzes the transfer of phospho-*N*-acetylglucoasmine from UDP-*N*-acetylglucosamine to specific mannose residues, yielding *N*-acetylglucosamine-1-phospho-6-mannose. Deficiency of this first enzyme results in mucolipidosis type I (I cell disease) (Reitman et al., 1981). Second, the *N*-acetylglucosamine is removed by *N*-acetylglucosamine-1-phosphodiester-α-*N*-acetylglucosaminidase to reveal the M6P moities.

Once phosphorylated, the enzymes travel to the trans Golgi network where they are recognized by one of two M6PRs (Hille-Rehfeld, 1995). The larger 300 kDa receptor is cation-independent and trafficks both intracellular and extracellular enzymes to lysosomes. It has also been identified as the insulin-like growth factor II receptor (Morgan et al., 1987). The smaller 46 kDa receptor is cation-dependent and apparently only recognizes intracellular enzymes (Hille-Rehfeld, 1995). Experiments involving fibroblasts from mice deficient in one or both M6PRs indicate that each receptor recognizes a different set of ligands (Pohlmann et al., 1995). Studies involving I cell disease have indicated that a M6P-independent pathway exists for lysosomal enzyme delivery in cell types other than fibroblasts. For example, various tissues from I cell disease patients, including liver

(Owada and Neufeld, 1982) and lymphocytes (Glickman and Kornfeld, 1993), contain near normal levels of lysosomal hydrolases. Normal cells also possess this lysosomal M6P-independent pathway, as acid β-glucosidase (Erickson et al., 1985), non-phosphorylated aspartylglucosaminidase (Tikkanen et al., 1995), and various other soluble proteins all reach their correct lysosomal destination.

Once the enzyme/receptor complexes are delivered to endosomes, the low pH causes dissociation of the ligands from their receptors (Von Figura et al., 1987). The receptors recycle back to the trans Golgi network for another round of ligand binding, and the enzymes, through vesicular transport, continue on their journey to lysosomes. Once in the lysosomes, the enzymes can go through a process of maturation, which may include intrachain, as well as N- and C-termini, proteolytic cleavage.

Lysosomal Storage Diseases: LSDs are a group of approximately 3 dozen heterogeneous human disorders characterized by accumulation of macromolecules within the lysosomes, resulting in an increase of the size and number of these organelles (Gieselmann, 1995). LSDs are generally classified by the the nature of the accumulated substrate (Glew et al., 1985). These include sphingolipidoses (Fig. 2), glycoproteinoses, mucolipidoses, mucopolysaccharidoses, and others. The concept of lysosomal storage diseases was first developed by Hers in 1965 (Hers, 1965) to explain the relationship between α-glucosidase and Pompe's disease. Originally, it was unclear whether LSDs arose by lack of degradation or increased synthesis of the accumulated substrates; however, experimentally-induced lysosomal storage of compounds such as sucrose and dextran seemed to suggest the former alternative (Lloyd, 1973). It is now known that the majority of LSDs result from a deficiency in a specific lysosomal enzyme; however, it is unclear how storage of accumulated substrates relates to pathology in the majority of of these disorders. For Fabry disease, it appears that the accumulated substrate physically occludes the endothelium, which ultimately causes the symptomology seen in the disease (Johnson and Desnick, 1978).

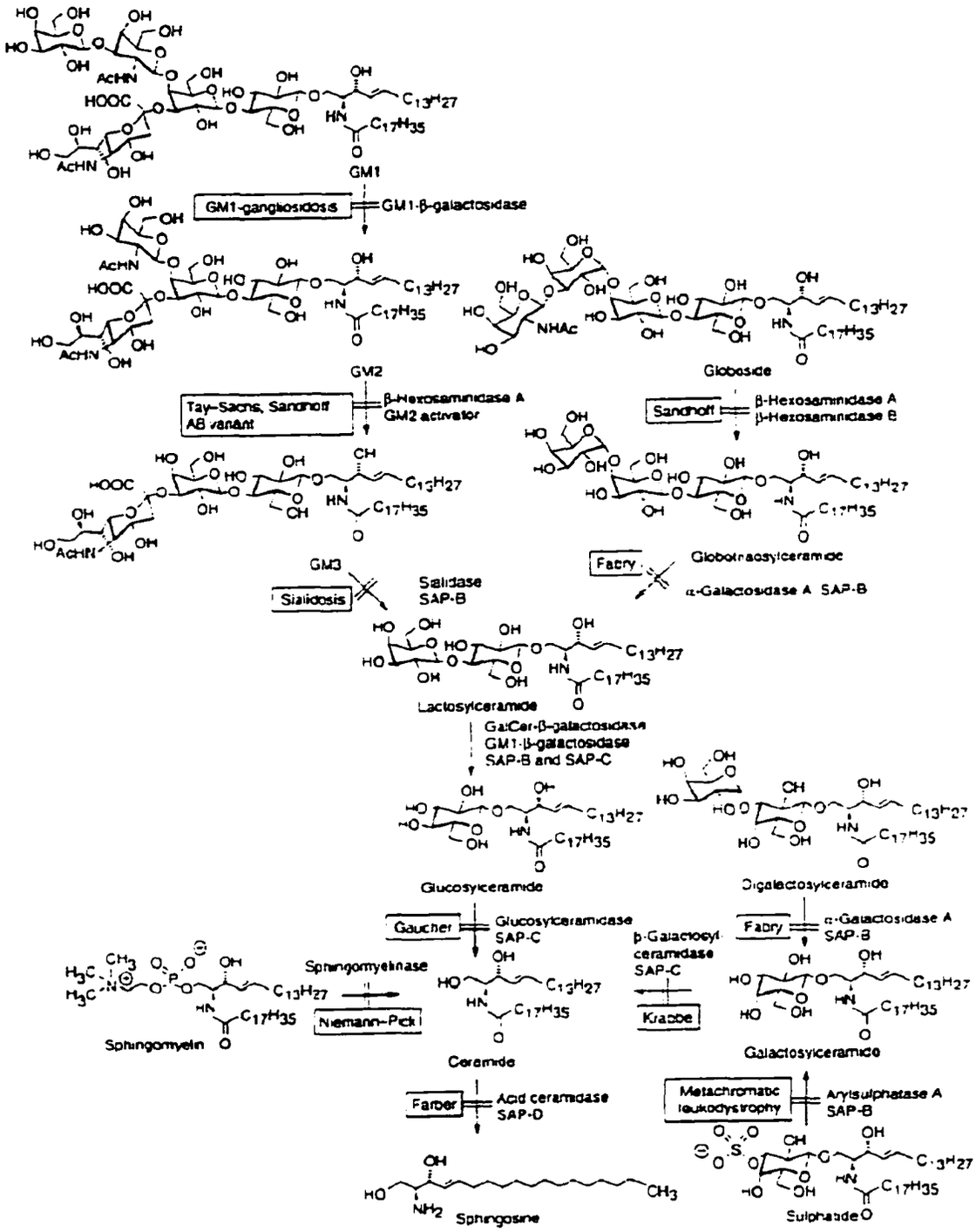

Figure 2. Lysosomal glycosphingolipid metabolism. Sphingolipid activator proteins, exohydrolases, and known storage diseases are shown. Taken from Sandhoff and Kolter, 1996.

Numerous animal models exist for specific LSDs and are the subject of extensive reviews (Bedell et al., 1997; Desnick et al., 1982; Patterson et al., 1982). Before the advent of gene-targeting technology, models of LSDs had to be identified in pre-existing animal colonies (Jezyk et al., 1982). The two prototypes for these naturally-occurring models are the *twitcher* mouse for Krabbe disease (Kobayashi et al., 1980) and the *gus*^{mps} mouse for mucopolysaccharidosis (MPS) type VII (Birkenmeier et al., 1989). Larger animal models of lysosomal storage diseases have also been identified. These include a feline model of G_{M2} gangliosidosis, type II (Cork et al., 1977), a bovine model of α-mannosidosis (Hocking et al., 1972), a canine model of MPS type I (Spellacy et al., 1983), a feline model of MPS type VI (Jezyk et al., 1977), and a caprine model of MPS type IIID (Thompson et al., 1992). With the advent of gene targeting technology, new murine models of lysosomal storage disease have been generated. These include α-*L*-iduronidase-deficient mice for MPS type I (Clarke et al., 1997); glycosylasparaginase-deficient mice for aspartylglycosaminuria (Kaartinen et al., 1996), arylsulfatase A-deficient mice for metachromatic leukodystrophy (Hess et al., 1996), arylsulfatase B-deficient mice for MPS VI (Evers et al., 1996), protective protein/cathepsin A-deficient mice for galactosialidosis (Zhou et al., 1995), acid-sphingomyelinase-deficient mice for Niemann-Pick disease, types A and B (Horinouchi et al., 1995; Otterbach and Stoffel, 1995), β-hexosaminidase A-deficient mice for Tay-Sachs disease (Yamanaka et al., 1994), β-hexosaminidase B-deficient mice for Sandhoff disease (Phaneuf et al., 1996; Sango et al., 1995), and glucocerebrosidase-deficient mice for Gaucher disease (Tybulewicz et al., 1992). These models should allow pathophysiology and treatment studies to be performed and provide the rationale for enzyme replacement therapy and gene therapy trials.

3. α-Galactosidase A

Biochemical Characterization. α-Gal A is the enzyme responsible for hydrolysis of terminal α-galactosyl moieties from glycoproteins and glycolipids (Desnick et al., 1995),

and was the enzyme found to be deficient in Fabry disease (Brady et al., 1967). The anomeric specificity of the enzyme was determined with synthestic substrates by Kint in 1970. Studies with synthetic substrates and tissues from Fabry patients revealed the presence of residual α-galactosidase activity (Desnick et al., 1995). Contrary to the α-galactosidase activity in normal individuals, which is heat labile and inhibited by myoinositol, the residual α-galactosidase activity was heat stable and not inhibited. α-Galactosidase was therefore thought to exist as two isozymes, A and B, differing in their levels of sialylation (Kint, 1970). This, along with early enzyme replacement trials for Fabry disease which were difficult to interpret (Mapes et al., 1970), led several investigators to question whether Fabry disease was due to a structural or regulatory gene defect (Beutler and Kuhl, 1971). The cloning of the α-Gal A genes (Bishop et al., 1986; Bishop et al., 1988; Kornreich et al., 1989) and detection of inactivating mutations (Desnick et al., 1989) ultimately confirmed that Fabry disease was due to a primary defect in α-Gal A. Also, α-Gal B was subsequently shown to be an α-*N*-acetylgalactosaminidase (Dean et al., 1977), encoded by a separate gene on chromosome 22 (DeGroot et al., 1978). This enzyme has been found to be deficient in the neuraxonal dystrophy, Schindler disease (Schindler et al., 1989), and the LSD Kanzaki disease (Kanzaki et al., 1993), in which glycoproteins and glycolipids with α-*N*-acetylgalactosaminyl moieties accumulate (Desnick and Wang, 1995), although it is now believed that Schindler disease may result from a different genetic lesion (Keulemans et al., 1996).

α-Gal A from various human tissues, including liver (Dean and Sweeley, 1979), spleen (Bishop and Desnick, 1981), kidney (Kano and Yamakawa, 1974), placenta (Mayes and Beutler, 1977), and plasma (Bishop and Sweeley, 1978) has been purified and characterized. Purification can be easily accomplished by chromatography on an α-galactosylamine resin (Harpaz et al., 1974). The enzyme is a homodimeric glycoprotein with a molecular weight of ~100 kDa which contains asparagine-linked complex and high mannose oligosaccharides. Biosynthetic studies with cultured human fibroblasts indicate

that a 51 kDa precursor is processed through intermediates to a mature 46 kDa lysosomal form over the course of several days (LeDonne et al., 1983; Lemansky et al., 1987). The enzyme has been shown to reach its lysosomal destination by means of the M6P receptor pathway (Lemansky et al., 1987). Post-lysosomal processing does not appear to be involved in the maturation of α-Gal A. The enzyme purified from plasma, with a pI of ~4.2, is more electronegative than its tissue counterpart, whose pI ranged from 4.3 to 5.1, due to the presence of multiple sialic acid resiudes (Bishop and Sweeley, 1978). The human enzyme has a pH optimum of 4.6 with the synthetic substrate 4MU-α-Gal, and a pH optimum of 3.9 with its natural substrate, GL-3 (Desnick and Bishop, 1989).

Expression. The human α-Gal A cDNA has been expressed in a variety of systems, including bacteria (Hantzopoulos and Calhoun, 1987; Ioannou, unpublished results), COS cells (Ioannou et al., 1998), Sf9 cells (Coppola et al., 1994; Ioannou et al., 1998), *Pichia pastoris* (Zeidner, unpublished results), BHK21 cells (Zeidner, unpublished results), and CHO cells (Ioannou et al., 1992). Biomedical interest in α-galactosidases and their recombinant forms is particularly strong, as these enzymes can be used to convert the type B blood groups to the more useful donor type O group (Zhu et al., 1996). α-Galactosidases can also be used to remove the Gal(α1-3)Gal antigen present on pig organs to decrease the immunological response associated with xenotransplantation (La Vecchio, 1995). The enzymes are also important in the biochemical production of sucrose and may be useful for control of flatulance (Zapater et al., 1990).

Expression of human α-Gal A in CHO cells has been reported to cause enzyme crystallization in lysosomes (Ioannou et al., 1992). The high expression also resulted in high levels of selective secretion from the cells, leading to the hypothesis that overexpression resulted in aggregation of the enzyme and avoidance of the M6PR (Ioannou et al., 1992). The secreted enzyme was purified to homogeneity and demonstrated to be similar to α-Gal A from human sources (Ioannou, unpublished results). It has been crytallized in the presence of a reversible, active-site directed inhibitor (Murali et al., 1994).

Expression studies have allowed detailed analyses of the oligosaccharide structures of α-Gal A to be performed. Site-directed mutagenesis and expression in COS-1 cells revealed that 3 of the 4 glycosylation consensus sequences were utililzed (Ioannou et al., 1998). This analysis also demonstrated that high mannose, hybrid, and complex oligosaccharides were present. It has previously been demonstrated that glycosylation of lysosomal enzymes is important for their activity. Expression of human α-Gal A in bacteria produced a highly unstable protein with little to no activity (Hantzopoulos and Calhoun, 1987; Ioannou, unpublished results), similar to human acid α-glucosidase expressed in bacteria (Martiniuk et al., 1992). Also, complete site-directed mutagenesis of all glycosylation sites in β-hexosamindase A (Weitz and Proia, 1992) and aspartylglucosaminidase (Tikkanen et al., 1995) resulted in aggregation in the endoplasmic reticulum (ER) with loss of activity. Similarly, mutagenesis of glycosylation site 3 in α-Gal A resulted in significant loss of activity with aggregation in the ER (Ioannou et al., 1998). It is thought that the hybrid oligosaccharide present at this site masks a hydrophobic patch which aids in the solubility of the enzyme (Ioannou et al., 1998).

Domains have been identified which may be involved in substrate recognition and enzyme regulation of α-Gal A. Recombinant fusions of human α-Gal A and α-Gal B identified regions encoded by exons 1-2 and exon 6 which may interact to promote hydrolysis of synthetic substrates (Ishii et al., 1994). Replacement of exons 1-2 of α-Gal A with those of α-Gal B did not greatly affect its activity, suggesting that the active site was still intact. Replacement of exon 6 of α-Gal A with that of α-Gal B greatly diminshed its activity due to an increased *Km*, suggesting a role for that protein region in substrate binding. Replacement of both areas led to a slight recovery in activity, which suggests an interaction between the two regions. That substrate binding may reside in the C-terminus portion of the protein is also supported by the recent finding that this region of human α-Gal A shares patches of significant homology with proteins known to bind GL-3 (Maloney and Lingwood, 1994) (Fig. 3). Another interesting potential domain in α-Gal A was

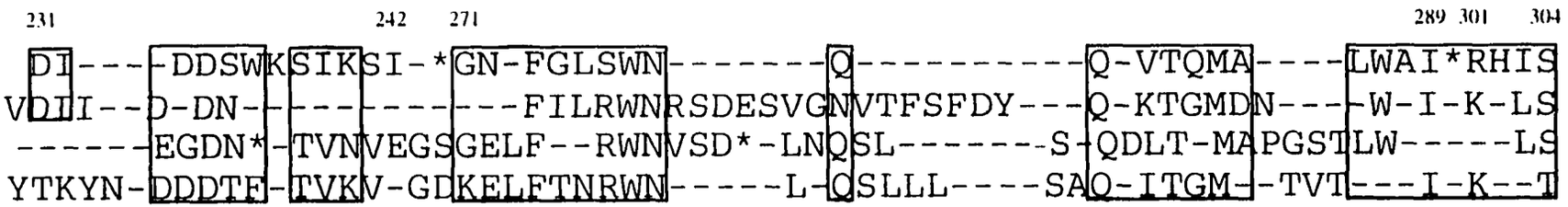

Figure 3. Alignment of proteins known to bind globotriaosylceramide. From top to bottom: amino acid sequences of human α-Gal A, human interferon (IFN)-α receptor, human CD19, and *E.coli* verotoxin B subunit (VTB). Human IFN-α receptor, human CD19, and *E.coli* VTB sequence alignment was taken from Maloney and Lingwood, 1994. Human α-Gal A alignment was performed manually. Numbering refers to the mature human α-Gal A amino acid sequence. Asterisks represent gaps in linear amino acid sequences.

discovered by C-terminal deletion mutagenesis (Miyamura et al., 1996). Removing up to 10 amino acids from α-Gal A greatly enhanced enzyme activity without increasing mRNA levels, suggesting this portion of the protein may partially mask the active site, whereas removal of more than 10 amino acids significantly reduced activity. A point that was not addressed is that the last four amino acids of α-Gal A are KDLL, similar to the ER retention signal, KDEL (Munro and Pelham, 1987). It is possible that removal of this sequence lead to greater amounts of mature enzyme reaching the lysosome, leading to an apparent increase in activity. Solving the α-Gal A crystal structure (Murali et al., 1994) should help confirm these results and identify new residues involved in catalysis and substrate recognition by α-galactosidases.

4. Fabry Disease

Nature of Accumulated Substrates. A deficiency of α-Gal A in Fabry disease leads to the progressive accumulation of α-galactosyl terminated neutral glycosphingolipids in the lysosomes of most tissues and fluids (Desnick et al., 1995) (Table 1). These accumulated glycosphingolipids are identical to those naturally found in the membranes of all cells. Four types of glycosphingolipids that accumulate in Fabry disease have been identified. These include globotriaosylceramide (also the blood group P^k glycolipid), digalactosylceramide, blood group B glycolipid, and blood group B1 glycolipid. Blood group P_1 glycolipid, which also contains a terminal α-Gal A moiety, has not been demonstrated to accumulate in Fabry disease (Desnick et al., 1995). Globotriaosylceramide (GL-3), the principle accumulated substrate in Fabry disease, has also been identified as the rare P^k antigen on human erythrocytes (Naiki and Marcus, 1974), as well as CD77, an antigen of germinal B cells undergoing apoptosis (Mangeney et al., 1993). Interestingly, the amount of accumulated blood group B glycolipids in the fluid of Fabry patients also depends on their secretor status, although the clinical relevance of this finding has not been determined (Ledvinova et al., 1997). Thus, it appears the

Table 1
Neutral Glycosphingolipids with terminal α-galactosyl moieties in human tissues

Chemical Structure	Trivial Name	Approved Nomenclature	Suggest Abbreviation
Gal(α1-4)Gal(β1-4)Glc(β1-1')Cer	Trihexosylceramide; Blood group P^k glycolipid; CD77	Globotriaosylceramide	GbOse3Cer
Gal(α1-4)Gal(β1-1')Cer	Digalactosylceramide	Galabiosylceramide	GaOse2Cer
Gal(α1-3)Gal(2-1αFuc)(β1-3)GlcNAc-(β1-3)Gal(β1-4)Glc(β1-1')Cer	Blood group B glycolipid	IV^2-α-Fucosyl-IV^3-α-galactosyl-lactotetraosylceramide	IV^2-α-Fuc-IV^3-α-Gal-$LcOse_4Cer$
Gal(α1-3)Gal(2-1αFuc)(β1-4)GlcNAc-(β1-3)Gal(β1-4)Glc(β1-1')Cer	Blood group B1 glycolipid	IV^2-α-Fucosyl-IV^3-α-galactosyl-neolactotetraosylceramide	IV^2-α-Fuc-IV^3-α-Gal-$LcnOse_4Cer$
Gal(α1-4)Gal(β1-4)GlcNAc (β1-3)Gal(β1-4)Glc(β1-1')Cer	Blood group P_1 glycolipid	IV^4-α-Galactosyl-neolacto-tetraosylceramide	IV^4-α-Gal-$LcnOse_4Cer$

Taken from Desnick et al., 1995.

metabolic load carried by a Fabry patient depends on multiple factors, such as residual α-Gal A activity and blood type.

Stepwise glycosylation of ceramide to form glycosphingolipids requires a series of glycosyltransferases which reside in the Golgi complex (Sandhoff and van Echten, 1993). The terminal α-galactosyl moieties of accumulated substrates in Fabry disease are attached by α-galactosyltransferases which appear to be tissue specific (Desnick et al., 1995). For example, UDPGal:Gal (β1-1')Cer(α1-4)galactosyltransferase activity, which synthesizes digalactosylceramide from galactocerebroside, is present in rat kidney, but not brain, liver, spleen, or lung, consistent with the tissue distribution of this glycosphingolipid in Fabry disease (Desnick et al., 1995). GL-3, the major accumulated substrate in Fabry disease, is synthesized from lactosylceramide by the action of UDPGal:LacCer(α1-4)galactosyltransferase (Stults et al., 1995). This enzyme has been purified from rat liver and shown to consist of two subunits which are highly specific for the synthesis of GL-3, as no digalactosylceramide, blood group B glycolipid, or blood group P_1 glycolipid synthesis was detected (Taniguchi et al., 1985).

In vitro step-wise degradation of glycosphingolipids by lysosomal hydrolases generally requires the addition of detergent to make the substrates available to the enzymes through the formation of mixed micelles (Desnick et al., 1995). *In vivo*, however, these enzymes need the assistance of sphingolipid activator proteins (SAPs) to degrade glycosphingolipids with short oligosaccharide chains (Sandhoff and Kolter, 1996). Two human genes code for SAPs (Van Echten and Sandhoff, 1993). One codes for the G_{M2} ganglioside activator protein, while another codes for a precursor SAP with four homologous domains which is proteolytically processed to SAP-A, B, C, and D (Sandhoff and Kolter, 1996). An activator of GL-3 hydrolysis by α-Gal A was originally identifed in 1976 (Li and Li, 1976). It was subsequently demonstrated that the same activator also stimulates the hydrolysis of cerebroside sulfate by arylsulfatase A and G_{M1} ganglioside by G_{M1}-β-galactosidase (Vogel et al., 1987). It is now known that SAP-B is necessary and

sufficient for the activation of hydrolysis of GL-3 by α-Gal A in detergent-free systems (Kase et al., 1996). This is supported by the observation that mice deficient in precursor SAP accumulate GL-3 in kidney and brain (Fujita et al., 1996).

Molecular Genetics. The genomic and cDNA clones for human α-Gal A have been isolated and sequenced (Bishop et al., 1986; Bishop et al., 1988; Kornreich et al., 1989). An unusual feature of the gene is the absence of a 3' untranslated region (Bishop et al., 1988). The genomic clone possesses 7 exons contained within 12 kb of DNA (Kornreich et al., 1989). The promoter contains a TAATAA sequence, five CCAAT sequences, two Sp1 binding sites, and a methylation-free island, indicative of a "housekeeping" gene (Kornreich et al., 1989). The cDNA predicts a protein of 429 amino acids, the first 31 of which correspond to the signal peptide (Bishop et al., 1988). Cleavage of the signal peptide following translocation into the endoplasmic reticulum appears to be the only proteolytic modification. Four *N*-linked glycosylation consensus sequences are present (Ioannou et al., 1998). Recent studies involving site-directed mutagenesis indicate that only the first three sites are utilized (Ioannou et al., 1998). The labeled cDNA clone has been used to map the gene to Xq22 (Desnick et al., 1995), thereby confirming the pedigree analysis of Opitz et al. (1965).

Over 100 mutations which cause Fabry disease have been identified in the α-Gal A gene (Desnick et al., 1995). These include partial gene duplications and deletions, small insertions and deletions, intron splice site mutations, mRNA editing mutations, coding region missense mutations, and nonsense mutations (Desnick et al., 1995). Most mutations causing Fabry disease are private (Desnick et al., 1995), although several mutations, including N215S, a glycosylation mutation resulting in mainly cardiac manifestations (Sakuraba et al., 1990), occur in unrelated families. So-called atypical Fabry hemizygotes, or cardiac variants, develop milder forms of the disease, usually restricted to the heart (Ogawa et al., 1990). It is now known that this is due to significant residual α-Gal A activity (Sakuraba et al., 1990). The majority of cardiac variant

mutations, including N215S, reside in exons 5 and 6 of the α-Gal A gene, suggesting that the C-terminus of the protein is not as essential to activity as the N-terminus. Recently, an R301Q mutation, which generally results in the cardiac variant of Fabry disease, presented in a case of Fabry disease with only nephropathy (Sawada et al., 1996). Attempts to establish genotype/phenotype correlations have been limited, as most mutations are private. Aside from the fact that all atypical hemizygotes have missense mutations, little correlation has been identified and the position and type of molecular lesion often fail to predict the clinical phenotype (Desnick et al., 1995). Future crystallographic studies may aid in these analyses.

Clinical Manifestations and Pathophysiology. Fabry disease, with an incidence of about 1 in 40,000 males, has one of the slowest clinical progressions of the LSDs, suggesting slow turnover of the principal accumulated substrate, GL-3 (Desnick et al., 1995). The manifestations associated with Fabry disease appear in adolescence and are due primarily to the accumulation of GL-3 in the vascular endothelium of most tissues (Desnick et al., 1995). In hemizygotes, these manifestations include excruciating pain, skin lesions, corneal dystrophy, strokes, myocardial infarctions, and renal failure (Table 2). Less common manifestations include joint problems (Paira et al., 1992), pulmonary involvement (Kariman et al., 1978), muscle pathology (Sima and Robertson, 1978) and intestinal symptoms (O'Brien et al., 1982). Premature death usually occurs in the fourth decade of life, generally due to impaired renal and cardiac function (Desnick et al., 1995).

Atypical hemizygotes have been identified who were essentially asymptomatic at the time when classical hemizygotes would manifest the disease (Desnick et al., 1995). Many were diagnosed after displaying cardiac manifestations, including hypertrophic cardiomyopathy and myocardial infarctions, and thus were termed cardiac variants. It is now known that these variants possess residual α-Gal A activity (Sakuraba et al., 1990), suggesting that small amounts of enzyme may protect the kidney, but not the heart, from failure (Sakuraba et al., 1990). Also, the myocardium is an early site of glycosphingolipid

Table 2
Major clinical manifestations in hemizygotes with Fabry disease.

Site of Glycolipid Deposition	Manifestation
Skin	Angiokeratome
Peripheral Nerves	Acroparethesias
Eyes	Corneal dystrophy
Heart	Ischemia and Infarctions
Brain	Strokes
Kidney	Renal Failure

Taken from Desnick et al., 1995.

accumulation, as evidenced by fetal post-mortem analysis (Desnick, unpublished results). Other types of variants have also been identified. For example, a patient with an α-Gal A point mutation characteristic of cardiac involvement presented with only nephropathy (Sawada et al., 1996), suggesting that factors other than residual activity influence the clinical course of the disease.

Fabry heterozygotes generally manifest less symtoms than Fabry hemizygotes, although their symptom severity can range from essentially undetectable to indistinguishable from hemizygotes (Desnick et al., 1995). This correlates well with the α-Gal A levels in these woman, which range from completely normal to undetectable (Desnick et al., 1989). This variation is presumably due to random X-chromosome inactivation (Lyon, 1961), which is supported by establishment of two populations of cells from obligate heterozygotes, one with normal and the other with deficient α-Gal A activity (Beaudet and Caskey, 1978). The most common finding in heterozygotes is corneal dystrophy, which occurs in approximately 70% of known cases (Desnick et al., 1995).

Morphologically, Fabry disease is characterized by the accumulation of birefringent, crystalline glycolipid deposits in the lysosomes of most cell types (Desnick et al., 1995). It appears that the cell types most involved in pathology, however, are endothelial and smooth-muscle cells of the vasculature (Johnson and Desnick, 1978). Numerous organ systems can be involved, such as skin, eye, nervous sytem, lung, gastrointestinal tract, and the reticuloendothelial system of liver and spleen (Desnick et al., 1995). Heart and kidney impairment, however, is the primary cause of the decreased life span of Fabry hemizygotes (Desnick et al., 1987). Cardiac disease occurs in most hemizygous males, manifesting as systemic hypertension, myocardial infarction, and congestive heart failure (Desnick et al., 1995). These symptoms appear to be caused by progressive lysosomal accumulation of glycosphingolipids in myocardial cells, valvular fibroblasts, and coronary vessels. Renal disease, with manifestations including proteinuria and uremia, also occurs in the majority of Fabry hemizygotes, ultimately resulting in renal

failure (Desnick et al., 1989). Renal lesions are thought to be caused by progressive lysosomal accumulation of glycosphingolipids in endothelial and epithelial cells of the glomerulus, Bowman's space, loops of Henli, and distal and proximal tubules.

GL-3 accumulation has been quantitated in some Fabry hemizygotes and heterozygotes. Hemizygotes accumulated GL-3 in heart, liver, and kidney to levels 100-fold, 20-50-fold, and 50-65-fold over normal, respectively (Hozumi et al., 1990). In a survey study of GL-3 levels in a Fabry hemizygote, all tissues including liver, spleen, heart, pancreas, muscle, and brain, showed 30-300-fold accumulation (Schibanoff et al., 1969). Accumulation in symptomatic heterozygotes was greatest in heart (32-fold) and less in liver (4-fold) and kidney (7-fold), reflecting the residual amounts of α-Gal A activity (Hozumi et al., 1990). Accumulation of GL-3 in atypical Fabry hemizygotes was restricted to the heart, with values 100-300 fold greated than normal (Ogawa et al., 1990). Thus, at least some heterozygotes resemble cardiac variant hemizygotes, strengthening the claim that low levels of α-Gal A protect the kidney, but not the heart, from accumulation of GL-3. These results also suggest that the total amount of tissue-specific accumulation is due to the amount of residual α-Gal A activity in each tissue, the amount of GL-3 naturally found in each tissue, and the susceptibility of each tissue to accumulation of GL-3. GL-3 is also elevated 5-200-fold in the urine of Fabry hemizygotes and heterozygotes (Desnick et al., 1970). The source of this GL-3 is thought to be exfoliated renal tubular epitlelial cells.

At least some accumulation of GL-3 in tissues of Fabry patients is due to endogenous synthesis, as the α-galactosyltransferase responsible for its synthesis has been detected in numerous cell types (Desnick et al., 1995). Also, catabolism of globoside (GL-4), the next lipid after GL-3 in the globo-series of glycosphingolipids may contribute to accumulation of GL-3. For example, globoside is the primary neutral glycosphingolipid found in kidney (Desnick et al., 1995), the principal organ affected in Fabry disease. But Fabry disease appears to be unique among the glycosphingolipidoses in that a substantial amount of accumulation may be due to deposition of circulating glycosphingolipids. This

view is supported by various findings. First, glycosphingolipids in circulation are associated with lipoprotein particles, primarily low density lipoproteins (LDL) and high density lipoproteins (HDL) (Chatterjee and Kwiterovich, 1984), which have efficient receptor-mediated endocytic pathways in numerous cell types (Desnick and Grabowski, 1981). Second, Fabry hemizygotes contain circulating levels of GL-3 up to 20-fold higher than normal (Vance et al., 1969). These first two points are linked by the fact that individuals homozygous for familial hypercholesterolemia (FH) have elevated levels of plasma and urinary GL-3 (Dawson et al., 1976). Third, endocytosis of LDL has been demonstrated in endothelial cells (Mommaas-Kienhaus et al., 1985), smooth muscle cells (Aulinskas et al., 1983), and glomerular epithelial cells (Rayner et al., 1990), which are all affected in Fabry disease. Endocytosis of LDL by renal epithelial cells has been shown to inhibit the transferase responsible for synthesis of lactosylceramide (GL-2), the precursor of GL-3 (Chatterjee et al., 1988). Fourth, hepatocytes, which synthesize LDL, show little accumulation of GL-3 (Desnick et al., 1995). Indeed, the liver is relatively unaffected in Fabry disease (Meuwissen et al., 1982). Finally, post-mortem analyses of confirmed Fabry fetuses revealed accumulation in liver but not kidney (Brady et al., 1971).

The final two points require clarification. Alhough the hepatocyte is the primary site for synthesis of LDL, isotopic tracer studies in pigs revealed that up to 80% of the daily circulating pool of GSL comes from catabolism of senescent erythrocyte membranes (Dawson and Sweeley, 1970). Similar results were also obtained in human studies (Vance et al., 1975). That it is the destruction of the erythrocytes which contributes to the pool is supported by the fact that erythrocyte GSLs with carbohydrate moieites longer than glucosylceramide (GL-1) do not exchange with lipoproteins in circulation (Dawson and Sweeley, 1970). Once the glycosphingolipids are released (presumably in the liver and spleen), they may be removed by the liver as micelles and directly incorporated into lipoprotein particles (Barkai and Di Cesare, 1975). As globoside is the primary glycosphingolipid in erythrocyte membranes (Vance and Sweeley, 1967), deposition and

subsequent hydrolysis to GL-3 in endocytic tissues which lack α-Gal A would lead to the accumulation seen in Fabry disease. This type of circulating deposition, as opposed to direct endogenous synthesis, may partially explain the slow progression of Fabry disease.

The physical deposition of lipoprotein-bound GL-3, as opposed to any abnormal metabolism of the particle, appears to be the primary cause of the vascular pathology associated with Fabry disease. Metabolism of LDL from Fabry hemizygotes appeared normal when injected into dogs (Clarke et al., 1980). Radiolabeled glycosphingolipid turnover times and rates did not differ significantly between normal individuals and Faby hemizygotes, although some differences were noted in the synthesis rates of GL-2 and GL-3 (Vance et al., 1975). Finally, GL-3 is distributed between LDL (~70%) and HDL (~20%) in the same ratio in normal and Fabry individuals (Clarke et al., 1976). When GL-3 is added to human serum *in vitro*, a very different ratio is produced (Clarke and Stoltz, 1976). HDL becomes the primary-bound lipoprotein, which suggests that endogenous glycosphingolipids are incorporated into lipoprotein particles at their site of synthesis and little exchange occurs between particles in circulation. Later studies however, indicated that exchange can occur between different lipoprotein fractions (Loeb and Dawson, 1982) and between lipoproteins and cells (Kwok et al., 1981), further strengthening the view that these molecules contribute to the accumulation of α-galactosyl-containing glycosphingolipids in Fabry disease.

Diagnosis. A presumptive clinical diagnosis in Fabry disease is most easily made by observation of skin lesions and corneal dystrophy, although these characteristics are also present in numerous other disorders (Desnick et al., 1995). Therefore, all suspected hemizygotes should be confirmed biochemically (Desnick et al., 1995). This can be accomplished by demonstration of accumulated substrate in various tissues and fluids, including fibroblasts (Wandall et al., 1982), plasma (Vance et al., 1969), cerebrospinal fluid (Kaye et al., 1992) and urine (Desnick et al., 1971). Enzymatic activity against the natural substrate, GL-3, can also be used as a diagnostic marker (Kobayashi et al., 1984),

although diagnosis is more easily accomplished by analysis of plasma using synthetic substrates containing *N*-acetylgalactosaminidase to inhibit α-Gal B activity (Mayes et al., 1981). This can be accomplished in fibroblasts, leukocytes, platelets, tears, plasma, and urine (Desnick et al., 1987). The biochemical identification of heterozygotes is less reliable due to random X-chromosome inactivation (Lyon, 1961). Many heterozygotes possess intermediate levels of α-Gal A activity, although some may express activites ranging from undetectable to normal (Desnick et al., 1987). This has led to attempts to accurately diagnosis heterozygotes by laborious enzymatic analysis of single hair roots (Beaudet and Caskey, 1978) or individual fibroblasts (Romeo and Migeon, 1970), or immunodetection of GL-3 accumulation in fibroblasts (Itoh et al., 1996) or urinary renal tubular cells (Chatterjee et al., 1984). With the recent advances in molecular technology, accurate diagnosis of heterozygotes has been achieved. This technology includes α-Gal A-specific (Desnick et al., 1987) and linked (Caggana et al., 1997) RFLP analysis, SSCP analysis (Takata et al., 1997), multiplex PCR (Kornreich and Desnick, 1993), and fluorescence-assisted mismatch analysis (Germain et al., 1996). Both the bichemical and molecular techniques can be used in conjunction with amniocentesis or chorionic villi sampling for accurate prenatal diagnosis of Fabry disease (Desnick et al., 1995).

Treatment. Treatment of Fabry disease centers on three areas: medical management, substrate depletion, and gene-product replacement. The most debilitating aspect of Fabry disease is pain (Desnick et al., 1995). Various drugs have been tested for relief. These include carbamazepine (Shibasaki et al., 1973), morphine (Gorden et al., 1995), and diphenyhydantoin (Lockman et al., 1973). Cosmetic treatment of angiokeratomas can be accomplished with argon laser (Newton and McGibbon, 1987). Renal insufficiency can be treated by hemodialysis and/or kidney transplantation (Donati et al., 1987). Care of patients with regard to cardiac, pulmonary, and neurological manifestations remains nonspecific and symptomatic (Desnick et al., 1995).

Substrate depletion generally involves one of two methods: phlebotomy or plasma exchange. As senescent erythrocytes are thought to be a major source of circulating GL-3 (Dawson and Sweeley, 1970), it was thought that phlebotomy would lessen the metabolic load on Fabry patients. In a phlebotomy trial involving a single Fabry patient, no decrease in plasma or urinary GL-3 levels was detected (Beutler et al., 1983). This suggested that other sources of GL-3, perhaps maturing reticulocytes, were more relevant to Fabry disease. In addition, since deposition of circulating GL-3 is thought to contribute to the accumulation in Fabry disease (Johnson and Desnick, 1978), plasma exchange, in which large volumes of plasma are removed and fresh plasma administered to avoid hypovolemic changes (Kolodny et al., 1981), was attempted. In a trial of plasma exchange involving a single Fabry patient, large amounts of GL-3 were removed; however, gradual reaccumulation of the glycolipid in the circulation occurred (Moser et al., 1980). The patient reported subjective improvement in his symptoms, although these were also reported in a sham series (Pyeritz et al., 1980). Also, renal function continued to deteriorate during and following this trial. It has been suggested that plasmapheresis with an anti-LDL affinity resin might be more selective and efficient (Desnick et al., 1995), as circulating GL-3 is primarily transported in the LDL particle (Chatterjee and Kwiterovich, 1984). It was also suggested that plasma exchange may be most effective as an adjunct to enzyme replacement (Kolodny et al., 1981).

Removal of GL-3 from plasma was also attempted using Sepharose-immobilized fig α-galactosidase (Schram et al., 1978). The immobilized enzyme was able to hydrolyze free GL-3 in the presence of taurocholate at pH 7.3 at about 20% efficiency of that at pH 4.4. However, the enzyme was not able to hydrolyze GL-3 in plasma, probably because the glycolipid present in lipoproteins was not accessible to the enzyme. A new type of substrate depletion, which can be termed substrate inhibition, has been attempted in the murine model for Tay-Sachs disease. Mice treated with *N*-butyldeoxnojirimycin, an inhibitor of the glycosyltransferase-catalyzed synthesis of glucosylceramide, showed less

accumulation of G_{M2} ganglioside in brain than untreated mice (Platt et al., 1997). As glucosylceramide is a biosynthetic precursor of GL-3, this type of treatment could potentially be of benefit to presymtomatic Fabry patients, although the long-term effects of the wholesale inhibition of globo-series glycosphingolipids remains unknown.

In gene-product replacement, the missing enzyme, in this case, α-Gal A, is replaced by some mechanism. In addition to treatment of renal failure, kidney transplantation has been undertaken to determine if the allograft could provide normal α-Gal A for substrate metabolism (Desnick et al., 1995). Although some clinical and biochemical improvements, including decreased plasma GL-3 levels, have been reported in some cases (Desnick et al., 1973; Philippart et al., 1972), no improvement has been found in others (Clarke et al., 1972; Spence et al., 1976). These improvements may only reflect the clinical course of the disease and appear to have little effect on the overall prognosis (Maizel et al., 1981). Most importantly, little evidence for increases in circulating levels of α-Gal A activity or tissue GL-3 catabolism was ever detected. This suggested that circulating levels of GL-3 were reduced by filtration through the normal kidney. It is now recommended that kidney tranplantation be used only for end-stage renal failure (Desnick et al., 1995). Fetal liver cells have also been transplanted into Fabry patients in the hopes of correcting the metabolic defect (Malik et al., 1979). Following transplantation, three hemizygotes noted improvement in their acroparesthesia, but no increase in α-Gal A levels or decrease in GL-3 levels were detected. This again suggested that improvement was due to fluctuations in clinical manifestations.

Gene therapy is the most recent attempt at treatment for Fabry disease. It is thought that enzyme levels can be returned to normal, either at the site of gene delivery or by enzyme secretion and uptake at distal sites. Retroviral vectors with drug resistance markers (Medin et al., 1996; Sugimoto et al., 1995) and adenoviral vectors (Ioannou, unpublished results) containing the human α-Gal A cDNA have been generated for this purpose. Retroviral vectors may be used in conjunction with bone marrow transplantation, although

the tissue involvement in Fabry disease may preclude its use (Desnick et al., 1995). These vectors may also be used to generate enzyme-secreting organoids (Salvetti et al., 1995). Direct intramuscular injection of α-Gal A cDNA vectors in mice has also been attempted (Novo et al., 1997), as has liposome-mediated gene delivery (Ioannou, unpublished results). Preliminary *in vitro* results demonstrate correction of the enzymatic defect, either directly or by secretion/endocytosis. *In vivo* results in mice demonstrated enzyme levels which range from barely detectable to normal. Still uncertain is the effect on the accumulated substrate or potential immunological responses.

5. Enzyme Replacement Therapy

Most of the above methods for substrate depletion and product replacement for Fabry disease suffer from ineffectiveness, difficulty in execution with great risks to the patient, or untested efficacy, precluding their use on a wide-spread scale. Many of these treatments, however, share a common objective: delivery of α-Gal A to tissues in order to reverse or prevent the metabolic storage. The most direct type of this enzyme replacement therapy (ERT) involves administration of exogenous enzyme to the patient. This type of therapy is relatively simple in terms of procedure and has proven effective for the treatment of other diseases. Several features of Fabry disease make it an attractive candidate for ERT (Desnick et al., 1995). First, Fabry disease and α-Gal A are well characterized; second, the disease has a slow progression of symptoms; third, only soft tissues are generally involved; fourth, neurological involvement is minimal; fifth, α-Gal A has been overexpressed and purified in quantities large enough to support a clinical ERT trial (Ioannou et al., 1992); and finally, a mouse model for Fabry disease has recently been produced (Ohshima et al., 1997; Wang, unpublished results).

ERT for LSDs was first suggested in 1964 by deDuve, who reasoned that normal enzyme would be endocytosed by deficient cells and be delivered to the site of substrate accumulation. This was confirmed by the cross-correction studies of Neufeld and

colleages (Fratantoni et al., 1968), who demonstrated that the accumulation of sulfated mucopolysaccharides could be reversed in fibroblasts from MPS type I and II patients by co-culture, presumably through the uptake of soluble "corrective factors." These factors were later identified as the missing lysosomal hydrolases. This type of *in vitro* metabolic correction has subsequently been demonstrated with other LSDs, including MPS type III (Kresse and von Figura, 1974), MPS type VII (Sly et al., 1973), multiple sulfatase deficiency (Eto et al., 1987), metachromatic leukodystrophy (Kihara et al., 1973), glycogenosis type II (Hug et al., 1973), and Fabry disease (Sifers et al., 1983).

Early trials of *in vivo* ERT for LSDs, however, were generally disappointing, due to such problems as immunological responses, short enzyme retention time in circulation, and lack of consistent catabolic effects (Desnick and Grabowski, 1981; Desnick et al., 1976; Rietra et al., 1974; Tager et al., 1980). These trials included administration of *Aspergillus niger* (Hug and Schubert, 1967; Huijing et al., 1973; Lauer et al., 1968) and human placental α-glucosidase (De Barsy et al., 1973) for glycogenosis type II, bovine brain (Greene et al., 1969) and human urine (Austin, 1967) arylsulfatase A for metachromatic leukodystrophy, human placental (Von Sprecht et al., 1979) and urine (Johnson et al., 1973) β-hexosaminidase A for G_{M2} gangliosidosis, human placental acid β-glucosidase (Brady et al., 1974) for Gaucher disease, and normal plasma for MPS type I (Di Ferrante et al., 1971), type II (Di Ferrante et al., 1971; Erickson et al., 1972), type III (Dean et al., 1973), and G_{M2} gangliosidosis (Desnick et al., 1972). These studies were important in identifying obstacles which must be overcome for successful ERT, which led to the development of a coscensus list of requisites which must be met before further human ERT studies could continue (Desnick and Grabowski, 1981; Desnick et al., 1976; Rietra et al., 1974; Tager et al., 1980). These requisites can be summarized as follows: 1) human enzymes should be available in large quantities; 2) the enzymes should be sterile and non-immunogenic; 3) the enzymes should be retained in the circulation as long as possible and delivered to the sites of pathology; 4) studies should be performed in non-human

mammalian systems; 5) methods must be established to monitor the effectiveness of treatment.

With the advent of DNA technology, requisites 1 (large quantities of human enzymes) and 4 (non-human mammalian systems) have become more uniformily achievable. Previously, the lack of sufficient amounts of purified human enzymes led researchers to infuse normal plasma into patients with LSDs, particularly mucopolysaccharidoses (Brown III et al., 1982; Dean et al., 1973; Di Ferrante et al., 1971; Di Ferrante et al., 1973; Erickson et al., 1972). Also, transplantation of normal tissues, including amnion membranes (Muenzer et al., 1992), fibroblasts (Dean et al., 1980), and fetal liver cells (Malik et al., 1979), was attempted in patients with LSDs to provide a source of continuous enzyme production. The results of these studies were often conflicting and their practice has generally been discontinued. When small amounts of purified human lysosomal enzymes were available, they were frequently tested only in normal animals (Desnick et al., 1976; Fiddler, 1980), including α-glucosidase (Huijing et al., 1973), β-glucuronidase (Achford et al., 1978), and acid β-glucosidase (Furbish et al., 1978). This obviously eliminated any chance of studying the effects of enzyme administration on substrate accumulation. Occasionally, purified human enzymes were tested in animal models of human LSDs. For example, the murine model of MPS type VII has been used to study the fate of intravenously administered β-glucuronidase in the native form (Thorpe et al., 1974) or entrapped in erythrocyte ghosts (Fiddler et al., 1977) and liposomes (Steger and Desnick, 1977). Also, the feline model of G_{M2} gangliosidosis has been used to study the effects of mannans on tissue distribution of exogenous β-hexosaminidase (Rattazzi et al., 1981). Now that molecular biology techniques have become available, large amounts of recombinant human enzymes and mammalian models to assess their effectiveness have been produced. Overexpressed human enzymes include α-Gal A (Ioannou et al., 1992), acid α-glucosidase (Van Hove et al., 1996), iduronate-2-sulfatase (Bielicki et al., 1993), α-*L*-iduronidase (Unger et al., 1994),

glycosylasparaginase (Mononen et al., 1995), α-*L*-fucosidase (Gramer et al., 1994). These have been studied in various animal model systems including feline MPS type VI (Crawley et al., 1996), canine MPS type I (Shull et al., 1994), and murine MPS type VII (Sands et al., 1994). The majority of these studies demonstrated that exogenous enzymes were quickly removed from the circulation by the liver but could correct the enzymatic defects in different tissues in a dose-dependent fashion. More importantly, the enzymes were capable of metabolizing accumulated substrate in many tissues, including liver, spleen, heart, kidney, and skin, although immunological complications often arose.

Problems associated with requisites 2 (enzymes should be non-immunogenic) and 3 (enzyme retention and targeting) still exist. To date, almost all lysosomal enzymes tested in animals or humans, even highly purified ones, elicit some type of immunological response. These responses are not predictable. It has generally been assumed that the presence of cross-reactive immunologic material (CRIM+) would ensure the lack of an antibody response (Boyer et al., 1973). In reality, all Gaucher patients receiving ERT are CRIM+, but approximately 15% develop antibodies to the administered acid β-glucosidase (Richards et al., 1993). Also, some CRIM+ MPS type VI cats (Yogalingam et al., 1996) develop high titers of clinically-relevant anti-*N*-acetylgalactosamine-4-sulfatase antibodies during ERT (Brooks et al., 1997). In fact, antibodies to normal protein can be made in CRIM+ patients which do not even recognize the mutant protein (Brooks et al., 1997). Species crossreactivity cannot explain the presence of antibodies to exogenous enzyme, as feline *N*-acetylgalactosamine-4-sulfatase (Brooks et al., 1997) and canine α-*L*-iduronidase (Brooks et al., 1997) also induced antibody production in MPS type VI cats and MPS type I dogs, respectively. It should be pointed out, however, that immune reactions which develop during the course of ERT are generally infrequent, relatively mild, and easily treated.

The intense study of LSDs, particularly the early cell and animal studies listed above, has led to the identification or clarification of various cell-surface receptors which are potentially important for ERT. These include the M6PR on fibroblasts, the LDL

receptor on fibroblasts, smooth muscle cells, and endothelial cells, the asialoglycoprotein receptor on hepatocytes, and the mannosyl/*N*-acetylglucosamine receptor on reticuloendothelial cells (Desnick and Grabowski, 1981). Attempts have been made to avoid or exploit these receptors during ERT studies. For example, liposomes and red cell ghosts have been used as carriers for lysosomal enzymes to increase retention time in the circulation and prevent rapid proteolytic degradation. Although these goals were accomplished (Desnick and Grabowski, 1981), it was determined that liposomes themselves were immunogenic (Hudson et al., 1980) and that both carriers were targeted to primarily the reticuloendothelial system (Fiddler, 1980). Long-term treatment of Gaucher patients with liposome-entrapped (Belchetz et al., 1977) or erythrocyte entrapped (Beutler et al., 1980) acid β-glucosidase was deemed unsuccessful due to the lack of clinical benefits. Polyethylene glycol has been coupled to sweet almond β-glucosidase (Wieder and Davis, 1983), green coffee bean α-Gal A (Wieder and Davis, 1983), and human α-Gal A (Coppola et al., 1994), although it was found to alter the enzymes' catalytic properties and mask specific determinant sites. An attempt to treat a patient with Tay-Sachs disease with polyvinylpyrrolidone (PVP)-conjugated β-hexosaminidase was largely unsuccessful (Von Sprecht et al., 1979), even though PVP was shown to prevent rapid circulation clearance (Geiger et al., 1977), possibly due to the fact that derivatized β-hexosaminidase may not be able to degrade G_{M2} ganglioside (Rattazzi, 1998). Coupling of enzymes to various receptor-specific ligands has also been attempted. For example, galaptin-α-*L*-fucosidase (Allen et al., 1990), α_2-macroglobulin-α-Gal A and α-glucosidase (Tsuji et al., 1994), and insulin-cholesterol esterase (Poznansky et al., 1989) conjugates have been tested in tissue culture and shown to degrade their respective accumulated substrates. Fragment C of tetanus toxin coupled to β-hexosaminidase was efficiently endocyosed by neuronal cultures from the feline model of G_{M2} gangliosidosis, with subsequent degradation of G_{M2} ganglioside (Dobrenis et al., 1992). Covalent linkage of trimannosyl-dilysyl residues to acid β-glucosidase resulted in a four-fold increase in Kupffer cell uptake in rat liver

(Doebber et al., 1982). Enzyme replacement in a patient with glycogenosis type II with an α-glucosidase-LDL conjugate revealed some evidence of enzyme activity in muscle, but little evidence for glycogen catabolism (Williams and Murray, 1980). Loss of enzymatic activity following conjugation is a major problem with these approaches. Also, it is unclear whether endogenous levels of ligands or competitors can be reduced enough to make these systems efficient. Obviously, it is best if these types of manipulations can be avoided.

In the 1970's a series of ERT trials for Fabry disease was attempted. In the first study, normal plasma was infused into two hemizygotes (Mapes et al., 1970). α-Gal A activity could be detected in their circulation for approximately one week post-infusion, and plasma GL-3 levels were maximally reduced at a time coincident with maximal α-Gal A activity. Interestingly, the amount of restored α-Gal A activity present in the plasma was 30-fold greater than expected, though it is questionable why the assay was performed at pH 7.2. This questionable result, and the fact that normal plasma has very low α-Gal A activity, has led other investigators to conclude that infusion of plasma for the treatment of Fabry disease is not recommended (Rietra et al., 1974). The second study involved administration of plasma, enriched plasma, and two doses of human placental α-Gal A into Fabry hemizygotes (Brady et al., 1973). Infusion of normal plasma did not cause an increase in α-Gal A activity or a decrease in circulating GL-3 levels. Administration of normal platelets and leukocytes suspended in plasma caused a 68% increase in α-Gal A activity, which returned to preinfusion level by 24 hr. By 40 minutes post-infusion, GL-3 levels had decreased 46%, which also returned to preinfusion level by 24 hr. Another two Fabry hemizygotes recieved either 5 mg or 3 mg placental α-Gal A. The enzyme was rapidly cleared from the circulation and was able to effect a large decrease in circulating GL-3 levels in a dose-dependent fashion, which gradually returned to preinfusion levels 48 hr after injection. Two important observations were made. First, maximal disappearance of GL-3 from the circulation occurred at a time when all of the administered enzyme had been removed from the circulation. Second, no increase in the amount of circulating GL-2,

the immediate product of GL-3 catabolism, was detected. This suggested that the site of action of α-Gal A was extracirculatory. The third study involved administration of two glycoforms of α-Gal A which differed in their level of sialylation (Desnick et al., 1979). The first non-sialylated form, purified from human spleen, disappeared from the circulation with a $t_{1/2}$ of ~10 min, whereas the second, highly sialylated, form, purified from human plasma, had a $t_{1/2}$ of ~70 min. Following administration of splenic α-Gal A, a 50% decrease in the level of circulating GL-3 was noted by 15 min, followed by a return to preinfusion levels by 3 hr. In contrast, administration of plasma α-Gal A caused a 60% decrease in plasma GL-3 levels by 2 hr, which returned to preinfusion levels by 72 hr. It was concluded that the increased amount of sialic acid associated with plasma α-Gal A was responsible for its longer retention in circulation and subsquent greater effect on GL-3 metabolism. A later study revealed that a large amount of both enzymes was rapidly removed from circulation by the liver, although less plasma α-Gal A was recovered than its splenic counterpart (Bishop et al., 1981). Subsequently, a metabolic labeling study was performed to determine the source of the reaccumulating plasma GL-3 (Desnick et al., 1980). The appearance of unlabeled substrate following enzyme administration suggested that GL-3 may equilibrate across the membranes of cells in which it is stored. The results of this study led to the development of the "equilibration-depletion" hypothesis, which states that efforts to deplete accumulated substrate in the circulation may prevent progressive deposition in other tissues, including the vascular endothelium, or may allow equilibration of substrate in these tissues with the plasma (Desnick and Grabowski, 1981). Thus, enzyme administration which results in the depletion of circulating substrate may provide effective therapy without the necessity to deliver enzyme to the sites of pathology. A common finding in all three studies is that exogenous α-Gal A could safely reduce the levels of circulating GL-3, thereby possibly preventing further deposition in extrahepatic tissues or providing a gradient for equilibration of tissue GL-3 stores with plasma. Even though the reason for these decreases remains obscure, this suggested that plasma GL-3

levels may be a reliable indicator of the efficacy of ERT for Fabry disease, though natural fluctuations in glycosphingolipid levels in plasma led some investigators to suggest that changes in plasma accumulation during ERT may not be a good assessment of its effectiveness (Groth et al., 1980). An obvious shortcoming of all three studies is the lack of analyses on changes in tissue GL-3 levels following enzyme administration. Therefore, requisite 5 for ERT has not been achieved in Fabry disease. Any future trials of ERT for Fabry disease must include tissue GL-3 studies.

The success of ERT for Gaucher, a related LSD, shows what can be accomplished when most, if not all, of the requisites are met. The disease and the deficient enzyme involved, acid β-glucosidase, are well characterized. Large amounts of the enzyme have been purified from human tissue or overexpressed in CHO cells (Morales, 1996). A small percentage of patients do produce antibodies to exogenous acid β-glucosidase (Richards et al., 1993), but the clinical effect is generally negligible and easily treatable (Ponce et al., 1997). The enzyme has been modified by sequential deglycosylation to better target to the site of pathology, the reticuloendothelial system (Brady et al., 1994). Accurate clinical evaluation of the effectiveness of acid β-glucosidase replacement includes liver, spleen, erythrocyte, and plasma glucocerbroside levels (Brady et al., 1974), hemoglobin and platelet concentrations (Barton et al., 1991), serum protein levels (Beutler et al., 1980), organomegaly and skeletal changes (Barton et al., 1991), and immunological responses (Ponce et al., 1997). Studies performed in both mammalian cell culture (Sato and Beutler, 1993) and rodents (Bijsterbosch et al., 1996; Xu et al., 1996) revealed that although the enzyme is clinically effective, it is still targeted to many sites of nonpathology with a lower intrinsic activity than the dose administered. In fact, it appears that liver endothelial cells are the main site of uptake of acid β-glucosidase (Bijsterbosch et al., 1996). Therefore, even effective treatments can ultimately be made more clinically beneficial and cost-efficient.

The previous human trials of α-Gal A replacement, along with the success of ERT for Gaucher disease, suggests that this modality of therapy will be an effective treatment for Fabry disease. Fabry disease possesses many qualities which make it an attractive candidate for ERT, and the presence of a murine model and large quantities of pure recombinant human α-Gal A now allow us to analyze this form of treatment for Fabry disease. Therefore, characterization of the murine model of Fabry disease and assessment of the effectiveness of α-Gal A replacement is now possible.

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Chapter 1

Expression and Single-Step Purification of Escherichia Coli Verotoxin B-Subunit: Application to Globotriaosylceramide Quantitation and Fabry Disease

ABSTRACT

Verotoxins (VTs) are periplasmic *Escherichia coli*., belonging in the family of Shiga-like toxins (SLTs) toxins, are responsible for hemolytic uremic syndrome and hemorrhagic colitis. The cell surface receptor for this family of toxins is the glycosphingolipid, globotriaosylceramide (Galα1-4Galβ1-4GlcCer; GL-3), the natural substrate of human lysosomal α-galactosidase A (α-Gal A) which accumulates to high levels in the plasma of Fabry patients. The non-cytotoxic pentameric B subunit of VT binds to GL-3 on cell membranes, a function that is retained even in the absence of the cytotoxic A subunit. The gene for the B subunit of VT (VTB) was cloned and expressed at high levels in *E. coli*. Single-step purification of VTB was achieved by immunoaffinity chromatography using a monoclonal anti-Shiga-like toxin I IgG-agarose column. The purified protein was homogoneous and shown to be capable of binding GL-3, and thus was used to develop an enzyme-linked immunosorbent assay (ELISA) for the quantitation of GL-3 in normal and Fabry plasmas. This GL-3 ELISA is rapid, sensitive, and specific, compared to traditional methods of glycosphingolipid quantitation. This technique should prove useful in analyzing Fabry patient plasmas and for monitoring the effects of enzyme replacement therapy for Fabry disease.

INTRODUCTION

Escherichia coli verotoxins (VTs), also known as Shiga-like toxins (SLTs) because of their homology to *Shigella dysenteriae* toxin, are the toxins responsible for hemolytic uremic syndrome and hemorrhagic colitis (Lingwood, 1993). VTs are composed of two subunits: the cytotoxic A subunit (VTA) which inhibits protein synthesis (Obrig et al., 1985) and the non-toxic B subunit (VTB) which binds to the VT receptor (Ramotar et al., 1990). Crosslinking studies have shown that the B subunit pentamerizes and associates with a single A subunit (Donohue-Rolfe et al., 1984). The B subunit is also able to pentamerize and bind the VT receptor in the absence of the A subunit (Head et al., 1991). VT has been crystallized (Boodhoo et al., 1991; Hart et al., 1991; Stein et al., 1992), and important amino acid residues have been identified by site-directed mutagenesis (Jackson et al., 1990; Jemal et al., 1995; Perera et al., 1991; Tyrrell et al., 1992).

Using thin-layer chromatography (TLC) overlays, the primary VT receptor was identified as the glycosphingolipid (GSL) globotriaosylceramide (Galα1-4Galβ1-4GlcCer; GL-3) (Jacewicz et al., 1986; Lindberg et al., 1987; Lingwood et al., 1987). Loss of VT binding following digestion of GL-3 with α-galactosidase indicates that the terminal Galα1-4Gal linkage is essential for recognition (Lingwood et al., 1987). The GL-3 oligosaccharide alone is not bound by VT (Lindberg et al., 1987), nor is digalactosyldiglyceride (Lingwood et al., 1987), which contains the same carbohydrate moieties as GL-3 but linked to glycerol instead of ceramide. These observations suggest that the lipid moiety of GL-3, ceramide, is essential for recognition by VTB (Lingwood et al., 1987) and may orient the oligosaccharide for maximal binding (Lingwood, 1993). Recent studies propose that the binding of VTB to GL-3 occurs at two sites and relies on a protein-carbohydrate interaction for specificity and a protein-lipid interaction for tight adhesion (Nyholm et al., 1996; St. Hilaire et al., 1994).

The importance of GL-3 for VTB binding is further supported by observations that mutant cell lines deficient in GL-3 are not susceptible to VT toxicity (Cohen et al., 1987; Jacewicz et al., 1994; Pudymaitis et al., 1991; Waddell et al., 1990). These cell lines can be made VT sensitive by adding liposomes containing GL-3 to the culture medium (Waddell et al., 1990), confirming the specificty of this toxin for GL-3.

Fabry disease is an X-linked disorder which is caused by mutations in the gene encoding lysosomal α-Gal A (E.C.3.2.1.22) (Desnick et al., 1995). The primary substrate of α-Gal A is GL-3, which accumulates at high levels in the plasma and vascular endothelium of patients with Fabry disease, leading to cardiac and kidney failure and early demise in adulthood. Accumulation of GL-3 in the plasma occurs in the form of overloaded lipoproteins, principally the low-density-lipoprotein (LDL) particle, which leads to subsequent endothelial cell deposition. Currently, no treatment exists for Fabry disease, although it is an excellent candidate for enzyme replacement therapy (ERT) (Mapes et al., 1970). Studies with patient fibroblasts indicate that α-Gal A from various sources can be endocytosed and correct the metabolic defect (Dawson et al., 1973; Dooley and Applegarth, 1980; Hasholt et al., 1988; Mayes et al., 1982; Osada et al., 1987; Sifers et al., 1983; Tsuji et al., 1994), whereas three studies involving α-Gal A replacement in Fabry patients indicate that the enzyme can reduce the circulating levels of GL-3 (Brady et al., 1973; Desnick et al., 1979; Mapes et al., 1970). However, the lack of sufficient amounts of pure α-Gal A and a lack of a suitable animal model have precluded further analysis.

With the cloning of the α-Gal A gene (Bishop et al., 1986; Kornreich et al., 1989) and its overexpression in Chinese hamster ovary (CHO) cells (Ioannou et al., 1992), it has become possible to produce and purify large quantities of recombinant human α-Gal A (Ioannou, unpublished results). In addition, the recent cloning of the murine α-Gal A gene (Gotlib et al., 1996; Ohshima et al., 1995) and the generation of an α-Gal A-deficient mouse model (Ohshima et al., 1997; Wang, unpublished results) offer the possibility of an ERT trial for Fabry disease. One of the major assessments of ERT success in this disease

is a decrease in circulating plasma GL-3 levels (Desnick and Grabowski, 1981). Thus, an ELISA has been developed to accurately quantitate GL-3 in human plasma. This GL-3 ELISA is rapid, sensitive, and specific, and should prove useful in assessing ERT for Fabry disease.

MATERIALS AND METHODS

Antibodies

The anti-SLT hybridoma, 13C4, was obtained from the American Type Culture Collection (Rockville, MD; ATCC # CRL-1794) and was grown in CO_2-gassed roller bottles containing DMEM with 10% FCS, 2 mM glutamine, and 100 μg/ml gentamicin sulfate at 37° C for ~2 wk. The cells were pelleted at 5,000 x *g*, and the clarified supernates collected and concentrated on a DC 2 hollow fiber concentrator (Amicon, Beverly, MA) with a 50 kDa MW cutoff cartridge. The concentrated anti-SLT I IgG_1 antibodies were purified using a protein A column on the Fast Protein Liquid Chromatography (FPLC; Pharmacia, Piscataway, NJ) system and the Immunopure Mouse IgG_1 kit (Pierce, Rockford, IL). Eluted anti-SLT I IgG antibodies were concentrated and washed with phosphate-buffered saline (PBS), pH 7.4 using an Amicon (Beverly, MA) stirred cell concentrator with a 30 kDa MW cutoff membrane under positive nitrogen pressure. Antibody concentrations were estimated by ELISA using purified anti-SLT I IgG quantitated by the fluorescamine method (Bohlen et al., 1973), as modified previously (Bishop et al., 1978), with bovine serum albumin (BSA) as the standard. The purified antibody was stored at -20° C.

VTB Cloning and Expression

Genomic DNA from lyophilized *E. coli* strain 0157:H7 (ATCC # 43890) was isolated by phenol-chloroform extraction, precipitated in ethanol, and resuspended in 50 mM Tris-HCl, 1 mM EDTA, pH 8. All oligonucleotides were synthesized with phosphoramidite chemistry on a 380BDNA Synthesizer (Applied Biosystems, Foster City, CA) and purified by precipitation with 5 volumes of ice-cold ethanol in 10 mM $MgCl_2$. Polymerase chain reactions (PCR) were in 100 μl volumes and contained 20 mM Tris-HCl, pH 8.8, 10 mM KCl, 0.1% Triton X-100, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 200 μM of

each dNTP, 100 ng *E. coli* 0157:H7 genomic DNA, 1 μg of sense and antisense primers, and 2 U Vent DNA polymerase. The sense and antisense primers for VTB amplification were 5'GCGAATTCATATGTTGTGGGATTCATCCACTCTG-3' and 5'GCGAATTCAG ATCTCCGCCTGCTATTTTCACTG-3', respectively. These primers correspond to a previously published sequence of the *slt* I operon (Calderwood et al., 1987), and amplify the last 16 amino acids of VTA, the Shine-Dalgarno sequence of VTB, the complete coding region, and 31 bp of 3' untranslated region of VTB. The amplification reaction was performed on a RoboCycler (Stratagene; La Jolla, Ca) using the following program: one cycle of 3 min at 94° C; thirty cycles of 40 s at 94° C, 40 s at 55° C, and 1 min at 72° C; and one cycle of 2 min at 72° C. The resulting ~0.4 kb PCR product was subcloned and confirmed by sequencing. It was subsequently cloned into the *E. coli* expression vector pET-11a (Invitrogen, Carlsbad, CA) and used to transform *E. coli* strain BL21(DE3)pLysS (Studier, 1991). BL21 cells with the expression plasmid were grown overnight at 37° C in M9 minimal media (Sambrook et al., 1989) containing 50 μg/ml ampicillin. The culture was diluted 1:100 into 2 liters of fresh M9 media containing 0.1% casamino acids and 50 μg/ml ampicillin, grown at 37° C to an OD_{600} of ~0.5, and induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 3 hrs. Cells were washed with Tris-buffered saline (TBS), pH 8 and the periplasmic fractions isolated by extraction with 100 ml of TBS containing 1 mg/ml polymyxin B sulfate at 37° C for 1 hr (Donohue-Rolfe and Keusch, 1983). Extracts were stored at 4° C until purification.

Verotoxin Subunit B Purification

All chromatographic steps were performed on the FPLC system. A 5 ml anti-SLT I IgG antibody column was generated by coupling 25 mg anti-SLT I IgG to Affi-Gel 10 (Biorad, Hercules, CA) according to manufacturer's recommendations. The affinity column was equilibrated with TBS and 50 ml of clarified VTB extract was applied. Following washing with 10 column volumes of TBS, the bound protein was eluted with

0.1 M glycine, pH 2.5 into a solution of 1 M Tris-HCl, pH 8 for neutralization. The eluant was concentrated and washed with TBS using a stirred cell concentrator with a 3 kDa MW cutoff membrane. Next, concentrated VTB was applied to a 2.5 x 100 cm column of Superose 12 (Pharmacia) preequilibrated with PBS, pH 7.4. The column was run at 1 ml/min and the eluted protein was concentrated with a Centricon 3 microconcentrator (Amicon).

Immunoprecipitation, SDS-PAGE, and Fluorography

Immunoprecipitations were performed according to standard procedures (Sambrook et al., 1989). [^{35}S]-methionine-labeled cytoplasmic and periplasmic extracts were prepared (Studier and Moffatt, 1986) and VTB immunoprecipitated (Sambrook et al., 1989) using anti-SLT I IgG antibody and Protein G-Sepharose (Pharmacia, Piscataway, NJ). Immunoprecipitates were washed and subjected to SDS-PAGE (Laemmli, 1970) using precast 20% acrylamide PhastGels run on a PhastSystem (Pharmacia). Radioactive gels were fixed, incubated with Amplify (Amersham, Arlington Heights, IL), and exposed to XAR 5 film (Eastman Kodak, Rochester, NY). Non-radioactive gels were stained with Coomassie Brilliant Blue tablets and destained according to manufacturer's recommendations (Pharmacia). Isoelectric focusing (IEF) was performed on precast pH 3-9 gels on the PhastSystem. Gels were fixed with 20% trichloroacetic acid and stained with Coomassie Brilliant Blue.

Receptor-Mediated Binding

VTB binding to Vero cells was assessed similarly to a previous method (Donohue-Rolfe et al., 1989). Small-scale radiolabelled pET-VTB periplasmic extracts were concentrated with Centricon 3 microconcentrators (Amicon) and washed extensively with PBS, pH 7.4 to remove unincorporated [^{35}S]-methionine. Radiolabelled extracts of *E. coli* strain BL21(DE3)pLysS transformed with pET-11a served as controls for nonspecific

binding and internalization. The counts of the radiolabelled extracts were determined in 10 ml of scintillation fluid with a 1219 RACKBETA liquid scintillation counter (Wallac, Gaithersburg, MD). Various amounts of radiolabelled extracts were incubated with semi-confluent monolayers of Vero cells for 1 hr at 4° C. Cells were washed extensively with ice-cold 1 X PBS, pH 7.4 and lysed in ice-cold lysis buffer (1 X PBS, pH 7.4 containing 1% NP40, 1 mM EDTA, 0.02% NaN_3) . Aliquots of each were counted in scintillation fluid as above. Cells not incubated with radiolabelled VTB determined background.

Plasma Neutral Glycosphingolipid Extraction and Purification

Freshly drawn heparinized blood was centrifuged at 3,000 x *g* for 5 min at 4° C to obtain plasma and subsequently recentrifuged at 14,000 x *g* for 5 min at 4° C to remove any debris. Frozen normal and Fabry heterozygote and hemizygote plasma were extracted and purified using a modification of a previously described method (Vance and Sweeley, 1967). Fifty µl plasma was extracted with 20 volumes chloroform-methanol (C-M) 2:1 for 15 min at 37° C with rocking. The samples were centrifuged at room temperature for 5 min at 14,000 rpm. The supernates were partitioned against 1/5 volume water (Folch et al., 1957) at 4° C until the phases separated. Following centrifugation, the bottom phase was dried under nitrogen using a Techne sample concentrator and heat block (Princeton, NJ) and resuspended in 2 ml of chloroform. These were applied to 500 mg LiChrolut RP-18 columns (EM Separations, Gibbstown, NJ) equilibrated with 2 ml chloroform. The bound glycolipids were washed with 2 ml of chloroform and eluted with 2 ml acetone-methanol 9:1. The eluted neutral glycolipids were dried under nitrogen and resuspended in 2.5 ml of 100% ethanol.

GL-3 Enzyme-Linked Immunosorbent Assay (GL-3 ELISA)

The GL-3 ELISA was performed as follows: doubling dilutions of pure human Fabry kidney GL-3 in 100 µl of 100% ethanol (0.1-100 ng) were plated in duplicate on

Immunopure Polysorp 96-well plates (Nunc, Napersville, IL). Plates were gently shaken for 1 hr at room temperature, and the ethanol was subsequently evaporated at 37° C. The plates were washed once with 400 µl TBS, then 400 µl blocking solution (5% BSA in TBS, pH 8) was added to each well and incubated at 37° C for 1 hr. All subsequent reagent additions were performed in a 100 µl volume. Plates were washed once with 400 µl TBS, incubated with purified VTB (0.2 µg/well) in 0.1% BSA-TBS at 37° C for 1 hr, and washed three times as above. Following incubation with anti-SLT I IgG antibody (0.5 µg/well) in 0.1% BSA-TBS at 37° C for 1 hr, they were washed and incubated with alkaline phosphatase-conjugated goat anti-mouse IgG (Boehringer Mannheim, Indianapolis, IN), diluted 1:1000 in 0.1% BSA-TBS at 37° C for 1 hr. Plates were washed and developed with *p*-nitrophenyl phosphate (*p*NPP) solution (100 µl/well) (Kirkegaard and Perry Laboratories, Gaithersburg MD) at 37° C for 15-30 min. Reactions were terminated by the addition of 100 µl 5% EDTA, pH 8 and gentle shaking. One hundred microliters of each reaction was removed to a clean plate and read at 405 nm on an ELX800 automated ELISA reader (Bio-Tek Instruments, Wisnooski, VT).

For the quantitation of human plasma GL-3, 100 µl of resuspended plasma glycolipids (equivalent to 2.5 µl of plasma) from normal, Fabry heterozygote, and Fabry hemizygote individuals was dried in duplicate on microtiter plates as above. Doubling dilutions of pure human Fabry kidney GL-3 were dried on the same plate in duplicate to provide a concentration standard. A scatter plot of average OD_{405} vs amount of pure GL-3 was used to generate a standard curve by linear regression and estimate GL-3 concentration in the various averaged plasma samples.

High-Performance Thin Layer Chromatography (HPTLC)

HPTLC was performed on 10 x 10 cm silica gel 60 plates (EM Separations) to visually confirm the results of the human plasma GL-3 ELISA. Neutral glycolipids were extracted from 1 ml of plasma from a representative Fabry heterozygote and hemizygote

individual and purified by scaling up the extraction procedure. Samples were resuspended in chloroform-methanol 2:1 and applied as thin streaks to the plates, which were developed in a closed atmosphere of chloroform-methanol-water 100:42:6 (Vance and Sweeley, 1967) for ~ 30 min. Purified glycosphingolipids (Sigma, St. Louis, MO and Supelco, Bellefonte, PA) were used as migration standards. Following separation, neutral glycosphingolipids were visualized with an acidic 0.5% ethanolic orcinol/1% ferric chloride solution for 5-10 min at 100° C.

The identity of the GL-3 band was confirmed by digestion of Fabry homozygote plasma glycolipids with recombinant human α-Gal A (Ioannou et al., 1992) prior to HPTLC as follows: 75 µl 0.5% sodium taurocholate was dried with the eluted glycolipids under nitrogen and resuspended in 80 µl 0.1 M acetate buffer, pH 3.9. The lipid-detergent mixture was sonicated for 2 min and 10 µl 1 mg/ml BSA in water was added followed by 10 µl ($5x10^4$ U; ~25 µg) recombinant human α-Gal A or 10 µl of water. The samples were incubated at 37° C for 24 hrs. Following incubation, the samples were extracted with 700 µl chloroform-methanol-water 2:4:1, 200 µl water, and 200 µl chloroform. The bottom layers were dried under nitrogen and HPTLC and staining performed as above.

RESULTS

Cloning, Expression, and Purification of VTB

Based on the sequence of the *slt* I operon from *E. coli* phage H19B (Calderwood et al., 1987) and potential translational coupling between VTA and VTB (Calderwood et al., 1990), the VTB gene was PCR amplified to include the VTA stop codon and VTB Shine-Dalgarno sequence. Following sequence confirmation, VTB was cloned into the *E. coli* expression vector pET-11a to generate pET-VTB. The expression construct was introduced into the *E. coli* strain BL21(DE3)pLysS. A positive clone was isolated, grown in M9 media until its OD_{600} reached ~0.6, and induced with IPTG. The culture was radiolabeled with [^{35}S]-methionine, and the extracts were immunoprecipitated with anti-SLT I IgG monoclonal antibody. SDS-PAGE of the immunoprecipitated extracts indicated that VTB was expressed and correctly targeted to the *E. coli* periplasmic space (Fig.1) (Donohue-Rolfe and Keusch, 1983). In addition, radiolabeled periplasmic extracts were used in binding studies with Vero cells. As shown in Fig. 2, the binding was saturable, suggesting that VTB was binding to its receptor, presumably GL-3. SDS-PAGE of Vero cell lysates following binding revealed the presence of a single band with a migration distance consistent with VTB (data not shown).

A two liter culture of *E. coli* harboring the pET-VTB plasmid was used to produce recombinant VTB. The protein was recovered from the periplasmic space fraction as described in Materials and Methods, with a maximal yield of active VTB protein of approximately 2 mg/L culture, similar to a previous report (Boulanger et al., 1994). Single-step purification was achieved using an anti-VTB monoclonal antibody affinity column. The resulting preparation was essentially homogeneous (Fig. 3), with a yield of >90%. Following purification, recombinant VTB migrated as a 5 kDa polypeptide on SDS-PAGE (Fig. 3), with a pI of 5.7 by isoelectric focusing gel electrophoresis (data not shown) (Ramotar et al., 1990). Gel filtration analysis of purified recombinant VTB

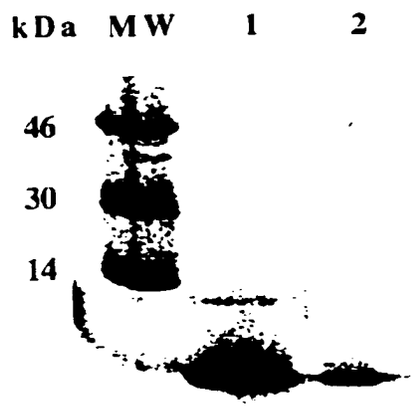

Figure 1. Localization of recombinant VTB in the periplasmic space of *E. coli*. MW, molecular weight standards; 1, periplasmic fraction; 2, cytosolic fraction.

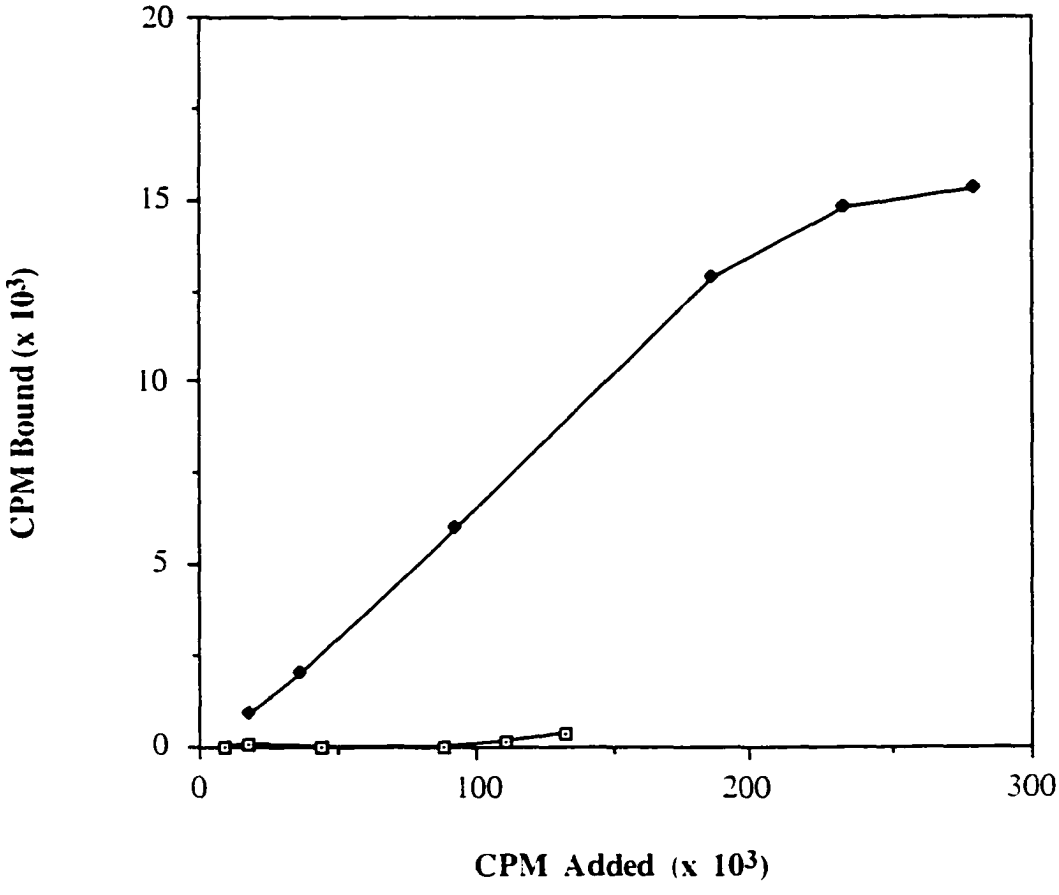

Figure 2. Binding of recombinant VTB to Vero cells. Each data point represents one incubation. Squares, periplasmic space of *E. coli* transformed with pET-11a; diamonds, periplasmic space of *E. coli* transformed with pET-VTB.

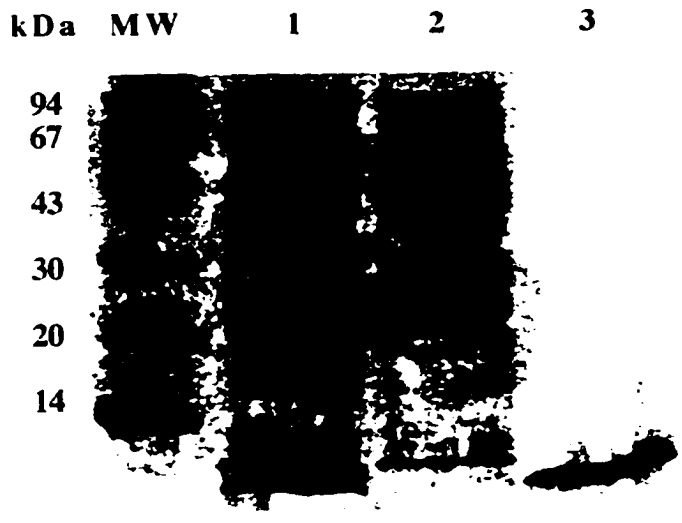

Figure 3. Immunoaffinity chromatography purification of recombinant VTB. MW, molecular weight standards; 1, crude periplasmic fraction; 2, column flow-through; 3, column eluate.

resulted in a recombinant protein complex that eluted with a molecular weight of approximately 25 kDa (data not shown), consistent with pentamerization (Donohue-Rolfe et al., 1984).

GL-3 ELISA

Utilizing the high affinity and specificity of VTB for GL-3, an ELISA was developed to quantitate plasma and tissue GL-3 using recombinant VTB. The detection of GL-3 was linear over a wide range (0.1-50 ng of GL-3) (Fig. 4). The assay was highly reproducible, had a very low background ($\leq$ 0.01 abs. units), and showed little standard deviation existed between the individual assays. The specificity of the VTB/GL-3 interaction in this assay was further analyzed by measuring the interaction of recombinant VTB with various GL-3 related lipids by ELISA. One hundred nanograms globotetraosylceramide (GL-4), lactosylceramide (GL-2), glucosylceramide (GL-1), disialoganglioside G_{D3}, sphingomyelin, phosphotidylcholine, cholesterol, and GL-3 were dried in duplicate on a 96-well plate and probed with VTB. As expected, VTB did not interact with GL-2, GL-1, GD3, sphingomyelin, phosphotidylcholine, or cholesterol (data not shown). However, at high amounts (>25 ng), GL-4 was recognized weakly by VTB.

The affinity of VTB for GL-4 was examined further by plating doubling dilutions of GL-3 and GL-4 and performing the ELISA. As shown in Fig. 5, the affinity of VTB for GL-3 is linear and is maximal at about 50 ng, whereas its affinity for GL-4 is much lower, being greatly decreased at 25 ng compared to GL-3 and essentially undetectable at 12.5 ng. The specificity of the VTB/GL-3 interaction was further confirmed by digesting 100 ng GL-3 with $5x10^4$ U of recombinant human α-Gal A prior to performing the ELISA. This amount of enzyme completely converts GL-3 to its product GL-2, as assessed by TLC (see Fig. 8B). Therefore, removal of the terminal α-galactosyl moiety completely abolished recognition by VTB, confirming the specificity of VTB for GL-3.

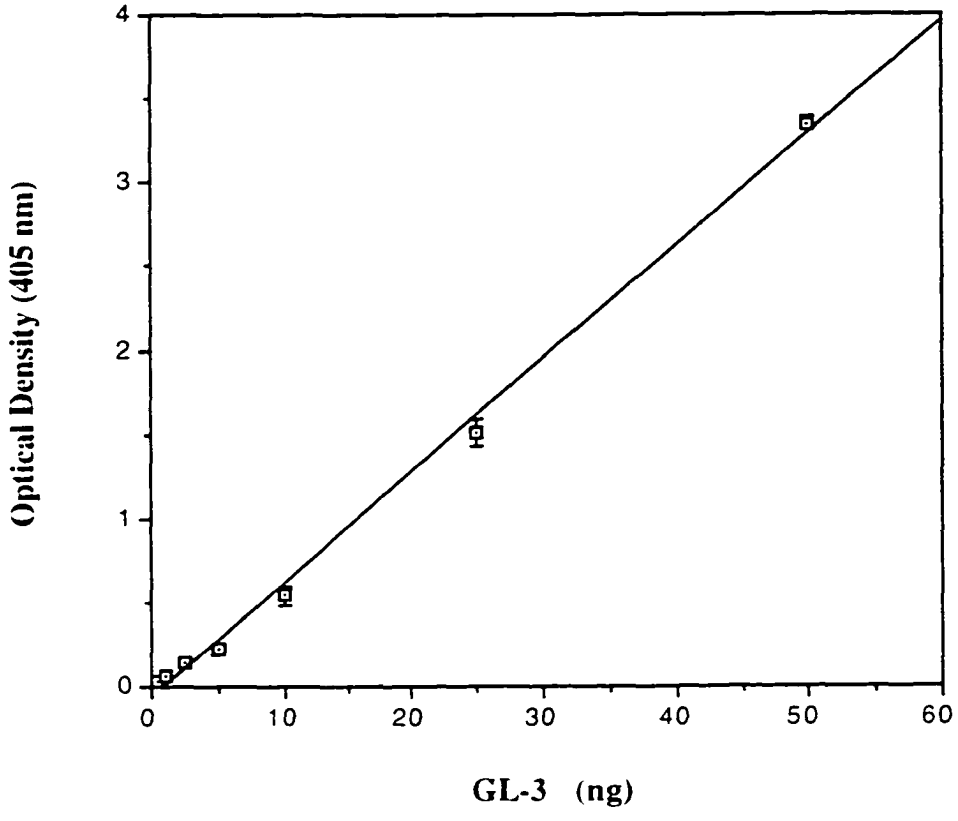

Figure 4. Reproducibility of GL-3 ELISA. Data points represent the mean ± SD of five independent assays.

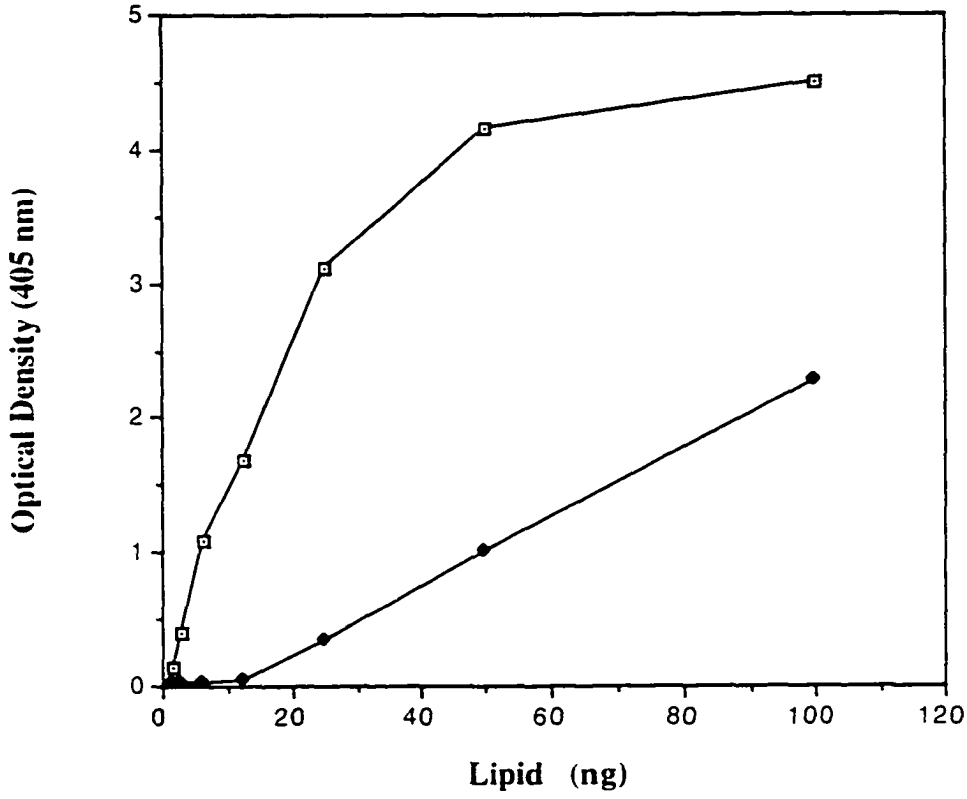

Figure 5. Comparison of affinities of recombinant VTB for GL-3 and GL-4. Data points represent the mean of duplicate wells. Squares, GL-3; diamonds, GL-4.

Since the extraction and purification protocols utilized in this sudy do not selectively isolate GL-3 from human plasma, but rather enrich for glycosphingolipids, their influence on the VTB/GL-3 interaction was analyzed by ELISA. Doubling dilutions of GL-3 were assayed in parallel with dilutions of GL-3 containing 8 ng GL-1, 5 ng GL-2, and 2.5 ng GL-4. These amounts of non-reactive GSLs are similar to those found in 2 µl human plasma (Clarke et al., 1976; Dawson et al., 1976; Vance et al., 1969; Vance and Sweeley, 1967), the equivalent of which is used for the ELISA (see below). At these concentrations, the non-reactive GSLs had a negligible effect, if any, on the interaction between VTB and GL-3 (Fig. 6).

Quantitation of Human Plasma GL-3

As the interaction between VTB and GL-3 was specific and reproducible, this ELISA was used to quantitate GL-3 extracted from human plasma. Plasma spiking experiments with a fluorescently-labeled GL-3 (lissamine rhodamine-GL-3; LR-GL-3) revealed that approximately 85% of the LR-GL-3 over a range of 0.025-2.5 µg was consistently recovered from plasma using the extraction and purification protocol described in the Materials and Methods. This extraction protocol was subsequently evaluated for reproducibility by performing the procedure on three 50 µl aliquots of human Fabry plasma and quantitating the recovered GL-3 by ELISA. The values obtained for the three GL-3 concentrations were 10.60, 10.45, and 9.45 µg/ml (10.17 ± 0.63 µg/ml), indicating that the extraction and quantitation of GL-3 from human plasma was reproducible. Therefore, ten normal, ten Fabry hemizygote, and four Fabry heterozygote plasma samples were assayed for their GL-3 levels. The average Fabry plasma GL-3 concentration (6.65 ± 2.35 µg/ml; 3.11-10.04 µg/ml) was ~7-fold higher than that for normal plasma (0.91 ± 1.13 µg/ml; 0.050-3.71 µg/ml) (Fig. 7). The GL-3 concentrations in the Fabry heterozygotes were quite variable (1.38 ± 1.94 µg/ml; 0.16-4.89 µg/ml) (Fig. 7), presumably due to random X-chromosome inactivation (Lyon, 1961), although the average is intermediate

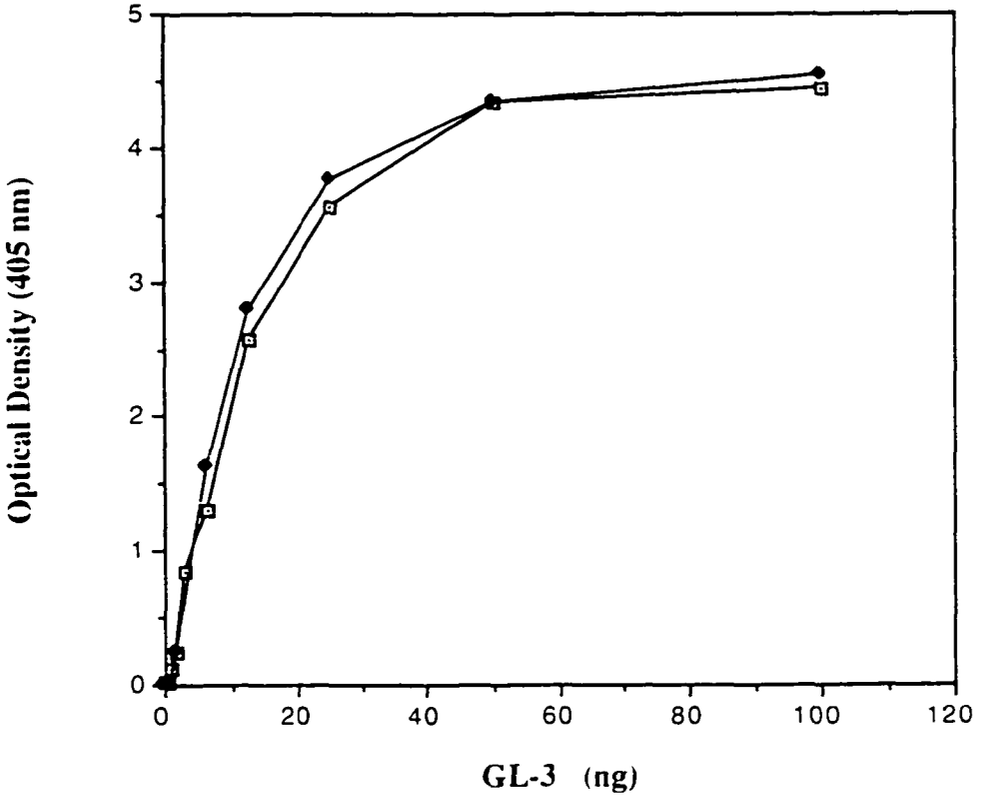

Figure 6. Effect of non-reactive glycosphingolipids on the affinity of recombinant VTB for GL-3. Data points represent the mean of duplicate wells. Squares, GL-3; diamonds, GL-3 + nonreactive GSLs.

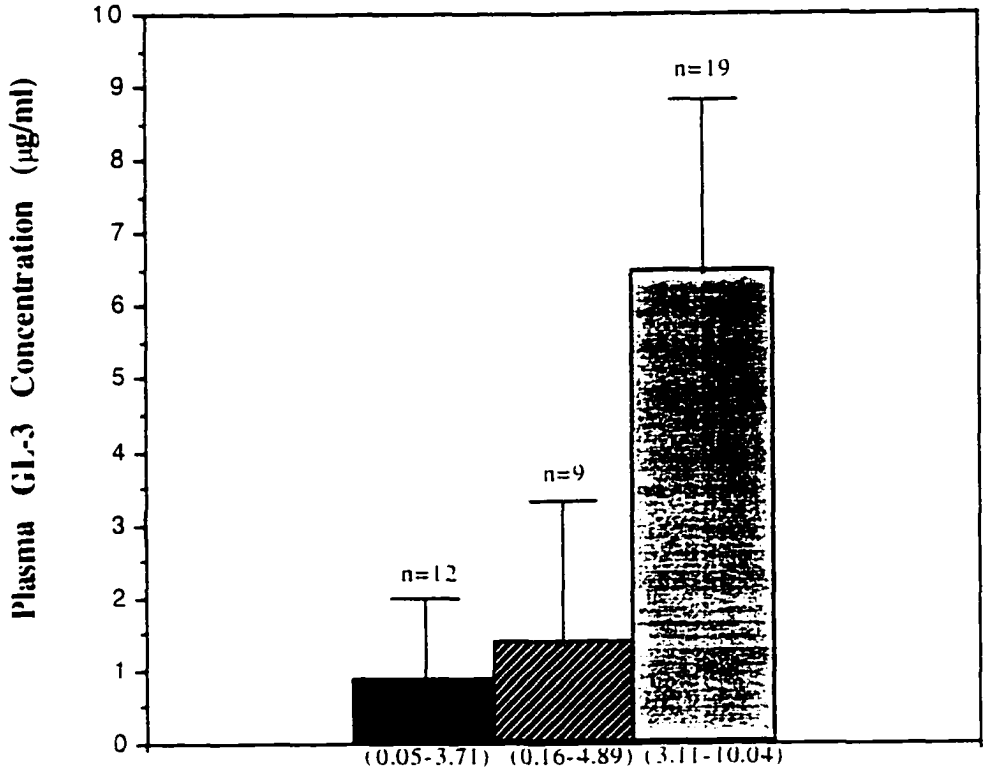

Figure 7. Quantitation of human plasma GL-3 levels using GL-3 ELISA Black column, normal; hatched column, Fabry heterozygote; gray column, Fabry hemizygote. Value represent means ± SD. Ranges are shown in parentheses.

between normal and Fabry hemizygote plasma. These results are in agreement with previous reports of GL-3 concentrations in the plasma of Fabry hemizygotes and heterozygotes (Brady et al., 1973; Clarke et al., 1976; Dawson et al., 1976; Mapes et al., 1970; Vance et al., 1969).

The results of the ELISA were qualitatively confirmed by HPTLC of representative plasma samples (Fig. 8A). Orcinol staining revealed a large increase in the GL-3 band between a Fabry hemizygote and an asymptomatic heterozygote (~10 μg GL-3/ml plasma vs. ~0.2 μg GL-3/ml plasma, respectively). Disappearance of this band in the Fabry sample, following digestion with α-Gal A, with a coinciding increase in the GL-2 band, confirmed its identity as GL-3 (Fig. 8B).

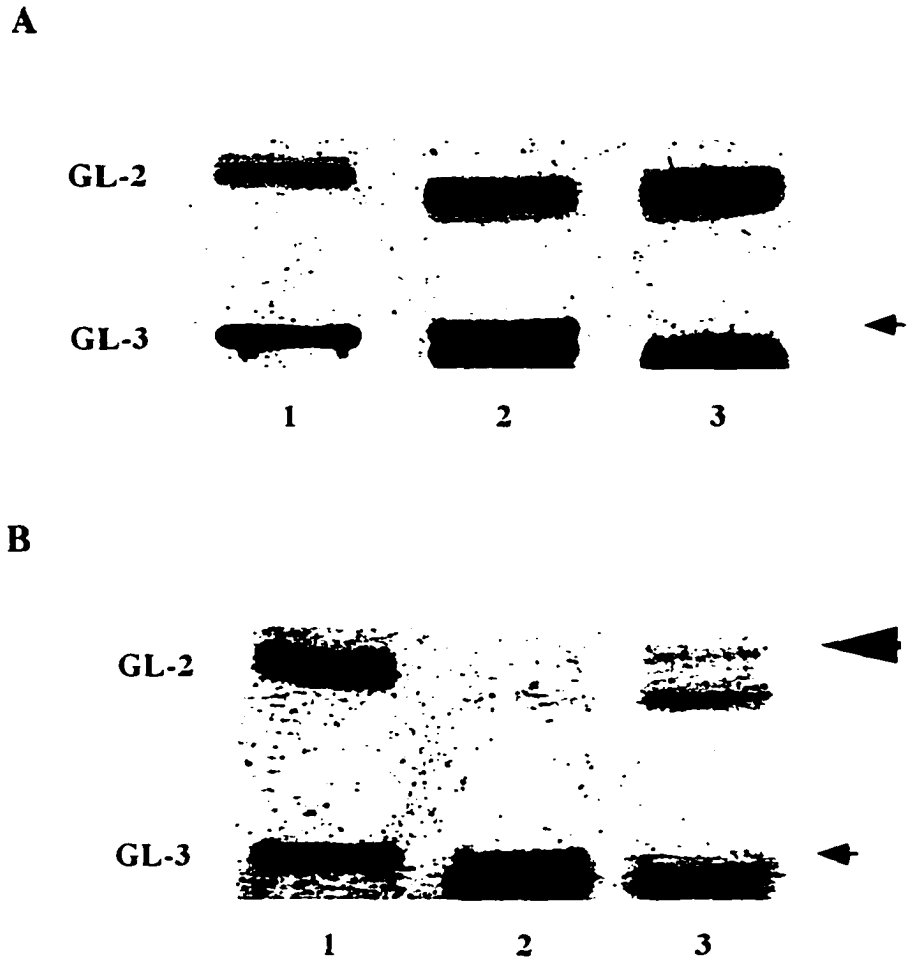

Figure 8. HPTLC of neutral GSLs purified from human plasma. A, untreated GSLs: 1, GSL standards; 2, GSLs from Fabry hemizygote plasma; 3, GSLs from normal plasma. B, GSLs treated with α-Gal A: 1, GSL standards; 2, GSLs from Fabry hemizygote plasma; 3, GSLs from 2 digested with $5x10^4$ U of α-Gal A. Small arrowheads denote position of GL-3; large arrowhead denotes position of GL-2.

DISCUSSION

Previous studies using Fabry patient fibroblasts indicated that α-Gal A from various sources could be endocytosed and correct the metabolic defect (Dawson et al., 1973; Dooley and Applegarth, 1980; Hasholt et al., 1988; Mayes et al., 1982; Osada et al., 1987; Sifers et al., 1983; Tsuji et al., 1994). Three studies involving α-Gal A replacement in Fabry patients indicated that the enzyme could reduce the circulating levels of GL-3 (Brady et al., 1973; Desnick et al., 1979; Mapes et al., 1970), although the lack of sufficient amounts of pure α-Gal A precluded further analysis. With the cloning of the human α-Gal A cDNA (Bishop et al., 1986) and its subsequent high-level expression in CHO cells (Ioannou et al., 1992), and the production of an α-Gal A-deficient mouse model (Ohshima et al., 1997; Wang et al., unpublished results], larger-scale ERT trials for Fabry disease have now become possible.

These trials will necessitate examining numerous plasma samples for changes in circulating substrate levels, as this will probably be the major indication that ERT for Fabry disease is successful. Current methods for quantitating GSLs, such as TLC, TLC immunoblotting, TLC immunostaining, HPLC, and GLC are either not sensitive enough or are too time-consuming to be of practical value in these types of trials. For example, TLC immunostaining often involves treating the plates with polyisobutylmethacrylate, a chemical which recently has been shown to induce artifactual binding of VT to GL-4, (Yiu and Lingwood, 1992), to prevent loss of silica. TLC immunoblotting avoids this problem, but transfer of glycolipids to nitrocellulose (Towbin et al., 1984) or polyvinylidene difluoride membranes (Taki et al., 1994) is less than complete. Therefore, an ELISA was developed to quantitate the accumulated substrate in Fabry disease, GL-3, using VTB as the detection molecule.

Expression of VTB was achieved using an inducible *E. coli* expression vector. High levels of recombinant VTB were easily obtained following induction with IPTG, and

were similar to those previously reported for recombinant VT (Boulanger et al., 1994; Calderwood et al., 1990; MacLeod and Gyles, 1990). Subsequent purification of VTB was achieved in a single step by immunoaffinity chromatography using the anti-SLT I IgG monoclonal antibody 13C4 coupled to an agarose matrix. Immunoaffinity chromatography using a polyclonal antibody has previously been used to purify VT in a single step (O'Brien et al., 1980), although generally this method is part of a larger purification scheme (Downes et al., 1988; MacLeod and Gyles, 1990; O'Brien and LaVeck, 1983). The protein was essentially pure following immunoaffinity chromatography, but most importantly, the polymyxin B sulfate, used to isolate the *E. coli* periplasmic fraction containing VTB, was removed from these preparations. This is critical, as polymyxin B was previously demonstrated to decrease the affinty of VT for GL-3, while producing an artifactual affinity for GL-4 (Head et al., 1990). Previously, single-step affinity purifications of VT were difficult, as they were achieved by chromatography using P1 glycoprotein (Donohue-Rolfe et al., 1989) and GL-3-silicic acid (Boulanger et al., 1994), matrices which are difficult to produce or obtain.

Based on physical properties, such as molecular weight and pI, and biochemical properties, such as receptor binding, cellular endocytosis, and immunolocalization (see Chapter 2), it was concluded that the VTB expressed and purified in this study was physically correct and biochemically functional and could be used for the development of a GL-3 ELISA. VTB was chosen as the detection molecule over the holotoxin VT1 for several reasons: 1) VTB expression in this study was consistently higher than VT1 (data not shown); 2) VTB can bind GL3 as efficiently as VT1 (Head et al., 1991); and 3) VTB has no *in vivo* cytotoxicity (Donohue-Rolfe et al., 1989). An anti-GL-3 antibody could have been used as the detection molecule (Clarke and Embil, 1979; Oosterwijk et al., 1991), but most antiglycolipid antibodies are of the IgM subtype and therefore of low affinity (Lingwood, 1993). Also, they often recognize only the carbohydrate moieties (Frey II et al., 1993) and therefore may cross-react with other molecules (McAlarney et al.,

1995). In addition, they may behave anomalously on solid supports (Frey II et al., 1993). A lectin recognizing terminal α-galactosyl moieties could also have been used as the detection molecule, although that would only recognize the terminal sugar and therefore would not be specific for GL-3 (Molin et al., 1986).

The ELISA developed in this study is sensitive (0.1 ng GL-3), rapid (100 samples analyzed in ~ 6 hrs) and highly reproducible. The ELISA is also specific for GL-3, as VTB did not interact with other lipids known to reside in lipoproteins (Chatterjee and Kwiterovich, 1984; Clarke et al., 1976; Dawson et al., 1976; Vance and Sweeley, 1967). GL-4 was recognized to a limited extent by VTB, though this affinity was avoided at low concentrations of the GSL. Digestion of GL-3 with recombinant human α-Gal A prior to ELISA abolished recognition by VTB, which indicates that the digested GL-3 resulting from successful ERT for Fabry disease will result in lower ELISA values. The influence of other GSLs had little effect on the VTB/GL-3 interaction as assessed by ELISA, which is important since the extraction and purification protocols utilized in this study do not purify GL-3 from human plasma, but rather enrich for GSLs.

Based on these results, the ELISA was subsequently used to quantitate GL-3 from human plasma. Analysis of 3 aliquots of a Fabry plasma sample yielded similar GL-3 concentrations, indicating that the extraction and purification schemes used in this study are reproducible. The sensitivity of the ELISA allowed very small amounts of plasma (2.5 μl) to be assayed, thereby ensuring that the small amounts of GL-4 present in this volume (Clarke et al., 1976; Dawson et al., 1976; Vance et al., 1969; Vance and Sweeley, 1967) would not artifactually interact with VTB.

The concentrations of GL-3 obtained by this method were similar to those obtained by more traditional methods (Brady et al., 1973; Clarke et al., 1976; Dawson et al., 1976; Mapes et al., 1970; Vance et al., 1969) For example, Vance et al. (1969) determined the GL-3 concentration in Fabry hemizygote plasma to be 7.6 ± 2.1 μg/ml (3.8-14.6 μg/ml) by GLC, whereas the concentration was determined to be 6.65 ± 2.35 μg/ml (3.11-10.04

μg/ml) using the VTB ELISA. The GL-3 concentration in normal plasma was determined to be 2.1 ± 0.7 μg/ml by GLC, whereas a value of 0.91 ± 1.13 μg/ml (0.050-3.71 μg/ml) was obtained by VTB ELISA. Female heterozygote plasma was found to contain 4.5 μg/ml by GLC, wheras the concentration as determined by VTB ELISA was 1.38 ± 1.94 μg/ml (0.16-4.89 μg/ml), an amount intermediate between that of normal and Fabry hemizygote plasma. The variable amount of GL-3 in the plasma of the four heterozygotes is presumably due to random X-chromosome inactivation (Lyon, 1961); this may also be the cause of the variable amounts of GL-3 in the heart, liver, and kidney of the four heterozygotes analyzed in an earlier study (Hozumi et al., 1990). The increase in GL-3 concentration in Fabry hemizygote plasma over normal plasma in the previous studies was ~3-4-fold, similar to the ~7-fold increase obtained in this study.

The rapid, sensitive, and specific ELISA described in this communication should prove useful for the assessment of ERT and gene therapy (Medin et al., 1996; Novo et al., 1997; Sugimoto et al., 1995) for Fabry disease. It may also be useful in examining the role of GL-3 in various biological processes and disorders, such as cell growth (Pudymaitis and Lingwood, 1992), B cell differentiation (Maloney and Lingwood, 1994) and apoptosis (Mangeney et al., 1993), cancer (Li et al., 1986; Mannori et al., 1990), α-interferon signaling (Cohen et al., 1987; Ghislain et al., 1994; Lingwood and Yiu, 1992), interleukin-1β, tumor necrosis factor-β, and hemolytic uremic syndrome (Harel et al., 1993; Kaye et al., 1993; Van de Kar et al., 1992), and familial dysautonomia (Strasberg et al., 1992). Similarly, ELISAs using other toxins could also be developed to quantitate minor accumulated substrates in Fabry disease. For example, the receptor for staphylococcal enterotoxin-B has recently been identified as digalactosylceramide (Chatterjee et al., 1995), which also accumulates in Fabry disease (Desnick et al., 1995).

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Chapter 2

Characterization of a Mouse Model for Fabry Disease Generated by Targeted Disruption of the Murine α-Galactosidase A Gene

ABSTRACT

The deficiency of α-galactosidase A (α-Gal A) in humans results in Fabry disease, an X-linked recessive lysosomal storage disorder. In order to characterize the pathophysiology of the disease and to evaluate potential treatment modalities such as enzyme replacement and gene therapies, a mouse model of α-Gal A deficiency was generated by targeted disruption of the α-Gal A gene in mouse embryonic stem (ES) cells. Tissues from adult hemizygous males and homozygous females for the null allele had no detectable α-Gal A activity and normal to elevated levels of a related enzyme, α-Gal B. Cultured skin fibroblasts from affected adult animals were unable to hydrolyze the natural α-Gal A glycosphingolipid substrate, globotriaosylceramide (GL-3), although some detergent-containing hydrolysis was detected using cell extracts in an *in vitro* assay. Adult α-Gal A-deficient mice had elevated levels of GL-3 in all tissues analyzed, including liver, spleen, heart, kidney, skin, and plasma, and this GL-3 accumulation was apparently age-dependent. Cultured skin fibroblasts from α-Gal A-deficient mice contained significantly higher levels of lysosomal GL-3 than cells from wild-type mice, although both cell lines lacked GL-3 in their plasma membranes. These α-Gal A-deficient animals provide an excellent model for evaluation of enzyme replacement therapy (ERT) for Fabry disease by allowing the determination of the effects of recombinant enzyme administration on GL-3 storage and reaccumulation.

INTRODUCTION

α-Galactosidase A (α-Gal A; E.C. 3.2.1.22) is the lysosomal glycosidase which hydrolyzes the terminal α-galactosyl moieties from glycolipids and glycoproteins (Desnick et al., 1995). The mature human enzyme has been biochemically characterized, and shown to be a homodimeric glycoprotein with a subunit molecular weight of about 48 kDa (Bishop and Desnick, 1981). The enzyme precursor is co-translationally glycosylated and modified in the Golgi complex and lysosomes to the mature polypeptide, which dimerizes to form the active 101 kDa lysosomal glycoprotein (LeDonne et al., 1983; Lemansky et al., 1987).

The deficient activity of α-Gal A results in Fabry disease, an X-linked recessive lysosomal storage disorder characterized by the accumulation of the enzyme's glycosphingolipid substrates, principally globotriaosylceramide (GL-3), in the plasma and vascular endothelium (Desnick et al., 1995). Recent studies reveal a variety of mutations from unrelated Fabry families (Eng and Desnick, 1994; Eng et al., 1994; Eng et al., 1993), which include point mutations, splicing mutations, small insertions and deletions, and gene deletions and rearrangements.

In classically affected hemizygous males, the major disease manifestations include angiokeratoma, acroparathesias, hyphohydrosis, corneal dystrophy, and vascular disease of the heart, kidneys, and brain leading to early demise in adulthood (Desnick et al., 1995). In heterozygous females, the manifestations are quite variable, and can range from mild to severe, presumably due to random X-chromosome inactivation (Lyon, 1961). The most common manifestation in these females is corneal opacity (Desnick et al., 1995). Some of the pathophysiology of the disease, such as the pattern and origin of the glycosphingolipid deposition, remains unknown.

At present, there is no treatment for Fabry disease. Recent experience with Gaucher disease (Brady et al., 1994), a related lysosomal glycosphingolipid storage disorder, suggests that enzyme replacement therapy may be an effective treatment for

Fabry disease. Previous enzyme replacement trials for Fabry disease (Brady et al., 1973; Desnick et al., 1979; Mapes et al., 1970) also indicated that this modality of treatment may be effective in treating the disease, although the lack of sufficient quantities of pure α-Gal A precluded further analysis. The human α-Gal A cDNA and genomic sequences have been isolated and characterized (Bishop et al., 1986; Bishop et al., 1988; Kornreich et al., 1989) and used to produce recombinant human α-Gal A in Chinese hamster ovary cells (Ioannou et al., 1992).

The availability of the mouse cDNA and genomic sequences (Gotlib et al., 1996; Ohshima et al., 1995) made it possible to develop a mouse model for α-Gal A deficiency by homologous recombination in embryonic stem (ES) cells (Ohshima et al., 1997; Wang, unpublished results). This model should increase the understanding of the pathophysiology of Fabry disease and allow various modalities of treatment, such as enzyme replacement therapy and gene therapy, to be evaluated.

MATERIALS AND METHODS

Mice

α-Gal A-deficient mice were produced by homologous recombination in 129/Sv ES cells using a replacement-type vector containing the neomycin-resistance gene for positive selection and the thymidine kinase gene for negative selection (Wang, unpublished results). Male mice, chimeric for the null α-Gal A allele , were bred with wild-type C57BL/6 females, and offspring screened for transmission of the null allele (Wang, unpublished results). Heterozygous F_1 females were identified by Southern hybridization analysis (Wang, unpublished results). These germ-line transmitting male chimeras were then bred with wild-type 129/Sv females to facilitate enzymatic and glycolipid analysis (Wang, unpublished results). Following Southern hybridization analysis, heterozygous females were bred with 129/Sv males to generate α-Gal A-deficient males. Hemizygous males and heterozygous females were bred to generate homozygous females (Wang, unpublished results), and these animals were used to maintain the colony. All mice analyzed in this study were adults and weighed between 20-30 g. α-Gal B-deficient mice were produced and maintained in a similar fashion (Wang, unpublished results) and were crossed with α-Gal A-deficient mice to produce a double knockout line (Wang, unpublished results). Wild-type 129/Sv mice were obtained from Taconic (Germantown, NY). Mice were given standard mouse chow and water*ad libidum*.

Cell Culture

Green monkey kidney (Vero) cells were obtained from American Type Tissue Culture (ATCC # CRL-1586, Rockville, Md.) and maintained in Dulbecco's Modified Eagles Medium (DMEM) containing 10% fetal calf serum (FCS), 2 mM glutamine, and 100 μg/ml gentamicin sulfate (Gibco BRL, Grand Island, NY). Chinese hamster ovary (CHO) DG44 cells were obtained from Dr. Larry Chasin (Columbia University, New

York, NY) and maintained in DMEM containing 10% FCS, 2 mM glutamine, 100 μM sodium hypoxanthine, 16 μM thymidine, and 100 μg/ml gentamicin sulfate. Human and mouse fibroblasts were obtained by skin biopsies from adult male individuals and maintained in DMEM with 10% FCS, 2 mM glutamine, and 100 μg/ml gentamicin sulfate. Cell lines were maintained at 37° C and 5% CO_2.

Tissue Collection and Homogenization

Blood samples from adult mice were obtained by retro-orbital eye bleeds using heparinized microhematocrit capillary tubes. Samples were centrifuged to obtain the supernatant plasma, which was either assayed immediately for enzyme activities or stored at -20° C for lipid analysis. Leukocytes were separated from red blood cells with the Puregene kit (Gentra Systems, Inc., Minneapolis, MN) according to manufacturer's recommendation. White blood cell pellets were lysed in ice-cold lysis buffer (citrate phosphate buffer, pH 4.6, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 0.02% sodium azide) for enzymatic analysis. Confluent mouse fibroblast cultures in 100 mm dishes were removed by trypsinization, washed with 0.9% saline, and lysed in ice-cold lysis buffer for enzymatic analysis.

For harvesting of organs, adult mice were anesthetized by intramuscular injection of 50 μl ketamine solution (Sigma, St Louis, MO, 100 mg/ml) and sacrificed by perfusion with 50 ml 0.9% saline. The organs were removed using clean, alcohol-disinfected surgical tools, snap-frozen on liquid nitrogen, and stored at -20° C prior to homogenization. For enzymatic analysis, freshly thawed tissue samples were homogenized on ice in buffer (citrate-phosphate buffer, pH 4.6, 0.15% Triton X-100) using a Tissue Tearor (Biospec Products, Inc., Bartlesville, OK), setting #3, for 2 min. Extracts were clarified by centrifugation at 14,000 x g for 5 min at 4° C. All samples were kept on ice until assayed for α-Gal A or α-Gal B activities.

Enzyme and Protein Assays

α-Gal A activity was determined artificially with 5 mM 4-methylumbelliferyl-α-D-galactopyranoside (4MU-α-Gal) containing 117 mM *N*-acetylgalactosamine (GalNAc) at pH 4.6, as previously described (Bishop et al., 1980). α-Gal B activity was determined using 1.25 mM 4-methylumbelliferyl-α-*N*-acetylgalactopyranoside (4MU-α-GalNAc) at pH 3.9, as previously described (Schindler et al., 1989). One unit (U) of α-Gal A and α-Gal B activities corresponds to one nmole 4MU hydrolyzed/hr. Protein concentrations were determined by the fluorescamine method (Bohlen et al., 1973).

α-Gal A activity in mouse fibroblasts against its natural substrate was determined with lissamine-rhodamine-GL-3 (LR-GL-3) as follows: for *in vitro* analysis, confluent mouse fibroblast cultures were lysed as described above. Protein extracts (50 μg) were incubated with 100 ng LR-GL-3 at 37° C for 24 hr as previously described (see Chapter 1). Control digestions contained no extract. Following lipid extraction, high-performance thin-layer chromatography (HPTLC) was performed as described below except the development solution was chloroform-methanol-water, 80:20:2. HPTLC plates were visualized under short-wave ultraviolet light, with lissamine rhodamine-labelled glycosphingolipids used as migration standards. For *in vivo* analysis, a modification of a previous method was used (Yeyati et al., 1995). Aliquots of 250 ng LR-GL-3 were dried with 2.5 μg phosphatidylcholine under nitrogen, resuspended in 1 ml cell culture medium (CHO-S-SFM II; Gibco BRL) containing glutamine and antibiotics, and sonicated for 1 min. The liposome solutions were added to confluent mouse fibroblast cultures in 100 mm dishes containing 4 ml CHO-S-SFM with glutamine and antibiotics. Following incubation for 24 hr at 37° C, the cells were chased with 5 ml complete DMEM for 24 hr. Cells were trypsinized and extracted without purification as described below. HPTLC and visualization were performed as described above.

Glycosphingolipid Analysis

For lipid analysis, each tissue sample was extracted in chloroform-methanol 2:1 (10 ml/100 mg wet weight). Twenty-five microliters of each plasma was extracted in 0.5 ml chloroform-methanol 2:1. Following a 15 min incubation at 37° C, samples were centrifuged to remove debris and 1/5 volume water was added (Folch et al., 1957) to the equivalent of 5 mg tissue or to the the total plasma samples. The phases were allowed to separate at 4° C for at least 1 hr and the bottom phases were dried under nitrogen for purification. Dried lipids were resuspended in chloroform, applied to 500 mg Lichrolut RP-18 columns (EM Separations, Gibbstown, NJ) pre-equlibrated with chloroform, washed with chloroform, and the neutral GSL fractions eluted with acetone-methanol 9:1 (Vance and Sweeley, 1967). Eluted GSLs were dried under nitrogen and resuspended in 0.5 ml ethanol for tissues and 0.5 ml for plasma.

The quantitation of GL-3 in the various tissues was performed using an ELISA based on the affinity of *E.coli* verotoxin B subunit (VTB) for the glycolipid. Lipids in ethanol (equivalent to 25 µg liver and heart, 12.5 µg spleen and kidney, and 2.5 µl plasma) were plated in 96-well PolySorp Immunoplates (Nunc, Napersville, IL) and dried completely by evaporation. The remainder of the assay was performed as described in Chapter 1.

HPTLC was performed on 10 x 10 cm silica gel 60 plates (EM Separations) to qualitatively confirm the results of the tissue and plasma GL-3 ELISAs. Neutral glycosphingolipids in ethanol, pooled from 3 mice and corresponding to about 20 mg tissue, were dried under nitrogen, resuspended in chloroform-methanol 2:1, and applied as thin streaks to the plates, which were developed in a closed atmosphere of chloroform-methanol-water 100:42:6 as described in Chapter 1. Purified glycosphingolipids (Sigma and Supelco, Bellefonte, PA) were used as migration standards. The identity of the GL-3 bands was occasionally confirmed by digestion with $5x10^{-4}$ U recombinant human α-Gal A (Ioannou et al., 1992) at 37° C for 24 hr prior to HPTLC, as described in Chapter 1.

Immunocytochemistry

Cells plated in 2-well chamber slides (Nunc, Naperville, IL) were rinsed with PBS and fixed in 4% paraformaldehyde in PBS, pH 7.4 for 10 min at room temperature. Cells were washed with PBS, pH 7.4 and permeabilized with 1% Triton X-100 in PBS for 10 min at room temperature, or left unpermeabilized. Cells were washed in PBS and blocked with 3% BSA in PBS (PBS-BSA) for 30 min at room temperature. One-half microgram purified VTB, diluted 1:250 in PBS-BSA, was added and the cells were incubated for 1 hr at room temperature. The cells were washed 3 times with PBS and incubated with 4 μg anti-VTB monoclonal antibody, diluted 1:250 in PBS-BSA, for 1 hr at room temperature. The cells were washed 3 times with PBS and incubated with fluorescein isothiocyanate (FITC)-labelled goat anti-rabbit IgG (Boehringer Mannheim), diluted 1:250 in PBS-BSA, for 1 hr at room temperature. Following the final washes, cells were fixed with Fluoromount G (Southern Biotechnology, Birmingham AL) on coverslips. The slides were viewed using an Axiophot fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY) and photographed with Kodak Ectochrome 400 film. As a control for specificity, cells were washed with PBS following fixation and permeablization, extracted with chloroform-methanol 2:1 for 10 min at room temperature, and washed again with PBS prior to blocking.

RESULTS

α-Gal A-Deficient Mice

No selection against male or female α-Gal A-deficient mice was noted; expected Mendelian ratios were obtained (data not shown). These mice appeared phenotypically normal at birth and had normal lifespans (~1.5-2.5 yrs), compared to wild-type 129/Sv mice. No gross anatomical abnormalities were noted in adult mice, and males and females were able to breed with each other, producing viable, normal-sized litters (~5-15 pups).

Enzymatic Analysis

α-Gal A and α-Gal B activities were determined in various tissue extracts from adult male and female mice (Table 1). α-Gal A activity was low to undetectable in all tissues of α-Gal A-deficient mice compared to wild-type mice, whereas α-Gal B activities were normal or elevated, indicating the correct gene had been disrupted. The residual α-Gal A activities detected in tissue extracts were attributed to α-Gal B activity, as this enzyme recognizes the artificial α-Gal A substrate 4MU-α-Gal to a limited extent, even in the presence of its inhibitor GalNAc. Note also that no α-Gal A activity is present in fibroblasts from α-Gal A/α-Gal B-deficient mice (Table 2; see below), indicating that the residual a-Gal A activity detected in α-Gal A-deficient mice is due to endogenous α-Gal B activity. In wild-type mice, higher α-Gal A and α-Gal B activities were detected in the liver, spleen, heart, lung, and kidney of female mice, compared to males. In α-Gal A-deficient mice, α-Gal B activities were elevated in the plasma, spleen, and white blood cells of α-Gal A-deficient mice, compared to wild-type activities. Fibroblasts from α-Gal A/α-Gal B-deficient mice were completely devoid of both enzyme activities (Table 2).

In vitro LR-GL-3 assays with detergent were performed on adult male mouse fibroblast extracts (Fig. 1A). As expected, wild-type extracts were able to completely hydrolyze the substrate (lane 2). This was also true for extracts of α-Gal B-deficient

Table 1
α-Gal A and α-Gal B activities in tissues of adult wild-type and α-Gal A-deficient mice.

	Wild-Type				α-GalA-Deficient			
	Male (n=5)		Female (n=7)		Male (n=6)		Female (n=6)	
Tissue	α-Gal A	α-Gal B	α-Gal A	α-Gal B	α-Gal A	α-Gal B	α-Gal A	α-Gal B
Liver	16.8±1.6 (15.1-18.7)	20.5±3.6 (16.3-24.5)	28.5±3.4 (23.4-33.3)	34.8±6.0 (27.3-41.7)	0.2±0.2 (0.1-0.3)	20.4±3.4 (16.5-25.2)	0.3±0.1 (0.2-0.4)	33.8±5.2 (25.6-40.1)
Spleen	39.2±8.3 (27.3-50.0)	31.5±4.4 (26.9-38.1)	68.9±10.1 (55.2-85.6)	70.8±6.1 (61.4-79.3)	0.9±0.6 (0.5-1.3)	68.8±28.0 (41.1-119.7)	0.8±0.3 (0.5-1.3)	89.6±25.0 (65.5-129.1)
Heart	3.2±0.6 (2.7-4.4)	10.4±1.8 (6.9-11.9)	6.2±1.0 (4.9-7.7)	17.7±4.4 (11.6-24.9)	1.0±0.8 (0.1-2.0)	19.3±6.7 (12.2-30.9)	0.8±0.8 (0.0-2.1)	21.9±4.1 (15.9-26.0)
Lung	29.8±4.7 (22.5-34.8)	31.3±8.0 (17.9-41.9)	42.0±10.4 (31.9-61.2)	40.4±4.1 (35.3-46.0)	1.0±1.0 (0.4-1.5)	38.3±1.1 (27.9-37.3)	0.4±0.4 (0.0-1.1)	44.8±7.0 (39.1-57.9)
Brain	26.9±2.9 (22.8-29.9)	14.7±2.6 (9.8-17.2)	34.9±4.2 (27.7-39.7)	19.5±3.9 (13.8-25.2)	0.4±0.3 (0.3-0.7)	21.0±7.8 (16.4-36.6)	0.2±0.1 (0.0-0.4)	21.9±2.2 (18.8-24.5)
Kidney	11.3±1.3 (10.0-13.2)	19.8±2.8 (14.6-22.3)	17.7±1.7 (15.9-20.9)	36.1±6.4 (29.8-48.4)	0.4±0.3 (0.2-0.6)	22.4±4.2 (17.5-28.4)	0.3±0.1 (0.2-0.5)	43.0±8.6 (29.9-56.1)
Plasma	5.7±1.2 (4.3-6.5)	12.6±2.6 (10.6-17.1)	5.9±1.7 (4.5-9.0)	13.7±2.2 (12.0-18.0)	1.2±0.3 (0.9-1.6)	30.9±4.5 (26.9-37.9)	1.9±0.4 (1.6-22)	25.0±4.6 (18.3-30.4)
WBC	9.6±3.0 (7.2-14.9)	31.9±20.2 (15.3-65.3)	12.2±3.9 (7.1-19.4)	31.4±18.5 (17.7-71.7)	1.4±0.4 (1.02-2.06)	43.1±10.4 (31.8-61.8)	1.5±0.3 (1.0-1.8)	47.4±16.4 (31.7-69.6)

Values are expressed as nmoles 4-MU/hr/mg protein ± SD, except for plasma, which is expressed as nmoles 4-MU/hr/ml plasma ± SD. Ranges are given in parentheses.

Table 2
Specific α-Gal A and α-Gal B activities in adult male mouse fibroblast extracts.

Cell Line	Specific Activity (U/mg)	
	α-Gal A	α-Gal B
Wild-Type	528	138
α-Gal A-Deficient	2	201
α-Gal B-Deficient	682	0
α-Gal A/α-Gal B-Deficient	0	0

Values are expressed as nmoles 4-MU/hr/mg protein.

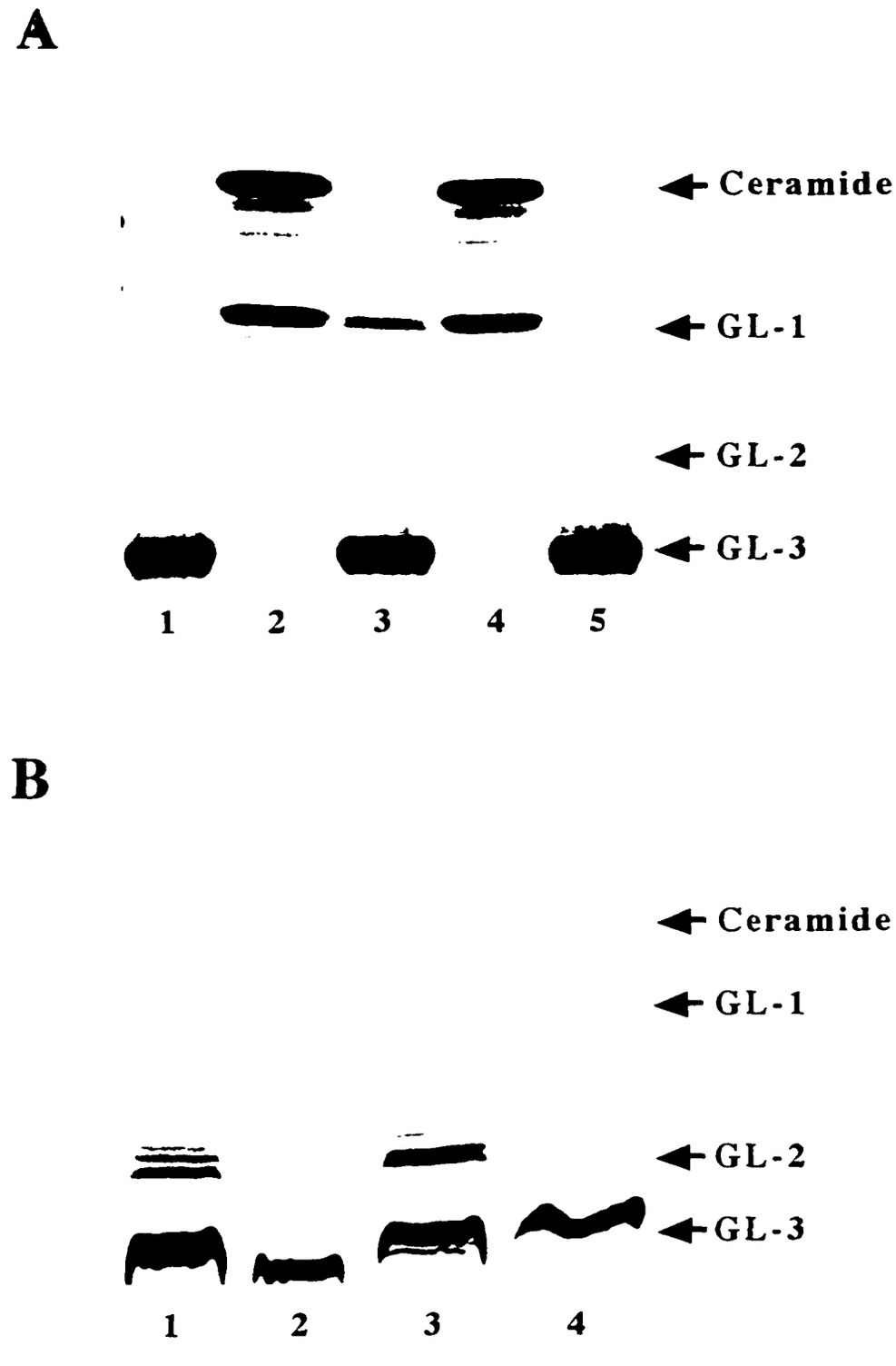

Figure 1. Hydrolysis of lissamine rhodamine-GL-3 by mouse fibroblast extracts. A, *In vitro* hydrolysis. 1, no extract; 2, wild-type mouse; 3, α-Gal A-deficient mouse; 4, α-Gal B-deficient mouse; 5, α-Gal A/α-Gal B-deficient mouse. B, *In vivo* hydrolysis. 1, wild-type mouse; 2, α-Gal A-deficient mouse; 3, α-Gal B-deficient mouse; 4; α-Gal A/α-Gal B-deficient mouse. Arrows denote positions of GL-3 and its hydrolysis products.

mouse fibroblasts (lane 4). Interestingly, α-Gal A-deficient fibroblasts were able to hydrolyze LR-GL-3 to a limited extent, suggesting that α-Gal B was responsible for this activity (lane 3). This idea was confirmed by the lack of hydrolysis by fibroblast extracts from α-Gal A/α-Gal B-deficient mice (lane 5). However, *in vivo* LR-GL-3 hydrolysis was different from the *in vitro* hydrolysis (Fig. 1B). Wild-type and α-Gal B-deficient fibroblasts were able to hydrolyze the substrate (lanes 1, 3). However, α-Gal A-deficient and α-Gal A/α-Gal B-deficient fibroblasts were unable to hydrolyze the LR-GL-3 (lanes 2, 4), confirming a previous report that α-Gal B does not act on GL-3 *in vivo* (Salvayre et al., 1985).

Glycosphingolipid Analysis

An ELISA based on the B subunit of verotoxin (see Chapter 1) was used to quantitate the GL-3 levels in the plasma, liver, spleen, heart, and kidneys of adult male mice (Table 3), as these are the primary tissues affected in Fabry disease (Desnick et al., 1995). All wild-type tissues contained essentially undetectable levels of GL-3, indicating that the 129/Sv strain of mouse had little GL-3 (Table 3). All tissues from α-Gal A-deficient mice contained greatly elevated levels of GL-3, with spleen possessing the highest amount and heart the lowest (Table 3). The amount of GL-3 present in the kidneys of α-Gal A-deficient mice is greater than the values reported for wild-type C57BL/6 mice, which contain high levels of GL-3 in their kidneys (McCluer et al., 1981). Plasma GL-3 levels were approximately 100-fold greater in α-Gal A-deficient mice than wild-type mice (Table 3), which is at least one order of magnitude higher than that reported for normal humans and Fabry patients (Clarke et al., 1976; Vance et al., 1969). The accumulation of GL-3 in tissues and plasma was age dependent, with a substantial increase in GL-3 concentration between 2 and 11 months of age (Fig. 2).

The results of the ELISAs were qualitatively confirmed by HPTLC. Liver, spleen, heart, and kidneys from adult male α-Gal A-deficient mice all contained greater amounts of

Table 3
GL-3 concentration in tissues from male adult wild-type and α-Gal A-deficient mice.

Tissue	Wild-Type	α-Gal A-Deficient
Liver	<8 (n=3)	378±67 (n=3) (334-454)
Spleen	15±8 (n=3) (10-25)	2792±400 (n=3) (2336-3082)
Heart	<8 (n=3)	196±28 (n=3) (169-224)
Kidney	9±7 (n=3) (4-17)	1096±93 (n=3) (997-1182)
Plasma	<0.05 (n=7)	5.06±1.97 (n=18) (2.28-10.25)

Values are expressed as ng GL-3/mg wet weight tissue ± SD except plasma, which is expressed as μg GL-3/ml plasma ± SD. Ranges are given in parentheses.

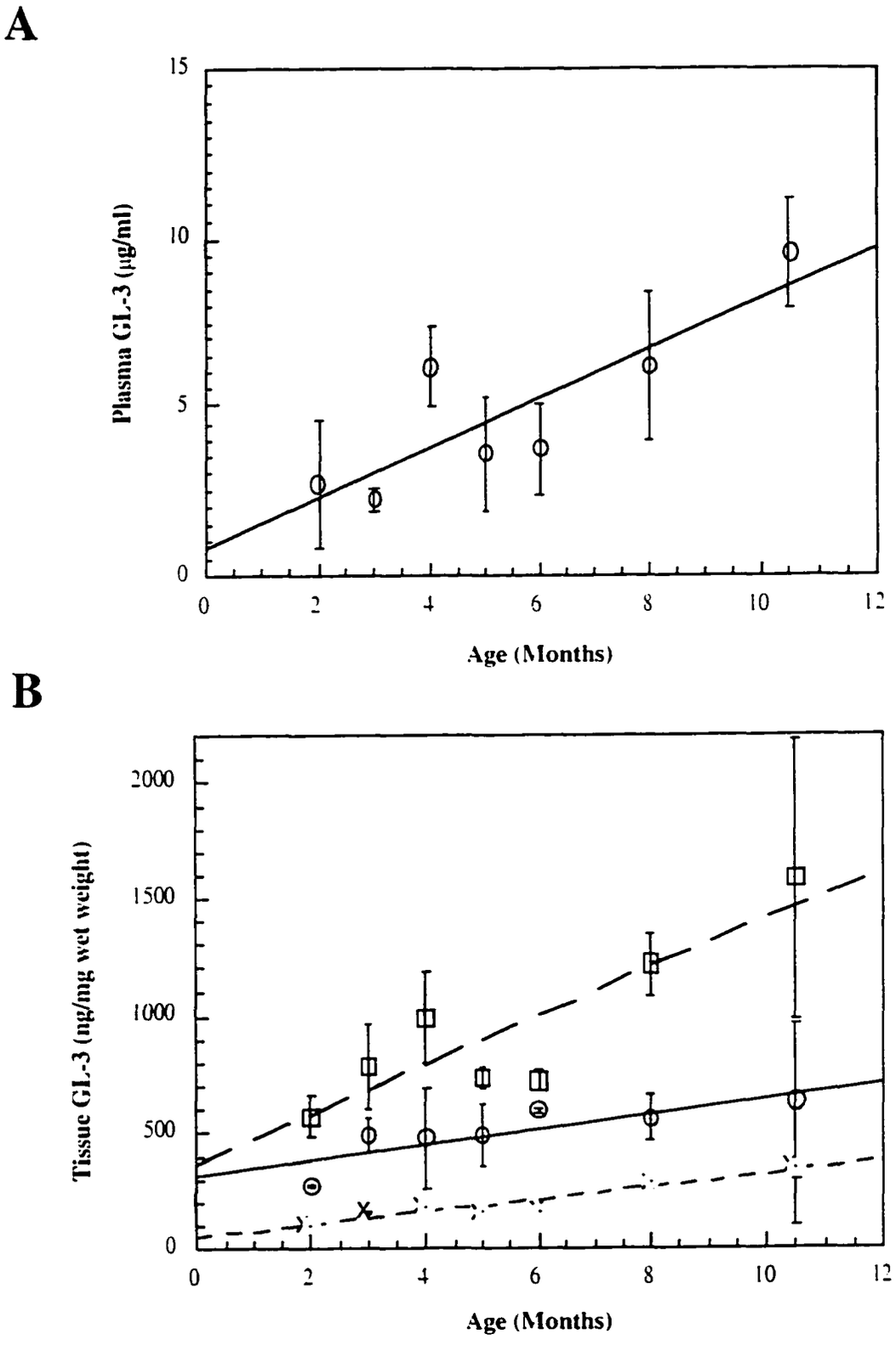

Figure 2. Effect of age on plasma and tissue GL-3 levels in α-Gal A-deficient mice. Each data point represents the mean ± SD of three mice. A, plasma GL-3. B, tissue GL-3. Squares, spleen; circles, kidney; x, liver.

GL-3 than their wild-type counterparts (Fig. 3). GL-3 from plasma, liver, spleen, and heart appeared as single bands (Fig. 3A, lane 3; 3B, lane 3; 3C, lane 3), whereas GL-3 from kidneys appeared as a separated doublet (Fig. 3A, lane 5). The slower migrating band is thought to be a testosterone-induced form normally excreted as multilamellar bodies in urine (McCluer et al., 1981). Glycosphingolipids from liver and kidneys of adult female mice were also analyzed by HPTLC and found to contain greater amounts of GL-3 than wild-type females (data not shown). No other glycosphingolipids were elevated in the α-Gal A-deficient mice, as assessed by HPTLC (data not shown).

Immunocytochemistry

VTB in the presence or absence of detergent was used to localize GL-3 in intracellular compartments and at the plasma membrane of various cell types, respectively (Fig. 4). GL-3 was identified on the plasma membrane of Vero cells (Fig. 4A) as well as in vesicles (Fig. 4B) (Sandvig et al., 1989). Little GL-3 was found on the membrane or in vesicles of CHO cells (Fig. 4C, D). The verotoxin interaction was specific for GL-3 and not just terminal α-galactosyl moieties, as chloroform-methanol extraction of Vero cells prior to addition of VTB eliminated staining (data not shown). Interestingly, VTB recognized what appeared to be the nucleoli of both Vero and CHO cells (Fig. 4B, D); this signal was also extractable by chloroform-methanol (data not shown). All cell lines studied thus far, including BHK-21, COS-1, human amniocytes, and primary human and mouse fibroblasts, have shown nucleolar staining (Zeidner, unpublished results). It is unclear what function nucleolar GL-3 possesses, but other glycosphingolipids, such as Forssman antigen and G_{M1} ganglioside, have also been shown to reside in the nucleus (Gillard et al., 1993).

VTB was also used to localize GL-3 in adult human fibroblasts. In human Fabry fibroblasts deficient in lysosomal α-Gal A, GL-3 was primarily found in intracellular vacuoles (Fig. 4F), consistent with the known site of storage in these cells

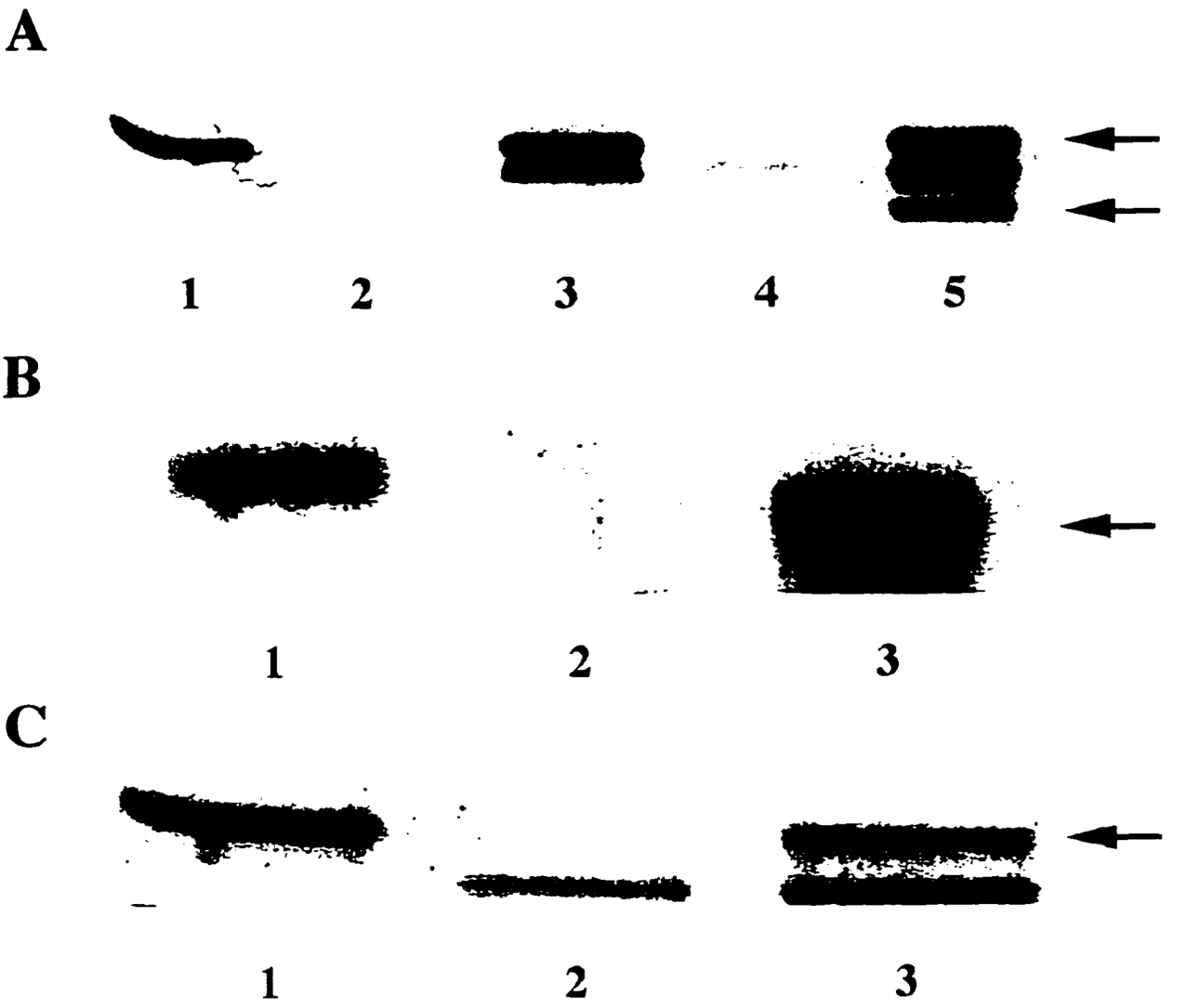

Figure 3. HPTLC of neutral glycosphingolipids purified from mouse tissues. A, liver and kidney GL-3. 1, GL-3 standard; 2, wild-type mouse liver; 3, α-Gal A-deficient mouse liver; 4, wild-type mouse kidney; 5, α-Gal A-deficient mouse kidney. B, spleen GL-3. 1, GL-3 standard; 2, wild-type mouse spleen; 3, α-Gal A-deficient mouse spleen. C, heart GL-3. 1, GL-3 standard; 2, wild-type mouse heart; 3, α-Gal A-deficient mouse heart. Each sample represents the pooled glycosphingolipids of three mice corresponding to 20 mg tissue wet weight. Arrows denote position of GL-3.

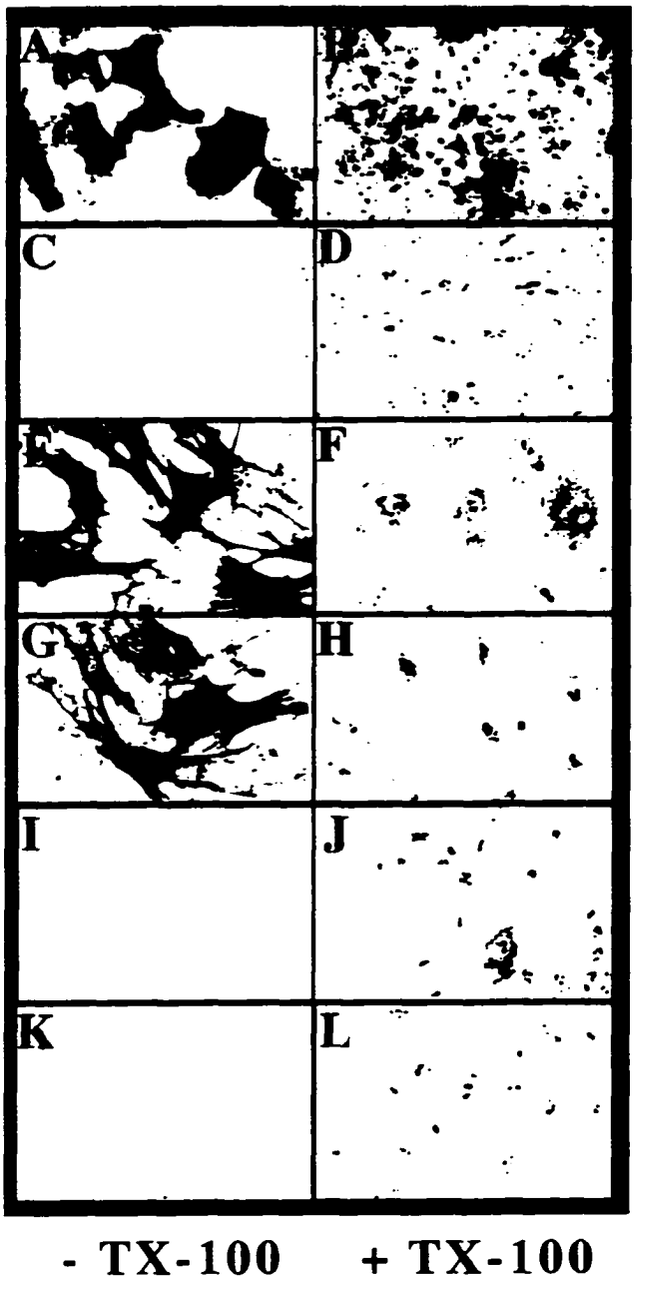

Fig 4. Localization of GL-3 in cultured cells using recombinant VTB. A, B, Vero cells; C, D, CHO cells; E, F, Fabry hemizygote fibroblasts; G, H, normal human fibroblasts; I, J, α-Gal A-deficient mouse fibroblasts; K, L, wild-type mouse fibroblasts.

(Desnick et al., 1995), and in the plasma membrane (Fig. 4G). In comparison, normal fibroblasts contained less GL-3 intracellularly (Fig. 4H) but similar amounts on the plasma membrane (Fig. 4G). Using this immunolocalization method, human Fabry fibroblasts are easily differentiated from control cells on the basis of accumulated intracellular subtrate, as was previously demonstrated using an anti-GL-3 antibody (Itoh et al., 1993).

Finally VTB was used to localize GL-3 in adult mouse fibroblasts. Little GL-3 was present in the plasma membrane of both wild-type (Fig. 4K) and α-Gal A-deficient mouse fibroblasts (Fig. 4I). Vacuolar GL-3 was elevated in the α-Gal A-deficient mouse fibroblasts (Fig. 4J), compared to the wild-type fibroblasts (Fig. 4L), though not to the same extent as human fibroblasts.

DISCUSSION

The mouse model for Fabry disease joins a growing list of animal models of lysosomal storage diseases (Bedell et al., 1997; Desnick et al., 1982; Patterson et al., 1982). Before the advent of gene targeting technology, models of human disease, in particular lysosomal storage diseases, had to be identified in pre-existing animal colonies (Jezyk et al., 1982). The two prototypes for these naturally-occurring models are the *twitcher* mouse for Krabbe disease (Kobayashi et al., 1980) and the *gus*^{mps} mouse for mucopolysaccharidosis (MPS) type VII (Birkenmeier et al., 1989). Larger animal models of lysosomal storage diseases have also been identified, including a feline model of G_{M2} gangliosidosis, type II (Cork et al., 1977), a bovine model of α-mannosidosis (Hocking et al., 1972), a canine model of MPS type I (Spellacy et al., 1983), a feline model of MPS type VI (Jezyk et al., 1977), and a caprine model of MPS type IIID (Thompson et al., 1992). With the advent of gene targeting technology, new murine models of lysosomal storage disease have been generated. These include α-*L*-iduronidase-deficient mice for MPS type I (Clarke et al., 1997); glycosylasparaginase-deficient mice for aspartylglycosaminuria (Kaartinen et al., 1996), arylsulfatase A-deficient mice for metachromatic leukodystrophy (Hess et al., 1996), arylsulfatase B-deficient mice for MPS VI (Evers et al., 1996), protective protein/cathepsin A-deficient mice for galactosialidosis (Zhou et al., 1995), acid-sphingomyelinase-deficient mice for Niemann-Pick disease, types A and B (Horinouchi et al., 1995; Otterbach and Stoffel, 1995), β-hexosaminidase A-deficient mice for Tay-Sachs disease (Yamanaka et al., 1994), β-hexosaminidase B-deficient mice for Sandhoff disease (Phaneuf et al., 1996; Sango et al., 1995), and glucocerebrosidase-deficient mice for Gaucher disease (Tybulewicz et al., 1992).

Germ-line transmitting males chimeric for the null α-Gal A allele were bred with 129/Sv females (Wang, unpublished results), resulting in a coisogenic strain, with the null mutation immediately present on a congenic 129 background (Bedell et al., 1997). This

was performed to facilitate the analysis of these mice, since large variations in lysosomal enzyme and glycolipid levels are known to exist between different mouse strains (Coles et al., 1970; Johnson et al., 1986; McCluer et al., 1983). α-Gal A-deficient mice were indistinguishable from wild-type mice in all respects, including *in utero* viability, appearance, life span, and fertility, indicating that the enzyme is not required for embryonic or sexual development. Humans with Fabry disease generally appear normal at birth and are able to reproduce, although in adult life they often develop outwardly-apparent symptoms, such as angiokeratoma, and have greatly diminished lifespans (Desnick et al., 1995). Normal fertility and life span have been demonstrated in arysulfatase A- (Hess et al., 1996) and arylsulfatase B-deficient mice (Evers et al., 1996), though these mice did possess many of the other characteristics of the diseases.

Mice were completely deficient in α-Gal A activity in all tissues examined (Table 1), indicating that the correct gene had been disrupted. Interestingly, the activity of α-Gal B, a related lysosomal enzyme derived by gene duplication of an ancestral α-galactosidase gene (Wang et al., 1990), was elevated in the plasma, spleen, and white blood cells of α-Gal A-deficient mice, compared to their wild-type counterparts. Humans with Fabry disease have also been noted with elevated levels of α-Gal B activity (Beutler and Kuhl, 1972). The reason for this elevation is unclear, although the disruption of the β-hexosaminidase A gene in mice also resulted in large increases in activity of the evolutionarily-related β-hexosaminidase B (Phaneuf et al., 1996; Yamanaka et al., 1994). The most likely explanation for these results is that the elevation is a non-specific result of lysosomal storage, as has been noted for many other lysosomal storage diseases (Hers, 1973). It has been suggested that storage material may stabilize certain lysosomal enzymes, leading to an apparent increase in activity (Van Hoof and Hers, 1972). That the increase in α-Gal B activity is due to the accumulated substrate of α-Gal A (see below) is further supported by the fact that administration of β-glucuronidase to deficient mice decreased the elevated levels of α-Gal A to near normal levels (Sands et al., 1994). This elevation of α-Gal B activity

suggests that the source of some circulating lysosomal enzymes may be the spleen and leukocytes (Table 1). Enzyme-deficient fibroblasts were also elevated in their remaining enzyme activities (Table 2). A more detailed examination of other lysosomal activities in these α-Gal A-deficient mice may help elucidate the cause of this phenomenon. Higher α-Gal A and α-Gal B activities were detected in the liver, spleen, heart, lung, and kidney of female wild-type mice, as compared to males. This is not uncommon, as numerous intersex differences in lysosomal enzyme activities of mice have been noted (Johnson et al., 1986).

As a consequence of α-Gal A deficiency, GL-3 was elevated in all tissues examined (Figs. 2, 3, 4; Table 3). These observations indicate that α-Gal B does not compensate for the missing enzyme *in vivo*. This increase of GL-3 occurred in an age-dependent fashion in plasma (Fig. 2A) and tissues (Fig. 2B). Spleen had the highest GL-3 concentration, while heart had the lowest (Table 3), correponding to their relative decrease in α-Gal A activity compared to wild-type mice (Table 1). These proportions are different from those found in human Fabry tissues, where kidney contains the largest amount of GL-3, followed by heart, liver, and spleen (Schibanoff et al., 1969), and are directly related to the amounts of GL-3 present in the tissues of normal individuals (Desnick et al., 1995). Most importantly, plasma GL-3 levels were significantly elevated in α-Gal A-deficient mice compared to wild-type mice (Table 3), even greater than the elevation of plasma GL-3 Fabry patient plasma when compred to normal individuals (Vance et al., 1969). This is important, since the pathology associated with Fabry disease is thought to occur by deposition of circulating GL-3 in extrahepatic endothelium, primarily heart and kidney (Johnson and Desnick, 1978).

Mouse fibroblasts were assayed for activity against GL-3. *In vitro* activity was present in α-Gal A-deficient fibroblasts but not in α-Gal A/α-Gal B-deficient fibroblasts (Fig. 1A), indicating that α-Gal B can hydrolyze GL-3 in the presence of detergent. Previous reports using purified α-Gal B or crude α-Gal B from Fabry tissue homogenates

have been contradictory regarding the hydrolysis of GL-3 (Desnick and Bishop, 1989). In early studies in which α-Gal B activity against GL-3 was detected, the absence of contaminating α-Gal A could not be confirmed. The use of α-Gal A/α-Gal B-deficient mouse fibroblast extracts provides the first conclusive proof that α-Gal B retains some activity against α-Gal A's natural substrate *in vitro*. Studies in our laboratory with purified recombinant secreted human α-Gal B from highly overexpressing CHO cells (Ioannou, unpublished results) have also detected activity against GL-3, even when the enzyme is heated at 55° C for 1 hr, which would inactivate any trace amounts of α-Gal A (Zeidner, unpublished results). This α-Gal B activity is inhibited by galactose but not GalNAc, whereas the hydrolysis of α-Gal A is not inhibited by either compound (Zeidner, unpublished results), suggesting a basic catalytic difference between the two enzymes. That α-Gal B can hydrolyze GL-3 further strengthens the hypothesis that α-Gal A and α-Gal B diverged from a common ancestor (Wang and Desnick, 1991).

No hydrolysis of GL-3 was detected in α-Gal A-deficient mouse fibroblasts *in vivo*, however (Fig. 1B), indicating that α-Gal B can not hydrolyze GL-3 in cells. This confirms an earlier report in which Epstein-Barr virus-transformed peripheral B-lymphocytes from a Fabry patient were incapable of hydrolyzing endogenously-labelled GL-3, even after 30 days (Salvayre et al., 1985). This may be due to the inaccesability of α-Gal B to the glycolipid within the lysosomal environment, due perhaps to lysosomal heterogeneity (Einstein and Gabel, 1991; Uchiyama et al., 1981; Wiener and Curelaru, 1975) or to the inability of α-Gal B to function with the GL-3 activator protein, saposin B (Kase et al., 1996). Li and Li (1976) demonstrated *in vitro* digestion of GL-3 with saposin B and purified α-Gal B, although small amounts of contaminating α-Gal A may have been present. The lack of hydrolysis in α-Gal A-deficient mouse fibroblasts is consistent with the fact that α-Gal A-deficient mice do accumulate GL-3, again indicating a lack of compensatory α-Gal B activity. Furthermore, it would be expected that α-Gal A/α-Gal B-deficient mice accumulate GL-3 at levels similar to, not greater than, α-Gal A-deficient

mice. It should be pointed out that the primary natural substrate of α-Gal B has not been determined. The α-Gal B-deficient mice should prove useful in identifying this substrate.

Fibroblasts were also analyzed by immunofluorescence microscopy. α-Gal A-deficient mouse cells were readily distinguished from wild-type mouse cells based on the amount of VTB-positive material present intracellularly (Fig. 4J, L), although it was considerably less than in human Fabry fibroblasts (Fig. 4F). Normal human and Fabry cells had similar amounts of GL-3 on their plasma membranes (Fig. 4E, G), suggesting a very slow membrane turnover and/or efficient low-density lipoprotein receptor (LDLR) system. Interestingly, no GL-3 was detected on the plasma membranes of mouse cells (Fig. 4I, K). Perhaps mouse cells are inefficient at endocytosing GL-3-containing LDL from the media (see below), which may explain the lower accumulation in mouse cells compared to human cells, or membrane turnover may be quicker in mouse cells than in human cells.

The main question remaining is why GL-3 accumulation in α-Gal A-deficient mice is nonpathogenic. One significant difference in GL-3 metabolism between humans and mice, as determined by verotoxin analysis, is the lack of GL-3 in murine glomeruli (Wadolkowski et al., 1990), which may partially explain the lack of renal pathology in α-Gal A-deficient mice. It is also possible that an alternative pathway of GL-3 metabolism exists in mice, a situation that exists for G_{M2} ganglioside metabolism and explains why β-hexosaminidase A-deficient mice suffer no neurological abnormalities (Phaneuf et al., 1996; Sango et al., 1995). An alternative metabolic pathway has also been proposed as a possible explanation for the mild phenotype in arylsulfatase A-deficient mice (Hess et al., 1996). A more likely explanation for the lack of pathogenicity, however, is the physiological difference between mouse and human lipoprotein metabolism. In humans, a substantial amount of GL-3 is synthesized in hepatocytes and transported in LDL particles (Attie et al., 1982) to tissues which possess the LDLR. A large amount of GL-3 may also be derived from the catabolism of senescent erythrocyte membranes (Dawson and Sweeley,

1970), which could then be reincorporated into LDL particles. This explains why hepatocytes from Fabry patients show little GL-3 storage, in contrast to Kuppfer cells (Meuwissen et al., 1982), and why plasma from Fabry patients (Vance et al., 1969) and individuals with familial hypercholesterolemia (Dawson et al., 1976) have elevated levels of GL-3. It is reasonable to assume that this applies to mice as well, as α-Gal A-deficient mice accumulate significant amounts of GL-3 in plasma (Table 3). Therefore, the pathology of Fabry disease is caused by a progressional deposit of GL-3-containing LDL particles by receptor-mediated endocytosis in the vascular endothelium, particularly of heart and kidney (Johnson and Desnick, 1978). A major ligand for the LDLR is apoB100, the primary apolipoprotein B in human very low density lipoprotein (VLDL) particles (Havel and Kane, 1995). In mice, however, the primary apoB is apoB48 (Higuchi et al., 1992), which is not recognized by the LDLR but may bind LDLR-related protein (LRP) through apoE (Beisiegel et al., 1989), and which may partially explain why mice are generally resistant to atherosclerosis (Jokinen et al., 1985). This also explains why LDLR-deficient mice accumulate plasma LDL to levels significantly lower than familial hypercholesterolemia patients (Ishibashi et al., 1993) and why apoE-deficient mice have higher cholesterol levels than LDLR-deficient mice (Ishibashi et al., 1994). In mice, this may lead to VLDL metabolism similar to that for chylomicron remnants, which are rich in apoB48 (Havel and Kane, 1995). Thus, if accumulated GL-3 is transported in apoB48-containing particles, the lack of apoB100 may result in a "shortcircuit" of GL-3 deposition, in which GL-3 synthesized in heptocytes is not deposited in endothelium, but rather is routed back to hepatocytes for uptake, perhaps by the LRP, for reincorporation into VLDL. Chylomicron remnants have also been shown to be phagocytosed by macrophages (Mamo et al., 1996). This may explain the relatively low levels of circulating cholesterol (Ishibashi et al., 1993) and the nearly undetectable levels of plasma GL-3 in wild-type mice (Table 3). If circulating GL-3 is not deposited to extrahepatic tissues in α-Gal A-deficient mice, the implication is that the source of accumulated GL-3 in the extrahepatic tissues of α-Gal A-

deficient mice is endogenous synthesis in the tissues themselves. Therefore, crossing of α-Gal A-deficient mice with various mutant mouse lines with increased plasma LDL levels, such as the apoE-deficient mouse (Plump et al., 1992), should not greatly affect the survival of the mice. Perhaps crossing the α-Gal A-deficient mice with transgenic mice expressing human apoB100 (Linton et al., 1993) or LDLR-deficient mice, which overproduce apoB100 (Ishibashi et al., 1993), would increase deposition of GL-3 in the vascular endothelium and present a better model of Fabry disease.

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Chapter 3

Enzyme Replacement in α-Galactosidase A-Deficient Mice

ABSTRACT

Fabry disease is an X-linked recessive lysosomal storage disorder in humans, resulting from a deficiency of α-galactosidase A (α-Gal A). The major site of pathology is the vascular endothelium, particularly in the heart and kidneys, due to accumulation of globotriaosylceramide (GL-3). To determine the efficacy of enzyme replacement therapy (ERT) for Fabry disease, α-Gal-A-deficient mice, generated by targeted gene disruption, were administered different recombinant α-Gal A glycoforms, each differing in their glycosylation and phosphorylation patterns. The biodistribution, pharmacokinetics, and stability of these glycoforms were analyzed *in vivo*. At a dose of 1 mg/kg body weight, the majority of each glycoform was recovered in the liver and spleen, with negligible amounts detected in heart, lung, kidney, and brain. The half-life in plasma was less than 5 min for each glycoform. A single α-Gal A glycoform, GF 1, was further analyzed for its ability to correct the enzyme deficiency in various tissues. The half-life of the enzyme in liver was ~40 hr, and in spleen and kidney was ~20 hr. Increased doses of this glycoform, up to 10 mg/kg, led to a redistribution of the enzyme, with readily detectable levels in heart and kidney. Multiple doses of GF 1 α-Gal A were able to completely correct the enzyme deficiency in all tissues except brain. These results provide the rationale for future studies on ERT for Fabry disease.

INTRODUCTION

α-Galactosidase A (α-Gal A; E.C. 3.2.1.22) is a lysosomal glycosidase capable of hydrolyzing terminal α-galactosyl moieties from glycolipids and glycoproteins (Desnick et al., 1995). The human α-Gal A cDNA and genomic sequences have been isolated and characterized (Bishop et al., 1986; Bishop et al., 1988; Kornreich et al., 1989). The mature human enzyme has been biochemically characterized and shown to be a homodimeric glycoprotein with a subunit molecular weight of about 48 kDa (Bishop and Desnick, 1981). The enzyme precursor is co-translationally glycosylated and modified in the Golgi complex and lysosomes, producing the mature polypeptide which homodimerizes to form the active 101 kDa lysosomal glycoprotein (LeDonne et al., 1983; Lemansky et al., 1987). Glycosylation analysis has revealed that three of the four N-linked glycosylation sites are utilized (Ioannou, unpublished results) and that complex, high mannose, and hybrid oligosaccharides are present (Matsuura et al., 1998).

A deficiency of α-Gal A results in Fabry disease, an X-linked recessive lysosomal storage disorder characterized by the accumulation of the enzyme's glycosphingolipid substrates, principally globotriaosylceramide (GL-3), in the plasma and vascular endothelium (Desnick et al., 1995). Recent molecular studies have revealed a variety of mutations from unrelated Fabry families (Eng and Desnick, 1994; Eng et al., 1994; Eng et al., 1993) which include point mutations, splicing mutations, small insertions and deletions, and gene deletions and rearrangements.

In classically affected hemizygous males, the major disease manifestations include angiokeratoma, acroparathesias, hyphohydrosis, corneal dystrophy, and vascular disease of the heart, kidneys, and brain leading to early demise in adulthood (Desnick et al., 1995). In heterozygous females, the manifestations are quite variable and can range from mild to severe, presumably due to random X-chromosome inactivation (Lyon, 1961).

Currently, there is no treatment for Fabry disease. Past experiences with kidney transplantation (Maizel et al., 1981), phlebotomy (Beutler et al., 1983), and plasmapheresis (Kolodny et al., 1981) have been ineffective. Early on, it was suggested that lysosomal storage diseases may be amenable to treatment by enzyme replacement therapy (ERT) (DeDuve, 1964). This was confirmed by the fact that enzyme administered to fibroblasts in culture from patients with various lysosomal storage diseases could correct their metabolic defect (Cantz and Kresse, 1974; Dawson et al., 1973; Di Ferrante et al., 1973; Fratantoni et al., 1968; Lagunoff et al., 1973; O'Brien et al., 1973; Porter et al., 1971). Early studies with human patients were encouraging (Desnick and Grabowski, 1981; Desnick et al., 1976; Rietra et al., 1974; Tager et al., 1980), but the lack of sufficient quantities of pure enzymes precluded further analyses. Recent experience with Gaucher disease (Brady et al., 1994), a related lysosomal glycosphingolipid storage disorder, indicates that sufficient quantities of correctly targeted, exogenously administered enzyme can be effective in treating lysosomal storage diseases.

Fabry disease appears to be well suited to treatment by enzyme replacement therapy. Crises appear relatively late in life, with minimal brain or hard tissue involvement (Desnick et al., 1995). In fact, previous studies with crude or pure α-Gal A demonstrated that the exogenous enzyme was effective at lowering circulating levels of GL-3 (Brady et al., 1973; Desnick et al., 1979; Mapes et al., 1970), which suggests that deposition of the glycolipid in vascular endothelium might be prevented. Recently, recombinant human α-Gal A has been produced in Chinese hamster ovary cells (Ioannou et al., 1992) and purified (Ioannou, unpublished results) in quantities sufficient to perform large-scale enzyme replacement trials. In addition, the mouse α-Gal A cDNA and genomic sequences have been isolated and characterized (Gotlib et al., 1996; Ohshima et al., 1995) and used to generate a murine model for Fabry disease by targeted gene disruption in embryonic stem cells (Ohshima et al., 1997; Wang, unpublished results). These mice are completely deficient in α-Gal A activity and accumulate GL-3 in all tissues examined in an age-

dependent fashion (see Chapter 2), thus allowing for an evaluation of enzyme replacement therapy in a background-free system. This study was undertaken to determine the biodistribution, pharmacokinetics, and stability of exogenously administered recombinant human α-Gal A in these mice. As previous studies of α-Gal A replacement in humans demonstrated differential tissue distribution clearance from the circulation and depending on the type of glycosylation present (Bishop et al., 1981; Brady et al., 1973; Desnick et al., 1979; Mapes et al., 1970), a variety glycoforms were analyzed *in vivo*.

MATERIALS AND METHODS

Mice

α-Gal A-deficient mice were produced by homologous recombination in 129/Sv ES cells as described in Chapter 2. All mice used in this study were adult males and weighed between 20-30 g. Mice were given standard mouse chow and water *ad libidum*.

Production and Characterization of α-Gal A Glycoforms

Four human α-Gal A glycoforms (GFs) were analyzed in this study for their biodistributions, pharmacokinetics, and stabilities in α-Gal A-deficient mice. GF 1 was stably expressed in DUKX B11 CHO cells and obtained from Genzyme Corporation (Framingham, MA). GF 2 was stably expressed in DG44 CHO cells (Ioannou et al., 1992). GF 3 was stably expressed in DG44 CHO cells also expressing the rat α2-6 sialyltransferase gene (Ioannou, unpublished results). GF 4 was transiently expressed in COS-1 cells (Ioannou et al., 1998). All glycoforms were expressed using the full-length wild-type human α-Gal A cDNA except GF 4, which possessed an Asn to Gln point mutation at amino acid 192 produced by site-directed mutagenesis. This abolishes the second glycosylation site in the enzyme, which is occupied by a high mannose oligosaccharide (Ioannou et al., 1998). All glycoforms were isolated from culture media, purified by α-D-galactosylamine chromatography (Harpaz et al., 1974), and stored concentrated (>0.5 mg/ml) in citrate-phosphate (C-P) buffer, pH 6.0 at 4° C.

Molecular weights and isoelectric points (pIs) were estimated by SDS-PAGE and isoelectric focusing (IEF) using precast 12.5 % polyacrylamide PhastGels and precast pH 4-6.5 gels, respectively, run on the PhastSystem (Pharmacia, Piscataway, NJ) according to manufacturer's recommendations. Sialylation was assessed by neuraminidase (Boehringer Mannheim, Indianapolis, IN) digestion according to manufacturer's recommendations, followed by IEF. Mannose-6-phosphorylation was assessed by incubation of Fabry

patient fibroblasts with $5x10^4$ U enzyme in the presence or absence of 5 mM mannose-6-phosphate (M6P. Following incubation for 16-24 hr, cells were lysed and assayed with 4MU-α-Gal as described below. Activity against the natural substrate, GL-3, was assessed *in vitro* using lissamine rhodamine-GL-3 as described in Chapter 2. Stability at pH 4.6 and 7.4 was assessed by incubations at 37° C for 15 min in appropriate buffer (acetate or Hepes, respectively), followed by 4MU-α-Gal assays as described below.

Enzyme Injections

Appropriate enzyme dilutions were made in C-P buffer, pH 6.0 containing 1 mg/ml bovine serum albumin (BSA). Mice were placed in an illuminated plexiglass restraining device (Dan-Kar Corp., Reading MA) with their tails extending out of the apparatus over a light source. Tails were swabbed with 70% ethanol to induce vein bulging. Each enzyme preparation was drawn into a 1 cc insulin syringe and air bubbles were expelled through the needle. Mice were given a single bolus tail vein injection of ~0.1 ml test substance over ~3 sec.

Tissue Collection and Homogenization

Blood samples were obtained by either tail or retro-orbital eye bleeds using heparinized microhematocrit capillary tubes. Samples were centrifuged at room temperature to obtain the plasma, which was stored on ice prior to enzyme analysis. Urine was collected by bladder puncture at the time of sacrifice and stored on ice prior to enzyme analysis. Mice were anesthetized by intramuscular injection of 50 µl of a 100 mg/ml ketamine solution (Sigma, St. Louis, MO) and perfused with 50 ml 0.9% saline to remove heme, which interferes with the α-Gal A enzyme assay. The liver, spleen, heart, lungs, brain, and kidneys were removed using clean, alcohol-disinfected surgical tools, weighed, and snap-frozen in petri dishes on a pool of liquid nitrogen. Organs were stored at -20° C until enzyme analysis.

For enzymatic analysis, freshly thawed tissue samples were placed in 50 ml polypropylene tubes containing lysis buffer (27 mM citric acid, 46 mM sodium phosphate dibasic, 0.15% Triton X-100, pH 4.6) and homogenized on ice using a Tissue Tearor (Biospec Products, Inc., Bartlesville, OK), setting #3, for 2 min. Debris was pelleted by centrifugation at room temperature, and soluble fractions were kept on ice until assayed for α-Gal A activity. Plasma and unfractionated urine were assayed for α-Gal A activity directly.

Enzyme and Protein Assays

α-Gal A activity was determined at 37° C using 5 mM 4-methylumbelliferyl-α-D-galactopyranoside (4MU-α-Gal) containing 117 mM *N*-acetylgalactosamine (an α-Gal B inhibitor) at pH 4.6, as previously described (Bishop et al., 1980). One unit (U) of α-Gal A activity corresponds to one nmole 4MU hydrolyzed/hr. Protein concentrations were determined by the fluorescamine method (Bohlen et al., 1973). Where indicated, activities were corrected by subtracting the mean residual α-Gal A activities for each organ from adult male α-Gal A-deficient mice (see Chapter 2).

RESULTS

Characterization of α-Gal A Glycoforms

The physical properties of the four α-Gal A glycoforms are given in Table 1. All glycoforms had similar molecular weights and pIs. GF 1 and GF 4 α-Gal A were sialylated, while GF2 α-Gal A was not sialylated, indicating that differences exist in the glycosylation apparatus between different CHO cell lines. These glycosylation patterns resemble α-Gal A purified from human plasma (Bishop and Desnick, 1981). Extensive characterization of the oligosaccharide on GF 2 by mass spectrophotometry revealed that this glycoform possesses small amounts of sialic acid (Matsuura et al., 1998). The sialylation in GF 1 α-Gal A is α2-3, as CHO cells apparently do not contain a functional α2-6 sialyltransferase gene (Smith et al., 1990). High levels of sialylation were restored in GF 3 α-Gal A by transfection of the rat α2-6 sialyltransferase gene in CHO DG44 cells expressing GF 2 (Ioannou, unpublished results). This form resembles human plasma α-Gal A more than GF1 α-Gal A, as the plasma enzyme possesses sialic acid exclusively in the α2-6 linkage (Ioannou, unpublished results). GF 4 α-Gal A was not mannose-6-phosphorylated, as mutating glycosylation site 2 results in the loss of the enzyme's high mannose oligosaccharide (Ioannou et al., 1998). This suggests that the hybrid oligosaccharide of α-Gal A contains little M6P (Matsuura et al., 1998). GF 1 and GF 2 were active against the enzyme's natural substrate, GL-3. GF 3 and GF 4 activity against GL-3was not determined, although there is no reason to believe that they do not hydrolyze the natural substrate. GF 1, GF 2, and GF 3 α-Gal A had similar physical properties and temperature and pH stabilities, also similar to those reported for human α-Gal A (Bishop et al., 1980; Bishop and Desnick, 1981; Bishop and Sweeley, 1978; Dean and Sweeley, 1979; Kusiak et al., 1978; Mayes and Beutler, 1977; Sweeley et al., 1972), whereas GF 4 was less stable, probably due to its lack of one of the three oligosaccharides present on the wild-type enzyme.

Table 1
Characterization of α-Gal A glycoforms

Glycoform	Source	Monomer MW (kDa)	pI	Sialylation	M6P	GL-3 Activity	Stability (% original activity) pH 4.6	pH 7.4
GF 1	Stable DUKXB11	~56	~3.9-4.7	+	+	+	100	50
GF 2	Stable DG44	~50	~4.4-4.9	-	+	+	100	52
GF 3	Stable DG44	~55	~4.0-4.6	+	+	ND	94	49
GF 4	Transient COS-1	~52	~4.2-4.6	+	-	ND	83	33

+, presence of characteristic; -, absence of characteristic; ND, not determined.

Biodistribution of α-Gal A Glycoforms

α-Gal A-deficient mice were administered a single bolus injection of between $8x10^3$ and $5x10^4$ (0.2-1 mg/kg) of each α-Gal A glycoform. One hour after injection, mice were sacrificed and perfused, and the liver, spleen, heart, lungs, brain, and kidneys were excised and analyzed for α-Gal A activity (Table 2). Of the activity recovered, 95-98% was found in the liver, 1-3% was detected in the spleen and kidneys, and little, if any, enzyme was recovered in the heart, lungs, or brain. GF 1 α-Gal A appeared to be the best glycoform as far as targeting to the kidney, whereas very little GF 4 α-Gal A was recovered in this tissue. Recoveries of injected material were between 25% and 35% for each glycoform, suggesting that the enzymes were either degraded during the one hour biodistribution period or they were targeted to sites not assayed in this study. Of the four glycoforms, GF 4 α–Gal A appeared to be the most liver-specific and least kidney-specific, which may reflect its lack of mannose-6-phosphorylation or its relative instability (Table 1). No significant differences in the biodistribution of the other three α-Gal A glycoforms were noted.

Pharmacokinetics of α-Gal A Glycoforms

Pharmacokinetic studies were performed by administering a single injection of between $1x10^4$ and $5x10^4$ U (0.2-1 mg/kg) of each α-Gal A glycoform. Plasma samples were obtained at various time points following injections and analyzed for α-Gal A activity (Fig. 1). All α-Gal A glycoforms had half-lives in circulation of less than 5 min, with GF 1 having the longest at ~ 4 min. The two sialylated glycoforms from CHO cells, GF 1 and GF 3, had slightly longer half-lives than the non-sialylated form, GF 2, suggesting that sialylation was important in delaying their clearance from the circulation. GF 4 from COS-1 cells had the shortest half-life, even though it is also sialylated. This is probably due to its relative instability at the neutral pH of blood (Table 1). The recovery of sialylated α-Gal A glycoforms at 20 min post-injection was slightly higher than the non-sialylated form.

Table 2
One hour biodistribution of α-Gal A glycoforms in α-Gal A-deficient mice

	GF 1		GF 2		GF 3		GF 4	
Organ	Total Units	% Recovered	Total Units	% Recovered	Total Units	% Recovered	Total Units	% Recovered
Liver	12088±2117	95.2±1.6	9813±451	95.7±0.2	10603±550	96.1±0.8	2356±284	98.5±0.9
Spleen	352±105	2.9±1.1	277±50	2.7±0.4	298±93	2.7±0.8	33±19	1.3±0.7
Kidney	167±39	1.4±0.5	115±35	1.1±0.4	13±4	0.8±0	0±0	0.2±0.2
Lung	45±64	0.4±0.6	37±18	0.4±0.2	27±4	0.2±0.1	0±0	0±0
Heart	26±9	0.2±0.1	16±7	0.2±0.1	0±0	0.1±0.1	0±0	0±0
Brain	0±0	0±0	0±0	0±0	88±7	0±0	6±6	0±0

Values are expressed as means ± SD of 3-4 adult male α-Gal A-deficient mice. Mice were administered $5x10^4$ U (1 mg/kg) of GF 1, 2, and 3, and $8x10^3$ U (0.2 mg/kg) of GF 4. Total units are expressed as nmoles 4-MU/hr and were corrected by subtracting the mean residual α-Gal A activities for each organ from adult male α-Gal A-deficient mice (see Chapter 2). Percent recovered was calculated by dividing the total units recovered for each organ by the sum of the total units recovered for the six organs.

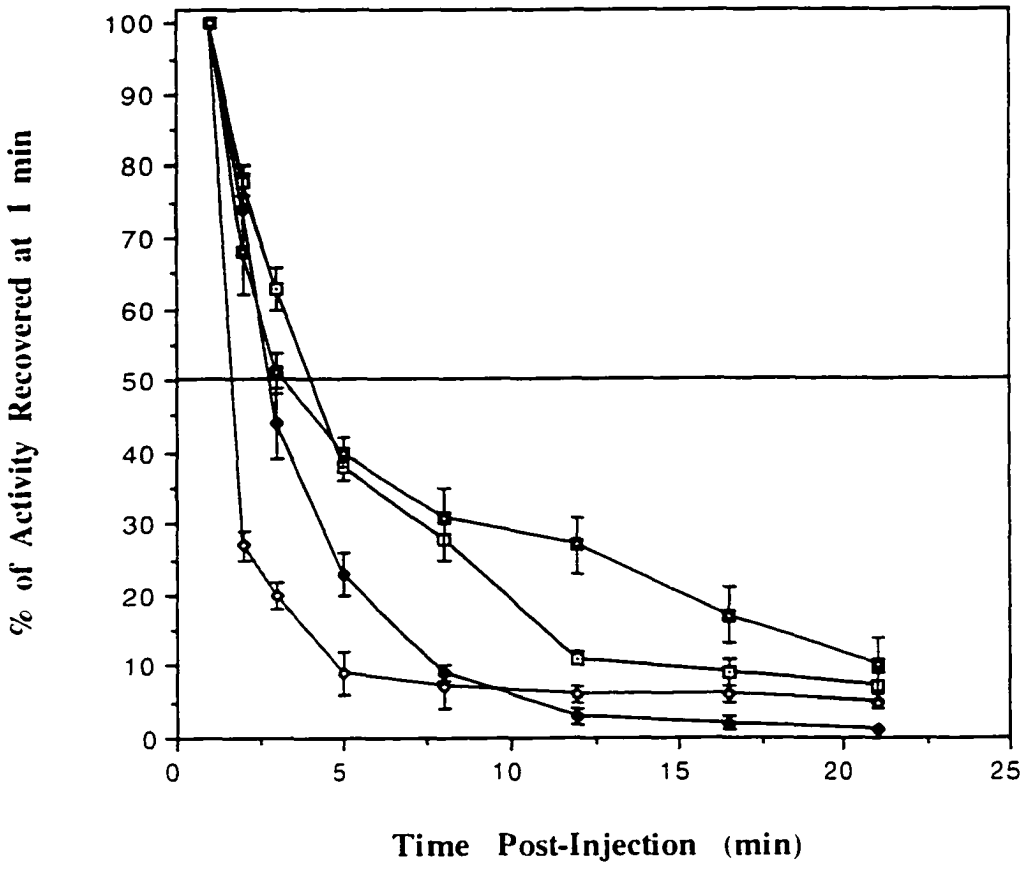

Figure 1. Pharmacokinetics of α-Gal A glycoforms in α-Gal A-deficient mice. Mice were administered between $1x10^{-4}$ (0.2 mg/kg) and $5x10^{-4}$ (1 mg/kg) of each glycoform. Each data point represents the mean ± SD of 3-4 mice. Open squares, GF1; filled diamonds, GF2; filled squares, GF3; open diamonds, GF4.

Approximately 40% of the injected GF 4 activity was present at 1 min post-injection, compared with ~10% for the other 3 glycoforms (data not shown). This suggests that removal of at least some of the high-mannose oligosaccharides by site-directed mutagenesis (Ioannou et al., 1998) increases the initial circulation retention of α-Gal A. The rapid clearance of enzymatic activity for all glycoforms from the circulation suggests that the enzymes were effectively taken up by specific tissues, were unstable in circulation, or a combination of both. Alhough slight differences in the pharmacokinetics of the four different α-Gal A glycoforms were detected, these differences were not expected to be clinically significant. Based on the above results of biodistribution and pharmacokinetic studies, GF 1 was selected for further analysis.

Biodistribution of GF 1 α-Gal A

Mice were administered a single injection of $5x10^5$ U (10 mg/kg) of GF 1 α-Gal A to determine if a different biodistribution to organs other than the liver could be achieved. One hour after the injection, mice were sacrificed and perfused and the tissues removed and analyzed for α-Gal A activity (Table 3). With the higher dose, increased levels of active α-Gal A enzyme were recovered in all tissues except the brain. More importantly, the percentage distributed to liver was decreased, whereas the percentage recovered in spleen, heart, and kidney was increased. This observation indicates that the amount of enzyme reaching each organ is dose dependent and that increasing the amount of administered enzyme can alter its biodistribution, perhaps by saturating certain tissue-specific receptors. This hypothesis was further supported by injecting a mouse with 2 mg mannans 20 min prior to administration of $2.5x10^4$ U GF 1 (0.5 mg/kg). The amount of α-Gal A activity remaining in the circulation 1 hr post-injection increased from <5% without mannans to 40% with mannans. In addition, the activity recovered in the kidneys increased from approximately 1% to 10% of the total enzyme recovered. These results indicated that the rapid removal of the enzyme from the circulation to the liver is partially due to the

Table 3

Comparison of one hour biodistribution of two doses of GF I α-Gal A in α-Gal A-deficient mice with endogenous α-Gal A levels in wild-type mice

	Wild-Type		$5x10^4$ U (~1 mg/kg)			$5x10^5$ U (~10 mg/kg)		
Organ	Total Units	% Recovered	Total Units	% Recovered	% of Wild-Type	Total Units	% Recovered	% of Wild-Type
Liver	3601±274	45.9±3.5	12088±2117	95.2±1.6	336	121505	93.2	3374
Spleen	1233±248	15.7±3.2	352±105	2.9±1.1	29	4739	3.7	384
Kidney	438±39	5.6±0.5	167±39	1.4±0.5	38	3262	2.5	745
Lung	729±39	9.2±0.5	45±64	0.4±0.6	6	350	0.3	48
Heart	33±2	0.4±0.1	26±9	0.2±0.1	79	534	0.4	1618
Brain	1753±241	22.3±3.1	0±0	0±0	0	37	0	2

Values are means ± SD and were calculated using 6 adult male wild-type mice, 3 adult male α-Gal A-deficient mice for the $5x10^4$ U dose, and 2 adult male α-Gal A-deficient mice for the $5x10^5$ U dose. Total units are expressed as nmoles 4-MU/hr and were corrected by subtracting the mean residual α-Gal A activities for each organ from adult male α-Gal A-deficient mice (see Chapter 2). Percent recovered was calculated by dividing the total units recovered for each organ by the sum of the total units recovered for the six organs.

reticuloendothelial mannose receptor and that by saturating this receptor, the enzyme can be diverted to other tissues.

Biostability of GF 1 α-Gal A

To determine the half-life of α-Gal A in tissue, mice were given a single injection of 1 mg/kg GF 1 a-Gal A, and pairs of mice were sacrificed and perfused at various time points. Livers, spleens, and kidneys were exised and analyzed for α-Gal A activity (Fig. 2). Only time points that differed by less than 15% were included in the analysis. Again, ~30% of the total enzyme activity was recovered at the 1 hr time point, with the majority of the enzyme recovered in the liver (~95%). The enzyme exhibited an exponential decay in liver with a $t_{1/2}$ of ~40 hr, and approximately 30% was recovered 96 hr post-injection. The stability was ~50% lower in spleen and kidney ($t_{1/2}$ ~20 hr), with 10-20% recovered 96 hr-post injection. These results indicated that the enzyme is fairly stable upon removal from the circulation and suggested that the enzyme was endocytosed into lysosomes, probably by the M6P receptor (M6PR), since the enzyme is quite unstable at neutral pH (Table 1).

Specific Activity of GF 1 α-Gal A Following Multiple Administrations

To determine whether α-Gal A-deficient tissues could accumulate wild-type levels of α-Gal A activity, mice were administered a total of 8 injections of 1 mg/kg or 10 mg/kg GF 1 α-Gal A at 48-hr intervals. Twenty-four hrs after the final injection, mice were sacrificed and perfused, and the tissues removed and analyzed for α-Gal A activity. As can be clearly seen in Table 4, recovered activities were dose dependent. As expected, liver was the most easily-corrected tissue, followed by heart, spleen, and lung. Most importantly, at the higer dose, kidney α-Gal A levels were completely corrected. The urine of mice treated with α-Gal A also showed increased activity compared to untreated α-Gal A-deficient mice, whereas brain showed no correction at either dose, as expected.

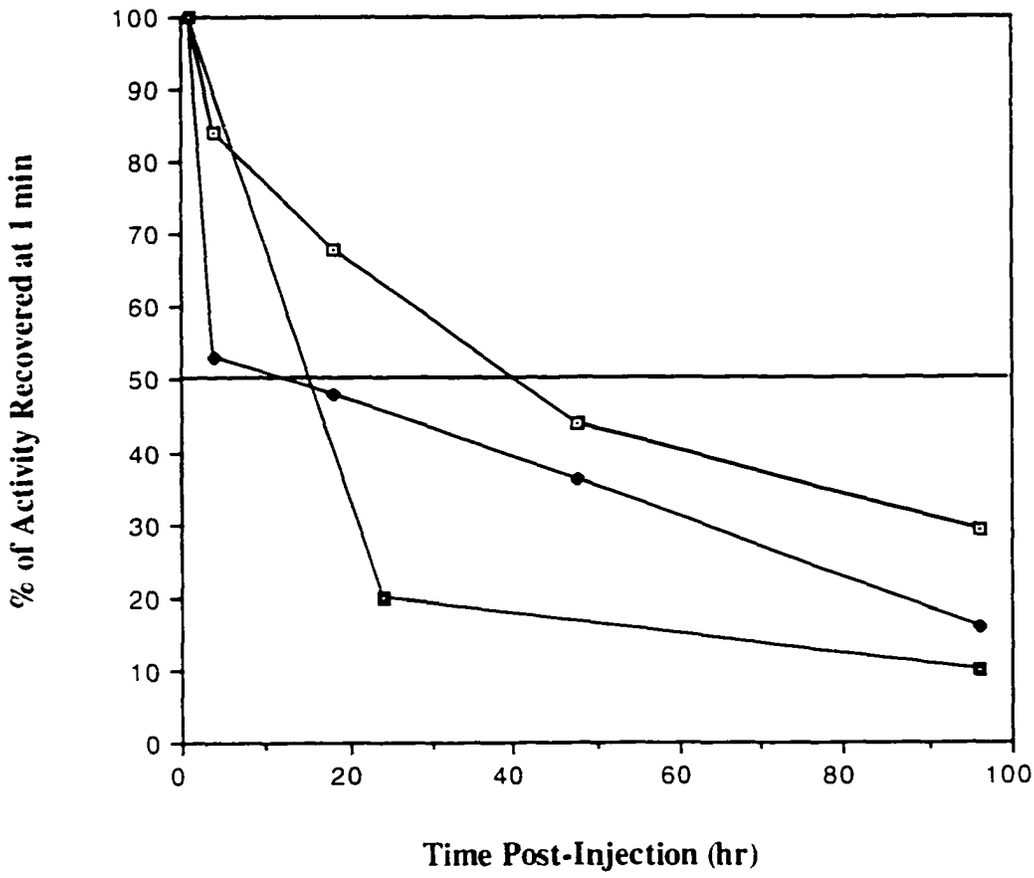

Figure 2. Stability of GF1 α-Gal A in various tissues of α-Gal A-deficient mice Mice were administered $5x10^4$ (1 mg/kg) of enzyme. Data points represent the mean of two mice which did not differ by more than 15%. Activities were corrected by subtracting the mean residual α-Gal A activities for each organ from adult male α-Gal A-deficient mice (see Chapter 2). Open squares, liver; filled diamonds, spleen; filled squares, kidney.

Table 4
Comparison of specific activities of different GF 1 α-Gal A doses following eight administrations to α-Gal A-deficient mice with endogenous α-Gal A levels in wild-type mice

	α-Gal A-Deficient					Wild-Type (n=6)
Tissue	Untreated (n=6)	1 mg/kg (n=3)	% of Wild-Type	10 mg/kg (n=3)	% of Wild-Type	
Liver	0.2±0.2 (0.1-0.3)	91.3±8.9 (85.4-101.6)	542	1162.7±161.0 (1014.4-1344.6)	6922	16.8±1.6 (15.1-18.7)
Spleen	0.9±0.6 (0.5-1.3)	28.6±5.4 (23.6-34.4)	73	44.7±14.4 (27.8-52.6)	114	39.2±8.3 (27.3-50.0)
Heart	1.0±0.8 (0.1-2.0)	2.4±0.4 (1.9-2.8)	75	17.7±8.7 (7.8-25.1)	553	3.2±0.6 (2.7-4.4)
Lung	1.0±1.0 (0.4±1.5)	4.1±1.8 (3.0-6.2)	14	24.7±14.8 (12.3-40.3)	82	29.8±4.7 (22.5-34.8)
Brain	0.4±0.3 (0.3-0.7)	0.4±0.2 (0.2-0.5)	1	0.5±0 (0.5-0.5)	2	26.9±2.9 (22.8-29.9)
Kidney	0.4±0.3 (0.2-0.6)	1.5±0.3 (1.1-1.7)	13	14.0±4.0 (9.6-17.9)	124	11.3±1.3 (10.0-13.2)
Plasma	1.2±0.3 (0.9-1.6)	3.2±2.1 (1.1-1.7)	56	6.3±0.2 (6.0-6.5)	111	5.7±1.2 (4.3-6.5)
Urine	26 (n=1)	35±11 (22-43)	-	290±176 (123-473)	-	-

Values represent means ± SD and are expressed as nmoles 4-MU/hr/mg protein, except for plasma and urine, which are nmoles 4-MU/hr/ml. Ranges are indicated in parentheses. Adult male mice were administered eight injections at 48 hr intervals and were sacrificed 24 hr following the last injection. Wild-type and untreated α-Gal A-deficient mouse data are taken from Chapter 2.

Accumulation of α-Gal A in plasma levels was also dose dependent and completely corrected at the higher dose.

DISCUSSION

Based on a number of observation, it appears that Fabry disease may be an excellent candidate for treatment by ERT, . First, patient fibroblasts can be corrected by the addition of α-Gal A from a number of different tissue sources (Dawson et al., 1973; Dooley and Applegarth, 1980; Hasholt et al., 1988; Mayes et al., 1982; Osada et al., 1987; Sifers et al., 1983; Tsuji et al., 1994); second, heterozygotes generally present with very few severe symptoms of the disease (Desnick et al., 1995); third, early trials with nomal plasma (Mapes et al., 1970), placental α-Gal A (Brady et al., 1973), and splenic and plasma α-Gal A (Desnick et al., 1979) were encouraging in that circulating plasma levels of GL-3 were reduced; and fourth, Gaucher disease, a related glycosphingolipid storage disease, has been successfully treated by ERT (Brady et al., 1994). Larger, more detailed analyses of the effectivenenss of ERT for Fabry disease awaited production of large amounts of pure enzyme and the development of a small animal model.

The cloning of the human α-Gal A genomic and cDNA sequences (Bishop et al., 1986; Kornreich et al., 1989) led to the production and purification of large quantities of purified recombinant α-Gal A in CHO cells (Ioannou et al., 1992; Ioannou, unpublished results). The enzyme has been characterized and shown to be similar to the enzyme purified from human sources. The cloning of the mouse α-Gal A gene (Gotlib et al., 1996; Ohshima et al., 1995) led to the development of an α-Gal A-deficient mouse by homologous recombination in embryonic stem cells (Ohshima et al., 1997; Wang, unpublished results). These mice are completely deficient in α-Gal A activity and accumulate large amounts of GL-3 in all tissues and plasma, but are otherwise phenotypically normal (see Chapter 2).

A previous study of α-Gal A replacement demonstrated a physiological difference between plasma and splenic α-Gal A when injected into Fabry patients (Desnick et al., 1979). The plasma form, which was significantly more sialylated than the splenic form

(Bishop and Desnick, 1981), had a circulating half-life of ~70 min, compared to ~10 min for its counterpart. Also, the plasma form was more effective at maintaining reduced plasma GL-3 levels than the splenic form. In another enzyme replacement study (Brady et al., 1973), placental α-Gal A also had a $t_{1/2}$ in plasma of ~10 min. These studies also revealed that a large amount of the administered enzyme was sequestered by the liver, although no other tissues were examined. We therefore tested four different recombinant human α-Gal A glycoforms for biodistribution, pharmacokinetics, and stability in α-Gal A-deficient mice, although, based on IEF, each glycoform is itself a heterogeneous population (Table 1). Since Fabry disease is primarily a disease of adult males (Desnick et al., 1995), and since in mice hormonally-induced glycosphingolipid differences exist between males and females (Coles et al., 1970; Johnson et al., 1986; McCluer et al., 1983), only adult male mice were used in this study.

These four α-Gal A glycoforms differed in their stabilities and glycosylation patterns (Table 1). They were first tested for 1 hr biodistributions following administration to α-Gal A-deficient mice (Table 2). Total recoveries of injected material were between 25% and 35% for each glycoform, suggesting that the enzymes were either degraded during the one hour biodistribution period or were targeted to sites not assayed in this study. In studies involving administration of acid β-glucosidase to normal mice, immunological material analysis revealed rapid loss of enzymatic activity in liver and spleen due to denaturation and proteolysis (Xu et al., 1996). The majority of GF 1, 2, and 3 α-Gal A was recovered in liver, while small amounts were recovered in spleen and kidney, and little to no activity was recovered in heart or lung. The lack of recovery in brain was expected, due to the presence of the blood-brain barrier (Rattazzi et al., 1980). The low recovery of GF 4 α-Gal A in kidney and its lack of recovery in heart and lung may reflect its lack of mannose-6-phosphorylation (Table 1), which has been shown to be important for extrahepatic tissue uptake of α-glucosidase (Van der Ploeg et al., 1991) and *N*-acetyl-galactosamine-4-sulfatase (Crawley et al., 1996), although the relative instability of the

glycoform (Table 1) cannot be discounted. The distributions of GF 1, 2, and 3 α-Gal A are similar to that seen with acid β-glucosidase in rats (Bijsterbosch et al., 1996) and mice (Xu et al., 1996), acid α-glucosidase in mice (Van der Ploeg et al., 1991), α-*L*-iduronidase in dogs (Kakkis et al., 1996; Shull et al., 1994), *N*-acetylgalactosamine-4-sulfatase in cats (Crawley et al., 1996), β-hexosaminidase in cats (Rattazzi et al., 1980), and β-glucuronidase in rats (Achford et al., 1977) and mice (Sands et al., 1994; Thorpe et al., 1974). The large hepatic recovery of exogenous recombinant α-Gal A was previously demonstrated with α-Gal A purified from human plasma and spleen when injected into Fabry patients (Bishop et al., 1981). This result is also similar to results involving other mammalian lysosomal enzymes. For example, acid α-glucosidase (De Barsy and Van Hoof, 1974; Van der Ploeg et al., 1991; Van Hove et al., 1996), β-hexosaminidase (Desnick, 1974; Johnson et al., 1973; Rattazzi et al., 1980), β-glucuronidase (Achford et al., 1977; Fiddler and Desnick, 1977; Sands et al., 1994; Thorpe et al., 1974), acid β-glucosidase (Bijsterbosch et al., 1996; Brady et al., 1974; Xu et al., 1996), arylsulfatase A (Greene et al., 1969), α-*L*-iduronidase (Kakkis et al., 1996; Shull et al., 1994), *N*-acetylgalactosamine-4-sulfatase (Crawley et al., 1996) all demonstrated high liver uptake when administered to human patients or animals. Even fungal α-glucosidase was shown to enter the livers of patients with type II glycogenosis (Badhuin et al., 1964; Hug and Schubert, 1967; Hug et al., 1973; Huijing et al., 1973; Lauer et al., 1968).

Uptake of human β-glucuronidase into the livers of rats was shown to be due to the reticuloendothelial mannose receptor (Achford et al., 1978). This was confirmed for GF 1 α-Gal A by injecting a mouse with 2 mg mannans 20 min prior to administration of 2.5×10^4 U of enzyme (0.5 mg/kg). The amount of α-Gal A activity remaining in the circulation 1 hr post-injection increased from <5% without mannans to 40% with mannans. Also, the activity recovered in the kidneys increased from approximately 1% to 10% of the total enzyme recovered. Mannans administration has also been used to prevent hepatic uptake and increase the circulation half-life of β-glucuronidase in rats (Achford et al.,

1978), β-hexosaminidase in cats (Rattazzi et al., 1980), and acid β-glucosidase in rats (Bijsterbosch et al., 1996) by saturating the mannose receptor. Thus, it can be assumed that all four α-Gal A glycoforms have at least some exposed mannose residues.

The four α-Gal A glycoforms were next tested for plasma clearance following administration to α-Gal A-deficient mice (Fig. 1). All four α-Gal A glycoforms were quickly removed from the circulation, with $t_{1/2}$s < 5 min. The two sialylated glycoforms from CHO cells, GF 1 and GF 3, had slightly longer $t_{1/2}$s than the non-sialylated form, GF 2, suggesting that this sugar moiety is important in delaying removal from the circulation. This is consistent with the study of Desnick et al. (1979), which demonstrated an increased $t_{1/2}$ of sialylated plasma α-Gal A (~70 min) over non-sialylated splenic α-Gal A (~10 min). The much higher difference in that study can probably be explained by the fact that α-Gal A purified from plasma has been "selected" for circulation retention and is completely complex and sialylated, whereas the CHO enzymes have at least some exposed mannose and β-galactose residues (Matsuura et al., 1998), making it a ligand for the mannose and asialoglycoprotein receptors, respectively. This was also thought to explain the increased $t_{1/2}$ of complex *N*-acetylgalactosamine-4-sulfatase (~45 min) over its high-mannose counterpart (~16 min) in the circulation of cats (Crawley et al., 1996). GF 4 α-Gal A from COS-1 cells had the shortest half-life, even though it is also sialylated and lacks the high mannose oligosaccharide (Table 1). This is probably due to its relative instability at the conditions present in circulation (Table 1). Recoveries of sialylated α-Gal A glycoforms at 20 min post-injection were slightly higher than the non-sialylated form. Approximately 40% of the injected GF 4 activity was present at 1 min post-injection, compared with ~10% for the other 3 glycoforms (data not shown), suggesting that removal of the high-mannose oligosaccharide increases the initial retention time of α-Gal A in circulation. The rapid removal from the circulation of enzymatic activity for all glycoforms suggested that the enzymes were effectively targeted to specific tissues, particularly the liver (Table 2), although some rapid denaturation in plasma was also likely (Table 1). The rapid clearance

of recombinant α-Gal A from the circulation of α-Gal A-deficient mice is similar to that obtained for other lysosomal enzymes. For example, acid α-glucosidase (De Barsy and Van Hoof, 1974), β-hexosaminidase A (Desnick, 1974; Johnson et al., 1973; Rattazzi et al., 1980; Von Sprecht et al., 1979), arylsulfatase A (Greene et al., 1969), acid β-glucosidase (Bijsterbosch et al., 1996; Brady et al., 1974; Furbish et al., 1978; Xu et al., 1996), β-glucuronidase (Achford et al., 1977; Thorpe et al., 1974), α-*L*-iduronidase (Shull et al., 1994), and *N*-acetylgalactosamine-4-sulfatase (Crawley et al., 1996) all had $t_{1/2}$s in circulation of 1-30 min when injected into human patients or animals. Three notable exceptions are total β-hexosaminidase activity in plasma concentrates, which had a $t_{1/2}$ in the circulation of a Sandhoff disease patient of 2-4 hr (Desnick et al., 1971), iduronate sulfatase activity in Hunter syndrome patients following plasma exchange, which had a $t_{1/2}$ in circulation of ~20 hr (Brown III et al., 1982), and *Aspergillus niger* α-glucosidase in the circulation of a patient with glycogenosis type II, which had a $t_{1/2}$ of ~2 hr (Hug and Schubert, 1967). Although slight differences in the pharmacokinetics of the four different α-Gal A glycoforms were detected, these differences were not expected to be clinically significant. Based on the biodistribution, pharamacokinetic, and stability studies, GF 1 was selected for further analysis.

The half-life of GF 1 α-Gal A in liver was ~40 hr, while in spleen and kidney the half-lives were ~20 hr (Fig. 2). This observation indicates that the enzyme is fairly stable once removed from circulation and suggests that the enzyme reaches the lysosome, probably via M6PR-mediated endocytosis, since the enzyme is quite unstable outside of an acidic environment (Table 1). The increased stability in liver may reflect the much higher concentration of the enzyme in that organ, compared to spleen and kidney. These results are similar to those obtained for human β-glucuronidase, which has a $t_{1/2}$ in rat liver of ~60 hr (Achford et al., 1977) and a $t_{1/2}$ in mouse liver of ~50 hr (Vogler et al., 1993), and human *N*-acetylgalactosamine-4-sulfatase, which has a $t_{1/2}$ in mouse liver of ~72 hr (Crawley et al., 1996). These values are significantly lower that those obtained for human

acid α-glucosidase in human liver ($t_{1/2}$~5 days) (De Barsy and Van Hoof, 1974), bovine acid α-glucosidase in mouse liver, spleen, and kidney ($t_{1/2}$~4 days) (Van der Ploeg et al., 1991), and human β-glucuronidase in mouse spleen and kidney ($t_{1/2}$~3 days) (Vogler et al., 1993) and rat spleen ($t_{1/2}$~6 days) (Achford et al., 1977), but significantly greater than *A. niger* α-glucosidase in rat liver ($t_{1/2}$~8 hr) (Huijing et al., 1973), bovine β-glucuronidase in mouse liver ($t_{1/2}$~10 hr) and kidney ($t_{1/2}$~1 hr) (Thorpe et al., 1974) and human acid β-glucosidase in mouse liver ($t_{1/2}$~1 hr) (Xu et al., 1996).

Mice were administered 10 mg/kg GF 1 α-Gal A to determine if a redistribution of the enzyme to other tissues could be achieved (Table 3). Except for brain, increased levels of α-Gal A were recovered in all tissues with the higher dose. This indicates that the amount of enzyme reaching a particular organ is dose-dependent, as was demonstrated for human *N*-acetylgalactosamine-4-sulfatase in cats (Crawley et al., 1996), bovine acid α-glucosidase in mice (Van der Ploeg et al., 1991), *A. niger* α-glucosidase in rats (Huijing et al., 1973), bovine β-glucuronidase in mice (Thorpe et al., 1974), and human α-*L*-iduronidase in dogs (Kakkis et al., 1996). More importantly, the percentage distributed to liver decreased, whereas the percentage recovered in spleen, heart, and kidney increased. These recoveries closely parallel the endogenous α-Gal A biodistribution in wild-type mice, indicating that increasing the amount of administered enzyme can alter its biodistribution, perhaps by saturating the reticuloendothelial mannose receptor.

To determine whether enzyme levels could be corrected long-term, mice were administered multiple injections of 1 or 10 mg/kg GF 1 α-Gal A (Table 4). Specific activities were again dose-dependent, with the higher dose completely correcting the enzyme deficiency in all tissues except brain. The lower α-Gal A dose was also capable of partially restoring normal activities in tissues and plasma. Similarly, multiple human α-*L*-iduronidase administration (~0.5 mg/kg) to deficient dogs has been shown to completely correct their deficiency in most tissues (Kakkis et al., 1996). Interestingly, only a single administration of human *N*-acetylgalactosamine-4-sulfatase (~1 mg/kg) to deficient cats

was needed to completely correct their deficiency in most tissues examined (Crawley et al., 1996). On the other hand, multiple human β-glucuronidase administration (~0.5 mg/kg) to deficient mice only partially corrected their deficiency in liver (~28% of normal), while barely effecting a change in spleen and kidney (3-5% of normal) (Sands et al., 1994).

Plasma α-Gal A levels were also corrected at 24 hr following the last injection, suggesting that with repeated injections, enzyme protein is selected for retention in the circulation, possibly due to subtle differences in glycosylation (Matsuura et al., 1998), similar to the human plasma enzyme (Desnick et al., 1979). Enzyme was also recovered from urine in a dose-dependent fashion (Table 4), suggesting that enzyme reached the lysosomal system of the kidney. Male mice normally excrete large daily amounts of lysosomal enzymes and glycosphingolipids in their urine as multilamellar bodies whose origin is the proximal tubules (Gross et al., 1991; McCluer et al., 1981). The enzyme's large size (M_r~100 kDa) makes it unlikely that it was simply excreted (Mattenheimer, 1971).

These results indicate that when administered to α-Gal A-deficient mice at doses comparable to those reported for other lysosomal enzymes, recombinant human α-Gal A can restore enzyme levels to those found in the tissues of wild-type mice in a dose-dependent fashion. In this respect, in addition to its pharmacokinetics and stability, α-Gal A behaves similarly to other lysosomal enzymes studied *in vivo* and should provide the rationale for ERT of Fabry disease.

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Chapter 4

Effect of α-Gal A Replacement on Globotriaosylceramide Storage in α-Gal A-Deficient Mice

ABSTRACT

Fabry disease is an X-linked recessive lysosomal storage disorder, resulting from a deficiency of α-galactosidase A (α-Gal A). Pathology occurs in the vascular endothelium, particularly of the heart and kidneys, which is due to the progressive accumulation of α-galactosyl-containing glycolipids, primarily globotriaosylceramide (GL-3). Previously, we demonstrated that recombinant human α-Gal A can correct the enzyme defect in α-Gal A-deficient mice in all tissues examined, including heart and kidney (see Chapter 3). This study was undertaken to determine the effect of enzyme replacement on GL-3 levels in these mice. Single doses of administered α-Gal A (0.3-10 mg enzyme/kg body weight) were capable of metabolizing liver, spleen, heart, and kidney GL-3 in a dose-dependent manner. A multiple high-level dose (8 x 3 mg/kg) reduced GL-3 to undetectable levels in liver, spleen, and heart and by 50% in kidney. GL-3 accumulation in skin was also reduced in a dose-dependent fashion following multiple administrations of α-Gal A, with a 5 x 10 mg/kg dose clearing ~80% of the stored lipid. Most importantly, all doses led to a depletion of circulating levels of GL-3 in a dose-dependent manner. Reaccumulation of depleted GL-3 following a single 3 mg/kg administration was seen at three weeks post-injection. Heart showed the greatest reaccumulation (~30% of untreated control) followed by spleen (~10%), with no reaccumulation detected in liver. Mice generally tolerated the injections well, and antibody production to α-Gal A was rare. When present, antibodies were not neutralizing and did not affect the enzyme's ability to metabolize GL-3. These results indicate that exogenous α-Gal A can safely catabolize accumulated GL-3 in α-Gal A-deficient mice and provide the rationale for ERT of Fabry disease.

INTRODUCTION

α-Galactosidase A (α-Gal A; E.C. 3.2.1.22) is a lysosomal glycosidase capable of hydrolyzing terminal α-galacatosyl moieties from glycolipids and glycoproteins (Desnick et al., 1995). The mature human enzyme has been biochemically characterized, and shown to be a homodimeric glycoprotein with a subunit molecular weight of about 48 kDa (Bishop and Desnick, 1981). The enzyme precursor is co-translationally glycosylated and modified in the Golgi complex and lysosomes to a mature homodimeric enzyme of 101 kDa (LeDonne et al., 1983; Lemansky et al., 1987).

A deficiency of α-Gal A results in Fabry disease, an X-linked recessive lysosomal storage disorder characterized by the accumulation of the enzyme's glycosphingolipid substrates, principally globotriaosylceramide (GL-3), in the plasma and vascular endothelium (Desnick et al., 1995). In classically affected hemizygous males, the major disease manifestations include angiokeratoma, acroparathesias, hyphohydrosis, corneal dystrophy, and vascular disease of the heart, kidneys, and brain leading to early demise in adulthood (Desnick et al., 1995). Some hemizygous males, the so-called cardiac variants, exhibit only heart manifestations (Ogawa et al., 1990), now known to be caused by mutations which result in residual α-Gal A activity (Sakuraba et al., 1990). In heterozygous females, the manifestations are quite variable, and can range from mild to severe, presumably due to random X-chromosome inactivation (Lyon, 1961).

Currently, there is no treatment for Fabry disease. Studies with patient fibroblasts indicated that exogenous α-Gal A could correct the metabolic defect in Fabry disease (Dawson et al., 1973; Dooley and Applegarth, 1980; Hasholt et al., 1988; Mayes et al., 1982; Osada et al., 1987; Sifers et al., 1983; Tsuji et al., 1994). Three studies of enzyme replacement therapy with crude or purified human α-Gal A in Fabry hemizygotes were successful in reducing circulating levels of GL-3 (Brady et al., 1973; Desnick et al., 1979; Mapes et al., 1970), although no effect on tissue storage was determined. With the

availability of large amounts of recombinant human α-Gal A (Ioannou et al., 1992) and an α-Gal A-deficient mouse model (see Chapter 2), the effectiveness of ERT for Fabry disease can now be assessed in ways which were previously impossible. This study was undertaken to determine the catabolic effect of α-Gal A replacement on GL-3 storage in these mice.

MATERIALS AND METHODS

Mice, Enzyme, and Injections

α-Gal A-deficient mice were produced by homologous recombination in 129/Sv ES cells as described in Chapter 2. All mice used in this study were adult males and weighed between 20–30 g. Mice were given standard mouse chow and water *ad libidum*. Recombinant human α-Gal A expressed in DUKXB11 CHO cells, provided by the Genzyme Corporation (Framingham, MA), was used for all injections. This enzyme, known as glycoform 1 (GF 1), was purified from the media of transfected cells and contains sialylated, complex oligosaccharides and mannose-6-phosphate (M6P) (see Chapter 3). Appropriate dilutions were made in citrate-phosphate (C-P) buffer, pH 6.0 containing 1 mg/ml BSA. Mouse injections were described in Chapter 3.

Tissue Collection, Extraction, and Glycosphingolipid Purification

Murine tissues were processed as described in Chapter 2, except skin was first homogenized in chloroform-methanol 2:1 using a Polytron homogenizer (Brinkmann, Westbury, NY).

Enzyme and Protein Assays

All enzyme, protein, and GL-3 assays were performed as described in Chapters 2 and 3.

Glycosphingolipid Analysis

GL-3 enzyme-linked immunosorbent assays (ELISAs) were performed as described in Chapters 1 and 2.

Antibody Analysis

Antibody response in mice to proteins present in the administered α-Gal A preparation was analyzed by ELISA. All reactions were in 100 µl volumes and were performed at room temperature. Immulon plates (Dynatech, Chantilly, VA) were coated with 1 µg GF 1 α-Gal A/well in 0.1 M sodium bicarbonate buffer, pH 9.6 for 3 hr. Wells were washed once with Tris-buffered saline (TBS), pH 8 and blocked with TBS containing 5% BSA for 1 hr. Wells were washed once with TBS and incubated with 2 µl mouse plasma in TBS containing 0.1% BSA and 0.05% Tween-20 (TBST) for 1 hr. Standard curves were generated with doubling dilutions of affinity purified polyclonal rabbit anti-α-Gal A IgG (Ioannou et al., 1992). Wells were washed three times with TBST and incubated with alkaline phosphatase-conjugated goat anti-mouse or anti-rabbit IgG (Boerhinger Mannheim) diluted 1:1000 in TBST for 1 hr. Wells were washed three times with TBST, once with TBS, and incubated with *p*NPP for 15-30 min. Reactions were terminated with 100 µl 5% EDTA, pH 8 and read at 405 nm as described in Chapter 2.

To determine whether the antibody response was to the α-Gal A enzyme itself, ELISAs were repeated on high-titer mouse plasma using two other α-Gal A glycoforms, GF 2 (see Chapter 3) and GF 5, which was produced in Sf9 cells using recombinant baculovirus (Ioannou et al., 1998). GF 2 α-Gal A contains complex non-sialylated oligosacchaidies and mannose-6-phosphate (see Chapter 3), whereas GF 5 α-Gal A contains only non-phosphorylated, high-mannose oligosaccharides (Ioannou et al., 1998). Western blot analysis was also performed to confirm the specificity of the response. α-Gal A (1 µg/lane) was separated by SDS-PAGE (Laemmli, 1970) in 10% polyacrylamide gels, followed by transfer to Westran PVDF membranes (Schleicher and Schuell, Keene, NH) according to manufacturer's recommendations. Membranes were blocked with phosphate-buffered saline (PBS), pH 7.4 containing 5% non-fat dry milk (Blotto) for 1 hr at room temperature, and then incubated with pooled mouse plasma diluted 1:100 in Blotto for 4 hr at room temperature. The membranes were treated with Vectastain Elite ABC kit (Vector

Laboratories, Burlingame, CA and stained for ~5 min with DAB substrate kit (Vector Laboratories) according to manufacturer's. Molecular weights were estimated using Rainbow markers (Amersham, Arlington Heights, IL).

Neutralization studies were performed to determine whether the murine antibodies were capable of inhibiting α-Gal A activity. GF 1 α-Gal A was diluted 1:5000 in PBS containing 1 mg/ml BSA. To 18 μl of enzyme solution (~5 ng α-Gal A), 2 ul PBS, pre- or post-injection high-titer mouse plasma, normal rabbit serum, or rabbit anti-α-Gal A was added and incubated for 1 hr at room temperature. α-Gal A activity was assayed for 30 min as described above.

RESULTS

Effect of α-Gal A on Tissue GL-3 Accumulation

Recombinant human α-Gal A was evaluated for its ability to catabolize the accumulated GL-3 in α-Gal A-deficient mice. Mice were administered either a single injection of 10, 3, 1, or 0.3 mg/kg α-Gal A or 8 injections of 10 or 3 mg/kg α-Gal A administered at 48 hr intervals. Mice administered single injections were sacrificed 14 days post-injection, whereas mice administered multiple injections were sacrificed 24 hr after the final injection on day 17. Animals were perfused, and the tissues removed and analyzed for GL-3 levels by ELISA. Controls recieved injections of buffer. As can be seen in Fig 1, all doses of enzyme were effective at catabolizing GL-3 in liver (A), spleen (B), heart (C), and kidney (D). The enzyme was most effective at clearing accumulated GL-3 in the liver, as all doses essentially reduced levels to those of wild-type mice. Spleen and heart GL-3 was catabolized in a dose-dependent fashion, with the 8 x 3 mg/kg dose reducing GL-3 levels in both organs to those of wild-type mice. Kidney GL-3 depletion did not appear strictly dose dependent, as all three single doses reduced the lipid by ~33%. The 8 x 3 mg/kg dose was able to further reduce the GL-3 to 50% of that found in untreated α-Gal A-deficient mice. This 50% reduction appears to be the maximum that can be achieved in kidney, as 8 x 10 mg/kg GF 1 yielded the same result (data not shown). However, it should be noted that kidney contains a second form of GL-3 which is specific for male mice (see Chapter 2) and is known to be testosterone induced (McCluer et al., 1981). This second band, shown as the slower migrating form of GL-3 in Fig. 2D, appears to be complexed as multilamellar bodies, seen under electron microscopy to be secreted in urine (Gross et al., 1991). Apparently this second form of GL-3 is not accessible by α-Gal A (McCluer et al., 1981). This form, however, is of no consequence, as it does not appear in human kidney (Boyd and Lingwood, 1989).

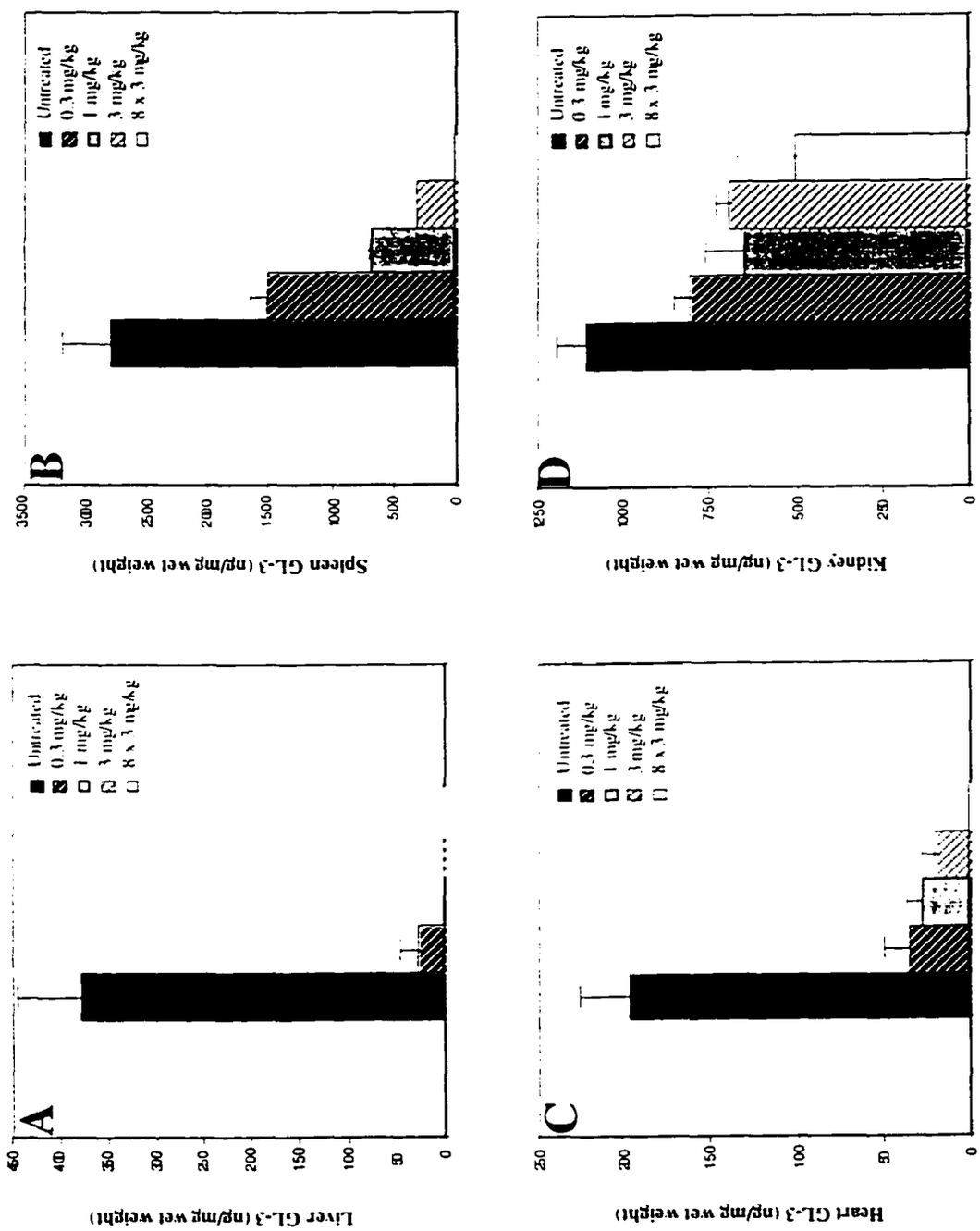

Figure 1. Effect of α-Gal A on tissue GL-3 levels in α-Gal A-deficient mice. A, liver GL-3 concentration; B, spleen GL-3 concentration; C, heart GL-3 concentration; D, kidney GL-3 concentration. Values represent the mean and standard deviation of 3 mice.

The above results were qualitatively confirmed by HPTLC (Fig. 2). Liver GL-3 was completely metabolized at all doses (Fig. 2A), while heart and spleen showed a dose-dependent decrease in GL-3 (Fig. 2B, C) , with the 8 x 10 mg/kg dose returning levels to normal or near-normal. HPTLC of kidney (Fig. 2D) revealed a semi-dose-dependent hydrolysis of the faster migrating GL-3 form, with little or no hydrolysis of the slower migrating form.

GL-3 accumulation in skin was also reduced in a dose-dependent fashion (Fig. 3). Mice administered 8 x 0.3 mg/kg had a 15% reduction in skin GL-3, as determined by ELISA, whereas mice administered 8 x 1 and 8 x 3 mg/kg had an ~50% reduction. To confirm the effect of exogenous α-Gal A on skin GL-3, mice were administered 10 mg/kg for 5 consecutive days and sacrificed on the sixth day. The GL-3 ELISA revealed an 80% reduction in accumulation of the stored lipid. Interestingly, the metabolism of skin GL-3 occurred in the absence or near absence of any detectable α-Gal A activity at all doses given (data not shown).

Effect of α-Gal A on Plasma GL-3 Accumulation

Since circulating GL-3 is thought to contribute to organ deposition in Fabry disease (Johnson and Desnick, 1978), GL-3 levels were quantitated in the plasma of treated and untreated mice (Fig. 4). Mice were administered either single or multiple injections of different α-Gal A doses. Plasma samples were obtained prior to administration of α-Gal A and subsequently at various time points following injections and were analyzed for GL-3 levels. As GL-3 levels were not appreciably affected up to 2 hr following administration of 10 mg/kg dose of α-Gal A (data not shown), 24 hr post-injection was taken as the first time point. Following an initial spiking in GL-3 concentration, all doses were effective in reducing circulating levels of GL-3. Maximal decrease occurred at approximately 7 days post-injection for all doses. The rapidity at which the decrease occurred was dependent upon the dose, as was the duration of the decrease. The 10 mg/kg and 8 x 10 mg/kg doses

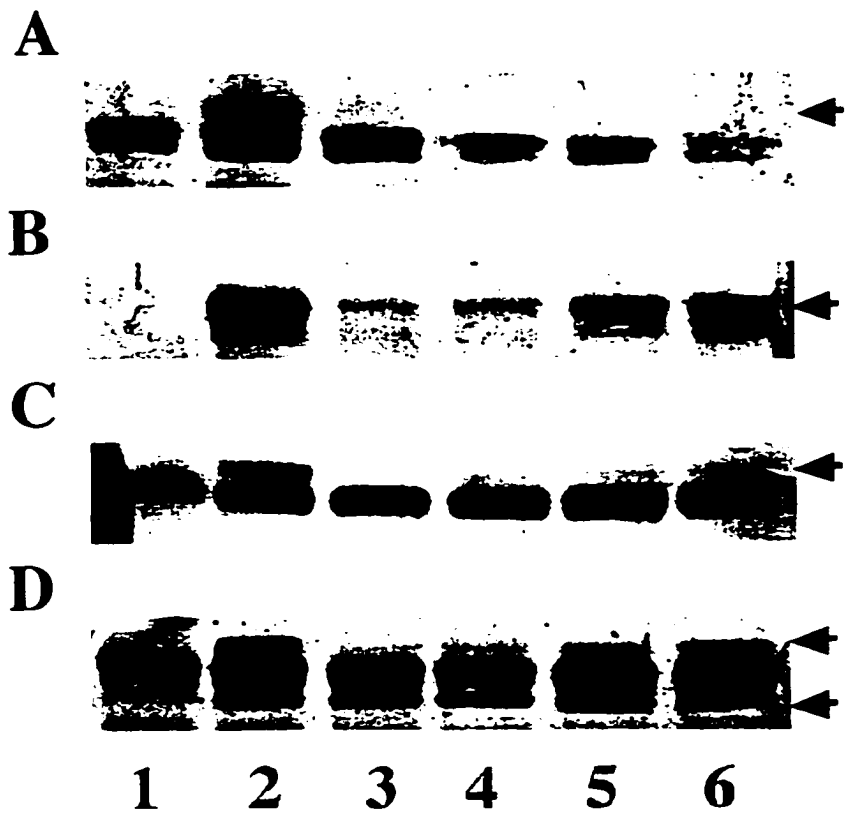

Figure 2. Effect of α-Gal A dose on GL-3 concentration in tissues of α-Gal A-deficient mice. A, liver GL-3; B, spleen GL-3; C, heart GL-3; D, kidney GL-3. 1, wild-type mice; 2, untreated α-Gal A-deficient mice; 3, α-Gal A-deficient mice administered 8 injections of 3 mg/kg α-Gal A; 4, α-Gal A-deficient mice administered 1 injection of 3 mg/kg α-Gal A; 5, α-Gal A-deficient mice administered 1 injection of 1 mg/kg α-Gal A; 6, α-Gal A-deficient mice administered 1 injection of 0.3 mg/kg α-Gal A. Each sample represents the pooled glycosphingolipids of three mice corresponding to 20 mg tissue wet weight. Arrows indicate position of GL-3.

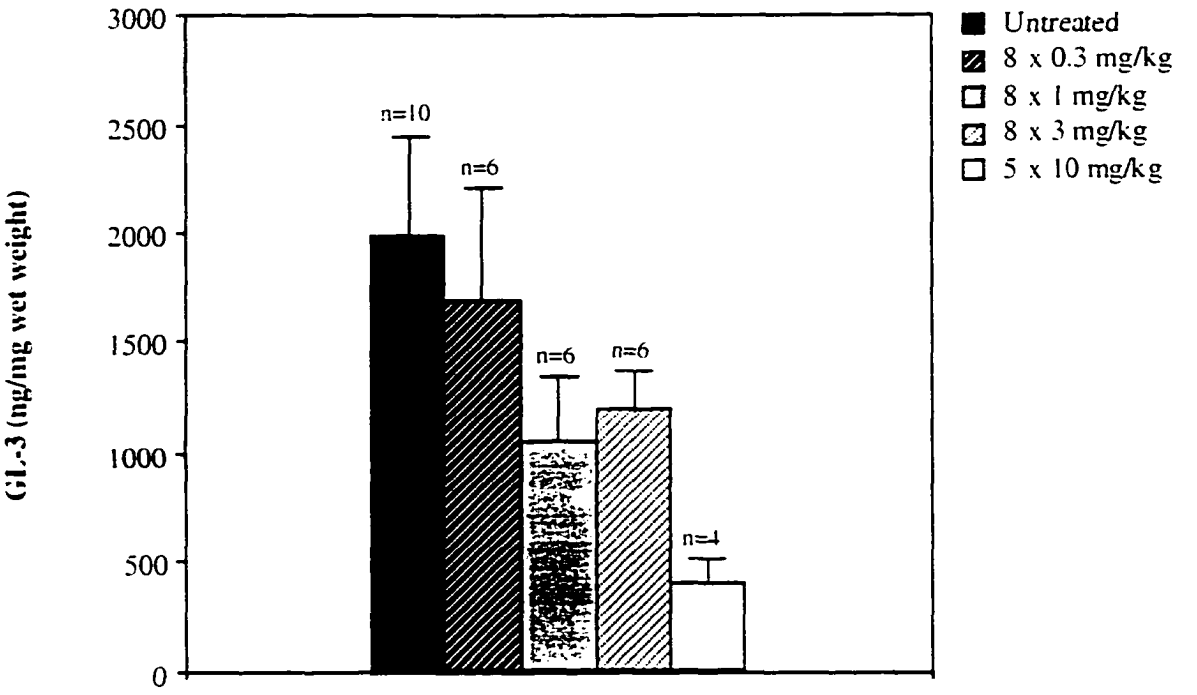

Figure 3. Effect of α-Gal A on skin GL-3 levels in α-Gal A-deficient mice. Values represent mean and standard deviation of 4-10 skin patches.

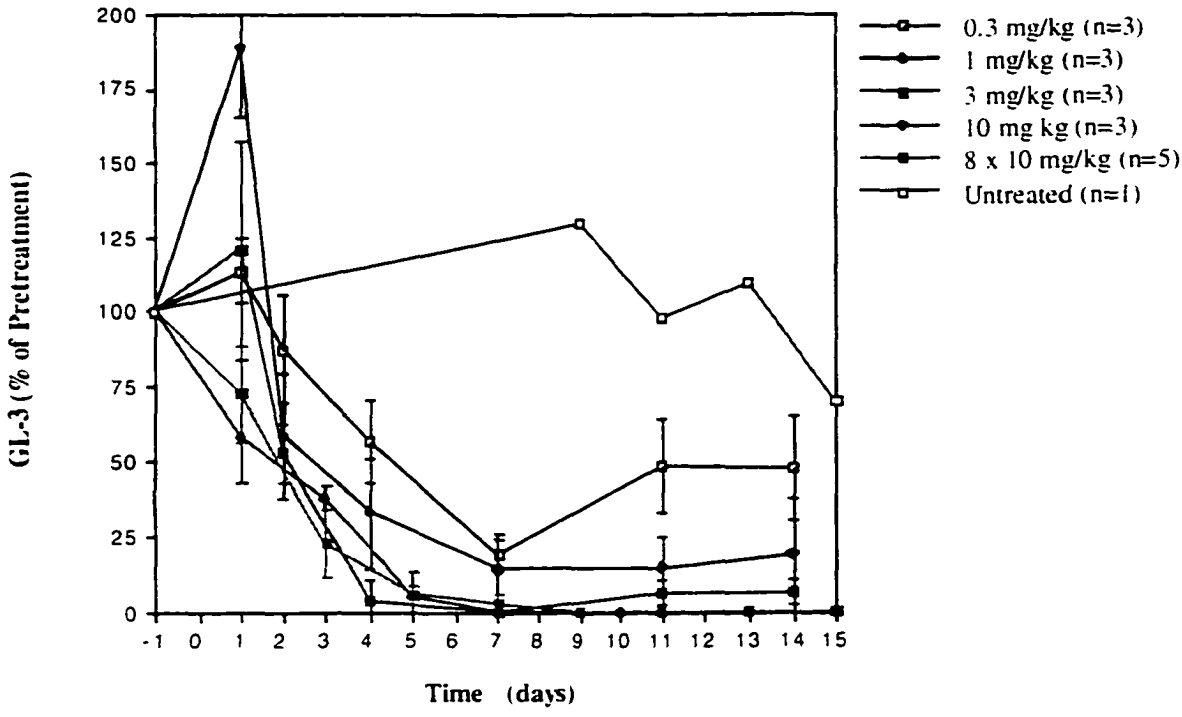

Figure 4. Effect of α-Gal A dose on plasma GL-3 concentration in α-Gal A-deficient mice. Values represent mean and standard deviation of 3-5 mice.

returned plasma GL-3 to wild-type levels, which were maintained for more than a week. GL-3 levels were not drastically affected in an untreated mouse which was bled for the duration of the experiment. HPTLC was performed to confirm the effect of α-Gal A on circulating plasma GL-3. As seen in Fig. 5, administration of 4 x 10 mg/kg α-Gal A completely metabolized the accumulated circulating plasma GL-3.

Reaccumulation of GL-3

To determine the rate of GL-3 accumulation following α-Gal A injection, 4 groups of mice were administered a single 3 mg/kg dose of α-Gal A. Each group was sacrificed at one week intervals and analyzed for changes in GL-3 levels (Fig. 6). Liver GL-3 was completely metabolized by 1 week post injection and remained below detection for 4 weeks. Spleen and heart GL-3 levels slowly decreased and reached a maximal decrease by 3 weeks. At week 4, GL-3 reaccumulation was evident in both spleen and heart.

Plasma showed maximal decrease (~5% of the prebleed values) at 1 week post-injection (Fig. 6). Reaccumulation was maximal by 3 weeks (~40% of prebleed values), indicating that reaccumulation of GL-3 in plasma precedes reaccumulation in tissues, suggesting that plasma may serve as an early detector of GL-3 accumulation.

Antibody Analysis

Mice generally tolerated the α-Gal A injections well, with no adverse reactions noted. An ELISA was developed to determine whether mice administered α-Gal A mounted an antibody response against the protein (Fig. 7). Wild-type mice (n=3) had a plasma anti-α-Gal A baseline concentration of 0.38 ± 0.08 μg/ml. Untreated α-Gal A-deficient mice (n=10) had a baseline of 0.46 ± 0.21 μg/ml, while pretreated α-Gal A-deficient mice (n=32) had a baseline of 0.30 ± 0.29 μg/ml. These final two values were combined to yield a baseline of 0.33 ± 0.28 μg/ml, which is not significantly different from wild-type mice. A concentration equal to the mean plus 2 standard deviations (0.90 μg/ml)

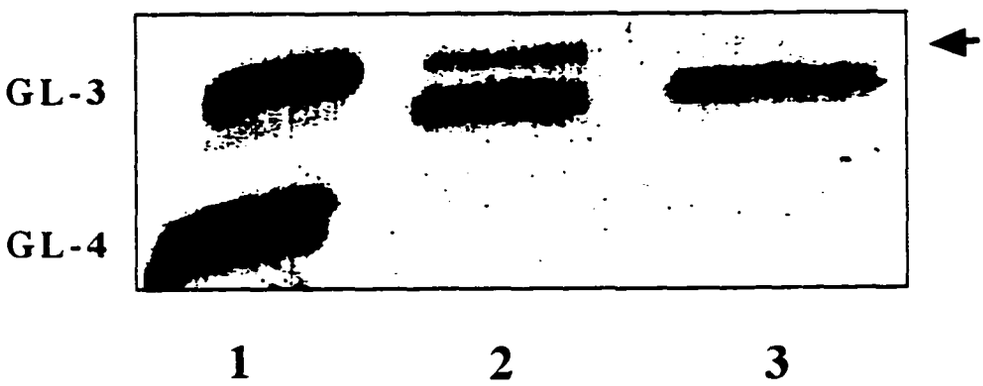

Figure 5. Effect of α-Gal A on GL-3 concentration in plasma of α-Gal A-deficient mice. 1. glycosphingolipid standards; 2. preteated plasma; 3. plasma pooled from 3 mice administered 4 injections of 10 mg/kg α-Gal A. Arrow indicates position of GL-3.

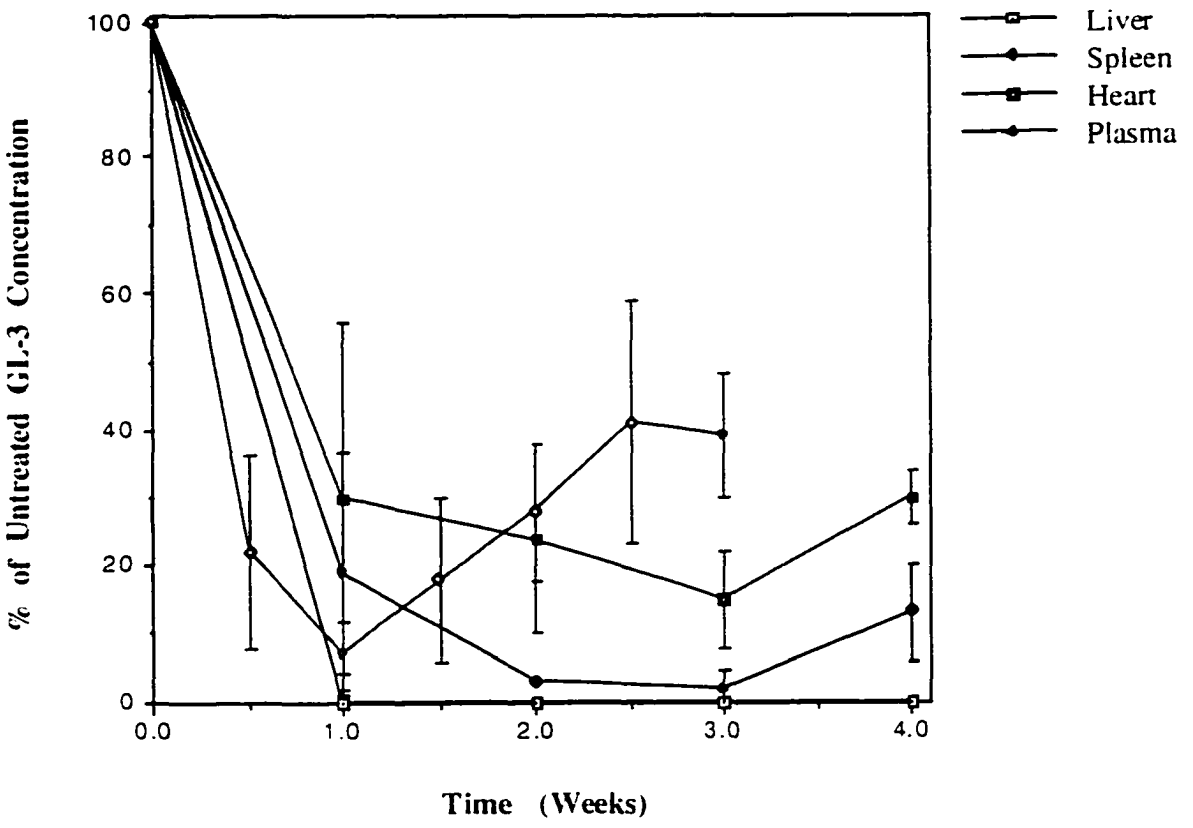

Figure 6. Effect of α-Gal A administration on the depletion and reaccumulation of GL-3 in tissues and plasma of α-Gal A-deficient mice. Values represent mean and SD of 3 mice, each receiving 1 injection of 3 mg/kg α-Gal A.

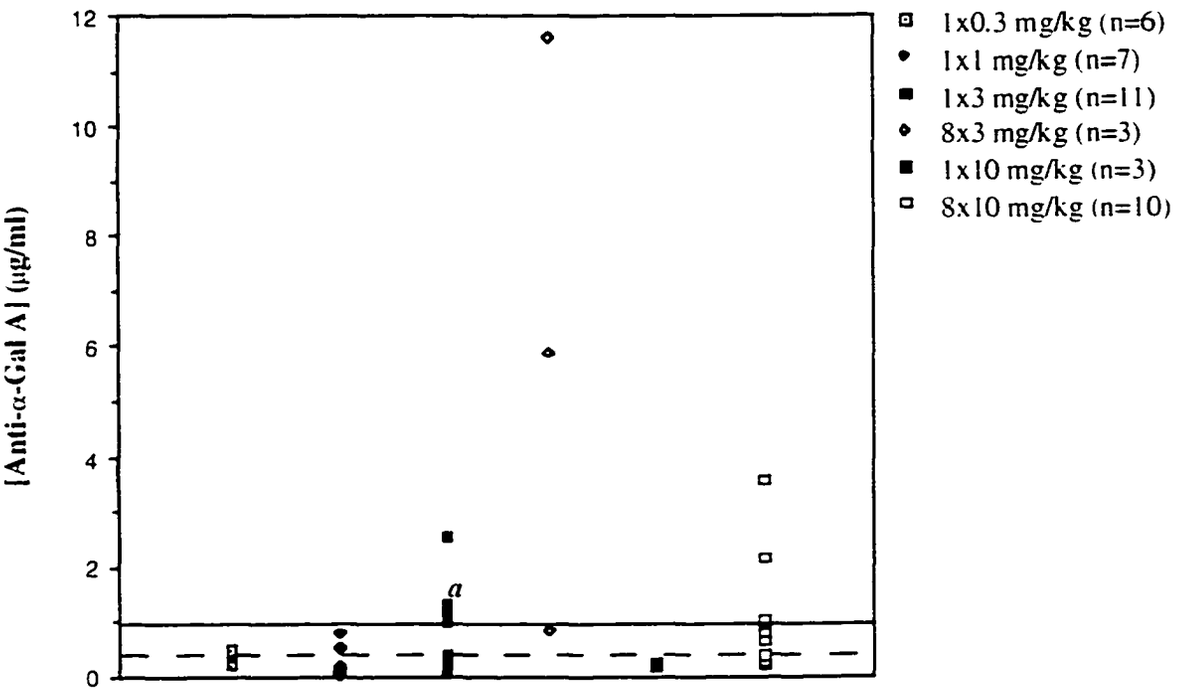

Figure 7. Antibody response in α-Gal A-deficient mice administered recombinant human α-Gal A. Dashed line represents mean plasma antibody concentration in untreated and pretreated α-Gal A-deficient mice; solid line represents this value + 2 SD. *a*, this mouse's pretreatment value was also significantly higher than those of untreated mice.

was taken to be positive for anti-α-Gal A antibodies. A single injection of 0.3 mg/kg α-Gal A did not promote an antibody response in 6 mice, nor did a single injection of 1 mg/kg in 7 mice. A single injection of 3 mg/kg α-Gal A produced a response in 5 of 11 mice, although one animal had a significantly high pretreatment antibody concentration, suggesting a non-specific reaction. Eight injections of 3 mg/kg α-Gal A produced a response in 2 of 3 mice. A single injection of 10 mg/kg α-Gal A did not produce a response in 3 mice, whereas eight injections of this dose produced a response in 3 of 10 mice. The results indicated that multiple injections led to higher and more frequent antibody responses than single injections. Doses under 3 mg/kg did not produce an antibody response, while the 3 mg/kg dose promoted higher antibody responses than the 10 mg/kg dose. The presence of anti-α-Gal A antibodies did not appear to affect the outcome of enzyme treatment.

Repeating the ELISA with high-titer mouse plasma using two other α-Gal A glycoforms revealed that a decrease in glycosylation complexity led to an increase in antigen recognition (data not shown), suggesting that mouse antibodies were recognizing α-Gal A, and not a contaminant in the preparation. This was confirmed by Western blot analysis, which revealed that pooled high-titer mouse plasma, as determined by ELISA, reacts with a single protein from the α-Gal A preparation (Fig. 8). The molecular weight of this protein is consistent with it being α-Gal A. Low-titer plasma did not react with any protein on the blot.

Neutralization studies detected no decrease in α-Gal A activity following incubation with high-titer mouse plasma, as compared to prebleed plasma or the PBS control (data not shown). It was noted that neither normal rabbit serum nor rabbit anti-α-Gal A IgG neutralizes α-Gal A activity, consistent with a previous report in which polyclonal anti-α-Gal A protected α-Gal A against thermal denaturation and protease degradation (Snyder et al., 1974).

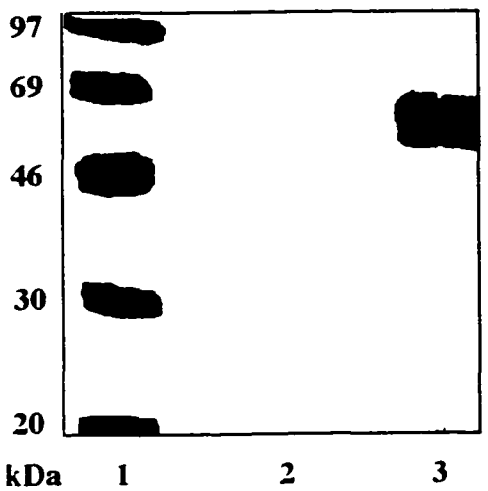

Figure 8. Western blot of recombinant human α-Gal A with pooled plasmas from α-Gal A-deficient mice administered α-Gal A. 1, molecular weight standards; 2, pooled plasma from low-titer mice; 3, pooled plasma from high-titer mice.

DISCUSSION

In Chapter 3, it was demonstrated that recombinant human α-Gal A could correct the enzyme deficiency in α-Gal A-deficient mice in most tissues examined, even though the primary site of uptake was liver. Previous α-Gal A replacement studies in humans (Bishop et al., 1981; Brady et al., 1973) revealed that the human enzyme is also rapidly sequestered by the liver, although no data on GL-3 metabolism in tissues other than plasma was obtained. Therefore, this study was undertaken to determine whether recombinant human α-Gal A could metabolize accumulated GL-3 in the tissues of α-Gal A-deficient mice.

Figures 1 and 2 demonstrate that all doses of α-Gal A were effective at metabolizing GL-3 in liver, spleen, heart, and kidney. As expected, liver GL-3 was the easiest to metabolize (Figs. 1A; 2A), since the majority of the enzyme is targeted to the liver (see Chapter 3). Spleen and heart GL-3 was completely metabolized only with the 8 x 3 mg/kg dose (Figs. 1B, C; 2B, C). Kidney GL-3 depletion did not appear strictly dose dependent, as all three single doses reduced the lipid by ~33% (Figs. 1D; 2D). The 8 x 3 mg/kg dose was further able to reduce the GL-3 to 50% of that found in untreated α-Gal A-deficient mice (Figs. 1D; 2D). This 50% reduction appears to be the maximum that can be achieved in kidney, as 8 x 10 mg/kg GF 1 yielded the same result (data not shown). HPTLC of kidney (Fig. 2D) revealed complete hydrolysis of the faster migrating GL-3 form, with little or no hydrolysis of the slower migrating form, which leads to an overall depletion of ~50% as determined by ELISA. It is believed that the slower form is in a compartment which is normally excreted, as it is present in urine, and which is inaccessable to the exogenous enzyme. This GL-3 form has been noted in male wild-type mice, which is also normally excreted and inaccessable to endogenous α-Gal A (McCluer et al., 1981). This is supported by the fact that the urine of male α-Gal A-deficient mice contains GL-3 levels comparable to their wild-type counterparts, and no dramatic changes in urinary GL-3 levels were detected following enzyme administration (data not shown). This type of

inaccessable compartment may also explain why glycosaminoglycan storage in the livers of dogs with MPS type I is never completely cleared, regardless of α-*L*-iduronidase dose (Kakkis et al., 1996). Thus, 50% depeletion may actually represent complete metabolism of kidney GL-3. This is in contrast to MPS type I dogs (Kakkis et al., 1996) and MPS type VI cats (Crawley et al., 1996; Crawley et al., 1997), which excreted significantly lower amounts of glycosaminoglycans (GAGs) following enzyme replacement. Decreased excretion of GAGs has also been demonstrated in some human patients with MPS types I, II, and III given plasma, leukocyte, or whole blood transfusions (Dean et al., 1973; Di Ferrante et al., 1971; Di Ferrante et al., 1973; Knudson et al., 1971).

The reduction in GL-3 accumulation in tissues of mice treated with α-Gal A is similar to results obtained with other animal models. MPS type VII mice receiving single or multiple injections of β-glucuronidase also exhibited marked reduction in liver and spleen accumulation, whereas heart and kidney reduction was seen only after multiple injections (Sands et al., 1994). Interestingly, no enzyme activity could be detected in heart or kidney. MPS type I dogs recieving multiple injections of α-*L*-iduronidase showed substantial decreases in storage in liver, spleen and kidney, but none in heart (Kakkis et al., 1996; Shull et al., 1994). At low doses only liver Kuppfer cells were cleared, whereas at high doses, both Kuppfer cells and hepatocytes were cleared (Kakkis et al., 1996). MPS type VI cats administered multiple injections of *N*-acetylgalactosamine-4-sulfatase showed reversal of lysosomal accumulation in liver and heart (Crawley et al., 1996), the extent of which in heart was dependent on dose (Crawley et al., 1997). Early studies of ERT in humans with lysosomal storage diseases also revealed evidence of catabolism of accumulated substrates in tissues of patients. For example, some patients with glycogenosis type II administered human or *Aspergillus niger* α-glucosidase showed decreases in glycogen accumulation in liver (Badhuin et al., 1964; De Barsy et al., 1973; Hug and Schubert, 1967; Hug et al., 1973; Huijing et al., 1973). Also, purified human acid β-glucosidase was capable of reducing storage in liver, red blood cells, lymphocytes,

and platelets in patients with Gaucher disease (Beutler et al., 1977; Brady et al., 1974). And most importantly, urinary globoside concentration was significantly decreased in a patient with Sandhoff disease following infusion of plasma, suggesting a catabolism of renal glycolipid by active hexosaminidases (Desnick et al., 1971).

As Fabry disease is primarily a disease of the vascular endothelium, it was important to analyze skin samples for reduction in GL-3 accumulation. Figure 3 revealed that skin GL-3 levels were reduced in a dose-dependent fashion, with the highest dose reducing accumulation to 20% of untreated values. That the metabolism of skin GL-3 possibly occurred in the absence of α-Gal A activity (data not shown) suggests that the lipid in capillaries may have equilibrated with plasma (Desnick and Grabowski, 1981), although the presence of low levels of enzyme over the large surface area of the skin cannot be ruled out. A dose-dependent decrease in skin glycosaminoglycan content was demonstrated following administration of *N*-acetylgalactosamine-4-sulfatase to MPS type VI cats (Crawley et al., 1996); however, readily detectable levels of enzyme were detected This result suggests that analysis of skin biopsies may be useful for evaluating the effectiveness of ERT for Fabry disease.

Because plasma lipoproteins synthesized in hepatocytes (Attie et al., 1982) carry circulating glycosphingolipids (Chatterjee and Kwiterovich, 1984) and α-Gal A is not active at neutral pH (Brady et al., 1973), it is reasonable to assume that the decrease in plasma GL-3 levels in α-Gal A-deficient mice (Figs. 4 and 5) is the result of effective clearance of hepatic storage. Figure 6 indicates that plasma GL-3 levels are maximally reduced only after liver GL-3 storage is depleted. Since the major pathology of Fabry disease is due to deposition of GL-3 in vascular endothelium (Johnson and Desnick, 1978), depletion of plasma stores is desirable so as to prevent low density lipoprotein (LDL)-mediated uptake of lipid into extrahepatic tissues. It appears that rapid uptake of α-Gal A by the liver, a site of minimal pathology in Fabry disease (Meuwissen et al., 1982), reduces the amount of circulating GL-3, thereby preventing deposition at sites which are

vulnerable to accumulation, such as the heart and kidney. Reduction of plasma glycosphingolipid levels has been demonstrated in previous human ERT trials. These include decreases in glucocerebroside in Gaucher disease (Brady et al., 1974), globoside and G_{M2} ganglioside in G_{M2} gangliosidosis (Johnson et al., 1973; Von Sprecht et al., 1979), and GL-3 in Fabry disease (Brady et al., 1973; Desnick et al., 1979; Mapes et al., 1970). These responses occurred very rapidly and were dependent on the amount of enzyme administered. In the case of Fabry disease, the maximal disappearance of GL-3 from plasma occurred when all administered enzyme had been removed from the circulation (Brady et al., 1973). Also, no increase in circulating levels of GL-2, the immediate product of GL-3 metabolism, was detected (Brady et al., 1973). Taken together, these results suggested that the site of action for these lysosomal enzymes was extracirculatory (Brady et al., 1975). As the liver, primarily the Kuppfer cell, is the primary site of uptake for exogenous lysosomal enzymes (see Chapter 2), it is possible that the monocyte/macrophage system is responsible for the plasma glycosphingolipid hydrolysis (Brady, 1984). This could occur through the scavenger receptor, which also functions as the high density lipoprotein (HDL) receptor (Acton et al., 1996), which might endocytose overloaded lipoproteins (Prokazova and Bergelson, 1994) into lysosomes already containing active enzyme. This is consistent with the finding that the splenic α-Gal A, which had a higher and more rapid uptake into liver than human plasma α-Gal A due to a lack of complex glycosylation (Bishop et al., 1981), exerted a more rapid effect on circulating GL-3 levels than its plasma counterpart when administered to a Fabry patient (Desnick et al., 1979). In the case of the α-Gal A-deficient mice, it is also possible that high levels of enzyme could have saturated the monocyte/macrophage system, leading to uptake by hepatocytes, as was previously demonstrated for β-glucuronidase replacement in MPS type VII mice (Sands et al., 1994). Thus, the accumulated GL-3 could be metabolized before incorporation into the LDL particle. Obviously, immunohistochemistry is needed to resolve this question.

No reaccumulation of GL-3 was detected in liver following administration of α-Gal A (Fig. 6). This finding is similar to that found in MPS type VII mice, who also showed little to no reaccumulation of storage in liver or spleen 1 month after multiple injections of β-glucuronidase (Vogler et al., 1996). Reaccumulation of GL-3 in plasma preceeded reaccumulation in spleen and heart by 2 weeks. This suggested that changes in plasma GL-3 levels may be a useful indicator of the effectiveness of ERT for Fabry disease. Urine is also easy to obtain and contains elevated GL-3 levels in Fabry disease (Desnick et al., 1970), but it is still unclear whether exogenous α-Gal A will have an effect on accumulation in this fluid (Brady et al., 1973). Also, urine GL-3 only reflects kidney levels, whereas plasma probably better reflects all tissue levels. Of note is that spleen and heart GL-3 continued to decrease after 2 weeks for an additional week. Enzyme analysis revealed that, whereas spleen α-Gal A specific activity was still at 20% of wild-type levels by day 14 post-administration, heart α-Gal A specific activity had decreased to levels below detection (data not shown). It would seem that heart GL-3 levels continued to decrease in the absence of active enzyme, suggesting a redistribution of lipid across cell membranes (Desnick and Grabowski, 1981). The data in Fig. 6 also suggest that a single administration of α-Gal A every month may maintain GL-3 at normal levels. Such a treatment plan has also been proposed for β-glucuronidase replacement in MPS type VII mice (Vogler et al., 1996).

These mice offer an excellent opportunity to test the "equlibration-depletion" hypothesis proposed by Desnick and colleagues (Desnick and Grabowski, 1981). This hypothesis states that circulating lysosomal enzyme substrates may be in dynamic equilibrium with those in tissues. Thus, efforts resulting in decreased levels of circulating substrate may reduce accumulation in certain cell types, particularly those in direct contact with the circulation, such as endothelial cells. In support of this hypothesis, metabolic labeling of a Fabry patient receiving exogenous α-Gal A revealed that the rate of deuterium incorporation into reaccumulating plasma GL-3 decreased from 2-36 hr, suggesting that

previously synthesized, that is, stored, substrate had re-entered the circulation (Desnick et al., 1980). In animal studies, MPS type VII mice administered β-glucuronidase exhibited a reduction in heart and kidney accumulation, although no enzyme activity could be detected in these tissues (Sands et al., 1994). Also, in the present study, GL-3 levels in the hearts of α-Gal A-deficient mice continued to decrease (Fig. 6) even after α-Gal A activity had disappeared (data not shown). In Gaucher disease, glucosylceramide decrease in red blood cells following administration of acid β-glucosidase is apparently due to decreases in lipoprotein levels, with rapid equilibration between the two compartments (Brady, 1984). This equilibration is apparently not possible for glycosphingolipids larger than glucosylceramide (Dawson and Sweeley, 1970). Therefore, a mechanism is needed to transfer tissue glycolipid stores to plasma. A likely candidate is the HDL particle, which is known to be involved in reverse cholesterol transport (Fielding and Fielding, 1994). Previous studies have demonstrated that glycolipids incorporated into artificial membranes (Shen et al., 1981) and cell membranes (Kwok et al., 1981) can become incorporated into HDL. Also, GL-3 specifically can become incorporated into HDL, at least *in vitro* (Clarke and Stoltz, 1976). Therefore, reaccumulation of plasma GL-3 levels in α-Gal A-deficient mice following administration of α-Gal A (Fig. 6) may reflect either LDL incorporation in hepatocytes or HDL uptake from extra-hepatic tissues. The fact that plasma GL-3 levels increased in the absence of hepatic GL-3 reaccumulation further supports the "equilibration-depletion" hypothesis, though small amounts of GL-3 incorporation into hepatocyte LDL cannot be ruled out, particularly because only a small amount of liver glycolipid is analyzed during ELISA. Thus, exogenous α-Gal A may function at the site of GL-3 accumulation directly, or by preventing or reversing extra-hepatic deposition, particulary in endothelium, by reducing plasma GL-3 levels. It should be pointed out that endothelial cell cultures established from Fabry hemizygotes are deficient in α-Gal A activity, while those established from normal individuals are rich in enzyme activity (Hasholt and Sorensen, 1986). Also, endothelial cells can endocytose α-Gal A, although this uptake appears to be

M6P independent (Hasholt et al., 1988). Acid β-glucosidase has been shown to be efficiently endocytosed by endothelial cells in a mannose-dependent fashion (Sato and Beutler, 1993). Thus, any future studies will need to differentiate between the effects of reverse GL-3 transport and direct tissue catabolism.

Mice generally tolerated the α-Gal A injections well. No obvious adverse effects were noted. Two mice died during the course of these experiments, but neither showed evidence of an antibody response as determined by ELISA (Fig. 7). ELISAs using different α-Gal A glycoforms (data not shown) and Western blot analysis (Fig. 8) indicated that the ELISA was specific for anti-α-Gal A antibodies. Multiple administrations were more effective than single injections at eliciting an antibody response. The fact that no antibody response was obtained with doses less than 3 mg/kg suggests a threshold effect, while the fact that the 3 mg/kg dose produced higher antibody concentrations than the 10 mg/kg dose suggests that tolerance was induced (Hay, 1974). Tolerance is also apparently induced in Gaucher patients receiving acid β-glucosidase treatment (Richards et al., 1993). Longer administrations of enzyme did not appear to increase the frequency or strength of the antibody response. Anti-α-Gal antibodies did not neutralize α-Gal A in solution (data not shown), explaining why no effect of anti-α-Gal A on catabolism of GL-3 was noted.

Regardless of the mechanism of GL-3 catabolism by α-Gal A, the results of this study suggest that ERT is a safe and effective treatment for Fabry disease. As plasmapheresis (Kolodny et al., 1981), phlebotomy (Beutler et al., 1983), and kidney transplantation (Maizel et al., 1981) have all proved ineffective in the treatment of Fabry disease, ERT appears to be the only method of treatment at this point. The results of these experiments also suggest that two of the original ERT requisites (see Introduction), maximal retention of enzyme in circulation and targeting to specific sites of pathology (Desnick and Grabowski, 1981; Desnick et al., 1976; Rietra et al., 1974; Tager et al., 1980), may not be indicated for Fabry disease. It appears that rapid uptake of α-Gal A by

the liver may be sufficient to maintain GL-3 at normal levels. Thus, these results provide the rationale for α-Gal A replacement therapy in Fabry patients.

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CONCLUSIONS

These studies were undertaken to evaluate the effectiveness of enzyme replacement therapy (ERT) for Fabry disease, a disorder of glycosphingolipid metabolism for which no adequate treatment currently exists.

In Chapter 1, an ELISA was developed to quantitate the glycosphingolipid, GL-3, the principle accumulated substrate in Fabry disease. This ELISA was based on the specific and strong affinity of the B-subunit of *E. coli* verotoxin (VTB) for GL-3. The gene for VTB was cloned and expressed in *E. coli*, and the protein purified to homogeneity using immunoaffinity chromatography. The recombinant VTB was characterized and demonstrated to be identical to native VTB. Utilizing purified VTB and a monoclonal antibody to the toxin, an ELISA was developed which was rapid, sensitive, and specific for GL-3, compared to traditional methods of glycosphingolipid quantitation. This ELISA should prove useful in evaluating the effectiveness of ERT for Fabry disease.

In Chapter 2, transgenic mice lacking a functional gene for the lysosomal enzyme α-galactosidase A (α-Gal A), which is deficient in Fabry disease, were characterized. These mice appeared normal at birth and had normal lifespans and fertility. Tissues from adult hemizygous males and homozygous females for the null allele had no detectable α-Gal A activity. No hydrolytic activity against GL-3 was detected in cultured skin fibroblasts from α-Gal A-deficient animals *in vivo*, though some hydrolysis was detected in detergent-containing *in vitro* assays due to the presence of α-galactosidase B (α-Gal B), a related lysosomal enzyme. α-Gal A-deficient mice were shown to accumulate GL-3 in all tissues examined, including liver, spleen, heart, kidney, skin fibroblasts, and plasma, in an age-dependent manner. The reason for the lack of pathology associated with GL-3 accumulation remains unclear, though it may be related to differences in lipoprotein metabolism between mice and humans. These α-Gal A-deficient animals, however,

provide an excellent model for Fabry disease, both in terms of determining the pathophysiology of GL-3 accumulation and evaluating various treatment modalities.

In Chapter 3, α-Gal-A-deficient mice were administered different recombinant α-Gal A glycoforms, each differing in their glycosylation and phosphorylation patterns. Negligible differences in tissue distribution and pharmacokinetics were detected between the different glycoforms. The majority of enzyme activity was recovered in the liver and spleen, with negligible amounts detected in heart, lung, kidney, and brain, while the half-life in plasma was less than 5 min for each glycoform. Therefore, only a single α-Gal A glycoform, GF 1, was further analyzed. Following administration, the enzyme was quite stable in various tissues, suggesting that it was correctly targeted to lysosomes. Increased doses of GF 1 α-Gal A led to a redistribution of the enzyme, with readily detectable levels in heart and kidney. Multiple administrations were able to restore enzyme levels to those of wild-type mice in all tissues examined, except brain. These results demonstrated that exogenous α-Gal A can correct the enzyme deficiency in all tissues examined in α-gal A-deficient mice.

In Chapter 4, the effect of enzyme replacement on GL-3 levels in α-Gal A-deficient mice was investigated. Single doses of administered α-Gal A were capable of metabolizing liver, spleen, heart, kidney, and skin GL-3 in a dose-dependent manner. Multiple high-level doses reduced GL-3 to nearly undetectable levels in these tissues. All doses led to a decrease in circulating levels of GL-3, the rapidity and extent of which was dose-dependent. Reaccumulation of depleted GL-3 in plasma preceded reaccumulation in organs, with levels still remaining well below those of untreated mice 1 month post-treatment, whereas GL-3 levels in heart continued to decrease in the absence of detectable α-Gal A activity. Antibody response in mice was rare, and, when present, anti-α-Gal A antibodies were not neutralizing and did not alter the clinical outcome of the treatment. These results indicated that exogenous α-Gal A can safely catabolize accumulated GL-3 in α-Gal A-deficient mice in a dose-dependent fashion and that changes in plasma GL-3 levels

may accurately reflect changes in organ levels. Also, only infrequent administrations of enzyme may be required to restore GL-3 levels to normal.

In conclusion, these studies have demonstrated that when administered to α-Gal A-deficient mice, recombinant human α-Gal A can correct the enzyme deficiency in numerous tissues and reverse the GL-3 accumulation in a dose-dependent fashion. Furthermore, the enzyme is well tolerated and does not elicit a neutralizing antibody response. These results also suggest that plasma GL-3 levels can be used as an indicator of the effectiveness of enzyme replacement. Therefore, ERT studies are warranted in patients with Fabry disease.

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IMAGE EVALUATION
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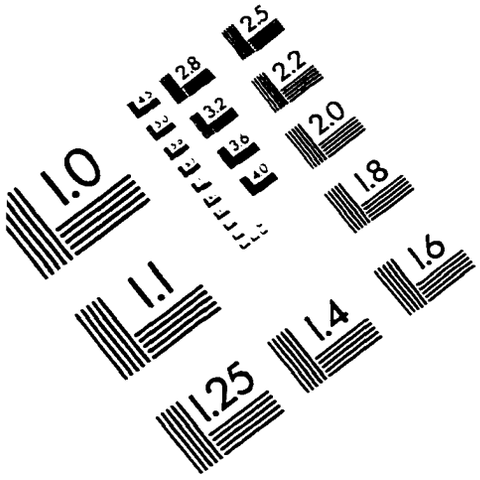

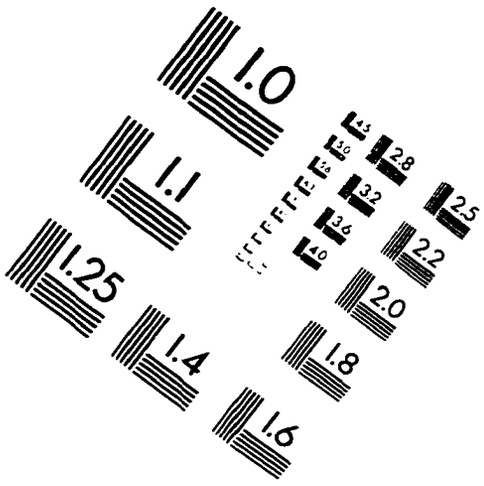

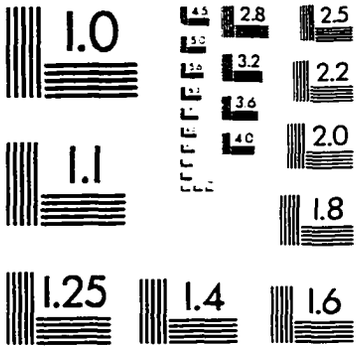

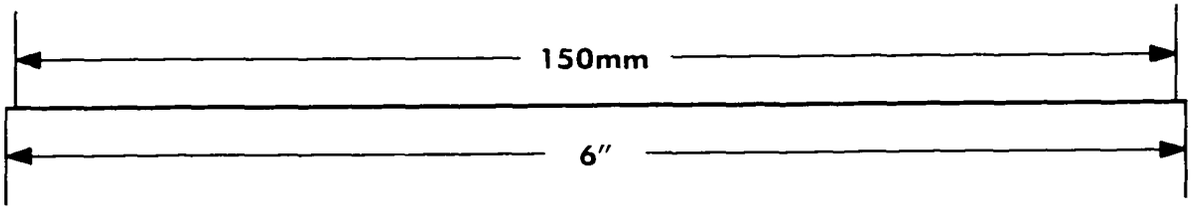

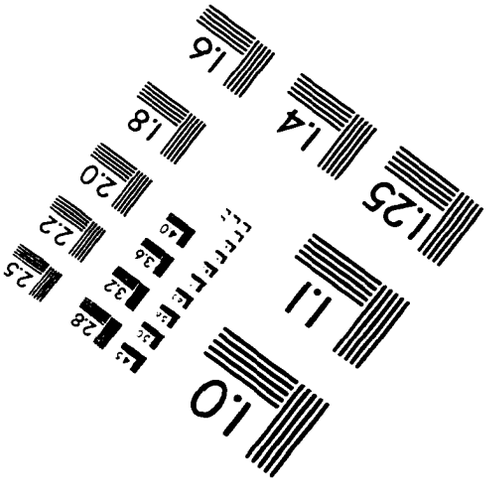

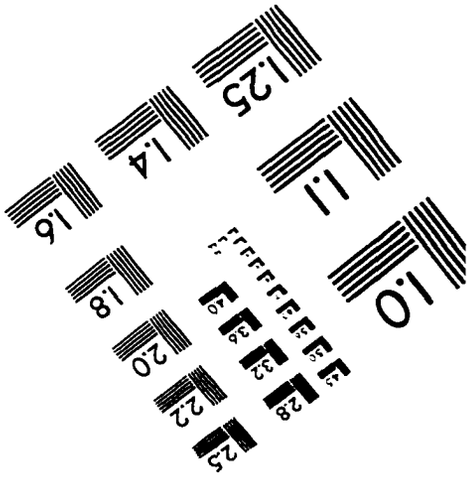

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