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Fabry Disease: Preclinical Studies Demonstrate the Effectiveness of α-Galactosidase A Replacement in Enzyme-Deficient Mice

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Preclinical studies of enzyme-replacement therapy for Fabry disease (deficient α-galactosidase A [α-Gal A] activity) were performed in α-Gal A–deficient mice. The pharmacokinetics and biodistributions were determined for four recombinant human α-Gal A glycoforms, which differed in sialic acid and mannose-6-phosphate content. The plasma half-lives of the glycoforms were ~2–5 min, with the more sialylated glycoforms circulating longer. After intravenous doses of 1 or 10 mg/kg body weight were administered, each glycoform was primarily recovered in the liver, with detectable activity in other tissues but not in the brain. Normal or greater activity levels were reconstituted in various tissues after repeated doses (10 mg/kg every other day for eight doses) of the highly sialylated AGA-1 glycoform; 4 d later, enzyme activity was retained in the liver and spleen at levels that were, respectively, 30% and 10% of that recovered 1 h postinjection. Importantly, the globotriaosylceramide (GL-3) substrate was depleted in various tissues and plasma in a dose-dependent manner. A single or repeated doses (every 48 h for eight doses) of AGA-1 at 0.3–10.0 mg/kg cleared hepatic GL-3, whereas higher doses were required for depletion of GL-3 in other tissues. After a single dose of 3 mg/kg, hepatic GL-3 was cleared for ⩾4 wk, whereas cardiac and splenic GL-3 reaccumulated at 3 wk to ~30% and ~10% of pretreatment levels, respectively. Ultrastructural studies demonstrated reduced GL-3 storage posttreatment. These preclinical animal studies demonstrate the dose-dependent clearance of tissue and plasma GL-3 by administered α-Gal A, thereby providing the in vivo rationale—and the critical pharmacokinetic and pharmacodynamic data—for the design of enzyme-replacement trials in patients with Fabry disease.

Introduction

Fabry disease (MIM 301500) is an X-linked inborn error of glycosphingolipid catabolism and results from the deficient activity of the lysosomal exoglycohydrolase, α-galactosidase A (α-Gal A) (Desnick et al. 1995, 2001). Classically affected males, who have little, if any, α-Gal A activity, progressively accumulate the neutral glycosphingolipid, globotriaosylceramide (GL-3), primarily in the lysosomes of vascular endothelial cells of the heart, liver, kidneys, skin, and brain. Onset of the classic disease phenotype occurs during childhood or adolescence and is characterized by severe acroparesthesias, angiokeratoma, corneal and lenticular opacities, and hypohidrosis. With advancing age, vascular disease of the heart, kidneys, and brain leads to early demise during the 4th or 5th decade of life (Colombi et al. 1967).

Current treatment for Fabry disease is nonspecific and supportive, primarily limited to symptomatic management of the excruciating pain and the complications of renal, cardiac, and/or cerebrovascular disease. Attempts to deplete the accumulated substrate and to alter disease progression by kidney transplantation (Maizel et al. 1981), phlebotomy (Beutler et al. 1983), or plasmapheresis (Kolodny et al. 1981), have not been not successful. Therefore, Fabry disease is an attractive candidate for enzyme-replacement therapy, particularly because classically affected patients lack neural involvement.

Initial efforts to replace the defective enzyme in classically affected males involved the administration of single doses of either normal plasma containing active, highly-sialylated α-Gal A (Bishop et al. 1981; Mapes et al. 1970) or partially purified enzyme from human placenta (Brady et al. 1973). The plasma glycoform, which was highly sialylated, was retained in the circulation longer (half-life [$T_{½}$] ~90 min) than was the placental glycoform (10 min), whose oligosaccharides were partially processed in placental lysosomes. Importantly, these single-dose studies indicated that intravenously infused enzyme from either source could decrease the level of accumulated plasma GL-3. A subsequent pilot study compared, over a 3-mo period, the effectiveness and safety of six doses (~0.05 mg/kg) of human α-Gal A glycoforms from splenic lysosomes or plasma (Desnick

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et al. 1979). The well-tolerated enzyme infusions demonstrated that the splenic-derived enzyme was cleared rapidly from the circulation ($T_{½}$ ~10 min) and transiently decreased the circulating GL-3 concentration (<1 h), whereas the more highly sialylated plasma-derived enzyme had a slower plasma clearance ($T_{½}$ ~70 min) and effected a longer and more profound depletion of the circulating GL-3 (>36 h). It is noteworthy that two doses of the plasma-derived enzyme, one infused at 0 h and the other infused at 48 h, reduced the plasma substrate level to within the normal range (Desnick et al. 1980). These studies demonstrated the feasibility of enzyme-replacement therapy for Fabry disease; however, the rate-limiting obstacles to clinical trials were the inability to produce sufficient amounts of purified normal enzyme and the absence of an animal model to evaluate the pharmacokinetics, biodistribution, and pharmacodynamics required for the design of clinical trials.

These obstacles were overcome by the isolation of the human α-Gal A cDNA (Bishop et al. 1986) and its high level expression in Chinese hamster ovary (CHO) cells (Ioannou et al. 1992), which provided a source of selectively secreted human recombinant enzyme that was sialylated and that had mannose-6-phosphate (M6P) residues for lysosomal targeting. In addition, the generation of an α-Gal A–knockout mouse model of Fabry disease permitted the preclinical evaluation of enzyme replacement (Wang et al. 1996). In this communication, we report the pharmacokinetics, biodistribution, and stability of various α-Gal A glycoforms after their infusion into the α-Gal A–deficient mouse model of Fabry disease. Use of a highly sialylated glycoform (i.e., AGA-1) demonstrated, biochemically and morphologically, the dose-dependent plasma and tissue clearance of accumulated GL-3. These animal-model studies provided the "proof of concept" and critical preclinical information for the design of clinical trials of α-Gal A replacement in patients with Fabry disease.

Material and Methods

Animal Model

α-Gal A–deficient mice were produced by homologous recombination in 129/Sv embryonic stem (ES) cells, by a replacement-type vector containing both the neomycin-resistance gene, for positive selection, and the thymidine kinase gene, for negative selection (Wang et al. 1996). Affected mice were totally deficient in α-Gal A activity and progressively accumulated GL-3 in both plasma and the lysosomes of most tissues (in particular, the liver, spleen, heart, skin, and kidneys). They had no clinical disease phenotype and survived a normal laboratory life span (~2 years). Hemizygous affected males were bred to homozygous affected females, thereby providing only affected offspring. For these studies, all mice were affected adult males ~5 mo old that weighed 20–30 g. Mice were provided standard mouse chow and water ad libidum.

Production, Purification, and Characterization of the Recombinant Human α-Gal A Glycoforms

Four human α-Gal A glycoforms were produced and purified, in order to compare their pharmacokinetics, biodistribution, and stability in α-Gal A–deficient mice. Two glycoforms, designated "AGA-1" and "AGA-2," were produced by stable, amplified expression of the normal human α-Gal A cDNA in DUKX B11 and DG44 CHO cells, respectively, as described elsewhere (Ioannou et al. 1992). To produce glycoform AGA-3, CHO DG44 cells overexpressing glycoform AGA-2 were stably transfected with a rat α-2,6-sialyltransferase cDNA generously provided by Dr. James Paulson (Los Angeles) (Wen et al. 1992). Enzyme produced by this modified cell line was highly sialylated, as shown by both its increased mobility on isolectric focusing (IEF) gels (fig. 1) and its binding to immobilized *Sambucus nigra* agglutinin (data not shown), a lectin specfic for α-2,6-sialic acid residues (Lee and Nathans 1988). Glycoform AGA-4 was constructed by site-specfic mutagenesis of the α-Gal A cDNA, to alter the consensus *N*-glycosylation site at N192 (asparagine to glutamine) and to delete one *N*-linked high–mannose oligosaccharide chain per monomer, thereby eliminating four M6P residues per active homodimeric enzyme (Ioannou et al. 1998; Matsuura et al. 1998). This construct was transiently overexpressed in COS-1 cells (Desnick et al. 1995, 2001). All four secreted glycoforms were purified by α-D-galactosylamine chromatography (Bishop et al. 1981), were concentrated (>0.5 mg/ml) in citrate-phosphate buffer (pH 6.0) containing 1 mg bovine serum albumin/ml, and were stored at 4°C.

Molecular weights and isoelectric points (pIs) were estimated by SDS-PAGE and IEF, on the PhastSystem

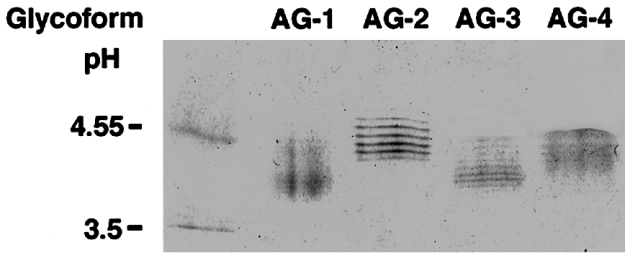

Figure 1 IEF gel of the four α-Gal A glycoforms used in the present study. The highly sialylated glycoforms, AGA-1 and AGA-3, had the lowest pI values.

Table 1

Characteristics of Human Recombinant α-Gal A Glycoforms

Glycoform	Overexpression Type/Cell	Molecular Weight of Monomer (kD)	pI	Degree of Sialylation[a]	M6P/ Dimer	GL-3 Activity[b]	Stability[c] (% of Initial Activity)	
							pH 4.6	pH 7.4
AGA-1	Stable/DUKX B11	~56	3.9–4.7	++++	6	+	100	50
AGA-2	Stable/DG44	~50	4.4–4.9	++	6	+	100	52
AGA-3	Stable/DG44	~55	4.0–4.6	++++	6	+	94	49
AGA-4	Transient/COS-1	~52	4.2–4.6	++	2	+	83	33

[a] Based on IEF (fig. 1) and α-neuraminidase studies (data not shown).
[b] In vitro hydrolysis of GL-3, assessed by HPTLC analysis.
[c] At 37°C, after incubation for 1 h.

(Pharmacia), by use of precast 12.5% polyacrylamide PhastGels and precast pH 4–6.5 gels, respectively, according to the manufacturer's recommendations. Sialylation was assessed by α-neuraminidase digestion according to the manufacturer's recommendations (Boehringer-Mannheim), followed by IEF. Mannose-6-phosphorylation was assessed by incubation of cultured human Fabry fibroblasts with 5×10^4 units (nmol/h) of each glycoform, in both the presence and the absence of 5 mM M6P, to determine enzyme uptake by the M6P receptor (data not shown).

Animal Injections and Specimen Collection

Individual mice were placed in an illuminated plexiglass restraining device, and the indicated dose of the enzyme was injected into the tail vein, ~0.1 ml during ~3 s. Blood samples were obtained by either tail bleed or retro-orbital eye bleed, by use of heparinized microhematocrit tubes. Mice were anesthetized by intramuscular injection of 50 μl of a solution of 100 mg ketamine/ml (Sigma) and were perfused with 50 ml of 0.9% saline, to remove heme, which interfered with the fluorometric α-Gal A enzymatic assay. The liver, spleen, heart, lungs, brain, kidneys, and dorsal and ventral skin biopsies from shaved mice were obtained and were snap-frozen in liquid nitrogen. Organs were stored at −20°C prior to enzyme or GL-3 analyses.

Study Design

To compare the pharmacokinetics of the four α-Gal A glycoforms, each was injected at a concentration of 1 mg/kg, and its plasma clearance rate was determined. The biodistribution of 1 or 10 mg of the glycoforms/kg of body weight was assessed in selected organs after the mice were killed and perfused with saline, 1 h after intravenous administration of the glycoform. After a dose of 1 mg/kg, the tissue stability of glycoform AGA-1 was determined in the liver and spleen, at 24-h intervals over 4 d. To determine the biodistribution and/or effect that the enzyme dose had on the tissue or plasma GL-3 levels, 0.3, 1.0, 3.0, or 10.0 mg of AGA-1/kg was injected as either a single dose or eight doses, at 48-h intervals. The mice were killed 24 h after the last injection, and the α-Gal A activity and/or GL-3 levels were determined. All reported values represent the mean in three to six mice for each time point and dose, unless otherwise indicated.

Enzymatic Activity, Protein, and GL-3 Assays

To determine the α-Gal A activity in tissues, freshly obtained or 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 were homogenized on ice by use of a Tissue Tearor (Biospec Products) for 2 min (at setting 3). Debris was pelleted by centrifugation at room temperature, and the soluble fractions were assayed for α-Gal A activity at 37°C, by use of 5 mM 4-methylumbelliferyl-α-D-galactopyranoside (or 4MU-α-Gal) con-

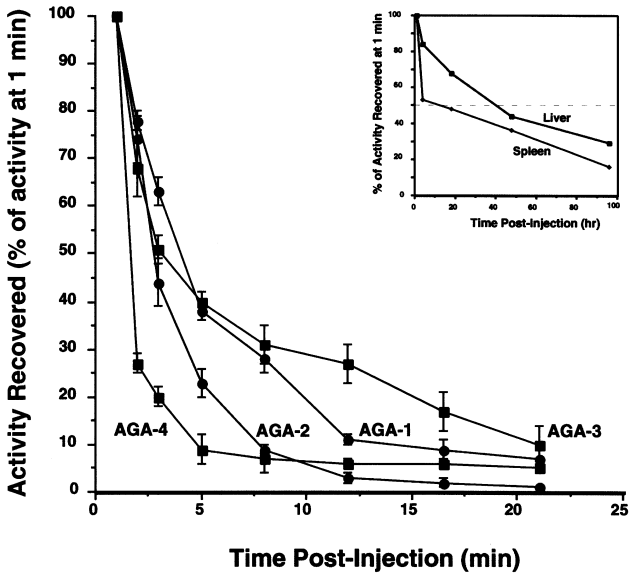

Figure 2 Pharmacokinetics of α-Gal A glycoforms (1mg/kg). *Inset,* Biostability of AGA-1 in the liver and spleen. Each value point represents the mean ± 1 SD for three or four mice.

Table 2

Mean Recovered Activity of Intravenously Administered Human α-Gal A Glycoforms in α-Gal A Deficient Mice

	Mean Recovered Activity of Glycoform [Dose][a] (%)				
Tissue	AGA-1 [1.0 mg/kg]	AGA-1 [10.0 mg/kg]	AGA-2 [1.0 mg/kg]	AGA-3 [1.0 mg/kg]	AGA-4 [1.0 mg/kg]
Liver	94.5	93.5	94.8	94.7	95.3
Spleen	2.8	3.5	2.8	2.0	2.7
Kidneys	1.3	2.7	1.3	1.0	1.0
Heart	ND	.4	ND	ND	ND
Lungs	ND	.3	ND	ND	ND
Brain	ND	ND	ND	ND	ND

Note.—Total activity recovered in the tissues listed was 25%–30% of administered dose.

[a] In at least three mice killed 1 h postinjection. ND = not detectable.

taining 117 mM *N*-acetylgalactosamine at pH 4.6, as described elsewhere (Bishop et al. 1980) (1 unit [U] of α-Gal A activity = 1 nmol of substrate hydrolyzed/h). Enzymatic activity was detectable in tissues at levels >0.01 U/mg. Protein concentrations were determined by the fluorescamine method (Bohlen et al. 1973). GL-3 analysis and quantitation were performed according to an enzyme-linked immunosorbent assay (ELISA) using the β-subunit of *Escherichia coli* verotoxin, which specifically binds GL-3 (Zeidner et al. 1999). GL-3 concentrations were expressed as nanograms of GL-3 per milligram of wet tissue. In addition, qualitative and semiquantitative analyses of tissue glycolipids were performed by high-performance thin-layer chromatography (HPTLC), as described elsewhere (Kupke and Zeugner 1978; Zeidner et al. 1999).

Morphological Studies

After mice were killed and perfused as described above, tissues were removed and minced into 2-mm cubes, which were immediately immersed in 0.2 M sodium cacodylate buffer, pH 7.4, containing 3% glutaraldehyde. Tissues were postfixed with 1% osmium tetroxide buffered with sodium cacodylate, for 1 h; were dehydrated in ethanol, through propylene oxide; and were embedded in Embed 812 resin (Electron Microscopy Sciences). One-micron plastic sections were cut, stained with methylene blue and azure II, and observed by light microscopy, to choose representative areas for ultrathin sectioning and examination by electron microscopy using a JEM 100CX electron-transmission microscope (Jeol).

Results

Characterization of the Human Recombinant α-Gal A Glycoforms

Table 1 compares the physical and kinetic properties of the four recombinant human α-Gal A secreted glycoforms. AGA-1 and AGA-2 were stably overexpressed by different CHO cell lines (Ioannou et al. 1992) and had similar specific activities toward the native glycolipid and artificial fluorogenic substrates. These two glycoforms differed only in the degree of sialylation, with both IEF migration (fig. 1) and α-neuraminidase digestion (data not shown) showing AGA-1 as being more highly sialylated. For comparison, the complete oligosaccharide structures on AGA-2 were determined (Matsuura et al. 1998). AGA-3, produced by stably introducing the rat α-2,6-sialyltransferase gene into the AGA-2–overexpressing CHO line, had properties similar to those of AGA-2, except that it was more highly sialylated and contained α-2,6-linked sialic acid residues, which are not normally synthesized by CHO cells. AGA-4 was designed, by site-specific mutagenesis, to eliminate the high-mannose oligosaccharide chain from each α-Gal A glycopeptide (Ioannou et al. 1998). The resultant active homodimeric enzyme, overexpressed in COS-1 cells, had only two M6P residues (one on each hybrid oligosaccharide chain at N215) and, at pH 7.4, was somewhat less stable than AGA-1 (33% vs. 50%, respectively, of initial activity at 37°C).

Pharmacokinetics and Biodistribution of the α-Gal A Glycoforms

After a single intravenous dose of 1 mg/kg, each α-Gal A glycoform was cleared from the circulation of the

Table 3

Mean Activity after Repeated Intravenous Administration of AGA-1 in α-Gal A–Deficient Mice

Tissue	Mean Activity in Wild-Type Mice[a] (U/mg)	Mean Activity in α-Gal A–Deficient Mice[a] (U/mg)	-Fold Greater than That in Wild-Type Mice
Liver	16.8	1,160	× 69.2
Spleen	39.2	44.7	× 1.14
Kidneys	11.3	14.0	1.24
Heart	3.2	17.7	× 5.40
Lungs	29.8	5.4	× .83
Brain	26.9	ND	

[a] In at least three mice injected with 10 mg/kg every other day for eight doses and killed after injection 8 on day 15. ND = not detectable.

α-Gal A–deficient mice in two phases—a rapid phase with a plasma $T_{1/2}$ of ~2–5 min and a second, longer phase (fig. 2). AGA-1 and AGA-3, the two most highly sialylated glycoforms, had the longest $T_{1/2}$ (~3–5 min) for the rapid-clearance phase and then circulated longer and at higher levels (activity at 20 min was ~10% and ~15%, respectively, of the activity at 1 min). AGA-3, engineered to have α-2,6-linked (as well as α-2,3-linked) sialic acid residues, circulated the longest of all four glycoforms. In contrast, the AGA-4 glycoform, in which the N192 glycosylation site was eliminated, was most rapidly cleared from the circulation, with a $T_{1/2}$ of ~2 min, but remained detectable for >20 min, at ~10% of its activity at 1 min.

The biodistribution of each glycoform was determined after a single injection of 1.0 mg/kg. For all four glycoforms, the total activity recovered, 1 h postinjection, from the brain, heart, kidneys, liver, lungs, and spleen was consistently 25%–39% (mean 29.3%; $n = 14$) of the administered dose. Surprisingly, ~95% of each glycoform was recovered in the liver (~28% of administered dose), and, for all four glycoforms, were similarly taken up by the spleen and kidneys (~2%–3% and ~1%, respectively, of recovered activity) (table 2). At this dose, none of the glycoforms was detected in the heart, lungs, or brain. On the basis of these remarkably similar findings, the highly sialylated AGA-1 glycoform was selected for further studies.

Biodistribution and Stability of AGA-1

To determine the effect that an increased dose of AGA-1 would have on the enzyme's biodistribution, 10 mg of AGA-1/kg was administered, and the α-Gal A activity in various tissues was assessed 1 h postinjection. The percentage of enzyme recovered in the liver decreased slightly, reflecting the increased activity detected in the spleen, in the heart, and, in particular, in the kidneys. Also, a low level of α-Gal A activity was detected in the lungs, but no activity crossed the blood-brain barrier, even at this high dose.

The stability of AGA-1 activity in the liver and spleen was determined after a dose of 1 mg/kg (fig. 2, *inset*). The $T_{1/2}$ of the enzyme activity in the liver and spleen was ~48 and ~36 h, respectively. Notably, 96 h postinjection, the liver and spleen retained ~30% and ~20% of the AGA-1 activity recovered at 1 h, respectively. These findings indicated that the enzyme was active and relatively stable after uptake, presumably in tissue lysosomes.

To assess the role that reticuloendothelial mannose receptors play in enzyme uptake, particularly by the liver and spleen, mice were intravenously injected with ~0.1 mg of yeast mannans/kg, 20 min prior to administration of 0.5 mg of AGA-1/kg. The mannan-treated mice retained 40% of the AGA-1 activity in the circulation 1 h postinjection, compared with <5% in untreated mice. With mannan pretreatment, the percentage of α-Gal A activity recovered in the kidneys 1 h after enzyme injection increased from ~1% to 10%. Thus, the distribution of injected enzyme was altered by increasing the dose or by blocking the mannose receptors on reticuloendothelial cells.

Repeated AGA-1 Dosing: Reconstitution of Tissue α-Gal A Activity

To determine the levels of α-Gal A activity that could be attained in various tissues, eight every-other-day doses of AGA-1 (10 mg/kg) were injected, and the mice were killed 24 h after the last dose, on day 15 (table 3). As expected, the α-Gal A activity recovered in the liver was markedly greater (~70-fold), whereas the activity in the heart was ~5-fold greater, and the activities in the spleen, kidneys, and lungs were near or slightly greater than the respective wild-type activity. In contrast, no activity was detected in the brain, even after repeated high doses of enzyme.

Depletion of Tissue GL-3: Dependence on AGA-1 Dose

Tissue GL-3 concentrations were determined for 7 d after a single intravenous injection of 0.3, 1.0, or 3.0 mg of AGA-1/kg (table 4). A single dose of 0.3 mg/kg cleared >90% of the accumulated hepatic substrate, and increased doses of 1.0 and 3.0 mg/kg completely cleared the glycolipid (<1.0 ng/mg). The GL-3 clearances in the spleen, heart, and kidneys also were dose dependent, with a single dose of 3 mg/kg resulting in ~90%, ~65%, and ~37% clearance, respectively.

Accumulated Tissue GL-3 Cleared by Repeated AGA-1 Doses

The effect that repeated AGA-1 doses had on the depletion of tissue GL-3 was determined 24 h after eight every-other-day intravenous administrations of 0.3, 1.0, 3.0, or 10 mg of AGA-1/kg (table 5). All doses cleared the accumulated GL-3 in the liver to nondetectable levels. A dose-dependent depletion of accumulated GL-3 was observed in the heart, spleen, and kidneys. Doses of 3 and 10 mg/kg reduced the GL-3 concentrations in the heart and spleen to nondetectable levels, whereas repeated doses of 10 mg/kg reduced the marked accumulation in the kidneys to ~40% of the untreated level. These reductions were determined by ELISA of GL-3 and were confirmed by HPTLC analysis (data not shown). It is noteworthy that only one of the two GL-3 bands observed in the kidneys of untreated α-Gal A–deficient mice was depleted in a dose-dependent manner by the administered enzyme. To confirm that both

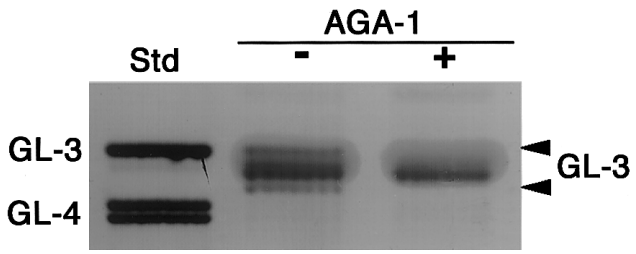

Figure 3 HPTLC of mouse kidney GL-3, before (−) and after (+) in vivo treatment with AGA-1.

bands were GL-3, a kidney homogenate from an untreated α-Gal A–deficient mouse was incubated, at 37°C and pH 4.6, with 10,000 U of purified AGA-1, and the neutral glycolipids were extracted and analyzed by HPTLC. As shown in figure 3, both bands were hydrolyzed by the exogenous enzyme.

Similarly, clearance of the accumulated GL-3 in the skin was dose dependent (fig. 4). Compared with the mean level of skin GL-3 in untreated mice (~1,990 ng/mg; $n = 10$), eight every-other-day doses of 0.3, 1.0, or 3.0 mg of enzyme/kg (followed by killing of the mice 24 h later) reduced the mean skin GL-3 level by ~85%, ~55%, and ~60%, respectively. Five daily doses of 10 mg/kg (following by killing of the mice 24 h later) further reduced skin GL-3 levels, to 450 ng/mg ($n = 4$), an average clearance of ~80%.

Depletion and Reaccumulation of Tissue and Plasma GL-3: Dose Dependence

The rate of depletion and reaccumulation of tissue GL-3 in α-Gal A–deficient mice was determined after a single dose of 3 mg of AGA-1/kg (fig. 5). As expected, liver GL-3 levels remained undetectable during the 4 wk of study. Splenic and cardiac GL-3 levels were maximally decreased at 3 wk, whereas reaccumulation of GL-3 was evident in both tissues at 4 wk. It is noteworthy that the plasma GL-3 decreased to nondetectable levels at 1 wk and then increased over time, to ~40% of pretreatment levels, at 2.5–3 wk.

Since it appeared that the plasma GL-3 increased as the tissue GL-3 reaccumulated, the plasma GL-3 levels were determined before and at 24-h intervals after single injections of 0.3, 1.0, 3.0, and 10 mg of AGA-1/kg. After an initial increase in plasma GL-3 concentrations at 24 h postinjection (except for the dose of 10 mg/kg), dose-dependent decreases in the levels of circulating GL-3 were observed (fig. 6). Not only the magnitude but the rapidity and duration of the decrease depended on the dose. For all doses, maximal decreases occurred ~7 d postinjection. The dose of 10 mg/kg reduced the plasma GL-3 to wild-type levels, which were maintained on day 11 postinjection. After doses of 0.3, 1.0 and 3.0 mg/kg, the levels of plasma GL-3 reaccumulated in a dose-dependent manner, and the levels 11 d postinjection were ~50%, ~15%, and ~5%, respectively. HPTLC analysis confirmed that AGA-1 had an effect on depletion and reaccumulation of plasma GL-3 (data not shown).

Ultrastructural Demonstration of Tissue GL-3 Clearance

Prior to treatment, the α-Gal A–deficient mice had numerous lamellar osmophilic-dense membrane-bound inclusions in the liver, spleen, heart, and kidneys (fig. 7, *a* and *b* series). After four every-other-day doses of AGA-1 (3 mg/kg), the number, size, and density of these inclusions were markedly decreased (fig. 7, *c* and *d* series). It is noteworthy that the posttreatment liver sinusoids were lined with numerous large empty-appearing, presumably fluid-filled vacuoles (fig. 7, *1c* and *1d*).

Discussion

Early pilot studies of enzyme-replacement therapy in Fabry disease demonstrated that intravenously administered α-Gal A could hydrolyze the accumulated circulating GL-3 (Mapes et al. 1970; Brady et al. 1973; Desnick et al. 1979; Bishop et al. 1981). These trials also indicated that the highly sialylated enzyme purified from human plasma was more effective in depleting the accumulated circulating GL-3 than was the processed enzyme purified from human splenic lysosomes (Desnick et al. 1979; Bishop et al. 1981). Subsequent trials were precluded because of the lack of sufficient human enzyme and of an animal model to assess the pharmaco-

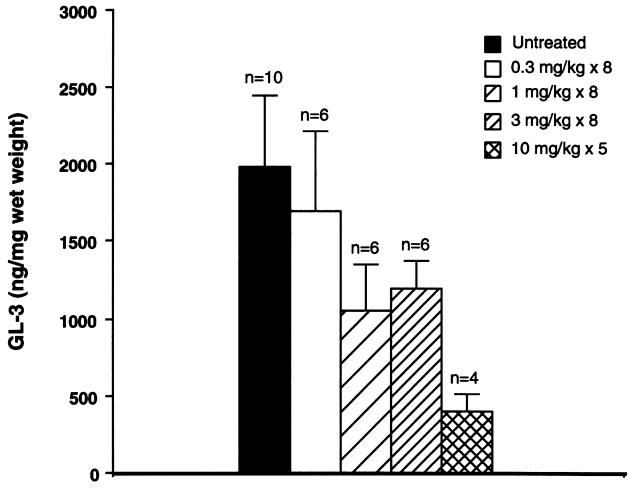

Figure 4 Effect of α-Gal A on skin GL-3 levels in α-Gal A–deficient mice. Values represent mean ± 1 SD for 4–10 skin patches per mouse.

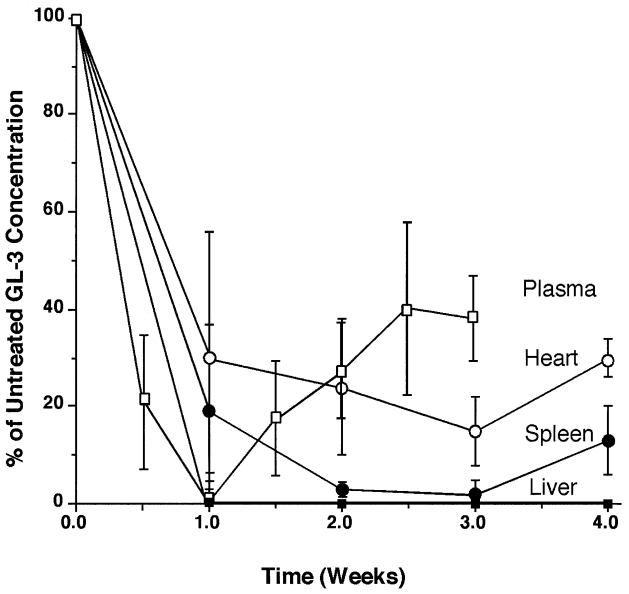

Figure 5 Effect of α-Gal A (3 mg/kg) on depletion and reaccumulation of GL-3 in tissues and plasma of α-Gal A–deficient mice. Values represent the mean ± 1 SD for three mice.

kinetics and biodistribution of the administered enzyme and the enzyme's ability to hydrolize the accumulated GL-3 in tissue sites of pathology. The recent availability of a novel method to overexpress and selectively secrete various recombinant human α-Gal A glycoforms (Ioannou et al. 1992, 1998), the generation of an α-Gal A–deficient mouse (Wang et al. 1996), and the development of a rapid and sensitive ELISA for GL-3 (Zeidner et al. 1999) permitted the systematic evaluation of four α-Gal A glycoforms, providing the first preclinical in vivo evidence that α-Gal A administration depletes the accumulated GL-3 in the plasma and in the lysosomes of pathogenic tissues.

Surprisingly, the four recombinant α-Gal A glycoforms, which varied in the number of sialic acid and M6P residues, had similar pharmacokinetics and tissue distributions. It was anticipated that the more highly sialylated forms would circulate longer and would have higher uptake by extrahepatic tissues. Although the more highly sialylated—AGA-1 and AGA-3—glycoforms were retained in the circulation longer (fig. 2), the biodistribution of all four glycoforms was remarkably similar, in that ~95% of each was recovered in the liver and the remainder of each was detected in the spleen and kidneys (table 2). A 10-fold–increased dose of the AGA-1 glycoform altered the biodistribution slightly, since activity also was detected in the heart and lungs. Even at the high dose (10 mg/kg), no enzymatic activity was detectable in the brain, consistent with the inability of the high-molecular-weight (~110 kD) enzyme to cross the blood-brain barrier. Presumably, the hepatic uptake was not influenced by the degree of sialylation or retention in the circulation but was mediated primarily by the mannose receptors for reticuloendothelial uptake and by the M6P receptors for lysosomal targeting (Jansen et al. 1991; Dini et al. 1992; Bijsterbosch et al. 1996). Consistent with this notion is the finding that the administration of mannans to block the mannose receptors prior to enzyme injection prolonged the circulatory $T_{½}$ of AGA-1 (from ~4 min to >60 min) and markedly increased the kidneys' uptake of the enzyme (from ~1% to 10% of recovered activity 1 h postinjection). Similar findings have been reported by others (Fiddler et al. 1977; Achord et al. 1978; Rattazzi et al. 1980; Bijsterbosch et al. 1996). Thus, it is likely that the mannose and M6P receptors are predominantly responsible for the biodistribution and lysosomal targeting of the exogenous α-Gal A glycoforms. It is noteworthy that murine antibodies to the heterologous human enzyme were not detected during these single- or multiple-dose short-term studies. However, in separate experiments, the immunogenicity of the human AGA-1 glycoform was assessed in mice by ELISA. Single doses of 0.3 mg/kg ($n = 6$ mice), 1 mg/kg ($n = 7$ mice), or 10 mg/kg ($n = 3$ mice) did not produce antibodies to human AGA-1 6 wk after administration. Eight injections of 10 mg of AGA-1/kg produced anti–α-Gal A antibodies in 3 of 10 mice at 6 wk. None of the antibodies were neutralizing.

Since ~95% of the recovered enzyme (i.e., ~30% of the dose) was in the liver, it is not surprising that most of the accumulated hepatic GL-3 was cleared after a single injection of the lowest dose, 0.3 mg/kg (table 4).

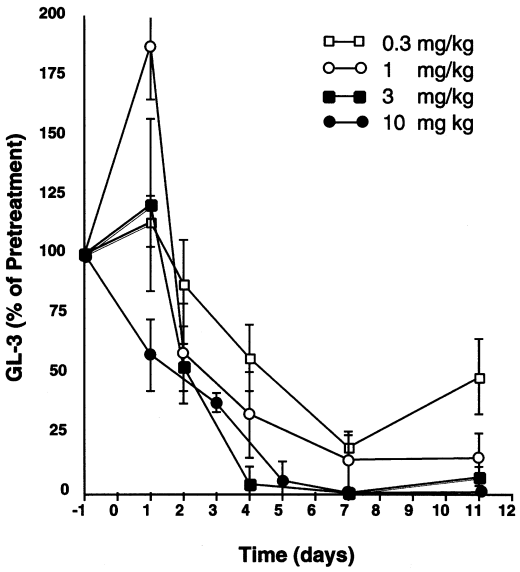

Figure 6 Effect of α-Gal A dose on plasma GL-3 concentration in α-Gal A–deficient mice. Values represent the mean ± 1 SD for three to five mice.

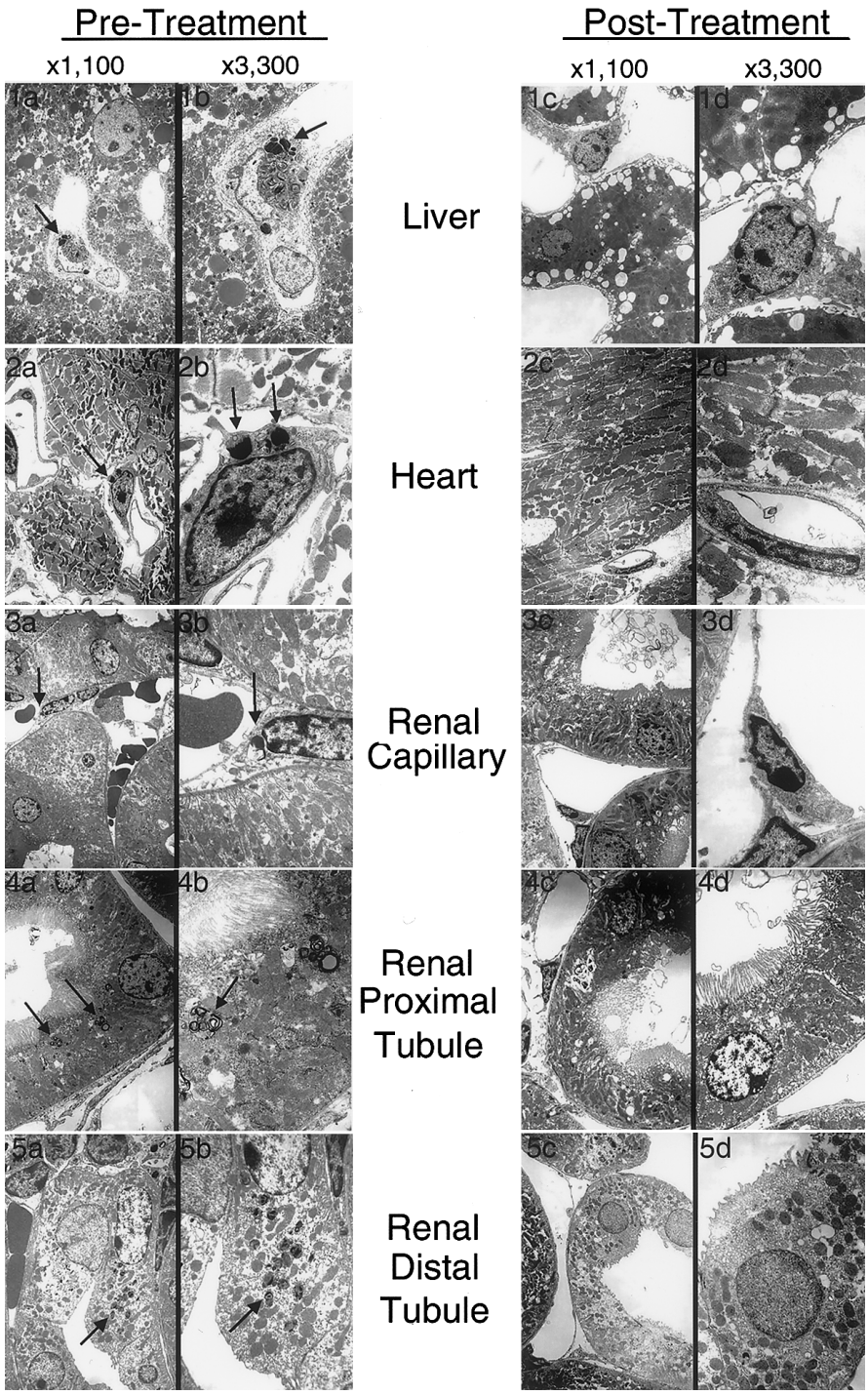

Figure 7 Representative electron micrographs of glutaraldehyde-fixed liver (row *1*), heart (row *2*), and kidneys (rows *3*–*5*) of α-Gal A–deficient mice, before (*a* and *b* series) and 7 d after (*c* and *d* series) enzyme treatment with 3 mg/kg every other day for four doses. Note the presence (*1a* and *1b*) and absence (*1c* and *1d*) of osmophilic dense storage material in Kupffer cell lysosomes in liver sinusoids. In the heart, the storage material was present in the lysosomes of cardiac pericytes and in the pericapillary histiocytes and fibroblasts (*2a* and *2b*, *arrows*), whereas these deposits were not observed posttreatment (*2c* and *2d*). In the kidneys, numerous lysosomes (*arrows*) containing storage material were present in the pericytes of capillaries (*3a* and *3b*), in the cells lining the proximal convoluted tubules (*4a* and *4b*), and in the apical cytoplasm of the cells lining the distal convoluted tubules (*5a* and *5b*). In contrast, the stored material was absent or markedly reduced in these renal cells posttreatment (*3c* and *3d*, *4c* and *4d*, and *5c* and *5d*, respectively). Ultrathin sections were stained with uranyl acetate and lead citrate.

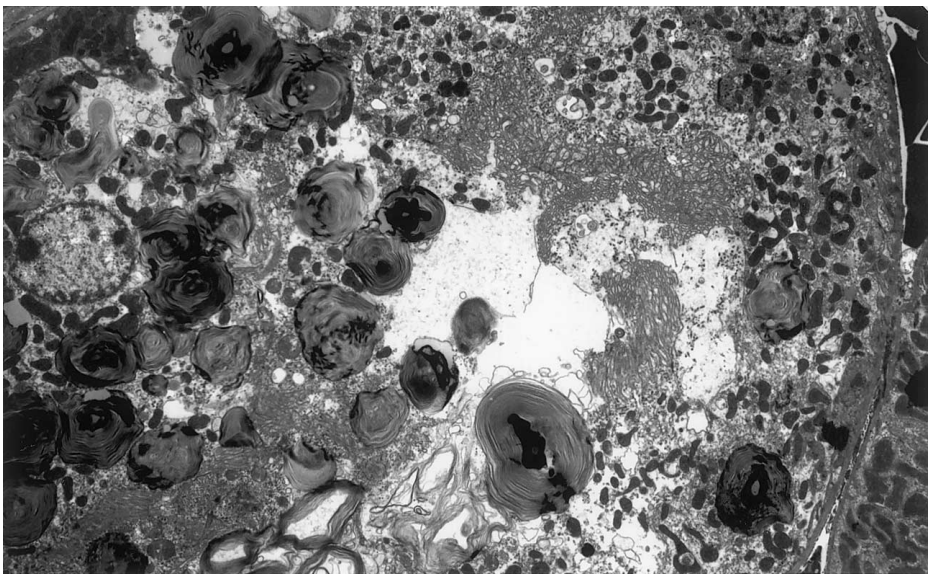

Figure 8 Electron micrograph of a kidney tubule from an α-Gal A–deficient mouse. Osmophilic dense lipid bodies can be seen secreted into the lumen of the tubule.

A 10-fold–higher dose (3 mg/kg) resulted in significant clearance in the heart, spleen, and kidneys, indicating that the amount of enzyme delivered to each tissue was critical for depletion of the substrate. When enzyme administration was increased to eight doses over 14 d, the level of GL-3 was remarkably reduced, as a function of dose, in all tissues analyzed (table 5 and fig. 4). Only the accumulated renal GL-3 was not completely cleared, even at the highest dose administered. Unlike humans (Boyd and Lingwood 1989), male wild-type mice have a testosterone-induced form of GL-3 (McCluer et al. 1981, 1983), which was one of the two GL-3 species observed in approximately equal quantities in the α-Gal A–deficient mice. The testosterone-induced GL-3 is secreted into the urine in multilammellar bodies, in both normal (Gross et al. 1991) and α-Gal A–deficient mice (fig. 8), and appears to be inaccessible to exogenously administered α-Gal A.

The rate of reaccumulation of GL-3 after a single enzyme dose of 3 mg/kg was assessed to indicate the dose frequency that would maintain reduced GL-3 levels. The accumulated hepatic GL-3 was rapidly cleared, and it remained at undetectable levels for ⩾4 wk, whereas the splenic and cardiac GL-3 concentrations were maximally decreased at 2 and 3 wk postinjection, respectively, after which both began to reaccumulate (fig. 6). These findings suggest that a biweekly dose of α-Gal A could both deplete the accumulated GL-3 and prevent its reaccumulation.

The biochemical demonstration of depletion of accumulated tissue GL-3 is consistent with the ultrastructural findings of absent, fewer, smaller, and/or less-dense lysosomes in the tissues of treated mice (fig. 7). Markedly decreased lysosomal glycolipid storage was observed in the pericytes of vascular smooth-muscle cells systemically, in fibroblasts and histiocytes of the heart, in Kupffer cells, and in the ducts of the kidneys. The finding of numerous translucent vacuoles lining the hepatic sinusoids after enzyme administration suggests that α-Gal A is readily endocytosed into endosomes, for subsequent delivery to lysosomes containing the stored substrate. These vacuoles were most prevalent in the liver, consistent with the fact that ~30% of dose was delivered to the liver. Similar ultrastructural findings have been observed in cultured rat hepatocytes after the incubation of gold-labeled lactosylated albumin or invertase (Dini et al. 1992). Thus, the hydrolysis of the accumulated substrates, the prolonged activity in tissues, and the morphological clearance of lysosomal deposits indicate that the exogenous α-Gal A was effectively endocytosed and delivered to tissue lysosomes.

It is noteworthy that the depletion and reaccumulation of plasma GL-3 also was dose dependent (fig. 6), suggesting that the plasma GL-3 levels reflected the tissue load. Since α-Gal A is not active at the neutral pH of blood (Desnick et al. 1973), and since circulating glycosphingolipids are carried by plasma lipoproteins synthesized in hepatocytes (Attie et al. 1982; Chatterjee and Kwiterovich 1984), it is likely that the decrease in plasma GL-3 levels after enzyme administration results from the clearance of stored tissue substrate. Thus, the plasma GL-3 levels may provide an easily accessible indicator of the tissue GL-3 load, as a way to monitor dose effectiveness in patients with Fabry disease.

Table 4

GL-3 Concentrations in α-Gal A–Deficient Mice after a Single Dose of AGA-1

	Liver		Spleen		Heart		Kidneys	
Dose (mg/kg)	Mean GL-3 Concentration [Range][a] (ng/mg wet wt)	% Untreated	Mean GL-3 Concentration [Range][a] (ng/mg wet wt)	% Untreated	Mean GL-3 Concentration [Range][a] (ng/mg wet wt)	% Untreated	Mean GL-3 Concentration [Range][a] (ng/mg wet wt)	% Untreated
Untreated	377 [344–454]	100	2,790 [2,340–3,080]	100	196 [169–224]	100	1,100 [997–1,180]	100
.3	26 [12–48]	6.9	1,350 [1,400–1,440]	48.3	191 [42–327]	97.4	804 [756–845]	73.4
1.0	<1. 0	0	683 [676–694]	24.5	95 [32–158]	48.5	650 [650–765]	59.3
3.0	<1. 0	0	310 [294–320]	11.1	68 [24–106]	34.7	689 [643–726]	62.9

[a] In at least three mice, 7 d postinjection.

Table 5

Effect of Eight AGA-1 Doses on Tissue GL-3 Concentrations in α-Gal A–Deficient Mice

	Liver		Heart		Spleen		Kidneys	
Dose (mg/kg)	Mean GL-3 Concentration[a] (ng/mg wet wt)	% Untreated	Mean GL-3 Concentration[a] (ng/mg wet wt)	% Untreated	Mean GL-3 Concentration[a] (ng/mg wet wt)	% Untreated	Mean GL-3 Concentration[a] (ng/mg wet wt)	% Untreated
Untreated	420	100	197	100	1,190	100	1,030	100
.3	<1.0	0	64.8	33	897	76	670	65
1.0	<1.0	0	52.4	27	747	63	484	47
3.0	<1.0	0	13.6	7	596	50	448	43
10.0	<1.0	0	<1.0	0	…	…	419	41

[a] In at least three mice injected every other day for eight doses and then killed 24 h later, on day 15.

In summary, these animal studies provide the first systematic in vivo evaluation of α-Gal A replacement for Fabry disease. The findings demonstrate that α-Gal A is delivered to the target sites of pathology in the murine model of Fabry disease and that the clearance of the accumulated GL-3 in tissues is dose dependent. These results provide critical preclinical data for the design of α-Gal A–replacement trials in patients with Fabry disease (Eng et al. 2000*a,* 2000*b;* Schiffmann et al. 2000*a,* Schiffmann et al. 2000*b*).

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Electronic-Database Information

The accession number and URL for data in this article are as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for Fabry disease [MIM 301500])

References

Achord DT, Brot FE, Bell CE, Sly WS (1978) Human beta-glucuronidase: in vivo clearance and in vitro uptake by a glycoprotein recognition system on reticuloendothelial cells. Cell 15:269–278

Attie AD, Pittman RC, Steinberg D (1982) Hepatic catabolism of low density lipoprotein: mechanisms and metabolic consequences. Hepatology 2:269–281

Beutler E, Westwood B, Dale GL (1983) The effect of phlebotomy as a treatment of Fabry disease. Biochem Med 30: 363–368

Bijsterbosch MK, Donker W, van de Bilt H, van Weeley S, van Berkel TJC, Aerts JMFG (1996) Quantitative analysis of the targeting of mannose-terminal glucocerebrosidase. Eur J Biochem 237:344–349

Bishop DF, Calhoun DH, Bernstein HS, Hantzopoulos P, Quinn M, Desnick RJ (1986) Human α-galactosidase A: nucleotide sequence of a cDNA clone encoding the mature enzyme. Proc Natl Acad Sci USA 83:4859–4863

Bishop DF, Dean KJ, Sweeley CC, Desnick RJ (1980) Purification and characterization of human α-galactosidase isozymes: comparison of tissue and plasma forms and evaluation of purification methods. In: Desnick RJ (ed) Enzyme therapy in genetic diseases. Vol 2. Alan R Liss, New York, pp 17–32

Bishop DF, Kovac CR, Desnick RJ (1981) Enzyme therapy. XX. Further evidence for the differential in vivo fate of human splenic andplasma forms of α-galactosidase A in Fabry disease: recovery of exogenous activity from hepatic tissue. In: Callahan JW, Lowden JA (eds) Lysosomes and lysosomal storage diseases. Raven Press, New York, pp 381–394

Bohlen P, Stein S, Dairman W, Undenfriend S (1973) Fluorometric assay of protein in the nanogram range. Arch Biochem Biophys 155:213–220

Boyd B, Lingwood C (1989) Verotoxin receptor glycolipid in human renal tissue. Nephron 51:207–210

Brady RO, Tallman JF, Johnson WG, Gal AE, Leahy WR, Quirk JM, Dekaban AS (1973) Replacement therapy for inherited enzyme deficiency: use of purified ceramidetrihexosidase in Fabry's disease. N Engl J Med 289:9–14

Chatterjee S, Kwiterovich POJ (1984) Glycosphingolipids and plasma lipoproteins: a review. Can J Biochem Cell Biol 62: 385–397

Colombi A, Kostyal A, Bracher R, Gloor F, Mazzi R, Tholen H (1967) Angiokeratoma corporis diffusum—Fabry's disease. Helv Med Acta 34:67–83

Desnick RJ, Allen KY, Desnick SJ, Raman MK, Bernlohr RW, Krivit W (1973) Fabry's disease: enzymatic diagnosis of hemizygotes and heterozygotes: α-galactosidase activities in plasma, serum, urine, and leukocytes. J Lab Clin Med 81: 157–171

Desnick RJ, Dean KJ, Grabowski G, Bishop DF, Sweeley CC (1979) Enzyme therapy in Fabry disease: differential in vivo plasma clearance and metabolic effectiveness of plasma and splenic α-galactosidase A isozymes. Proc Natl Acad Sci USA 76:5326–5330

——— (1980) Enzyme therapy. XVII. Metabolic and immunologic evaluation of α-galactosidase A replacement in Fabry disease. Birth Defects 16:393–413

Desnick RJ, Ioannou YA, Eng CM (1995) α-galactosidase A deficiency: Fabry disease. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds) The metabolic and molecular basis of inherited diseases, 7th ed. McGraw-Hill, New York, pp 2741–2784

——— (2001) α-Galactosidase A deficiency: Fabry disease. In: Scriver CR, Beaudet AL, Sly WS, Valle D, Kinzler KE, Vogelstein B (eds) The metabolic and molecular basis of inherited diseases, 8th ed. McGraw-Hill, New York, pp 3733–3774

Dini L, Lentini A, Mantile G, Massimi M, Devirgiliis LC (1992) Receptor-mediated endocytosis of galactose and mannose exposing ligands: an electron microscopic study on adult and neonatal cultured rat hepatocytes. Biol Cell 74: 217–224

Eng CM, Cochat P, Wilcox WR, Germain DP, Lee P, Waldek S, Caplan L, Heymans H, Braakman T, Fitzpatrick MA, Huertas P, O'Callaghan MW, Richards S, Tandon P, Desnick RJ (2000*a*) Enzyme replacement therapy in Fabry disease: results of a placebo-controlled phase 3 trial. Am J Hum Genet 67 Suppl 2:38

Eng CM, Phelps R, Kim L, Goldman M, Gordon R, Glass A, Winston J, Brodie S, Mehta D, Dikman S, Fallon J, Parsons R, Stacy C, Shinnar M, Rosenberg M, O'Callaghan M, Fitzpatrick M, Huertas P, Desnick RJ (2000*b*) Enzyme replacement therapy in Fabry diseases: results of a phase I/II clinical trial. J Inherit Metab Dis 23:222

Fiddler MB, Hudson LDS, Desnick RJ (1977) Enzyme therapy.

VIII. Immunologic evaluation of repeated administration of erythrocyte-entrapped β-glucuronidase to β-glucuronidase deficient mice. Biochem J 168:141–145

Gross SK, Daniel PF, Evans JE, McCluer RH (1991) Lipid composition of lysosomal multilamellar bodies in male mouse urine. J Lipid Res 32:157–164

Ioannou YA, Bishop DF, Desnick RJ (1992) Overexpression of human α-galactosidase A results in its intracellular aggregation, crystallization in lysosomes and selective secretion. J Cell Biol 119:1137–1150

Ioannou YA, Zeidner KM, Grace ME, Desnick RJ (1998) Human α-galactosidase A: glycosylation site 3 is essential for enzyme solubility. Biochem J 332:789–797

Jansen RW, Molema G, Ching TL, Oosting R, Harms G, Moolenaar F, Hardonk MJ, Meijer DK (1991) Hepatic endocytosis of various types of mannose-terminated albumins: what is important, sugar recognition, net charge, or the combination of these features. J Biol Chem 266:3343–3348

Kolodny EH, Ullman MD, Pyeritz RE, Moser HW (1981) Plasmapheresis therapy for the lipidoses. In: Callahan JW, Lowden JA (eds) Lysosomes and lysosomal storage diseases. Raven Press, New York, pp 395–403

Kupke IR, Zeugner S (1978) Quantitative high-performance thin-layer chromatography of lipids in plasma and liver homogenates after direct application of 0.5-microliter samples to the silica-gel layer. J Chromatogr 146:261–271

Lee S-J, Nathans D (1988) Proliferin secreted by cultured cells binds to mannose-6-phosphate receptors. J Biol Chem 263: 3521–3527

Maizel SE, Simmons RL, Kjellstrand C, Fryd DS (1981) Ten-year experience in renal transplantation for Fabry's disease. Transplant Proc 13:57–59

Mapes CA, Anderson RL, Desnick RJ, Krivit W, Sweeley CC (1970) Enzyme replacement as a possible therapy for Fabry's disease. Science 169:987–989

Matsuura F, Ohta M, Ioannou YA, Desnick RJ (1998) Human α-galactosidase A: characterization of the *N*-linked oligosaccharides on the intracellular and secreted glycoforms overexpressed by Chinese hamster ovary cells. Glycobiology 8:329–339

McCluer RH, Deutsch CK, Gross SK (1983) Testosterone-responsive mouse kidney glycosphingolipids: developmental and inbred strain effects. Endocrinology 113:251–258

McCluer RH, Williams MA, Gross SK, Meisler MH (1981) Testosterone effects on the induction and urinary excretion of mouse kidney glycosphingolipids associated with lysosomes. J Biol Chem 256:13112–13120

Rattazzi MC, Lanse SB, McCullough RA, Nester JA, Jacobs EA (1980) Towards enzyme replacement in G_{M2} gangliosidosis: organ deposition and induced central nervous system uptake of β-hexosaminidase in the cat. In: Desnick RJ (ed) Enzyme therapy in genetic diseases. Vol 2. Alan R Liss, New York

Schiffmann R, Kopp JB, Austin H, Moore DF, Sabnis S, Weibel T, Balow JE, Brady RO (2000*a*) Efficacy and safety of enzyme replacement therapy for Fabry disease demonstrated by a double-blind placebo-controlled trial. Am J of Hum Genet 67 Suppl 2:38

Schiffmann R, Murray GJ, Treco D, Daniel P, Sellos-Moura M, Myers M, Quirk JM, Zirzow GC, Borowski M, Loveday K, Anderson T, Gillespie F, Oliver KL, Jeffries NO, Doo E, Liang TJ, Kreps C, Gunter K, Frei K, Crutchfield K, Selden RF, Brady RO (2000*b*) Infusion of alpha-galactosidase A reduces tissue globotriaosylceramide storage in patients with Fabry disease. Proc Natl Acad Sci USA 97:365–370

Wang AM, Ioannou YA, Zeidner KM, Gotleb RW, Dikman S, Stewart CL, Desnick RJ (1996) Generation of a mouse model with α-galactosidase A deficiency. Am J Hum Genet 59:A208

Wen DX, Svensson EC, Paulson JC (1992) Tissue-specific alternative splicing of the beta-galactoside alpha 2,6-sialyltransferase gene. J Biol Chem 267:2512–2518

Zeidner K, Desnick R, Ioannou Y (1999) Quantitative determination of globotriaosylceramide by immunodetection of glycolipid-bound recombinant verotoxin B subunit. Anal Biochem 267:104–113