Long-Term Intravenous Treatment of Pompe Disease With Recombinant Human α-Glucosidase From Milk

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ABSTRACT. *Objective.* Recent reports warn that the worldwide cell culture capacity is insufficient to fulfill the increasing demand for human protein drugs. Production in milk of transgenic animals is an attractive alternative. Kilogram quantities of product per year can be obtained at relatively low costs, even in small animals such as rabbits. We tested the long-term safety and efficacy of recombinant human α-glucosidase (rhAGLU) from rabbit milk for the treatment of the lysosomal storage disorder Pompe disease. The disease occurs with an estimated frequency of 1 in 40 000 and is designated as orphan disease. The classic infantile form leads to death at a median age of 6 to 8 months and is diagnosed by absence of α-glucosidase activity and presence of fully deleterious mutations in the α-glucosidase gene. Cardiac hypertrophy is characteristically present. Loss of muscle strength prevents infants from achieving developmental milestones such as sitting, standing, and walking. Milder forms of the disease are associated with less severe mutations and partial deficiency of α-glucosidase.

Methods. In the beginning of 1999, 4 critically ill patients with infantile Pompe disease (2.5–8 months of age) were enrolled in a single-center open-label study and treated intravenously with rhAGLU in a dose of 15 to 40 mg/kg/week.

Results. Genotypes of patients were consistent with the most severe form of Pompe disease. Additional molecular analysis failed to detect processed forms of α-glucosidase (95, 76, and 70 kDa) in 3 of the 4 patients and revealed only a trace amount of the 95-kDa biosynthetic intermediate form in the fourth (patient 1). With the more sensitive detection method, ^{35}S-methionine incorporation, we could detect low-level synthesis of α-glucosidase in 3 of the 4 patients (patients 1, 2, and 4) with some posttranslation modification from 110 kDa to 95 kDa in 1 of them (patient 1). One patient (patient 3) remained totally deficient with both detection methods (negative for cross-reactive immunologic material [CRIM negative]). The α-glucosidase activity in skeletal muscle and fibroblasts of all 4 patients was below the lower limit of detection (<2% of normal). The rhAGLU was tolerated well by the patients during >3 years of treatment. Anti-rhAGLU immunoglobulin G titers initially increased during the first 20 to 48 weeks of therapy but declined thereafter. There was no consistent difference in antibody formation comparing CRIM-negative with CRIM-positive patients. Muscle α-glucosidase activity increased from <2% to 10% to 20% of normal in all patients during the first 12 weeks of treatment with 15 to 20 mg/kg/week. For optimizing the effect, the dose was increased to 40 mg/kg/week. This resulted, 12 weeks later, in normal α-glucosidase activity levels, which were maintained until the last measurement in week 72. Importantly, all 4 patients, including the patient without any endogenous α-glucosidase (CRIM negative), revealed mature 76- and 70-kDa forms of α-glucosidase on Western blot. Conversion of the 110-kDa precursor from milk to mature 76/70-kDa α-glucosidase provides evidence that the enzyme is targeted to lysosomes, where this proteolytic processing occurs. At baseline, patients had severe glycogen storage in the quadriceps muscle as revealed by strong periodic acid-Schiff–positive staining and lacework patterns in hematoxylin and eosin–stained tissue sections. The muscle pathology correlated at each time point with severity of signs. Periodic acid-Schiff intensity diminished and number of vacuoles increased during the first 12 weeks of treatment. Twelve weeks after dose elevation, we observed signs of muscle regeneration in 3 of the 4 patients. Obvious improvement of muscular architecture was seen only in the patient who

learned to walk. Clinical effects were significant. All patients survived beyond the age of 4 years, whereas untreated patients succumb at a median age of 6 to 8 months. The characteristic cardiac hypertrophy present at start of treatment diminished significantly. The left ventricular mass index decreased from 171 to 599 g/m² (upper limit of normal 86.6 g/m² for infants from 0 to 1 year) to 70 to 160 g/m² during 84 weeks of treatment. In addition, we found a significant change of slope for the diastolic thickness of the left ventricular posterior wall against time at $t = 0$ for each separate patient. Remarkably, the younger patients (patients 1 and 3) showed no significant respiratory problems during the first 2 years of life. One of the younger patients recovered from a life-threatening bronchiolitis at the age of 1 year without sequelae, despite borderline oxygen saturations at inclusion. At the age of 2, however, she became ventilator dependent after surgical removal of an infected Port-A-Cath. She died at the age of 4 years and 3 months suddenly after a short period of intractable fever of >42°C, unstable blood pressure, and coma. The respiratory course of patient 1 remained uneventful. The 2 older patients, who both were hypercapnic (partial pressure of carbon dioxide: 10.6 and 9.8 kPa; normal range: 4.5-6.8 kPa) at start of treatment, became ventilator dependent before the first infusion (patient 2) and after 10 weeks of therapy (patient 4). Patient 4 was gradually weaned from the ventilator after 1 year of high-dose treatment and was eventually completely ventilator-free for 5 days, but this situation could not be maintained. Currently, both patients are completely ventilator dependent. The most remarkable progress in motor function was seen in the younger patients (patients 1 and 3). They achieved motor milestones that are unmet in infantile Pompe disease. Patient 1 learned to crawl (12 months), walk (16 months), squat (18 months), and climb stairs (22 months), and patient 3 learned to sit unsupported. The Alberta Infant Motor Scale score for patients 2, 3, and 4 remained far below p5. Patient 1 followed the p5 of normal.

Conclusion. Our study shows that a safe and effective medicine can be produced in the milk of mammals and encourages additional development of enzyme replacement therapy for the several forms of Pompe disease. Restoration of skeletal muscle function and prevention of pulmonary insufficiency require dosing in the range of 20 to 40 mg/kg/week. The effect depends on residual muscle function at the start of treatment. Early start of treatment is required. *Pediatrics* 2004;113:e448–e457. URL: http://www.pediatrics.org/cgi/content/full/113/5/e448; *transgenesis, enzyme therapy, lysosomal, muscular dystrophy, glycogen storage disease.*

ABBREVIATIONS. rhAGLU, recombinant human α-glucosidase; CHO, Chinese hamster ovary; PAS, periodic acid-Schiff; Ig, immunoglobulin; LVPWd, left ventricular posterior wall diastole; LVMI, left ventricular mass index; AIMS, Alberta Infant Motor Scale; BSIDII, Bayley Scales of Infant Development II; CRIM, cross-reactive immunologic material; MDI, Mental Developmental Index.

Production of therapeutic proteins in milk of transgenic animals is a promising new technology in light of the rapidly expanding market for protein drugs and the ever-increasing costs of health care.[1] As the transgenic protein concentration in milk is very high, kilogram quantities of product per year can be obtained at relatively low costs, even in small animals such as rabbits.[2]

Since the first report in 1988 on α-1-antitrypsin production in sheep milk,[3] a variety of transgenic proteins have been produced. These range from small unstable peptides (salmon calcitonin peptide[4]) to large proteins with complex posttranslational modifications (factor VIII[5,6] and lactoferrin[7]). However, despite the great promises of this novel production platform, there is not yet a single transgenic medicine on the market. Three recombinant proteins from milk are being tested in clinical trials: antithrombin III from goats, α-1-antitrypsin from sheep, and α-glucosidase from rabbits.[8–10]

We have focused on the production of recombinant human α-glucosidase (rhAGLU) for the treatment of Pompe disease, an autosomal recessive muscle disorder. The classic infantile form of the disease leads to death at a median age of 6 to 8 months[11] and is diagnosed by absence of α-glucosidase activity and presence of fully deleterious mutations in the α-glucosidase gene.[12] Cardiac hypertrophy is characteristically present.[11] Loss of muscle strength prevents infants from achieving developmental milestones such as sitting, standing, and walking.[11–13] Milder forms of the disease caused by less severe mutations and partial deficiency of α-glucosidase are also known. Pompe disease is a lysosomal glycogen storage disorder with an estimated frequency of 1:40 000 births[14,15] and is designated an orphan disease.

We have successfully explored the feasibility of enzyme therapy for Pompe disease in preclinical studies.[16–19] The principle of treatment is based on the capacity of lysosomes to engulf exogenous proteins via endocytosis.[20] After cloning the human α-glucosidase gene, we have focused on production of the enzyme in Chinese hamster ovary (CHO) cells and milk of transgenic animals.[18,19,21–25] The high yield of 2 g/L and the short reproduction time of rabbits has led to the pharmaceutical production of rhAGLU in milk of transgenic rabbits. The product's safety and efficacy were tested in phase I and phase II clinical studies.[10] Here we report how 4 patients with infantile Pompe disease responded in a follow-up study to >3 years of intravenous treatment.

METHODS

Enzyme Purification and Characterization

A line of transgenic rabbits producing rhAGLU was obtained.[19] Rabbit milk was collected and stored at −20°C until use. An α-glucosidase containing whey fraction was prepared from skimmed milk by tangential flow filtration using a Biomax 1000 membrane cassette (Millipore, Bedford, MA) and subsequently concentrated by ultrafiltration using a Biomax 30 membrane (Millipore). After a virus inactivation step with Tween-80 in 1% and tri-n-butylphosphate in 0.3% concentration for 6 hours at 25°C, the α-glucosidase was subsequently captured by Q Sepharose Fast Flow chromatography (Amersham Pharmacia Biotech, Uppsala, Sweden). After intermediate purification on a Phenyl Sepharose HP column (Amersham Pharmacia Biotech), α-glucosidase was polished by Source Phenyl 15 chromatography (Amersham Pharmacia Biotech). After a second viral removal step by nanofiltration, purified α-glucosidase was concentrated by ultrafiltration (Biomax 30 membrane) and sterilized by microfiltration (0.2-μm dead-end filter). The enzyme has a specific activity of >250 μmol/mg/hour for 4-methylumbelliferyl-α-D-glucopyranoside and is >95% pure. Toxicity studies were performed in mice, rats, and dogs in doses up to 100 mg/kg. A phase I study was completed

successfully in humans. All information is contained in the Investigator's Brochures.

Biochemical-Genetic Studies

Skin fibroblasts and muscle specimens were homogenized in water, and the $2000 \times g$ supernatants were used to determine α-glucosidase activity, glycogen content, and protein concentration.[19,26] Mutation analysis was performed on genomic DNA and cDNA, as described previously.[27] The functional effects of mutations were studied by assay of α-glucosidase synthesis and catalytic activity in transiently transfected COS cells.[27,28]

Study Design

The study was a single-center, open-label, phase II study approved by the institutional review board. Written informed consent was obtained from the parents of all patients. The objective of the study was to evaluate the safety and efficacy of rhAGLU.

Inclusion Criteria

Patients qualified for inclusion when they had symptoms characteristic of the infantile form of Pompe disease, including a hypertrophic cardiomyopathy. The upper age limit was 10 months. Confirmation of the diagnosis was required by an open biopsy from the quadriceps muscle, revealing a virtual absence of α-glucosidase activity and the presence of lysosomal glycogen storage.

Clinical Studies

RhAGLU was administered intravenously as a 1- to 2-mg/mL solution in saline with 5% glucose and 0.1% human serum albumin, in single starting doses of 20 mg/kg weekly for patients <6.5 kg and 15 mg/kg for patients >6.5 kg. After 14 to 23 weeks of treatment, the dose was increased to 40 mg/kg weekly for all infants. During infusions, heart rate, temperature, transcutaneous oxygen saturation, and blood pressure were recorded continuously.

Before the start of rhAGLU treatment, there was a period of up to 2 weeks in which baseline assessments were performed. Thereafter, patients were assessed at regular intervals. Muscle biopsies were taken from the quadriceps muscle via an open muscle biopsy 1 day after rhAGLU infusion. Tissue specimens for measurement of α-glucosidase were frozen immediately in liquid nitrogen and stored at $-80°C$ until use. For histology purposes, tissue specimens were fixed in 4% glutaraldehyde and embedded in glycolmethacrylate. Tissue sections (4 μm) were stained with periodic acid-Schiff (PAS). Slides that were prepared at different time points were stained in 1 session.

Immunoglobulin E (IgE) and IgG antibody titers were detected using a standard enzyme-linked immunosorbent assay in which the plates were coated with antigen (1 μg/mL) and the samples were diluted 5-fold for IgE and 100-fold for IgG. Samples from healthy volunteers served as negative controls.

Left ventricular dimensions were determined by M-mode echocardiography, in compliance with the guidelines of the American Society of Echocardiography, using a Hewlett Packard Sonos 5500.[29] The diastolic thickness of the left ventricular posterior wall (LVPWd) and the calculated left ventricular mass index (LVMI) were used as measures of hypertrophic cardiomyopathy.[30] Psychomotor development was assessed using the Alberta Infant Motor Scale (AIMS),[31] the Bayley Scales of Infant Development II (BSIDII),[32] and regular standardized neurologic examinations.

RESULTS

RhAGLU From Rabbit Milk

To achieve high-level expression of rhAGLU in the mammary gland, we placed the entire α-glucosidase gene under control of the bovine α_{S1}-casein gene promoter. The gene construct was injected into fertilized rabbit oocytes, and these were implanted in foster mothers. Thus, we obtained a line of transgenic rabbits with high-level expression of rhAGLU during lactation.[18] The production line yields on average of 2 g of crude rhAGLU per liter of milk during

the first 3 weeks of lactation. The molecular mass of α-glucosidase from milk is 110 kDa (Fig 1A), similar to the mass of the acid α-glucosidase precursor produced in genetically engineered CHO cells, and both enzyme species contain uncleaved N- and C-terminal pro-peptides.[18,24,33]

Clinical Condition and Molecular Delineation of Patients

RhAGLU from rabbit milk was tested for its safety and therapeutic efficacy in an open-label study in 4 patients who had the most severe infantile form of Pompe disease and fulfilled the inclusion criteria as described in the Methods section. The clinical status of the 4 patients at baseline is summarized in Table 1. To sustain the diagnosis of classic infantile Pompe disease, the patients were characterized with regard to the degree of acid α-glucosidase deficiency, the pattern of α-glucosidase synthesis, and the type of α-glucosidase gene mutations. The α-glucosidase activity in skeletal muscle and fibroblasts of all 4 patients was below the lower limit of detection (<2% of normal; Table 2). The 95-, 76-, and 70-kDa biosynthetic forms of α-glucosidase were missing in 3 of the 4 patients, when investigated by Western blot analysis (Fig 2A). A trace amount of the naturally occurring 95-kDa biosynthetic intermediate was seen in cultured fibroblasts from the fourth patient (patient 1). When we used a more sensitive detection method, ^{35}S-methionine incorporation, we could detect low-level synthesis of α-glucosidase in 3 of the 4 patients (patients 1, 2, and 4) and some posttranslation modification from 110 kDa to 95 kDa in 1 of them (patient 1; Fig 2B). Patient 3 remained totally deficient with both detection methods (negative for cross-reactive immunologic material [CRIM negative]). As a third mode of molecular delineation, we analyzed the genotype of the patients (Table 2). The CRIM-negative patient (patient 3) turned out to be homozygous for ΔT525, a mutation known to lead to unstable mRNA, frame shift, and absence of CRIM.[34] Both of her parents are carriers of this mutation. Each of the other 3 patients has at least 1 α-glucosidase allele with a mutation permitting low-level synthesis of the
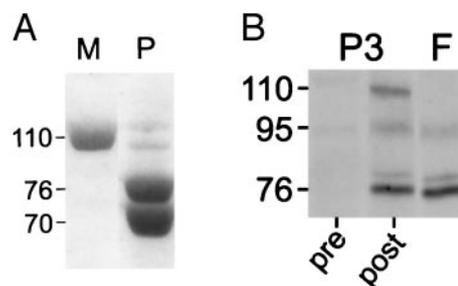

Fig. 1. Characterization of rhAGLU from rabbit milk. A, Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; 8%) analysis under reducing conditions. The gel was loaded with 50 μg of protein per lane and stained with Coomassie brilliant blue. The 110-kDa precursor from rabbit milk (M) is compared with the 76- and 70-kDa mature forms of acid α-glucosidase from human placenta. B, Western blot analysis of α-glucosidase derived from muscle of patient 3 (P3) before ($t = 0$) and after ($t = 3$) treatment. α-Glucosidase from fibroblasts of a healthy individual is used to mark and identify the various molecular forms.

TABLE 1. Clinical Characteristics of Patients at Baseline

Patient	Gender/Age at Start of Treatment	Clinical Appearance
1	M/3 mo	Cardiac hypertrophy, head lag, slipping through, axial hypotonia
2	F/7 mo	Cardiac hypertrophy, heart failure, hypercapnia, oxygen need, head lag, slipping through, axial hypotonia, poor head balance, paresis arms, paralysis legs, nasogastric tube feeding
3	F/2.5 mo	Cardiac hypertrophy, heart failure, tachypnea, borderline oxygen saturations, extreme perspiration, head lag, slipping through, axial hypotonia, poor head balance, nasogastric tube feeding
4	F/8 mo	Cardiac hypertrophy, heart failure, oxygen need, atelectasis left lung, head lag, slipping through, axial hypotonia, poor head balance, paresis arms, paralysis legs, nasogastric tube feeding, growth retardation, intractable fever

TABLE 2. Biochemical-Genetic Delineation of Patients

Patient	α-Glucosidase* Activity	Genotype	Amino Acid Substitution
1	0.6	G1799A/del exon 18	R600H/del55AA
2	0.5	A1115T/delT525	H372L/−†
3	0.1	delT525/delT525	−/−†
4	0.7	G1913T/silent	G638V/−‡
Control	40–160		

* The activities are expressed as nmol 4-methylumbelliferon formed per hour per milligram of cellular protein.
† The delT525 is not expressed at the protein level.
‡ The G1913T mutation was heterozygous in genomic DNA and homozygous in cDNA obtained by reverse transcriptase–polymerase chain reaction, indicating that only the G1913T allele is expressed.
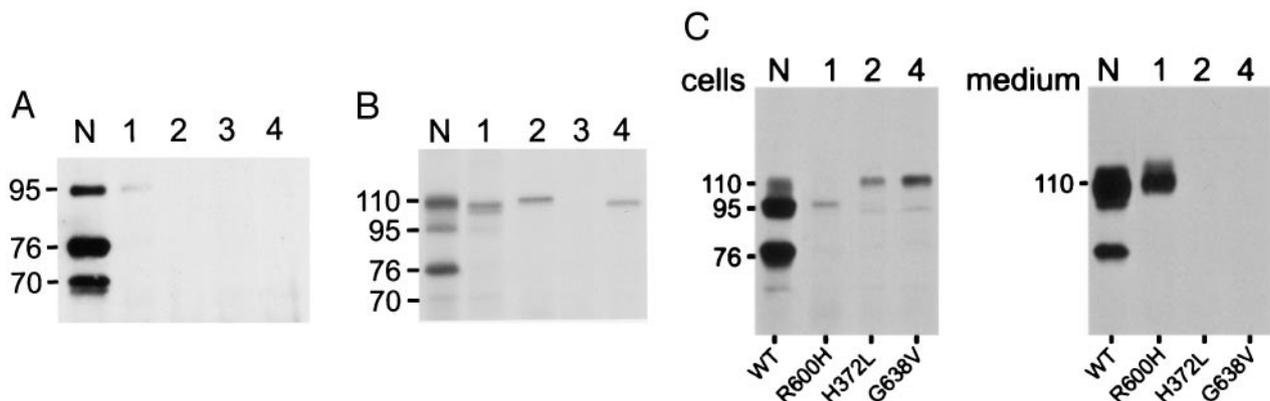

Fig. 2. Molecular forms of α-glucosidase. A, Western blot analysis. α-Glucosidase was immunoprecipitated from fibroblast lysates with rabbit anti-human α-glucosidase antibodies and subjected to SDS-PAGE. α-Glucosidase on the Western blots was visualized with mouse anti-human α-glucosidase antibodies and chemoluminescence. The numbers above the lanes correspond to the patient number. N are cells from a healthy individual. Each lane shows the relative amount and molecular mass of the α-glucosidase species present in an aliquot of lysate containing 1 mg of total cellular protein. B, Synthesis and posttranslational modification of α-glucosidase. Fibroblasts were incubated for 8 hours in medium containing ^{35}S-methionine and then harvested. α-Glucosidase was immunoprecipitated from cell lysates with rabbit anti-human α-glucosidase antibodies and subjected to SDS-PAGE. The autoradiogram shows the processing forms of α-glucosidase. The numbers above the lanes correspond to the patient number. N are cells from a healthy individual. Equal numbers of cells were labeled per lane. C, Functional effects of novel mutations. Mutations were introduced in wild-type cDNA by site-directed mutagenesis and transiently expressed in COS cells. α-Glucosidase was immunoprecipitated from cells and media at 72 hours after transfection and analyzed by Western blotting, as described above. The numbers above the lanes correspond to the patient number. N is the wild-type construct. Equal numbers of cells and equal volumes of media were used per lane.

110-kDa precursor when overexpressed in COS cells (Fig 2C). In 2 cases (patients 2 and 4), the precursor is degraded, but in 1 (patient 1), the Arg600His substitution is compatible with the formation of some 95-kDa intermediate. Importantly, none of the mutant alleles generates catalytically active α-glucosidase, despite overexpression. Thus, the genotype of all patients enrolled in the study is consistent with the classic infantile phenotype of Pompe disease.

RhAGLU Tolerability

RhAGLU was administered intravenously, in an initial dose of 15 to 20 mg/kg/week. The dose was later increased to 40 mg/kg/week. The duration of the infusions ranged from 4 to 8 hours. Infusion-associated reactions were observed in the initial phase of treatment (starting at weeks 5–7) as reported previously. They comprised fever, malaise, erythematous rash, sweating, hypoxia, flushing, and

tachycardia.[10] Corticosteroids and antihistamines were given in this period but did not have a significant effect and were discontinued. The reactions disappeared and did not occur when low infusion rates were applied (2–10 mL/hour) for the first 2 hours. They did not recur when the dose was doubled. In the later phase of treatment, infusion reactions occurred only sporadically in the form of low-grade fever and rash. They were transient and mild. None of the patients receives at present antihistamines or corticosteroids. The infusions are easily manageable on an outpatient basis. One patient receives the infusions at home.

The IgE titer did not rise above background levels during the study. Anti-rhAGLU IgG titers increased to levels between 5 and 13 times baseline values during the first 20 to 48 weeks and declined to 1 and 8 times baseline values during the following infusions. IgG titers against rabbit whey also increased (4–12 times baseline values) during the first 24 weeks and then stabilized between 3 and 9 times baseline values. There was no consistent difference in antibody formation comparing the CRIM-negative with the CRIM-positive patients. Notably, we measured the highest IgG titer in a 34-year-old patient with 10% to 20% of normal CRIM who received the same enzyme preparation.

α-Glucosidase Activity in Tissues

During the first 12 weeks of treatment, muscle α-glucosidase activity increased from <2% to 10% to 20% of normal in all patients (Table 3). To optimize the therapeutic effect, we increased the rhAGLU dose in all infants to 40 mg/kg, and this resulted, 12 weeks later, in normal α-glucosidase activity levels. These were maintained until the last measurement in week 72. Importantly, all 4 patients, including the patient without any endogenous α-glucosidase (CRIM negative), revealed mature 76- and 70-kDa forms of α-glucosidase on Western blot after treatment with the 110-kDa (precursor) rhAGLU (Fig 1). As the final steps of enzyme maturation occur in lysosomes, this observation provides evidence that rhAGLU reaches its target.

Muscle Morphology

At baseline, all patients had severe glycogen storage in the quadriceps muscle as revealed by strong PAS-positive staining and lacework patterns in hematoxylin and eosin–stained tissue sections. The 2 older patients had more severely affected muscle at inclusion than the 2 younger ones. The muscle pathology correlated at each time point with the severity of signs. After 12 weeks of treatment with 15 or 20 mg/kg rhAGLU, the PAS intensity had diminished, whereas the number of vacuoles had increased. Twelve weeks after dose elevation, we observed signs of muscle regeneration in 3 of the 4 patients. An obvious improvement of muscular architecture was seen only in the patient who learned to walk (Fig 3A). A detailed account of the morphologic changes in muscle is given.[35] The glycogen concentration in muscle stabilized in patients 2, 3, and 4 and decreased in patient 1 during the course of treatment (Table 3).

Cardiac Morphology and Function

All 4 patients had the characteristic cardiac hypertrophy at start of treatment, with an LVMI exceeding 170 g/m² (normal value $P_{97.5}$ 86.6 g/m² for infants from 0 to 1 year of age[36]). During 84 weeks of treatment, the LVMI decreased from 171, 203, 308, and 599 g/m² at baseline to 70, 160, 104, and 115 g/m² for patients 1, 2, 3, and 4, respectively. The decrease of the LVMI along time appeared linear ($r = -.86, -.91, -.89,$ and $-.80$ for the 4 respective patients; all $P < .01$). The LVPWd was increased before start of rhAGLU treatment. We found a significant change of slope for LVPWd against time at $t = 0$ for each separate patient ($P < .01$; Fig 3B). The decrease in LVPWd after the start of treatment leveled out in 2 patients (patients 3 and 4) when the P_{95} of normal was approached, as evidenced by a second significant change of slope using the "broken stick" method ($P < .05$). The systolic function normalized and the diastolic function improved in all patients.

Respiratory Condition

During the first 2 years of life, the 2 younger patients (patients 1 and 3) remarkably showed no

TABLE 3. Uptake of α-Glucosidase in Muscle and Correction of Glycogen Storage

Patient	$t = 0$	$t = 1$	$t = 2$	$t = 3$
α-Glucosidase activity*				
1	0.2/0.4	4.9/3.7	27.4/29.3	27.1/25.8
2	0.3/0.3	2.7/3.1	8.0/9.5	28.9/12.1
3	0.2/0.7	2.1/3.8	13.0/7.4	8.2/8.9
4	0.4/0.2	2.7/1.8	16.2/11.3	5.8/4.4
Control	8–40			
Glycogen concentration*				
1	747/1022	755/1351	892/1440	86/503
2	2810/2543	3270/3199	2450/3190	2460/3020
3	1650/1397	2330/1847	2020/2440	2300/2550
4	3690/2720	4630/3700	3060/2720	2130/2310
Control	30–180			

* Multiple measurements of α-glucosidase activity (nmol/mg/hour) and glycogen concentration (μg/mg) in muscle at baseline ($t = 0$) and after 12 weeks of treatment with 15 to 20 mg/kg ($t = 1$), 12 more weeks of treatment with 40 mg/kg ($t = 2$), and 72 more weeks of treatment ($t = 3$). Values at each time point were obtained from 2 different pieces of muscle.
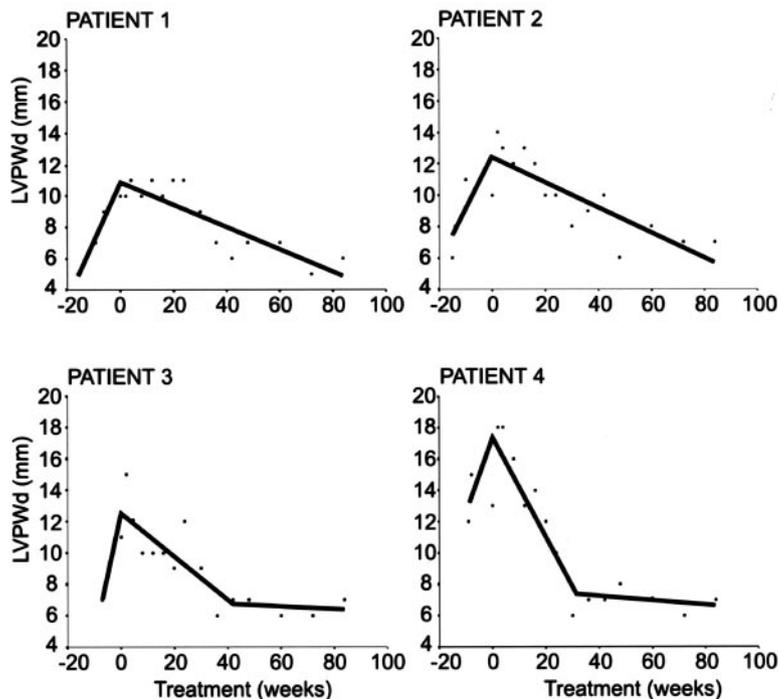
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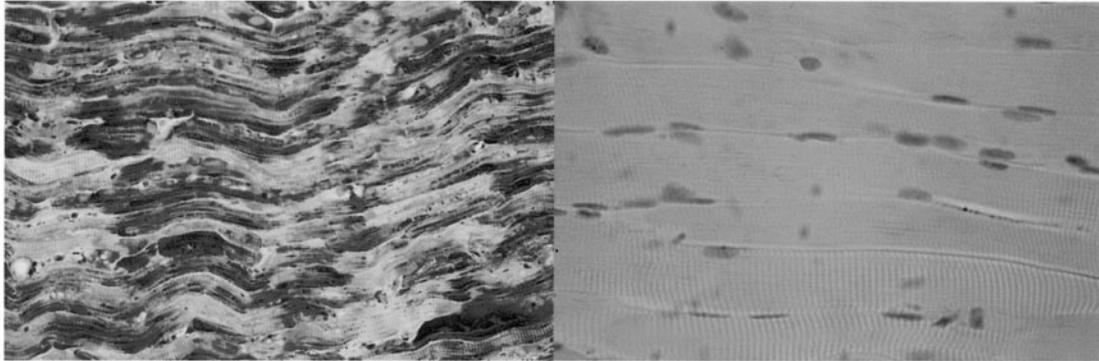
B.

Fig. 3. Correction of muscle pathology and improvement of cardiac and skeletal muscle function. A, Longitudinal sections of a muscle biopsy from patient 1 at baseline (left) and 72 weeks of treatment (right). Sections are stained with PAS to visualize lysosomal glycogen. B, Changes in LVPWd during treatment for patients 1 to 4. Piece-wise linear regression ("broken-stick" method) is used to illustrate the statistically significant change in slope after start of treatment.

significant respiratory problems. Patient 3 recovered from a life-threatening bronchiolitis at the age of 1 year without sequelae, despite borderline oxygen saturation at inclusion. At the age of 2, she became ventilator dependent after surgical removal of an infected Port-A-Cath. The respiratory course of patient 1, now 4 years of age, remained uneventful. Patients 2 and 4, who were older and more severely affected at inclusion, had a marginal respiratory condition from the start of treatment, and both required oxygen. Patient 2 (partial pressure of carbon dioxide: 10.6 kPa equivalent to 80 mm Hg at start; normal range: 4.5–6.8 kPa) became ventilator dependent before the first rhAGLU infusion and remained fully ventilated. Patient 4 (partial pressure of carbon dioxide: 9.8 kPa equivalent to 75 mm Hg at start) became ventilator dependent during a bout of pneumonia after 10 weeks of low-dose treatment. She could

gradually be weaned from the ventilator after 1 year of high-dose treatment and was eventually completely ventilator-free for 5 days, but this situation could not be maintained. At the age of 4, her ventilator needs are 24 hours per day.

Motor Development

The most remarkable progress in motor development was seen in the younger patients (patients 1 and 3; Fig 4). Patient 1 learned to crawl (12 months), walk (16 months), squat (18 months), and climb stairs (22 months). Patient 3 learned to sit unsupported (Fig 4), and her condition further improved until the age of 2, when she became ventilator dependent. At the age of 4 years and 3 months, she died suddenly after a short period of intractable fever of >42°C, unstable blood pressure, and coma. At baseline, the older patients (patients 2 and 4) could hardly lift their arms

Fig. 4. Motor development. A, Patient 1 stands on 1 leg at 4.5 years of age. B, Patient 3 sits without support at the age of 19 months.

while in a supine position, and their legs lay immobile on the surface. During treatment, the muscle function of the arms improved significantly. Patient 2, however, lost her regained muscle strength after a series of airway infections. In her case, restoration of function seems to be a difficult process. Patient 4 maintained the strength in her arms. When she was 24 months of age, she could roll over onto her side and play for longer periods while seated in a wheel chair. Her condition has not improved since then. Over time, there was a mild decline in the active elevation of the upper arms from 90 degrees to 45 degrees. The AIMS score for patients 2, 3, and 4 remained far below the p5. Patient 1 followed the p5

of normal (Fig 5). For patient 1, the psychomotor development on the BSIDII, based on raw score equivalents, progressed from 2 to 37 months' developmental ages at chronological ages of 3 to 37 months. The Psychomotor Developmental Index progressed from 79 to 97. In patients 2, 3, and 4, the Psychomotor Developmental Index was <50, and a motor developmental age could not be calculated.

Mental Development

As rhAGLU is unlikely to pass the blood-brain barrier, we had concerns about long-term mental development and neurologic performance. Neurologic examinations performed from the start of treatment at preset time points showed no signs of central nervous system involvement so far. As an unexpected finding, we measured an elevated response threshold of brainstem auditory evoked potentials in all patients at inclusion. This did not change during treatment.

For patient 1, the mental development on the BSID II, based on raw score equivalents, progressed from 3 to 31 months' developmental ages at chronological ages of 3 to 37 months. The Mental Developmental Index (MDI) was 101 and 79 at 3 months and 37 months, respectively. The BSIDII was inadequate to
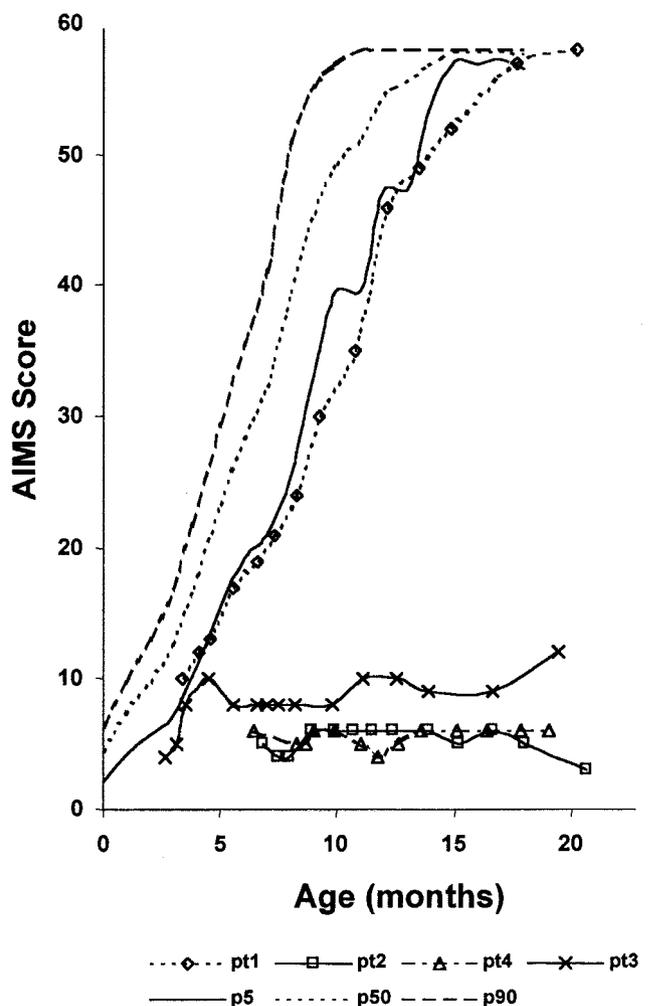

Fig. 5. The AIMS scores of the patients during enzyme replacement therapy. The upper age limit of the test is 18 months.

test the mental development of patient 2 because of her severe hypotonia. For patient 3, the mental development progressed from 2 to 18 months at chronological ages of 3 to 22 months. The MDI was 81 and 72, respectively. The mental development of patient 4 progressed from 5 to 27 months at chronological ages of 8 to 37 months. The MDI of patient 4 was 76 and 67, respectively.

In practice, the BSIDII scores are not a true reflection of mental development. Items were missed through motor handicaps and hearing problems rather than through mental delay. All patients were interested in their environment, interacted with their parents, and were able to attend school.

Survival

Three of the 4 patients are alive. All 4 patients reached the age of 4 years, whereas the life expectancy of untreated patients with the classic infantile form of Pompe disease is typically <1 year.[10–13]

DISCUSSION

Our study demonstrates that a safe and effective medicine for intravenous treatment of human disease can be produced in the milk of transgenic animals, in this case rhAGLU from rabbit milk for patients with Pompe disease. The safety of the product is proved by the fact that >700 infusions were well tolerated by critically ill patients. The efficacy of the therapy is evident from several observations. First, all 4 patients have reached the age of 4 years, and 1 patient remained ventilator-free, whereas infants with classic infantile Pompe disease typically succumb before they are 1 year of age.[10–13] Second, the cardiac hypertrophy diminished and cardiac function improved, whereas cardiac failure is a major cause of death in infantile Pompe disease. Third, patients gained muscle strength, whereas there normally is a progressive loss of muscle function. Two patients achieved developmental milestones that are unmet by untreated patients. Notably, the motor score on the BSIDII of 1 patient normalized at the age of 2 years.

The efficacy of treatment is further supported by biochemical findings. Correction of α-glucosidase deficiency occurred in skeletal muscle of all 4 patients. After 3 months of single weekly doses of 15 to 20 mg/kg, the correction was partial. A 100% correction was achieved after 3 additional months on a weekly dose of 40 mg/kg. Conversion of the 110-kDa precursor from milk to mature 76/70-kDa α-glucosidase provides evidence that the enzyme is targeted to lysosomes, where this proteolytic processing occurs.[37] Moreover, we observed a decrease of the PAS-staining intensity and the appearance of empty vacuoles in the quadriceps muscle of all patients, suggestive of an effect on the lysosomal glycogen pool. In the best performing patient, this resulted in greatly improved muscle morphology and glycogen content.[35]

It is evident that the 4 patients respond differently to the treatment, and we have tried to find an explanation. All patients seemed to have molecular-genetic defects and clinical characteristics consistent with classic infantile Pompe disease.[11–13] Notably, the best performing patient had the most severe hearing deficit (50–80 dB). This makes it unlikely that the difference in response is explained by inclusion of patients with milder nonclassic infantile phenotypes.

We find it equally unlikely that an inhibitory immune response, as described in a study by Amalfitano et al[38] with α-glucosidase from CHO cells, explains the difference in efficacy. That study claimed that neutralizing antibodies caused the loss of treatment benefit at the age of 8 months in 2 CRIM-negative patients. This counter-effect did not occur in our study. On the contrary, our truly CRIM-negative patient was the second best responder. Enzyme therapy studies for Gaucher disease, Fabry disease, and mucopolysaccharidosis type I do not indicate either that a CRIM-negative status per se or an antibody formation interferes with the efficacy of enzyme replacement therapy.[39–43]

In our view, the degree of impairment of skeletal muscle function and the age of the patient at start of treatment play the decisive roles in outcome. The 2 patients, included at 2.5 and 3 months of age, had milder symptoms at inclusion and responded clearly better than the 7- and 8-month-old patients who were in an end stage of the disease at the time of inclusion. The patient who had the best muscle function at start of treatment learned to walk. This may indicate that active muscle movement is required to correct the lysosomal glycogen storage and restore the muscle morphology.[35]

Despite the significant effects of our therapy, we warn that patients with infantile Pompe disease are at risk of developing residual disease with contractures, scoliosis, or respiratory insufficiency if treatment is started too late or with a too low a dose. The therapeutic window is small. We may have lost precious time by treating the patients for 3 to 6 months with a suboptimal dose.

The results of our study are encouraging for the additional exploration of enzyme replacement therapy. This is true for infants but even more so for patients with milder forms of Pompe disease. The therapeutic window is larger as we experienced in our pilot study with 3 patients aged 12 to 33 years. One of them, who was wheelchair-bound for 4 years, started to walk again after 2 years of treatment with rhAGLU, 20 mg/kg/week.[44]

The effective dose of rhAGLU is high compared with the 1-mg/kg dose of glucocerebrosidase used for the treatment of type 1 Gaucher disease and the 0.2- to 1-mg/kg dose of α-galactosidase used for treatment of Fabry disease.[39–41,43,45] These differences are explainable by the different target tissues. In Pompe disease, the target tissue (skeletal muscle) is shielded from the enzyme by the capillary endothelium and the interstitial tissue. In contrast, the targets in Gaucher disease (liver and spleen macrophages) and Fabry disease (endothelial cells) are directly exposed to circulating enzyme. Notably, in Fabry disease, 3 mg/kg α-galactosidase is sufficient to reach the endocardium but insufficient to reach the cardiomyocytes.[40] The higher dose required to

correct enzyme activity in skeletal muscle is supported by studies in animal models and was demonstrated for various enzyme species among which was rhAGLU from both transgenic mammals and genetically engineered CHO cells.[19,33,46,47]

CONCLUSION

The results of our study demonstrate both the safety and the efficacy of enzyme replacement therapy in Pompe disease as well as the feasibility of producing medicines in the milk of transgenic animals. This novel production platform can potentially reduce the costs of therapeutics, but scale-up persists as a major challenge in the rhAGLU production process. The currently used transgenic rabbit production line supplies ~10 g of rhAGLU per animal per year. At a dose of 10 to 40 mg/kg per patient per week, the production of rhAGLU in rabbit milk is feasible in the initial phase of product development but falls short of supplying patients with Pompe disease worldwide. The more conventional production system in genetically modified CHO cells can be used as an alternative but also has a capacity limitation.[1] Thus, timely investments need to be made in the development of alternative production platforms. Our studies show that production in milk of sheep, goat, or cow is the option of choice.

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