Cerebroside-β-Glucosidase Encapsulation in Liposomes for Gaucher's Disease Treatment Revisited

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Received June 29, 1998; accepted December 1, 1998

KEY WORDS: cerebroside-β-glucosidase; Gaucher's disease; liposomes; enzyme replacement therapy.

INTRODUCTION

Gaucher's disease is an autosomal, recessively-inherited disorder of sphingolipid metabolism, characterized by hepatosplenomegaly, anemia, thrombocytopenia, and bone lesions, having various degrees of severity in different patients. Biochemically, this disease is characterized by a deficiency of cerebroside β-glucosidase (β-D-glucosyl-N-acylsphingosine glucohydrolase), which results in the accumulation of the glycolipid glucocerebroside in reticuloendothelial cells, particularly of spleen, bone marrow, and liver (1).

Enzyme replacement has been under consideration as a therapeutic strategy for patients with Gaucher's disease for more than two decades (2).

In 1991 an enzymatic preparation, Ceredase®, (Genzyme Corporation, Cambridge, MA) received approval of the US FDA for use in the treatment of Gaucher's disease, type 1. Ceredase® (alglucerase injection) is a modified form of the enzyme. Alglucerase, which is human placenta-derived, is a monomeric glycoprotein of 497 amino acids, with carbohydrates making up approximately 6% of the molecular mass (Ceredase,® Genzyme, package insert).

The second generation preparation, Cerezyme™, (Genzyme Corporation, Cambridge, MA) was introduced as a β-glucocerebrosidase analog in 1994. This preparation contains imiglucerase, which is produced by recombinant DNA technology. The purified imiglucerase is a monomeric glycoprotein of 497 amino acids, containing four N-linked glycosylation sites. Imiglucerase differs from the placental glucocerebrosidase by one amino acid at position 495, where histidine is substituted for arginine (Cerezyme™, Genzyme, package insert).

The central problem with the current use of β-glucocerebrosidase analogs to treat Gaucher's disease by enzyme replacement therapy is the high cost. The cost of one year of therapy for a single patient is in the range of $100,000–380,000 (4). It should be noted that a large percentage of the enzyme in the i.v. injection is lost in the systemic circulation and only a small percentage is taken up by the reticuloendothelial cells (5). Therefore it is important to look for ways to improve the bioavailability of the enzymatic preparation to the target tissue and also in this way reduce the cost of the treatment.

Another obstacle of the enzyme injection is its immunogenicity (6). During the clinical trials, 16% of patients treated with Cerezyme™, and 40% of patients treated with Ceredase® developed IgG antibodies to the injected enzyme (Ceredase,® Description list). A delivery system that will help to reduce hypersensitivity to the treatment should be of great clinical importance.

Recently liposomes have become increasingly significant as carriers of biologically important molecules in living systems (7,8). The high affinity of most liposomes to reticuloendothelial cells enables their use as carriers of the cerebroside β-glucosidase that is deficient in these cells. The first trial use of liposomes as the β-glucosidase carriers was made by Gregoriadis and coworkers more than 20 years ago (9). The lack of therapeutic activity of the preparation can be attributed to the low quality of liposomes and insufficient enzyme concentration used.

In the present work our aim was to take advantage of the availability of pure and efficacious β-glucosidase and of greatly improved understanding of liposome technology and liposome biofate. Together, these developments make the revisit of the feasibility of using liposome-encapsulated β-D-glucosyl-N-acylsphingosine glucohydrolase analogs to improve enzyme replacement therapy for Gaucher's disease worthwhile.

MATERIALS AND METHODS

Assay of Enzyme Activity

The assay is based on hydrolysis of a fluorogenic substrate, 4-methylumbelliferyl-β-D-glucopyranoside, to release the fluorescent product 4-methylumbelliferone (4-MU), which is determined spectrofluorimetrically (10). All reagents, except the enzyme, were from Sigma Chemical Co. (St. Louis, MO).

Reagents: citrate-phosphate buffer (0.20 M), pH 5.0; glycine-KOH (0.25 M), pH 10.3;
Detergent: sodium taurocholate, 2%w/v. The detergent was added to solubilize the liposomes;
Substrate: 4-methylumbelliferyl-β-glucupyranoside.

Enzyme analogs:

1) Cerezyme™, imiglucerase for injection. Cerezyme™ is supplied as a sterile, non-pyrogenic, white to off-white lyophilized product. The quantitative composition of the lyophilized drug per vial is: imiglucerase 212 international units (total amount), which gives 40 U/ml when diluted with 5.1 ml of sterile water for injection. This enzyme preparation is stable for more than 1 month, at 4°C.

2) Ceredase®, aglucerase injection. Ceredase® is supplied as a clear sterile non-pyrogenic solution of aglucerase in a citrate-buffered solution containing 1% albumin (human, USP). The enzyme is supplied at 400 international units per bottle (80 U/ml).

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ABBREVIATIONS: 4-MU, 4-methylumbelliferone; PC, phosphatidylcholine; MLV, multilamellar vesicles.

0724-8741/99/0300-0466$16.00/0

The two enzyme solutions were stored at 4°C. In each experiment an aliquot of the solution was removed from the bottle under sterile conditions and diluted to the desired concentration. For the assay about 0.02 mU enzyme was used. The fluorescence intensity of the liberated 4-MU was determined spectrofluorimetrically by a Perkin Elmer luminescence spectrometer LS50B (excitation: 366 nm, slit: 2.5 nm; emission: 446 nm, slit: 5 nm). Results are expressed as fluorescence intensity arbitrary units.

Encapsulation of the Enzyme

All liposomes were composed of egg phosphatidylcholine (PC II, Lipoid, KG, Ludwigshafen, Germany).

Among the many methods available for liposome preparation, we selected two which have good potential for high encapsulation of an active enzyme, as well as being good candidates for industrial preparation (8). Both methods are based on the dehydration-rehydration approach. In both, the enzyme was added before lyophilization. We used 1–0.8 U per 0.14 mmoles egg PC. The first method is based on the Kirby and Gregoriadis procedure (11). The second is based on lyophilization from *tert*-butanol (12). Stability of the enzyme in *tert*-butanol was checked first. The lipids were dissolved in *tert*-butanol and the enzyme in buffered solution was added to the same flask. Then the preparation was lyophilized overnight and hydrated at room temperature. The hydration was performed stepwise, first a small amount of double distilled water was added (10–20% of the final volume) followed by 0.9% NaCl, with continuous shaking of the preparation. Large multilamellar vesicles (MLV) were formed by this method. The final liposomal preparation contained 10% lipid w/v. Mean diameter of the receiving vesicles was 1310 nm (S.D. = 760 nm). For size measurements a Coulter submicron particle analyzer (Model N45D, Coulter Electronics, Luton, England) instrument was used.

In order to check encapsulation efficiency, the liposomes were separated from the medium by centrifugation in a Hemle Z 230 MA centrifuge for 10 min (10,000 rpm) and diluted to the desired concentration with sterile saline. Due to major losses (see below) in enzyme activity during preparation, we quantified the adsorption of the enzyme to various surfaces.

Adsorption on the Plasticware and Glassware

In the present study we tested the susceptibility of the β-glucosidase analogs to be adsorbed on glassware, siliconized glassware, and different types of plasticware. The influence of inactive protein (bovine serum albumin) addition on adsorption onto glass was also investigated.

Adsorption to six kinds of plastic surfaces was evaluated:

1) Nunclon, 50-ml cell-counting flasks, made of polystyrene;
2) Sarstedt, 13-ml test tube, made of polypropylene;
3) Falcon, 14-ml test tube made of polystyrene;
4) Nunc Immuno™ Tubes MiniSorb, 4 ml, made of polyethylene;
5) Sigma freezing vials, 2 ml, made of polypropylene;
6) Elcam Plastic, 1.5-ml microtube, made of polypropylene.

The Cerezyme™ (imiglucerase) was diluted 1:100 with isotonic saline. One-milliliter aliquots of this solution were placed in the above plasticware and stored for 24 h at 4°C.

All the adsorption experiments were performed in triplicate.

RESULTS

Adsorption of Enzyme to Glassware

One problem attracted our attention during the work with the β-glucosidase analogs: after dilution (1:80) with saline, the preparations lost 30 to 40% of their activity during the first 24 hours at 4°C, without further loss of enzymatic activity. The nondiluted preparations showed only minimal loss of enzymatic activity during 72 h at 4°C. For liposome preparations we used diluted enzyme preparations, and in this case too we observed the loss of enzymatic activity in the total preparation. One of the reasons responsible for this loss in enzyme activity is enzyme adsorption onto the glassware surface.

This was evaluated using 1:100 dilutions of both enzymatic preparations in the following media:

(1) double distilled water (DDW);
(2) saline (0.15M NaCl);
(3) saline containing bovine serum albumin (1% w/v);

Samples 1–3 were placed in untreated glass tubes.

A fourth sample consisted of (2) placed in test tubes that were pre-treated with Sigmacote.®

The four samples were incubated for 24 hours at 4°C. The results describing the residual enzyme activity are summarized in Table 1.

Table 1 shows that both imiglucerase and alglucerase are adsorbed onto the glassware surface. This adsorption could be eliminated by addition of 1% albumin to the medium. Lower albumin concentrations are less efficient (see below). This suggests that 1% w/v of albumin provides saturation of all the adsorption sites on the glassware. The higher adsorption of imiglucerase to the glassware in the DDW can be attributed to the small difference in molecular structure and to the presence of carbohydrate residues which make it different from the placental aglucerase (Cerezyme™, package insert). Surprisingly only the placental aglucerase but not the recombinant imiglucerase adsorption to the glassware could be prevented by siliconization of the glassware by Sigmacote.®

Table 1. Percent Residual Enzyme Activity in Different Media After Incubation in Glassware for 24 h at 4°C

	Alglucerase % value ± S.D.	Imiglucerase % value ± S.D.
DDW	69.2 ± 7.9	50.3 ± 7.4
Saline	70.1 ± 9.5	60.1 ± 15.9
Albumin 1%	100.4 ± 6.6	100.2 ± 9.9
Sigmacote®	100.2 ± 8.5	59.4 ± 13.8

Table 2. Percent Residual Imiglucerase Activity After Incubation in Different Plasticware for 24 h at 4°C

Plasticware	% of initial activity value ± S.D.
Control (freshly diluted preparation)	100.0 ± 3.8
1) Nunclon, 50 ml, cell counting flask (polystyrene)	4.4 ± 6.2
2) Sarstedt, 13 ml test tube (polypropylene)	66.1 ± 6.0
3) Falcon, 14 ml test tube (polystyrene)	59.7 ± 4.8
4) Nunc, Immuno™ Tubes MiniSorb, 4 ml (polyethylene)	65.3 ± 13.0
5) Sigma freezing vials, 2 ml (polypropylene)	91.9 ± 9.6
6) Elcam Plastic, 1.5 ml microtube (polypropylene)	65.5 ± 4.4

Adsorption to Plasticware

Adsorption of β-glucosidase onto glassware during the encapsulation process is a serious obstacle that increases significantly the cost of the preparation. Therefore the possibility of replacing glassware by plasticware was explored.

The ability of different plasticware to adsorb β-glucosidase analogs was tested using imiglucerase, whose adsorption to glass could not be reduced by using siliconization.

Table 2 shows the residual enzyme activity in different plasticware after incubation for 24 h at 4°C.

Table 2 shows that most types of plasticware used adsorbed 35–40% of imiglucerase, with two exceptions: 1 and 5.

(1) Nunclon flasks made of polystyrene are characterized by very high adsorption—more than 90% of the initial enzyme was adsorbed.

(5) Polypropylene freezing vials are characterized by very low (less than 10%) adsorption, and therefore it is convenient for experiments where low enzyme concentrations are used and the enzyme loss due to adsorption should be minimized.

Enzyme Stability Under Conditions Used for Liposome Preparation

The first step was to prove that the enzyme analogs didn't lose their enzymatic activity during the encapsulation process. Full recovery of activity was observed in the two β-glucosidase analogs after lyophilization and rehydration, when undiluted enzyme preparations were used. Addition of *tert*-butanol before the lyophilization does not alter the enzyme recovery.

Encapsulation of the Enzymes into Liposomes

Good encapsulation efficiency was achieved using both kinds of β-glucocerebrosidase analogs, when the dry lipid cake (after dehydration) was hydrated with saline. All dilutions were performed using glassware. The results of the two encapsulation methods were very similar, therefore only the results of the rehydration-dehydration method are shown in Table 3.

Table 3. Enzyme Distribution Between Liposomes and Extraliposomal Liquid (%)

	Extraliposomal liquid, value ± S.D.	Liposomes value ± S.D.	Total value ± S.D.
Alglucerase	8.2 ± 3.2	88.0 ± 7.0	96.5 ± 10.0
Imiglucerase	10.4 ± 7.0	90.1 ± 8.0	100.4 ± 3.4

Effect of Albumin on Encapsulation Efficiency

The presence of 1% w/v albumin which protects the active enzyme from adsorption onto glassware (Table 1), may interfere (compete) with β-glucosidase encapsulation in liposomes. This possibility was checked using alglucerase (Table 4).

The results described in Table 4 demonstrate that indeed albumin at 1% w/v prevents adsorption to glassware; however, the cost is a major reduction in encapsulation. A lower level of albumin, 0.1%w/v, which has only minimal effect on the encapslulation did not reduce loss of enzyme activity data not shown. Therefore other alternatives to use of albumin were tried for prevention of enzyme loss due to adsorption to surfaces (as described above).

DISCUSSION

Adsorption to Glassware

A major obstacle to encapsulation of β-glucosidase analogs is their adsorption to glassware and/or plasticware.

Both enzyme analogs are extensively adsorbed onto the glassware. In diluted enzyme preparations the adsorption reduces the total enzyme activity by about 30%. Addition of 1% w/v of inactive protein like albumin can decrease this adsorption. The fact that albumin concentration significantly higher than the enzyme concentration is needed for prevention of activity loss suggested to us that the affinity of β-glucosidase analogs to glass is much higher than that of albumin. Siliconization could also be regarded as a possible way to eliminate the enzyme's adsorption. Use of Sigmacote^{R}, as was shown in the case of alglucerase, prevented enzyme loss. But in the case of imiglucerase, sililiconization did not prevent the enzyme adsorption. Therefore the solution is to find suitable materials which do not induce adsorption of the enzyme to their surfaces.

Table 4. Influence of Albumin on Encapsulation Efficiency of Alglucerase (%)

	Liposomes, value ± S.D.	Extraliposomal liquid, value ± S.D.	Total, value ± S.D.	Lost, value ± S.D.
Saline	57.7 ± 6.5	4.7 ± 2.7	69.3 ± 6.3	30.7 ± 2.3
Albumin 0.1%	53.2 ± 1.5	13.2 ± 1.6	71.8 ± 2.1	28.2 ± 8.2
Albumin 1%	15.7 ± 2.0	84.3 ± 7.2	100 ± 6.3	0

The adsorption of proteins to synthetic surfaces has been extensively investigated. The majority of studies concentrated on fibrinogen and albumin adsorption on plasticware (polyethylene) and glassware (13). Whereas polyethylene represented a typical hydrophobic inert surface on which proteins bind mainly by dispersion forces, glassware is capable of forming ionic bonds with proteins due to its polar, hydrophilic nature.

It was shown that adsorption patterns of siliconized glass were similar to those of polyethylene for albumin, fibrinogen, and IgG (13).

Different patterns of adsorption have been shown for different proteins (13). Fibrinogen showed transient adsorption onto all studied artificial surfaces when the experiment was carried out at plasma concentration higher than 0.25% (14). This fact was attributed to the presence of high molecular weight kininogen (HMWK) which rapidly replaces fibrinogen from its adsorption sites (15). Albumin, however did not show transient adsorption, which suggests a different kind of surface interaction (14).

Brush at al. (14) showed that in the binary system (albumin and fibrinogen), the surface concentration of each species is considerably less than in the single protein system. And, what is more surprising, the total molar surface concentration is also less than for albumin or fibrinogen alone. These data could be applied to our study where we tried to reduce the enzyme adsorption using bovine serum albumin.

Adsorption to the Plasticware

Use of plasticware instead of glassware in order to decrease the enzyme adsorption was tested. Our studies proved that the majority of the examined plasticware possessed adsorption similar to glass (30–40%); polystyrene, however, is worse, giving the highest adsorption (90%).

The only type of plasticware which gave a very low level of adsorption is Sigma's freezing vials, 2-ml, of polypropylene. This gives hope that suitable plasticware will be found.

Albumin as Protection Against Enzyme Adsorption During the Encapsulation Process

Albumin in concentration 1% w/v eliminates the enzyme adsorption onto glassware. A lower concentration of albumin (0.1%, w/v) did not eliminate the enzyme adsorption. However, at 1% albumin, the enzyme encapsulation is very poor. Therefore it is clear that albumin could not serve as a protectant against enzyme adsorption during the process of encapsulation in liposomes. Other methods, such as siliconization of glassware or using test tubes with low adsorption capacity, should be used.

Selection of Lipsosome Lipid Composition

We selected egg PC as the sole lipid component of the liposomes because this lipid was used extensively in high levels in humans as part of nutritional emulsions. Also the liposomes are aimed to quickly reach the liver macrophages, where they have to be processed in order to release the enzyme. Including cholesterol or "solid" lipid in the liposomes may slow down this process. Also it is expected that release of large molecules such as this enzyme in the short resident time in plasma will be slow enough to deliver enough enzyme to the liver.

Feasibility of Encapsulation

Our results indicate that when enzyme adsorption to glassware or plasticware is minimized there is no loss of activity during the encapsulation process.

The data reported here demonstrate good encapsulation potential of β-glucocerebrosidase analogs, the placental alglucerase as well as recombinant imiglucerase. This is a good starting point to begin animal efficacy and toxicity studies.

One major point of concern is the adjuvant effect of the liposomes which may enhance immune responses against the enzyme. As liver macrophages are not professional antigen presenting cells the delivery to these cells may actually reduce the immune response when compared with the free enzyme.

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