Incorporation of glucocerebrosidase into Gaucher’s disease monocytes in vitro
(enzyme replacement therapy/β-glucosidase/erythrocyte entrapment of enzyme)

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ABSTRACT Several carriers were evaluated for use in the delivery of exogenous glucocerebrosidase to monocytes from Gaucher’s disease patients. Only gamma globulin-coated, resealed erythrocytes proved to be an effective vehicle for enzyme delivery. Glucocerebrosidase added in this manner normalized intracellular enzyme levels for at least 18 hr. In this model system for the study of enzyme replacement therapy, soluble enzyme, enzyme in uncoated resealed erythrocytes, and enzyme incorporated into liposomes were ineffective.

Gaucher’s disease is a glycolipid storage disorder characterized by the accumulation of glucocerebroside in tissue macrophages of the spleen, liver, and bone marrow (1). The molecular basis of this disease is an inherited deficiency of the enzyme glucocerebrosidase (2). In 1964, de Duve (3) postulated that lysosomal disorders such as Gaucher’s disease could be treated by administering exogenous enzyme to catabolize storage deposits. The success of such enzyme replacement therapy for Gaucher’s disease will depend upon the availability of partially purified glucocerebrosidase and its efficient and effective delivery to the appropriate cell (4, 5).

Several clinical trials of enzyme replacement have already been initiated. Purified placental enzyme has been infused, directly intravenously in solution (6), precipitated on albumin (7), encapsulated in erythrocytes (8), or incorporated into liposomes (8). Although some favorable biochemical and clinical changes have been reported, the results of treatment have been far from satisfactory. In order to administer enzyme in an optimal fashion, it is desirable to be able to study its uptake by the target cell, its persistence in these cells after uptake, and the effect of the enzyme on the stored glucocerebroside. An appropriate model for the study of these factors is the blood monocyte, a cell that serves as precursor of tissue macrophages and shares several cell surface markers with Gaucher’s disease cells (9–11). We have now devised a model system in which we study the uptake and persistence of purified human glucocerebrosidase delivered in several different vehicles to Gaucher’s disease monocytes.

MATERIALS AND METHODS

Monocyte Isolation. Approximately 100 ml of blood was drawn from patients with Gaucher’s disease; 1 ml of neutralized 0.27 M EDTA served as anticoagulant. The blood was divided into four 25-ml portions, each of which was pipetted over 12 ml of Ficoll/dextran (12) and centrifuged at 400 × g for 25 min at 4°C. The monocyte/lymphocyte/platelet layer at the interface was washed twice in 0.154 M sodium chloride; the first time the suspension was centrifuged at 1000 × g for 10 min and the second time, at 800 × g for 5 min to remove most of the platelets. The cells were resuspended in Eagle’s minimal essential medium (13) containing 20% fetal calf serum, 100 units of penicillin per ml, and 100 µg of streptomycin per ml. Three-milliliter portions of cell suspension, containing 1.2–1.5 × 10^6 cells were distributed into 35-mm-diameter plastic petri dishes. The dishes were allowed to stand at 37°C for 2 hr under 1% CO₂ in air to maintain the pH at 7.4 and to allow the monocytes to attach to the plastic dish. The unattached cells were then gently rinsed from the attached monocytes with 0.154 M sodium chloride at 37°C. Three milliliters of fresh suspending medium was added to each dish.

The monocytes were harvested by adding 400 µl of 2.7 mM EDTA in 0.154 M sodium chloride and gently scraping the dishes. The cells were disrupted by freeze-thawing three times and briefly sonicated to disperse cell fragment aggregates.

Glucocerebrosidase. The enzyme was purified as described (14) with the following modifications: a 50–64% ethanol fractionation was substituted for the previous 0–90% ethanol step, and hydrophobic chromatography was performed on an oleoyl-agarose rather than a phosphatidylinerine-agarose column.

Activity was measured as β-glucosidase activity with the artificial substrate 4-methylumbelliferyl-β-D-glucopyranoside and oleic acid as a cofactor. Because we have previously demonstrated that the purified enzyme preparation has both glucocerebrosidase and β-glucosidase activity, only activity against the artificial substrate was measured. The complete assay system contained 400 µl of 2 mM 4-methylumbelliferyl-β-D-glucopyranoside, 0.2 mM oleic acid, 0.25% taurocholate, 0.1 M citric acid/Na₂HPO₄ (pH 5.0), and 50 µl of enzyme solution. One unit of enzyme activity forms 1 µmol of product per minute at 37°C.

Enzyme Carriers. Type O Rh-positive erythrocytes from a normal donor were loaded with glucocerebrosidase at a hematocrit value of 50% as described (15). An average incorporation of approximately 20% of the enzyme was achieved. A portion of the resealed erythrocytes were coated with human IgG by adding 20 µl of RhoGam per milliliter of packed cells, incubating at room temperature for 15 min, and then washing three times. The IgG-coated erythrocytes were strongly agglutinated by anti-human serum (rabbit) whereas the untreated, resealed cells were not.

Glucocerebrosidase was entrapped into liposomes by the method of Gregoriadis (16); the incorporation was 8%.

Taurocholic acid (catalog number 580217), used in the enzyme assay, was obtained from Calbiochem. Sigma supplied 4-methylumbelliferyl-β-D-glucopyranoside, phosphatidylcholine, phosphatidic acid, cholesterol, and Ficoll. Eagle’s minimal essential medium was from Flow Laboratories (Rockville, MD), and Gibco supplied the 20% fetal calf serum. RhoGam (human anti-Rh globulin) was obtained from Ortho Diagnostics (Raritan, NJ). MCB Chemical (Norwood, OH) supplied the oleic acid.

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RESULTS AND DISCUSSION

Purified human glucocerebrosidase was added to Gaucher's disease monocyte layers in the following forms: (i) soluble enzyme; (ii) encapsulated in type O Rh-positive human erythrocytes; (iii) encapsulated in type O Rh-positive human erythrocytes coated with IgG; (iv) incorporated into liposomes. At various time periods after the addition of enzyme, the plates were washed free of uningested carrier and the monocytes were harvested for assay of glucocerebrosidase. The uptake of enzyme into Gaucher's disease monocytes from these four sources of enzyme is shown in Fig. 1. Only minimal amounts of soluble enzyme, enzyme in liposomes, or enzyme in uncoated erythrocyte were incorporated into the monocytes; however, excellent uptake of glucocerebrosidase was observed when IgG-coated erythrocytes served as the carrier. In fact, under the conditions of these experiments, the uptake of enzyme in IgG-coated, resealed erythrocytes was sufficient to raise the specific activity of glucocerebrosidase in the Gaucher's disease monocytes to within the normal range (168 ± 22 μunits/mg of protein; see legend Fig. 2).

To determine how long ingested enzyme persisted in Gaucher's disease monocytes, plates with adherent monocytes were allowed to phagocytose glucocerebrosidase-loaded IgG-coated erythrocytes for 2 hr. The uningested erythrocytes were then washed from the dishes, fresh medium was added, and plates were sacrificed at subsequent intervals and assayed for glucocerebrosidase activity. The results of three separate experiments are shown in Fig. 2. At the time the uningested erythrocytes were removed, the monocyte preparations had incorporated considerable quantities of glucocerebrosidase. However, the fate of the ingested enzyme during the next 6 hr varied: in experiment 1 there was a rapid decrease in glucocerebrosidase level; in experiment 2 there was a more modest decrease; and in experiment 3 there was no change. Nonetheless, the behavior of the ingested enzyme for the next 10 hr was similar in all three experiments with only a gradual loss of activity, the total activity remaining within the range for normal monocytes. Even though the enzyme levels in the early phases of the experiment varied dramatically, at the end of these incubations all three monocyte preparations that had ingested enzyme-loaded erythrocytes had similar levels of glucocerebrosidase. After the early fluctuations in activity, the intracellular half-life of glucocerebrosidase was at least 24 hr in all three experiments.

Although there is no way of refuting rigidly the possibility that the association of enzyme with monocyte represented mere adherence of erythrocytes to the membrane, the monocytes rather than true ingestion, the erythrocytes did appear to be inside the phagocytic cells on microscopic examination. Moreover, the red color that the monocyte layer assumed initially after being fed IgG-coated resealed erythrocytes faded during incubation, suggesting that the hemoglobin was being catabolized within the monocytes at a time when the enzyme activity was at high levels.

Several studies have shown that resealed erythrocytes and liposomes are phagocytosed by macrophages in vitro (17, 18) and by the reticuloendothelial system in vivo (5, 19). However, the results of this report indicate that IgG-coated, resealed erythrocytes are phagocytosed more avidly by monocytes in vitro than are either uncoated erythrocytes or liposomes. They therefore appear to be a superior delivery system for glucocerebrosidase in the treatment of Gaucher's disease. Indeed, studies in Gaucher's disease patients have shown that IgG-coated, resealed erythrocytes are cleared more quickly from the circulation than are uncoated, resealed erythrocytes (20).

The uptake of free glucocerebrosidase after intravenous injection has also been studied in humans (6) and in rats (21); the latter study involved both carbohydrate-modified glucocerebrosidase and native enzyme. Enzyme was predominantly localized in the liver, with hepatocytes sequestering the majority of the activity (21). However, the Kupffer cell, rather than the hepatocyte, is the target cell in the treatment of Gaucher's disease (1). The results obtained in our in vitro model system support these findings in that little free glucocerebrosidase entered monocytes, cells that closely resemble the phagocytic Kupffer cell.

A second key finding is that exogenous glucocerebrosidase persisted in Gaucher's disease monocytes for a relatively long

Fig. 1. Comparison of carriers for delivery of glucocerebrosidase to Gaucher's disease monocytes. (A) Concentrations of glucocerebrosidase in the medium were 310 μunits/ml for IgG-coated erythrocytes (△), 410 μunits/ml for uncoated erythrocytes (○), and 0 for the control (●). The ratio of erythrocytes to monocytes was 10:1. (B) Concentrations of glucocerebrosidase in the medium were 230 μunits/ml for IgG-coated erythrocytes (△), 228 μunits/ml for liposomes (○), and 0 for the control (sham-loaded) erythrocytes (●). The ratio of erythrocytes to monocytes was 10:1; the liposome-to-monocyte ratio was approximately 13:1.
FIG. 2. Intracellular survival of added glucocerebrosidase. Gaucher’s disease monocytes were exposed to IgG-coated erythrocytes with entrapped glucocerebrosidase for 2 hr. Fresh medium was then added and the decay of added glucocerebrosidase was monitored. The glucocerebrosidase activity in the medium for the first 2 hr was 813 \( \text{\mu} \text{units/ml} \) in experiment 1 (O, ●), 574 \( \text{\mu} \text{units/ml} \) in experiment 2 (△), and 388 \( \text{\mu} \text{units/ml} \) in experiment 3 (□, ▼). Open symbols represent the control for each experiment. In all three experiments the erythrocyte-to-monocyte ratio was 10:1. The hatched area represents the monocyte glucocerebrosidase levels from five normal individuals, 168 ± 44 \( \text{\mu} \text{units/mg of protein} \) (mean ± 2 SD).

By the end of the experiment, the intracellular enzyme levels were approximately 400 units/mg of protein. This shows that glucocerebrosidase is essential for the removal of glucocerebroside deposits in Gaucher’s disease cells.

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