Mannose 6-Phosphate Receptor-mediated Uptake Is Defective in Acid Sphingomyelinase-deficient Macrophages

Rajwinder Dhami and Edward H. Schuchman

Progressive accumulation of lipid-laden macrophages is a hallmark of the acid sphingomyelinase (ASM)-deficient forms of Niemann-Pick disease (i.e. Types A and B NPD). To investigate the mechanisms underlying enzyme replacement therapy for this disorder, we studied the uptake of recombinant, human ASM (rhASM) by alveolar macrophages from ASM knock-out (ASMKO) mice. The recombinant enzyme used for these studies was produced in Chinese hamster ovary cells and contained complex type, N-linked oligosaccharides. Binding of radiolabeled, rhASM to the ASMKO macrophages was enhanced as compared with normal macrophages, consistent with their larger size and increased surface area. However, internalization of the enzyme by the ASMKO cells was markedly reduced when compared with normal cells. Studies using receptor-specific ligands to inhibit enzyme uptake revealed that in normal cells rhASM was taken up by a combination of mannose and mannose 6-phosphate receptors (MR and M6PR, respectively), whereas in the ASMKO cells the M6PR had a minimal role in rhASM uptake. Expression of M6PR mRNA was normal in the ASMKO cells, although Western blotting revealed more receptors in these cells when compared with normal. We therefore hypothesized that lipid accumulation in ASMKO macrophages led to abnormalities in M6PR trafficking and/or degradation, resulting in reduced enzyme uptake. Consistent with this hypothesis, we also found that, when rhASM was modified to expose terminal mannose residues and target mannose receptors, the uptake of this modified enzyme form by ASMKO cells was 10-fold greater when compared with the “complex” type rhASM. These findings have important implications for NPD enzyme replacement therapy, particularly in the lung.

Types A and B Niemann-Pick disease (NPD)\(^1\) are lysosomal storage disorders resulting from an inherited deficiency of acid sphingomyelinase (ASM) activity (EC 3.1.4.12) (1). ASM is responsible for hydrolyzing the lipid, sphingomyelin, to ceramide and phosphocholine, and both forms of the disease are characterized by extensive lipid storage in various cells and tissues. Patients with Type A NPD follow a rapid, neurodegenerative course that leads to death by about 3 years of age, whereas Type B NPD patients have a less severe form of the disease with little or no neurological involvement. This latter form is characterized mainly by visceral organ complications, including hepatosplenomegaly and progressive pulmonary compromise (1, 2). Intermediate forms also have been described (e.g. Ref. 3), revealing that ASM deficiency can lead to a wide spectrum of disease.

Enzyme replacement therapy was first suggested as an approach for the treatment of lysosomal storage disorders over 30 years ago (4), although difficulties in purifying the human enzymes limited its early evaluation (5–7). However, during the past decade the isolation of genes encoding most lysosomal enzymes and the development of expression systems that produced large quantities of recombinant proteins led to the successful treatment of several lysosomal storage diseases in animal models, providing “proof of principle” for this approach (e.g. Refs. 8 and 9). In addition, enzyme therapy has become available for three human lysosomal storage diseases, Type 1 Gaucher disease, Fabry disease, and mucopolysaccharidosis type I (10–12), and clinical trials are underway for several other disorders (e.g. Refs. 13 and 14).

Most lysosomal enzymes are glycoproteins containing N-linked oligosaccharides. The principle underlying enzyme replacement therapy for lysosomal storage disorders is that intravenous infusion of these enzymes will lead to cell-type specific uptake via receptors that recognize these carbohydrate structures. In particular, mannose 6-phosphate (M6P) and mannose residues present on these sugar chains are instrumental in determining selectivity and efficiency of uptake by specific cell types (15, 16).

Macrophages are important therapeutic targets for many lysosomal storage diseases. These phagocytic cells have a complicated endocytosis mechanism involving several different receptors for internalization of specific lysosomal enzymes (17–19). Among these receptors, the mannose and the M6P receptors are the best characterized, and their role in the uptake of lysosomal enzymes has been extensively investigated. A 175-kDa mannose receptor (MR) and the 275-kDa, cation-independent M6P receptor (also known as the M6P/IGF-II receptor, herein designated M6PR) are present on the cell surface and recycle constitutively from intracellular compartments (20, 21). The MR is a pattern recognition receptor for various microorganisms and has been shown to play an important role in host defense and scavenger function (22), whereas the primary function of the M6PR appears to be the recapture of secreted lysosomal hydrolases and transport to the lysosomal compartment.
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A 46-kDa, cation-dependent M6P receptor also has been identified in macrophages, but this receptor has not been shown to be present on the cell surface and has no endocytic function.

A hallmark of NPD is the accumulation of large numbers of lipid-filled macrophages (“foam cells”) in various tissues, including the lung (23). Previously, we treated a mouse model of NPD (i.e., acid sphingomyelinase knock-out mice; ASMKO) by enzyme replacement therapy using recombinant human ASM (rhASM) containing “complex type,” N-linked oligosaccharides (24, 25). This treatment could prevent the accumulation of lipid-laden macrophages in visceral organs when the enzyme injections were begun early in life and also led to clearance of these macrophages from the tissues of older, affected mice.

The goal of the current study was to investigate the mechanism(s) governing ASM uptake by NPD macrophages, with the hope that these mechanistic insights would lead to a better understanding of these successful preclinical studies, as well as improved and/or alternative therapeutic strategies. Our focus was on alveolar macrophages, because these are important therapeutic targets in NPD patients. In this study we report an unexpected and novel abnormality in macrophages from the ASMKO mice; i.e. reduced enzyme uptake via M6PRs. The implications of these findings for NPD enzyme replacement therapy are discussed within.

EXPERIMENTAL PROCEDURES

Materials—RPMI 1640 medium and phosphate-buffered saline (PBS) were obtained from Cellgro (Herndon, VA). Mannose 6-phosphate and yeast mannans were purchased from Sigma Aldrich (St. Louis, MO). Purified recombinant human ASM (rhASM) and a modified form of rhASM with exposed mannose residues (modified rhASM) were provided by the Genzyme Corp. (Cambridge, MA). Mannosylated bovine serum albumin (mannoseBSA) was isolated from EY Labs (San Mateo, CA). Recombinant human IGF-II and IGF-I were purchased from Cell Sciences (Norwood, MA). Polyclonal, anti-rat M6P/IGF-II receptor antibody (29), followed by a 60-min incubation at room temperature, was used to detect the mannose or M6P receptor on ASM uptake. For the inhibitory studies, 2 mg/ml yeast mannans or 10 μg M6P were added to the macrophage culture media at the same time as rhASM or modified rhASM. After 24 h, cells were washed twice with PBS, and the ASM activities were determined in the cell lysates as described above. Receptor binding studies were analyzed using the Scion Image Beta (version 4.0) as described above for immunoblot analysis (3). Northern blots were quantified using Scion Image Version 4.0 as described above for immunoblot analysis (30). The sequence of the antisense (5′-CTTACGACATGTGAGGTCG-3′, and antisense, 5′-GACCA- TTACAGAGTCGG-3′). Northern blots were quantified using Scion Image Version 4.0 as described above for immunoblot analysis (n = 3).

Binding and Uptake of 125I-Labeled Ligands—For the binding and uptake studies using non-radio-labeled enzyme, 1 μg of rhASM (specific activity = 5.4 × 10^4 pmol/mg) or modified rhASM (specific activity = 5.2 × 10^4 pmol/mg) was added to the culture medium, and macrophages were incubated at 37°C. After 3 or 24 h, the medium was removed, and cells were washed twice with PBS and subjected to three freeze-thaw cycles in 200 μl of 0.1 M Tris-HCl buffer, pH 7.0, containing 0.25% Triton X-100. Protein concentrations were measured using the Bio-Rad protein assay method, and ASM activities were determined as described above.

Acid Sphingomyelinase Knock-out Mice—The ASMKO mouse colony was established from heterozygous (+/−) breeding pairs (26). Mice were housed in a pathogen-free facility according to IACUC guidelines, and routine histological evaluation of mouse lungs showed no evidence of infection. Normal (+/+) mice were littermates of homozygous (−/−) ASMKO mice. Genotypes of the mice were identified as previously described (26).

Bronchoalveolar Lavage and Macrophage Cell Culture—Alveolar macrophages were obtained from 16- to 24-week-old ASMKO mice and age-matched normal littermates by bronchoalveolar lavage. Mice were sacrificed by halothane overdose, and the lungs were removed from the chest cavity en bloc, with the trachea intact. An 18-gauge catheter was inserted and tied into the trachea, and the lungs were washed 8–10 times by gentle massaging with 0.8 ml of cold normal PBS to obtain the maximum number of cells from the lungs. The lavage fluid was collected in plastic tubes and centrifuged at 800 × g for 10 min. The cell pellet was resuspended in 1 ml of cold PBS and used for total cell counts with a Neubauer hemacytometer.

Cell culture, alveolar cells obtained by bronchoalveolar lavage were washed once with sterile PBS, and 5 × 10⁶ cells were then added to individual wells of 24-well tissue culture plates and grown in 1 ml of RPMI 1640 medium containing 2% fetal calf serum and 1% penicillin/streptomycin at 37°C in 5% CO₂. Non-adherent cells were removed after 3 h by washing twice with warm PBS. Fresh medium was then added, and the adherent cells (macrophages) were grown overnight prior to the enzyme binding/uptake experiments.

For uptake studies using non-labeled rhASM, 2 mg/ml yeast mannans or 10 μg M6P were added to the macrophage culture media at the same time as rhASM or modified rhASM. After 24 h, cells were washed twice with PBS, and the ASM activities were determined in the cell lysates as described above. Receptor binding assays, cells were incubated for 4 h with radiolabeled ligand at 4°C, washed 3 × with PBS, and solubilized in 0.1% Triton X-100. Counts were determined with an ISODATA gamma counter (Polymedco, Inc.).

ASM Activity Assays—A fluorescence-based, high performance liquid chromatographic method was used to measure ASM activity in macrophage lysates (27). Briefly, equal volumes of cell lysates (prepared by 3 cycles of freeze-thaw in 0.1 M Tris-HCl buffer, pH 7.0, containing 0.25% Triton X-100) and 0.2 μM BODIPY-labeled C₂₃-sphingomyelin (Molecular Probes, Eugene, OR) diluted in assay buffer (0.1 μl sodium phosphate, pH 5.0, containing 0.1 mA, 0.5% Triton X-100), were incubated at 37°C for 60 min. The reaction was stopped by the addition of ethanol, and the hydrolytic product (BODIPY® C₂₃-ceramide), was detected and quantified by chromatographic analysis using a reverse phase column (Aquasil C-18, Keystone Scientific Inc., St. Marys, PA).

Inhibition Studies Using Receptor-specific Ligands—For the inhibitor studies, 2 mg/ml yeast mannans or 10 μg M6P were added to the macrophage culture media at the same time as rhASM or modified rhASM. After 24 h, cells were washed twice with PBS, and the ASM activities were determined in the cell lysates as described above. Results were compared between experiments with or without mannans or M6P in the culture media to determine the inhibitory effect of each compound on ASM uptake.

Immunoblotting Experiments—To detect the mannose or M6P receptors by Western blotting, 15 μg of total protein from normal or ASMKO cell lysates (obtained by mixing cells with 0.25% Triton X-100 in PBS followed by three cycles of freezing and thawing) was added to an equal volume of native loading buffer (without boiling), and electrophoresed on a 4–20% pre-cast Tris-glycine gel (Invitrogen Technologies, Carlsbad, CA) using standard SDS running buffer (National Diagnostics, Atlanta, GA). Transfer to a Hybond-ECL nylon membrane (Amersham Biosciences, Piscataway, NJ) was carried out at 4°C for 100 min at 40 V with transfer buffer containing 0.1% SDS. Proteins were detected by overnight incubation at 4°C with a 1:400 dilution of a polyclonal antibody against the rat M6PR (28) or a 1:2000 dilution of a polyclonal anti-MR antibody (29), followed by a 60-min incubation at room temperature with a horseradish peroxidase-conjugated anti-rabbit secondary antibody (Amersham Biosciences) at a dilution of 1:2000. Blocking was done with 0.05% Tween 20 and 5% skim milk powder in PBS. 0.1% BSA was added during the primary antibody incubation. Autoradiograms were scanned with Scion Image Beta (version 4.0), an image processing and analysis program. The density of bands in lanes loaded with equal concentrations of protein (as determined by colloidal blue staining) was calculated as the area under the individual peaks of a plotted histogram. Ratios of the values for ASMKO to normal bands on the same gel were calculated.

RNA Expression Analysis—Total RNA was isolated from alveolar macrophages using the RNeasy Mini kit from Qiagen (Valencia, CA) and separated on a denaturing gel. RNA was transferred to a nylon membrane (Bio-Rad Zeta-Blotting membrane) using the standard capillary transfer method, prehybridized for 1 h with Quikhyb solution (Stratagene, Cedar Creek, TX), and hybridized overnight at 68°C. The 14.5 kb MR transcript was identified by hybridization to a 32P-labeled 650-bp probe amplified from murine DNA using primers described in Lane et al. (30). The ~9-kb M6PR transcript was identified using a 686-bp probe amplified from the murine DNA using primers derived from the murine CI-M6PR/IGFII receptor mRNA sequence as follows: sense, 5′-CATTACACATGTGAGGTCG-3′, and antisense, 5′-GACCA-TTACAGTGTCGG-3′. Northern blots were quantified using Scion Image Version 4.0 as described above for immunoblot analysis (n = 3).
RESULTS

Binding and Uptake of rhASM by Alveolar Macrophages—Concentration-dependent binding (4 °C) and uptake (37 °C) of 
125I-labeled rhASM were studied using normal or ASMKO alveolar macrophages (Fig. 1). Although binding of [125I]rhASM 
was increased in ASMKO macrophages compared with normal macrophages (Fig. 1A), consistent with their larger size and 
increased surface area (23), uptake of the enzyme by the ASMKO cells was significantly decreased, particularly when 
higher concentrations were used (Fig. 1B). To confirm these observations, uptake of rhASM also was measured by activity assays 
using 1 μg of unlabeled rhASM added to macrophage cultures for 3 or 24 h (C) and for 24 h with the addition of receptor-specific ligands (5 mg/ml mannan or 10 mM M6P) (D). The percent inhibition was calculated from the ratio of values obtained with and without inhibitors. The graphs show the mean results of three individual experiments and the S.D. values. (*, significant difference between ASMKO and normal macrophages, p = 0.005.)

rhASM alone, or in the presence of excess mannan or M6P (Fig. 1D). Mannan and M6P can specifically bind to MRs and 
M6PRs, respectively, and act as competitive inhibitors of receptor-mediated uptake (31). ASM activity in the cells was 
measured after 24 h. As can be seen in Fig. 1D, addition of mannan almost completely inhibited rhASM uptake by normal 
macrophages, whereas addition of M6P resulted in ~60% inhibition. This indicated that rhASM was taken up by a combina-
tion of both receptors in normal cells, consistent with the fact that rhASM contains five N-linked oligosaccharides, some of 
which are of the “complex” type (25, 32). In contrast, in ASMKO macrophage cultures mannan inhibited uptake of rhASM by 
~60%, but M6P did not affect rhASM uptake at all. These results suggested that M6PRs were either not present on the 
ASMKO cell surface or were not functional. In addition, there was reduced utilization of MRs in the ASMKO cells.

Analysis of MRs in Normal and ASMKO Macrophages—MRs 
are abundant on the surface of macrophages and involved in the
uptake of several lysosomal enzymes. Commercial availability of the MR-specific ligand, mannoseBSA (18), allowed us to further investigate whether there was a difference in MR-mediated uptake between ASMKO and normal macrophages. As can be seen in Fig. 2, binding (panel A) and uptake (panel B) studies performed using $^{125}$I-mannoseBSA revealed only slight differences between normal and ASMKO macrophages. Northern blot analysis (C), total RNA was isolated from alveolar macrophages and a MR-specific probe was derived from PCR-amplified murine cDNA fragments. Murine glyceraldehyde-3-phosphate dehydrogenase mRNA was detected on the same membranes to quantify RNA loading. For Western blots (D), equal amounts of total cell lysates (15 μg) from normal and ASMKO alveolar macrophages were loaded onto gels and reacted with antibodies specific for the MR. The density of the bands in lanes loaded with equal concentrations of protein (as determined by colloidal blue staining of the gels; not shown) was measured. Autoradiograms were analyzed with Scion Image Beta 4 Version, an image processing and analysis program. The density of the bands in individual lanes was calculated as the area under the individual peaks of a plotted histogram. Representative Northern and Western blots from three independent experiments are shown.

**Fig. 2. Evaluation of mannose receptor function/expression in alveolar macrophages.** $5 \times 10^6$ normal (black circles) or ASMKO (white circles) mouse alveolar macrophages were incubated with varying concentrations of $^{125}$I-mannoseBSA. For binding (A), cells were incubated for 4 h at 4 °C. For uptake assays (B), cells were incubated for 24 h at 37 °C. The results are representative of two independent experiments. For Northern blot analysis (C), total RNA was isolated from alveolar macrophages and a MR-specific probe was derived from PCR-amplified murine cDNA fragments. Murine glyceraldehyde-3-phosphate dehydrogenase mRNA was detected on the same membranes to quantify RNA loading. For Western blots (D), equal amounts of total cell lysates (15 μg) from normal and ASMKO alveolar macrophages were loaded onto gels and reacted with antibodies specific for the MR. The density of the bands in lanes loaded with equal concentrations of protein (as determined by colloidal blue staining of the gels; not shown) was measured. Autoradiograms were analyzed with Scion Image Beta 4 Version, an image processing and analysis program. The density of the bands in individual lanes was calculated as the area under the individual peaks of a plotted histogram. Representative Northern and Western blots from three independent experiments are shown.

rhASM uptake defect in ASMKO macrophages (Fig. 1) was due to abnormalities in other receptor-mediated pathways.

**Analysis of M6PRs in Normal and ASMKO Macrophages**—We next used $^{125}$I]IGF-II as a M6PR-specific ligand to determine whether there were any differences in cell surface expression of this receptor between normal and ASMKO macrophages. This study revealed that ASMKO macrophages bound ~50% less $^{125}$I]IGF-II compared with their normal counterparts (3260 ± 553 cpm compared with 5721 ± 680 cpm, n = 4, p < 0.005), suggesting reduced cell surface expression of M6PRs. Northern blot analyses revealed no differences in M6PR RNA expression between normal and ASMKO macrophages (Fig. 3A), but Western blotting studies showed a dramatic increase in the total amount of M6PRs in the ASMKO
macrophages compared with normal (Fig. 3B). Because our inhibition studies using M6P (Fig. 1) had indicated that the M6PR system did not participate in the uptake of rhASM by the ASMKO cells, and receptor-binding studies with [125I]IGF-II showed reduced binding to the ASMKO cell surface, it was surprising to observe more M6PRs in the mutant cells compared with normal. Thus, we hypothesized that these receptors were either not located in the proper cellular compartments and/or were not functional.

**Evaluation of Modified rhASM Uptake by Alveolar Macrophages**—In an effort to test our prediction that the MR uptake system in ASMKO alveolar macrophages was functional whereas the M6PR system was not, uptake of a highly mannosylated form of rhASM was evaluated. This modified enzyme form was prepared by sequential deglycosylation of rhASM to expose surface mannose residues, a procedure identical to that used to prepare the mannosylated terminated form of β-glucocerebrosidase (i.e. Cerezyme) currently used to treat patients with Type 1 Gaucher disease (35).

As can be seen in Fig. 4A, by 24 h both normal and ASMKO macrophages internalized the modified rhASM effectively. Internalization by ASMKO macrophages was ∼5-fold less than normal macrophages, consistent with the Northern and Western blotting data demonstrating somewhat lower expression of MRs in ASMKO macrophages compared with normal cells (see Fig. 2). Importantly, however, when the uptake of modified rhASM was compared with non-modified rhASM (see Fig. 1C), uptake by the modified enzyme form was ∼10-fold greater using either normal or ASMKO cells. This was consistent with our hypothesis that the MR uptake system was functional in ASMKO cells, and demonstrated that the modification procedure had produced a rhASM form that was targeting these receptors. Inhibition studies using mannan and M6P further confirmed these findings by revealing almost complete inhibition of modified rhASM uptake by mannan, but not M6P (Fig. 4B).

**DISCUSSION**

ASM-deficient NPD is considered an excellent candidate for enzyme replacement therapy, particularly in those patients without primary neurological involvement (1). Previous studies have shown that intravenous injection of “complex” type, rhASM obtained from Chinese hamster ovary cells into ASMKO mice reduced sphingomyelin storage and improved macrophage infiltration in various visceral organs, including the liver, spleen, heart, and, to a lesser degree, the lung (24). However, the one or more mechanisms underlying this preclinical, therapeutic success are not certain, nor do we have a full understanding of how rhASM is taken up by important cellular targets of pathology in this disease, including macrophages. Previous data from our laboratory demonstrated that direct lung delivery of rhASM into NPD mice was no more efficient than systemic delivery in improving lung pathology, despite the fact that a much greater concentration of the enzyme was directly available to the lung macrophages by the former method.2 This led us to propose that there may be a defect in the ability of NPD alveolar macrophages to endocytose exogenous enzyme, a hypothesis that formed the basis for the current study.

Most lysosomal enzymes are internalized by cells via surface receptors that recognize N-linked oligosaccharides. Macrophages express a variety of such cell surface receptors used for endocytosis, including the M6PRs, MRs, asialoglycoprotein receptors, and various scavenger receptors (36). Depending on the carbohydrate moieties of an enzyme, all of these receptors, individually or more likely in combination, may be expected to contribute to enzyme uptake by macrophages. Among these, the M6PR and MR are the best characterized. Many cell types possess M6PRs, but MRs are restricted primarily to cells of the reticuloendothelial system. Our studies using radiolabeled ligands and receptor-specific inhibitors demonstrated that both of these receptors play a role in rhASM uptake by normal, alveolar macrophages. This is consistent with previous work showing that both receptors are involved in the uptake of β-glucuronidase by peritoneal macrophages (19). However, our studies also showed that in ASM-deficient macrophages uptake

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2 R. Dhami and E. H. Schuchman, unpublished results.
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It was not within the scope of this study to determine the ultrastructural localization of the M6PRs in the alveolar macrophages, however, preliminary investigation by microscopic immunofluorescent analysis suggests that M6PRs in ASMKO macrophages may be distributed abnormally compared with normal macrophages. Further studies are warranted to determine exactly where the accumulating M6PRs are within the ASMKO cells.

It is also notable that, although there was normal or only moderately reduced binding of either $^{125}\text{I}$-labeled mannosylated BSA or IGF-II to the ASMKO macrophages, the binding of $^{125}\text{I}$-rhASM to these same cells was markedly enhanced. The enhanced enzyme binding may be due to its interactions with other receptor types, because it has been previously suggested that, even in cells lacking MRs (e.g., skin fibroblasts), M6PRs are only partially responsible for ASM uptake and other receptors are involved (37). Perhaps the normally small influence of these other receptors (e.g., asialoglycoprotein receptor, etc.) becomes more important in the disease state but is still insufficient to restore normal enzyme uptake.

The data presented in this manuscript also have provided new insights into the mechanism underlying our successful preclinical enzyme replacement therapy studies in ASMKO mice (24). We now know that the clearance of alveolar macrophages from the airways of the treated mice we observed in these studies was almost certainly not due to direct uptake of rhASM by these cells. Instead, previous analysis of the lung pathology in these animals had revealed that there was an overexpression of inflammatory cell chemotactants by epithelial cells in the ASMKO lungs (23), presumably resulting in enhanced recruitment of macrophages. Therefore, we hypothesize that the intravenous delivery of rhASM most likely resulted in uptake by these epithelial cells (which appear normal even in older mice), rather than the macrophages themselves, leading to subsequent correction of the chemokine levels and a reduction in inflammatory cell recruitment.

With regards to enzyme replacement therapy, our data also suggested that it may be possible to improve uptake by NPD alveolar macrophages with a highly mannosylated form of rhASM (similar to the commercially available form of $\beta$-glucocerebrosidase, Cerezyme, in use for Type 1 Gaucher disease), because the MR appeared to retain function in the ASMKO cells. We were able to test and confirm this hypothesis using such a modified enzyme form and indeed found that ASMKO macrophages were capable of taking up $\sim 10$-fold more of this enzyme form when compared with the non-modified enzyme. Although this is an important observation that could be used to influence potential therapies for NPD, it does not necessarily follow that macrophase-targeted enzyme replacement therapy for NPD would result in greater clinical efficacy than treatment with "complex type," rhASM. Although this is certainly true for Type 1 Gaucher disease, in the murine model of MPS VII (Sly disease; $\beta$-glucuronidase deficiency), treatment with phosphorylated $\beta$-glucuronidase resulted in better clinical efficacy than treatment with non-phosphorylated enzyme (38). Ultimately, each lysosomal disease will prove to be unique and require enzyme targeting to specific cell types, and although macrophages are important cellular targets for NPD, they are not the exclusive cellular sites of pathology. Because MRs are restricted to cells of the monocyte/macrophage lineage, enzyme therapy directed at this cell population alone might be counterproductive. In addition, we do not know how the results reported here with alveolar macrophages compare with other macrophage populations in NPD patients. Ultimately, successful treatment of NPD individuals by enzyme replacement therapy may be one that includes enzyme with a variety of oligosaccharides designed to take advantage of the many cellular receptors involved in endocytosis.

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Rajwinder Dhami and Edward H. Schuchman

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