# Sphingolipidoses: molecular manifestations and biochemical strategies

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Among the estimated 20,000 to 40,000 human gene pairs (1) approximately 2000 single-gene alterations are now recognized (2). A specific protein alteration has been established or suggested for about 10% of these mutations (3). Most lysosomal storage disorders fall into this class of inherited metabolic disorders. The sphingolipidoses represent those lysosomal storage disorders in which the predominating storage product is a specific sphingolipid. The diseases have been classified according to the lipid that accumulates (**Table 1**). Chemical identification of the accumulated product led to the description of the specific enzymatic lesion in Gaucher's disease (4), metachromatic leukodystrophy (5), and Niemann-Pick disease (6). However, as was predicted by Hers (7), the storage material of certain lysosomal storage disorders can be very heterogeneous, resulting sometimes in an ambiguous classification of lipidoses. Although we have chosen to include α-fucosidosis in the sphingolipidoses, it has been argued, with some justification, that the complex nature of the numerous products accumulating in this particular disorder make such a designation restrictive (8). In Krabbe's disease, it is not product heterogeneity but rather lack of overt lysosomal storage that has limited its unequivocal classification as a sphingolipidosis; however, the primary enzyme deficiency in this disorder has clearly been established as being lysosomal (9–11).

The prevalence of the sphingolipidoses is similar to that of other common inborn errors of metabolism. In Ashkenazi Jews the gene frequency of Gaucher's disease has been estimated to be 1:50 (12), that of Tay-Sachs disease 1:30 (13), that of type A Niemann-Pick disease 1:200 (14). In the Swedish population, the estimates of gene frequencies of metachromatic leukodystrophy and Krabbe's disease have been placed at 1:200 (15) and 1:230 (16), respectively. In compari-

son, the gene frequency of sickle cell anemia in blacks is 1:25 (17), of cystic fibrosis in Europeans is 1:50 (18), and of phenylketonuria in Europeans is 1:125 (19).

All genetic disorders are by definition characterized by aberrant biochemical processes. However, phenotypic expression often is observed at some metabolic point distant from the primary genetic lesion. The lysosomal storage disorders offer the unique opportunity of studying phenotypic expression (lysosomal storage) in close proximity to the altered gene product (enzyme deficiency). Scientists have not overlooked this advantage and remarkable progress has been made in the study of the sphingolipidoses. The popularity of this field can be judged by the 4,395 publications that have appeared during the last 5 years on the sphingolipidoses and related topics (20). Numerous review articles on the subject have appeared (21–24). The purpose of the present discussion will be to highlight some of the clinical, biochemical, and therapeutic studies of these disorders in terms of both past accomplishments and future directions.

## ENZYMOLOGY

In general, sphingolipid hydrolases are glycoproteins (25). Many of the enzymes have been characterized and some have been successfully isolated. β-Hexosaminidases (26), glucocerebrosidase (27), sphingomyelinase (28), ceramidetrihexosidase (29), and galactosylceramide sulfate sulfatase (30, 31) can now be obtained in a state of purity sufficient to carry out critical chemical and physical analyses.

One of the major complexities in this field of enzymology has been the heterogeneity of enzymatic

TABLE 1. Diseases classified by accumulating lipid

| Sphingolipidosis | Enzymatic Lesion | Major Accumulating Sphingolipid | Primary Organ Involvement |
|---|---|---|---|
| Tay Sach's disease | Hexosaminidase A | Ganglioside $G_{M2}$ | Brain |
| Sandhoff's disease | Hexosaminidase A and B | Ganglioside $G_{M2}$ and Globoside | Brain |
| Fabry's disease | Ceramidetrihexoside $\alpha$-galactosidase | Ceramidetrihexoside | Kidney |
| Gaucher's disease | Glucocerebroside $\beta$-glucosidase | Glucocerebroside | Liver, spleen, bone, brain (infantile form) |
| Metachromatic Leukodystrophy | Galactocerebroside Sulfate Sulfatase | $\beta$-Sulfogalactocerebroside | Brain |
| Niemann-Pick disease | Sphingomyelinase | Sphingomyelin | Brain, liver, spleen |
| Krabbe's disease | Galactoscerebroside $\beta$-galactosidase | Galactocerebroside | Brain |
| General gangliosidosis | $\beta$-Galactosidase | Ganglioside $G_{M1}$ | Brain, liver, bone |
| Fucosidosis | $\alpha$-Fucosidase | H-Isoantigen | Brain |

activities associated with many of these hydrolases. Certainly part of this is a reflection of the general lack of specificity of the artificial substrates often used for enzymatic assay. In addition, the tendency of many of these enzymes to aggregate and/or associate with subcellular components can artifactually contribute to the heterogeneity. However, a multiplicity of enzyme activities has often been found under very carefully controlled conditions so that the existence of complex isozyme systems for many of these hydrolases is well established. Various lysosomal forms of sphingomyelinase have been reported (32, 33). The heterogenous quaternary structure of this enzyme supports the possibility of isozymes (28). Under specific assay conditions, both galactosyl ceramide $\beta$-galactosidase and $G_{M1}$-$\beta$-galactosidase will hydrolyze lactosylceramide and each of these two genetically distinct enzymes also has some ability to hydrolyze the major substrate of the other enzyme (34).

The most rigorous study to date on an integrated correlation of the molecular forms of an enzyme system and the specific clinical manifestations resulting from genetically determined malfunctions of that system has been carried out with the $\beta$-hexosaminidases and the variants of Tay-Sachs disease. Utilizing techniques of immunological resolution (35, 36), genetic complementation (37–39), and chemical analysis (36, 40), it is now generally accepted that the major isozymes of hexosaminidase are coded by two separate cistrons. It appears that hexosaminidase A is a hetero-oligomeric protein made up of both $\alpha$ and $\beta$ subunits and that hexosaminidase B is composed of only the $\beta$-subunits. In addition it has been postulated that an additional specific protein bestows $G_{M2}$-$\beta$-$N$-acetylgalactosaminidase activity on the A isozyme (41, 42). The various forms of Tay-Sachs disease are thought to occur because of mutations affecting one of these three protein components.

Considering the lipid nature of the substrates and the particulate and hydrophobic character of the enzymes, it is not surprising to find that detergents greatly influence the hydrolysis of sphingolipids in vitro. For most enzymes, a set of optimal detergent concentrations resulting in maximal hydrolyzing activity has been determined empirically. Surprising and perplexing, however, have been the reports that factors can be found in tissue extracts that, although themselves not catalytically active, can nevertheless significantly stimulate enzymatic hydrolysis of natural substrates in vitro. Popularly referred to as activators or specifiers, these factors for the most part have been shown to be heat-stable, low molecular weight glycoproteins (41–44). The physiological significance of these factors has been questioned (45, 46). The problem reflects the tenuous position of attempting to transpose in vitro conditions to an in vivo system. A very promising direction in resolving this difficult problem has been taken by Mraz, Fischer, and Jatzkewitz (47, 48) and Fischer and Jatzkewitz (49), who have shown the existence of such activators in highly purified lysosomes. With the acidic pH and ionic strength thought to occur in vivo, purified activators stimulate the catalytic activities of a number of hydrolases. It has been suggested that the activators might serve as intralysosomal detergents which interact with water-insoluble lipid substrates so as to present them to the enzymes in a form more compatible for hydrolysis (50). With the availability of

purified activators, it will be of considerable interest to observe if antibodies directed against them can interfere with normal lysosomal function. The feasibility of such a study has been established by Tulkens, Trouet, and Van Hoof (51), who showed that antibodies to soluble lysosomal components can induce a storage syndrome in cultured fibroblasts. It will also be of considerable interest to see what influence such activators might have on ganglioside-$G_{M2}$ hydrolysis by tissue extracts from Tay-Sachs AB variant patients.[1]

Molecular mechanisms underlying genetically determined enzyme deficiencies deal with mutations that affect either the rate of gene expression (regulatory mutations) or the quality of gene expression (structural mutations). The apparent lack of cross-reacting material to ceramidetrihexosidase in Fabry's disease (52) and the apparent absence of the unique subunit component of hexosaminidase A in the B variant of Tay-Sachs disease (53–55) suggest regulatory mutations. The presence of cross-reacting material to arylsulfatase A in metachromatic leukodystrophy and qualitative differences in the small amounts of residual sulfatase found in such pathological tissues strongly suggest a structural mutation (56–58). When significant residual enzymatic activity is found in a disease, it is crucial to determine, whenever possible, whether a structural or regulatory mechanism is involved. Preliminary investigations of the residual enzyme activity in Gaucher's disease have been carried out (59–61). These studies indicate alterations in the catalytic and/or stability properties of the residual enzyme, suggesting a structural mutation of the glucocerebrosidase. Unfortunately, these studies were carried out with crude tissue extracts. The limitations of comparative enzymatic characterizations in crude systems are many; for example, Tanaka and Suzuki (62), drawing precedence from their studies of $G_{M1}$ ganglioside $\beta$-galactosidase and galactosyl ceramide galactosidase, have cautioned against attributing characteristics of residual enzyme activities in disease states to mutant enzymes until the possibility of genetically autonomous enzyme interference has been ruled out. Now that glucocerebrosidase can be isolated in high yields (63), the opportunity of a rigorous and comprehensive comparison of normal and abnormal enzyme systems can be pursued.

## DIAGNOSIS

A highly developed phase of sphingolipidosis research has been in the area of disease detection. The

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[1] Sandhoff, K. Personal communication.

documentation of a specific enzyme deficiency is necessary for confirming the diagnosis of a particular disorder. The prenatal and postnatal detection of homozygotes and heterozygotes for all the sphingolipidoses either has been confirmed or should now be possible. The quantitative measurement of lysosomal hydrolase activity has been carried out with radioactive natural substrates or synthetic substrate analogues. The latter have generally consisted of 4-methylumbelliferone and $p$-nitrophenyl conjugates of specific glycosides which, when hydrolyzed by the appropriate enzymes, will form fluorogenic or chromogenic products, respectively (64, 65). The advantages of these substrates are their commercial availability and ease of assay; for example, the feasibility of a continuous-flow system of automatic analysis of 11 acid hydrolases with fluorogenic substrates has recently been established (66). However, the disadvantage of these synthetic substrate analogues is their inherent lack of specificity. Since multiple glycosidases often exist for a single glycosidic linkage, specificity problems can sometimes be overcome with the use of assay conditions favoring a particular enzyme. Heat inactivation (67), physical separation (68), and use of very specific pH (69) have been used to improve specificity. Recently a notable breakthrough in the use of synthetic substrates has been achieved. Rather than approaching the problem from the standpoint of manipulating the assay conditions, Brady and co-workers (70) set out to synthesize a type of substrate that incorporates the convenience of a chromogenic marker and the reliability and specificity of a natural substrate. An analogue of ceramide, the chromogenic product 2-hexadecanoyl-amino-4-nitrophenol was synthesized and coupled to one of the three naturally occurring conjugates of ceramide: phosphorylcholine, $\beta$-D-galactose, and $\beta$-D-glucose. For Niemann-Pick (71) and Krabbe (72) diseases the newly synthesized phosphorylcholine and galactose derivatives, respectively, offered for the first time the use of chromogenic substrates with the specificity needed for reliable diagnosis. The $\beta$-glucoside derivative showed more specificity than the commercially available glucosides and consequently offers greater reliability in the assay of glucocerebrosidase for Gaucher's disease (73).

A variety of body fluids and tissues has been employed in the assay of enzyme levels for diagnostic purposes. Plasma, urine, leukocytes, cultured fibroblasts, hair follicles (74), and tears (75) have all been used to facilitate quantitation. Some intriguing recent developments in ultramicro assays have taken place. Using assay volumes in the submicroliter range and extended incubation times in order to minimize back-

ground interference, quantitative measurements of enzyme activities on single cells have been achieved (76). With these conditions, prenatal diagnosis can be carried out before the 17th week of pregnancy (77). These microtechniques have also facilitated genetic complementation studies in that single somatic cell hybrids can now be isolated and analyzed (78).

## PATHOGENESIS

Lysosomal storage disorders are characterized by a genetically determined deficiency of specific lysosomal hydrolases and the consequential lysosomal storage of unmetabolized material. The normal turnover of the cellular components of blood and tissues gives rise to a variety of glycosphingolipids which pass through the digestive system of the cell for catabolism. Lysosomal blockage will manifest itself in those deficient cells that are offered indigestible material. Since degradable products enter the lysosomes in several ways and since mechanical and functional barriers exist between different cell types and organs, it is not surprising that a variety of morphological and clinical abnormalities appear in these disorders. Often the phagocytized material originates from the circulating fluids (heterophagy). The extensive accumulation of lipid material within the reticuloendothelial system in Niemann-Pick and Gaucher's diseases reflects the high heterophagic activities of these cells towards red and white blood corpuscles (79). In cells that have less endocytic activity, such as neurons, the undigested material probably results from the normal turnover of endogenous cellular material (autophagy). It has been well documented that the marked overloading of the lysosomal system results in the morphological hallmark of lysosomal hypertrophy. This pathological phenomenon implies that there is no effective homeostatic control limiting lysosomal uptake (80). The progression of lysosomal hypertrophy to clinical disease remains speculative. One can envision mechanical crowding of the cytoplasm by the larger and more numerous lysosomes which leads to critical alterations in fine cellular architecture and disruption of normal functional integrity (81). In Gaucher's and Niemann-Pick diseases there can be massive encroachment of liver, spleen, and lymph node parenchyma by engorged storage cells. In an organ that is greatly distensible, such as the liver, marked enlargement might occur before any apparent compromise of function takes place. However, in organs that have no capacity for expansion only relatively small amounts of storage material could produce significant disturbances of spatial relationships and function. This is obviously the case in the central nervous system where any increase in size within the confined space of the skull puts direct compression on surrounding tissue. Specialized cells are apparently more affected by architectural disturbances. For example, in metachromatic leukodystrophy the appearance of inclusions within glial and Schwann cells could alter their function so that myelin assembly is either faulty or deficient, resulting in the characteristic demyelination of the cerebral white matter (82).

Experimental models of induced lysosomal storage have shown changes in cellular function. By allowing cells to take up indigestible substances such as dextran (83), sucrose (84) or polystyrene latex (85), lysosomal loading has been shown to lead to increased lysosomal enzyme synthesis. Profuse excretion of these enzymes into the culture medium has also been observed (86). Release of lysosomal material caused by a rupture of distended lysosomes may be involved. Although lysosomal rupture has not yet been shown for any of the sphingolipidoses, such a pathogenic mechanism has been suggested for silicosis (87) and acute gouty arthritis (88).

In Krabbe's disease an accumulating lipid has itself been strongly implicated as a cytotoxic agent. It has been found that galactosylsphingosine levels of Krabbe brain are approximately 100-fold greater than normal (89). This lipid with its free amino group has been shown to be cytotoxic (90). It has been suggested that as a result of the specific enzyme deficiency of a galactosidase the normally trace amounts of galactosylsphingosine build up to toxic levels in the oligodendroglial cells, causing cell death and cessation of myelination (10).

A mechanistic and molecular interpretation of the pathophysiological developments in these disorders can only be as complete as our comprehension of normal and abnormal biochemistry and physiology. Consequently, the pathogenesis of the sphingolipidoses remains largely a descriptive characterization along the guidelines predicted by Hers (7) in 1965 for lysosomal storage disorders in general. This working hypothesis has served the useful purpose of unifying a very heterogeneous body of knowledge and has provided a theoretical basis for experimentation.

## MOLECULAR AND THERAPEUTIC LINKS

Specific treatment of the sphingolipidoses has not yet been established; supportive care has been the sole means of intervention. The firm establish-

ment of the sphingolipidoses as lysosomal storage disorder has, however, stimulated a considerable and now optimistic effort towards therapy. It has been assumed that an amelioration of a disorder can be achieved through a reversal of the sometimes massive accumulation of uncatabolized products. An obvious avenue for attempting to mobilize this lipid is through a supplementation of the deficient endogenous enzyme levels with exogenous sources. As predicted by de Duve (91) in his generalized concept of lysosomal action, the exogenously supplied enzymes could be expected to be targeted directly to the stored substrates because of the normal fusion of lysosomes (containing the accumulating material) and endocytic vesicles (containing the exogenous enzymes). The ability of exogenous enzymes to reverse lysosomal accumulation has been established with several experimental models (84, 92).

A good deal of information has also now been obtained concerning the parenteral administration of purified enzyme preparations to human patients. In general when highly purified sterile human placental enzyme preparations have been administered intravenously, patients have shown no ill effects (21). Although the potential hazards of antibody formation and anaphylactic reaction to the administration of exogenous enzymes should be closely monitored, careful consideration of experimental conditions will reduce risks. As exemplified with Gaucher's disease, the existence of residual enzyme activity in patients, the use of highly purified human enzymes for infusion, and the choice of the least immunologically provocative route and schedule of administration can minimize the risks in such clinical investigations (21). After enzyme infusion the deficient enzyme activities in the livers of recipients have been shown to rise significantly, paralleling a rapid clearance of the exogenous enzymes from the circulation (21). Studies with rhesus monkeys and rats have shown that exogenous glycosidases are rapidly incorporated into the lysosomal compartment of liver (93). Administration of hexosaminidase to Tay-Sachs patients (94) and of $\alpha$-galactosidase to Fabry patients (95) have shown that the elevated levels of glycolipids in the circulation could be specifically and transiently lowered to the normal range. Studies with Gaucher patients have shown that the clearance of the elevated erythrocyte glucocerebroside levels could be maintained for several months after the infusion of purified glucocerebrosidase (96). In addition, the critical observation of partial clearance of glucocerebroside from the liver itself was made in these Gaucher patients (97).

Direct infusion of enzyme has not been the only means of attempting enzyme replacement. Organ transplantations have been performed with spleen (98) and kidney (99) in Gaucher's disease and with kidney (100) in Fabry's disease. The conceptual basis for these transplants is the presence of a healthy organ that is able to function both as a continuous source of endogenous enzyme for general tissue distribution and as a target organ for the in situ metabolism of products accumulating in the blood. The inherent medical problems of organ transplantation and the uncertainty and variability of the biochemical data gathered from such studies have prompted the suggestion that such therapeutic attempts be limited to crisis situations such as renal failure in Fabry's disease (101).

The scientific advances of the past few years offer realistic solutions to many problems once considered major obstacles to effective enzyme replacement trials. The availability of a suitable enzyme source has been resolved with the finding that human placental tissue contains high levels of most lysosomal hydrolases. Development of streamlined methods for the large-scale preparation of enzymes has provided enzymes in quantity with a high degree of purity (63). Chemical (102) and physiological (93) means of protecting and stabilizing the enzyme for optimal therapeutic effect in vivo have been suggested. The carbohydrate moieties of the lysosomal enzymes known to play important roles in receptor–membrane interactions can very likely be manipulated in directing the enzymes to various sites of action (103–105). Enzymes may be encapsulated in erythrocyte ghosts (106) or artificial lipid vesicles (107) for possible protection from immunological surveillance or for enhanced target delivery. A reversible osmotic opening of the blood–brain barrier (108) has provided the means of having intravenously administered enzymes reach the interstitial fluid of the brain (109). Thus, the stage seems set for the second and critical phase of enzyme replacement trials—a comprehensive survey of clinical as well as biochemical parameters during and following a sustained regimen of enzyme administration.

One can speculate on several different fronts. To what extent are the accumulated lipids cleared from tissues, assuming an adequate supply of enzyme? The sphingolipidoses are progressive disorders with continual lysosomal hypertrophy and formation of residual bodies. Some recent evidence suggests that aged lysosomes and residual bodies showed a lowered capacity for fusion with newly formed lysosomes containing exogenous material (110). Thus, there may be inherent limits to the ability of exogenous enzymes to mobilize accumulated lipid. It is possible that enzyme supplementation may prove more effective

in controlling the progression of these disorders than in achieving major reversals.

How sustained is the enzymatic clearance of lipid? In Gaucher's disease the clearance of circulating glucocerebroside was sustained for over 2½ years following enzyme infusion (23); however, re-examination of the patient's liver at that time revealed that the hepatic glucocerebroside levels had returned to slightly higher than preinfusion levels.[2] The rate of lipid accumulation may determine whether enzyme replacement will require frequent or occasional treatment.

What relationships exist between the extent of lipid accumulation and the clinical and pathological manifestations? In Gaucher's disease we have observed a considerable variation in concentration of accumulating liver lipid from patient to patient.[2] Consequently, lipid clearance induced through enzyme infusion may be more beneficial to some patients than to others. Moreover, whether lipid clearance will correlate with a change in the course of the disease remains to be answered. Research in the sphingolipidoses has progressed to the point where one is now on the threshold of answering many of these critical questions.∎

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