GAUCHER DISEASE

AN IMMUNOELECTRON MICROSCOPIC AND BIOCHEMICAL STUDY

ROB WILLEMSEN

Front cover: Lysosomal glucocerebroside deposits in a splenic macrophage of a patient with type 1 Gaucher disease. Inset: "Scientist" by Birgit Willemsen-Horsten (photography Ruud Koppenol; lay-out Tar van Os)

GAUCHER DISEASE

AN IMMUNOELECTRON MICROSCOPIC AND BIOCHEMICAL STUDY

DE ZIEKTE VAN GAUCHER
EEN IMMUNOELECTRONENMICROSCOPISCHE EN BIOCHEMISCHE STUDIE

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
AAN DE ERASMUS UNIVERSITEIT ROTTERDAM
OP GEZAG VAN DE RECTOR MAGNIFICUS
PROF. DR. P.W.C. AKKERMANS M.A.
EN VOLGENS BESLUIT VAN HET COLLEGE VOOR PROMOTIES

DE OPENBARE VERDEDIGING ZAL PLAATSVINDEN OP
WOENSDAG 27 SEPTEMBER 1995 OM 13.45 UUR

door

ROBERT WILLEMSEN

geboren te Breda

PROMOTIECOMMISSIE

PROMOTOR:	Prof. Dr. H. Galjaard
OVERIGE LEDEN:	Prof. Dr. J.F. Jongkind
	Prof. Dr. W. van Ewijk
	Dr. E.I. Ginns

Gedrukt door: Drukkerij Haveka B.V., Alblasserdam

Dit proefschrift werd bewerkt binnen de vakgroep Celbiologie en Genetica van de Faculteit der Geneeskunde en Gezondheidswetenschappen van de Erasmus Universiteit te Rotterdam. Het onderzoek werd financieel gesteund door de Stichting Klinische Genetica regio Rotterdam. Subsidie voor de drukkosten van dit proefschrift werd verleend door Philips Nederland afdeling BU-Analytical, Leica Rijswijk en door Aurion Wageningen.

Vision is the art of seeing things invisible
(Jonathan Swift 1667-1745)

Voor mijn ouders
Aan Birgit en de kinderen

CONTENTS

CHAPTER I.

THE LYSOSOMAL SYSTEM

I.1. FUNCTION OF LYSOSOMES

Lysosomes are part of the vacuolar system of the cell and these organelles are mainly responsible for the degradation of a variety of biological macromolecules derived from both extracellular and intracellular constituents. In a mammalian cell there can be several hundred lysosomes, most of which are located in the perinuclear area of the cell. The heterogeneous shape, size and inner structure of lysosomes are reflected in the morphological nomenclature (residual bodies, dense bodies, multivesicular bodies). Certain stimuli, such as storage material, inflammation, ischemia, trauma or hormones can activate the lysosomal system.

Lysosomes have a complement of over 40 enzymes, including proteases, nucleases, glycosidases, lipases, phosphatases and sulfatases each responsible for hydrolyzing a well-defined chemical linkage. The lysosomal membrane provides protection against degradation of the cytosol by the cell's own acid hydrolases. Lysosomal enzymes have in common an acidic pH optimum between 3.5 and 5.0. This pH environment is established by an ATP-dependent proton pump in the lysosomal membrane.

Following the discovery of the lysosome by biochemical fractionation of cell extracts by de Duve and coworkers 40 years ago (de Duve et al., 1955), the subcellular visualization of lysosomes using enzyme cytochemistry (acid phosphatase) suggested that lysosomes form as vesicles budding off the GERL-compartment (**G**olgi-**E**ndoplasmic **R**eticulum-**L**ysosomes; Novikoff et al., 1964). Over the last decade this view has changed as a result of the identification of the mannose 6-phosphate receptor (MPR), the definition of lysosomal membrane proteins, more specific markers of the Trans-Golgi Network (TGN) and the various compartments of the endocytic system in combination with the development of advanced immunoelectron microscopic techniques (reviewed by Kornfeld, 1992). In the current view the lysosome is a dynamic cell organelle closely related to the biosynthetic and endocytic systems (reviewed by Sahagian and Novikoff, 1994).

I.2. SUBSTRATE DELIVERY PATHWAYS TO LYSOSOMES

The entry of substrates e.g. (glyco)proteins, (glyco)lipids, polysaccharides and nucleic acids into lysosomes is essential for their effective

degradation. The uptake and digestion of extracellular materials is called "heterophagy" (also referred to as endocytosis), whereas degradation of intracellular constituents is called "autophagy" (see figure 1).

Heterophagy

In the process of heterophagy there are several mechanisms, including phagocytosis, pinocytosis and receptor-mediated endocytosis. In all three processes the plasma membrane plays a crucial role in enclosing the extracellular material.

Phagocytosis is restricted to specialized cells such as macrophages, eosinophils and neutrophils, which ingest micro-organisms, senescent cells and tumor cells. This process requires a specific interaction between material to be digested and the cell surface, a process mediated by cell surface receptors (Griffin et al., 1975; Silverstein, 1989). After binding to the plasma membrane the engulfed particulate material forms a phagosome, which is transformed into a phagolysosome. Characteristic of phagocytosis is that the size of the phagosome is determined by the dimension of the engulfed material. The transformation of phagosomes into phagolysosomes is a dynamic process in which fusion events with early and late endosomes (see figure 1) are involved (Pitt et al., 1992; Desjardins et al., 1994).

In contrast, vacuoles derived from **pinocytosis** (also referred to as fluid-phase endocytosis) have a more homogeneous size. Pinocytosis is a non-selective uptake of extracellular material by fluid-phase endocytosis (Watts and Marsh, 1992; Sandvig and VanDeurs, 1994), which in special cases can give rise to distinct endosome populations (Hewlett et al., 1994). Both phagocytosis and pinocytosis uptake often leads to lysosomal degradation of the engulfed material.

The process of **receptor-mediated endocytosis** is involved in many cellular functions, including nutrient uptake, clearance of plasmaproteins, uptake of viruses, binding and uptake of hormones and growth factors and subsequent mediating signal transduction (reviewed by Smythe and Warren, 1991; Trowbridge et al., 1993; Bu and Schwartz, 1994). It provides a selective uptake mechanism for efficient (1000 fold increase compared to non-selective uptake) internalization of particular ligands. More than 25 different receptors have been described that can take part in receptor-mediated endocytosis (Browne and Greene, 1991). In general, receptor-mediated endocytosis starts with the binding of the ligand to surface receptors and subsequent clustering in clathrin-coated pits. The latter is dependent on interactions between the receptors and a multisubunit complex called "HA-II adaptors" (Pearse and Robinson, 1990; Robinson,

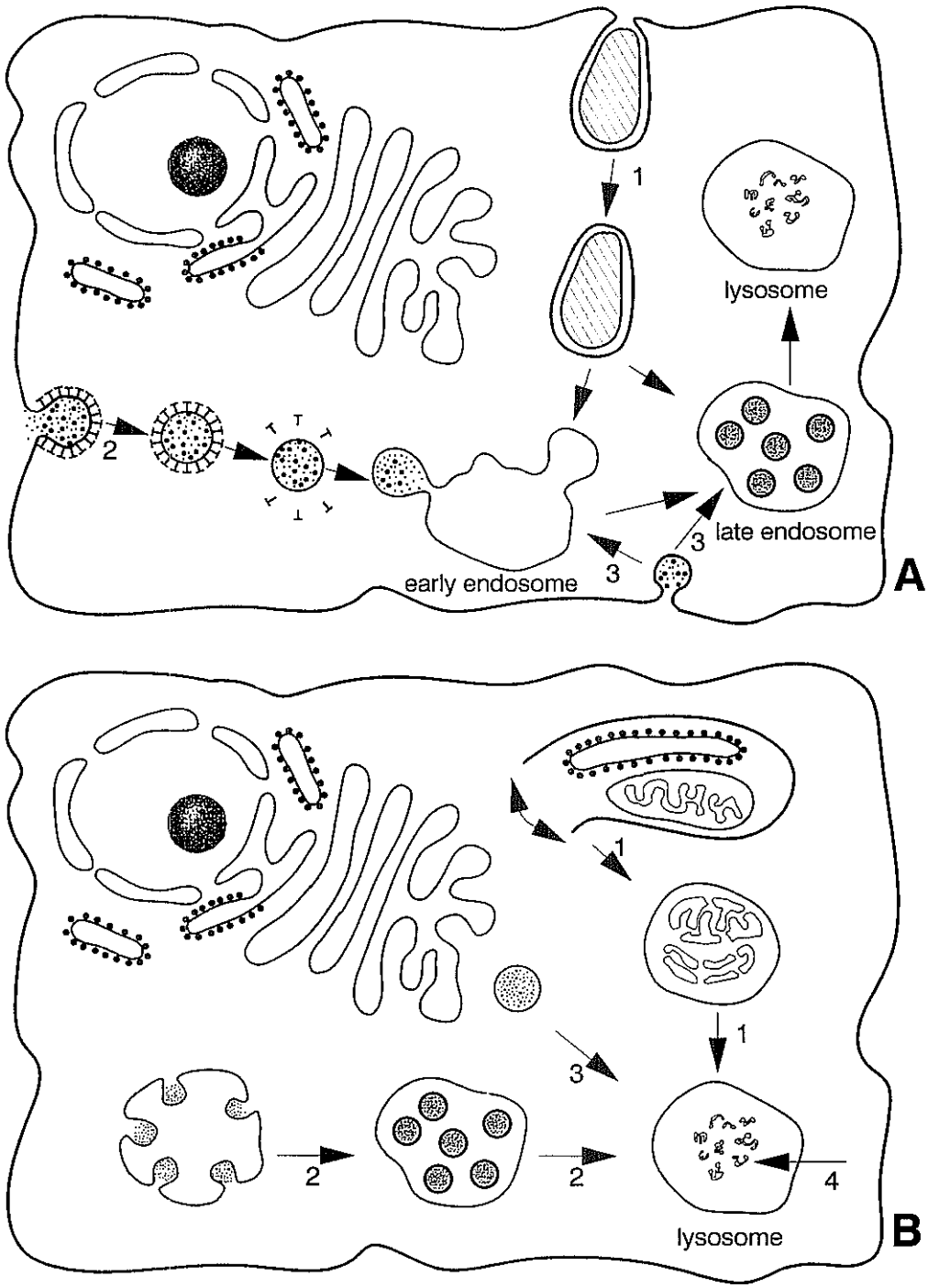


Figure 1. Schematic diagram of a cell depicting the pathways of substrate delivery to lysosomes from extracellular sources (heterophagy; A) and intracellular constituents (autophagy; B). A: 1=phagocytosis, 2=receptor-mediated endocytosis, 3=pinocytosis. B: 1=macroautophagy, 2=microautophagy, 3=crinophagy, 4=direct delivery via specific transporters.

1994). This complex binds to the cytoplasmic tail of the receptor, in which the presence of a tyrosine residue in a generic sequence pattern of 4- and 6- residue signals is essential. It is suggested that this tyrosine internalization signal surrounded by hydrogen-bonding residues gives rise to a type I ß tight turn, which might be needed for a receptor to recognize HA-II adaptors (Collawn et al., 1990; Ktistakis et al., 1990; Eberle et al., 1991). Membrane proteins that lack a tyrosine-containing signal (FcIIB2-receptor, CD4, GLUT4) can participate in receptor-mediated endocytosis by means of a di-leucine sequence motif in their cytoplasmic domain (Trowbridge et al., 1993; Corvera et al., 1994).
Recently, it was shown in polarized MDCK cells that LDL and Fc receptors possess either tyrosine or di-leucine containing signals for basolateral sorting whereby the amino acids in the vicinity of these motifs determine whether they function for endocytosis, basolateral sorting or both (Matter et al., 1994).
The receptor-ligand complex enters the cell via invagination of the coated-pit, forming a clathrin-coated vesicle. These vesicles lose their clathrin and form an endosome by fusion with each other. Since extracellular fluid is also trapped by this pathway in a non-selective manner in the endosomes, it implies that receptor-mediated endocytosis is also involved in fluid-phase endocytosis.

On the basis of the final destination of both receptor and ligand five intracellular routes can be distinguished (see figure 2):

1. Receptor and ligand dissociate in the endosome, receptor is recycled to the surface or to the TGN and the ligand is degraded in lysosomes (LDL-receptor, MPR, asialoglycoprotein-receptor).
2. Receptor and ligand both recycle to the plasma membrane (transferrin-receptor).
3. Both receptor and ligand are transported to lysosomes (EGF-receptor, PDGF-receptor, insulin-receptor).
4. Ligand-receptor complex is delivered to the opposite side of polarized cells, where the ligand is released and the receptor is either degraded or recycled. This process is known as "transcytosis" (IgA-receptor, IgG-receptor).
5. Ligand and receptor dissociate in the endosome, receptor is recycled to the surface or TGN and ligand is released from endosomes (viruses, toxins).

The endocytic pathway to be followed is cell type specific and determined by factors, such as receptor distribution, valency and type of ligand.

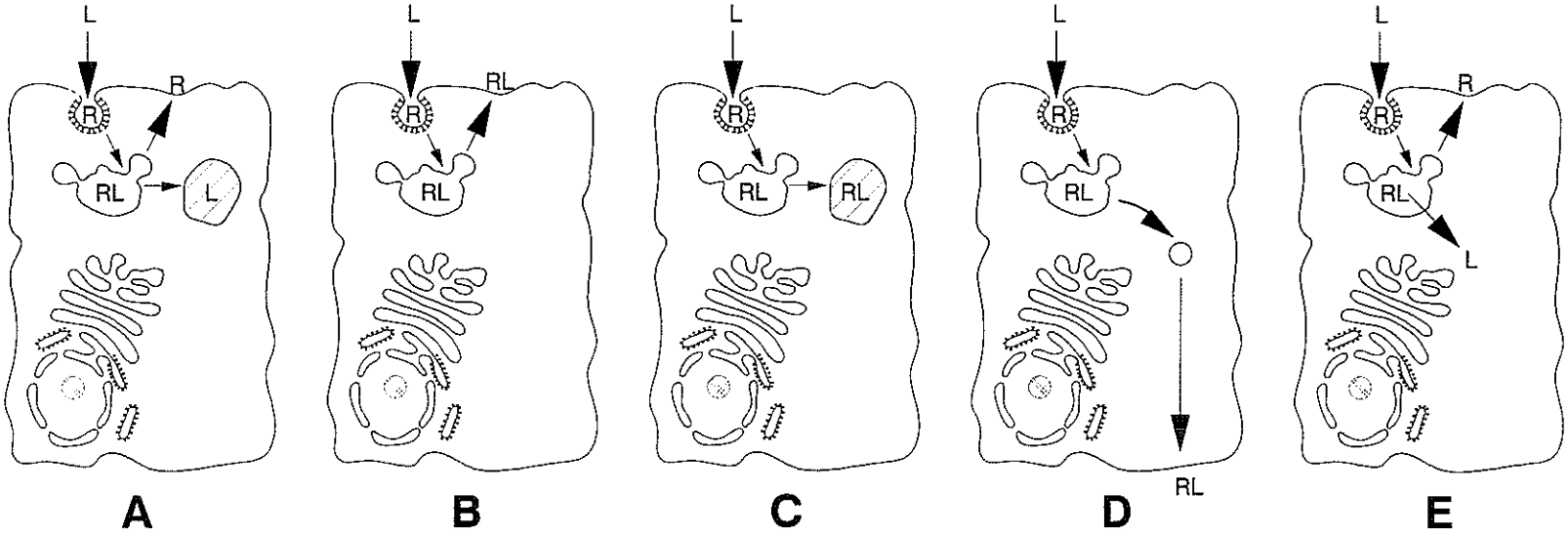


Figure 2. Schematic diagram showing the intracellular sorting pathways of receptor-mediated endocytosis. All five different pathways have in common the clustering in coated pits of receptor (R) and ligand (L), internalization of receptor-ligand complex and subsequent transport to endosomes. A; receptor recycles and ligand is degraded within lysosomes, B; receptor and ligand recycle, C; receptor and ligand are degraded, D; transcytosis of receptor and ligand, E; receptor recycles and ligand is released in cytoplasm. Lysosomes are hatched. (Bu and Schwartz, 1994)

The mechanism by which endosomes are transformed into lysosomes is still controversial. In the "vesicle shuttle" model, vesicles budding from the early endosomes recycle to the cell surface or deliver their contents into late endosomes. Movement from the late endosome to lysosomes could occur in the same fashion. Thus, in this model early endosomes, late endosomes and lysosomes exist as stable, functional organelles, hence the name vesicle shuttle model (Griffiths and Gruenberg, 1991). Alternatively, in the "maturation" model early endosomes are transformed in late endosomes and then into lysosomes by a remodeling process. The endosome and lysosome identity is transient, but the vesicles they interact with and that modify them are stable (Stoorvogel et al., 1991; Murphy, 1991; Dunn and Maxfield, 1992). A third model was proposed by Hopkins (Hopkins et al., 1990) who visualized a continuous endosomal reticulum by fluorescence microscopy in combination with video recording. Endocytosed material moves via multivesicular bodies along this reticulum.

Autophagy

Cells also need lysosomes to degrade their own components. This process, termed autophagy, is as complex as heterophagy. On the basis of the size of the segregated material a distinction can be made between "macroautophagy" and "microautophagy".

In the early stage of macroautophagy, large intracellular components (mitochondria, membrane fragments) are sequestered in autophagosomes by enclosing the cellular material. The limiting membranes of these autophagosomes are derived from the endoplasmic reticulum (Dunn, 1990a), subsequently a fusion with endosomes occurs that are transformed to lysosomes (Tooze et al., 1990; Dunn, 1990b; Punnonen et al., 1993). Smaller cytosolic components, like glycogen particles and soluble proteins appear to be taken up by microautophagy, a process whereby the endosomal/lysosomal membrane invaginates. Multiple events of microautophagy result in endosomes referred to as "multivesicular bodies" (Marzella and Glaumann, 1987). Studies from Kopitz and coworkers (Kopitz et al., 1990) yielded similar sequestration and degradation rates for normal cytosolic proteins with varying half-lives, suggesting that microautophagy occurs by a nonselective bulk process. Furthermore, it has been proposed that degradation of internalized EGF-receptor and perhaps other selected integral proteins of the endocytic boundary is established by multiple events of microautophagy (Hopkins, 1992), mediated by distinct sequences in the carboxyl terminus regulatory region (Opresko et al., 1995). Advantages of autophagy are cell rejuvenation and adaptability, but this process is

primarily a response to food deprivation.
A special kind of autophagy is named "crinophagy". This process occurs in gland cells as a result of fusion between secretory granules and lysosomes. The destruction of secretory material may serve as a mechanism for modulating the level of secretory material available for export (Farquhar, 1969). Secretory proteins can also be degraded at the level of the endoplasmic reticulum (ER) by direct conversion of ER cisternae into lysosomes (Noda and Farquhar, 1992).
An alternative route for entering lysosomes was recently reported for proteins that contain a KFERQ-like peptide sequence. It was suggested that a specific transporter in the lysosomal membrane recognizes these signals and transports these cytosolic proteins in a highly selective manner directly across the lysosomal membrane (Chiang et al., 1989; Dice, 1990).
A second alternative substrate entry pathway was described by Rome and coworkers (Rome et al., 1983; Bame and Rome, 1985). The lysosomal membrane enzyme, acetyl CoA:α-glucosaminide N-acetyltransferase (Klein et al., 1978) is responsible for transferring acetyl groups from cytosolic acetyl CoA across the lysosomal membrane whereafter they are bound to the terminal α-linked glucosamine residues of heparan sulfate that are localized in the lysosomal lumen.

Acid hydrolases in the lysosomes convert their substrates by irreversible reactions. This degradation occurs often by the sequential stepwise removal of components such as sugars from glycolipids, glycoproteins and proteoglycans. The basic building blocks formed by the digestive process, such as amino acids, sugars and nucleotides, diffuse or are transported by special carrier systems across the lysosomal membrane into the cytoplasm, where they can be reutilized (reviewed by Pisoni and Thoene, 1991). The products of digestion that cannot be cleared ultimately accumulate in the lysosomes forming structures called "dense bodies".

I.3. BIOSYNTHESIS AND TARGETING OF LYSOSOMAL PROTEINS

Lysosomal enzymes are glycoproteins which are synthesized on membrane-bound ribosomes and translocated into the lumen of the endoplasmic reticulum. For the majority of the lysosomal enzymes this process is mediated by an amino-terminal signal peptide (reviewed by Rapoport, 1991; Ng and Walter, 1994). Proteins lacking signal peptides can be translocated across the endoplasmic reticulum membrane by

means of a nascent-polypeptide-associated complex (NAC; Wiedmann et al., 1994). After translocation the signal peptide is proteolytically removed cotranslationally by a signal peptidase (Von Heyne, 1983). In the rough endoplasmic reticulum (RER) N-linked glycosylation starts with cotranslational transfer en bloc of a large high mannose oligosaccharide ($Glc_3Man_9GlcNAc_2$) from a lipid carrier (dolichol). During transport through the RER, the precursor is folded and the oligosaccharide undergoes a first trimming modification i.e. removal of glucose. In the Golgi complex further posttranslational modifications occur (Goldberg, 1987).

mannose 6-phosphate dependent pathway

Most (soluble) lysosomal enzymes obtain subsequently phosphorylated mannose residue(s). The latter is accomplished in a two step reaction: N-acetylglucosamine-1-phosphate binding to mannose residues and removal of N-acetylglucosamine. The enzymes responsible for these reactions are N-acetyl-glucosamine-phosphotransferase and N-acetylglucosamine-1-phosphodiester N-acetylglucosaminidase, respectively (Goldberg, 1987). The arising mannose 6-phosphate residue acts as a recognition marker for the mannose 6-phosphate receptor (MPR) (Kornfeld and Mellman, 1989; Kornfeld, 1992).

Two MPR's have been identified (reviewed by Kornfeld, 1992); a large (275 kDa) receptor, independent of divalent cations (CI-MPR; Sahagian, 1981) which is identical to the insulin-like growth factor II receptor and a small (46 kDa) receptor, that binds ligands in a divalent cation dependent manner (CD-MPR; Hoflack and Kornfeld, 1985). The receptors share structural homology and their coding sequences are derived from a common ancestor gene. Both MPR's are localized predominantly in the TGN, however, their steady state distribution may differ between different cell types (Geuze et al., 1984 and 1985; Willemsen et al., 1988; Griffiths et al., 1988; Bleekemolen et al., 1988). MPR's are also present at the plasma membrane and in endosomes, although differences for both receptors in endosomal distribution are reported (Klumperman et al., 1993). Targeted disruption of the CD-MPR gene in mice exhibited partial missorting of lysosomal enzymes and subsequent increased secretion of multiple lysosomal enzymes in serum (Köster et al., 1993; Ludwig et al., 1993). This hypersecretion was partly compensated by significant uptake mediated by carbohydrate-specific receptors (Köster et al., 1994). Fibroblasts prepared from embryos lacking the CI-MPR also exhibit a partial missorting of several lysosomal enzymes. However, the secreted hydrolases belong to a different subset of lysosomal enzymes compared

to the secreted hydrolases from CD-MPR-negative fibroblasts (Ludwig et al., 1994). These results clearly demonstrate that both receptors are required for an efficient intracellular targeting of lysosomal enzymes to lysosomes, and that they interact with different but overlapping classes of hydrolases. Embryonic fibroblasts lacking both MPR's revealed completely impaired targeting of multiple lysosomal enzymes (Ludwig et al., 1994).

It has now been generally accepted that both MPR's bind mannose 6-phosphate containing lysosomal proteins in the TGN. Receptor-ligand complexes are clustered in clathrin-coated pits, that pinch off from the TGN. Clustering of both MPR's and thus efficient sorting in TGN is dependent of di-leucine motifs in the cytoplasmic tail, that are believed to mediate the interaction with HA-1 adaptor complexes of clathrin-coated pits of the TGN (Johnson and Kornfeld, 1992a and 1992b). Interestingly, these di-leucine signals were unable to promote receptor-mediated endocytosis at the plasma membrane (Lobel et al., 1989). In addition, the tyrosine containing internalization signal of the CI-MPR can mediate targeting to coated pits of the TGN as well (Johnson and Kornfeld, 1992a). This phenomenon of two sorting signals for lysosomal targeting has also been described for CD3 chains (Letourneur and Klausner, 1992). Recently a third function of cytoplasmic tyrosine-containing motifs was assigned in mediating TGN localization of TGN38, a resident TGN protein (Humphrey et al., 1993).
The coated vesicles lose their coat and fuse with the acidified endosomal compartment, where the receptor and ligand dissociate at the acidic pH. MPR's recycle to the TGN or the plasma membrane, while the lysosomal enzymes remain in the lumen of the endosome. The enzymes are then together with endocytosed proteins transported to lysosomes (Kornfeld and Mellman, 1989; Ludwig et al., 1991). Thus, lysosomes are virtually devoid of MPR's.
Newly synthesized lysosomal enzymes with mannose 6-phosphate residues that were not sorted out in the TGN by the MPR's and instead secreted can bind to the CI-MPR present at the plasmamembrane (Kornfeld, 1986). These lysosomal enzymes are captured via receptor-mediated endocytosis and transported by the endosomal system to lysosomes. The CD-MPR is unable to bind ligands at neutral pH at the plasma membrane and is therefore not involved in this process (Stein et al., 1987; Tong and Kornfeld, 1989).

mannose 6-phosphate independent pathways

As described above MPR's play a major role in targeting of newly synthesized lysosomal enzymes, however, additional or alternative transport routes have been considered.

The following observations suggested the existence of alternative MPR independent pathways. In cultured fibroblasts from Mucolipidosis II (I-cell disease) and Mucolipidosis III patients (reviewed by Kornfeld and Sly, 1995) a near normal activity (Leroy et al., 1972; Hickman and Neufeld, 1972; Vladutiu and Rattazzi, 1975) and localization (Van Dongen et al., 1984; Van Dongen et al., 1985; Parenti et al., 1987) was observed for some lysosomal enzymes (e.g. acid phosphatase, glucocerebrosidase). In these disorders the formation of the mannose 6-phosphate recognition marker is defective, because the enzyme N-acetylglucosamine-1-phosphotransferase is deficient. A characteristic feature of both disorders is the secretion of many lysosomal enzymes, since these proteins are not effectively sorted in the TGN by the MPR's (Kornfeld, 1986). For acid phosphatase, an integral membrane protein, it was demonstrated in transfected BHK cells that molecules are probably transported to the plasma membrane via the constitutive secretory pathway. On arrival they cycle on average 15 times between the plasma membrane and the endosomal compartment before entering the lysosomes (Braun, 1989). Intralysosomal proteolytic cleavage revealed a soluble form of the enzyme (Gottschalk et al., 1989). The presence of a tyrosine residue in the cytoplasmic domain of acid phosphatase is necessary for a correct lysosomal targeting, presumably because the enzyme needs this aromatic residue to enter clathrin coated pits at the plasma membrane (Peters et al., 1990; Sosa et al., 1993).

The mechanisms by which glucocerebrosidase, a membrane-associated enzyme, is transported to lysosomes are not well characterized. Quantitative immunocytochemical studies on intestinal epithelial cells suggest a direct pathway from TGN to lysosomes (Willemsen et al., 1991), but details of this targeting are still unknown.

Importantly, liver tissue and leucocytes from I-cell disease patients have near normal activities of lysosomal enzymes, suggesting that there is another pathway for directing lysosomal enzymes to lysosomes in these cells and other tissues (Owada et al., 1982; Waheed et al., 1982). Perhaps other receptors , like mannose receptors or asialoglycoprotein receptors are used to capture secreted lysosomal enzymes and target them via receptor-mediated endocytosis to lysosomes.

A second indication for the existence of a mannose 6-phosphate independent pathway is the fact that glucocerebrosidase and integral

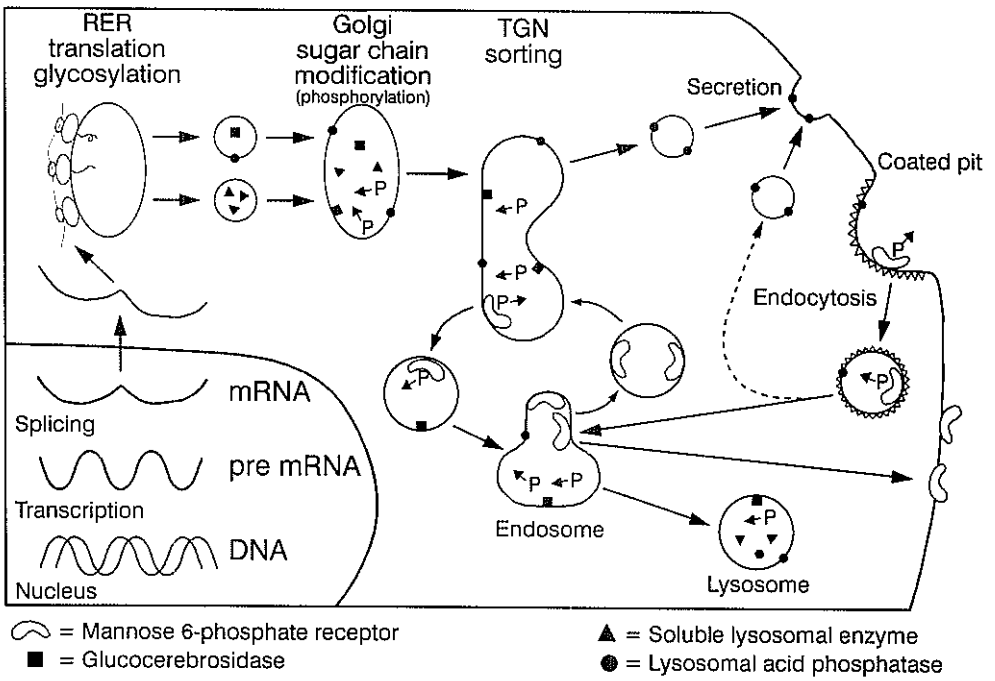


Figure 3. Simplified scheme showing the proposed intracellular transport pathways for soluble, membrane bound and membrane-associated lysosomal enzymes. (Adapted from Van der Ploeg, 1989).

lysosomal membrane proteins (lgp-A and lgp-B group, acid phosphatase) do not contain a mannose 6-phosphate recognition marker on their N-linked oligosaccharides in normal cells (Krentler et al., 1986; Barriocanal et al., 1986; Waheed et al., 1988; Howe et al., 1988; Aerts et al., 1988; Granger et al., 1990).

The pathway by which lysosomal membrane proteins are delivered to lysosomes is still controversial (reviewed by Fukuda, 1991; Peters and Von Figura, 1994; Sandoval and Bakke, 1994a). The presence of lysosomal membrane proteins from the lgp-A as well as the lgp-B group at the plasma membrane (Lippincott-Schwartz and Fambrough, 1987; Carlsson and Fukuda, 1992; Mathews et al., 1992) and the fact that the plasma membrane expression was increased by mutations affecting the cytoplasmic tyrosine domain (Williams and Fukuda, 1991; Harter and Mellman, 1992) would favor a transport pathway for at least part of the lgp's from the TGN via the plasma membrane and endosomal system to lysosomes, as described for lysosomal acid phosphatase. However, it can

not be excluded that this tyrosine internalization residue is also necessary for sorting in clathrin-coated buds from the TGN. Mutation of this tyrosine might result in missorting and thus transport via the constitutive pathway to the plasma membrane. Lysosomal targeting of LimpII, an integral lysosomal membrane glycoprotein lacking a tyrosine residue, is mediated by a specific extended configuration of a leucine-isoleucine motif in the cytoplasmic tail (Ogata and Fukuda, 1994; Sandoval et al., 1994b). This motif can function also for rapid internalization.
Alternatively, kinetic studies on lysosomal membrane proteins have suggested a direct pathway from the TGN to lysosomes via endosomes (D'Souza and August, 1986; Green et al., 1987). Altering the tyrosine internalization signal in lgp-A blocks the direct intracellular route to lysosomes too, which implicates that sorting in the TGN for lgp-A requires also tyrosine containing signals (Harter and Mellman, 1992).

I.4. Lysosomes and storage diseases

The concept of a lysosomal storage disease was for the first time described in 1963 by Hers (Hers, 1963) for a disorder called glycogenosis type II, caused by the deficiency of acid α-glucosidase. The deficiency of this enzyme leads to accumulation of glycogen in lysosomes from skeletal muscle and heart. At the present time more than 30 different lysosomal storage disorders have been reported, nearly all of which are inherited in an autosomal recessive manner. Thusfar, two X-linked lysosomal storage disorders are known i.e. mucopolysaccharidosis II (Hunter's disease) and Fabry's disease. The incidence at birth of lysosomal disorders varies from 1 in 100,000 to 1 in 250,000. However, in certain ethnic groups like Ashkenazi Jews the incidence of some lysosomal disorders like Gaucher disease (1 in 625) and Tay-Sachs disease (1 in 3,000) is much higher. The total group of lysosomal storage disorders has an incidence of approximately 1:5,000 to 1:10,000 (Galjaard and Reuser, 1984; Reuser et al., 1994).
Most lysosomal enzyme deficiencies result in the dysfunction of many organs, though in a different degree. Microscopic studies often reveal abnormalities in shape, size and content of the lysosomal system. These abnormalities are sometimes specific for a certain group or a single lysosomal storage disease (Scriver et al., 1995).
The clinical presentation among lysosomal disorders is extremely heterogeneous. Also within each of the diseases clinical heterogeneity is often observed. Some patients have an early onset form with very severe

symptoms leading to serious handicaps and early death. Others have a milder variant of the disease with onset later in childhood or even (late) adulthood. A patient with a lysosomal enzyme deficiency may have an asymptomatic course. The etiology of clinical diversity has been an interesting area of research in lysosomal storage diseases.
In the past a correlation between clinical heterogeneity and biochemical parameters was sought. This has led to the "critical threshold" theory (Conzelmann and Sandhoff, 1983). In this model it is assumed that lysosomal storage develops when the residual activity of the mutant enzyme is below a critical threshold. Above the threshold the residual activity is by definition just sufficient to cope with the influx of substrate, and hence no storage, cell damage or clinical abnormalities occur. Differences in the residual enzyme activity below a critical threshold could significantly affect the severity of pathological and clinical features, but differences in levels of enzyme activity above the threshold would have no effect.
More recently, studies on clinical heterogeneity have focused on the allelic diversity by analysis of the genotype of patients with different variants of a specific lysosomal storage disease. The majority of the gene defects in lysosomal storage diseases are point mutations. Most patients appear to be compound heterozygotes i.e. they have different mutations in their alleles. However, in some patients deletions of nucleotide(s) or exons, insertions of nucleotides and splicing defects have been observed. Furthermore, other factors such as genetic background or non-genetic factors may influence the clinical heterogeneity observed in patients suffering from a lysosomal storage disease.
As described in section I.3., synthesis, maturation and correct targeting of lysosomal enzymes involve transcription, translation and often post-translational modifications. In principle, dysfunction of a lysosomal enzyme can be caused by defects in any of these steps. These defects can be caused by mutations in the gene of a lysosomal enzyme, that leads to:

1. No or erroneous transcription, resulting in absence or reduced levels of mRNA or unstable mRNA and as a consequence no protein synthesis.
2. Other mutations might result in enhanced degradation of newly synthesized protein in the RER or lysosomes, because the protein is not folded correctly.
3. Mutations that result in impaired post-translational modification can lead to incorrect compartmentalization or secretion, enhanced degradation, or impaired function in the lysosomes.
4. Dysfunction of proteins involved in post-translational modification of

lysosomal enzymes can also give rise to a lysosomal storage disorder (Kornfeld and Sly, 1995).

5. The primary defects of most lysosomal storage diseases involve the deficiency of a specific lysosomal enzyme. However, defects in proteins that influence either the stability of lysosomal enzyme(s), as in the case of the protective protein (D'Azzo et al., 1982), or the activity as a result of abnormalities of activatorproteins (Fürst and Sandhoff, 1992) may also result in a lysosomal storage disease.
6. The impaired export of low molecular weight degradation products out of the lysosomes by dysfunction of the carrier proteins which are located in the lysosomal membrane have recently been identified as a new group of lysosomal storage diseases (Mancini, 1991; Pisoni and Thoene, 1991).

In the next chapter the most common lysosomal storage disorder i.e. Gaucher disease will be discussed in more detail.

I.5. REFERENCES

Aerts JMFG, Schram AW, Strijland A, Van Weely S, Jonsson LMV, Tager JM, Sorrell SH, Ginns EI, Barranger JA, Murray GJ (1988). Glucocerebrosidase, a lysosomal enzyme that does not undergo oligosaccharide phosphorylation. Biochem Biophys Acta 964: 303-308.

Bame KJ, Rome LH (1985). Acetyl coenzyme A:alpha-glucosaminide N-acetyltransferase. Evidence for a transmembrane acetylation mechanism. J Biol Chem 260: 11293-11299.

Barriocanal JG, Bonifacino JS, Yuan L, Sandoval IV (1986). Biosynthesis, glycosylation, movement through the Golgi system, and transport to lysosomes by an N-linked carbohydrate-independent mechanism of three lysosomal integral membrane proteins. J Biol Chem 261: 16755-16763.

Bleekemolen JE, Stein M, Von Figura K, Slot JW, Geuze HJ (1988). The two mannose 6-phosphate receptors have almost identical subcellular distributions in U937 monocytes. Eur J Cell Biol 47: 366-372.

Braun M, Waheed A, Von Figura K (1989). Lysosomal acid phosphatase is transported to lysosomes via the cell surface. EMBO J 8: 3633-3640.

Brown VI, Greene MI (1991). Molecular and cellular mechanisms of receptor-mediated endocytosis. DNA and Cell Biol 10: 399-409.

Bu G, Schwartz AL (1994). Receptor-mediated endocytosis. In: The Liver: Biology and Pathobiology, third edition. Arias IM, Boyer JL, Fausto N, Jakoby WB, Schachter DA, Shafritz DA (eds.) Raven Press Ltd., New York pp. 259-275.

Carlsson SR, Fukuda M (1992). The lysosomal membrane glycoprotein lamp-1 is transported to lysosomes by two alternative pathways. Archives Biochem Biophys 296: 630-639.

Chiang H-L, Terlecky SR, Plant CP, Dice JF (1989). A role for a 70-kilodalton heat shock protein in lysosomal degradation of intracellular proteins. Science 246: 382-385.

Collawn JF, Stangel M, Kuhn LA, Esekogwu, Jing S, Trowbridge IS, Tainer JA (1990). Transferrin receptor internalization sequence YXRF implicates a tight turn as the structural recognition motif for endocytosis. Cell 63: 1061-1072.

Conzelmann E, Sandhoff K (1983/1984). Partial enzyme deficiencies: Residual activities and the development of neurological disorders. Dev. Neurosci 6: 58-71.

Corvera S, Chawla A, Chakrabarti R, Joly M, Buxton J, Czech MP (1994). A double leucine within the GLUT4 glucose transporter COOH-terminal domain functions as an endocytosis signal. J Cell Biol 126: 979-989.

D'Azzo A, Hoogeveen AT, Reuser AJJ, Robinson D, Galjaard H (1982). Molecular defect in combined ß-galactosidase and neuraminidase deficiency in man. Proc Natl Acad Sci USA 79: 4535-4539.

De Duve C, Pressman BC, Gianetto R, Wattiaux R, Appelmans F (1955). Tissue fractionation studies. Intracellular distribution patterns of enzymes in rat liver tissue. Biochem J 60: 604-716.

Desjardins M, Huber LA, Parton RG, Griffiths G (1994). Biogenesis of phagolysosomes proceeds through a sequential series of interactions with the endocytic apparatus. J Cell Biol 124: 677-688.

Dice JF (1990). Peptide sequences that target cytosolic proteins for lysosomal proteolysis. TIBS 15: 305-309.

D'Souza MP, August JT (1986). A kinetic analysis of biosynthesis and localization of lysosome-associated membrane glycoprotein. Archives Biochem Biophys 249: 522-532.

Dunn WA (1990a). Studies on the mechanisms of autophagy: Formation of the autophagic vacuole. J Cell Biol 110: 1923-1933.

Dunn WA (1990b). Studies on the mechanisms of autophagy: Maturation of the autophagic vacuole. J Cell Biol 110: 1935-1945.

Dunn KW, Maxfield FR (1992). Delivery of ligands from sorting endosomes to late endosomes occurs by maturation of sorting endosomes. J Cell Biol 117: 301-310.

Eberle W, Sander C, Klaus W, Schmidt B, Von Figura K, Peters C (1991). The essential tyrosine of the internalization signal in lysosomal acid phosphatase is part of a beta turn. Cell 67: 1203-1209.

Farquhar MG (1969). Lysosome function in regulating secretion: disposal of secretory granules in cells of anterior pituitary gland. In: Lysosomes in Biology and Pathobiology. Dingle JT, Tell HB (eds.). North Holland, Amsterdam pp. 462-482.

Fukuda M (1991). Lysosomal membrane glycoproteins. J Biol Chem 266: 21327-21330.

Fürst W, Sandhoff K (1992). Activator proteins and topology of lysosomal sphingolipid catabolism. Biochim Biophys Acta 1126: 1-16.

Galjaard H, Reuser AJJ (1984). Genetic aspects of lysosomal storage diseases. In: Lysosomes in biology and pathology. Dingle JT, Dean RT, Sly W (eds.). Elsevier Science Publishers BV pp. 315-345.

Geuze HJ, Slot JW, Strous GJAM, Hasilik A, Von Figura K (1984). Ultrastructural localization of the mannose 6-phosphate receptor in rat liver. J Cell Biol 98: 2047-2054.

Geuze HJ, Slot JW, Strous GJAM, Hasilik A, Von Figura K (1985). Possible pathways

for lysosomal enzyme delivery. J Cell Biol 101: 2253-2262.
Goldberg DE (1987). Biogenesis of lysosomal enzymes: oligosaccharide chains. In: Lysosomes their role in protein breakdown. Academic Press Inc., London. pp 163-191.
Gottschalk S, Waheed A, Schmidt B, Laidler P, Von Figura K (1989). Sequential processing of lysosomal acid phosphatase by a cytoplasmic thiol proteinase and a lysosomal aspartyl proteinase. EMBO J 8: 3215-3219.
Granger BL, Green SA, Gabel CA, Howe CL, Mellman I, Helenius A (1990). Characterization and cloning of lgp110, a lysosomal membrane glycoprotein from mouse and rat cells. J Biol Chem 265: 12036-12043.
Green SA, Zimmer K-P, Griffiths G, Mellman I (1987). Kinetics of intracellular transport and sorting of lysosomal membrane and plasma membrane proteins. J Cell Biol 105: 1227-1240.
Griffin FM, Griffin JA, Leider JE, Silverstein SC (1975). Studies on the mechanism of phagocytosis. I. Requirements for circumferential attachment of particle-bound ligands to specific receptors on the macrophage plasma membrane. J Exp Medicine 142: 1263-1282.
Griffiths G, Hoflack B, Simons K, Mellman I, Kornfeld S (1988). The mannose 6-phosphate receptor and the biogenesis of lysosomes. Cell 52: 329-341.
Griffiths G, Gruenberg J (1991). The arguments for pre-existing early and late endosomes. Trends in Cell Biol 1: 5-9.
Harter C, Mellman I (1992). Transport of the lysosomal membrane glycoprotein lgp 120 (lgp-A) to lysosomes does not require appearance on the plasma membrane. J Cell Biol 117: 311-325.
Hers HG (1963). Alpha-glucosidase deficiency in generalized glycogen storage disease (Pompe's disease). Biochem J 86: 11-16.
Hewlett LJ, Prescott AR, Watts C (1994). The coated pit and macropinocytic pathways serve distinct endosome populations. J Cell Biol 124: 689-703.
Hickman S, Neufeld EF (1972). A hypothesis for I-cell disease: Defective hydrolases that do not enter lysosomes. Biochem Biophys Res Commun 49: 992-999.
Hoflack B, Kornfeld S (1985). Purification and characterization of a cation dependent mannose 6-phosphate receptor from murine $P338_1$ macrophages and bovine liver. J Biol Chem 260: 12008-12014.
Hopkins CR, Gibson A, Shipman M, Miller K (1990). Movement of internalized ligand-receptor complexes along a continuous endosomal reticulum. Nature 346: 335-339.
Hopkins CR (1992). Selective membrane protein trafficking: vectorial flow and filter. TIBS 17: 27-31.
Howe CL, Granger BL, Hull M, Green SA, Gabel CA, Helenius A, Mellman I (1988). Derived protein sequence, oligosaccharides, and membrane insertion of the 120-kDa lysosomal membrane glycoprotein (lgp120): Identification of a highly conserved family of lysosomal membrane glycoproteins. Proc Natl Acad Sci, USA 85: 7577-7581.
Humphrey JS, Peters JP, Yuan LC, Bonifacino (1993). Localization of TGN38 to the trans-Golgi network: involvement of a cytoplasmic tyrosine-containing sequence. J Cell Biol 120: 1123-1135.
Johnson KF, Kornfeld S. (1992a). The cytoplasmic tail of the mannose 6-

phosphate/insulin-like growth factor-II receptor has two signals for lysosomal enzyme sorting in the Golgi. J Cell Biol 119: 249-257.
Johnson KF, Kornfeld S. (1992b). A His-Leu-Leu sequence near the carboxyl terminus of the cytoplasmic domain of the cation-dependent mannose 6-phosphate receptor is necessary for the lysosomal enzyme sorting function. J Biol Chem 267: 17110-17115.
Klein U, Kresse H, Von Figura K (1978). Sanfilippo syndrome type C: Deficiency of acetyl-CoA:alpha-glucosaminide N-acetyltransferase in skin fibroblast. Proc Natl Acad Sci USA 75: 5185-5189.
Klumperman J, Hille A, Veenendaal T, Oorschot V, Stoorvogel W, Von Figura K, Geuze HJ (1993). Differences in the endosomal distributions of the two mannose 6-phosphate receptors. J Cell Biol 121: 997-1010.
Kopitz J, Kisen GO, Gordon PB, Bohley P, Seglen PO (1990). Nonselective autophagy of cytosolic enzymes by isolated rat hepatocytes. J Cell Biol 111: 941-953.
Kornfeld S (1986). Trafficking of lysosomal enzymes in normal and disease states. J Clin Invest 77: 1-6.
Kornfeld S (1992). Structure and function of the mannose 6-phosphate/insulinlike growth factor II receptors. Annu Rev Biochem 61: 307-330.
Kornfeld S, Mellman I (1989). The biogenesis of lysosomes. Annu Rev Cell Biol 5: 4 83-525.
Kornfeld S, Sly WS (1995). I-cell disease and pseudo-Hurler polydystrophy: Disorders of lysosomal enzyme phosphorylation and localization. In: The metabolic and molecular bases of inherited disease, 7th edition. Scriver, Beaudet, Sly, Valle (eds.), McGraw-Hill pp.2495-2508.
Köster A, Saftig P, Matzner U, Von Figura K, Peters C, Pohlmann R (1993). Targeted disruption of the M_r 46.000 mannose 6-phosphate receptor gene in mice results in misrouting of lysosomal proteins. EMBO J 12: 5219-5223.
Köster A, Von Figura K, Pohlmann R (1994). Mistargeting of lysosomal enzymes in M_r 46.000 mannose 6-phosphate receptor-deficient mice is compensated by carbohydrate-specific endocytotic receptors. Eur J Biochem 22: 685-689.
Krentler C, Pohlmann R, Hasilik A, Von Figura K (1986). Lysosomal membrane proteins do not bind to mannose 6-phosphate-specific receptors. Biol Chem Hoppe-Seyler 367: 141-145.
Ktistakis NT, Thomas D'Nette, Roth MG (1990). Characteristics of the tyrosine recognition signal for internalization of transmembrane surface glycoproteins. J Cell Biol 111: 1393-1407.
Leroy JG, Ho MW, MacBrinn MG, Zielke K, Jacob J, O'Brien JS (1972). I-cell disease: Biochemical studies. Pediat Res 6: 752-757.
Letourneur F, Klausner RD (1992). A novel di-leucine motif and a tyrosine-based motif independently mediate lysosomal targeting and endocytosis of CD3 chains. Cell 69: 1143-1157.
Lippincott-Schwartz J, Fambrough DM (1987). Cycling of the integral membrane glycoprotein LEP100, between plasma membrane and lysosomes: kinetic and morphological analysis. Cell 49: 669-677.
Lobel P, Fujimoto K, Ye RD, Griffiths G, Kornfeld S (1989). Mutations in the cytoplasmic domain of the 275 kd mannose 6-phosphate receptor differentially alter lysosomal enzyme sorting and endocytosis. Cell 57: 787-796.

Ludwig T, Griffihs G, Hoflack B (1991). Distribution of newly synthesized lysosomal enzymes in the endocytic pathway of normal rat kidney cells. J Cell Biol 115: 1561-1572.

Ludwig T, Ovitt CE, Bauer E, Hollinshead M, Remmler J, Lobel P, Ruther U, Hoflack B (1993). Targeted disruption of the mouse cation-dependent mannose 6-phosphate receptor results in partial missorting of multiple lysosomal enzymes. EMBO J 12: 5225-5235.

Ludwig T, Munier-Lehmann H, Bauer U, Hollinshead M, Ovitt C, Lobel P, Hoflack B (1994). Differential sorting of lysosomal enzymes in mannose 6-phosphate receptor-deficient fibroblasts. EMBO J 13: 3430-3437.

Mancini GMS (1991). Lysosomal membrane transport. Thesis, Rotterdam.

Marzella L, Glaumann H (1987). Autophagy, microautophagy and crinophagy as mechanisms for protein degradation. In: Lysosomes their role in protein breakdown. Academic Press Inc. (London) Ltd. pp. 319-367.

Mathews PM, Martinie JB, Fambrough DM (1992). The pathway and targeting signal for delivery of the integral membrane glycoprotein LEP100 to lysosomes. J Cell Biol 18: 1027-1040.

Matter K, Yamamoto EM, Mellman I (1994). Structural requirements and sequence motifs for polarized sorting and endocytosis of LDL and Fc receptors in MDCK cells. J Cell Biol 126: 991-1004.

Murphy RF (1991). Maturation models for endosome and lysosome biogenesis. Trends in Cell Biol 1: 77-82.

Ng DTW, Walter P (1994). Protein translocation across the endoplasmic reticulum. Current Opinion in Cell Biology 6:510-516.

Noda T, Farquhar MG (1992). A non-autophagic pathway for diversion of ER secretory proteins to lysosomes. J Cell Biol 119: 85-97.

Novikoff AB (1964). GERL, it's form and function in neurons of rat spinal ganglia. Biol Bull 127: 358.

Ogata S, Fukuda M (1994). Lysosomal targeting of Limp II membrane glycoprotein requires a novel Leu-Ile motif at a particular position in its cytoplasmic tail. J Biol Chem 269: 5210-5217.

Opresko LK, Chang CP, Will BH, Burke PM, Gill GN, Wiley HS (1995). Endocytosis and lysosomal targeting of epidermal growth factor receptors are mediated by distinct sequences independent of the tyrosine kinase domain. J Biol Chem 270: 4325-4333.

Owada M, Neufeld EF (1982). Is there a mechanism for introducing acid hydrolases into liver lysosomes that is independent of mannose 6-phosphate recognition? Evidence from I-cell disease. Biochem Biophys Res Commun 105: 814-820.

Parenti G, Willemsen R, Hoogeveen AT, Verleun-Mooyman M, Van Dongen JM, Galjaard H (1987). Immunocytochemical localization of lysosomal acid phosphatase in normal and "I-cell" fibroblasts. Eur J Cell Biol 43: 121-127.

Pearse BMF, Robinson MS (1990). Clathrin, adaptors and sorting. Annu Rev Cell Biol 6: 151-171.

Peters C, Braun M, Weber B, Wendland M, Schmidt B, Pohlmann R, Waheed A, Von Figura K (1990). Targeting of a lysosomal membrane protein: A tyrosine-containing endocytosis signal in the cytoplasmic tail of lysosomal acid phosphatase is necessary and sufficient for targeting to lysosomes. EMBO J 9: 3497-3506.

Peters C, Von Figura K (1994). Biogenesis of lysosomal membranes. FEBS Letters 346: 108-114.

Pisoni RL, Thoene JG (1991). The transport systems of mammalian lysosomes. Biochim Biophys Acta 1071: 351-373.

Pitt A, Mayorga LS, Schwartz AL, Stahl PD (1992). Transport of phagosomal components to an endosomal compartment. J Biol Chem 267: 126-132.

Punnonen E-A, Autio S, Kaija H, Reunanen H (1993). Autophagic vacuoles fuse with the prelysosomal compartment in cultured rat fibroblasts. Eur J Cell Biol 61: 54-66.

Rapoport TA (1991). Protein transport across the endoplasmic reticulum membrane: facts, models, mysteries. FASEB J 5: 2792-2798.

Reuser AJJ, Kroos MA, Visser WJ, Willemsen R (1994). Lysosomal storage disease: Cellular pathology, clinical and genetic heterogeneity, therapy. Ann Biol Clin 52: 721-728.

Robinson MS (1994). The role of clathrin, adaptors and dynamin in endocytosis. Current Opinion Cell Biol 6: 538-544.

Rome L, Hill DF, Bame KJ, Crain LR (1983). Utilization of exogenously added acetyl coenzyme A by intact isolated lysosomes. J Biol Chem. 258: 3006-3011.

Sahagian GG, Distler J, Jourdian GW (1981). Characterization of a membrane-associated receptor from bovine liver that binds phosphomannosyl residues of bovine testicular beta-galatosidase. Proc Natl Acad Sci, USA 78: 4289-4293.

Sahagian GG, Novikoff PM (1994). Lysosomes. In: The Liver: Biology and Pathobiology, third edition. Arias IM, Boyer JL, Fausto N, Jakoby WB, Schachter DA, Shafritz DA (eds.). Raven Press Ltd., New York pp. 275-291.

Sandoval IV, Bakke O (1994a). Targeting of membrane proteins to endosomes and lysosomes. Trends in Cell Biol 4: 292-297.

Sandoval IV, Arredondo JJ, Alcalde J, Noriega AG, Vandekerckhove J, Jimenez MA, Rico M (1994b). The residues Leu(Ile)^{475}-Ile(Leu, Val, Ala)^{476}, contained in the extended carboxyl cytoplasmic tail, are critical for targeting of the resident lysosomal membrane protein LIMP II to lysosomes. J Biol Chem 269: 6622-663.

Sandvig K, Van Deurs B (1994). Endocytosis without clathrin. Trends in Cell Biol 4: 275-277.

Scriver CR, Beaudet AL, Sly WS, Valle D eds. (1995). The metabolic and molecular bases of inherited disease, 7th edition, McGraw-Hill, New York.

Silverstein C, Greenberg S, Di Virgilio F, Steinberg H (1989). Phagocytosis. In: Fundamental immunology, second edition. Paul WE (ed.) Raven Press Ltd., New York pp. 703-720.

Smythe E, Warren G (1991). The mechanism of receptor-mediated endocytosis. Eur J Biochem 202: 689-699.

Sosa MA, Schmidt B, Von Figura K, Hille-Rehfeld A (1993). In vitro binding of plasma membrane-coated vesicle adaptors to the cytoplasmic domain of lysosomal acid phosphatase. J Biol Chem 268: 12537-12543.

Stein M, Zijderhand-Bleekemolen JE, Geuze H, Hasilik A, Von Figura K (1987). M_r 46.000 mannose 6-phosphate specific receptor: its role in targeting of lysosomal enzymes. EMBO J 6: 2677-2681.

Stoorvogel W, Strous GJ, Geuze HJ, Oorschot V, Schwartz AL (1991). Late endosomes derive from early endosomes by maturation. Cell 65: 417-427.

Tong PY, Kornfeld S. (1989). Ligand interactions of the cation-dependent mannose 6-

phosphate receptor. J Biol Chem 264: 7970-7975.
Tooze J, Hollinshead M, Ludwig T, Howell K, Hoflack B, Kern H (1990). In exocrine pancreas, the basolateral endocytic pathway converges with the autophagic pathway immediately after the early endosome. J Cell Biol 111: 329-345.
Trowbridge IS, Collawn JF (1993). Signal-dependent membrane protein trafficking in the endocytic pathway. Annu Rev Cell Biol 9: 129-161.
Van der Ploeg AT (1989). Glycogenosis type II. Thesis, Erasmus University Rotterdam
Van Dongen JM, Barneveld RA, Geuze HJ, Galjaard H (1984). Immunocytochemistry of lysosomal hydrolases and their precursor forms in normal and mutant human cells. Histochem J 16: 941-945.
Van Dongen JM, Willemsen R, Ginns EI, Sips HJ, Tager JM, Barranger JA, Reuser AJJ (1985). The subcellular localization of soluble and membrane-bound lysosomal enzymes in I-cell fibroblasts: a comparative immunocytochemical study. Eur J Cell Biol 39: 179-189.
Vladutiu GD, Rattazzi MC (1975). Abnormal lysosomal hydrolases excreted by cultured fibroblasts in I-cell disease (mucolipidosis II). Biochem Biophys Res Commun 67: 956-964.
Von Heijne G (1983). Patterns of amino acids near signal-sequence cleavage sites. Eur J Biochem 133: 17-21.
Waheed A, Pohlmann R, Hasilik A, Von Figura K, Von Elsen A, Leroy JG (1982). D efficiency of UPD-N-acetylglucosamine: lysosomal enzyme N-acetylglucosamine-1-phosphotransferase in organs of I-cell patients. Biochem Biophys Res Commun 105: 1052-1058.
Waheed A, Gottschalk S, Hille A, Krentler C, Pohlmann R, Braulke T, Hauser H, Geuze H, Von Figura K (1988). Human lysosomal acid phosphatase is transported as transmembrane protein to lysosomes in transfected baby hamster kidney cells. EMBO J 7: 2351-2358.
Watts C, Marsh M (1992). Endocytosis:what goes in and how? J Cell Science 103: 1-8.
Wiedmann B, Sakai H, Davis TA, Wiedmann M (1994). A protein complex required for signal-sequence sorting and translocation. Nature 370: 434-440.
Willemsen R, Kroos M, Hoogeveen AT, Van Dongen JM, Parenti G, Van der Loos CM, Reuser AJJ (1988). Ultrastructural localization of steroid sulphatase in cultured human fibroblasts by immunocytochemistry: a comparative study with lysosomal enzymes and the mannose 6-phosphate receptor. Histochem J 20: 41-51.
Willemsen R, Brünken R, Sorber CJW, Hoogeveen AT, Wisselaar HA, Van Dongen JM, Reuser AJJ (1991). A quantitative immunoelectronmicroscopic study on soluble, membrane-associated and membrane-bound lysosomal enzymes in human intestinal epithelial cells. Histochem J 23: 467-473.
Williams MA, Fukuda M (1990). Accumulation of membrane glycoproteins in lysosomes requires a tyrosine residue at a particular position in the cytoplasmic tail. J Cell Biol 111: 955-966.

CHAPTER II

GLUCOCEREBROSIDASE AND GAUCHER DISEASE

II.1. HISTORICAL PERSPECTIVE

In 1882, the French physician Philippe Charles Ernest Gaucher described the autopsy findings in a 32 year old patient with an enlarged spleen, that he concluded resulted from a primary splenic neoplasm (Gaucher, 1882). The familial occurrence of the disorder, which subsequently became known as Gaucher disease, was reported in 1901 (Brill, 1901). Three years later, the occurrence of Gaucher cells in the bone marrow was described (Brill, 1904), and 30 years later the "hyaline substance" in these cells was identified as glucosylceramide or glucocerebroside (Aghion, 1934). In 1920 the first case of Gaucher disease in an infant was reported (Kraus, 1920) and the severe neurologic involvement of the infantile form of the disease was emphasized in 1927 (Oberling and Woringer, 1927).

The primary metabolic defect of the disease was demonstrated in 1965 (Brady et al., 1965; Patrick, 1965) when the deficiency of the lysosomal enzyme glucocerebrosidase was identified in tissues from patients with Gaucher disease. The description of the primary defect and the availability of natural and artificial substrates provided the basis for enzymatic diagnosis of affected individuals and prenatal diagnosis of affected fetuses (Kampine et al., 1966; Beutler and Kuhl, 1970; Schneider et al., 1972). Increased levels of the neurotoxin glucosylsphingosine, a deacylated analogue of glucocerebroside, in neuronopathic variants was demonstrated in 1982 by Svennerholm and coworkers (Nilsson and Svennerholm, 1982).

Purification of glucocerebrosidase and the preparation of monospecific antibodies against the enzyme enabled investigators to discriminate the different molecular forms of the enzyme in healthy individuals and Gaucher patients (Ginns et al., 1982 and 1983; Barneveld et al., 1983) and to confirm its lysosomal membrane association in vivo (Willemsen et al., 1987). Cloning of the human gene encoding for glucocerebrosidase (Ginns et al., 1984; Sorge et al., 1985; Tsuji et al., 1986) made it possible to identify a large number of mutations that cause Gaucher disease (Beutler, 1993; Horowitz and Zimran, 1994). In addition, enzyme replacement protocols were developed using modified human placental glucocerebrosidase (Barton et al., 1991).

The identification of the human gene permitted the development of gene therapy strategies. The mouse glucocerebrosidase cDNA was described in 1989 (O'Neill et al., 1989). A mouse model for type 2 Gaucher disease has been generated by targeted disruption of the mouse glucocerebrosidase gene, also referred to as a "knock-out" or "null-allele" mouse (Tybulewicz et al., 1992).

II.2. CHARACTERISTICS OF GLUCOCEREBROSIDASE AND ITS INTERACTION WITH SAPOSINS

The human glucocerebrosidase gene is located on chromosome 1q21 and is comprised of 11 exons and 10 introns contained within 7604 base pairs. The promotor region is located approximately 150 base pairs upstream to exon 1. The gene has five potential N-glycosylation sites, but only four of the asparagine residues are glycosylated (Takasaki et al., 1984). Studies of normal and mutated cDNA's in Sf9 and COS-1 cells showed that Asn-462 was not glycosylated. In addition, these studies demonstrated that glycosylation of the first asparagine residue (Asn-19) is critical for the synthesis of a catalytically active enzyme (Berg-Fussman et al., 1993). Two functional ATG initiator codons have been identified (Sorge et al., 1987). The downstream ATG was translated more efficiently resulting in a 19-amino acid signal polypeptide which contains a hydrophobic core (Reiner et al., 1987). The cDNA contains 1548 base pairs encoding functional glucocerebrosidase. No transmembrane domains are identified in the protein, but short hydrophobic stretches in the carboxy-terminal part of the enzyme probably play a role in the membrane association of glucocerebrosidase (Barranger and Ginns, 1989). The enzyme's specific activity varies enormously between different cell types. This may reflect variation in protein synthesis or degradation (Doll and Smith, 1993). A glucocerebrosidase pseudogene has been identified 16 kB downstream from the functional gene. This pseudogene has many missense mutations and deletions that prevent synthesis of active enzyme (Horowitz et al., 1989).

The enzyme is synthesized in the endoplasmic reticulum and the signal peptide is cotranslationally cleaved by signal peptidase. The asparagine residues are core glycosylated (Erickson et al., 1985). The apparent molecular weight of the 63 kDa nascent polypeptide is increased to 66 kDa by converting the oligosaccharides predominantly to complex-type structures with terminal sialic acid residues. On the average 20% of human placental enzyme contain high-mannose chains (Takasaki et al., 1984). Glucocerebrosidase processing with complex carbohydrate occurs as the enzyme moves through Golgi cisternae. Differential permeabilization of pulse-labelled cells with saponin initially revealed a more soluble glucocerebrosidase that became membrane associated as the higher molecular form appeared (Rijnboutt et al., 1991). This would suggest that the membrane association occurs between exit from the endoplasmic reticulum and arrival in the TGN.

In the lysosomes further modifications of the enzyme oligosaccharide

chains by exoglycosidases occur, resulting in a decrease of the molecular weight to 59 kDa. Thus, after cotranslational cleavage of the signal peptide no additional proteolytic processing occurs in the post-translational modification of glucocerebrosidase. The transport mechanism from the TGN to the lysosomes is unknown. It has been demonstrated for other hydrolases that targeting to lysosomes can occur via a MPR-independent pathway (see chapter I). The membrane association of glucocerebrosidase might be essential for its correct lysosomal localization as was speculated in studies of the subcellular localization of soluble and membrane-bound lysosomal enzymes in I-cell fibroblasts (VanDongen et al., 1985). Comparative quantitative studies of soluble, membrane associated and membrane-bound lysosomal enzymes in human intestinal epithelial cells revealed a labelling pattern that was most compatible with a transport pathway leading directly from the TGN to the lysosomes (Willemsen et al., 1991).

There have been reports of glucocerebrosidase isoenzymes (Glew et al., 1988), including a non-lysosomal membrane-bound ß-glucosidase (CBE-insensitive) that hydrolyzes glucocerebroside at less acidic conditions than lysosomal glucocerebrosidase (CBE-sensitive). This CBE-insensitive enzyme is not deficient in patients with Gaucher disease (VanWeely et al., 1993). Discrimination between lysosomal and non-lysosomal glucocerebrosidase activity was achieved by pretreatment of cells with CBE. The biological function of the non-lysosomal enzyme is thusfar unknown.

The acid exohydrolases that degrade sphingolipids in the lysosomes work in concert with small nonenzymic glycoproteins, called "sphingolipid activator proteins" (SAP's) for effective interaction with the substrates (Sandhoff et al., 1995). The SAP's are encoded by two genes on chromosome 5 and 10, carrying the genetic information for the ganglioside G_{M2} activator and prosaposin, respectively. Proteolytic and glycosidic processing of the 70 kDa molecular form of prosaposin generates saposin A, B, C, and D. All four of these peptides contain conserved cysteine and proline residues (reviewed by O'Brien et al., 1991; Fürst and Sandhoff, 1992; Kishimoto et al., 1992). Saposin C enhances the in vivo hydrolysis of glucocerebroside by glucocerebrosidase and is one of the four heat-stable glycoproteins involved in spingolipid hydrolysis. The other three, saposin A, B and D, stimulate the hydrolysis of gluco- and galactocerebroside, sulfatides and ceramides (Klein et al., 1994), respectively. Effective reconstitution of glucocerebrosidase activity in vitro requires negatively charged phospholipids (e.g. bile salts or anionic lipids) or saposin C (spingolipid activator protein-2)(Ho and O'Brien, 1971). Saposin C stimulates glucocerebrosidase by forming a complex with the

enzyme. The formation of such aggregates might lead to conformational changes, thereby resulting in a more active enzyme. Other acidic lipids (e.g. phosphatidylserine) are also capable of forming aggregates and activate glucocerebrosidase, though to a lesser extent than saposin C. Using phosphatidylserine-containing vesicles it has been found that saposin C is also capable of destabilizing and fusing phospholipid-containing bilayers, thereby creating a situation by which glucocerebrosidase can insert into the membranes (Vaccaro, 1994).
Prosaposin is transported to the lysosomes in a MPR-dependent manner, which explains the deficiency of saposin C in I-cell fibroblasts (Fujibayashi and Wenger, 1985). Immunoelectronmicroscopic studies on the subcellular localization of saposin C demonstrated association of the activator protein with the lysosomal membrane (Paton et al., 1990). The importance of saposin C in the degradation of glucocerebroside is illustrated by the severe clinical manifestations resulting from the deficiency of the activator protein (Christomanou et al., 1989; Harzer et al., 1989).

II.3. CLINICAL PHENOTYPE

Gaucher disease is the most prevalent inherited lysosomal storage disease and belongs to the sphingolipidoses. This group of disorders results from deficiencies of lysosomal enzymes that degrade glyco-sphingolipids and sphingomyelin. On the basis of the presence and degree of neurologic involvement (Table 1), Gaucher disease has been classified into three main clinical types: type 1 (chronic, non-neuronopathic), type 2 (acute, neuronopathic) and type 3 (subacute, neuronopathic) (Beutler and Grabowski, 1995; Grabowski, 1993). All these clinical types are caused by a deficiency of glucocerebrosidase. In other instances a deficiency of saposin C has been reported to result in a glucocerebroside accumulation. Identification of the primary metabolic defect permitted studies in which a correlation was sought between protein defect and clinical phenotype. Cloning of the human gene led to the identification of at least 50 different mutations within the gene for glucocerebrosidase. Mutation analysis enabled investigators to study the relation between genotype and clinical phenotype.
The incidence of type 1 Gaucher disease is approximately 1:50,000 newborns in the general population, with variable onset of symptoms that may appear in childhood or remain unnoticed until far into adulthood. In general, the earlier the onset of the clinical symptoms the more severe

Table 1. Clinical manifestations of Gaucher disease.

	Type I (chronic, non neuronopathic)	Type 2 (acute, neuronopathic)	Type 3 (subacute neuronopathic)
Presentation	very heterogeneous	more stereotypic	heterogeneous
Age	presents at any age	presents at 0-6 months	usually in childhood
Progression	variable	death by age 2-3 years	neurologic abnormalities by adolescence
Common signs	hepatosplenomegaly bone involvement anemia/thrombocytopenia	hepatosplenomegaly seizures trismus strabismus hyperreflexia	hepatosplenomegaly bone involvement supranuclear ophthalmoplegia *rare:* slowly progressive dementia, seizures and spasticity
Genetics	. autosomal recessive . increased incidence . among Ashkenazim	autosomal recessive no ethnic predilection rare	autosomal recessive panethnic with a Norrbottnian subgroup rare

the course of the disease. The phenotypic variability of type 1 Gaucher disease ranges from asymptomatic or mildly affected patients to children with massive hepatosplenomegaly, skeletal abnormalities and sometimes fatal complications. The development of a "knock-out" mouse model of Gaucher disease led to the emphasis of a severely affected type 2 Gaucher phenotype in human neonates, with clinical manifestations that may include ichthyotic skin and/or hydrops fetalis as associated symptoms (Sidransky et al., 1992).

Clinical phenotype in relation to protein defect

Glucocerebroside (or glucosylceramide), the major natural substrate for glucocerebrosidase, is synthesized from ceramide and UDP-glucose by glucosylceramide synthase (Glew et al., 1988) and is primarily present in cell membranes. The sphingolipid also arises as an intermediate in the degradation pathway of glycosphingolipids, such as globosides and gangliosides.

Glucocerebrosidase catalyzes the hydrolyses of the ß-glucosidic bond in glucosylceramide (figure 4, arrow) to yield glucose and ceramide, the latter being degraded by lysosomal acid ceramidase to sphingosine and fatty acid (Beutler and Grabowski, 1995; Grabowski, 1993a). The fatty acid chain length varies, the most abundant component being stearic acid (C_{18}) in brain, and C_{20} to C_{24} fatty acids in spleen, liver and blood cells.

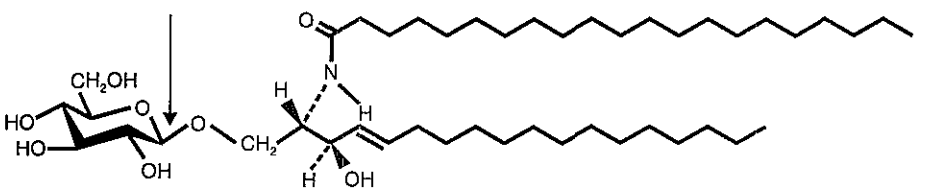


Figure 4. Structure of glucocerebroside.

Although every cell in Gaucher patients is deficient in glucocerebrosidase the accumulation of glucocerebroside is most pronounced in macrophages of the mononuclear phagocyte system (reticulo-endothelial system). An important reason for this selective storage is the degradation of membranes of senescent red and white blood cells by tissue macrophages. However, it can not be excluded that uptake of lipoproteins, the carrier

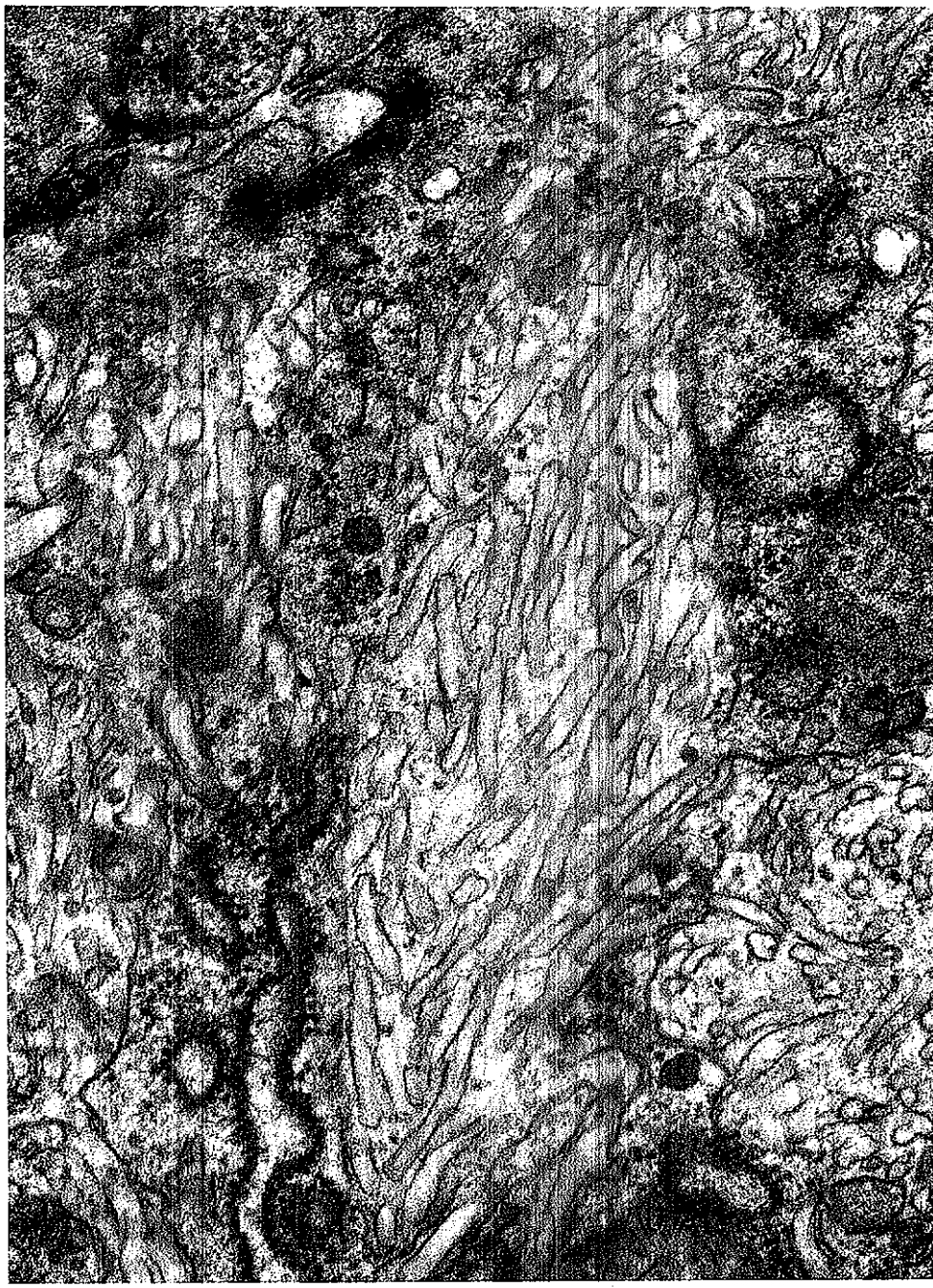


Figure 5. High magnification electronmicrograph illustrating typical Gaucher cell inclusion bodies which are filled with the characteristic tubule-like structures.

of plasma glucocerebroside, by macrophages contributes to the high level of storage material in these cells. The glucocerebroside that accumulates in macrophages gives the cytoplasm of these cells a characteristic striated appearance characteristic of "Gaucher cells". These cells, which are the hallmark of the disease, are 20-100 µm in diameter and contain one or more nuclei often in an eccentric location. At the subcellular level it was demonstrated that the highly hydrophobic glucocerebroside molecules stored in the lysosomes give rise to bilayers that stack up as membranous sheets (see figure 5). It has been suggested that storage material can leak out from lysed Gaucher cells into the extracellular space (Pennelli et al., 1969; Hibbs et al., 1970) and in this way be the source of 2-20-fold elevated plasma levels of glucocerebroside in Gaucher patients. It has been demonstrated that glucocerebroside can act as a growth factor (Radin, 1994). This stimulated cell division might explain the enlargment of spleen and liver in Gaucher patients. Increased secretion of lysosomal hydrolases and cytokines by stimulated macrophages as a response to extensive storage may contribute to the pathogenesis of the disease (Moffitt et al., 1978; Gery et al., 1981). Furthermore, it has been observed that hypersplenism can result from the striking increase in glycolipid turnover from blood cell membranes. It is likely that the rate of influx of substrate, the half-life of the enzyme, the lysosomal pH and the expression of saposin C in lysosomes (VanWeely et al., 1993) all influence the quantity of stored material and in this way contribute to the heterogeneity of clinical manifestations. Also the inability of Gaucher cells to perform the normal functions of macrophages may contribute to the severity of the disease.

Studies on the processing of glucocerebrosidase in cultured fibroblasts from Gaucher patients indicate that in type 1 Gaucher disease normal processing occurs, producing a lysosomal 59 kDa mature form with reduced activity to artificial substrate. In type 2 and 3 Gaucher fibroblasts only precursor (63 kDa and 66 kDa) and no mature (59 kDa) form of the enzyme could be detected. Lysosomes contained very little cross-reactive material, while RER and TGN were normally labelled (Ginns et al., 1982 and 1983; Willemsen et al., 1987). These results suggest that in type 2 and 3 Gaucher fibroblasts the enzyme either fails to reach the lysosome or is rapidly degraded within the lysosomes, and support the hypothesis of a deficient processing of glucocerebrosidase in type 2 and type 3 Gaucher disease. It is also suggested that the presence of sufficient residual activity in the lysosomes might preclude or postpone development of neurologic disease (Tsuji et al., 1987; Willemsen et al., 1987). In this respect it is of interest that a correlation between the residual glucocerebrosidase activity

in cultured fibroblasts and the clinical type of Gaucher disease was demonstrated, using short-acyl-chain sphingolipid analogues as substrate (Meivar-Levy et al., 1994).
Many patients develop skeletal manifestations with episodic bone crises during childhood and adolescence. A characteristic feature of skeletal changes is the "Erlenmeyer flask" appearance resulting from expansion of the bony cortex. The pathophysiology of bone lesions is largely unknown, but it is suggested that infiltration of Gaucher cells into the bone marrow may cause mechanical obstruction, replacement of normal marrow cells and toxic foci (Beutler and Grabowski, 1995). Alternatively, an imbalance in calcium homeostasis by reduced absorption of vitamin D and calcium has been suggested to play a role in the pathogenesis of bone disease (Mankin et al., 1990).
Reports on the central nervous system involvement in Gaucher disease are limited. In type 2 Gaucher patients microscopic abnormalities, including gliosis, neuronal loss and free Gaucher cells within brain parenchyma and perivascular spaces has been demonstrated (Norman et al., 1956; Banker et al., 1962; Adachi et al., 1967; Espinas and Faris, 1969; Hernandez et al., 1973; Kaga et al., 1982; Lacey and Terplan, 1984; Kaye et al., 1986). It is notable that the fatty acid of stored glucocerebroside in type 2 brain is predominantly stearic acid (C_{18}), which is the major fatty acid in gangliosides and probably derives from ganglioside turnover. In type 3 Gaucher patients less severe central nervous system manifestations than seen in type 2 are generally observed (Conradi et al., 1984; Kaye et al., 1986). In cases with numerous Gaucher cells in the brain of type 3 patients the proportion of C_{18} fatty acids is less than normal while C_{24} fatty acids are increased. This might be explained by circulating glucocerebroside from extracerebral sources being taken up by perivascular Gaucher cells (Nilsson and Svennerholm, 1982). This would be in agreement with the observation that increased concentrations of glucocerebroside in plasma caused by splenectomy in type 3 patients result in enhanced accumulation of storage material and a larger number of Gaucher cells in brain (Conradi et al., 1984) and impaired neurological function (Blom and Erikson, 1983). Neurons with characteristic storage material were encountered only sporadically (Banker et al., 1962; Adachi et al., 1967; Conradi et al., 1984).
In the brain the presence of cytotoxic substances may play a major role in the pathogenesis of type 2 and 3 Gaucher disease. In particular, the role of the neurotoxin glucosylsphingosine (glucosyl-psychosine), mainly derived from deacylation of glucocerebroside (Yamaguchi et al., 1994) has been studied extensively. Nilsson and Svennerholm (1982) found

significant elevated levels of this neurotoxin in brain from type 2 and 3 patients. Furthermore, lysosphingolipids, including glucosylsphingosine, inhibit protein kinase C activity and phorboldiester binding at very low concentrations, and might interfere with signal transduction and cellular differentiation processes (Hannun and Bell, 1987). This may disrupt neuronal function and cause neuronal cell death. It is conceivable that the failure to visualize significant amount of storage material in lysosomes of neurons is the result of low concentrations of the neurotoxin causing neuronal dysfunction or neuronal cell death before discrete pathological changes are seen. Also the extrusion of storage material from Gaucher cells within the brain parenchyma might contribute to local neuronal loss. The absence of neurological manifestations in patients with type 1 Gaucher disease may be, because they have sufficient residual enzyme activity within the lysosomes of neurons to cope with the ganglioside turnover in the brain during development.

Clinical phenotype in relation to genotype

All three phenotypes of Gaucher disease are caused by a deficiency of glucocerebrosidase. In addition, glucocerebroside storage has been reported as the result of mutations in saposin C and prosaposin (Harzer et al., 1989; Christomanou et al., 1989; Schnabel et al., 1991). The broad clinical heterogeneity among Gaucher patients is attributed to more than 50 different mutations within the gene for glucocerebrosidase, including missense, splice site, frame shift, and deletion mutations (reviewed by Beutler, 1993; Horowitz and Zimran, 1994). Thusfar, mutations have been found throughout the glucocerebrosidase gene. In the next section the four most common mutations will be discussed.

Mutation N370S

This G to A mutation at nucleotide 1226 of the cDNA of glucocerebrosidase, results in the substitution of the amino acid serine for asparagine. Accounting for 70-75% of the mutated alleles among Jewish Gaucher patients and 23% in non-Jewish patients, it is the most prevalent mutation among Gaucher patients. The presence of the N370S mutation in one or both alleles seems to preclude the development of neurologic involvement and is therefore most predictive of type 1 Gaucher disease. Approximately 90% of the patients who are homozygous for this mutation have a late onset of symptoms and a mild clinical course (Zimran et al., 1989 and 1992). It has been estimated that up to 66% of individuals homozygote for the N370S mutation are asymptomatic

(Zimran et al., 1991; Beutler et al., 1993). A moderate to severe clinical course was found in only 5% of the patients homozygous for N370S. In a recent study using lymphoblasts and fibroblasts from type 1 patients the relationship between intralysosomal pH and catalytic efficiency of the mutant N370S enzyme was investigated. Near normal kinetic properties of the enzyme were found if the assay was performed at the appropriate pH and in the presence of saposin C and phosphatidylserine, indicating a role of the pH in determining the state of activation of mutant glucocerebrosidase (VanWeely et al., 1993). Overexpression of cDNA carrying the N370S mutation in Sf9 insect cells revealed a stable glucocerebrosidase protein with decreased catalytic rate constants and reduced binding to active site-directed inhibitors (Grace et al., 1994).

Mutation 84GG

This insertion of an extra G at nucleotide 84 of the cDNA is the second most common mutation among Ashkenazi Jewish patients (13%), whereas in the non-Jewish populations it account for less than 1% of the mutations. This produces a frameshift causing a premature stopcodon, and results in the production of unstable mRNA and complete lack of enzyme protein. In the heteroallelic state this mutation is usually associated with type 1 Gaucher disease with severe clinical manifestations (Horowitz and Zimran, 1994). Patients homozygous for this "null" mutation have not been found. Homozygosity for 84GG may therefore be lethal in utero or shortly after birth, like the phenotype of the type 2 Gaucher mouse created by targeted disruption of the murine glucocerebrosidase gene (see appendix paper III).

Mutation L444P

This T to C substitution at nucleotide 1448 of the cDNA, results in a proline instead of leucine. It accounts for 50% of the mutated alleles in patients with neuronopathic Gaucher disease, but this mutation is also found in 30% of alleles in type 1 non-Jewish Gaucher patients, mostly in a heteroallelic state. All patients homozygous for the L444P mutation developed type 2 or type 3 Gaucher disease (Dahl et al., 1988), whereas type 2 patients can also be heterozygous for the mutation. Homozygosity of L444P in the Japanese population is associated with non-neuronopathic Gaucher disease (Masuno et al., 1990).

Overexpression of the mutant cDNA in Sf9 insect cells resulted in the synthesis of unstable enzyme with very low catalytic activity and lack of stimulation by saposin C (Grace et al., 1994).

Mutation IVS2+ 1
This splice site mutation due to a G to A transition of the first nucleotide of the second intron destroys the 5' donor splice site. The low levels of aberrant mRNA's do not result in synthesis of active enzyme. It accounts for 1-3% of the mutated alleles among Jewish and non-Jewish Gaucher patients. The mutation is found in patients with both non-neuronopathic and neuronopathic Gaucher disease. Most patients with type 1 Gaucher disease carrying the genotype N370S/IVS2+ 1 developed moderate to severe Gaucher disease.

It has been hoped that mutation analysis might permit both the reliable detection of carriers and the prediction of the clinical phenotype and course from the genotype. The first application is important for genetic counseling in families at risk, particular in the Ashkenazi Jewish population. The second application is arduous due to the phenotypic variability that exists among patients with the same genotype and vice versa. This variability should be appreciated in counseling Gaucher patients (Sidransky et al., 1994).

II.4. THERAPY

For the vast majority of heritable diseases, including the lysosomal storage disorders, there is no cure or adequate therapeutic treatment. The ultimate goal in treating a patient with a lysosomal storage disorder would be to provide sufficient enzymic activity in the lysosomes of affected cells. During a century of research on Gaucher disease several attempts have been made to successfully treat patients. Often the treatment has been directed toward symptomatic relief rather than correcting the enzyme deficiency, and has included total or partial removal of the spleen, transfusions and orthopedic procedures. However, over the last decade a number of procedures have been attempted to address the accumulation of lipid per se. In this section some of these procedures such as organ transplantation, cell transplantation, enzyme replacement therapy and somatic gene therapy will be discussed.

Organ Transplantation
A number of attempts to treat Gaucher patients by organ transplantation have been undertaken. Kidney, spleen and liver transplantation were not successful in improving clinical symptoms of the disease (Groth et al., 1971; Desnick et al., 1973; Carlson et al., 1990; Starzl et al., 1993; Smanik

et al., 1993). In the case of other lysosomal storage diseases like Fabry disease it was also demonstrated that the transplanted organ did not create a source of sufficient amounts of exogenous enzyme that could be targeted to storage cells in the body or act as a substrate sink (Van denBergh et al., 1976). Organ transplantation may still be worthwhile in some cases.

Cell Transplantation

Transplantation of non-immunogenic amniotic membranes producing large quantities of lysosomal hydrolases, has been attempted as a therapy for several lysosomal storage disorders including mucopolysaccharidosis, GM_1 Morquio B, I-cell disease, metachromatic leukodystrophy, Farber disease, GM_1 gangliosidosis, Tay-Sachs disease, Niemann-Pick type B disease and type 3 Gaucher disease (Tylki-Szymanska et al., 1987; Yeager et al., 1985; Scaggiante et al., 1987; Sakuragawa et al., 1992). The clinical effects resulting from amniotic tissue transplantation have been variable. Clinical improvements were reported in patients with Niemann-Pick type B disease (Scaggiante et al., 1987) and type 3 Gaucher disease (Sakuragawa et al., 1992), whereas no significant improvements were observed in patients with other lysosomal storage disorders. Although there may be some clinical benefit for patients with certain lysosomal storage disorders the clinical use of amniotic tissue transplantats is still controversial and more research seems to be required to explore the efficacy of the procedure.

Bone marrow transplantation (BMT) has also been used as a source of normal enzyme. BMT was initially used for the treatment of hematologic malignancies and aplastic anemias. Over the last decade, however, there have been reports of approximately 150 patients with a lysosomal storage disease who underwent BMT (Krivit et al., 1992). In principle, BMT from a normal donor should provide a continuous source of a missing enzyme and should correct a metabolic defect by replacement of enzyme deficient cells with normal cells of hematopoietic origin. Uptake of secreted enzymes via receptor molecules at the plasma membrane or via enzyme transfer by cell to cell contact could occur (Bou-Gharios et al., 1993).

Since type 1 Gaucher disease primarily results from the accumulation of glucocerebroside in bone marrow-derived macrophages this type of Gaucher disease had been a good candidate for BMT, until enzyme replacement therapy became successful (Barton et al., 1991). Until now seven type 1 patients were reported with bone marrow transplants from histocompatible siblings. Two patients died and five less severely affected patients are doing well (Rappeport and Ginns, 1984; Hobbs et al., 1987).

Long-term follow up studies of BMT in six patients with type 3 Gaucher disease revealed a stable neurologic status (IQ between 112-120) (Erikson et al., 1990; Ringden et al., 1995). Even patients who became chimeras (20-80%) showed an excellent clinical outcome (Erikson et al., 1994; Chan et al., 1994). Autopsy of a type 3 patient, who died of sepsis two years after BMT, demonstrated few Gaucher cells in liver and lung and absence of these storage cells in brain. Neuropathic manifestations had not progressed and a complete reconstitution of enzymatic activity in visceral organs was observed. However, glucocerebrosidase activity in brain did not increase (Tsai et al., 1992).

There is no evidence that membrane-associated glucocerebrosidase can cross the blood-brain barrier and subsequently be taken up by neurons. Therefore the underlying mechanisms that cause arrest of nervous system deterioration are not understood. It cannot be ruled out that the presence of large numbers of Gaucher cells in the perivascular space may cause leakiness in the blood-brain barrier. Entrance into the brain parenchyma of cells from hematopoietic origin that secrete lysosomal hydrolases might also contribute to the stabilization of the neurologic status. Electron-microscopic studies of mucopolysaccharidosis type VII mice, twitcher mice, fucosidase deficient dogs and α-mannosidosis cats showed significant reduction after BMT in storage material in cells of non-hemopoietic origin in brain such as neurons (Hoogerbrugge et al., 1988; Birkenmeier et al., 1992; Taylor et al., 1992; Sands et al., 1993; Walkley et al., 1994; Poorthuis et al., 1994). Secreted normal enzyme may be taken up by cells of non-hemopoietic origin, like neurons and glia, as demonstrated by enzyme cytochemistry (Hoogerbrugge et al., 1988; Walkley et al., 1994), despite the low endocytotic activity of neuronal cells in vitro (Rattazzi et al., 1987). In addition, autologous transplanted retroviral marked mouse hematopoietic stem cells were identified in regions throughout the brain in which donor derived cells were shown to populate brain parenchyma as perivascular macrophages and microglia (Krall et al., 1994).

Reduction of storage material in blood might also lead to decreased levels of storage material in tissues as a result of a gradient of lipid between plasma and tissues (Barranger, 1984). Recent studies using lethally irradiated normal mice that were engrafted with fetal liver cells derived from the glucocerebrosidase-null mouse displayed no storage material in macrophages of the mononuclear phagocyte system one year after transplantation. This suggests that storage of glucocerebroside in lysosomes of glucocerebrosidase deficient macrophages can be prevented either by uptake of enzyme from the blood circulation or by maintenance of normal

levels of glucocerebroside in plasma (McKinney et al., 1995).
There are no data available on BMT in type 2 Gaucher patients. However, BMT in patients with other infantile forms of lysosomal storage disorders with central nervous system involvement, did not show clinical improvement or arrest of neurologic manifestations (Krivit et al., 1992). It is conceivable that irreversible neuronal damage develops during the first months of life.
Interestingly, very recently it was shown that engraftment of neural progenitor cells, after injection into the lateral ventricles, corrects lysosomal storage throughout the brain in the mucopolysaccharidosis type VII mouse (Snyder et al., 1995).

Enzyme Replacement Therapy

The suggestion by de Duve in 1964 that enzyme replacement might be useful in the treatment of lysosomal storage disorders has become reality for individuals with type 1 Gaucher disease (De Duve,1964; Brady and Barton, 1994a, 1994b and 1994c). The first attempts at enzyme therapy for Gaucher disease began with the development of purification techniques for glucocerebrosidase from human placenta (Pentchev et al., 1973). The initial clinical trials using human placental glucocerebrosidase were encouraging, but the subsequent experience was disappointing (Brady et al., 1974, 1977, 1980; Beutler et al., 1982). Uptake studies in rat liver showed that the purified human placental enzyme was predominantly targeted to parenchymal cells instead of the non-parenchymal cells (Furbish et al., 1978; Morrone et al.,1981). Further animal studies demonstrated that modification of the sugar chains into terminal mannose residues with exoglycosidases led to a significant increase of uptake by non-parenchymal liver cells, including Kupffer cells, via a mannose-specific receptor (Furbish et al., 1981). These observations resulted in the development of alglucerase (Ceredase™) and the effect of this modified enzyme on clinical manifestations was examined in twelve type 1 patients (Barton et al., 1991). In addition to reduction in organomegaly and improvement of anemia, skeletal improvement has been suggested, albeit the latter lags behind the other parameters (Mankin, 1993). Analysis of more than 250 sera of patients treated with alglucerase displayed that approximately 13% of the patients developed antibodies against the enzyme, although, the clinical effectiveness of alglucerase was retained (Richards et al., 1993).
The clinical trials with recombinantly produced glucocerebrosidase from CHO cells (Cerezyme™) have only recently started and the initial results

are promising, suggesting that it is as effective as alglucerase (Grabowski et al., 1995).

Alglucerase therapy has also been tried for neuronopathic Gaucher disease. In one case of type 2 Gaucher disease treated at 5 months of age with alglucerase infusions, no improvement of severe neurological manifestations were observed. Enzyme infusion therapy was stopped after seven months and the patient died four weeks later (Erikson et al., 1993). The severe neuronal damage developed in the first five months of life could not be reversed by alglucerase treatment, suggesting that enzyme therapy in type 2 patients if tried should be given well before neurological symptoms develop. The initial reports of alglucerase administration to type 3 Gaucher patients are encouraging (Zimran et al., 1993; Bembi et al., 1994) and clinical trials with recombinantly produced glucocerebrosidase in these patients have recently started.

More than 900 individuals worldwide are now successfully receiving alglucerase treatment. However, limited information is available about tissue, cellular and subcellular distribution of the enzyme. The half-life of alglucerase in Gaucher cells after infusion in humans is not well characterized (Whittington and Goa, 1992). This lack of pharmacokinetic and pharmacodynamic data is reflected in a controversy about the optimum dosage regimen and the efficacy of the treatment (Figueroa et al., 1992; Pastores et al., 1993; Hollak et al., 1993; Zimran et al., 1994). In a recent in vitro study it was reported that uptake of alglucerase at high enzyme concentrations by cultured macrophages is not efficient, and that endothelial cells are more effective in uptake of alglucerase (Sato and Beutler, 1993). The uptake of mannose terminated ligands by hepatic endothelial cells has already been reported using electronmicroscopic autoradiographic techniques (Hubbard et al., 1979).

It has also been suggested that the beneficial response to alglucerase therapy may be due at least in part, to the clearance of the storage material from the blood circulation (Sidransky et al., 1993). Electron-microscopic studies in mice and rats following intravenously administered alglucerase demonstrated that alglucerase is targeted to the endosomal/lysosomal system of both Kupffer cells and endothelial cells. The contribution of endothelial cell uptake of alglucerase to the beneficial response to therapy observed in Gaucher patients is still unknown, but the issue appears to be more complex than previously recognized (Willemsen et al., 1995; Murray and Jin, 1995). Major disadvantages of alglucerase therapy include high cost ($200,000 per patient per year), contamination with placenta proteins and a requirement for life-long intravenous injections.

These uncertainties in our understanding of pathophysiologic mechanisms in Gaucher disease have stimulated the development of other approaches for enzyme replacement therapy in Gaucher disease e.g. poly-ethylene glycol (PEG) modified glucocerebrosidase. PEG modification of glucocerebrosidase prolongs the survival of the enzyme in blood and should permit use of lower doses and alternative routes of administration (Allen et al., 1994). This strategy has been successful in the treatment of severe combined immunodeficiency (SCID) with PEG-modified adenosine deaminase (Hershfield et al., 1987). A clinical trial of PEG-glucocerebrosidase for the treatment of type 1 and 3 Gaucher disease is now in progress (Ginns, personal communication).
The experience with enzyme replacement therapy in Gaucher disease may serve as a prototype for the treatment of other metabolic disorders, such as Pompe disease, Fabry's disease and Niemann-Pick disease type B. Delivery of the missing enzyme to the target (storage) cell may require other delivery strategies than those used in Gaucher disease. So far, various carrier proteins such as insulin (Poznansky et al., 1989), low density lipoprotein (Williams and Murray, 1980), galaptin (Allen et al., 1990), fragment C of tetanus toxin (Dobrenis et al., 1992) and a_2-macroglobulin (Tsuji et al., 1994) have been proposed for the targeting of lysosomal enzymes. Much more research will be needed to develop effective treatment for inherited metabolic disorders with central nervous system involvement, particularly passage of the administered protein through the blood-brain barrier is required (Brownlees and Williams, 1993). Interestingly, a recent study has demonstrated incorporation of mannose labelled liposomes into the mouse brain after intraperitoneal injection (Umezawa and Eto, 1988). In vitro targeting of ß-N-acetylhexosaminidase A (Hex A) to neuronal lysosomes has been achieved in neuronal cell cultures by coupling Hex A to fragment C of tetanus toxin (Dobrenis et al., 1992). However, in vivo studies are required to extend these findings. Enzyme replacement therapy with recombinant ß-glucuronidase in murine mucopolysaccharidosis type VII at 5 weeks of age resulted in partial correction of the lysosomal storage within specific neuronal cell populations (Sands et al., 1994).

Somatic Gene Therapy

The introduction of normal disease-related genes including those coding for α-D-galactose-1-phosphate uridyl transferase, ß-globin and hypoxanthine guanine phosphoribosyltransferase into the genome of target cells were the first experimental attempts at somatic gene therapy for heritable disorders (Merril and Geier, 1971; Mulligan and Berg, 1981;

Green et al., 1982). The transfer of genes for somatic gene therapy has been attempted by two main approaches (Morsy et al., 1993; Moseley and Caskey, 1993; Kay and Woo, 1994). One approach involves in vivo transfer of the gene directly into the affected cell or tissue. This method requires specific targeting in sufficient quantities to the appropriate cell type. Alternatively, the ex vivo delivery of the gene to target host cells followed by autologous cell transplantation is also being developed. For both approaches viral vectors and nonviral methods are used to achieve efficient gene transfer.

Viral vectors

The therapeutic gene is inserted in replication-defective viral vectors and propagated in helper cell lines that complement the deleted essential genes of the vector. Retroviruses, adenoviruses, adeno-associated viruses and herpes simplex viruses have been explored for somatic gene therapy (Ali et al., 1994). Retroviruses have been used extensively because of their high efficiency of low copy number integration and permanent expression of the therapeutic gene. A major disadvantage of this virus system is the poor infectivity of nonreplicating cells (e.g. neurons) and the restriction to relatively short genes (Vile and Russell, 1995). Adenoviral vectors do not integrate into the genome and are gradually lost with cell division. As a consequence the therapeutic gene is expressed only in a transient manner. Adenoviral vectors are able to infect dividing and nondividing cells (Kremer and Perricaudet, 1995). Adeno-associated vectors (AAV) appear to integrate efficiently into a specific site on chromosome 19 in human cells. Recently, long-term expression of human tyrosine hydroxylase in mammalian brain was obtained using the AAV approach (Kaplitt et al., 1994). Herpes simplex viruses are neuronotropic DNA viruses that do not integrate into the host genome, but exist as an episome that can be reactivated in infected neurons. Herpes simplex virus vectors are usually cytopathic and more studies are needed before they can be used for clinical trials (Efstathiou and Minson, 1995).

Nonviral methods

Alternatively, gene delivery can be accomplished using nonviral methods (Schofield and Caskey, 1995). Receptor-mediated endocytosis has been proposed as a mechanism to transfer genes into specific cell types (Wagner et al., 1994; Frese et al., 1994). Proteins that are ligands for specific receptors at the plasma membrane of the target cell are conjugated with DNA and these complexes can be subsequently internalized. To overcome lysosomal degradation and the low and

transient gene expression, the ligand- DNA-protein complex can be linked to inactivated adenovirus or fusogenic peptides that lysis endosomes (Curiel et al., 1991; Wagner et al., 1992). The production of high expression levels in vitro has been accomplished with this strategy using cultured hepatocytes. Recently, in vivo success using the strategy has also been demonstrated (Peralis et al., 1994). Proteins that have been utilized for this approach include orosomucoid and transferrin, targeting the asialoglycoprotein receptor and transferrin receptor, respectively (Cristiano et al., 1993; Wagner et al., 1994; Frese et al., 1994). Liposomes (Felgner et al., 1987; Frese et al., 1994), direct injection of DNA (Wolff et al., 1990) and pneumatically delivered DNA-coated gold particles (Yang et al., 1990) have also been used to deliver DNA to the target cells.

Successful somatic gene therapy depends not only on the molecular identification of a disease-related gene and the efficacy of gene transfer, but also on the understanding of the pathogenesis of the disease. For the lysosomal storage disease galactosialidosis as a good example, it was initially thought that this disease was a rare variant of ß-galactosidase deficiency. Later an additional neuraminidase deficiency was observed in patients and ultimately it was demonstrated that this combined lysosomal enzyme deficiency was due to a primary defect in a third protein (D'Azzo et al., 1995).

Another important aspect that must be considered is the type of cells, tissues and organs that are most profoundly affected. If a transferred normal gene cannot reach the major target organs, therapy may be very inefficient. In lysosomal storage diseases passage through the blood-brain barrier may also present a major problem.

In type 1 Gaucher disease, accumulation of glucocerebroside is mainly restricted to bone-marrow derived macrophages and this disease is a good candidate for somatic gene therapy. The observed beneficial response after allogenic BMT in patients with type 1 and type 3 Gaucher disease makes the transfer of the normal glucocerebrosidase gene by retroviral vectors into hematopoietic stem cells followed by autologous BMT an attractive approach to consider.

Several groups have reported protein expression in macrophages differentiated from mouse hematopoietic stem cells that were infected with a retroviral vector containing the human glucocerebrosidase gene (Weinthal et al., 1991; Ohashi et al., 1992; Correll et al., 1992; Krall et al., 1994). A retroviral vector based on the Moloney murine leukemia virus long terminal repeat has been effective (Freas-Lutz et al., 1994). Immunohistochemical analysis of tissues of transplanted mice revealed that expression of human glucocerebrosidase was not only restricted to

macrophages in visceral tissues, but also in brain. In the central nervous system of these mice, human glucocerebrosidase-positive macrophages were observed, predominantly in perivascular spaces, but surprisingly also in microglia in close proximity with blood vessels. These findings suggest that circulating monocytes can enter the brain as perivascular cells and subsequently traverse the basement membrane and enter the brain parenchyma. The reconstitution with mouse glucocerebrosidase-positive macrophages is however only partial i.e. on the average in 20% of the Kupffer cells, splenic macrophages and microglia cells by 8 months after transplantation (Krall et al., 1994). Long-term bone-marrow cultures of hematopoietic cells of Gaucher patients were also successfully transduced with a retroviral vector encoding normal glucocerebrosidase cDNA partially (30-50%) (Nolta et al., 1992; Xu et al., 1994).
Similar observations were obtained in ß-glucuronidase deficient mice in which the therapeutic gene (normal ß-glucuronidase) was transferred into bone-marrow progenitors by either retrovirally mediated gene transfer (Wolfe et al., 1992a) or by herpesvirus vector gene transfer into the central nervous system (Wolfe et al., 1992b). In addition, retroviral transduction of the human ß-glucuronidase cDNA into primary mouse skin fibroblasts which were engrafted into neo-organs in syngeneic recipient mice yielded engrafted fibroblasts expressing the human enzyme and resulted in a reduction of the lysosomal storage in these animals (Moullier et al., 1993). Thus, the secretion-recapture mechanism with low expression of vector-transferred genes in vivo can result in partial correction of pathology.
Prior to initiation of human clinical trials, the safety and efficacy of somatic gene therapy should be determined in appropriate animal models. Currently, most of the approved human protocols have focused on retroviral and adenoviral vectors to transfer genes into hepatocytes (OTC-deficiency, hypercholesterolemia), hematopoietic cells (ADA-deficiency, Gaucher disease, cancer), lung (Cystic Fibrosis) and muscle (Duchenne type muscular dystrophy) (reviewed by Morsy et al., 1993; Correll and Karlsson, 1994). None of these somatic gene therapy attempts have led to a permanent correction of the enzyme/protein deficiencies. However, further development of highly efficient gene delivery systems that lead to permanent expression of stable and sufficient amounts of the gene product that are necessary to alleviate all clinical manifestations of the disorder will hopefully become available in the next decade, making somatic gene therapy a useful therapeutic option for heritable diseases.
The correction or prevention of human genetic deficiences through the transfer of normal genes into reproductive cells (germ-line gene modification) is currently not possible. Even if the technical obstacles are

overcome many ethical questions remain to be resolved (Wivel and Walters, 1993).

II.5. ANIMAL MODELS

The availability of appropriate animal models of lysosomal storage disorders facilitates examination of the pathogenesis of the disease and the investigation of new experimental therapeutic strategies. Animal models for lysosomal storages diseases can be classified into three categories:

1. Several spontaneously occurring lysosomal storage diseases in animals have been recognized and documented (Farrow et al., 1980).
2. Chemical induced animal models have been produced by administration of inhibitors of lysosomal enzymes.
3. Gene-specific disruption in mice by gene-targeting in embryonic stem cells has resulted in "knock-out" or "null"-allele mice (reviewed by Doi et al., 1992; Melton, 1994). These mice can provide an adequate model of the analogous human disease for studying the loss of specific gene function.

In Table 2 the reported "knock-out" mouse models of lysosomal storage diseases are listed. More subtle gene alterations by introduction of specific point mutations by homologous recombination (hit and run method and double replacement method) have been devised to model a specific phenotype of the disease (Melton et al., 1994).

A naturally occurring canine model of Gaucher disease in the Australian Silkie Terrier had been reported but not propagated (Farrow et al., 1982). Inhibition of glucocerebrosidase by conduritol B epoxide (CBE) in mice generated an animal model having the biochemical characteristics of Gaucher disease (Kanfer et al., 1982). However, ultrastructurally, no Gaucher cells were observed in either the viscera or brain, although neurons displayed inclusion bodies similar to those seen in human Gaucher disease (Adachi and Volk, 1977). Thus, inhibition of glucocerebrosidase by CBE has been inadequate in creating an animal model for Gaucher disease.

Targeted disruption of the mouse glucocerebrosidase gene has resulted in a mouse model with a phenotype analogous to severely affected neonates with type 2 Gaucher disease (Sidransky et al., 1992).

Homozygous "null" allele mutant mice die within 24 hours after birth and have no residual enzyme activity, no detectable glucocerebrosidase cross-

Table 2. Knock-out mice for lysosomal storage diseases

Defective enzyme or receptor	Disease	Phenotype analogue	Reference
glucocerebrosidase	Gaucher disease	severe type 2	Tybulewicz et al.,1992
CD-MPR	?	normal	Köster et al., 1993 Ludwig et al., 1993
CI-MPR	?	lethal at birth	Wang et al., 1994
aryslsulfatase B	Mucopoly-saccharidosis VI	infantile	Peters et al., 1994
lysosomal acid phosphatase	?	?	Saftig et al., 1994
cathepsin D	?	?	Peters et al., 1994
cathepsin A	Galactosialidosis	infantile/juvenile	d'Azzo et al., 1994
hexosaminidase A	Tay-Sachs	late-onset	Yamanaka et al., 1994
arylsulfatase A	Metachromatic leucodystrophy	normal	Gieselmann personal comm.

?=corresponding disease not defined

reactive material and store glucocerebroside in lysosomes of macrophages located in the mononuclear phagocyte system, microglia and in brainstem and spinal cord neurons (Tybulewicz et al., 1992; Willemsen et al., 1995). In addition, examination of the cellophane-like skin of these mice, showed severe disruption of the lamellar bilayer structure of the outer stratum corneum (Holleran et al., 1994). The cause of the early and rapid demise of these Gaucher mice is unknown. At birth they are underweight and show feeding and respiratory complications. Surprisingly, the cellular pathology of tissues from these Gaucher mice was very mild, suggesting that a toxic metabolite may be responsible for their rapid postnatal

demise. It has been hypothesized that glucosylsphingosine, a neurotoxic metabolite, could disrupt neuronal activity by interfering with signal transduction and cellular differentiation. However, an incompetent epidermal barrier, dehydration and poor feeding, may all contribute to their early death (Willemsen et al., 1995).

Despite the many positive expectations for genetically produced animal models it is important to consider possible biochemical and cell biological differences between man and mice. This phenomenon is well illustrated by the mouse model for Lesch-Nyhan syndrome, an inherited neurologic disorder in man caused by hypo-xanthine-guanosine phosphoribosyl transferase (HPRT) deficiency. Despite the severe and characteristic clinical features in affected children the mouse model did not show any features of the characteristic human phenotype (Hooper et al., 1987; Kuehn et al., 1987). In this instance it has been shown that mice might be less dependent than man on HPRT in their cellular biochemical pathways (Wu and Melton, 1993).

On the other hand, homozygous "knock-out" mutant mice have, however, provided new insights into the pathogenesis of the most severe form of Gaucher disease. A Gaucher mouse created by the introduction of specific missense mutations associated with milder presentations of Gaucher disease should provide a longer lived mouse with less severe symptoms. These mildly affected mice should be valuable for testing novel therapeutic strategies and for the study of the underlying pathogenetic mechanisms causing different clinical variants of Gaucher disease.

II.6. REFERENCES

Adachi M, Wallace BJ, Schneck L, Volk BW (1967). Fine structure of central nervous system in early infantile Gaucher's disease. Arch Path 83: 513-526.

Adachi M, Volk BW (1977). Gaucher disease in mice induced by Conduritol-B-Epoxide. Arch Pathol Lab 101: 255-259.

Aghion H (1934). La maladie de Gaucher dans l'enfance. Paris, these.

Ali M, Lemoine NR, Ring CJA (1994). The use of DNA viruses as vector for gene therapy. Gene Therapy 1: 367-384.

Allen TM (1994). The use of glycolipids and hydrophilic polymers in avoiding rapid uptake of liposomes by the mononuclear phagocyte system. Advanced Drug Delivery Reviews 13: 285-309.

Allen HJ, Ahmed H, DiCoccio RA (1990). Metabolic correction of fucosidoses lymphoid cells by Galaptin-alpha-L-fucosidase conjugates. Biochem Biophys Res Commun 172: 335-340.

Banker BQ, Miller JQ, Crocker AC (1962). The cerebral pathology of infantile Gaucher's disease. In: Cerebral Sphingolipidosis. Aronson SM and Volk BW (eds)

Academic, New York pp. 73-99.
Barneveld RA, Tegelaers FPW, Ginns EI, Visser P, Laanen EA, Brady RO, Galjaard H, Barranger JA, Reuser AJJ, Tager JM (1983). Monoclonal antibodies against human beta-glucocerebrosidase. Eur J Biochem 134: 585-589.
Barranger JA (1984). Marrow transplantation in genetic disease. N Engl J Med 311: 1629-1631.
Barranger JA, Ginns EI (1989). Glucosylceramide lipidoses: Gaucher disease. In: The metabolic basis of inherited disease II, vol 6. Scriver et al. (eds), McGraw-Hill, New York, NY, pp. 1677-1698.
Barton NW, Brady RO, Dambriosa JM, DiBisceglie AM, Doppelt SH, Hill SC, Mankin HJ, Murray GJ, Parker RI, Argoff CE, Grewal RP, Yu K-T (1991). Replacement therapy for inherited enzyme deficiency-macrophage-targeted glucocerebrosidase for Gaucher's disease. New Engl J Med 324: 1464-1470.
Bembi B, Zanatta M, Carrozzi M, Baralle F, Gornati R, Berra B, Agosti E (1994). Enzyme replacement treatment in type 1 and type 3 Gaucher's disease. Lancet 344:1679-1682
Berg-Fussman A, Grace ME, Ioannou Y, Grabowski GA (1993). Human acid beta-glucosidase. N-glycosylation site occupancy and the effect of glycosylation on enzymatic activity. J Biol Chem 268: 14861-14866.
Beutler E, Kuhl W (1970). Detection of the defect of Gaucher's disease and its carrier state in peripheral-blood leucocytes. Lancet 612-613.
Beutler E, Dale GL (1982). Enzyme replacement therapy: a model and clinical studies. In: Gaucher disease: a century of delineation and research. Desnick et al. (eds.). Alan R Liss, New York, pp. 703-716.
Beutler E (1993). Gaucher disease as a paradigm of current issues regarding single gene mutations of humans. Proc Natl Acad Sci USA 90: 5384-5390.
Beutler A, Nguyen NJ, Henneberger MW, Smolec JM, McPherson RA, West C, Gelbart T (1993). Gaucher disease: Gene frequencies in the Ashkenazi Jewish population. Am J Hum Genet 52: 85-88.
Beutler E, Grabowski GA (1995). Gaucher disease. In: The metabolic and molecular bases of inherited disease II, vol 7. Scriver et al. (eds), McGraw-Hill, New York, NY, pp.2641-2670.
Birkenmeijer EH, Barker JE, Vogler CA, Kyle JW, Sly WS, Gwynn B, Levy B, Pegors C (1991). Increased life span and correction of metabolic defects in murine mucopolysaccharidosis type VII after syngeneic bone marrow transplantation. Blood 78: 3081-3092.
Blom S, Erikson A (1983). Gaucher disease - Norbottnian type. Eur J Pediatr 140: 316-322.
Bou-Gharios G, Abraham D, Olsen I (1993). Lysosomal storage diseases: mechanisms of enzyme replacement therapy. Histochem J 25: 593-605.
Brady RO, Kanfer JN, Shapiro D (1965). Metabolism of glucocerebrosidases. II. Evidence of an enzymatic deficiency in Gaucher's disease. Biochem Biophys Res Commun 18: 221-225.
Brady RO, Pentchev PG, Gal AE, Hibbert SR, Dekaban AS (1974). Replacement therapy for inherited enzyme deficiency. N Engl J Med 291: 989-993.
Brady RO (1977). Heritable catabolic and anabolic disorders of lipid metabolism. Metabolism 26: 329-345.

Brady RO, Barranger JA, Gal AE, Pentchev PG, Furbish FS (1980). Status of enzyme replacement therapy for Gaucher's disease. In: Enzyme therapy in genetic diseases, vol 2. Desnick (ed.). Alan R Liss, New York, pp. 361-338.

Brady RO, Barton NW (1994a). Enzyme replacement therapy for Gaucher disease: Critical investigations beyond demonstration of clinical efficacy. Biochem Med Metabol Biol 52: 1-9.

Brady RO, Murray GJ, Barton NW (1994b). Modifying exogenous glucocerebrosidase for effective replacement therapy in Gaucher disease. J Inher Metab Dis 17: 510-519.

Brady RO, Barton NW (1994c). Development of effective enzyme therapy for metabolic storage disorders. Int Ped 9: 175-180.

Brill NE (1901). Primary splenectomy with a report of three cases occurring in one family. Am J Med Sci 121: 377-392.

Brill NE (1904). A case of "splenomegalie primitif" with involvement of haemopoietic organs. Proc NY Path Soc 4: 143.

Brownlees J, Williams CH (1993). Peptidases, peptides and the mammalian blood-brain barrier. J Neurochem 60: 793-803.

Carlson DE, Busuttil RW, Giudici TA, Barranger JA (1990). Orthotopic liver transplantation in the treatment of complications of type I Gaucher disease. Transplantation 49: 1192-1194.

Chan KW, Wong LTK, Applegarth D, Davidson AGF (1994). Bone marrow transplantation in Gaucher's disease: effect of mixed chimeric state. Bone Marrow Transplantation 14: 327-330.

Christomanou H, Chabas A, Pamplos T, Guardiola A (1989). Activator protein deficient Gaucher's disease. Klin Wochenschr 67: 999-1003.

Conradi NG, Sourander P, Nilsson O, Svennerholm L, Erikson A (1984). Neuropathology of the Norrbottnian type of Gaucher disease. Acta Neuropathol 65: 99-109.

Correll PH, Colilla S, Dave HPG, Karlsson S (1992). High levels of human glucocerebrosidase activity in macrophages of long-term reconstituted mice after retroviral infection of hematopoietic stem cells. Blood 80: 331-336.

Correll PH, Karlsson S (1994). Towards therapy of Gaucher disease by gene transfer into hematopoietic cells. Eur J Haematol 53: 253-264.

Cristiano RJ, Smith LS, Woo SLC (1993). Hepatic gene therapy: Adenovirus enhancement of receptor-mediated gene delivery and expression in primary hepatocytes. Proc Natl Acad Sci USA 90: 2122-2126.

Curiel DT, Agarwal S, Wagner E, Cotten M (1991). Adenovirus enhancement of transferrin-polylysine-mediated gene delivery. Proc Natl Acad Sci USA 88: 8850-8854.

D'Azzo A, Morreau H, Bonten E, Rottier R, Gillemans N, Grosveld G, Zhou XY (1994). Mouse model for the lysosomal storage disorder galactosialidosis. Am J Hum Genet 55:1268

D'Azzo A, Andria G, Strisciuglio P, Galjaard (1995). Galactosialidosis. In: The metabolic and molecular bases of inherited disease, 7th edition. Scriver, Beadet, Sly, Valle (eds), McGraw-Hill, New-York, pp 2825-2837.

Dahl N, Lagerstrom M, Erikson A, Pettersson U (1990). Gaucher disease type III (Norrbottnian type) is caused by a single mutation in exon 10 of the glucocerebrosidase gene. Am J Hum Genet 47: 275-278.

De Duve C (1964). From cytases to lysosomes. Fed Proc 23: 1045-1049.
Desnick SJ, Desnick RJ, Brady RO, Pentcha PG, Simmons RL, Narjarian JS, Swaiman K, Sharp MI, Krivit W (1973). Renal transplantation in type II Gaucher disease. Birth defects 1x(2): 109-119.
Dobrenis K, Joseph A, Rattazzi MC (1992). Neuronal lysosomal enzyme replacement using fragment C of tetanus toxin. Proc Natl Acad Sci USA 89: 2297-2301.
Doi S, Campbell C, Kucherlapati (1992). Directed modification of genes by homologous recombination in mammalial cells. In: Transgenic animals (Grosveld F and Kollias G, eds), pp 27-46, Academic Press, London.
Doll RF, Smith FI (1993). Regulation expression of the gene encoding human acid beta-glucosidase in different cell types. Gene 127: 255-260.
Efstathiou S, Minson AC (1995). Herpes virus-based vectors. British Med Bull 51: 45-55.
Erickson AH, Ginns EI, Barranger JA (1985). Biosynthesis of the lysosomal enzyme glucocerebrosidase. J Biol Chem 260: 14319-14324.
Erikson A, Groth CG, Mansson J-E, Percy A, Ringden O, Svennerholm L (1990). Clinical and biochemical outcome of marrow transplantation in Gaucher disease of the Norrbottnian type. Acta Paed Scand 79: 680-685.
Erikson A, Johansson K, Mansson JE, Svennerholm L (1993). Enzyme replacement therapy of infantile Gaucher disease. Neuroped 24: 237-238.
Erikson A, Ringden O, Groth C-G, Granqvist S, Mansson JE, Sparreled E (1994). Ten years experience of bone marrow transplantation for Gaucher disease. In: Workshop of the European Working Group on Gaucher disease. Abstract, p 49.
Espinas OE, Faris AA (1969). Acute infantile Gaucher's disease in identical twins. Neurology 19: 133-140.
Farrow BRH (1980). Storage diseases. In: Current Veterinary Therapy VII. Kirk (ed.). W.B. Saunders, Philadelphia, London, Toronto, p. 821.
Farrow BRH, Hartley WJ, Pollard AC, Fabbro D, Grabowski GA, Desnick RJ (1982). Gaucher disease in the dog. In: Gaucher disease: A century of delineation and research. Desnick RJ, Gatt S, Grabowski GA (eds.). Alan R Liss Inc, New York, pp. 645-653.
Felgner PL, Gadek TR, Holm M, Roman R, Chan HW, Wenz M, Northrop JP, Ringold GM, Danielsen M (1987). Lipofection: A highly efficient, lipid mediated DNA-transfection procedure. Proc Natl Acad Sci USA 84: 7413-7417.
Figueroa ML, Rosenbloom BE, Kay AC, Garver P, Thurston DW, Koziol JA, Gelbart T, Beutler E (1992). A less costly regimen of alglucerase to treat Gaucher's disease. N Eng J Medicine 327: 1632-1636.
Freas-Lutz DL, Correll PH, Dougherty SF, Xu L, Pluznik DH, Karlsson S (1994). Expression of human glucocerebrosidase in murine macrophages: identification of efficient retroviral vectors 22: 857-865.
Frese J, Wu CH, Wu GY (1994). Targeting of genes to the liver with glycoprotein carriers. Advanced Drug Delivery Reviews 14: 137-152.
Fujibayashi S, Wenger DA (1985). Studies on a sphingolipid activator protein (SAP-2) in fibroblasts from patients with lysosomal storage diseases, including Niemann-Pick disease type C. Clin Chim Acta 146: 147-156.
Furbish FS, Steer CJ, Barranger JA, Jones EA, Brady RO (1978). The uptake of native and desialylated glucocerebrosidase by rat hepatocytes and Kupffer cells. Biochem Biophys Res Commun 81: 1046-1053.

Furbish FS, Steer CJ, Krett NL, Barranger JA (1981). Uptake and distribution of placental glucocerebrosidase in rat hepatic cells and effect of sequential deglycosylation. Biochem Biophys Acta 673: 425-434.

Fürst W, Sandhoff K (1992). Activator proteins and topology of lysosomal sphingolipid catabolism. Biochim Biophys Acta 1126: 1-16.

Gaucher PCE (1882). De l'epithelioma primitif de la rate hypertrophie idiopathique de la rate sans leucemie. Paris, these.

Gery I, Zigler S, Brady RO, Barranger JA (1981). Selective effect of glucocerebroside (Gaucher's storage material) on macrophage cultures. J Clin Invest 68: 1182-1189.

Ginns EI, Brady RO, Pirrucello S, Moore C, Sorrell S, Furbish FS, Murray GJ, Tager J, Barranger JA (1982). Mutations of glucocerebrosidase: Discrimination of neurologic and non-neurologic phenotypes of Gaucher Disease. Proc Natl Acad Sci USA 79: 5607-5610.

Ginns EI, Tegelaers FPW, Barneveld R, Galjaard H, Reuser AJJ, Brady RO, Tager JM, Barranger JA (1983). Determination of Gaucher's disease phenotypes with monoclonal antibody. Clin Chim Acta 131: 283-287.

Ginns EI, Choudary PV, Martin BM, Winfield S, Stubblefield B, Mayor J, Merkle-Lehman D, Murray GJ, Bowers LA, Barranger JA (1984). Isolation of cDNA clones for human ß-glucocerebrosidase using the λgt11 expression system. Biochem Biophys Res Comm 123: 574-580.

Glew RH, Basu A, LaMarco KL, Prence EM (1988). Biology of disease. Mammalian glucocerebrosidase: Implication for Gaucher's disease. Lab Invest 58: 5-25.

Grabowski GA (1993). Gaucher disease. Enzymology, genetics and treatment. In: Advances in human genetics. Harris H, Hirschhorn K (eds.). Plenum Press, New York, pp. 377-441.

Grabowski GA, Barton NW, Pastores G, Dambrosia JM, Banerjee TK, McKee MA, Parker C, Schiffmann R, Hill SC, Brady RO (1995). Enzyme therapy in type 1 Gaucher disease:comparative efficacy of mannose-terminated glucocerebrosidase from natural and recombinant sources. Ann Intern Med 122: 33-39.

Grace ME, Newman KM, Scheinker V, Berg-Fussman A, Grabowski GA (1994). Analysis of human acid beta-glucosidase by site-directed mutagenesis and heterologous expression. J Biol Chem 269: 2283-2291.

Green MR, Treisman R, Maniatis T (1983). Transcriptional activation of cloned human beta-globin genes by viral immediate-early gene products. Cell 35: 137-148.

Groth CG, Dreborg S, Ockerman PA, Svennerholm L, Hagenfeldt L, Lofstrom B, Samuelson K, Werner B (1971). Splenic transplantation in a case of Gaucher's disease. Lancet: 1260-1264.

Hannun YA, Bell RM (1987). Lysosphingolipids inhibit protein kinase C: implications for the sphingolipidoses. Science 235: 670-674.

Harzer K, Paton BC, Poulos A, Kustermann-Kuhn, Grisar T, Popp M (1989). Sphingolipid activator protein deficiency in a 16-week-old atypical Gaucher disease patient and his fetal sibling: biochemical signs of combined sphingolipidoses. Eur J Pediatr 149: 31-39.

Hernandez F, Bueno M (1973). Infantile neurological Gaucher's disease in three siblings. An ultrastructural study. Virchows Arch Abt A Path Anat 360: 27-32.

Hershfield MS, Buckley RH, Greenberg ML, Melton AL, Schiff R, Hatem C, Kurtzberg J, Markert ML, Kobayashi AL (1987). Treatment of adenosine deaminase deficiency

with polyethylene glycol-modified adenosine deaminase. N Engl J Med 316: 589-596.
Hibbs RG, Ferrans VJ, Cipriano PR, Tardiff KJ (1970). A histochemical and electron microscopic study of Gaucher cells. Arch Path 89: 137-153.
Ho MW, O'Brien JS (1971). Gaucher's disease : Deficiency of 'acid' beta-glucosidase and reconstitution of enzyme activity in vitro. Proc Nat Acad Sci USA 68: 2810-2813
Hobbs Jr, Shaw PJ, Jones KH, Lindsay I (1987). Beneficial effect of pre-transplant splenectomy on displacement bone marrow transplantation for Gaucher's syndrome. Lancet: 1111-1116.
Hollak CEM, Aerts JMFG, Van Oers MHJ (1993). Letter. N Engl J Medicine 328: 1565.
Holleran WM, Ginns EI, Menon GK, Grundmann J-U, Fartasch M, McKinney CE, Elias PM, Sidransky E (1994). Consequences of beta-glucocerebrosidase deficiency in epidermis. J Clin Invest 93: 1757-1764.
Hoogerbrugge PM, Suzuki K, Suzuki K, Poorthuis JHM, Kobayashi T, Wagemaker G, Van Bekkum DW (1988). Donor-derived cells in the central nervous system of twitcher mice after bone marrow transplantation. Science 239: 1035-1038.
Hooper M, Hardy K, Handyside A, Hunter S, Monk M (1987). HPRT-deficient (Lesch-Nyhan) mouse embryos derived from germline colonization by cultured cells. Nature 326: 292-294.
Horowitz M, Wilder S, Horowitz Z, Reiner O, Gelbar T, Beutler E (1989). The human glucocerebrosidase gene and pseudogene: Structure and evolution. Genomics 4: 87-96.
Horowitz M, Zimran A (1994). Mutations causing Gaucher disease. Human Mutation 3: 1-11.
Hubbard AL, Wilson G, Ashwell G, Stukenbrok H (1979). An electron microscope autoradiographic study of the carbohydrate recognition systems in rat liver. I. Distribution of ^{125}I-Ligands among the liver cell types. J Cell Biol 83: 47-64.
Kaga M, Azuma C, Imamura T, Murakami T (1982). Auditory brainstem response (ABR) in infantile Gaucher's disease. Neuropediatrics 13: 207-210.
Kampine JP, Brady RO, Kanfer JN, Feld M, Shapiro D (1966). Diagnosis of Gaucher's disease and Niemann-Pick disease with small samples of venous blood. Science 155: 86-88.
Kanfer JN, Stephens MC, Singh H, Legler G (1982). The Gaucher mouse. In: Gaucher disease: A century of delineation and research. Desnick RJ, Gatt S, Grabowski GA (eds.). Alan R Liss Inc, New York, pp. 627-644.
Kaplitt MG, Leone P, Samulski RJ, Xiao X, Pfaff DW, O'Malley KL, During MJ (1994). Long-term gene expression and phenotypic correction using adeno-associated virus vectors in the mammalian brain. Nature Genetics 8: 148-154.
Kay MA, Woo SL (1994). Gene therapy for metabolic disorders. TIG 10: 253-257.
Kaye EM, Ullman D, Wilson ER, Barranger JA (1986). Type 2 and type 3 Gaucher disease: a morphologial and biochemical study. Annals of Neurology 20: 223-230.
Kishimoto Y, Hiraiwa M, O'Brien JS (1992). Saposins: structure, function, distribution, and molecular genetics. J Lipid Res 33: 1255-1267.
Klein A, Henseler M, Klein C, Suzuki K, Harzer K, Sandhoff K (1994). Sphingolipid activator protein D (sap-D) stimulates the lysosomal degradation of ceramide in vivo. Biochem Biophys Res Commun 200: 1440-1448.

Köster A, Saftig P, Matzner U, Von Figura K, Peters C, Pohlmann R (1993). Targeted disruption of the M_r 46.000 mannose 6-phosphate receptor gene in mice results in misrouting of lysosomal proteins. EMBO J 12: 5219-5223.

Krall WJ, Challita PM, Perlmutter LS, Skelton DC, Kohn DB (1994). Cells expressing human glucocerebrosidase from a retroviral vector repopulate macrophages and central nervous system microglia after murine bone marrow transplantation. Blood 83: 2737-2748.

Kraus EJ (1920). Zur kenntnis der splenomegalie Gaucher, insbesondere der histogenese der grosszellen wucherung. Z. Angew Anat 7: 186.

Kremer EJ, Perricaudet (1995). Adenovirus and adeno-associated virus mediated gene transfer. British Med Bull 51: 31-43.

Krivit W, Shapiro E, Hoogerbrugge PM, Moser HW (1992). State of the art review bone marrow transplantation treatment for storage diseases. Bone Marrow Transplant 10 Suppl 1: 87-96.

Kuehn MR, Bradley A, Robertson EJ, Evans MJ (1987). A potential animal model for Lesch-Nyhan syndrome through introduction of HPRT mutations into mice. Nature 326: 295-298.

Lacey DJ, Terplan K (1984). Correlating auditory evoked and brainstem histologic abnormalities in infantile Gaucher's disease. Neurology 34: 539-541.

Ludwig T, Ovitt CE, Bauer U, Hollinshead M, Remmler J, Lobel P, Ruther U, Hoflack B (1993). Targeted disruption of the mouse cation-dependent mannose 6-phosphate receptor results in partial missorting of multiple lysosomal enzymes. EMBO J 12: 5225-5235.

Mankin HJ, Doppelt SH, Rosenberg AE, Barranger JA (1990). Metabolic bone disease in patients with Gaucher's disease. In: Metabolic bone disease. Avioli LV, Krane SM (eds). Saunders Co., Philadelphia, pp. 730-752.

Mankin HJ (1993). Gaucher's disease: a novel treatment and important breakthrough. J Bone and Joint Surgery 75B: 2-3.

Masuno M, Tomatsu S, Sukegawa K, Orii T (1990). Non-existence of a tight association between a ^{444}leucine to proline mutation and phenotypes of Gaucher disease:high frequency of a NciI polymorphism in the non-neuronopathic form. Hum Genet 84: 203-206.

McKinney CE, Sidransky E, LaMarca ME, Riviere I, Holleran WM, Martin BM, Willemsen R, Mulligan RC, Ginns EI (1995). Gaucher disease: A tale of two species. Mental retardation and development disabilities research reviews. In press.

Meivar-Levy I, Horowitz M, Futerman AH (1994). Analysis of glucocerebrosidase activity using N-(1-[14C]hexanoyl)-D-erythroglucosylsphingosine demonstrates a correlation between levels of residual enzyme activity and the type of Gaucher disease. Biochem J 303:377-382.

Melton DW (1994). Gene targeting in the mouse. BioEssays 16: 633-638.

Merril CR, Geier MR (1971). Bacterial virus gene expression in human cells. Nature 233: 398.

Moffitt KD, Chambers JP, Diven WF, Glew RH, Wenger DA, Farrell DF (1978). Characterization of lysosomal hydrolases that are elevated in Gaucher's disease. Archives of Biochem and Biophys 190: 257-280.

Morrone S, Pentchev PG, Baynes J, Thorpe S (1981). Studies in vivo of the tissue uptake, cellular distribution and catabolic turnover of exogenous glucocerebrosida-

se in rat. Biochem J 194: 733-742.
Morsy MA, Mitani K, Clemens P, Caskey T (1993). Progress toward human therapy. JAMA 270: 2338-2345.
Moseley AB, Caskey CT (1993). Human genetic disease and the medical need for somatic gene therapy. Advanced Drug Delivery Reviews 12: 131-142.
Moullier P, Bohl D, Heard J-M, Danos O (1993). Correction of lysosomal storage in the liver and spleen of MPS VII mice by implantation of genetically modified skin fibroblasts. Nature Genet 4: 154-159.
Mulligan RC, Berg P (1981). Selection for animal cells that express the Escherichia coli gene coding for xanthine-guanine phosphoribosyltransferase. Proc Natl Acad Sci USA 78: 2072-2076.
Murray GJ, Jin FS (1995). Immunoelectron microscopic localization of mannose-terminal glucocerebrosidase in lysosomes of rat liver Kupffer cells. J Histochem Cytochem 43: 148-158.
Nilsson O, Svennerholm L (1982). Accumulation of glucosylceramide and glucosylsphingosine (Psychosine) in cerebrum and cerebellum in infantile and juvenile Gaucher disease. J Neurochemistry 39: 709-718.
Nolta JA, Yu XJ, Bahner I, Kohn DB (1992). Retroviral-mediated transfer of the human glucocerebrosidase gene into cultured Gaucher bone marrow. J Clin Invest 90: 342-348.
Norman RM, Urich H, Lloyd OC (1956). The neuropathology of infantile Gaucher's disease. J Path Bact 72: 121-131.
O'Brien JS, Kishimoto Y (1991). Saposin proteins: structure, function, and role in human lysosomal storage disorders. FASEB 5: 301-308.
O'Neill RR, Tokoro T, Kozak CA, Brady RO (1989). Comparison of the chromosomal localization of murine and human glucocerebrosidase genes and of the deduced amino acid sequences. Proc Natl Acad Sci USA 86: 5049-5053.
Oberling C, Woringen P (1927). La maladie de Gaucher chez la nourrison. Rev Franc de Pediat 3: 475.
Ohashi T, Boggs S, Robbins P, Bahnson A, Patrene K, Wei F-S, Wei J-F, Li J, Lucht L, Fei Y, Clark S, Kimak M, He H, Mowery-Rushton P, Barranger JA (1992). Efficient transfer and sustained high expression of the human glucocerebrosidase gene in mice and their functional macrophages following transplantation of bone marrow transduced by a retroviral vector. Proc Natl Acad Sci USA 89: 11332-11335.
Pastores GM, Sibille AR, Grabowski GA (1993). Enzyme therapy in Gaucher disease type I: Dosage efficacy and adverse effects in 33 patients treated for 6 to 24 months. Blood 82: 408-416.
Paton BC, Hughes JL, Harzer K, Poulos A (1990). Immunocytochemical localization of sphingolipid activator protein 2 (SAP-2) in normal and SAP-deficient fibroblasts. Eur J Cell Biol 51: 157-164.
Patrick AD (1965). A deficiency of glucocerebrosidase in Gaucher's disease. Biochem J 97: 17C-18C
Pennelli N, Scaravelli F, Zacchello F (1969). The morphogenesis of Gaucher cells investigated by electron microscopy. Blood 34: 331-347.
Pentchev PG, Brady RO, Hibbert SR, Gal AE, Shapiro D (1973). Isolation and characterization of glucocerebrosidase from human placental tissue. J Biol Chem 248: 5256-5261.

Perales JC, Ferkol T, Beegen H, Ratnoff OD, Hanson RW (1994). Gene transfer in vivo: Sustained expression and regulation of genes introduced into the liver by receptor-targeted uptake. Proc Natl Acad Sci USA 91: 4086-4090.

Peters C, Saftig P, Hetman M, Hess B, Schmahl W, Heine L, Gradehandt G, Milbradt S, Von Figura K (1994). Cathepsin D deficient mice exhibit atrophies of intestinal mucosa and multiple lymphatic organs. Biol Chem Hoppe Seyler 375: S80.

Peters C, Evers M, Saftig P, Hess B, Von Figura K (1994). Mucopolysaccharidosis VI (Maroteaux-Lamy-Syndrome): Generation and characterization of a mouse model. Gene Therapy 1: S18.

Poorthuis BJHM, Romme AE, Willemsen R, Wagemaker G (1994). Bone marrow transplantation has a significant effect on enzyme levels and storage of glycosaminoglycans in tissues and in isolated hepatocytes of mucopolysaccharidosis type VII mice. Ped Res 36: 187-193.

Poznansky MJ, Hutchison K, Davis PJ (1989). Enzyme replacement therapy in fibroblasts from a patient with cholesteryl ester storage disease. FASEB 3: 152-156.

Radin NS (1994). Glucosylceramide in the nervous system. Neurochem Res 19: 533-540.

Rappeport JM, Ginns EI (1984). Bone-marrow transplantation in severe Gaucher's disease. N Engl J Medicine 311: 84-88.

Rattazzi MC, Dobrenis K, Joseph A, Schwartz P (1987). Modified beta-D-N-acetyl-hexosaminidase isozymes for enzyme replacement in GM2 gangliosidosis. Isozymes Curr Top Biol Med Res 16: 49-65.

Reiner O, Wilder S, Givol D, Horowitz M (1987). Efficient in vitro and in vivo expression of human glucocerebrosidase cDNA. DNA 6: 101-108.

Richards SM, Olson TA, McPherson JM (1993). Antibody response in patients with Gaucher disease after repeated infusion with macrophage-targeted glucocerebrosidase. Blood 82: 1402-1409.

Ringden O, Groth C-G, Erikson A, Granqvist S, Mansson J-E,Sparrelid E (1995). Ten years experience of bone marrow transplantation for Gaucher disease. Transplantation 59: 864-870.

Rynboutt S, Aerts HMFG, Geuze HJ, Tager JM, Strous GJ (1991). Mannose 6-phosphate-independent membrane-association of Cathepsin D, glucocerebrosidase and sphingolipid activating protein in HepG2 cells. J Biol Chem 266: 4862-4868.

Saftig P, Evers M, Hess B, Schmahl W, Von Figura K, Peters C (1994). Investigation of in vivo functions of lysosomal acid phosphatase in gene targeted mice. Biol Chem Hoppe Seyler 375: S87.

Sakuragawa N, Yoshikawa H, Sasaki M (1992). Amniotic tissue transplantation: Clinical and biochemical evaluations for some lysosomal storage diseases. Brain Dev 14: 7-11.

Sandhoff K, Harzer K, Fürst W (1995). Sphingolipid activator proteins. In: The metabolic and molecular bases of inherited disease II, vol 7. Scriver et al. (eds), McGraw-Hill, New York, NY, pp.2427-2441.

Sands MS, Barker JE, Vogler C, Levy B, Gwynn B, Galvin N, Sly WS, Birkenmeijer E (1993). Treatment of murine mucopolysaccharidosis type VII by syngeneic bone marrow transplantation in neonates. Lab Invest 68: 676-686.

Sands MS, Vogler C, Kyle JW, Grubb JH, Levy B, Galvin N, Sly WS, Birkenmeijer EH (1994). Enzyme replacement therapy for murine mucopolysaccharidoses type VII.

J Clin Invest 93: 2324-2331.
Sato Y, Beutler E (1993). Binding, internalization, and degradation of mannose-terminated glucocerebrosidase by macrophages. J Clin Invest 91: 1909-1917.
Scaggiante B, Pineschi A, Sustersich M, Andolina M, Agosti E, Romeo D (1987). Successful therapy of Niemann-Pick diseases by implantation of human amniotic membrane. Transplantation 44: 59-61.
Schnabel D, Schroder M, Furst W, Klein A, Hurwitz R, Zenk T, Weber J, Harzer K, Paton BC, Poulos A, Suzuki K, Sandhoff K (1992). Simultaneous deficiency of sphingolipid activator proteins 1 and 2 is caused by a mutation in the initiation codon of their common gene. J Biol Chem 267: 3312-3315.
Schneider EL, Ellis WG, Brady RO, McCulloch JR, Epstein CJ (1972). Infantile (type II) Gaucher's disease: In utero diagnosis and fetal pathology. J Ped 81: 1134-1139.
Schofield JP, Caskey CT (1995). Non-viral approaches to gene therapy. British Med Bull 51: 56-71.
Sidransky E, Sherer DM, Ginns EI (1992). Gaucher disease in the neonate: A distinct Gaucher phenotype is analogous to a mouse model created by targeted disruption of the glucocerebrosidase gene. Ped Res 32: 494-498.
Sidransky E, Martin B, Ginns EI (1993). Treatment of Gaucher's disease (letter). N Engl J Med 328: 1566-1567.
Sidransky E, Bottler A, Stubblefield B, Ginns EI (1994). DNA mutation analysis of type 1 and type 3 Gaucher patients: How well do mutations predict phenotype? Human Mutation 3: 25-28.
Smanik EJ, Tavill AS, Jacobs GH, Schafer IA, Farquhar L, Weber FL, Mayes JT, Schulak JA, Petrelli M, Zirzow GC, Oliver KL, Miller SPF, Brady RO (1992). Orthotopic liver transplantation in two adults with Niemann-Pick and Gaucher's diseases: implications for the treatment of inherited metabolic diseases. Hepatology 17: 42-49.
Snyder EY, Taylor RM, Wolfe JH (1995). Neural progenitor cells engraftment corrects lysosomal storage throughout the MPS VII mouse brain. Nature 374: 367-370.
Sorge J, West C, Westwoord B, Beutler E (1985). Molecular cloning and nucleotide sequence of human glucocerebrosidase cDNA. Proc Natl Acad Sci USA 82: 7289-7293.
Sorge JA, West C, Kuhl W, Treger L, Beutler E (1987). The human glucocerebrosidase gene has two functional ATG initiator codons. Am J Hum Genet 41: 1016-1024.
Starzl TE, Demetris AJ, Trucco M, Ricordi C, Ildstad S, Terasaki PI, Murase N, Kendall RS, Kocova M, Rudert WA, Zeevi A, Van Thiel D (1993). Chimerism after liver transplantation for type IV glycogen storage disease and type 1 Gaucher's disease. New Engl J Medicine 328: 745-749.
Takasaki S, Murray GJ, Furbish FS, O'Brady RO, Barranger JA, Kobata A (1984). Structure of the N-Asparagine-linked oligosaccharide units of human placental beta-glucocerebrosidase. J Biol Chem 259: 10112-10117.
Taylor RM, Farrow BRH, Stewart GJ (1992). Amelioration of clinical disease following bone marrow transplantation in fucosidase-deficient dogs. Am J Med Genet 42: 628-632.
Tsai P, Lipton JM, Sadhev I, Najfeld V, Rankin LR, Slyper AH, Ludman M, Grabowski GA (1992). Allogenic bone marrow transplantation in severe Gaucher's disease. Ped Res 31: 503-507.

Tsuji A, Oda R, Sakiyama K, Nagamune H, Itoh K, Kase R, Sakuraba H, Suzuki Y, Matsuda Y (1994). Lysosomal enzyme replacement using alpha2-macroglobulin as a transport vehicle. J Biochem 115: 937-944.

Tsuji S, Choudary SV, Martin BM, Winfield S, Barranger JA, Ginns EI (1986). Nucleotide sequence of cDNA containing the complete coding sequence for human lysosomal glucocerebrosidase. J Biol Chem 261: 50-53.

Tsuji S, Choudary PV, Martin BM, Stubblefield BK, Mayor JA, Barranger JA, Ginns EI (1987). A mutation in the human glucocerebrosidase gene in neuronopathic Gaucher disease. N Engl J Med 316: 570-575.

Tybulewicz VLJ, Tremblay ML, LaMarca ME, Willemsen R, Stubblefield BK, Winfield S, Zablocka B, Sidransky E, Martin BM, Huang SP, Mintzer KA, Westphal H, Mulligan RC, Ginns EI (1992). Animal model of Gaucher's disease from targeted disruption of the mouse glucocerebrosidase gene. Nature 357: 407-410.

Tylki-Szymanska A, Maciejko D, Kidawa M, Jablonska-Budaj U (1985). Amniotic tissue transplantation as a trial of treatment in some lysosomal storage diseases. J Inher Metab Dis 8: 101-104.

Umezawa F, Eto Y (1988). Liposome targeting to mouse brain: mannose as a recognition marker. Biochem Biophys Res Comm 153: 1038-1044.

Vaccaro AM, Tatti M, Ciaffoni F, Salvioli R, Serafino A, Barca A (1994). Saposin C induces pH-dependent destabilization and fusion of phosphatidylserine-containing vesicles. FEBS Letters 349: 181-186.

Van Dongen JM, Willemsen R, Ginns EI, Sips HJ, Tager JM, Barranger JA, Reuser AJJ (1985). The subcellular localization of soluble and membrane-bound lysosomal enzymes in I-cell fibroblasts: a comparative immunocytochemical study. Eur J Cell Biol 39: 179-189.

Van den Berg RAJTM, Pietra PJGM, Kolk-Vegter AJ, Bosch E, Tager JM (1976). Therapeutic implications of renal transplantation in patients with Fabry's disease. Acta Med Scand 200: 249-256.

Van Weely S, Brandsma M, Strijland A, Tager JM, Aerts JMFG (1993). Demonstration of the existence of a second non-lysosomal glucocerebrosidase that is not deficient in Gaucher disease. Biochim Biophys Acta 1181: 55-62.

Van Weely S, Van den Berg M, Barranger JA, Sa Miranda MC, Tager JM, Aerts JFMG (1993). Role of pH in determining the cell-type-specific residual activity of glucocerebrosidase in type 1 Gaucher disease. J Clin Invest 91: 1167-1175.

Vile RG, Russell (1995). Retroviruses as vectors. British Med Bull 51: 12-29.

Wagner E, Plank C, Zatloukal K, Cotten M, Birnstiel ML (1992). Influenza virus hemagglutinin HA-2 N-terminal fusogenic peptides augment gene transfer by transferrin-polylysine-DNA complexes: Toward a synthetic virus-like gene-transfer vehicle. Proc Natl Acad Sci USA 89: 7934-7938.

Wagner E, Curiel D, Cotten M (1994). Delivery of drugs, proteins and genes into cells using transferrin as a ligand for receptor-mediated endocytosis. Advanced Drug Delivery Reviews 14: 113-135.

Walkley SU, Thrall MA, Dobrenis K, Huang M, March PA, Siegel DA, Wurzelmann S (1994). Bone marrow transplantation corrects the enzyme defect in neurons of the central nervous system in a lysosomal storage disease. Proc Natl Acad Sci USA 91: 2970-2974.

Wang ZQ, Fung MR, Barlow DP, Wagner EF (1994). Regulation of embryonic growth

and lysosomal targeting by the imprinted Igf2/Mpr gene. Nature 372: 464-467.
Weinthal J, Nolta JA, Yu XJ, Lilley J, Uribe L, Kohn DB (1991). Expression of human glucocerebrosidase following retroviral vector-mediated transduction of murine hematopoietic stem cells. Bone Marrow Transplant 8: 403-412.
Whittington R, Goa KL (1992). Alglucerase. A review of its therapeutic use in Gaucher's disease. Drugs 44: 72-93.
Willemsen R, Van Dongen JM, Ginns EI, Sips HJ, Schram AW, Tager JM, Barranger JA, Reuser AJJ (1987). Ultrastructural localization of glucocerebrosidase in cultured Gaucher's disease fibroblasts by immunocytochemistry. J Neurol 234: 44-51.
Willemsen R, Brunken R, Sorber CJW, Hoogeveen AT, Wisselaar HA, Van Dongen JM, Reuser AJJ (1991). A quantitative immunoelectronmicroscopic study on soluble, membrane-associated and membrane-bound lysosomal enzymes in human intestinal epithelial cells. Histochem J 23: 467-473.
Willemsen R, Tybulewicz V, Sidransky E, Eliason WK, Martin BM, LaMarca ME, Reuser AJJ, Tremblay M, Westphal H, Mulligan RC, Ginns EI (1995). A biochemical and ultrastructural evaluation of the Gaucher phenotype developing from targeted disruption of the murine glucocerebrosidase gene. Molec and Chem Neuropath in press
Willemsen R, Tibbe JJM, Kroos MA, Martin BM, Reuser AJJ, Ginns EI (1995). A biochemical and immunocytochemical study on the targeting of alglucerase. (in press).
Williams JC, Murray AK (1980). Enzyme replacement in Pompe disease with an alpha-glucosidase-low density lipoprotein complex. In: Enzyme therapy in genetic diseases: 2. Desnick RJ (ed.). Alan R Liss, New York, pp. 415-423.
Wivel NA, Walters L (1993). Germ-line gene modification and disease prevention: Some medical and ethical perspectives. Science 262: 533-538.
Wolfe JH, Sands MS, Barker JE, Gwynn B, Rowe LB, Vogler CA, Birkenmeier EH (1992a). Reversal of pathology in murine mucopolysaccharidosis type VII by somatic cell gene transfer. Nature 360: 749-753.
Wolfe JH, Deshmane SL, Fraser NW (1992b). Herpesvirus vector gene transfer and expression of ß-glucuronidase in the central nervous system of MPS VII mice. Nature genetics 1:379-384.
Wolff JA, Malone RW, Williams P, Chong W, Acsadi G, Jani A, Felgner PL (1990). Direct gene transfer into mouse muscle in vivo. Science 247: 1465-1468.
Wu CL, Melton DW (1993). Production of a model for Lesch-Nyhan syndrome in hypoxanthine phosphoribosyltransferase-deficient mice. Nature Genetics 3: 235-240.
Xu L, Stahl SK, Dave HPG, Schiffmann R, Correll PH, Kessler S, Karlsson S (1994). Correction of the enzyme deficiency in hematopoietic cells of Gaucher patients using a clinically acceptable retroviral supernatant transduction protocol. Experimental Hematology 22: 223-230.
Yamaguchi Y, Sasagasako N, Goto I, Kobayashi T (1994). The synthetic pathway for glucosylsphingosine in cultured fibroblasts. J Biochem 116: 704-710.
Yamanaka S, Johnson MD, Grinberg A, Westphal H, Crawley JN, Taniike M, Suzuki K, Proia RL (1994). Targeted disruption of the Hexa gene results in mice with biochemical and pathologic features of Tay-Sachs disease. Proc Natl Acad Sci USA 91: 9975-9979.
Yang N-S, Burkholder J, Roberts B, Martinell B, McCabe D (1990). In vivo and in vitro

gene transfer to mammalian somatic cells by particle bombardment. Proc Natl Acad Sci USA 87: 9568-9572.

Yeager AM, Singer HS, Buck JR, Matalon R, Brennan S, O'Donnell O'Toole S, Moser HW (1985). A therapeutic trial of amniotic epithelial cell implantation in patients with lysosomal storage diseases. Am J Med Genet 22: 347-355.

Zimran E, Gross E, West C, Sorge J, Kubitz M, Beutler E (1989). Prediction of severity of Gaucher's disease by identification of mutations at DNA level. Lancet: 349-352.

Zimran A, Gelbart T, Westwood B, Grabowski GA, Beutler E (1991). High frequency of the Gaucher disease mutation at nucleotide 1226 among Ashkenazi jews. Am J Hum Genet 49: 855-859.

Zimran A, Kay A, Gelbart T, Garver P, Thurston D, Saven A, Beutler E (1992). Gaucher disease. Clinical, laboratory, radiologic, and genetic features of 53 patients. Medicine 71: 337-353.

Zimran A, Hadas-Halpern I, Zevin S, Levy-Lahad E, Abrahamov A (1993). Low-dose high-frequency enzyme replacement therapy for very young children with severe Gaucher disease. British J Haematology 85: 783-786.

Zimran A, Elstein D, Kanna R, Zevin S, Hadas-Halpern I, Levy-lahad E, Cohen Y, Horowitz M, Abramahov A (1994). Low-dose enzyme replacement therapy for Gaucher's disease: Effects of age, sex, genotype and clinical features on response to treatment. Am J Medicine 97: 3-13.

CHAPTER III

THE EXPERIMENTAL WORK

III.1. OBJECTIVES

Gaucher disease is an inherited sphingolipid lysosomal storage disease caused by a deficiency of the lysosomal enzyme glucocerebrosidase. The clinical presentation of Gaucher disease varies considerably. The question is whether this is related to the more than 50 different mutations that have so far been found within the gene for glucocerebrosidase or whether epigenetic or environmental factors play a major role. To approach this question studies were performed on the biosynthesis, subcellular localization and function of normal and mutant enzyme, because these parameters determine primarily the capability of cells to catabolize glucocerebroside, the natural substrate of glucocerebrosidase. A second approach was to study an animal model for Gaucher disease, offering new perspectives for the examination and understanding of the pathogenesis. Animal models were employed in addition, to gather information on the fundamental mechanisms of existing therapies and to device novel experimental therapeutic strategies for Gaucher disease. The experimental work of this thesis is focused on these topics.

III.2. RESULTS AND DISCUSSION

Appendix paper I describes the ultrastructural localization of glucocerebrosidase in cultured skin fibroblasts from normal individuals and patients with the three types of Gaucher disease using the indirect immunogold method on ultrathin frozen sections. We report the presence of glucocerebrosidase in different compartments of the biosynthetic pathway and demonstrate in vivo association of the enzyme with the lysosomal membrane. In fibroblast cell lines from 3 of the 4 patients with type 1 Gaucher disease not only was a similar localization pattern for glucocerebrosidase observed, but the intensity of lysosomal labelling was identical to that in controls. Biochemical characterization of the cell lines was established by Western blotting and enzyme activity assay. Three different molecular forms of glucocerebrosidase could be distinguished in cell homogenates of type 1 patients as in control cells, with molecular weights of 66 kDa, 63 kDa and 59 kDa (Erickson et al., 1985). The membrane association was confirmed for the 66 kDa and 59 kDa molecular forms of glucocerebrosidase, using differential permeabilization of pulse-labelled HepG2 cells (Rijnboutt et al., 1991). The near normal presence of all three molecular forms of glucocerebrosidase as detected by Western blotting and the low residual activity towards 4-MU-substrate in

these cell lines confirms the hypothesis that in type 1 Gaucher disease normal processing occurs, resulting in a lysosomal 59 kDa mature enzyme with a reduced catalytic activity (Ginns et al., 1982 and 1983).
In contrast, the mature 59 kDa molecular form of glucocerebrosidase was absent in fibroblasts from patients with type 2 and type 3 Gaucher disease which is indicative for a defect in the post-translational processing of the enzyme (Ginns et al., 1982 and 1983; Beutler et al., 1984). The absence of cross-reactive material in lysosomes of these mutant cells suggests that the 59 kDa form of glucocerebrosidase is the lysosomal form. The observed normal labelling of RER and Golgi complex in type 2 and type 3 fibroblasts is suggestive of either a rapid lysosomal degradation of the mutant enzyme, obstruction of transport followed by pre-lysosomal degradation or loss by secretion. The presence of mature enzyme in lysosomes of non-neuronopathic Gaucher patients, on the one hand, and the absence of mature enzyme in lysosomes of neuronopathic patients, on the other, has led us to speculate that the presence of sufficient residual glucocerebrosidase activity in type 1 disease precludes development of neurologic symptoms. This principle of a "critical threshold" for lysosomal storage diseases was suggested by Conzelmann and Sandhoff (1983), and it might explain how differences in residual activity within lysosomes can influence the clinical presentation of the disease to a great extent.
In **appendix paper II** the combined technologies of immunoelectron-microscopy and biochemistry are applied to study the lipid-loaden Gaucher cells in the spleen from a patient with type 1 Gaucher disease, because these cells are the primary site of glucocerebroside storage. The specific activity of glucocerebrosidase in splenic extracts from normal individuals and the type 1 patient was determined by measuring glucocerebrosidase activity towards 4-MU-substrate and the molecular forms were identified by Western blotting. In splenic extracts from the type 1 patient a significantly reduced (20X) activity was measured. Western blot analyses revealed three molecular forms of 66 kDa, 63 kDa and 59 kDa with similar intensity for both control spleen extracts and extracts of the type 1 patient. These results suggest a decreased activity per molecule of glucocerebrosidase (specific activity) in the spleen from this type 1 patient and thereby confirm the results obtained with fibroblasts as described in appendix paper I.
Subcellular localization was studied by performing immunoelectron-microscopy on ultrathin frozen sections of the spleen tissue using polyclonal antibodies against glucocerebrosidase. The enzyme was present in the characteristic lipid-loaden Gaucher cells. Since the storage material in the lysosomes of these Gaucher cells was well preserved it could be

demonstrated that the enzyme was associated with the twisted glycolipid tubules within the lysosomes. Thus, mutant enzyme is targeted correctly to lysosomes of Gaucher cells in the spleen of this type 1 patient, however, the enzyme is catalytically defective. Co-localization with other lysosomal enzymes in double labelling experiments, using gold particles of different sizes (5 and 10 nm) supported the view that glucocerebroside does not accumulate in specific storage lysosomes that have lost their normal function.

The experimental work on the "knock-out" Gaucher mouse in the **appendix papers III and IV** has enabled us to study the cell biology and ultrastructural pathology of severe type 2 Gaucher disease in more detail than possible with human patients. This animal model of type 2 Gaucher disease was generated by targeted disruption of the mouse glucocerebrosidase gene in embryonic stem (ES) cells through homologous recombination. These genetically modified ES cells were used to generate a mouse strain homozygous for a null glucocerebrosidase allele.

The type 2 Gaucher mouse, had a striking and rapidly deteriorating phenotype resulting in death within 24 hours after birth. Some characteristic features were: feeding and movement difficulties, abnormal respiration and a cellophane-like appearance of the skin. Homozygous mutant mice had no residual glucocerebrosidase activity towards 4-MU-substrate and thin-layer chromatography revealed elevated glucocerebroside levels in liver, lung and brain tissue. Western blot analyses of brain and liver tissue from homozygous mutant mice showed absence of glucocerebrosidase protein, whereas Western blot analyses of tissue from normal and heterozygous littermates showed a major molecular form with an apparent molecular weight of 56 kDa. Surprisingly, the cellular pathology of tissues from these homozygous mutant mice was very mild. Ultrastructural studies of tissues from the type 2 Gaucher mouse revealed the characteristic twisted tubular structures of accumulated glucocerebroside within lysosomes of macrophages from liver, brain, bone-marrow and spleen, although typical Gaucher cells, the hallmark of Gaucher disease, were not observed by light-microscopy. Co-localization with both ingested carbon particles and cathepsin D confirmed the lysosomal nature of these storage vacuoles. No lipid accumulation was found in macrophages from normal or heterozygous mouse litter mates. Electronmicroscopic study of central nervous system neurons revealed the presence of stored glucocerebroside in brainstem and spinal cord neurons, but absence of glucocerebroside storage in neurons of the cerebellum and cortex. A similar lipid storage pattern is observed in type 2 human Gaucher patients (Banker et al., 1962) and suggests varying

glycosphingolipid catabolism within different brain regions. The rapid demise of these newborn mutant mice is intriguing and several causes were proposed as discussed below.

Despite the rapid deterioration of the type 2 Gaucher mice the cellular pathology of liver, spleen, bone marrow and the central nervous system was very mild. Most neurons did not show lipid storage and only a very limited quantity of stored glucocerebroside was found in certain groups of neurons. These results suggest that a neurotoxic metabolite, such as glucosylsphingosine, may contribute to the early and rapid demise of the type 2 Gaucher mice. This would suggest that neuronal dysfunction appears before gross cellular pathology is observable. In the brain of type 2 patients elevated levels of glucosylsphingosine were recorded (Nilsson and Svennerholm, 1982). Recently, it has been postulated that lysosphingolipids, such as glucosylsphingosine, are potent inhibitors of protein kinase C activity, even at low concentrations (Hannun and Bell, 1987). Hence they could interfere with signal transduction and cellular differentiation and disrupt neuronal activity. Similarly, a correlation was found between the concentration of galactosylsphingosine in the brain of the twitcher mouse, a murine analogue of Krabbe's disease, and the observed pathology. Tissues containing higher concentrations of galactosylsphingosine show earlier and more severe pathological changes (Shinoda et al., 1987).

Recent studies with a conduritol B epoxide induced animal model of Gaucher disease have demonstrated an important role for glucocerebrosidase in the epidermal permeability barrier function (Holleran et al., 1993). Lipids enriched in ceramides, which are localized in the intercellular spaces of the stratum corneum, are critical components in epidermal permeability barrier function. Conversion of glucocerebroside to ceramide is essential to form normal intercellular membrane structures. Thus, absence of glucocerebrosidase activity in the epidermis results in an incorrect processing of epidermal glucocerebroside and as a consequence an abnormal epidermal permeability barrier function. The skin abnormalities observed in the type 2 Gaucher mice is attributed to abnormal intracellular lamellar bilayers as a result of deficient glucocerebrosidase activity (Holleran et al., 1994). This disruption of the skin barrier may also contribute to the rapid demise of the type 2 Gaucher mice. In addition, these observations in mice led to the appreciation of a subset of neonates that were initially diagnosed with an collodion-like skin and/or hydrops fetalis (Sidransky et al., 1992). Newborn homozygous mutant mice were frequently separated from the nest and ignored by their mothers. This ejection resulted in a lack of feeding of these mutant mice. Therefore,

dehydration and hypoglycemia may also contribute to the observed rapid deterioration.

A mouse model of Gaucher disease should be a valuable tool to investigate the potential effect of currently applied and newly developed therapeutic interventions. Unfortunately, the short-lived mice described in appendix paper III and IV have very limited use for evaluating therapeutic strategies for Gaucher disease. Generation of mouse strains with specific point mutations, associated with a milder presentation of the disease, should produce longer lived mice and thus permit development of successful therapeutic interventions.

Successful enzyme replacement therapy with modified human placental glucocerebrosidase (alglucerase, Ceredase™) in patients with type 1 Gaucher disease has been developed. The strategy of alglucerase administration is based on the modification of the carbohydrate chains of glucocerebrosidase by exoglycosidases into terminal mannose residues, mediating efficient delivery to macrophages via the mannose receptor system (Furbish et al., 1981; Brady et al., 1994). However, the dose regimen and clinical efficacy is still controversial. The work reported in **appendix paper V** involves the characterization of enzyme replacement therapy in Gaucher disease in normal mice. In this study the biochemical identification, targeting and intracellular trafficking in mice of intravenously infused human unmodified glucocerebrosidase and alglucerase (60 IU/kg body weight) were determined in the absence or presence of mannan. Immunoprecipitation studies, using monoclonal antibodies against human glucocerebrosidase enabled discrimination between mouse (endogenous) and human enzyme (Barneveld et al., 1983). We observed a significant increase of human glucocerebrosidase activity towards 4-MU-substrate in liver and spleen tissue with a highest activity at 15 minutes after administration of alglucerase.

This targeting to liver and spleen tissue occurs through mannose receptor-mediated binding as was demonstrated by simultaneous administration of mannan to the infusion sample, resulting in dramatically less immunoprecipitable glucocerebrosidase activity in both organs. The targeting of alglucerase to liver tissue was almost three fold higher compared to the targeting of alglucerase to spleen tissue at 15 minutes after administration. Characterization of the molecular forms of alglucerase in both liver and spleen tissue homogenates by immunoprecipitation followed by Western blotting, revealed one band of cross-reactive material of approximately 60 kDa. This form was decreased in intensity when mannan was infused simultaneously. These data illustrate the presence of mature, active glucocerebrosidase in liver and

spleen tissue following alglucerase infusion.
The targeting of alglucerase in liver was visualized by light- and electronmicroscopic techniques, using rabbit polyclonal antibodies against human placental glucocerebrosidase. For light-microscopy we applied the indirect immuno-peroxidase technique on cryostat sections and for immunoelectronmicroscopy we used the indirect immunogold method on ultrathin frozen sections. Cross reactivity of the antibodies with endogenous mouse glucocerebrosidase in liver sections was negligible. At the light-microscopic level a strong labelling in Kupffer cells and along the lining of the sinusoids was observed. It is clear that Kupffer cells are not the only target cells for alglucerase. Uptake inhibition with mannan suggests a mannose receptor mediated binding and uptake in both Kupffer cells and the endothelial cells lining the sinusoids. At the subcellular level, alglucerase was targeted to the endosomal/lysosomal system of both Kupffer cells and endothelial cells.
These results confirm the biochemical findings in rats that showed an eight-fold increase in the delivery of glucocerebrosidase to non-parenchymal cells after modification of the carbohydrate chains to expose terminal mannose residues (Furbish et al., 1981). However, in these studies no discrimination was made between Kupffer cells and endothelial cells. Our in vivo results document that alglucerase is directed to both endothelial and Kupffer cells. Approximately two-thirds of the non-parenchymal liver cells are endothelial cells and the fact that sinusoidal endothelial cells are the most active in binding and internalizing of mannose-terminated ligands (Hubbard et al., 1979; Stang et al., 1990) suggest that the majority of alglucerase is directed to endothelial cells, rather than the intended Kupffer cells. Recently, similar findings were reported in vitro, using cultured macrophages and cultured endothelial cells (Sato and Beutler, 1993).
Our study would suggest that the cellular mechanisms responsible for clinical improvement following alglucerase treatment of patients with Gaucher disease, may be more complex than previously recognized. Further studies using a mouse model with type 1 Gaucher disease created by introduction of a "mild" mutation (like the N370S mutation) into the murine glucocerebrosidase gene, should produce longer-lived mice with accumulation of glucocerebroside in Gaucher cells. The opportunity to perform further biochemical and immunoelectron microscopic studies in these mice, and to especially be able to study the uptake of administered enzyme in Gaucher cells, should provide better insights into the cellular mechanisms responsible for efficacy of enzyme replacement therapy in patients with Gaucher disease.

III.3. REFERENCES

Banker BQ, Miller JQ, Crocker AC (1962). The cerebral pathology of infantile Gaucher's disease. In: Cerebral Sphingolipidosis. Aronson SM and Volk BW (eds) Academic, New York pp. 73-99.

Barneveld RA, Tegelaers FPW, Ginns EI, Visser P, Laanen EA, Brady RO, Galjaard H, Barranger JA, Reuser AJJ, Tager JM (1983). Monoclonal antibodies against human beta-glucocerebrosidase. Eur J Biochem 134: 585-589.

Beutler E, Kuhl W, Sorge J (1984). Cross-reacting material in Gaucher disease fibroblasts. Proc Natl Acad Sci USA 81:6506 6510.

Brady RO, Murray GJ, Barton NW (1994). Modifying exogenous glucocerebrosidase for effective replacement therapy in Gaucher disease. J Inher Metab Dis 17: 510-519.

Conzelmann E, Sandhoff K (1983/1984). Partial enzyme deficiencies: Residual activities and the development of neurological disorders. Dev. Neurosci 6: 58-71.

Furbish FS, Steer CJ, Krett NL, Barranger JA (1981). Uptake and distribution of placental glucocerebrosidase in rat hepatic cells and effect of sequential deglycosylation. Biochem Biophys Acta 673: 425-434.

Ginns EI, Brady RO, Pirrucello S, Moore C, Sorrell S, Furbish FS, Murray GJ, Tager J, Barranger JA (1982). Mutations of glucocerebrosidase: Discrimination of neurologic and non-neurologic phenotypes of Gaucher Disease. Proc Natl Acad Sci USA 79: 5607-5610.

Ginns EI, Tegelaers FPW, Barneveld R, Galjaard H, Reuser AJJ, Brady RO, Tager JM, Barranger JA (1983). Determination of Gaucher's disease phenotypes with monoclonal antibody. Clin Chim Acta 131: 283-287.

Hannun YA, Bell RM (1987). Lysosphingolipids inhibit protein kinase C: implications for the sphingolipidoses. Science 235: 670-674.

Holleran WM, Takagi Y, Menon GK, Legler G, Feingold KR, Elias PM (1993). Processing of epidermal glucosylceramides is required for optimal mammalian cutaneous permeability barrier function. J Clin Invest 91: 1656-1664.

Holleran WM, Ginns EI, Menon GK, Grundmann J-U, Fartasch M, McKinney CE, Elias PM, Sidransky E (1994). Consequences of beta-glucocerebrosidase deficiency in epidermis. J Clin Invest 93: 1757-1764.

Hubbard AL, Wilson G, Ashwell G, Stukenbrok H (1979). An electron microscope autoradiographic study of the carbohydrate recognition systems in rat liver. I. Distribution of ^{125}I-Ligands among the liver cell types. J Cell Biol 83: 47-64.

Nilsson O, Svennerholm L (1982). Accumulation of glucosylceramide and glucosylsphingosine (Psychosine) in cerebrum and cerebellum in infantile and juvenile Gaucher disease. J Neurochemistry 39: 709-718.

Rynboutt S, Aerts HMFG, Geuze HJ, Tager JM, Strous GJ (1991). Mannose 6-phosphate-independent membrane-association of Cathepsin D, glucocerebrosidase and sphingolipid activating protein in HepG2 cells. J Biol Chem 266: 4862-4868.

Sato Y, Beutler E (1993). Binding, internalization, and degradation of mannose-terminated glucocerebrosidase by macrophages. J Clin Invest 91: 1909-1917.

Shinoda H, Kobayashi T, Katayama M, Goto I, Nagara H (1987). Accumulation of galactosylsphingosine (psychosine) in the Twitcher mouse: determination by HPLC. J Neurochem 49:92-97.

Sidransky E, Sherer DM, Ginns EI (1992). Gaucher disease in the neonate: A distinct

Gaucher phenotype is analogous to a mouse model created by targeted disruption of the glucocerebrosidase gene. Ped Res 32: 494-498.
Stang E, Kindberg GM, Berg T, Roos N (1990). Endocytosis mediated by the mannose receptor in liver endothelial cells. An immunocytochemical study. Eur J Cell Biol 52: 67-76.

SUMMARY

The human body is composed of some few hundred different cell types, which are integrated into tissues and organs. Individual cells consist of distinct cellular compartments separated by membranous partitions, like the nucleus, mitochondria, lysosomes, Golgi complex and endoplasmic reticulum. Many cell functions depend upon the interaction between different compartments and a proper metabolic processing within these compartments.
Lysosomes are membrane-enclosed acidic vesicles that play a major role in the degradation of a variety of extracellular and intracellular macromolecules. The intralysosomal catabolism of substrates is catalysed by lysosomal enzymes (hydrolases). Some human genetic disorders are known to be the result of a deficient function of one or more lysosomal proteins. The more than thirty lysosomal storage disorders known today are based either on a deficiency or dysfunction of a lysosomal enzyme, a lysosomal activatorprotein or are due to a defective lysosomal membrane transport system. The intralysosomal accumulation of (macromolecular) degradation products is a characteristic phenomenon and affects the function of specific cell types and organs. An overview on lysosomal storage disorders and the functioning of the lysosomal system is provided in Chapter I.
Gaucher disease is the most common lysosomal storage disorder and is caused by a deficiency of glucocerebrosidase. At present over 50 different mutations have been identified in the gene encoding glucocerebrosidase. Some of the mutations are common others are rare. Patients can be homozygous for one particular mutation, but most patients have different mutations in each of their two alleles (compound heterozygosity). The stored compound in Gaucher disease is glucocerebroside, which is mainly found in cells of the mononuclear phagocyte system. They have a characteristic morphology and are named "Gaucher cells". The clinical phenotype of Gaucher disease is very heterogeneous, ranging from asymptomatic or mildly affected adult patients to children who die after a severe clinical course. Patients without neurologic involvement are classified as type 1, whereas patients with neurologic involvement are classified as type 2 (acute) or type 3 (subacute). Only recently has therapeutic intervention (enzyme replacement therapy) by intravenous administration of modified glucocerebrosidase (Alglucerase, Ceredase™) become effective, albeit only for patients with type 1 Gaucher disease. A historical perspective of Gaucher disease, related research and future prospects are given in Chapter II.

Immunoelectron microscopy was one of the major tools in the experimental work described in this thesis (Chapter III), which was aimed at understanding the cause of clinical heterogeneity in Gaucher disease by delineating more precisely the nature of glucocerebrosidase deficiency with respect to the biosynthesis, the subcellular location and function of the mutant glucocerebrosidase. The results of these studies are presented in appendix papers I and II.
A second aim was to gain further insight into the pathogenesis of the disease. To this end a mouse model of Gaucher disease type 2 was studied. This "Gaucher mouse" was generated by creating a null glucocerebrosidase allele in embryonic stem cells through homologous recombination (knock-out mouse). The homozygous mutant mice are the first example of a mouse model of a human inherited metabolic disease produced using this technology. Appendix paper III reports on the technological details of the targeted mutagenesis of this mouse gene, documents the glucocerebrosidase deficiency in the homozygous mutant mouse, and illustrates the storage of glucocerebroside in Kupffer cells of the liver and macrophages in the bone marrow. A more detailed investigation into the distribution of lysosomal storage in the liver, spleen, bone marrow and in different parts of the central nervous system follows in appendix paper IV. The paradox of early lethality and minor morphological changes in our mouse model raises questions concerning the pathogenic mechanisms.
The mechanism by which enzyme replacement therapy is thought to result in a beneficial effect is addressed in appendix paper V using a normal mouse model.
In the small series of patients investigated in appendix paper I and II the clinical phenotype of Gaucher disease correlates with the molecular phenotype of mutant glucocerebrosidase. In patients with the mildest form of the disease (type 1), cells synthesize a normal amount of glucocerebrosidase, which matures normally and associates with the lysosomal membrane. It lacks however activity to catabolize the substrate at a rate sufficient to prevent storage and clinical symptoms. In patients with the more severely affected forms of Gaucher disease (type 2 and type 3) a defect in maturation and a marked deficiency of lysosomal glucocerebrosidase is observed. These findings led us to speculate that sufficient residual glucocerebrosidase activity within lysosomes of neurons of type 1 patients prevents lysosomal storage and consequently precludes neurologic symptoms.
The mouse model of human Gaucher disease type 2, as described in appendix papers III and IV, revealed pathological features that earlier on

were not associated with the acute form of Gaucher disease in man. The cellular pathology of the mouse, however, appeared unexpectedly mild. Typical Gaucher cells were not identified. Lysosomal storage of glucocerebroside in the central nervous system was localized in microglia (macrophages) and was observed only sporadically in certain neurons. These findings could not explain the rapid demise of the Gaucher mouse 24 hours after birth. It was therefore hypothesized that death is the result of a neurotoxic metabolite.

The study of enzyme replacement therapy in mice, as reported in appendix paper V, illustrates that at least a portion of the mannose-terminated human placental glucocerebrosidase (Ceredase™) is taken up by Kupffer cells via the mannose receptor system and is delivered to the lysosomal system. However, our study also demonstrates the potential "loss" of enzyme by uptake by hepatic endothelial cells lining the blood sinusoids. It may well be that uptake of Ceredase™ by these endothelial cells contributes to the therapeutic effect by clearing the blood plasma of glucocerebroside. Genetic mouse models, representing the different clinical phenotypes of human Gaucher disease, are presently under development and they should provide a means to study the pathogenic mechanisms and to test novel therapeutic strategies.

SAMENVATTING

Het menselijk lichaam is opgebouwd uit enige honderden verschillende celtypen, die geintegreerd zijn in weefsels en organen. Individuele cellen bevatten afzonderlijke compartimenten die begrensd zijn door een membraan zoals de kern, mitochondria, lysosomen, het Golgi complex en het endoplasmatisch reticulum. Veel celfuncties zijn afhankelijk van de samenwerking tussen de verschillende compartimenten en van correcte metabole processen in deze compartimenten.
Lysosomen zijn compartimenten met een hoge zuurgraad. Zij spelen een belangrijke rol bij de afbraak van een groot aantal extracellulaire en intracellulaire macromoleculen. De intralysosomale afbraak van substraten wordt gekatalyseerd door lysosomale enzymen (hydrolasen). Een aantal genetische ziekten wordt veroorzaakt door een functioneel verlies van een of meerdere lysosomale eiwitten, hetgeen leidt tot accumulatie (stapeling) van bepaalde moleculen in de lysosomen. Momenteel zijn bij de mens meer dan dertig lysosomale stapelingsziekten bekend. Zij zijn gebaseerd op een deficientie of dysfunctie van een lysosomaal enzym of een lysosomaal activatoreiwit, of zij worden veroorzaakt door een defect in het transportsysteem in de lysosomale membraan. De intralysosomale stapeling van (macromoleculaire) afbraakprodukten is een karakteristiek fenomeen en beinvloedt de functie van specifieke celtypen en organen. Informatie over het functioneren van het lysosomale systeem en een overzicht van lysosomale stapelingsziekten wordt gegeven in Hoofdstuk I.
De ziekte van Gaucher is de meest voorkomende lysosomale stapelingsziekte en wordt veroorzaakt door een deficientie van glucocerebrosidase. Tot op heden zijn 50 verschillende mutaties in het gen dat codeert voor glucocerebrosidase gevonden. Sommige mutaties komen vaak voor, andere zijn zeldzaam. Patienten kunnen homozygoot voor een bepaalde mutatie zijn, maar de meeste patienten hebben verschillende mutaties in de twee copieën van het glucocerebrosidase gen. Het gestapelde produkt bij de ziekte van Gaucher is glucocerebroside dat voornamelijk gevonden wordt in cellen van het mononucleaire fagocytensysteem. De afwijkende cellen hebben een karakteristieke morfologie en worden "Gaucher cellen" genoemd. Het klinisch fenotype van de ziekte van Gaucher is erg heterogeen en varieert van mild aangedane volwassen patienten tot kinderen die overlijden na een ernstig klinisch verloop. Patienten zonder neurologische afwijkingen worden aangeduid als type 1, terwijl patienten met neurologische afwijkingen aangeduid worden als type 2 (acuut) of type 3 (subacuut). Sinds kort is therapeutische interventie

(enzymvervangingstherapie) door middel van intraveneuze toediening van gemodificeerd glucocerebrosidase effectief gebleken, echter alleen voor patienten met de type 1 vorm van de ziekte van Gaucher. In hoofdstuk II wordt een historisch overzicht van de ziekte van Gaucher gegeven, gerelateerd onderzoek wordt vermeld en toekomstige verwachtingen worden bediscussieerd.
Het experimentele werk, beschreven in dit proefschrift, is voornamelijk gebaseerd op immunoelectronenmicroscopie. Dit onderzoek had als doel de oorzaken van klinische heterogeniteit nader te bestuderen. Speciale aandacht werd besteed aan de biosynthese, de subcellulaire locatie en de functie van het normale en het gemuteerde glucocerebrosidase. De resultaten van deze studies zijn beschreven in appendix papers I en II.
Een tweede doelstelling was om meer inzicht te verkrijgen in de pathogenese van de ziekte. Hiertoe werd een muismodel voor de ziekte van Gaucher type 2 bestudeerd. Deze "Gaucher muis" werd verkregen door het glucocerebrosidase gen van de muis uit te schakelen via homologe recombinatie in embryonale stamcellen ("knock-out" technologie). De Gaucher muizen zijn het eerste voorbeeld van een succesvolle poging om een muismodel van een humane erfelijke stofwisselingsziekte op deze wijze te verkrijgen. Appendix paper III beschrijft de technische details van de procedure, documenteert de glucocerebrosidase deficientie in de homozygoot mutante muis en laat de stapeling van glucocerebroside in Kupffer cellen in de lever en macrofagen in het beenmerg zien. Een meer gedetailleerd onderzoek naar het voorkomen van lysosomale stapeling in de lever, milt, beenmerg en in verschillende delen van het centraal zenuwstelsel van de Gaucher muis volgt in appendix paper IV. De tegenstelling tussen vroege dood en geringe morfologische veranderingen in ons muismodel roept vragen op ten aanzien van het pathogenetische mechanisme.
Het mechanisme van enzymvervangingstherapie wordt beschreven in appendix paper V. Hierbij werd gebruik gemaakt van een normaal muismodel.
Met de kleine aantallen patienten, die bestudeerd zijn in appendix paper I en II werd een correlatie gevonden tussen het klinische fenotype van de ziekte van Gaucher en het moleculaire fenotype van het gemuteerde glucocerebrosidase. Cellen van patienten met de mildere vorm van de ziekte (type 1) synthetiseren een normale hoeveelheid glucocerebrosidase. De enzym precursor ondergaat de normale post-translationele veranderingen en bereikt het lysosoom, waar het geassocieerd is met de lysosomale membraan. De activiteit van het mutante enzym is echter onvoldoende om het substraat in die mate af te breken dat stapeling en

klinische symptomen worden voorkomen. Bij patienten aangedaan met de meer ernstige vormen van de ziekte van Gaucher (type 2 en type 3) wordt een verstoring van de maturatie en een duidelijke deficientie van het lysosomale glucocerebrosidase gevonden. Deze resultaten hebben geleid tot de speculatie dat er bij Gaucher type 1 voldoende restactiviteit van het glucocerebrosidase is om lysosomale stapeling in neuronen en daarmee neurologische symptomen te voorkomen.
Het muismodel van de humane ziekte van Gaucher type 2, zoals beschreven in appendix paper III en IV, leverde pathologische beelden op die eerder niet geassocieerd werden met de acute vorm van de ziekte van Gaucher bij de mens. De cellulaire pathologie van de muis bleek onverwacht mild. Typische Gaucher cellen werden niet gevonden. Lysosomale stapeling van glucocerebroside in het centrale zenuwstelsel werd in microglia gelocalizeerd en werd slechts zelden in bepaalde neuronen waargenomen. Deze resultaten konden de snelle dood van de Gaucher muis, binnen 24 uur na de geboorte, niet verklaren. Er wordt daarom verondersteld dat de dood veroorzaakt wordt door een neurotoxisch stofwisselingsproduct.
De studie naar het mechanisme van enzymvervangingstherapie bij muizen, zoals beschreven in appendix paper V, illustreert dat tenminste een deel van het toegediende gemodificeerde humane glucocerebrosidase (Ceredase™) via de mannose receptor opgenomen wordt door Kupffer cellen en vervolgens getransporteerd wordt naar het lysosomale systeem. Echter, onze studies tonen ook het potentiele "verlies" van enzym aan, door opname in lever endotheel cellen gelegen langs de bloed sinusoiden. Anderzijds zou opname van Ceredase™ door deze endotheelcellen kunnen bijdragen aan het therapeutische effect door het verwijderen van glucocerebroside uit het bloedplasma. Genetisch gemodificeerde muizen, die de verschillende klinische fenotypen van de humane ziekte van Gaucher vertegenwoordigen zijn momenteel in ontwikkeling en deze maken het mogelijk de pathogenetische mechanismen nader te bestuderen en nieuwe therapeutische strategieën uit te testen.

DANKWOORD

Het beoefenen van succesvol wetenschappelijk onderzoek is afhankelijk van een goede samenwerking tussen veel verschillende disciplines. Dit proefschrift zou dan ook niet tot stand zijn gekomen zonder de inspanningen van velen.

De belangrijkste bijdrage aan dit proefschrift is door het thuisfront geleverd. Lieve Birgit, het was voor mij vanzelfsprekend dat wanneer het ooit zover zou komen jij als paranimf naast mij zou staan. Zonder jou zou er geen boekje zijn gekomen. Jouw enthousiasme, geduld, steun en persoonlijke inbreng zijn niet te evenaren. Dit boekje is dan ook voor ons en door ons. Op een toekomst vol met nieuwe uitdagingen.
Onze kinderen Anne, Guido en Pieter zorgden voor de ongevraagde, relativerende opmerkingen waardoor papa weer met beide benen op de grond stond. Jongens, ik ben bevoorrecht om vader van zulke kinderen te zijn.
Mijn ouders ben ik zeer erkentelijk voor hun zorgzame en liefdevolle opvoeding en voor de gegeven mogelijkheid dat ik heb kunnen studeren.

Mijn promotor professor Galjaard ben ik zeer dankbaar voor de positie die hij mij gegeven heeft binnen de Stichting Klinische Genetica Rotterdam. Door uw vertrouwen in mij heb ik me als analist kunnen ontplooien in het wetenschappelijke wereldje. Het grote aantal publicaties toont aan dat uw vertrouwen in het gebruik van electronen-microscopische technieken binnen het huidige wetenschappelijk onderzoek terecht is gebleken.
De leden van de promotie commissie professor Jongkind, professor van Ewijk en Dr. Ginns ben ik dank verschuldigd voor hun zorgvuldigheid bij het kritisch doorlezen van het concept proefschrift. Daarnaast ben ik professor Jongkind erkentelijk voor zijn positieve bijdrage, als voorzitter van de EM-commissie, met betrekking tot de stimulering van de electronenmicroscopie. Onder uw voorzitterschap is het oude "machine-park" totaal vervangen. Hiervan kunnen wij de komende 15 jaar de vruchten plukken.

De gezellige werksfeer op lab 791 en later 2210 is mede te danken aan Pim en Han. Aan humor ontbreekt het jullie zeker niet. Beste Han, ik waardeer het ten zeerste dat jij mijn tweede paranimf wil zijn. Naast een fantastische collega beschouw ik je ook als een van mijn beste vrienden.

Ik denk met bijzonder veel plezier terug aan onze heftige discussies over politiek of wetenschap of aan onze dagelijkse bami-hap op het station van Den Bosch toen jij als gast bij ons op het lab in Utrecht werkte.
Arnold Reuser heeft gedurende de afgelopen 11 jaar de wetenschappelijke begeleiding verzorgd. Beste Arnold, ook zonder jou zou dit boekje er niet zijn geweest. Jij hebt mij in het Gaucher wereldje (jungle) geintroduceerd en er voor gezorgd dat mijn resultaten omgezet werden in wetenschappelijke publicaties. Jouw onbaatzuchtige houding, creativiteit en tomeloze inzet zijn een voorbeeld voor iedere promovendus. Verder is jouw creatie van Sinterklaas onnavolgbaar.
I am very grateful to Dr. Ginns who gave me the opportunity to study the pathology of the genetically modified mouse model for Gaucher disease. Dear Ed, I am very pleased that you are a member of the promotion committee since your contribution was of great importance for this thesis. Furthermore, I enjoyed our collaboration very much and appreciated your hospitality during my short stay at NIH.

Met enige weemoed denk ik nog wel eens terug aan de lysosomenclub. Hier lag de basis van mijn wetenschappelijke vorming. Een aantal namen uit die beginperiode wil ik hier noemen: Andre, Arnold, Herman, Elly, Marian, Frans, Han, Ans, Lies, Marianne, Heleen, Bert, Grazia, Cecile, Joke, Otto, Henk, Monique en Nynke. Bedankt allemaal.
Arthur, Rini, Tanneke, Rick en Rien wil ik bedanken voor de prettige samenwerking tijdens mijn aanwezigheid in de HDL-groep. Dit geldt uiteraard ook voor de mensen van de FRA-X en de TS werkgroep.
In de afgelopen jaren heb ik verschillende stageaires van de Hogeschool West-Brabant tijdens hun stage mogen begeleiden. Robert, Monique, Maud, Lies-Anne en Constant, jullie bijdrage en samenwerking heb ik zeer op prijs gesteld.
John Tibbe wil ik bedanken voor zijn deskundige hulp bij het inspuiten van de muizen.
Voor een electronenmicroscopist is een goede fotografische weergave van zijn resultaten essentieel. In de beginjaren werd de fotografie perfect verzorgd door Joop Fengler en Tar van Os. Met name aan de gezamenlijke uren in de doka met Joop bewaar ik goede herrineringen. Later is het fotografische werk voortreffelijk overgenomen door Tom, Mirko en Ruud. Een speciaal woord van dank aan Tar van Os voor zijn nauwkeurig uitgevoerde aandeel in het digitaliseren van de electronen-microscopische foto's en de lay-out van het proefschrift is zeker op zijn plaats.
Beste Marian en Elly, de vanzelfsprekendheid waarmee jullie altijd klaar

staan om de receptie te verzorgen is uniek. Het bepaalt in grote mate het sociale klimaat op een afdeling.
De secretariële ondersteuning door Marike, Rita, Nelly en zeker niet op de laatste plaats Jeanette wordt door mij zeer gewaardeerd. Even babbelen, schertsen of gewoon een kopje koffie drinken met jullie is altijd een welkome onderbreking van het werk.
De verzorging van de bestellingen was in goede handen bij Rein, Mieke en Madeleine. Datzelfde geldt ook voor Jopie, Elly, Rob en Joke voor wat betreft het glaswerk en de koffie. Beste Piet, ik mis de discussies tijdens de koffie. De computerondersteuning door Jan, Peter en Ton was voor mij zeker geen overbodige luxe.

Ik realiseer mij dat niet iedereen die heeft bijgedragen aan het tot stand komen van dit proefschrift met naam genoemd is. Jullie bijdrage wordt door mij zeker niet minder gewaardeerd. Dit geldt met name voor de mensen die werkzaam zijn op de afdelingen Klinische Genetica, Celbiologie, Immunologie, Pathologie en Anatomie.
Tenslotte wil ik nog een speciaal woord van dank richten aan mijn carpoolers. Sylvia, Lydia, Constant en Han, realiseren jullie je dat wij ieder jaar gemiddeld 300 uur in een ruimte van 2X3X1 meter opgesloten zitten? Dat schept een speciale band. Voor mij geldt in ieder geval dat het woon-werkverkeer een extra dimensie heeft gekregen. De rust die Constant uitstraalt, de aanstekelijke lach van Sylvia, de humor van Han en het gezellige gebabbel van Lydia maken het begin en het einde van de werkdag de moeite waard.

CURRICULUM VITAE

Robert Willemsen Geboren te Breda	17 april 1956
Examen MAVO-4 Nuts-mavo te Breda	1972
Examen HBO-B medisch-biologische studierichting Dr. Struycken-Instituut te Breda Afstudeeropdracht: Electronenmicroscopische lokalisatie van zure fosfatase in de exocriene pancreascel van de rat. (Prof. Dr. J.J. Geuze, Rijksuniversiteit Utrecht)	1976
Militaire dienstplicht	1977
Aanstelling als analist Hubrecht laboratorium (KNAW) te Utrecht	1978
Aanstelling als laboratorium-assistent Lab. voor Histologie en Celbiologie Rijksuniversiteit Utrecht	1982
Aanstelling als analist-b Stichting Klinische Genetica, Rotterdam	1984
Aanstelling als hoofd-analist Stichting Klinische Genetica, Rotterdam	1989-heden

LIST OF PUBLICATIONS

Strous, G.J., P. Van Kerkhof, R. Willemsen, H.J. Geuze, E.G. Berger:Transport and topology of galactosyltransferase in endomembranes of HeLa cells. J. Cell Biol. 97, 723-727 (1983).

Strous, G.J., R. Willemsen, P. Van Kerkhof, J.W. Slot, H.J. Geuze, H.F. Lodish: VSV glycoprotein, albumin, and transferrin are transported to the cell surface via the same Golgi vesicles. J. Cell Biol. 97, 1815-1822 (1983).

Strous, G.J., P. Van Kerkhof, R. Willemsen, J.W. Slot, H.J. Geuze:Effect of monensin on the metabolism, localization, and biosynthesis of N- and O-linked oligosaccharides of galactosyltransferase. Eur. J. Cell Biol. 36, 256-262 (1985).

De Vries, J.W.A., R. Willemsen, H.J. Geuze: Immunocytochemical localization of acrosin and hyaluronidase in epididymal and ejaculated porcine spermatozoa. Eur. J. Cell Biol. 37, 81-88 (1985).

Van Dongen, J.M., R. Willemsen, E.I. Ginns, H.J. Sips, J.M. Tager, J.A. Barranger, A.J.J. Reuser: The subcellular localization of soluble and membrane-bound lysosomal enzymes in I-cell fibroblasts: a comparative immunocytochemical study. Eur. J. Cell Biol. 39, 179-189 (1985).

Sips, H.J., A.H.W. Claass, J.M. Van Dongen, R. Willemsen, A.T. Hoogeveen, H. Galjaard, M. Sinaasappel, H.P. Hauri, E.E. Sterchi: Sucrase-isomaltase and Cystic Fibrosis. J. Inher. Metab. Dis. 8, 163-168 (1985).

Willemsen, R., A.T. Hoogeveen, H.J. Sips, J.M. Van Dongen, H. Galjaard: Immunoelectron microscopical localization of lysosomal ß-galactosidase and its precursor forms in normal and mutant human fibroblasts. Eur. J. Cell Biol. 40, 9-15 (1986).

Van Dongen, J.M., M. Vermey, W.J. Visser, R. Willemsen: De celbiologie van wondgenezing. T.G.O. 11, 46-56 (1986).

Galjaard, H., A.T. Hoogeveen, F.W. Verheyen, R. Willemsen, S. Palmeri, G. Mancini, L. Svennerholm, J.E. Mansson: The ß-galactosidase-sialidase complex. In: L. Freysz, H. Dreyfus, R. Massarelli, S. Gatt (eds): Enzymes of lipid metabolism II.pp. 753-770. Plenum Publishing Corporation, 1986.

Willemsen, R. J.M. Van Dongen, E.I. Ginns, H.J. Sips, A.W. Schram, J.M. Tager, J.A. Barranger, A.J.J. Reuser: Ultrastructural localization of glucocerebrosidase in cultured Gaucher's disease fibroblasts by immunocytochemistry. J. Neurol. 234, 44-51 (1987).

Reuser, A.J.J., M. Kroos, R. Willemsen, D. Swallow, J.M. Tager, H. Galjaard: Clinical diversity in Glycogenosis type II. J. Clin. Invest. 79, 1689-1699 (1987).

Van Dongen, J.M., A.T. Hoogeveen, A.J.J. Reuser, R. Willemsen: Immunoelectron microscopical localization of lysosomal hydrolases in normal and I-cell fibroblasts. Ultramicroscopy 21, 188 (1987).

Willemsen, R., M. Kroos, A.T. Hoogeveen, J.M. Van Dongen, A.J.J. Reuser: Common pathways for cellular transport of steroid sulfatase, lysosomal enzymes, and the mannose 6-phosphate receptor: an immunocytochemical appraisal. Ultramicroscopy 21, 205 (1987)

Parenti, G., R. Willemsen, A.T. Hoogeveen, M. Verleun-Mooyman, J.M. Van Dongen, H. Galjaard: Immunocytochemical localization of lysosomal acid

phosphatase in normal and I-cell fibroblasts. Eur J. Cell Biol. 43, 121-127 (1987).
Willemsen, R., A.T. Hoogeveen, A.J.J. Reuser, J.M. Van Dongen: Immunoelectron microscopical localization of lysosomal hydrolases in normal and I-cell fibroblasts. In: E. Reid, G.M.W. Cook, J.P. Luzio (eds): Cells, Membranes, and Disease, including Renal. pp.135-137 Plenum Publ. Corp. (1987).
Galjaard, H., R. Willemsen, A.T. Hoogeveen, G.M.S. Mancini, S. Palmeri, F.W. Verheyen, A. D'Azzo: Molecular heterogeneity in human ß-galactosidase and neuraminidase deficiency. Enzyme 38, 132-143 (1987).
Willemsen, R., M. Kroos, A.T. Hoogeveen, J.M. Van Dongen, G. Parenti, C.M. Van der Loos, A.J.J. Reuser: Ultrastructural localization of steroid sulfatase in cultured human fibroblasts by immunocytochemistry: a comparative study with lysosomal enzymes and the mannose 6-phosphate receptor. Histochemical J. 20, 41-51 (1988).
Willemsen, R., J.M.F.G. Aerts, J.M. Van Dongen, R. Goudsmit, A.W. Schram, J.M. Tager, A.J.J. Reuser: An immunoelectron microscopic study of glucocerebrosidase in type I Gaucher's disease spleen. Ultrastruct. Pathol. 12, 471-478 (1988).
Van Diggelen, O.P., D. Schindler, R. Willemsen, M. Boer, W.J. Kleijer, J.G.M. Huijmans, W. Blom, H. Galjaard: α-N-acetylgalactosaminidase deficiency, a new lysosomal storage disorder. J. Inher. Metab. Dis. 11, 349-357 (1988).
Reuser, A.J.J., R. Willemsen, A.T. Van der Ploeg, E.H. Hoefsloot. B.A. Oostra: Clinical diversity in lysosomal storage disorders. In: R. Salvayre, L. Douste-Blazy, S. Gatt (eds): Inserm Symposium on Lipid Storage Disorders: Biological and Medical Aspects, vol. 150, p.547-558. Plenum Publishing Corp. (1988).
Scholte, B.J., J. Bijman, A.T. Hoogeveen, R. Willemsen, J.S. Rhim, A.W.M. Van der Kamp: Immortalization of nasal epithelial cells from cystic fibrosis patients. Exp. Cell Res. 182, 559-571 (1989).
Morreau, H., N.J. Galjart, N. Gillemans, R. Willemsen, G.T.J. van der Horst, A. d'Azzo: Alternative splicing of ß-galactosidase mRNA generates the classic lysosomal enzyme and a ß-galactosidase-related protein. J.Biol. Chem. 264, 20655-20663 (1989).
Van der Ploeg, A.T., A.M.M. van der Kraay, R. Willemsen, M.C.B. Loonen, J.F.Koster, A.J.J. Reuser : Rat heart perfusion as model for enzyme replacement therapy in glycogenosis type II. Pediatric Research 28, 344-347 (1990).
Willemsen, R., H.A. Wisselaar, A.T. van der Ploeg: Plasmalemmal vesicles are involved in transendothelial transport of albumin, lysosomal enzymes and mannose 6-phosphate receptor fragments in capillary endothelium. Eur. J. Cell Biol. 51, 235-241 (1990).
Hoefsloot, L.H., R. Willemsen, M.A. Kroos, M. Hoogeveen-Westerveld, M.M.P. Hermans, A.T. Van der Ploeg, B.A. Oostra, A.J.J. Reuser: Expression and routeing of human lysosomal α-glucosidase in transiently transfected mammalian cells. Biochem. J. 272, 485-492 (1990).
Van der PLoeg, A.T., R. Willemsen, N.H.C. Brons, A.J.J. Reuser: Intravenous administration of phosphorylated acid α-glucosidase leads to uptake of enzyme in heart and skeletal muscle of mice. J. Clin. Invest. 87, 513-518 (1991).
Hoogeveen, A.T., J. Keulemans, R. Willemsen, B.J. Scholte, J. Bijman, M.J. Edixhoven, H.R. De Jonge, H. Galjaard: Immunological localization of Cystic Fibrosis candidate gene products. Exp. Cell Res. 193, 435-437 (1991).
Willemsen, R., R. Brunken, C.W.J. Sorber, A.T. Hoogeveen, H.A. Wisselaar, J.M.

Van Dongen, A.J.J. Reuser: A quantitative immuno-electronmicroscopic study on soluble, membrane-associated and membrane-bound lysosomal enzymes in human intestinal epithelial cells. Histochemical J. 23, 467-473 (1991).

Galjart, N.J., H. Morreau, R. Willemsen, N. Gillemans, E. Bonten, A. d'Azzo: Human lysosomal protective protein has cathepsin A-like activity distinct from its protective function. J. Biol. Chem. 266, 14754-14762 (1991).

Versnel, M.A., L. Claesson-Welsh, A. Hammacher, M.J. Bouts, Th. Vanderkwast, A. Eriksson, R. Willemsen, S.M. Weima, H.C. Hoogsteden, A. Hagemeijer, C.-H Heldin: Human malignant mesothelioma cell lines express PDGF ß-receptors whereas cultured normal mesothelial cells express predominantly PDGF α-receptors. Oncogene 6, 2005-2011 (1991).

Yan Zhou, X., N.J. Galjart, R. Willemsen, N. Gillemans, H. Galjaard, A. d'Azzo: A mutation in a mild form of Galactosialidosis impairs dimerization of the protective protein and renders it unstable. EMBO J. 10, 4041-4048 (1991).

DeCrom, R.P.G., R. VanHaperen, R. Willemsen, A.W.M. VanderKamp: High Density lipoprotein-binding proteins in porcine liver. Isolation and histological localization. Arteriosclerosis and Thrombosis 12, 325-331 (1992).

Tybulewicz, V.L.J., M.L. Tremblay, M.E. LaMarca, R. Willemsen, B.K. Stubblefield, S. Winfield, B. Zablocka, E. Sidransky, B.M. Martin, S.P. Huang, K.A. Mintzer, H. Westphal, R.C. Mulligan & E.I. Ginns: Animal model of Gaucher's disease from targeted disruption of the mouse glucocerebrosidase gene. Nature 357, 407-410 (1992).

Morreau, H., N. Galjart, R. Willemsen, N. Gillemans, X. Yan Zhou, A. d'Azzo: Human lysosomal protective protein. Glycosylation, intracellular transport and association with ß-galactosidase in the endoplasmic reticulum. J. Biol. Chem. 267, 17949-17956 (1992).

DeCrom, R.P.G., R. VanHaperen, R. Willemsen, A.W.M. VanderKamp: High density lipoprotein interactions with liver. In: Hepatic endocytosis of lipids and proteins. (E. Windler & H. Greten, eds) p. 406-412. W. Zuckschwerdt Verlag (1992).

Hermans, M.M.P., E. deGraaff, M.A. Kroos, H.A. Wisselaar, R. Willemsen, B.A. Oostra, A.J.J. Reuser: The conservative substitution Asp-645→ Glu in lysosomal α-glucosidase affects transport and phosphorylation of the enzyme in an adult patient with glycogen-storage disease type II. Biochem. J. 289, 687-693 (1993).

Verheij, C., C.E. Bakker, E. deGraaff, J. Keulemans, R. Willemsen, A.J.M.H. Verkerk, H. Galjaard, A.J.J. Reuser, A.T. Hoogeveen, B.A. Oostra: Characterization and localization of the FMR-1 gene product. Nature 363, 722-724 (1993).

Willemsen, R., A.T. vanderPloeg, H.F.M. Busch, P.E. Zondervan, C.J.F.vanNoorden, A.J.J. Reuser: Synthesis and in situ localization of lysosomal α-glucosidase in muscle of an unusual variant of glycogen storage disease type II. Ultrastr. Pathol. 17, 515-527 (1993).

Hoogeveen, A.T., R. Willemsen, N. Meyer, K.E. deRooij, R.A.C. Roos, G-J.B. vanOmmen, H. Galjaard: Characterization and localization of the Huntington disease gene product. Human Molec. Genetics 12, 2069-2073 (1993).

DeCrom, R.P.G., R. VanHaperen, P. Visser, R. Willemsen, A.W.M. VanderKamp: A structural relation between high density lipoprotein-binding proteins in porcine liver. Arterioscler. Thromb. 14, 305-312 (1994).

Reuser, A.J.J., M.A. Kroos, W.J. Visser, R. Willemsen: Lysosomal storage diseases:

cellular pathology, clinical and genetic heterogeneity, therapy. Ann. Biol.Clin. 52, 721-728 (1994).

Hermans, M.M.P., E. DeGraaff, M.A.Kroos, S. Mohkamsing, B.J. Eussen, M. Joosse, R. Willemsen, W.J. Kleijer, B.A. Oostra, A.J.J. Reuser: The effect of a single base pair deletion and a C1634T missense mutation on the expression of lysosomal α-glucosidase in patients with glycogen storage disease type II. Hum. Molec. Genet. 12, 2213-2218 (1994).

Poorthuis, B.J.H.M., E.A. Romme, R. Willemsen, G. Wagemaker: Bone marrow transplantation has a significant effect on enzyme levels and storage of glycosaminoglycans in tissues and in isolated hepatocytes of mucopolysaccharidosis type VII mice. Pediatr. Res. 36, 187-193 (1994).

Defresne, M.P., B. Nabarra, E. vanVliet, R. Willemsen, H. vanDongen, W. vanEwijk:The ER-TR4 monoclonal antibody recognizes murine thymic epithelial cells (type 1) and inhibits their capacity to interact with immature thymocytes: immuno-electron microscopic and functional studies. Histochem. 101, 355-363 (1994).

DeJong, J., C VandenBerg, H. Wijburg, R. Willemsen, O. VanDiggelen, F. Hoevenaars, , R. Wevers:N-acetylgalactosaminidase deficiency with mild clinical manifestations and difficult biochemical diagnosis. J. Pediatr. 125, 385-391 (1994).

DeJong, J.P., J.S.A. Voerman, A.J. Vandersluijsgelling, R. Willemsen, R.E. Ploemacher: A monoclonal antibody (ER-HR3) against murine macrophages; ontogeny, distribution and enzyme histochemical characterization of ER-HR3 positive cells. Cell and Tissue Research 275, 567-576 (1994).

The Dutch-Belgian Fragile X Consortium: Fmr1 knockout mice: a model to study fragile X mental retardation. Cell 78, 23-33 (1994).

Willemsen, R., V. Tybulewicz, E. Sidransky, W.K. Eliason, B.M. Martin, M.E. LaMarca, A.J.J. Reuser, M. Tremblay, H. Westphal, R. Mulligan, E.I. Ginns: A biochemical and ultrastructural evaluation of the type 2 Gaucher mouse developed from targeted disruption of the murine glucocerebrosidase gene. Molec. Chem. Neurol. Pathol. in press (1995).

Willemsen, R., S. Mohkamsing, B. DeVries, D. Devys, A. VandenOuweland, J.L. Mandel, H. Galjaard, B. Oostra: Rapid antibody test for fragile X syndrome. Lancet 345, 1147-1148 (1995).

Willemsen, R., J.J.M. Tibbe, M.A. Kroos, B.M. Martin, A.J.J. Reuser, E.I. Ginns: A biochemical and immunocytochemical study on the targeting of alglucerase. Histochem. J. in press (1995).

McKinney, C.E., E. Sidransky, M.E. LaMarca, I. Riviere, W.M. Holleran, B.M. Martin, R. Willemsen, R.C. Mulligan, E.I. Ginns: Gaucher disease: A tale of two species. Ment. Ret. Dev. Dis. Res. Rev. in press (1995).

Verheij, C., E. DeGraaff, C.E. Bakker, R. Willemsen, P.J. Willems, N. Meijer, H. Galjaard, A.J.J. Reuser, B.A. Oostra, A.T. Hoogeveen: Characterization of FMR1 proteins isolated from different tissues. Hum. Molec. Genet. 4, 895-901 (1995).

DeGraaff, E., R. Willemsen, N. Zhong, C.E.M. Die-Smulders, W.T.Brown, G. Freling, B.A. Oostra: Instability of the CGG repeat and expression of the FMR1 protein in a male fragile X patient with a lung tumour. Am. J. Hum. Genet. in press (1995).

ABBREVIATIONS

AAV	adeno associated virus
ADA	adenosine deaminase
Asn	asparagine
ATP	adenosine triphosphate
BHK	baby hamster kidney
BMT	bone marrow transplantation
CBE	conduritol B epoxide
CD-MPR	cation dependent mannose 6-phosphate receptor
cDNA	complementary DNA
CHO	chinese hamster ovary
CI-MPR	cation independent mannose 6-phosphate receptor
DNA	desoxyribonucleic acid
EGF	epidermal growth factor
ER	endoplasmic reticulum
ES	embryonic stem
GERL	Golgi-endoplasmic reticulum-lysosomes
GLUT	glucose transporter
HA	hydroxyl-apatite
HPRT	hypo-xanthine-guanosine phosphoribosyl transferase
Ig	immunoglobulin
kB	kilo base
kDa	kilo dalton
LDL	low density lipoprotein
lgp	lysosomal membrane glycoprotein
MDCK	Madin-Darby canine kidney
MPR	mannose 6-phosphate receptor
mRNA	messenger ribonucleic acid
NAC	nascent-polypeptide-associated complex
OTC	ornithine transcarbamylase
PDGF	platelet derived growth factor
PEG	polyethylene glycol
RER	rough endoplasmic reticulum
SAP	sphingolipid activator protein
SCID	severe combined immunodeficiency
Sf	Spodoptera frugiperda
TGN	trans Golgi-network

APPENDIX PAPER I

J Neurol (1987) 234:44-51

ULTRASTRUCTURAL LOCALIZATION OF GLUCO-CEREBROSIDASE IN CULTURED GAUCHER'S DISEASE FIBROBLASTS BY IMMUNOCYTOCHEMISTRY

R. Willemsen, J.M. van Dongen, E.I. Ginns, H.J. Sips, A.W. Schram, J.M. Tager, J.A. Barranger, A.J.J. Reuser.

ABSTRACT

The subcellular localization of glucocerebrosidase was studied in cultured skin fibroblasts from eight patients with Gaucher's disease. The enzyme, in situ, was visualized under the electron microscope by incubating ultrathin frozen sections of fibroblasts with antibodies against glucocerebrosidase, followed by a second incubation with goat anti-(rabbit IgG) coupled to colloidal gold. In control cells, most of the gold label was found in lysosomes, associated with the membrane. Labeling of the rough endoplasmic reticulum (RER) and Golgi complex was also observed. In fibroblasts from three Gaucher's disease patients without neurological symptoms (type 1 disease), a near normal amount of cross-reactive material (CRM) was detected in lysosomes, but in a fourth such patient, the lysosomal CRM was reduced. Little lysosomal glucocerebrosidase was detected in cells from patients with the acute neuronopathic form (Type 2) or the subacute neuronopathic form (Type 3) of Gaucher's disease. CRM in lysosomes correlates with amount of mature, 59 kDa glucocerebrosidase which is undetectable in type 2 and type 3 Gaucher's disease cell lines. These findings demonstrate that different mutations in the gene for glucocerebrosidase result in mutant proteins that have different intracellular localization. They also suggest that there is a relationship between the amount of cross-reactive material in the lysosomes and the phenotypic expression of the disease.

INTRODUCTION

An inherited deficiency of glucocerebrosidase in Gaucher's disease leads to accumulation of glucosylceramide in reticuloendothelial cells of various organs and tissues.[2,15]. The clinical presentation of Gaucher's disease varies considerably and has led to classification of patients into three subtypes or phenotypes. Type 1 (chronic, non-neuronopathic) is the most common with an estimated carrier frequency of 1 in 13 among Ashkenazi

Jews.[13] Storage of glucosylceramide in this form of Gaucher's disease is restricted to the viscera and perivascular space in brain and is not observed within the brain parenchyma.[3] Type 2 (acute, neuronopathic) and type 3 (subacute, neuronopathic) Gaucher's disease are distinct from type 1, in that there is also involvement of the central nervous system. In type 3, signs of neurological damage appear later than in type 2.[3]
Polymorphism of the enzyme protein has permitted discrimination of the different phenotypes.[9,10] In brain and cultured fibroblasts from control subjects, three different molecular species of glucocerebrosidase are recognized immunochemically on Western blots. These represent biosynthetic forms of the enzyme.[5,9] In type 1 Gaucher's disease all three molecular forms are present, whereas the mature form of the enzyme is characteristically deficient in phenotypes 2 and 3.[9] Using a monoclonal antibody 8E4, a distinction between type 2 (cross reactive material absent) and type 3 (cross reactive material present) can be made.[10]
Since the mature forms of most lysosomal enzymes are derived from their precursor after extensive modification, the apparent absence of mature glucocerebrosidase in type 2 and type 3 Gaucher's disease could reflect improper processing and/or routing of the mutant enzyme.[9] It is conceivable that the degree of maturation of a mutant enzyme and the cellular compartmentation together determine the capability of cells to catabolize glucosylceramide. We have now studied the subcellular localization of glucocerebrosidase in cultured skin fibroblasts of the three Gaucher's disease phenotypes using immunocytochemical methods.

MATERIALS AND METHODS

Cell lines and culture conditions
Fibroblast cell lines 83RD257, 543LAD, TRI, GRA and 81RD238 were provided by Prof. M.F. Niermeijer, Department of Clinical Genetics, University Hospital, Rotterdam, The Netherlands. Dr. M.T. Zabot and Dr. M. Mathieu, Hospital Debrousse, Lyon, France, supplied cell line 01674S. The cell lines DMN 83.102, 5519, PRW and JF83AD were started from skin biopsy specimens taken by Dr. E. Ginns and Dr. J.A. Barranger from the Molecular and Medical Genetics Section, National Institutes of Health, Bethesda, USA. Cell line GM877 was obtained from the Human Mutant Cell Repository (Institute for Medical Research), Camden, New Jersey, USA. Early passages of each cell line were grown in Dulbecco's Modified Eagles Medium supplemented with 10% fetal calf serum and antibiotics (penicillin, 120 IU/ml and streptomycin, 0.12 mg/ml).

Immunocytochemistry

Immunocytochemistry was performed with affinity-purified rabbit polyclonal antibodies against homogeneous human placental glucocerebrosidase.[9] For light microscopy, the fibroblasts were grown on coverslips, fixed, and then incubated with antibodies exactly as described previously.[19] The indirect immunocytochemical procedure was performed with goat anti-(rabbit IgG) conjugated to fluorescein isothiocyanate (Nordic, Tilburg, The Netherlands) for the second step, as described by Van Dongen et al.[19]

For ultrastructural studies, confluent cultures were harvested with trypsin and washed twice with fresh medium containing 10% fetal calf serum. The cells were resuspended in fresh medium and kept in a rotating tube for 2 h at 37°C to recover from possible typsinization damage. They were then collected from suspension by centrifugation and taken up in 0.1 M phosphate buffer (PB), pH 7.3, containing 1% acrolein and 0.4% glutaraldehyde. After fixation for 60 min at 4°C, the cells were rinsed twice in 0.1 M PB and resuspended in the same buffer containing 10% gelatin, at 37°C. The cells were directly pelleted and solidified on ice. The same fixative was used for postfixation to cross-link the gelatin, and the pellet was stored at 4°C in 0.1 M PB containing 1% paraformaldehyde and 1 M sucrose.[7] Frozen sections were made as described by Tokuyasu[16] using a LKB III ultra microtome equipped with the 14800 cryokit.

The methods used for immunocytochemistry were those described by Geuze et al.[8] with the following minor modifications. Prior to incubation with the first antibody, the sections were treated for 10 min. with sodium borohydride (2 mg/ml PB) to reduce residual aldehyde groups of the fixative.[4,20] Antigen-antibody complexes were visualized through binding of goat anti-(rabbit IgG) coupled to 10 nm colloidal gold (Janssen-Pharmaceutica, Belgium). After these incubations, the sections were stained as described by Tokuyasu[17] and embedded in 1.5% methylcellulose.[11] They were studied with a Phillips EM 400 at 80 KV.

Immunoblotting

Proteins were separated in 10% polyacrylamide gels in the presence of sodium dodecylsulphate as described by Laemmli[14] , transferred to nitrocellulose filters[18], and then incubated with rabbit anti-glucocerebrosidase immunoglobulins. Immune complexes were detected using goat anti-(rabbit IgG) coupled to horse radish peroxidase (Biorad), according the manufacturers instructions.

Table 1. Some characteristic data on cell lines of healthy individuals and patients with Gaucher's disease

Code	Phenotype	Glucocerebrosidase activity[a] (nmol/h/mg)	Glucosidase / Hexosaminidase x10^3	Blotting pattern		
				66	63	59
GRA	Normal	275	109	+	+	+
84RD133	Normal	396	160	+	+	+
FC	Normal	180	71	+	+	+
81RD238	Normal	318	126	+	+	+
5519	Type 1	12	4	+	+	+
543LAD	Type 1	13	5	+	+	+
PRW	Type 1	22	4	+	+	+
83RD257	Type 1	8	2	+	+	+
DMN	Type 2	5	2	+	-	-
GM877	Type 2	10	3	+	+	-
JF83AD	Type 3	20	9	+	-	-
01674S	Type 3	50	7	+	-	-

[a]Mean value of three assays

Biochemical assays

Glucocerebrosidase activity in fibroblast homogenates was assayed with 4-methylumbelliferyl-ß-D-glucoside in a reaction mixture (final volume, 1 ml) containing 5mM substrate, 0.1% (w/v) Triton X-100, 0.2% (w/v) sodium taurocholate, 100 mM citrate-phosphate buffer (pH 5.2) and fibroblast homogenate. The N-acetyl-ß-D-hexosaminidase activity was assayed as described by Galjaard.[6]

RESULTS

Fibroblast cell lines were used from patients who had been classified on the basis of clinical characteristics as having type 1, 2 or 3 Gaucher's disease (Table 1). To characterize the cell lines, cell extracts were prepared and molecular forms of glucocerebrosidase were separated in SDS-polyacrylamide gels. Proteins were subsequently transferred to a nitrocellulose filter and glucocerebrosidase was visualized immunochemically. The results are illustrated in Fig. 1. There are 3 bands

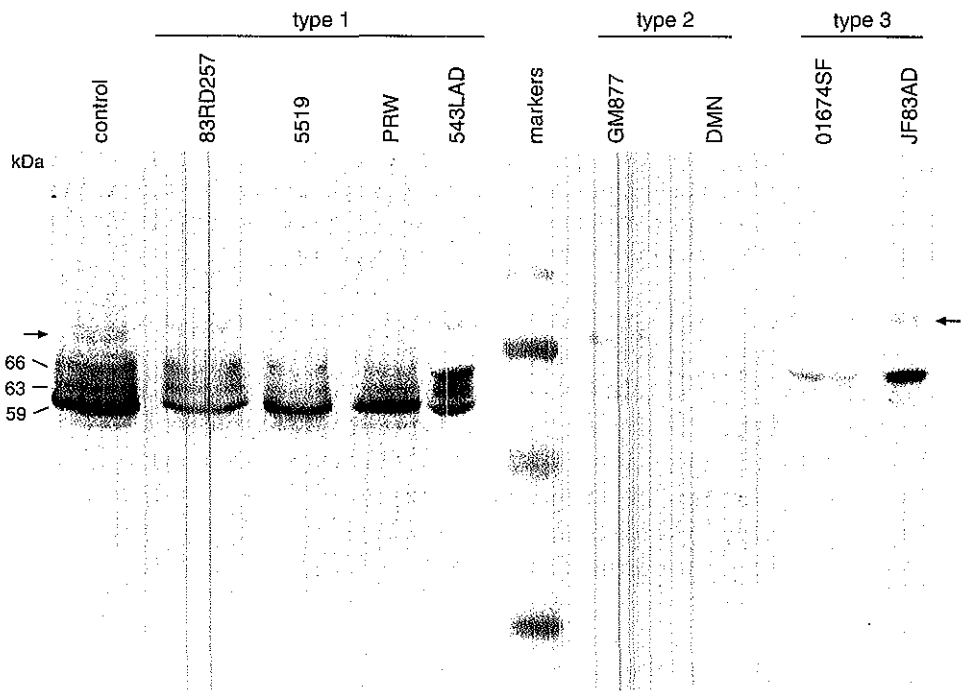


Figure 1: Molecular forms of glucocerebrosidase in normal and mutant fibroblasts as revealed by immunoblotting.

of cross reactive material in control fibroblasts with apparent molecular weights of 66 kDa, 63 kDa and 59 kDa. These bands correspond to the molecular species of 63 kDa, 61 kDa an 56 kDa, respectively, which were described previously by Ginns et al. using a slightly different gel system.[9,10] The band at about 72 kDa (see arrows) is contaminating protein that is infrequently seen on immunoblots and does not cross react with

monoclonal antibodies raised against glucocerebrosidase.[10] All three molecular forms of glucocerebrosidase are seen in fibroblasts from type 1 patients, but the 59 kDa and sometimes also the 63 kDa forms are deficient in type 2 and type 3 cells.

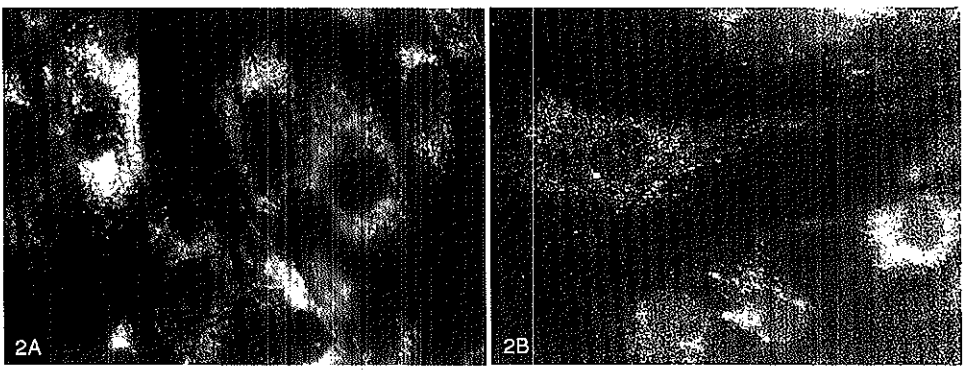


Figure 2: Localization of glucocerebrosidase in control (81RD238) (A) and Gaucher's disease type 1 (PRW) (B) skin fibroblasts. The enzyme is visualized by incubation with rabbit polyclonal antibodies against glucocerebrosidase and subsequent incubation with goat anti-(rabbit IgG) (GAR) conjugated to fluorescein.

The cell lines used in these studies were also assayed for glucocerebrosidase activity with the artificial substrate 4-methyl-umbelliferyl-ß-D-glucopyranoside in the precence of Triton X-100 and taurocholate (Table 1). By immunoprecipitation of glucocerebrosidase with monoclonal antibodies it was estimated that the contribution of non-specific ß-glucosidase to the total activity in Gaucher's disease cells was approximately 15% (data not shown). The ratio of ß-glucosidase activity to ß-N-acetyl-hexosaminidase activity is listed in Table 1.

Subcellullar localization of glucocerebrosidase

Using light microscopy, glucocerebrosidase could be visualized immunocytochemically only in type 1 fibroblasts. However, the signal appeared somewhat weaker than in control cells (Fig. 2.) The label is seen in distinct particles that were previously identified by double labeling as lysosomes.[19] The intracellular compartmentation of the enzyme was examined at the ultrastructural level in frozen sections of fibroblasts and the results of these studies are illustrated in Figs. 3-8. In control fibroblasts (Fig. 3 and 4), immunoreactive material is predominantly detected along the lysosomal membrane. Frequently, however, labeling

is also found on intralysosomal membraneous arrays in secondary lysosomes (Fig. 3). The average number of gold particles observed in lysosomes of control cells is approximately eight. Labeling of the rough endoplasmic reticulum (RER) and Golgi complex is also observed (Fig. 4), but is much less in comparison with the lysosomal labeling.

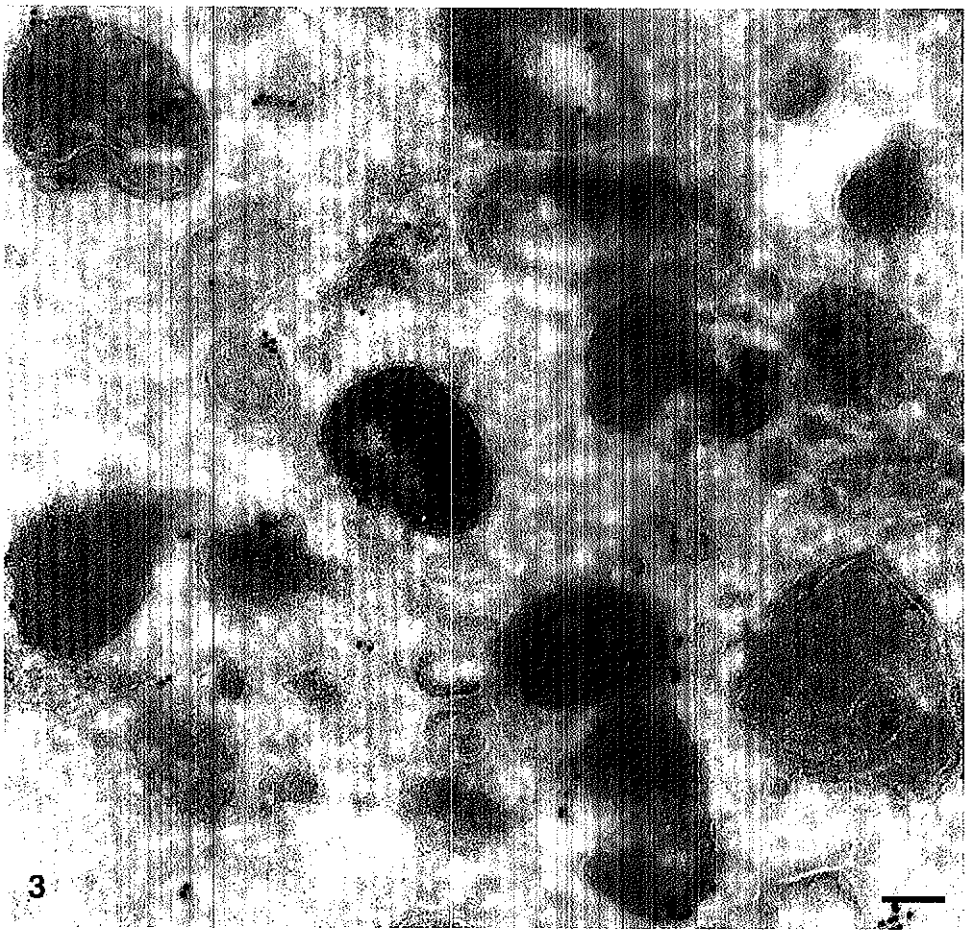


Figure 3: Subcellular localization of glucocerebrosidase in normal human skin fibroblasts (GRA), using GAR conjugated with 10 nm gold particles as immunomarker. Bar 0.1 μm.

In three of the four type 1 cell lines, the number of gold particles in lysosomes is about the same as in control cells (Figs. 5A, 5C and 5D). The RER (Fig. 5B) and Golgi complex are labeled too. In the fourth type 1 cell line the number of gold particles found in the lysosomes is clearly reduced (Fig. 6). However, the RER and Golgi labeling are comparable to

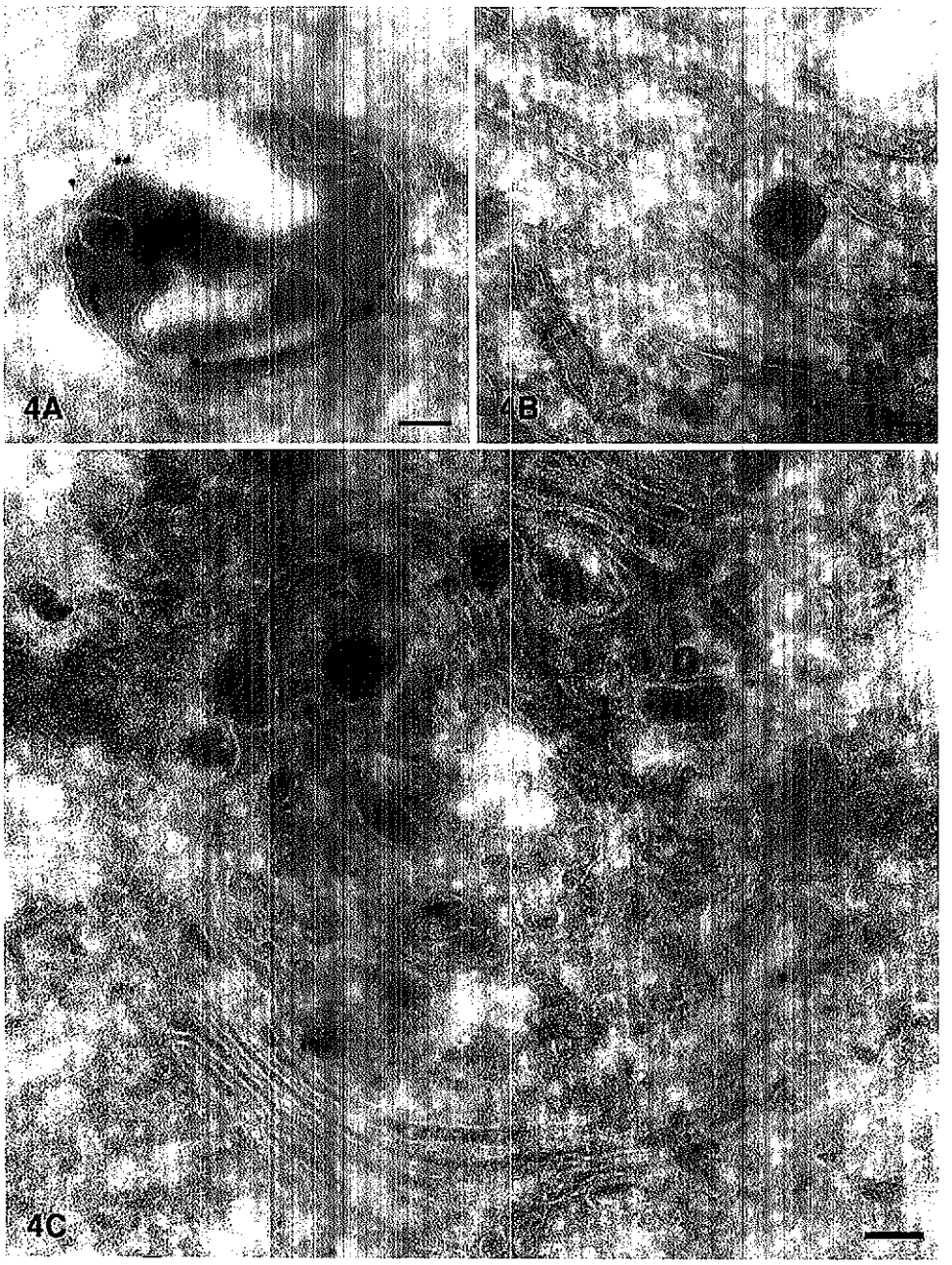


Figure 4: Subcellular localization of glucocerebrosidase in normal human skin fibroblasts (81RD238), using GAR conjugated with 10 nm gold particles as immunomarker. A; labeling of the lysosomal membrane. B; labeling of the rough endoplasmic reticulum. C; labeling of the Golgi complex. Bars 0.1 μm.

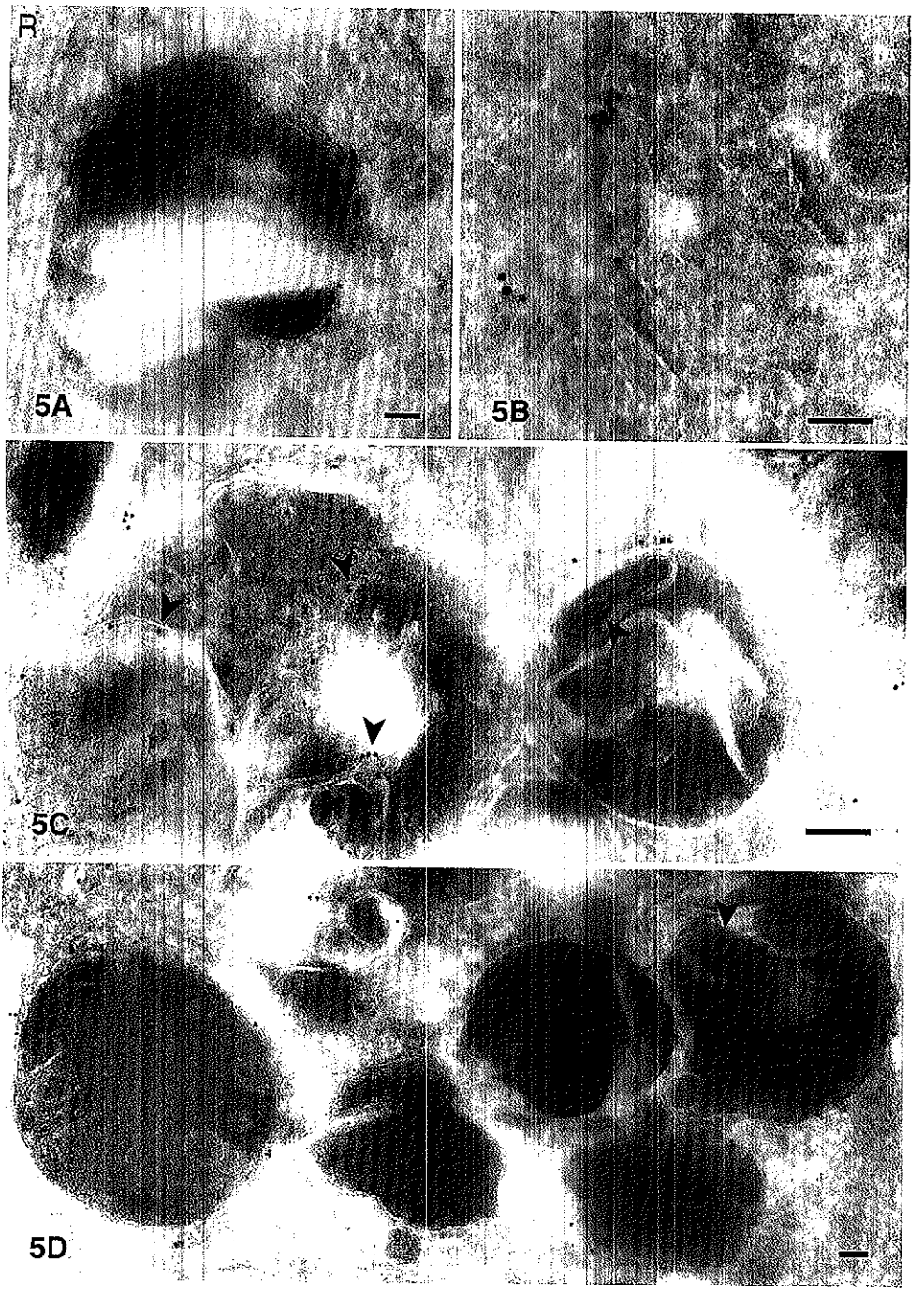


Figure 5: Subcellular localization of glucocerebrosidase in fibroblasts from patients with type 1 Gaucher's disease. A; cell line 83RD257; labeling of the lysosomal membrane and the rough endoplasmic reticulum (R). B; cell line 543 LAD; labeling of the rough endoplasmic reticulum. C; cell line 543 LAD; labeling of lysosomes. Gold particles are localized on the lysosomal membrane and on intralysosomal membraneous material (arrow heads). D; cell line PRW; labeling of the lysosomal membrane and intralysosomal membraneous material (arrow heads). Bars 0.1 μm.

those in control cells.
The labeling pattern in type 2 and type 3 fibroblasts is significantly different from that in control cells and the majority of type 1 cells. Very little cross-reactive material is present in lysosomes, but the number of gold particles in RER and Golgi complex appears normal (see Fig. 7 for type 2 cells and Fig. 8 for type 3 cells).
A semi-quantitative estimate of the number of gold particles per lysosome in the most informative cell lines is given in Table 2.

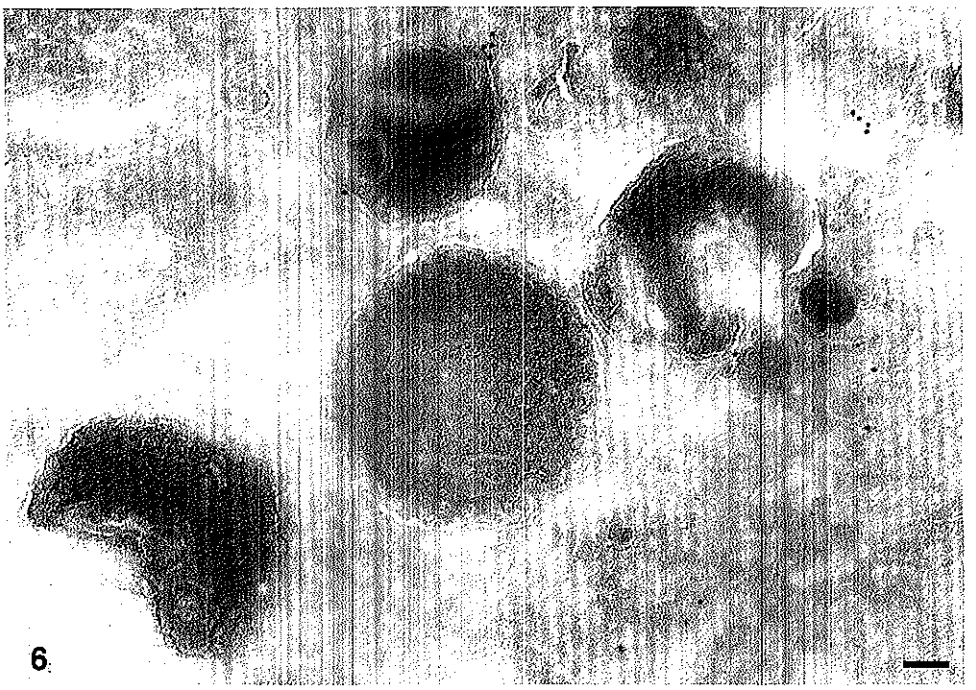


Figure 6: Subcellular localization of glucocerebrosidase in type 1 Gaucher's disease ; cell line 5519. Bar 0.1 μm.

DISCUSSION

Using monospecific antibodies for immunocytochemistry on ultrathin frozen sections of cultured skin fibroblasts, we were able to determine for the first time the actual, in situ, localization of glucocerebrosidase in cells from eight different patients with Gaucher's disease. In control fibroblasts, most enzyme appeared to be associated with the lysosomal membrane. Approximately eight gold particles were detected per lysosome. The four

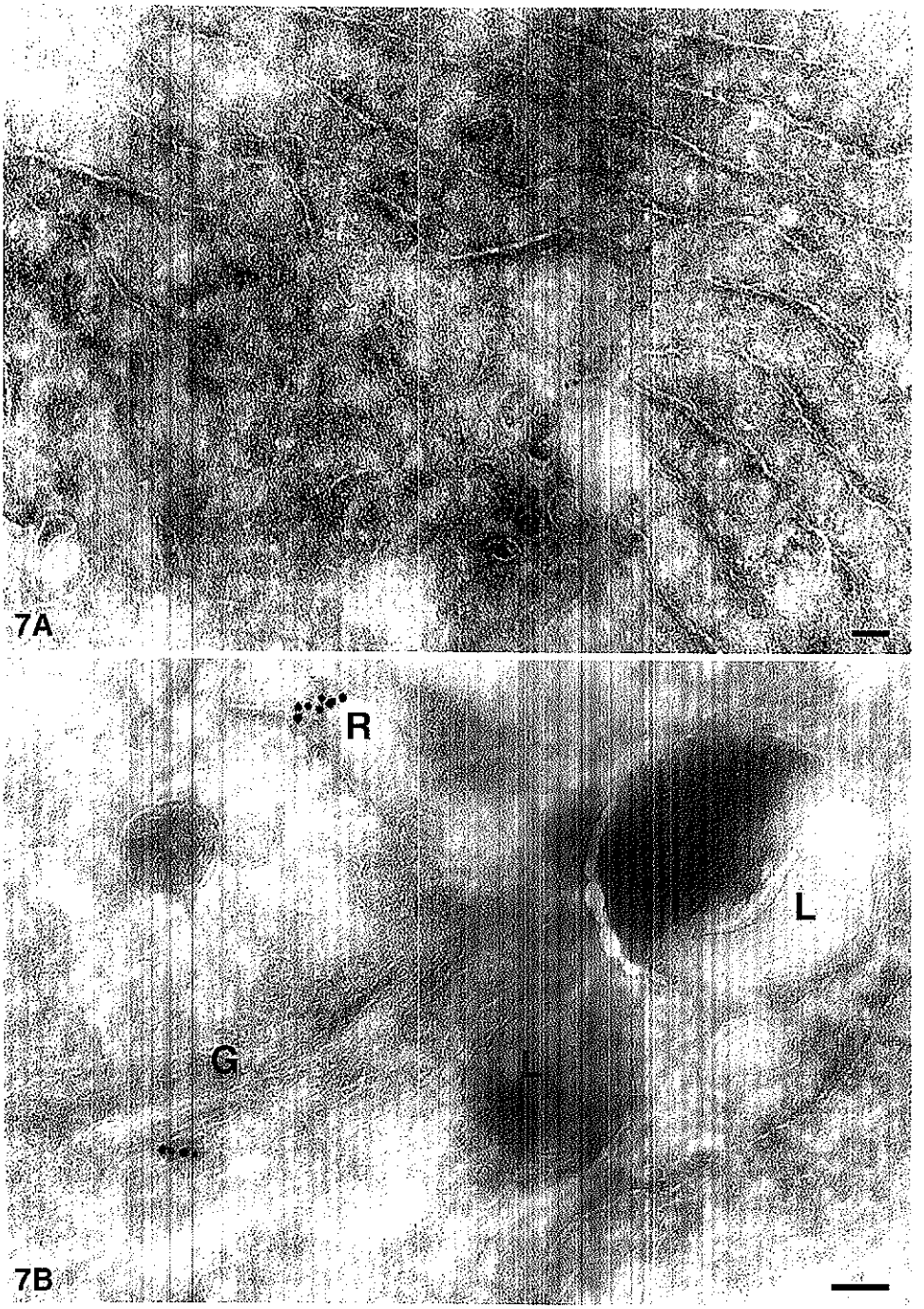


Figure 7: Subcellular localization of glucocerebrosidase in fibroblasts from a patient with Gaucher's disease type 2 (GM877). A; labeling of the rough endoplasmic reticulum (RER). B; labeling of the Golgi-complex (G) and RER (R). Lysosomes (L) are not labeled. Bars 0.1 µm.

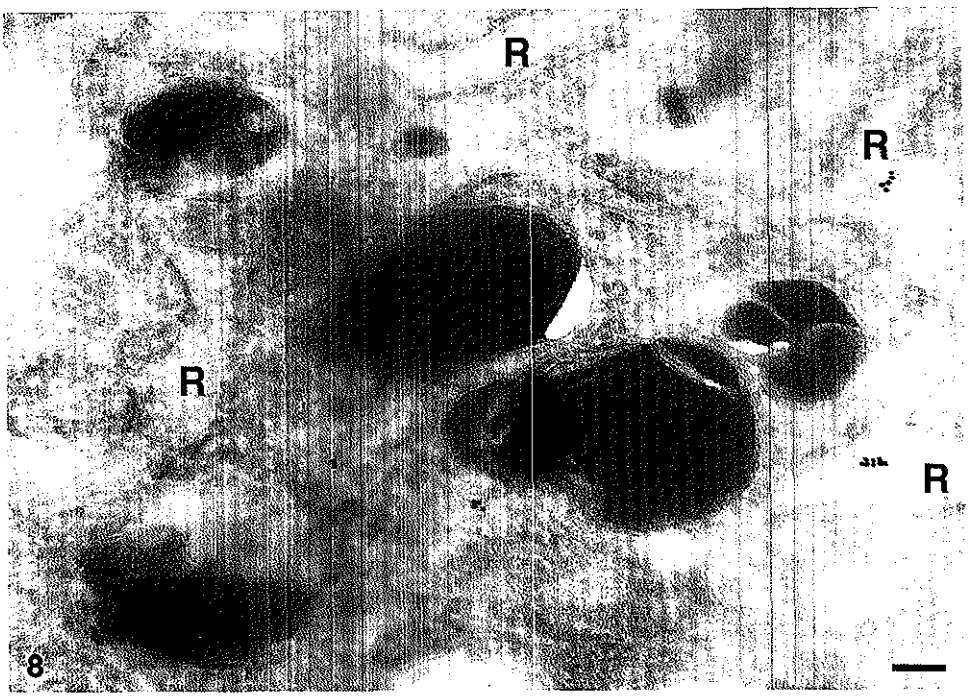


Figure 8: Subcellular localization of glucocerebrosidase in fibroblasts from a patient with type 3 Gaucher's disease (01674S). Lysosomes are weakly labeled, while the labeling of the rough endoplasmic reticulum (R) is normal. Bar 0.1 μm.

type 1 cell lines showed distinct labeling of the lysosomal membrane, but we observed heterogeneity with respect to the amount of immunoreactive material detectable. In three of the four type 1 cases the amount was almost normal, but in the fourth case it was reduced. Interestingly, immunoblotting did not distinguish the various type 1 patients. The different results may relate to the way in which each mutant enzyme is specifically exposed to antibodies in both procedures. Hardly any labeling of lysosomes was observed in type 2 or type 3 cases of Gaucher's disease, whereas RER and Golgi complex in all the different mutant cell lines were normally labeled.

The near absence of immunoreactive material in lysosomes in type 2 and type 3 cells is instructive. Since these cells lack the mature 59 kDa

Table 2. Semi-quantitative data on the lysosomal labeling in different phenotypes.

Code	Phenotype	Average number of gold particles[a]	SEM[b]
5519	type 1	1.3	0.2
JF83AD	type 3	1.0	0.1
83RD257	type 1	7.7	0.5
81RD238	normal	7.1	0.6
GM877	type 2	0.8	0.5

[a)]Frozen fibroblast sections were examined with the electron microscope, and gold particles were counted in randomly chosen lysosomes. In each case 125 lysosomes were inspected.

[b)]SEM; standard error of the mean.

molecular form of glucocerebrosidase on immunoblots, it is reasonable to conclude that this form of glucocerebrosidase is the lysosomal form. Indeed, Western blots of lysosomes prepared from fibroblasts on percoll gradients reveal the 59 kDa to be the predominant CRM in the lysosome (Ginns and Barranger, unpublished data). The 66 kDa and 63 kDa biosynthetic forms of the enzyme, which are presumably in transit to the lysosomes, and which are detectable on immunoblots, probably account for the labeling of RER and Golgi complex.

Since the 59 kDa form of glucocerebrosidase is the most abundant molecular species in control cells, it is obvious that deficiency of this form will lead to a substantial reduction in the total amount of cross-reactive material. Our results are, in this respect, in line with recent data of Beutler et al.[1] showing a severe reduction of immunologically detectable glucocerebrosidase in fibroblasts of patients with type 2 and type 3 Gaucher's disease.

The present information on the subcellular localization of glucocerebrosidase in cultured fibroblasts from different clinical variants strongly supports a previous hypothesis by Ginns et al.[9,10] in which deficient processing of glucocerebrosidase was postulated in type 2 and type 3 Gaucher's disease. This has recently been confirmed by direct measurement of the biosynthesis of enzyme in mutant cell lines by pulse-labeling studies.[12]

The difference in lysosomal localization of glucocerebrosidase in fibroblasts from three non-neuronopathic (type 1) patients, on the one hand, and patients with neuronal involvement (type 2 and type 3), on the other, is striking. It provides a logical basis for understanding the very different appearance of these several phenotypes of Gaucher's disease in relation to the genetic defect. We speculate that neuronal damage is prevented or postponed when a sufficient amount of mutant enzyme with some residual activity is present in the lysosome. To substantiate this hypothesis it is of importance to extend these studies to other cell types, and in particular, to those from neuronal tissues.

ACKNOWLEDGEMENTS

This study was supported by a grant from The Netherlands Organization for the Advancement of Pure Research (ZWO) under the auspices of The Netherlands Foundation for Fundamental Research (FUNGO) and, in part, by a grant from the National Gaucher's Foundation. Prof. Dr. J.F. Jongkind and Prof. Dr. H. Galjaard are thanked for their continuing interest in the work. The help of J. Fengler with the preparation of the micrographs, the technical assistance of M. Kroos and the secretarial assistance of D. Heinsius and L. Metz are greatly appreciated.

REFERENCES

1. Beutler E, Kuhl W, Sorge J (1984) Cross-reacting material in Gaucher's disease fibroblasts. Proc Natl Acad Sci USA 81:6506-6510
2. Brady RO, Kaufer JN, Shapiro D (1965) Metabolism of glucocerebrosidase. II. Evidence of an enzymatic deficiency in Gaucher's's disease. Biochem Biophys Res Commun 18:221-225
3. Brady RO, Barranger JA (1983) Glucosylceramide lipidosis: Gaucher's's disease. In:Stanbury JB, Wijngaarden JB, Frederickson DS, Goldstein JL, Brown MS (eds) The metabolic basis of inherited disease , 4th edn. McGraw-Hill, New York pp 842-856
4. De Vries JWA, Willemsen R, Geuze HJ (1985) Immunocytochemical localization of acrosin and hyaluronidase in epididymal and ejaculated porcine spermatozoa. Eur J Cell Biol 37: 81-88
5. Erickson AH, Ginns EI, Barranger JA (1985) Biosynthesis of the lysosomal enzyme, glucocerebrosidase. J Biol Chem 260: 14319-14324
6. Galjaard H (1980) Genetic metabolic disease; early diagnosis and prenatal analysis. Elsevier/North Holland, Amsterdam, pp 825-826
7. Geuze HJ, Slot JW (1980) Disproportional immunostaining patterns of two secretory proteins in guinea pig and rat exocrine pancreatic cells. An

immunoferritin and fluorescence study. Eur J Cell Biol 21:93-100
8. Geuze HJ, Slot JW, Van der Ley PA, Scheffer RCT, Griffith JM (1981) Use of colloidal gold particles in double-labeling immunoelectronmicroscopy of ultrathin frozen tissue sections. J Cell Biol 89:653-665
9. Ginns EI, Brady RO, Pirrucello S, Moore C, Sorrell S, Furbish FS, Murray GJ, Tager JM, Barranger JA (1982) Mutations of glucocerebrosidase: Discrimination of neurologic and non-neurologic phenotypes of Gaucher's disease. Proc Natl Acad Sci USA 79:5607-5610
10. Ginns EI, Tegelaers FPW, Barneveld R, Galjaard H, Reuser AJJ, Brady RO, Tager JM, Barranger JA (1983) Determination of Gaucher's's disease phenotypes with monoclonal antibody. Clin Chim Acta 131:283-287
11. Griffiths G, Brands R, Burke B, Louvard D, Warren G (1982) Viral membrane proteins acquire galactose in trans Golgi cisternae during intracellular transport. J Cell Biol 95:781-792
12. Jonsson LV, Murray GJ, Ginns EI, Strijland A, Schram AW, Tager JM, Barranger JA (1985) Processing of ß-glucocerebrosidase in normal human fibroblasts and fibroblasts from patients with Gaucher's disease Fed Proc 44:1742
13. Kolodny EH, Ullman MD, Mankin HJ, Raghaven SS, Topal J, Sullivan J (1982) Phenotypic manifestations of Gaucher's disease: clinical features in 48 biochemically verified type 1 patients and comment on type 2 patients. In: Desnick RJ, Gatt S, Grabowski GA (eds) Gaucher's Disease: A century of Delineation and Research Alan R. Liss, New York, pp 33-65.
14. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685
15. Patrick AD (1965) A deficiency of glucocerebrosidase in Gaucher's's disease. Biochem J 97: 17c-18c
16. Tokuyasu KT (1973) A technique for ultracryotomy of cell suspensions and tissues. J Cell Biol 57:551-565
17. Tokuyasu KT (1978) A study of positive staining of ultrathin frozen sections. J Ultrastruct Res 63:287-307
18. Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 76:4350-4354
19. Van Dongen JM, Barneveld RA, Geuze HJ, Galjaard H (1984) Immunocytochemistry of lysosomal hydrolases and their precursor forms in normal and mutant human cells. Histochem J 16:941-954
20. Weber K, Rathke PC, Osborn M (1978) Cytoplasmic microtubular images in glutaraldehyde-fixed tissue culture cells by electronmicroscopy and by immunofluorescence microscopy. Proc Natl Acad Sci 75:1820-1824

APPENDIX PAPER II

Ultrastr Pathol (1988) 12:471-478

AN IMMUNOELECTRON MICROSCOPIC STUDY OF GLUCOCEREBROSIDASE IN TYPE I GAUCHER'S DISEASE SPLEEN

R. Willemsen, J.M. van Dongen, J.M.F.G. Aerts, A.W. Schram, J.M. Tager, R. Goudsmit, A.J.J. Reuser

ABSTRACT

An immunogold labelling procedure was applied to ultrathin cryosections and used to study the subcellular localization of glucocerebrosidase in lipid-laden "Gaucher cells" in spleen from a patient with type 1 Gaucher's disease. Glucocerebrosidase protein was associated with the characteristic stored lipid material in large, irregularly shaped vacuoles. As shown by double labelling the storage vacuoles contained not only glucocerebrosidase protein but also other lysosomal enzymes. Thus, the storage vacuoles can be considered to be secondary lysosomes. The findings indicate that although glucocerebrosidase was present in secondary lysosomes in this patient the activity of the mutant enzyme was insufficient to prevent storage of glucocerebroside in the spleen.

INTRODUCTION

Gaucher's disease is an inherited sphingolipid storage disorder caused by a deficiency of the lysosomal enzyme glucocerebrosidase[1,2]. The lipid accumulates predominantly in macrophages, in all parts of the reticulo-endothelial system[3,4]. Storage cells have a characteristic morphology and are called Gaucher cells. Their identification can be used as a diagnostic criterion for Gaucher's disease (for review see Ref. 5).
Among the different clinical phenotypes of Gaucher's disease, type 1 (chronic, nonneuronopathic) is the most common. It has a particularly high incidence among Ashkenazic Jews. Type 2, the acute, neuronopathic form, and type 3, the chronic, neuronopathic form of Gaucher's disease are more rare[5,6]. Clinical differences have been attributed to distinct abnormalities in the formation, function and subcellular localization of glucocerebrosidase in the various forms of this disease[7-11]. In fibroblasts from normal individuals, glucocerebrosidase is synthesized as a 63 kD precursor and converted via intermediate forms to a mature enzyme of

59kD[11,12]. This mature form of glucocerebrosidase is often deficient in phenotypes 2 and 3[7,8,10,11]. In type 1 Gaucher's disease the maturation process is completed, but the enzyme is catalytically deficient[7,10], and in some instances more labile[11].
Recently, we succeeded in visualizing glucocerebrosidase in situ in control and Gaucher's disease fibroblasts by performing immunocytochemistry at the electronmicroscopic level[10] .
Colloidal gold was used as electron-dense marker. In three out of four type 1 cases studied, a lysosomal localization of glucocerebrosidase was observed and the amount of cross-reactive material was near normal. But, in the fourth case and in fibroblasts from type 2 and type 3 patients, little cross-reactive material was detected in lysosomes[10]. In a recent study encompassing a total of 36 patients it has been shown that different allelic mutations occur in the various clinical phenotypes of Gaucher's disease[13].
One of the characteristic features of type 1 Gaucher's disease is the splenomegaly caused by massive accumulation of glucocerebroside in macrophages in the spleen. We have, therefore used the immunogold labelling procedure to establish the intracellular localization of glucocerebrosidase in the lipid-laden Gaucher cells in the spleen of a patient with type 1 Gaucher's disease. For the first time it is shown that the mutant enzyme is present in the characteristic lipid-containing storage vacuoles (secondary lysosomes) in the Gaucher cells.

MATERIALS AND METHODS

Case history
Patient D (Ashkenazic Jewish) was born in 1947. In 1954 the diagnosis of M. Gaucher was tentatively made and was confirmed a few years later by bone marrow aspiration. In 1974 the patient underwent a thorough medical investigation. At that time he had no special complaints. He was not anemic, although subecteric. The liver was palpable 3 cm below the costal margin, the spleen reached just above the pelvic inlet. The hemoglobin concentration was 8.1 mmol/L, leucocytes 2.9 x 10^9/L, platelets 52 x 10^9/L. At a later examination, in 1985, the abdomen was markedly distended. The liver was palpable 4 cm below the costal margin, the spleen was enormous and filled almost the whole abdomen. There was a femoral hernia bilaterally. The hemoglobin concentration was 5.8 mmol/L, hematocrit 0.3, reticulocytes 70°/oo, leucocytes 1.7 x 10^9/L, platelets 54 x 10^9/L. The haptoglobin content was low.
Splenectomy was planned because of the risk of incarceration of the

herniae, mechanical complaints and the hypersplenism. After a very careful preparation the splenectomy was performed in November 1986, and a spleen weighing 13 Kg was removed. The postoperative course was undisturbed. After the operation the patient felt much better. In July 1987 the Hb concentration was 9.9 mmol/L, hematocrit 0.49, leucocytes 13.4 x 10^9/L and platelets 320 x 10^9/L.

Light and Immunoelectron microscopy

Immediately after splenectomy, small pieces of the spleen were fixed in 0.1 M phosphate buffer (PB) pH 7.3, containing 1% acrolein and 0.4% glutaraldehyde. After fixation for 24 hrs at 4°C, the specimens were stored in 0.1 M PB containing 2% paraformaldehyde and 1 M sucrose. Frozen sections were made as described before[10], using an LKB Nova ultratome equipped with the Cryo Nova, at -110°C. Thick sections (0.5 µm) were stained with 0.4% Toluidine blue. Immunolabelling on ultrathin sections (approximately 60 nm) was performed as described before[10]. Antigen-antibody complexes were visualized through binding of goat anti-(rabbit IgG) antibodies conjugated with 10 nm colloidal gold (GAR 10, Janssen Pharmaceutica, Belgium). To localize two antigens simultaneously, the first antibody was labelled with a 5 nm protein A-gold probe and the second with a 10 nm probe, according to Geuze et al.[14]

Sections were stained with uranylacetate, embedded in 1.5 % methylcellulose, and examined with a Philips EM 400 at 80 kV. As a check on the specificity of the labelling procedure, sections were incubated with normal rabbit serum as substitution for the primary antibody, GAR-10 conjugate alone or with protein A-gold (ϕ 5 nm) only. Background labelling was negligible.

Antibodies

Immunocytochemistry was performed with affinity-purified rabbit polyclonal antibodies against homogeneous human placental glucocerebrosidase[7]. For double labelling studies, a mixture of affinity-purified rabbit polyclonal antibodies against human acid α-glucosidase[15], N-acetyl-ß-hexosaminidase[15], ß-galactosidase[16] and acid phosphatase[17] (tartrate inhibitable) were used.

Biochemical procedures

Immediately after splenectomy pieces of the spleen were quickly frozen

in liquid nitrogen and stored at -70°C until used. Tissue was homogenized in 50 mM potassium phosphate buffer (pH 6.5) containing 0.25% (v/v) Triton X-100, and glucocerebrosidase activity was determined as described by Jonsson et al.[11] using 4-methylumbelliferyl-ß-glucoside as substrate. Glucocerebrosidase was immunoprecipitated from an extract prepared from 50 mg of either control spleen or Gaucher spleen. Molecular species of the glucocerebrosidase were separated by polyacrylamide gel electrophoresis according to Laemmli[18], and electrophoretically transferred to nitrocellulose[19]. Glucocerebrosidase was visualized with enzyme-specific rabbit antibodies[7] in combination with goat anti-(rabbit IgG) coupled to horse radish peroxidase[11].

RESULTS AND DISCUSSION

Biochemical analysis

The activity of glucocerebrosidase in spleen tissue of the patient was 0.8 nmol/h/mg protein as compared with a mean activity of 15.4 ± 1.9 nmol/h/mg protein in specimens of three different control spleens measured at the same time.

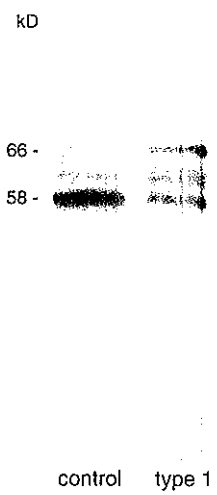


Fig. 1 Molecular mass forms of glucocerebrosidase in splenic extracts from a control subject and the patient with type 1 Gaucher's disease. For experimental details see Materials and Methods. Enzyme was isolated from 50 mg wet weight of spleen. The activity applied to the gel was 2.6 nmol/h in the case of the Gaucher spleen preparation and 78 nmol/h in the case of the control spleen preparation.

Fig. 1 shows an analysis by immunoblotting of the molecular mass species of glucocerebrosidase present in extracts of the patient's spleen and of control spleen. In the control spleen, forms of Mr 62-63, 66 and 59 kD are seen, corresponding to the precursor, intermediate and mature

forms of glucocerebrosidase, respectively (see ref. 20). The same forms are seen in the spleen from the patient.
It should be noted that the glucocerebrosidase in each lane was immunoprecipitated from extracts prepared from identical amounts of tissue. Since the intensity of the bands containing cross-reactive material was about the same in the Gaucher spleen as in the control spleen, and since the enzymic activity was about 20-fold less in the case of the type 1 spleen extract, the specific molecular activity (i.e. the activity per molecule of glucocerebrosidase protein) must be markedly decreased in the patient's spleen.

Light and Immunoelectromicroscopy
By light microscopy, numerous large lipid-laden Gaucher cells were found in the red pulp of the spleen (Fig. 2). Typically, the nucleus of the cells is eccentric and the cytoplasm is structured irregularly, because of deposits of glucocerebroside. The fine structure of the storage material in Epon embedded tissue sections has been described as arrays of twisted tubules [21] . The characteristic appearance of the Gaucher cell is well

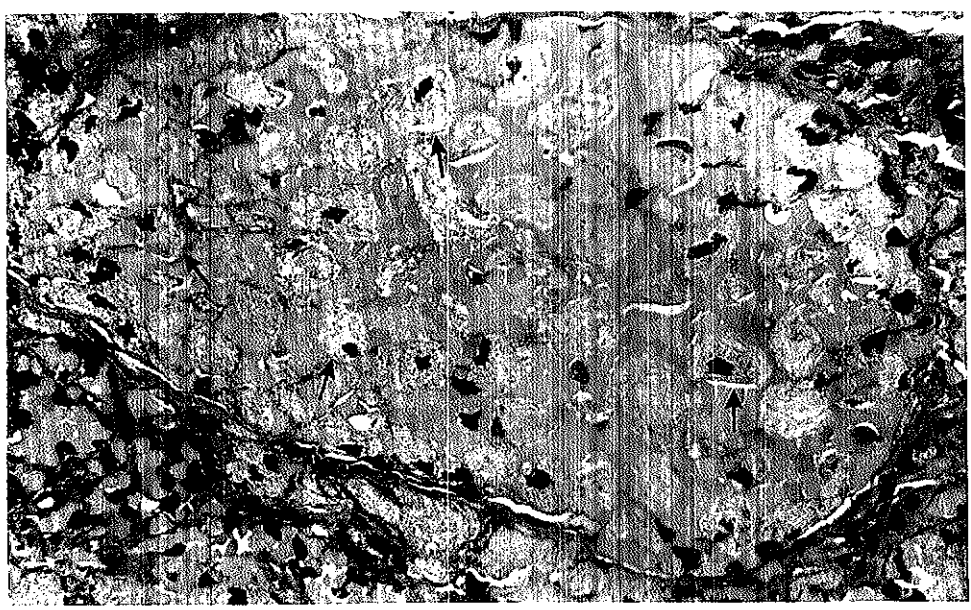


Fig. 2 Semithin (0.5 µm) cryosection of the red pulp of the spleen from the patient with type 1 Gaucher's disease. Numerous lipid-laden Gaucher cells are present (arrows). 550x.

Fig. 3 Subcellular localization of lysosomal enzymes in a Gaucher cell using a mixture of antibodies to α-glucosidase, N-acetyl-ß-hexosaminidase, ß-galactosidase and acid phosphatase in combination with goat anti-(rabbit IgG) antibodies conjugated with 10 nm gold particles as immunomarker. Lysosomal enzymes are present in irregular, enlarged lysosomes (L) with characteristic storage material. 50,000x. N = nucleus

preserved in ultrathin frozen sections, as illustrated in Figs. 3-5. There is massive storage throughout the cytoplasm. Clearly, the storage compound is surrounded by a membrane (Fig. 4, arrows), and the irregular fields of storage material are enlarged lysosomes. This was demonstrated by incubating the cryosections with a mixture of affinity purified antibodies against four different lysosomal enzymes and, subsequently, with goat anti-(rabbit IgG) antibodies coupled to colloidal gold. Fig. 3 shows heavy labelling of the storage vacuoles whereas hardly any gold particles are seen in the nucleus or cytoplasmic areas between the storage material. Apparently, the lysosomes are still intact although their normal shape is completely distorted.

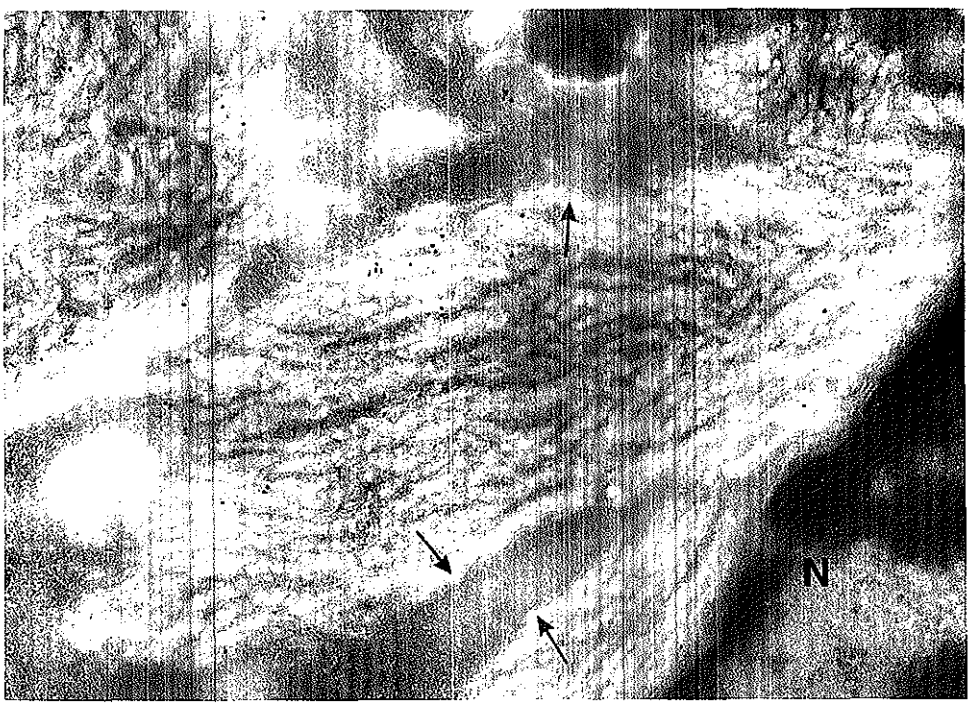


Fig. 4 Subcellular localization of glucocerebrosidase in a Gaucher cell using anti-(glucocerebrosidase) and goat anti-(rabbit IgG) antibodies conjugated with 10 nm gold particles as immunomarker. Glucocerebrosidase is localized in lysosomes in association with storage material. The arrows indicate the limiting membrane of the lysosome. 45,000x. N = nucleus

The localization of glucocerebrosidase in these Gaucher cells is illustrated in Fig. 4 using the same immunocytochemical procedure. Gold particles are aligned along the twisted glycolipid tubules. The labelling pattern

Fig. 5 Simultaneous subcellular localization of glucocerebrosidase and other lysosomal enzymes in a Gaucher cell using protein A coupled to 5 nm and 10 nm colloidal gold particles as immunomarkers. Glucocerebrosidase (10 nm gold) and other lysosomal enzymes (5 nm gold) are present within the same lysosome (L). 82,500x. M=mitochondrion.

suggests that mutant glucocerebrosidase is associated with its natural substrate but is not able to degrade glucocerebroside at a sufficient rate to prevent storage of the glycolipid. Thus the picture illustrates exactly the situation predicted by biochemical data obtained with spleen (J.M.F.G. Aerts, unpublished observations) and biochemical[11] and immunocytochemical[10] data obtained with fibroblasts: glucocerebrosidase protein is present in near normal amounts and is localized in lysosomes in the spleen of this type 1 Gaucher's disease patient, but the mutant enzyme is catalytically defective.
A double labelling procedure with protein A coupled to gold complexes of different sizes was used to visualize glucocerebrosidase (10 nm) and other lysosomal enzymes (5 nm) simultaneously. Particles of both sizes are found in the same organelle (Fig. 5). It suggests that glucocerebroside does not accumulate in specific storage vacuoles that have lost their lysosomal function, but in secondary lysosomes with probably a normal content of lysosomal enzymes.
In conclusion, immunoelectronmicroscopy on ultrathin frozen sections of Gaucher organs can serve as an additional tool to study and characterize the different clinical forms of Gaucher's disease.

ACKNOWLEDGEMENTS

The authors are very grateful to the patient (Mr. D.) for his interest in the studies and for agreeing to allow his spleen to be used for this purpose. We thank Dr. Ed I. Ginns and Dr. John A. Barranger for supplying antibodies against glucocerebrosidase. The help of J. Fengler and T. van Os with the preparation of the micrographs and the secretarial assistance of D. Heinsius are greatly appreciated.
Supported by a grant to J.M.T. and A.W.S. from the National Gaucher Foundation, U.S.A. (Grant no. 19).

REFERENCES

1. Brady, R.O., Kanfer, J.N., Shapiro, D.: Metabolism of glucocerebrosidase. II. Evidence of an enzymatic deficiency in Gaucher's disease. Biochem. Biophys. Res. Commun. 18:221-225, 1965.
2. Patrick, A.D.: A defiency of glucocerebrosidase in Gaucher s disease. Biochem. J. 97:17c-18c, 1965.
3. Burns, G.F., Cawly, J.C., Flemans, R.J., Higgy, K.E., Worman,C.P. Barker, C.R., Roberts, B.E., Hayhoe, F.G.J.: Surface marker and other characteristics of Gaucher cells. J. Clin.Path. 30:981-988, 1977.
4. Djaldetti, M., Fishman, P., Bessler, H.: The surface ultra-structure of Gaucher

cells. Am. J. Clin. Pathol. 71:146-150, 1979.
5. Brady, R.O., Barranger, J.A.: Glucosylceramide lipidosis:Gaucher's Disease. In: The Metabolic Basis of Inherited Disease, edited by J.B. Stanbury, J.B. Wijngaarden, D.S.Fredrickson, J.L. Goldstein, M.S. Brown, 4th edn., pp 842-856. New York: McGraw-Hill, 1983.
6. Fredrickson, D.S., Sloan, H.R.: Glucosylceramide lipidosis:Gaucher's disease. In: The Metabolic Basis of Inherited Disease, edited by J.B. Stanbury, J.B. Wijngaarden, D.S.Fredrickson, 3d ed., pp 730-759. New york: McGraw-Hill, 1972.
7. Ginns, E.I., Brady, R.O. Pirrucello, S., Moore, C., Sorrell, S., Furbish, F.S., Murray, G.J., Tager, J.M., Barranger, J.A. Mutations of glucocerebrosidase: discrimination of neurologic and non-neurologic phenotypes of Gaucher disease. Proc. Natl. Acad. Sci. U.S.A. 79:5607-5610, 1982.
8. Ginns, E.I., Tegelaers, F.P.W., Barneveld, R., Galjaard, H., Reuser, A.J.J., Brady, R.O., Tager, J.M., Barranger, J.A. Determination of Gaucher's disease phenotypes with monoclonal antibody. Clin. Chim. Acta. 131:281-287, 1983.
9. Beutler, E., Kuhl, W. Glucocerebrosidase processing in normal fibroblasts and in fibroblasts from patients with type I, type II, and type III Gaucher disease. Proc. Natl. Acad. Sci. U.S.A. 83:7472-7474, 1986.
10. Willemsen, R., Van Dongen, J.M., Ginns, E.I., Sips, H.J., Schram, A.W., Tager, J.M., Barranger, J.A., Reuser, A.J.J. Ultrastructural localization of glucocerebrosidase in cultured Gaucher's disease fibroblasts by immunocytochemistry. J. Neurol. 234:44-51, 1987.
11. Jonsson, L.M.V., Murray, G.J., Sorrell, S.H., Strijland, A., Aerts, J.M.F.G., Ginns, E.I., Barranger, J.A., Tager, J.M., Schram, A.W. Biosynthesis and maturation of glucocerebrosidase in Gaucher fibroblasts. Eur. J. Biochem. 164:175-179, 1987.
12. Erickson, A.H., Ginns, E.I., Barranger, J.A. Biosynthesis of the lysosomal enzyme glucocerebrosidase J.Biol.Chem. 260:14319-14324, 1985.
13. Tsuji, S., Choudary, P.V., Martin, B.M., Stubblefield, B.K., Mayor, J.A., Barranger, J.A., Ginns, E.I. A mutation in the human glucocerebrosidase gene in neuronopathic Gaucher's disease. N. Engl. J. Med. 316:570-575, 1987.
14. Geuze, H.J., Slot, J.W., Van der Ley, P.A., Scheffer, R.C.T., Griffith, J.M. Use of colloidal gold particles in double labelling immuno-electronmicroscopy of ultrathin frozen tissue sections. J. Cell Biol. 89:653-665, 1981.
15. Reuser, A.J.J., Kroos, M., Oude Elferink, R.P.J., Tager, J.M. Defects in synthesis, phosphorylation and maturation of acid α-glucosidase in glycogenosis Type II. J. Biol. Chem. 263:7336-8341, 1985.
16. D Azzo, A., Hoogeveen, A.T., Reuser, A.J.J., Robinson, D., Galjaard, H. Molecular defect in combined ß-galactosidase and neuraminidase deficiency in man. Proc. Natl. Acad. Sci. U.S.A. 69:4535-4539, 1982.
17. Parenti, G., Willemsen, R., Hoogeveen, A.T., Verleun-Mooyman, M., van Dongen, J.M., Galjaard, H. Immunocytochemical localization of lysosomal acid phosphatase in normal and "I-cell" fibroblasts. Eur. J. Cell Biol. 43:121-127, 1987.
18. Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685, 1970.
19. Towbin, H., Staehelin, T., Gordon, J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications.

Proc. Natl. Acad. Sci. U.S.A. 76:4350-4354, 1979.
20. Aerts, J.M.F.G., Donker-Koopman, W.E., Koot, M., BrouwerKelder, E.M., Murray, G.J., Barranger, J.A., Tager, J.M., Schram, A.W. Forms of glucocerebrosidase present in tissues and urine. In: Enzymes of lipid Metabolism II. Edited by L. Freyse, H. Dreyfus, R. Massarelli and S. Gatt. pp 747-752. New York: Plenum, 1986.
21. Lee, R.E. The fine structure of the cerebroside occurring in Gaucher's disease. Proc. Natl. Acad. Sci. U.S.A. 61:484-489, 1978.

APPENDIX PAPER III

Nature (1992) 357:407-410

ANIMAL MODEL OF GAUCHER'S DISEASE FROM TARGETED DISRUPTION OF THE MOUSE GLUCOCEREBROSIDASE GENE

VLJ Tybulewicz, ML Tremblay, ME LaMarca, R Willemsen, BK Stubblefield, S Winfield, B Zablocka, E Sidransky, BM Martin, S Huang, KA Mintzer, H Westphal, RC Mulligan, and EI Ginns

ABSTRACT

Gaucher disease is the most prevalent lysosomal storage disorder in humans and results from an autosomally inherited deficiency of the enzyme glucocerebrosidase (β-D-glucosyl-N-acylsphingosine glucohydrolase)[1-6], which is responsible for degrading the sphingolipid glucocerebroside. An animal model for Gaucher's disease would be important for investigating its phenotypic diversity and pathogenesis and for evaluating therapeutic approaches. A naturally occurring canine model has been reported but not propagated[7]. Attempts to mimic the disease in animals by inhibiting glucocerebrosidase have been inadequate[8]. Here we generate an animal model for Gaucher's disease by creating a null allele in embryonic stem cells through gene targeting and using these genetically modified cells to establish a mouse strain carrying the mutation[9,10]. Mice homozygous for this mutation have < 4% of normal glucocerebrosidase activity, die within twenty four hours of birth, and store glucocerebroside in lysosomes of cells of the reticuloendothelial system.

To disrupt the murine glucocerebrosidase gene, a targeting plasmid was constructed containing a neomycin-resistance gene inserted into exons 9 and 10 (Fig. 1a), which encode part of the active site of the enzyme[2,11]. A herpes simplex virus thymidine kinase gene flanks the construct, allowing the use of a positive-negative selection scheme[12]. This construct was introduced into embryonic stem cells by electroporation and the cells were subjected to selection with the drugs G418 and gancyclovir[12]. The correct gene targeting event in doubly resistant individual clones was identified by Southern blot analysis (Fig. 1b). Of 528 clones screened, 19 had underdone the homologous recombination event. As gancyclovir selection enriched the recombinants by 8.5-fold, the ratio of correctly

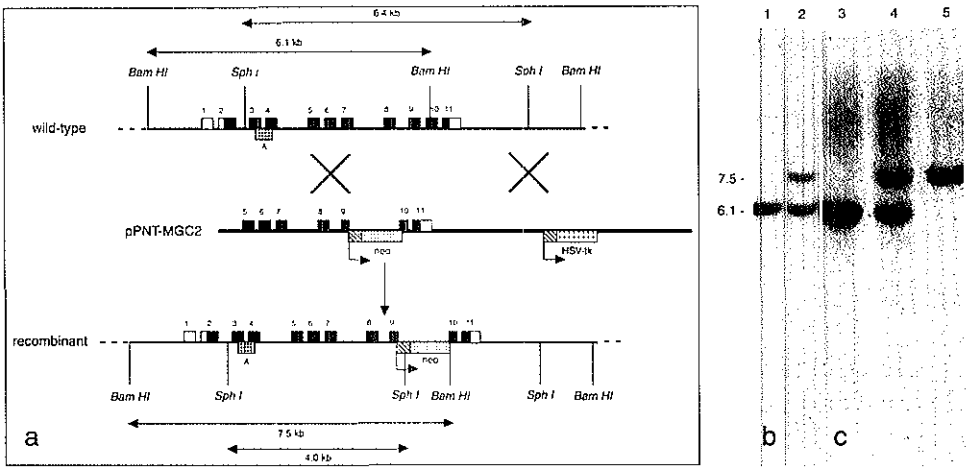


Figure 1. a, Strategy for disruption of the murine glucocerebrosidase gene. The line labelled "wild-type" represents the normal genomic structure of the mouse glucocerebrosidase gene[11]. Exons 1 to 11 are shown as boxes above the line, with filled boxes representing coding regions. The middle line labelled pPNT-MGC2 represents the targeting plasmid linearized at a unique NotI site. Neo-mycin-resistance (neo) and herpes simplex virus thymidine kinase (HSV-tk) genes are shown, both transcribed from the phosphoglycerate kinase-1 gene promoter (shaded boxes with arrows); thick line represents the plasmid backbone. The lower line labelled "recombinant" shows the structure of the glucocerebrosidase gene after a correct targeting event. Parts of exons 9 and 10 and all of intron 9 are replaced with the *neo* gene. The shaded box labelled "A" represents a 325-bp HindIII/NarI fragment used as probe to identify gene targeting events. Predicted sizes (in kilobases, kb) of BamH1 digested and SphI-digested fragments hybridizing to this probe are shown. b and c, Southern blot analysis of BamHI-digested genomic DNA from embryonic stem (ES) cell clones and from progeny of a germ-line chimaera generated from a correctly targeted clone. In b, lane 1 contains normal ES cell DNA from cell line CCE[20] and lane 2 contains DNA of clone 23-2A-1b, showing a correct targeting into the glucocerebrosidase gene. In c, Lanes 3, 4, and 5 contain DNA extracted from tails of mice that are normal, heterozygous and homozygous mutant, respectively. DNA in all lanes was digested with BamHI and was hybridized with probe A (see a). Sizes of fragments are shown in kilobases. Digests of DNA with SphI gave fragments of the expected sizes on Southern blot analysis (data not shown).

METHODS

Construct pPNT-MGC2 was made by subcloning a 2,797-bp SspI-SalI fragment containing all of exons 5 to 8 and part of exon 9 of the murine glucocerebrosidase gene into the XhoI site of pPNT (ref. 21) to create pPNT-MGC1. The glucocerebrosidase gene was isolated from a BALB/c DNA phage library and has a map corresponding to that reported in ref. 11. A 3,205-bp BamHI fragment containing part of exon 10 and all of exon 11 was subcloned into the BamHI site of pPNT-MGC1 to give

pPNT-MGC2. ES cell line CCE[20] was grown, electroporated, and selected as described[21], then injected into C57BL/6 blastocysts[13]. DNA was prepared from ES clones and mouse tails and analyzed by Southern blotting as before[21].

targeted genes was one per 236 stably transformed cells.

Cells from four embryonic stem cell clones containing the targeted mutation in the glucocerebrosidase gene of one allele were injected into blastocysts from C57BL/6 mice and transferred to FVB/N foster mice[13]. From these injections, male offspring with more than 30% chimaerism (as judged by coat color) were test-bred against C57BL/6 females. One chimaeric male, derived from embryonic stem cell line 23-2A16, transmitted the glucocerebrosidase mutation to his progeny. Mice heterozygous for the disrupted glucocerebrosidase gene were mated and homozygous mutant progeny were identified by Southern blot analysis (Fig. 1c). As expected, the targeted mutation was transmitted in a mendelian fashion. Assay of glucocerebrosidase activity from mouse tails demonstrated that in heterozygous mutants the mean activity was 44.4% (s.d. $\pm$ 2.2%) of that in normal littermates (Fig. 2a). The glucocerebrosidase activity in homozygous mutant mice was less than 4% of control, confirming that the targeted gene disruption had resulted in a null allele. Thin-layer chromatography of the neutral glycosphingolipids in tissues of normal and homozygous mutant mice demonstrated increased glucocerebroside levels only in the mutants (Fig. 2b).

Homozygous mutant mice are severely compromised at birth, are under weight and respire abnormally with rapidly progressing cyanosis, and their feeding and movement are decreased (Fig. 3). All homozygous mutant mice (n>100) died within 24 h of birth. Using electron microscopy, macrophages with lysosomal lipid accumulation were found in the liver (Fig. 4a), bone marrow (Fig. 4b), spleen and brain of homozygous mutant mice (n=4), but not in normal littermates (Fig. 4c; n=3) or a heterozygous mutant mice. Twenty-five macrophages were examined in each tissue specimen. The overall architecture of the liver of the homozygous mutant mice appeared appropriate for the early neonatal stage of development, demonstrating extensive hepatic hematopoiesis. As in the human disease, hepatocytes were not affected and storage was restricted to the Kupffer cells in the liver sinusoids[1]. Stored lipid in the macrophages of the homozygous mutant mice has the same physical appearance as the tubular lysosomal deposits of glucocerebroside in cells of human patients with Gaucher's disease (Fig. 4d)[14,15].

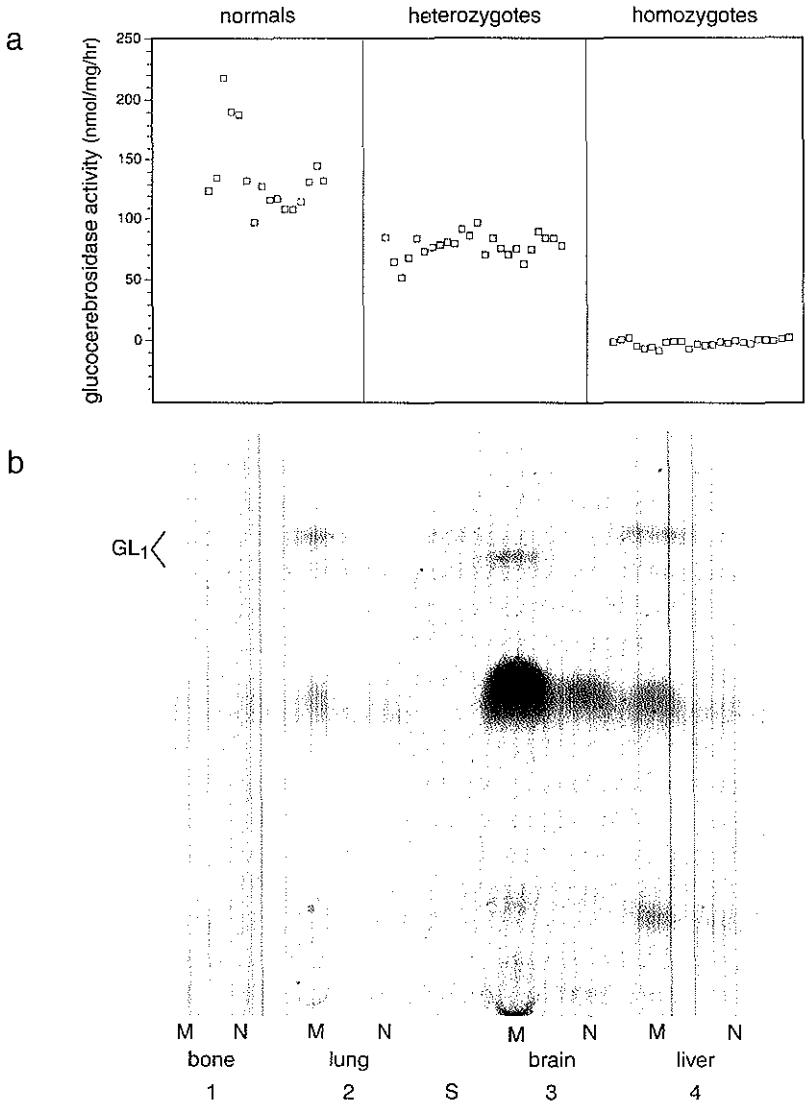


Figure 2. a, Glucocerebrosidase activity in normal, heterozygous, and homozygous mutant mice. Enzyme activity was assayed in mouse tail extracts using 4-methylumbelliferyl-β-D-glucopyranoside as a substrate as described[22]. b, Thin-layer chromatographic of neutral glycosphingolipids in tissues from normal and homozygous mutant mice. Bone marrow (lanes 1), lung (lanes 2), brain (lanes 3), and liver (lanes 4) from homozygous mutant (M) or normal (N) mice were extracted and analyzed (see Methods). Results shown are from normal and homozygous mutant littermates and are representative of data from four homozygous mutant and normal

pairs of littermates. The lane marked "S" contains 4 μg of standard glucocerebroside (GL_1). The position of glycosphingolipid GL_1, running as two bands on the chromatogram, is indicated. Glucocerebroside in brain differs from that in extraneural tissues, having predominantly C_{18} rather than C_{22} fatty acids ceramide moieties[23]. As a result of these differences in the fatty acid component, GL_1 resolves into two components.

METHODS

Tissues from normal and homozygous mutant mice were extracted with 9.5 ml (v/w) of chloroform/methanol (2:1 v/v) with sonication for 10 min at room temperature followed by addition of 0.2 volumes water. After vortexing, samples were centrifuged for 2 min. A 100-μl aliquot of the lower phase containing the neutral glycosphingolipids from each tissue was evaporated to dryness, resuspended in 50 μl chloroform/methanol (2:1 v/v) and applied to precoated silica gel-60 high-performance thin-layer chromatographic plates (Merck) that had been activated at 100°C for one h[24]. The solvent mixture used for development was chloroform-methanol-water, 65:25:4 (v/v/v). After development for 30 min, the plates were air-dried, and the glycolipids visualized with orcinol-ferric chloride (Bial's reagent)[24].

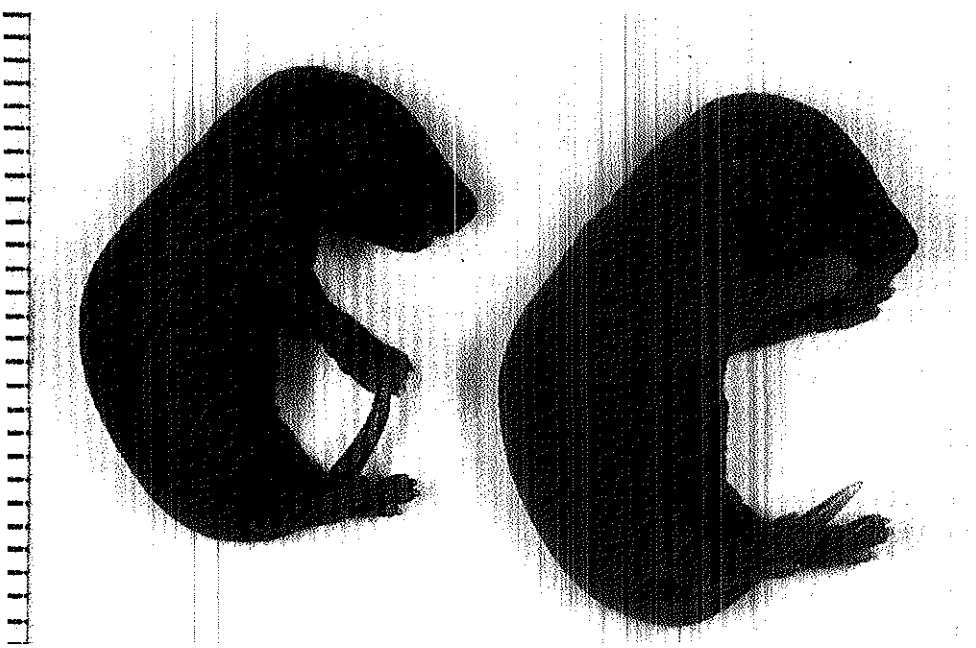


Figure 3. Photograph of a homozygous mutant mouse (left) and a normal littermates (right) ~6 h after birth. In contrast to the normal mouse, the mutant mouse is cyanotic, akinetic, has not fed, and has abnormal skin (scale graduated in millimeters).

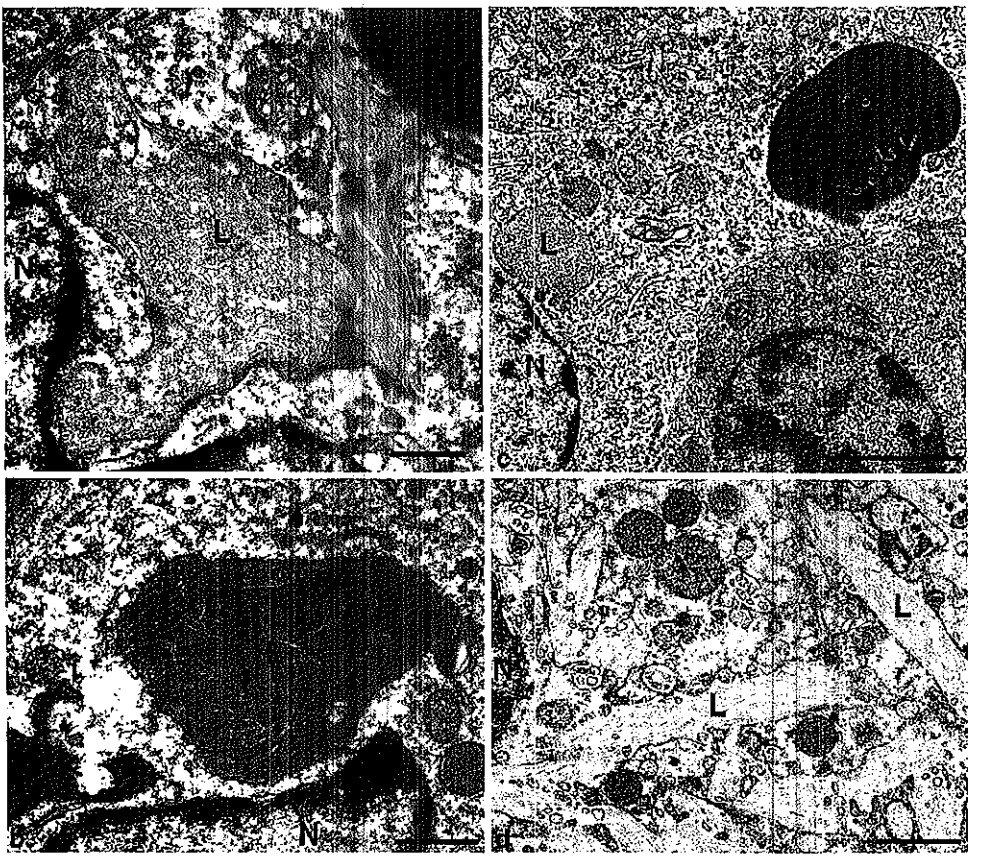


Figure 4. Electron micrographs showing the characteristic morphology of lipid storage material in lysosomes of macrophages in the liver (a) and bone marrow (b) of homozygous mutant mice with glucocerebrosidase deficiency. In contrast to the more rounded lysosome morphology in normal newborn mice (c), the tubular and elongated shape of stored lipid gives the lysosomes in homozygous mutant mice a more irregular shape. Stored glucocerebroside in the lysosome of a macrophage in the spleen of an affected human fetus is shown in d (kindly provided by V.D. Vuzevski). L, lysosome; E, phagocytosed erythrocyte; N, nucleus. Scale bars, 0.5 μm.

METHODS

Tissues were fixed in phosphate buffer, pH 7.3, containing 1% acrolein and 0.4% glutaraldehyde and postfixed according to ref. 25. Standard procedures were used for Epon embedding.

Although all homozygous mutant mice succumb within the first day of life, no single factor causing death has been identified. Glucocerebroside is estimated by thin-layer chromatography to be 5-10μg per mg wet weight of liver, lung and brain tissue of mutant homozygous mice at birth (Fig. 2b). Less striking amounts of glucocerebroside are seen in bone

marrow (Fig. 2b) and plasma. Electron microscopy has demonstrated that the storage in lysosomes of macrophages is present but not severe. Thus, despite the consistent finding of increased tissue glucocerebroside and storage within macrophages in the liver and spleen, there does not appear to be sufficient hepatosplenomegaly to explain the rapid deterioration in these newborn mutant mice. Soon after birth the homozygous mutant mice are always separated from healthy littermates by their mothers and neglected, so both the underlying pathology and the maternal perception of abnormality probably contribute to the early demise of the newborn mutant mice.

The irregular respiration, poor feeding and decreased movement of the mutant mice are consistent with the nervous system dysfunction responsible for the fulminant clinical course seen in the more severely affected subset of type-2 (acute neuronopathic) infant Gaucher's patients[1,16,17]. Our results may therefore indicate that null mutations similar to the one created here could be responsible for the early mortality in this subset of infants with type-2 Gaucher's disease who die of neurological disease shortly after birth, so we would predict that individuals homozygous for these null mutations would not survive beyond early infancy. Mutant mice carrying less deleterious mutations could therefore be models for the more frequently observed milder presentations of Gaucher's disease.

Mice negative for the enzyme hypoxanthine ribosyl phosphoryl transferase have been generated by gene targeting, but these were asymptomatic and were not adequate as a model for Lesch-Nyhan disease[18,19]. The mice described here may thus be the first example of a mouse model of a human genetic disease generated by gene targeting. These mice should be useful for studying the pathogenesis and the phenotypic diversity seen in Gaucher's disease and will provide a model in which enzyme replacement, cellular transplantation and gene transfer therapies can be evaluated.

REFERENCES

1. Barranger, J.A., & Ginns, E.I. in *The Metabolic Basis of Inherited Disease* (ed. Scriver, C.R., Beaudet, A.L., Sly, W.S. and Valle, D.) 1677-1698 (McGraw-Hill, New York, 1989).
2. Martin, B.M., Sidransky, E. & Ginns, E.I. *Adv. Pediatr.* **36**, 277-306.(1989)
3. Tsuji, S., et al. *New Engl. J. Med.* **316**, 570-575 (1987).
4. Tsuji, S., et al. *Proc. Natl. Acad. Sci. U.S.A.* **85**, 2349-2352 (1988).
5. Beutler, E., Gelbart, T., Kuhl, W., Sorge J., and West, C., *Proc. Natl. Acad. Sci. U.S.A.* **81**, 10544-10547 (1991).

6. Grabowski, G.A., Gatt, S. & Horowitz, M. *Crit. Rev. in Biochem. mol. Biol.* **25**, 385-414 (1991).
7. Hartley, W.J. & Farrow, R.R.H. in *Handbook: Animal Models of Human Disease.* Fasc II. (ed. Capen, C.C. et al.) Model No. 241, Registry of Comparative Pathology (Armed Forces Institute of Pathology, Washington, DC, 1982).
8. Kanfer, J.N., Stephens, M.C., Singh, H & Legler, G. *Prog. Clin. Biol. Res.* **95**, 627-644 (1982).
9. Robertson, E. in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach.* (ed. Robertson, E.J.) 71-112 (IRL, Oxford, 1987).
10. Capecchi, M.R., *Trends Genet.* **5**, 70-76 (1989).
11. O'Neill, R., Tokoro, T., Kozak, C.A. & Brady, R.O., *Proc. Natl. Acad. Sci. U.S.A.* **86**, 5049-5053 (1989).
12. Mansour, S.L., Thomas, K.R., and Capecchi, M.R., *Nature* **336**, 348-352 (1988).
13. Bradley, A., in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach.* (ed. Robertson, E.J.) 113-151 (IRL, Oxford, 1987).
14. Willemsen, R. et al. *Ultrastr. Path.* **12**, 471-478 (1988).
15. Lee, R.E. *Prog. clin. biol. Res.* **95**, 177-218 (1982).
16. Fredrickson, D.S. & Sloan, H.R. in *The Metabolic Basis of Inherited Disease* (eds. Stanbury, J.B., Wyngaarden, J.B. and Fredrickson, D.S.) 731-759 (McGraw-Hill, New York, 1978).
17. Sun, C.C., Panny, S., Combs, J. & Gutberlett, *R. Path. Res. Pract.* **179**, 101-104 (1984).
18. Hooper, M.L., Hardy, K., Handyside, A., Hunter, S. & Monk, M. *Nature* **326**, 292-294 (1987).
19. Kuehn, M.R., Bradley, A., Robertson, E.J. & Evans, M. J. *Nature* **326**, 295-298 (1987).
20. Robertson, E., Bradley, A., Kuehn, M., and Evans, M., *Nature* **323**, 445-448 (1986).
21. Tybulewicz, V.L.J., Crawford, C.E., Jackson, P.K., Bronson, R.T., and Mulligan, R.C., *Cell* **65**, 1153-1163 (1991).
22. Beutler, E. and Kuhl, W.L. *Lancet* **1**, 612-613 (1970).
23. Svennerholm, L., Hakansson, G., Mansson, J.E. & Nilsson, O. in *Gaucher's Disease: A Century of Delineation and Research* (eds Desnick, R.J. et al.) 231-252 (Liss, New York, 1982).
24. Kundu, S.K., in *Meth Enzym.* (ed. Lowenstein, J.M.) 185-204 (Academic Press, New York, 1981).
23. Debruyn, W.C. and Denbreejen, P., *Histochem. J.* **8**, 121-142 (1976).

ACKNOWLEDGEMENTS

We thank H. Stewart, A. Liebelt, T. O'Neill, R. Lee and M. Amver for, histopathological consultations, A. Reuser, W. Eliason, G. Bingham, K. Mahon, J. LeGross and B. Martin for help and discussion, and E. Alzona for typing and editorial assistance. This work was in part supported by the National Gaucher Foundation, by a postdoctoral fellowship (V.L.J.T.) from the Irvington Institute for Medical Research, a grant from the NIH (R.C.M.), and by a postdoctoral fellowship from the National Cancer Institute of Canada (M.L.T.).

APPENDIX PAPER IV

Molec Chem Neuropathol (1995) in press

A BIOCHEMICAL AND ULTRASTRUCTURAL EVALUATION OF THE TYPE 2 GAUCHER MOUSE DEVELOPED FROM TARGETED DISRUPTION OF THE MURINE GLUCOCEREBROSIDASE GENE

R. Willemsen, V. Tybulewicz, E. Sidransky, W.K. Eliason, B.M. Martin, M.E. LaMarca, A.J.J. Reuser, M. Tremblay, H. Westphal, R.C. Mulligan, E.I. Ginns.

ABSTRACT

Gaucher mice, created by targeted disruption of the glucocerebrosidase gene, are totally deficient in glucocerebrosidase and have a rapidly deteriorating clinical course analogous to the most severely affected type 2 human patients. An ultrastructural study of tissues from these mice revealed glucocerebroside accumulation in bone marrow, liver, spleen and brain. This glycolipid had a characteristic elongated tubular structure and was contained in lysosomes as demonstrated by co-localization with both ingested carbon particles and cathepsin D. In the central nervous system glucocerebroside was diffusely stored in microglia cells and in brainstem and spinal cord neurons, but not in neurons of the cerebellum or cerebral cortex. This rostral-caudal pattern of neuronal lipid storage in these Gaucher mice replicates the pattern seen in type 2 human Gaucher patients and clearly demonstrates that glycosphingolipid catabolism and/or accumulation varies within different brain regions. Surprisingly, the cellular pathology of tissue from these Gaucher mice was relatively mild, and suggests that the early and rapid demise of both Gaucher mice and severely affected type 2 human neonates may be the result of both a neurotoxic metabolite such as glucosylsphingosine and other factors such as skin water barrier dysfunction secondary to the absence of glucocerebrosidase activity.

INTRODUCTION

Gaucher disease in humans and null-allele mice is caused by the inherited deficiency of glucocerebrosidase, the lysosomal enzyme that hydrolyses the β-glucosidic bond in glucocerebroside. The major source of this glycosphingolipid is the turnover of senescent white and red blood cell

membranes. While glucocerebroside is predominantly degraded within lysosomes of normal macrophages, accumulation of this lipid in Gaucher disease transforms macrophages throughout the mononuclear phagocyte system into the characteristic "Gaucher cells". The Gaucher cells have a relatively small eccentrically located nucleus, and the cytoplasm contains numerous irregularly-shaped or branching lysosomes filled with twisted tubular deposits of glucocerebroside (Barranger & Ginns, 1989; Martin, Sidransky & Ginns, 1989).

Gaucher disease has been classified into three subtypes, type 1 (chronic, non-neuronopathic), type 2 (acute, neuronopathic) and type 3 (subacute, neuronopathic) on the basis of the presence and degree of neurologic involvement (Barranger & Ginns, 1989; Martin, Sidransky & Ginns, 1989). The clinical manifestations encountered in type 2 Gaucher disease include failure to thrive, hepatosplenomegaly, and neurologic deterioration which often includes the triad of strabismus, trismus and retroflexion of the head. The most severely affected type 2 patients may also have ichthyotic skin and/or hydrops fetalis as associated symptoms (Sidransky, Sherer & Ginns, 1992). In brains from the few Gaucher patients with neurologic involvement that have been studied, perivascular Gaucher cells are found and a variable degree of neuronal loss is observed. Neurons with the characteristic lysosomal inclusions typically observed in Gaucher cells are encountered only sporadically (Banker, Miller & Crocker, 1962; Adachi, Wallace, Schneck & Volk, 1967; Conradi, Sourander, Nilsson, et al., 1984; Kaye, Ullman, Wilson, et al., 1986; Hernandez & Bueno, 1973). The pathophysiologic mechanisms leading to the neurologic symptoms observed in these patients remain unknown, largely because of the rarity of severely affected type 2 human neonates and the very limited opportunities for extensive pathological studies in these patients.

A Gaucher mouse line recently created by targeted disruption of the glucocerebrosidase gene (Tybulewicz, Tremblay, LaMarca, et al., 1992) has provided a relevant animal model for investigation of the pathophysiologic mechanisms occurring in type 2 Gaucher disease. In the initial report we presented limited pathologic results on these Gaucher mice, demonstrating that the deficiency of glucocerebrosidase did result in accumulation of glucocerebroside in macrophages. This present report describes the more detailed ultrastructural pathology in a variety of tissues, including the liver, spleen, bone-marrow and brain from type 2 Gaucher mice having clinical features characteristic of the more severely affected type 2 Gaucher patients. Particular attention is focused on the central nervous system, including frontal and occipital cortex, brainstem, cerebellum and spinal cord, in an attempt to explain the rapid postnatal

demise of these type 2 Gaucher mice and severely affected type 2 human neonates.

MATERIALS AND METHODS

The Gaucher Mouse

A Gaucher mouse line was developed by targeted disruption of the mouse glucocerebrosidase gene via homologous recombination in ES cells (Tybulewicz et al., 1992). The neomycin resistance cassette replaces part of the exons 9 and 10 and abolishes glucocerebrosidase activity in the homozygous mutant mice.

Western Blot Analyses

Tissue samples were stored at -20°C until use. Prior to extraction, the tissue samples were frozen in liquid nitrogen and then crushed into a fine powder. The powdered tissue was resuspended in 200-250 µl of 60mM potassium phosphate, pH 5.9, containing 0.1% Triton X-100, and sonicated three times each for 10 seconds (50 Watts, Heat Systems Ultrasonics Inc., Cell Disrupter Model W225R) at 4°C. After sonication the samples were centrifuged at 12,000xg at 4°C for 5 minutes, and the supernatant used for enzyme assay and Western Blot analyses. Protein concentration was determined with the Pierce BCA reagent using the recommended protocol with BSA as a standard. Enzyme assay was performed using 4-methylumbelliferyl-β-D-glucopyranoside as substrate (Beutler & Kuhl, 1970).

Polyacrylamide gel electrophoresis was performed using precast 12% minigels (NOVEX, 1.0mm thickness) at constant current of 40ma using reducing, denaturing conditions. Each lane contained 36 µg of tissue. After electrophoretic separation, the proteins were transferred to Immobilon-P (Millipore) using 25mM tris, 192 mM glycine, 20 mM methanol as buffer. The membrane was briefly rinsed with water and incubated in a blocking solution (5% milk powder, 0.05% Tween-20, PBS pH 7.2) at 37°C for 1 hr. The membrane was then incubated with rabbit antibody specific for mouse glucocerebrosidase (diluted 1:5000 in blocking solution) for 1 hr. at 37°C, washed five times for 5 minutes at 37°C with PBS pH 7.2 containing 0.05% Tween-20, and finally incubated with goat anti-rabbit IgG-HRP (human serum absorbed, Sigma Chemical Co.) at a dilution of 1:5000 in blocking solution for 30 minutes at 37°C. The wash was then repeated 5 times each for 5 minutes in PBS pH 7.2 containing 0.05% Tween-20 at 37°C. The antibody-protein conjugate was detected

with the ECL Detection Kit (Amersham) using the recommended protocol. After the antibody-protein signals were developed, the membrane was rinsed with water and protein standards (Nova Wide range SDS-PAGE protein standards) were visualized with Sulforhodamine B (50 mg/liter in 30% methanol, 2% acetic acid, Molecular Probes Inc., S1307).

Glycosphingolipid Analyses
Liver and brain tissue from normal, heterozygous and mutant homozygous mice were extracted with 18 v/w of 2:1 chloroform-methanol by sonication for 10 minutes at RT, followed by addition of 0.2 volumes of water. After vortexing, samples were centrifuged for 2 minutes at 12,000xg. A 100 ul aliquot of the lower phase was evaporated to dryness, resuspended in 2:1 chloroform-methanol, and applied to precoated silica gel-60 high-performance thin-layer chromatography plates (Merck) that had been activated at 100°C for 1 hour (Kundu, 1981). Development was performed with a solvent mixture of chloroform-methanol-water, 65:25:4 (v/v/v) for 45 minutes. The plates were then air dried and the glycolipids visualized with orcinol-ferric chloride (Bial's) reagent.

Tissue processing for Epon embedding
Liver, spleen, bone-marrow and brain from normal and homozygous mutant mice were fixed in 0.1 M phosphate buffer (PB), pH 7.3, containing 1% acrolein and 0.4% glutaraldehyde. After fixation for 24 hrs. at 4°C, the tissues were stored in 0.1 M PB, containing 2% paraformaldehyde. Postfixation was performed according to De Bruyn et al., (DeBruyn & DenBreejen, 1976). Specific areas of the brain were selected for epon embedding i.e. the frontal and occipital cerebral cortex, the cerebellum, the brainstem and the cervical part of the spinal cord. After epon embedding a further selection was made for the red nucleus, the vestibular nucleus and the facial nerve nucleus of the brainstem. Ultrathin sections were cut with a LKB ultratome NOVA and stained with uranylacetate and lead citrate.

Macrophage marking
A suspension of carbon particles (India ink) was injected intravenously into normal and homozygous glucocerebrosidase deficient mice seconds prior to sacrificing the animals. The liver was fixed as described above.

Immunocytochemistry
Spleen from normal and affected mice was fixed and stored as described

above, but no postfixation was performed. Specimens were embedded in Lowicryl K4M according to standard procedures (DeBruyn & DenBreejen, 1976). Immunolabelling of Cathepsin D on ultrathin sections was performed as previously described (Armbruster, Carlemalm & Chiovetti, 1982). Sections were stained with uranylacetate and lead citrate. Sections incubated with normal rabbit serum showed negligible labelling.

RESULTS

Biochemical Findings

The type 2 Gaucher mouse described in this report was produced by targeted disruption of the glucocerebrosidase gene. The affected mice are "null mutants" of Gaucher disease, where glucocerebrosidase is completely deficient. This is illustrated in Figures 1a by the absence of glucocerebrosidase protein in brain tissue of affected animals. Western blot analyses of brain and liver (not shown) from normal and heterozygous mice (Figure 1a) show that the major form of crossreacting material (CRM) to mouse glucocerebrosidase has an apparent molecular weight of 56 kDa.

Thin-layer Chromatographic Analyses for Glucocerebroside

Elevated glucocerebroside levels were demonstrated in brain (Figure 1b) and liver (Figure 1c) of homozygous mutant mice. The difference in mobility of glucocerebroside from brain compared to that from liver or the standard is consistent with the heterogeneity in fatty acid moieties of the lipid, being C_{24} in liver or standard and C_{18} in brain (Svennerholm, Hakansson, Mausson & Nilsson, 1982). No elevation of glucocerebroside is observed in heterozygous mice.

Macrophages in Bone Marrow, Liver and Spleen

Except for the dramatic changes observed in the skin (Sidransky, Sherer & Ginns, 1992), at the light-microscopic level no definite pathological changes were observed. Typical Gaucher cells, easily observed in bone-marrow, liver and spleen of affected humans were not readily identified in the neonatal mouse organs. Therefore, we performed electron-microscopy to obtain information about the presence and precise localization of stored glycolipid. The overall cellularity of the bone marrow was identical in normal and mutant mice. Bone marrow from

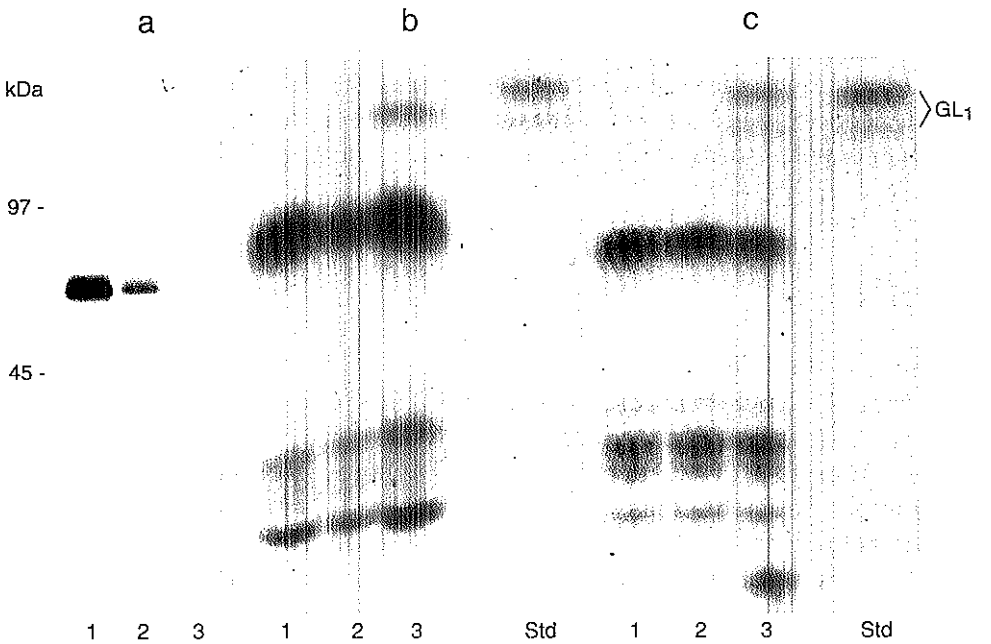


Figure 1. **(A)** Western blot analysis of glucocerebrosidase in brain of normal (lane 1), heterozygote (lane 2), and homozygous mutant mice (lane 3). Sample preparation, electrophoresis, and Western blot analyses were performed as described in Materials and Methods. Each lane contains 36ug of protein; **(B and C)** Thin-layer chromatography of neutral glycosphingolipids from brain (B) and liver (C) of normal (lane 1), heterozygote (lane 2), and homozygous mutant (lane 3) mice. The lane marked Std contains 4 ug of glucocerebroside (GL_1) standard.

both normal and mutant mice is shown in Figure 2. Storage of glucocerebroside was observed only in macrophages from homozygous mutant mice, where the glucocerebroside is deposited in elongated tubular arrays which gives the lipid laden lysosomes their characteristic irregular shape.

In these type 2 Gaucher mice the lysosomal accumulation of glycolipid was most obvious in the liver. A high power magnification of a Kupffer cell is shown in Fig. 3. The lysosomes were marked in this experiment by intravenous injection of carbon particles prior to sacrificing the animals. In Fig. 4 the lysosomal nature of the storage organelles is demonstrated by the co-localization of the lipid deposits and the lysosomal marker enzyme cathepsin D. The figure shows a Lowicryl K4M ultrathin section of the spleen from a Gaucher mouse on which immunocytochemistry was

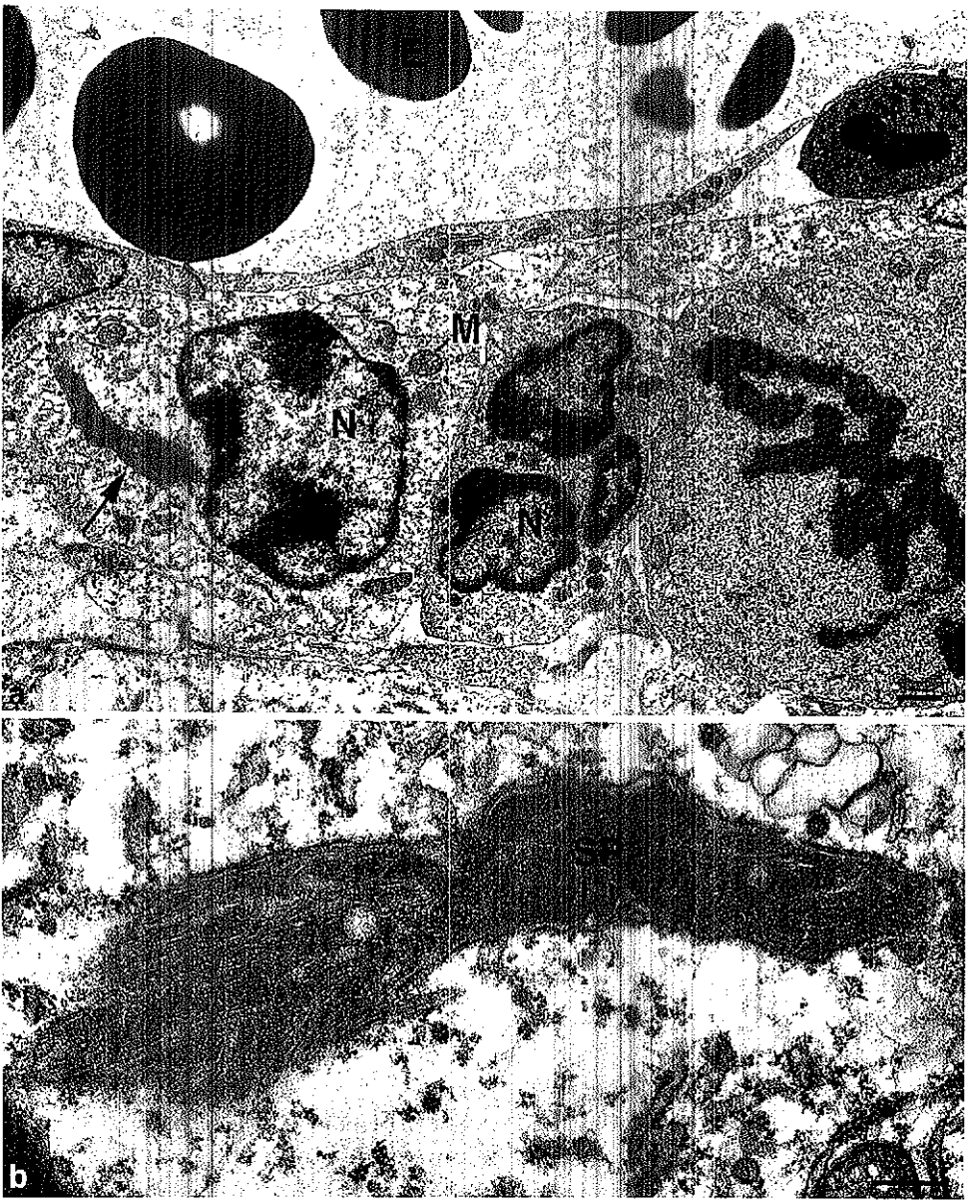


Figure 2. (a) Ultrathin epon section of bone marrow of homozygous mutant glucocerebrosidase deficient mice; **(b)** Stored glucocerebroside (SP) is observed within a lysosome (arrow). N, nucleus; M, mitochondrion; E, erythrocyte. Bar represents 1.0μm in 2a and 0.25μm in 2b.

performed. The gold particles marking cathepsin D were located along the twisted glycolipid tubules in the lysosomes in spleen. The accumulation of lipid was consistently seen in homozygous mutant mice (n=10), but never observed in macrophages from normal (n=5) or heterozygous (carrier) mice (n=2).

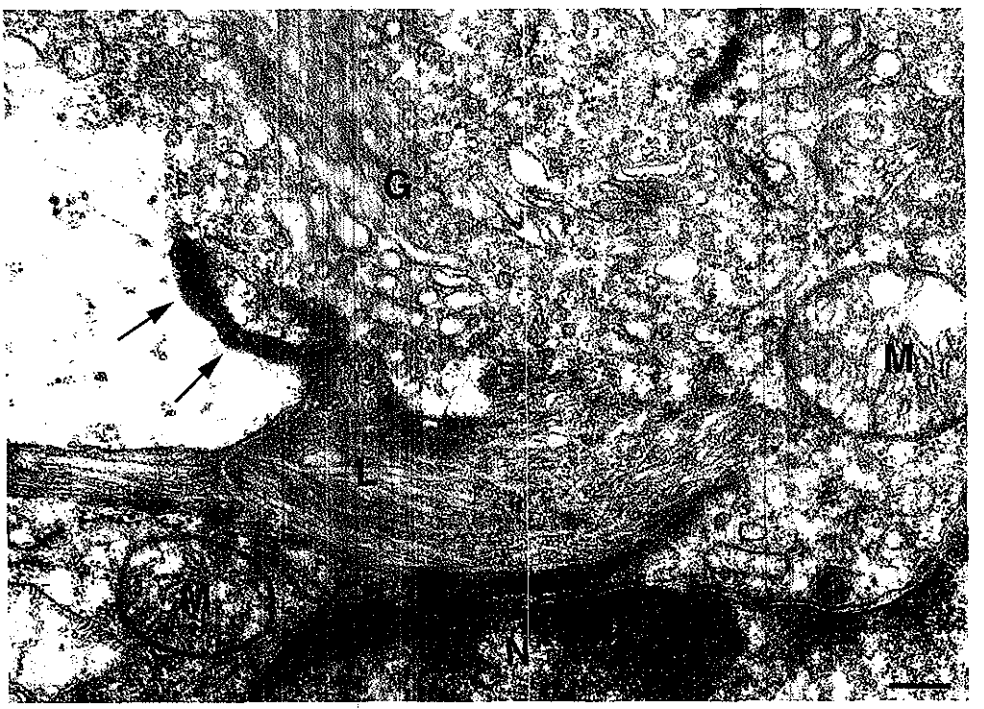


Figure 3. Ultrathin epon section of liver of homozygous mutant glucocerebrosidase deficient mice sacrificed after intravenous injection of carbon particles. The lysosomes (L) of the Kupffer cells are marked by the ingested carbon particles (arrows) that colocalize with the stored material. G, Golgi complex; N, nucleus; M, mitochondrion; bar represents 0.2µm.

Macrophages in the central nervous system

Typical Gaucher cells were not observed in histological serial sections of the brain of glucocerebrosidase deficient mice. However, examination of the selected areas of the brain (n=6; see materials and methods) revealed at the subcellular level microglia with lysosomal accumulation of lipid in tubular arrays which was not seen in normal mice (Figs 5A and B). Macrophages throughout the central nervous system did contain dense material, but in this respect the glucocerebrosidase deficient mice were not different from the normal. No lipid storage was observed in either astroglia cells or oligodendrocytes. The various cell types were distinguished by morphological criteria.

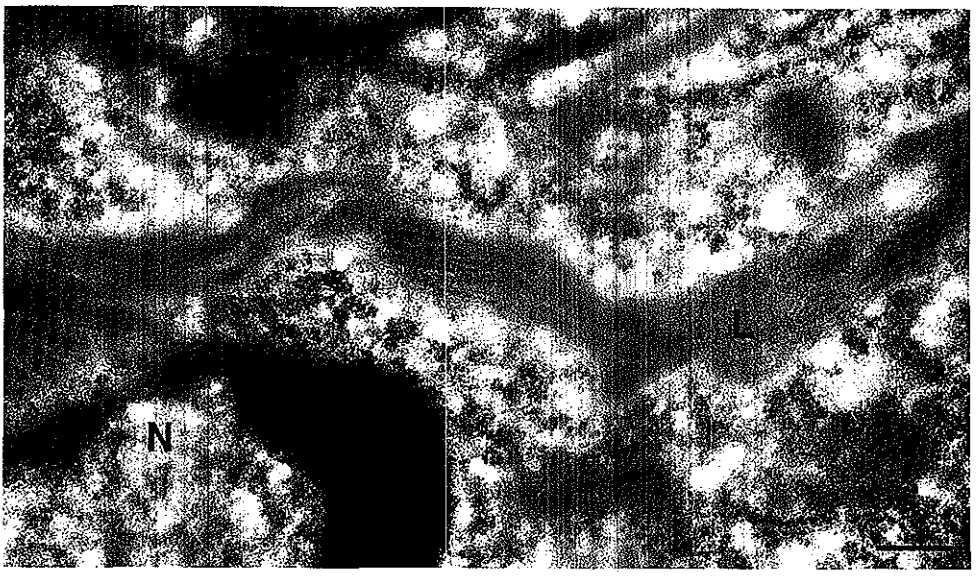


Figure 4. Ultrathin Lowicryl K4M section of spleen of homozygous mutant mice immunogold labeled for lysosomal cathepsin D. The labeled structure is an elongated lysosome (L). Cathepsin D is associated with the twisted tubular deposits. N, nucleus; bar represents 0.2μm.

Neurons

Lipid storage was not detected at the light-microscopic level in neurons in brainstem, cerebellum or spinal cord, and hence electronmicroscopic studies were performed. In brainstem of mutant mice (n=2), glucocerebroside was seen within neurons of the red nucleus (Fig 6a) and the vestibular nucleus (Fig. 6b) but not in the neurons of the facial nerve nucleus (Fig. 6c). Lysosomal storage was also observed in both sensory and motor neurons of the spinal cord (n=2)(Fig. 7), but not in the cerebellum or the occipital and frontal cerebral cortex (n=3, data not shown).

DISCUSSION

The ultrastructural pathology of the type 2 Gaucher mouse was studied in an attempt to explain the rapid postnatal death of these mice and severely affected type 2 neonates. The cellular and tissue pathology was

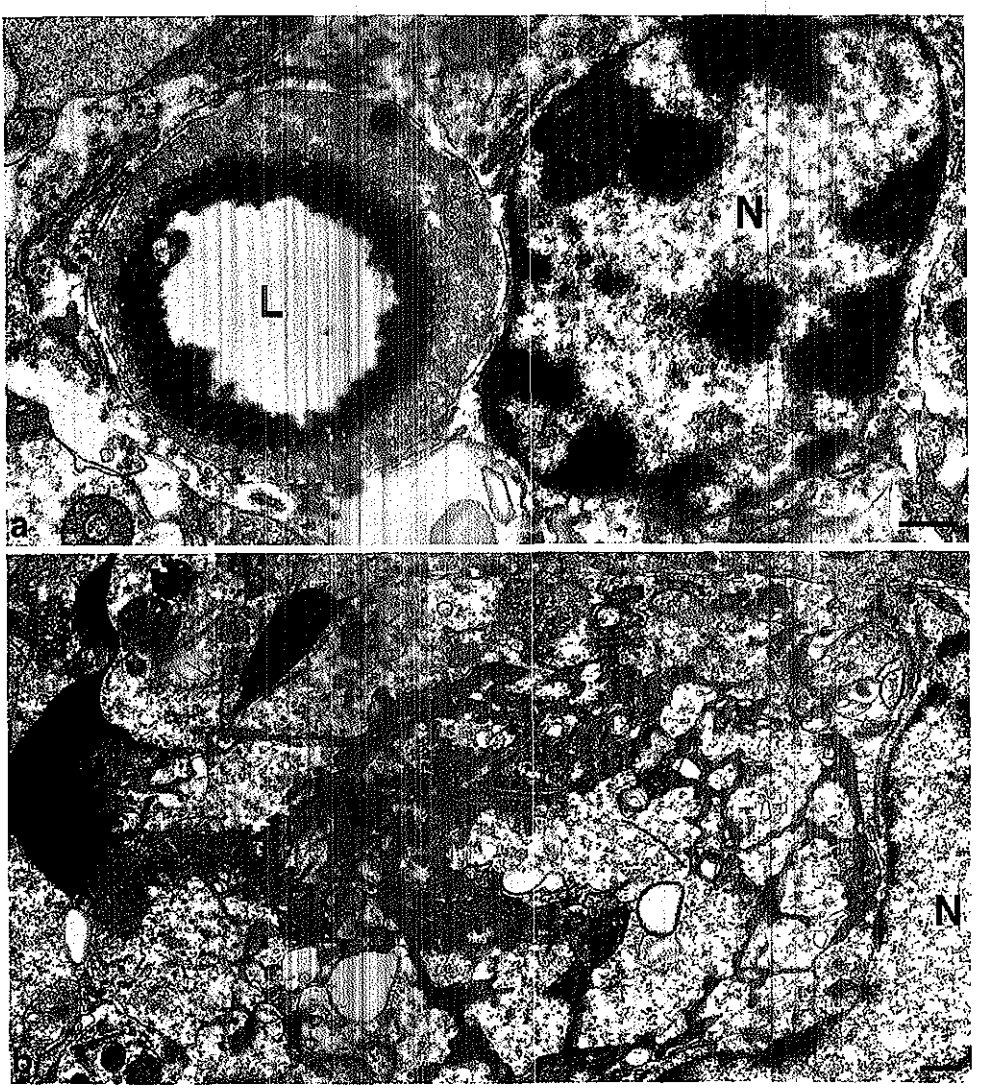


Figure 5. Ultrathin epon section of cerebellum of normal (a) and homozygous mutant mice (b). Microglia in the central nervous system of healthy animals do contain occasionally enlarged lysosomes (L) which are associated with normal function (5a). Lysosomes (L) filled with the characteristic deposits of glucocerebroside are only found in microglia of affected animals. The storage material gives the lysosome the irregular shape of a network spreading throughout the cytoplasm (5b). N, nucleus; bar represents 0.5µm in 5a and 5b.

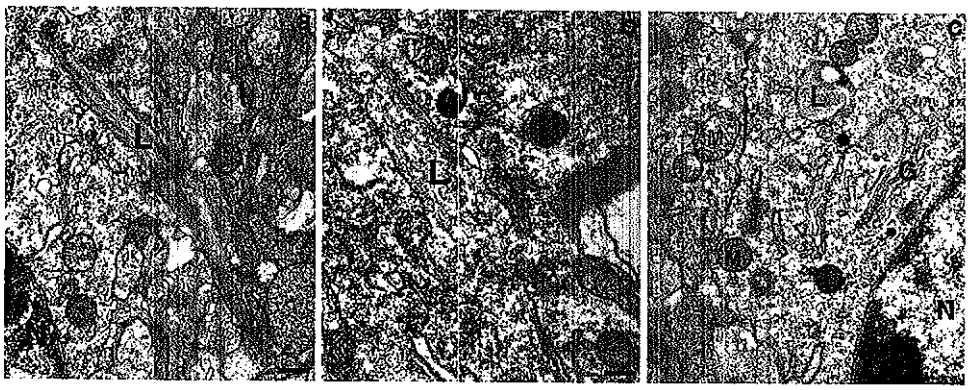


Figure 6. Ultrathin epon section of brainstem of homozygous mutant mice. Neurons of the red nucleus (a) and vestibular nucleus (b) have lysosomes (L) with storage material, but lysosomal storage was not observed in neurons of the facial nerve nucleus (c). M, mitochondrion; G, Golgi complex; N, nucleus; bar represents 0.2μm in 6a, 6b and 6c.

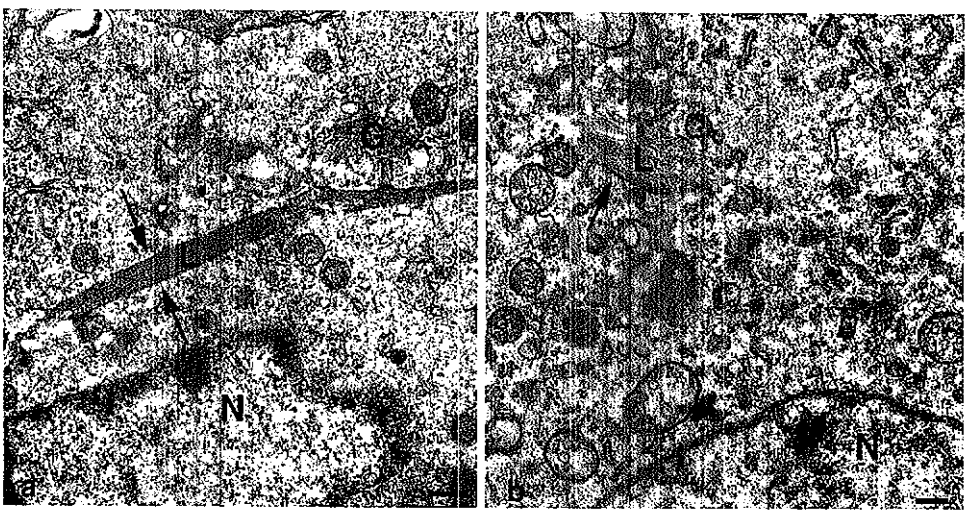


Figure 7. Ultrathin epon sections of sensory (a) and motor (b) neurons in the spinal cord of homozygous mutant mice. The arrows indicate the array of lipid tubules present in the lysosomes (L). G, Golgi complex; N, nucleus; bar represents 0.2μm in 7a and 7b.

remarkably mild, despite the complete deficiency of glucocerebrosidase. Although the overall architecture of the liver, bone marrow, spleen and brain appeared appropriate for age as judged by light microscopy, accumulation of glucocerebroside was clearly demonstrated by electronmicroscopy. The stored lipid in macrophages of the liver and spleen had the characteristic elongated tubular structure (Willemsen, VanDongen, Aerts, et al., 1988; Lee, Worthington & Glew, 1973; Lee, 1982) and was contained in organelles identified as lysosomes by the presence of ingested carbon particles and lysosomal cathepsin D. Interestingly, even though these storage vacuoles did occasionally occupy significant cellular volume, typical Gaucher cells were not observed by light microscopy.

The cellular pathology in the central nervous system of the homozygous mutant mice similarly is surprisingly limited, particularly in light of the rapid clinical deterioration that occurs. Glucocerebroside is stored predominantly in microglia cells and only to a very limited extent in neurons. While certain sensory and motor neurons of the brain stem and spinal cord were affected, Purkinje cells and neurons of the cerebellar and cerebral cortex were free of storage product. This rostral-caudal pattern of neuronal lipid storage suggests that glycosphingolipid catabolism and/or accumulation varies between neurons in different brain regions. A comparison of the neuropathology of the glucocerebrosidase deficient mouse with the human type 2 and 3 patients suggests that in the Gaucher patients there is also more deposition of glucocerebroside in microglia than in neurons (Adachi, Wallace, Schneck & Volk, 1967; Banker, Miller & Crocker, 1962; Conradi, Sourander, Nilsson, Et al., 1984; Kaye, Ullman, Wilson, et al., 1986). The limited number of neuropathology studies in Gaucher patients consistently demonstrates that Gaucher cells are predominantly located in the adventitia of vessels in the cerebral and cerebellar subcortical white matter, but the reported involvement of microglia and neurons in the different regions of the brain is more varied. An increasing gradient of Gaucher cells was observed from frontal to occipital cerebral cortex by Kaye et al. (1986), whereas Banker et al. (1962) report a comparable density of Gaucher cells in the frontal, temporal, parietal and occipital cortex. A similar comparison could not be made in the mouse model because of the lack of histologically detectable Gaucher cells. Neuronal storage was only sporadically found in humans throughout the central nervous system albeit with an increasing gradient from rostral to caudal, as in the Gaucher mice (Banker, Miller & Crocker, 1962; Adachi, Wallace, Schneck & Volk, 1967; Conradi, Sourander, Nilsson, et al., 1984; Hernandez & Bueno, 1973).

The degree of neuronal loss and neuronophagia in humans varied in different nuclei (Banker, Miller & Crocker, 1962; Adachi, Wallace, Schneck & Volk, 1967; Conradi, Sourander, Nilsson, et al., 1984; Kaye, Ullman, Wilson, et al., 1986; Hernandez & Bueno, 1973). No such dramatic pathological changes were observed in the mouse.

This Gaucher mouse line with a complete deficiency of glucocerebrosidase, provides us with a relevant animal model of the most severe neonatal form of Gaucher disease, in which we can conveniently study the clinical-pathological progression of this disease. From the studies reported here, we conclude that the early demise of affected mice is probably not caused by liver, spleen or bone marrow dysfunction, nor by neuronal degeneration unless the observed amount of lysosomal glucocerebroside accumulation results in neuronal dysfunction before gross cellular pathology occurs. Nilsson and Svennerholm have suggested that the neurotoxin glucosylsphingosine (glucosylpsychosine) is elevated in the nervous system of type 2 patients (Nilsson & Svennerholm, 1982). Hannun et. al, have recently reported that lysosphingolipids, including glucosylsphingosine, are potent inhibitors of both protein kinase C activity and phorboldiester binding (Hannun & Bell, 1987), suggesting that these sphingolipid derivatives can interfere with signal transduction and cellular differentiation (Zeller & Marchase, 1992; Ballou, 1992) and thus disrupt neuronal function.

The unusual skin texture, turgor and lipid composition of type 2 Gaucher skin (Sidransky, Sherer & Ginns, 1992; Tybulewicz, Tremblay, LaMarca, et al., 1992; Holleran, Takagi, Menon, Legler, Feingold & Elias, 1993) may also be an important factor contributing to the rapid deterioration observed in severely affected mice and human neonates. Ceramides are critical components in skin permeability barrier homeostasis (Holleran, Takagi, Menon, Legler, Feingold & Elias, 1993), and the functional disruption of the skin barrier as a consequence of the absence of glucocerebrosidase could result in fluid or electrolyte imbalance (Holleran, Takagi, Menon, Legler, Feingold & Elias, 1993; Holleran, Menon, Elias, Ginns & Sidransky, 1993; Holleran, Ginns, Menon, Grundmann, Fartasch, McKinney, Elias & Sidransky, 1994). Lastly, the homozygous mutant mice are rapidly identified by their mothers and are ejected from the nest. Thus they have been poorly fed, and as a consequence dehydration and hypoglycemia may further hasten their demise.

The opportunity to perform further biochemical and pathological studies using this mouse model of type 2 Gaucher disease should further our understanding of the basis of neurologic involvement observed in these

Gaucher mice and type 2 infants. Investigations using these mice may also provide more details of the pathophysiologic mechanisms of systemic involvement seen in Gaucher disease and permit the more rational development of successful therapeutic interventions.

ACKNOWLEDGEMENTS

The authors thank L. Carmon for technical assistance with animal procedures, Professor J.M. Tager (University of Amsterdam) and coworkers for providing rabbit polyclonal antibodies against cathepsin D, and Dr. J.C. Holstege (Department of Anatomy, Erasmus University, Rotterdam) for stimulating discussion. R.M. Koppenol, M. Kuit and J. de Vries Lentsch are acknowledged for photography, and J. Lokker, L. Syed and E. Alzona for manuscript preparation.

REFERENCES

Adachi M., Wallace B.J., Schneck L., and Volk B.W. (1967) Fine structure of central nervous system in early infantile Gaucher's disease. *Arch. Pathol.* **83,** 513-526.

Armbruster B.L., Carlemalm E., and Chiovetti R. (1982) Specimen preparation for electron microscopy using low temperature embedding resins. *J. Microsc.* **126,** 77-85.

Ballou L.R. (1992) Sphingolipids and cell function. *Immunology Today* **13,** 339-341.

Banker B.Q., Miller J.O., and Crocker A.C. (1962) The cerebral pathology of infantile Gaucher's disease, in *Cerebral Sphingolipidosis,* (Aronson S.M., Volk B.W., eds.), pp. 73-99, Academic Press, New York, NY.

Barranger J.A., and Ginns E.I. (1989) Glucosylceramide lipidosis: Gaucher disease, in *The Metabolic Basis of Inherited Disease II,* vol. 6 (Scriver C.R., Beaudet A.L., Sly W.S., Valle D., eds.), pp. 1677-1698, McGraw-Hill, New York, NY.

Beutler E., and Kuhl W.L. (1970) Detection of the defect of Gaucher's disease and its carrier state in peripheral blood leukocytes. *Lancet* **1,** 612-613.

Conradi N.G., Sourander P., Nilsson O., Svennerholm L., and Erikson A. (1984) Neuropathology of the Norrbottnian type of Gaucher disease: morphological and biochemical studies. *Acta Neuropathol. (Berl)* **65,** 99-109.

DeBruyn W.C., and DenBreejen P. (1976) Glycogen, its chemistry and morphological appearance in the electron microscope. *Histochem. J.* **8,** 121-142.

Hannun Y.A., and Bell R.M. (1987) Lysosphingolipids inhibit protein kinase C: implications for the Sphingolipidoses. *Science* **235,** 670-674.

Hernandez F., and Bueno M. (1973) Infantile neurological Gaucher's disease in three siblings: an ultrastructural study. *Virchows Arch (Pathol Anat)* **360,** 27-32.

Holleran W.M., Ginns E.I., Menon G.K., Grundmann J.U., Fartasch M., McKinney C.E., Elias P.M., and Sidransky E. (1994) Consequences of β-glucocerebrosidase deficiency in epidermis: ultrastructure and permeability barrier alterations in Gaucher disease. *J. Clin. Invest.* **93,** 1756-64.

Holleran W.M., Menon G.K., Elias P.M., Ginns E.I., and Sidransky E. (1993) ß-

glucocerebrosidase-deficient transgenic mice have abnormal stratum corneum lamellar bilayers. *Clin. Res.* **41(2),** 402A.

Holleran W.M., Takagi Y., Menon G.K., Legler G., Feingold K.R., and Elias P.M. (1993) Processing of epidermal glucosylceramides is required for optimal mammalian cutaneous permeability barrier function. *J. Clin. Invest.* **91,** 1656-1664.

Kaye E.M., Ullman M.D., Wilson E.R., and Barranger J.A. (1986) Type 2 and type 3 Gaucher disease: a morphological and biochemical study. *Ann. Neurol.* **20,** 223-230.

Kundu S.K. (1981) In *Meth. Enzym.*, (Lowenstein J.M., ed.), pp. 185-204, Academic Press, New York, NY.

Lee R.E. (1982) The pathology of Gaucher disease, in *Gaucher's Disease: A Century of Delineation and Research,* (Desnick R.J., Gatt S., Grabowski G.A., eds.), pp. 177-218, Alan R. Liss, New York, NY.

Lee R.E., Worthington C.R., and Glew R.H. (1973) The bilayer nature of deposits occurring in Gaucher's disease. *Arch. Biochem. Biophys.* **159,** 259-266.

Martin B.M., Sidransky E., and Ginns E.I. (1989) Gaucher disease: advances and challenges. *Adv. Pediatr.* **36,** 277-306.

Nilsson O., and Svennerholm L. (1982) Accumulation of glucosylceramide and glucosylsphingosine in cerebrum and cerebellum in infantile and juvenile Gaucher disease. *J. Neurochem.* **39,** 709-718.

Sidransky E., Sherer D.M., and Ginns E.I. (1992) Gaucher disease in the neonate: a distinct Gaucher phenotype is analogous to a mouse model created by targeted disruption of the glucocerebrosidase gene. *Pediatric Res.* **32,** 494-498.

Svennerholm L., Hakansson G., Mansson J.E., and Nilsson O. (1982) Chemical differentiation of the Gaucher subtypes, in *Gaucher's Disease: A Century of Delineation and Research,* (Desnick R.J., Gatt S., Grabowski G.A., eds.), pp. 231-252, Alan R. Liss Inc., New York, NY.

Tybulewicz V.L.J., Tremblay M.L., LaMarca M.E., Willemsen R., Stubblefield B.K., Winfield S., Zablocka B., Sidransky E., Martin B.M., Huang S.P., Mintzer K.A., Westphal H., Mulligan R.C., and Ginns E.I. (1992) Animal model of Gaucher's disease from targeted disruption of the mouse glucocerebrosidase gene. *Nature* **357,** 407-410.

Willemsen R., VanDongen J., Aerts J.M.F.G., et al. (1988) An immunoelectron microscopic study of glucocerebrosidase in type 1 Gaucher's disease spleen. *Ultrastr. Pathol.* **12,** 471-478.

Zeller C.B., and Marchase R.B. (1992) Gangliosides as modulators of cell function. *Am. J. Physiol.* **262,** C1341-C1355.

APPENDIX PAPER V

Histochem J in press.

A BIOCHEMICAL AND IMMUNOCYTOCHEMICAL STUDY ON THE TARGETING OF ALGLUCERASE IN MURINE LIVER

R. Willemsen, J.J.M. Tibbe, M.A. Kroos, B.M. Martin, A.J.J. Reuser, E.I. Ginns

ABSTRACT

A current hypothesis is that functional glucocerebrosidase needs to be delivered to the lysosomes of tissue macrophages to guarantee successful enzyme therapy for Gaucher disease. In this study, biochemical and immunohistochemical techniques were applied to identify in mice the localization of intravenously administered alglucerase (human modified placental glucocerebrosidase). Only in liver and spleen a significant increase of glucocerebrosidase activity was measured with a maximum level at 15 minutes after enzyme infusion. The uptake of enzyme by liver was sufficiently high to allow more detailed studies on the (sub)cellular distribution of human alglucerase. The enzyme in liver is localized both in the endosomal/lysosomal system of the Kupffer cells and the endothelial cells lining the lumen of the sinusoids. Uptake by both these cell types is prevented by mannan. The results suggest that the cellular mechanisms responsible for improvement of Gaucher patients receiving alglucerase treatment is probably more complicated than previously recognized.

INTRODUCTION

Glucocerebrosidase (EC 3.2.1.45) is a membrane-associated lysosomal enzyme involved in glycolipid metabolism, and is deficient in patients with Gaucher disease. This autosomal recessive disorder is characterized by accumulation of glucocerebroside in lysosomes throughout the mononuclear phagocyte system. The membranes of senescent red and white blood cells are the major catabolic source of the stored glucocerebroside. Enzyme replacement therapy has been developed for the treatment of the non-neurological type 1 form of Gaucher disease (Barranger & Ginns,1989; Grabowski, 1993; Brady & Barton, 1994a; Brady & Barton, 1994b).

In the initial human clinical study, injection of unmodified human placental glucocerebrosidase resulted in biochemical but not clinical improvement (Brady, 1984; Beutler *et al.*, 1980). Furbish et al. observed in rats that intravenously injected native glucocerebrosidase was targeted to both hepatocytes and non-parenchymal cells (Furbish *et al.*, 1978). Subsequently, animal studies revealed that modification of the carbohydrate chains of placental glucocerebrosidase to expose mannose residues enhanced the uptake of glucocerebrosidase by non-parenchymal cells (Furbish *et al.*, 1981). These conclusions about cell type specific targeting of enzyme eventually resulted in the development of alglucerase (Ceredase, Genzyme Inc.), a modified placental glucocerebrosidase, the terminal mannose residues of which facilitate mannose specific receptor uptake (reviewed by Pontow et al., 1992). Enzyme replacement therapy with alglucerase in type 1 Gaucher patients has been very successful (Barton *et al.* 1991), but it is currently controversial whether a high-dose (60 IU/kg body weight, every 2 weeks) or low-dose (2.3 IU/kg body weight, 3 times weekly) regimen is more appropriate (Figueroa *et al.*, 1992; Zimran *et al.*, 1994). Also, despite the clinical efficacy of alglucerase, targeting to macrophages has been suggested to be very low (Sato & Beutler, 1993). It has been suggested that the striking improvement of Gaucher patients may therefore be the result of uptake of alglucerase by other cells than macrophages (Sato & Beutler, 1993). In this present work we have studied the in vivo distribution of intravenously administered alglucerase in the mouse liver.

MATERIALS AND METHODS

Enzyme infusion and tissue preparation

Female BALB/C mice (8 weeks old) were injected with 0.5 ml of 0.1 M phosphate buffered saline (PBS), pH7.4, containing alglucerase (Ceredase, Genzyme Inc., 60 IU/kg body weight) or unmodified human placental glucocerebrosidase (60 IU/kg body weight). In the uptake competition studies, 3 mg/ml Mannan (Sigma, St Louis) was added to the enzyme preparation before administration. Mice were sacrificed by CO_2 exposure. The liver and spleen were immediately removed, frozen in liquid nitrogen and stored at -70° C until used.

Biochemical procedures

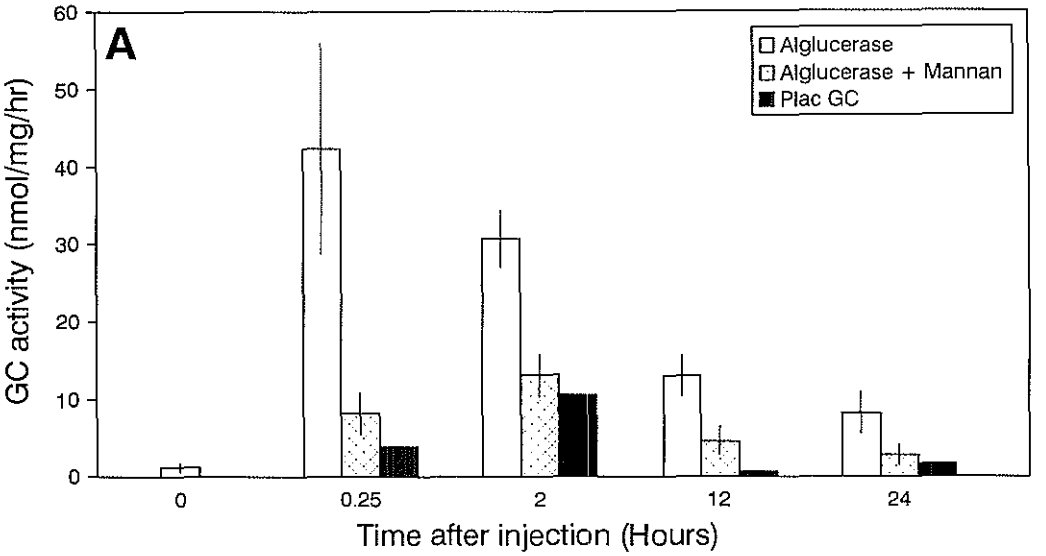
Liver and spleen were homogenized and sonicated in 1% (v/v) Triton X-

100:water. Glucocerebrosidase activity was measured as described by Barneveld et al., using 4-methylumbelliferyl-ß-glucopyranoside as substrate (Barneveld *et al.*, 1983a). Monoclonal antibody 8E4 was used to discriminate between the administered human and endogenous mouse glucocerebrosidases (Barneveld *et al.*, 1983b). Alglucerase was immunoprecipitated from 1 ml of a tissue homogenate (82 µg protein/ml) in 0.1 M PBS containing 1 mg/ml BSA with 8E4 and protein G Sepharose, and the activity on the beads was measured as described (Barneveld *et al.*, 1983b). Alglucerase was immunoprecipitated from a tissue homogenate containing 820 µg protein/ml and subsequently analyzed by SDS-PAGE and blotting using rabbit polyclonal antibody in combination with ^{125}I-labeled protein A for detection (Willemsen *et al.*, 1987).

Immunocytochemistry
For light-microscopic localization of alglucerase in liver, thick (7 µm) cryostat sections were fixed and incubated as previously described with rabbit polyclonal antibody against human placental glucocerebrosidase (Hoogeveen *et al.*, 1993). The indirect immunoperoxidase procedure was performed with swine anti-(rabbit Ig) conjugated with peroxidase (DAKO, Denmark) for the second step. Peroxidase was visualized using 3,3'-diaminobenzidine.HCl (Serva) as a substrate. Endogenous peroxidases were inhibited by a 30 min incubation in PBS-hydrogen peroxide(1%)-sodium-azide(1%) solution. Sections were counterstained with haematoxyline (Gill's). Immunoelectronmicroscopy was performed on ultrathin frozen sections using the immunogold method (Willemsen *et al.*, 1988). Briefly, small pieces of liver were fixed in 0.1 M PBS, containing 3% paraformaldehyde for one week and stored at 4° C in 0.1M PBS, containing 1% paraformaldehyde and 1 M sucrose. Ultrathin frozen sections were incubated with rabbit polyclonal antibodies against glucocerebrosidase. Antigen-antibody complexes were visualized through binding of goat anti-(rabbit Ig) coupled to 10 nm colloidal gold particles (Aurion, Wageningen, The Netherlands). Sections were stained with uranyl salts and examined in a Philips CM100 at 80 kV. Liver sections from mice that were injected with PBS were used as controls.

RESULTS

Biochemical identification of Alglucerase
Intravenous injection of alglucerase (60 IU/kg body weight) resulted in a two fold increase of the total glucocerebrosidase activity in mouse liver



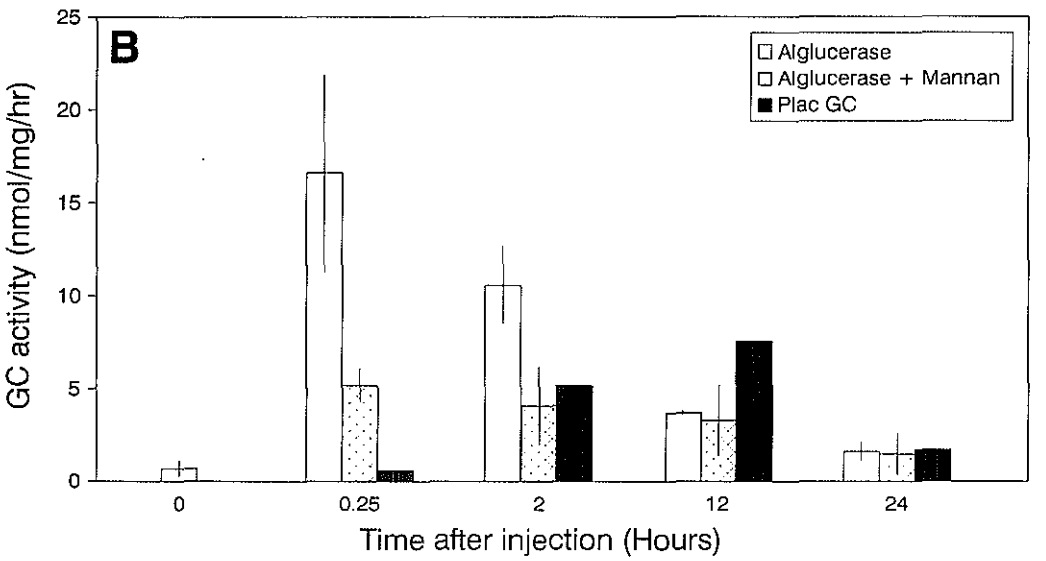
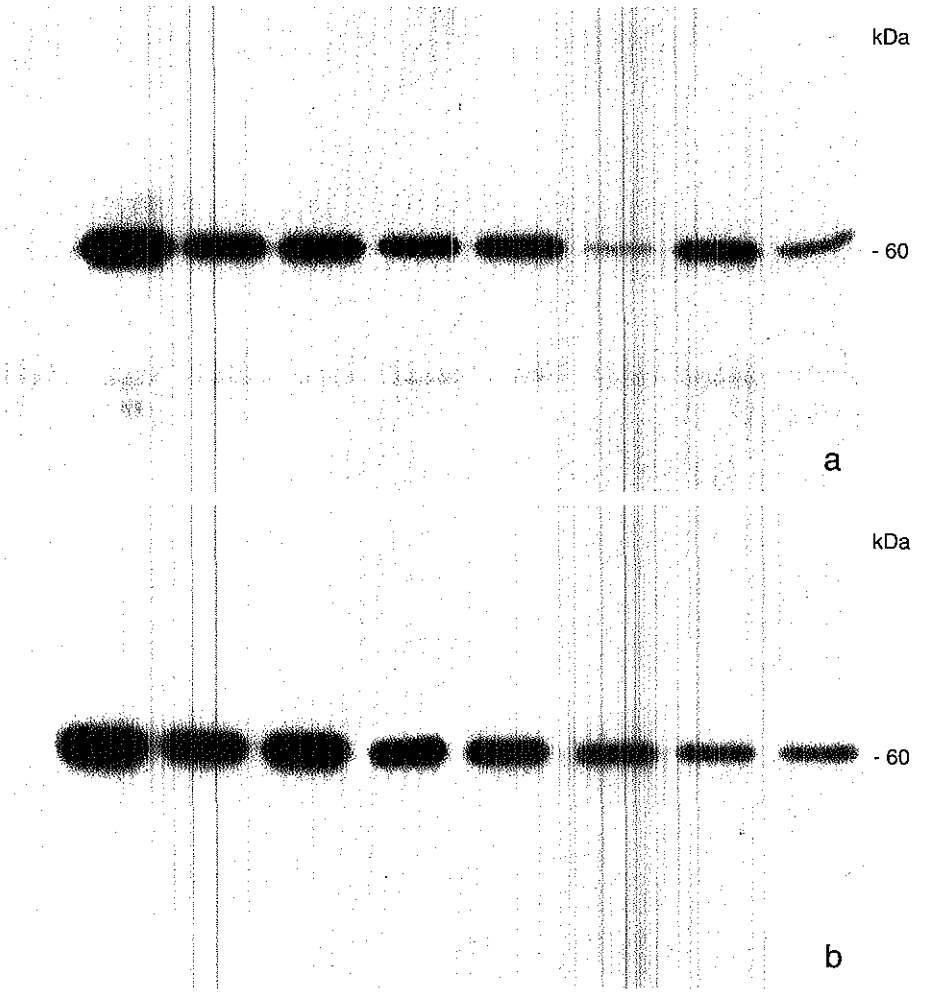


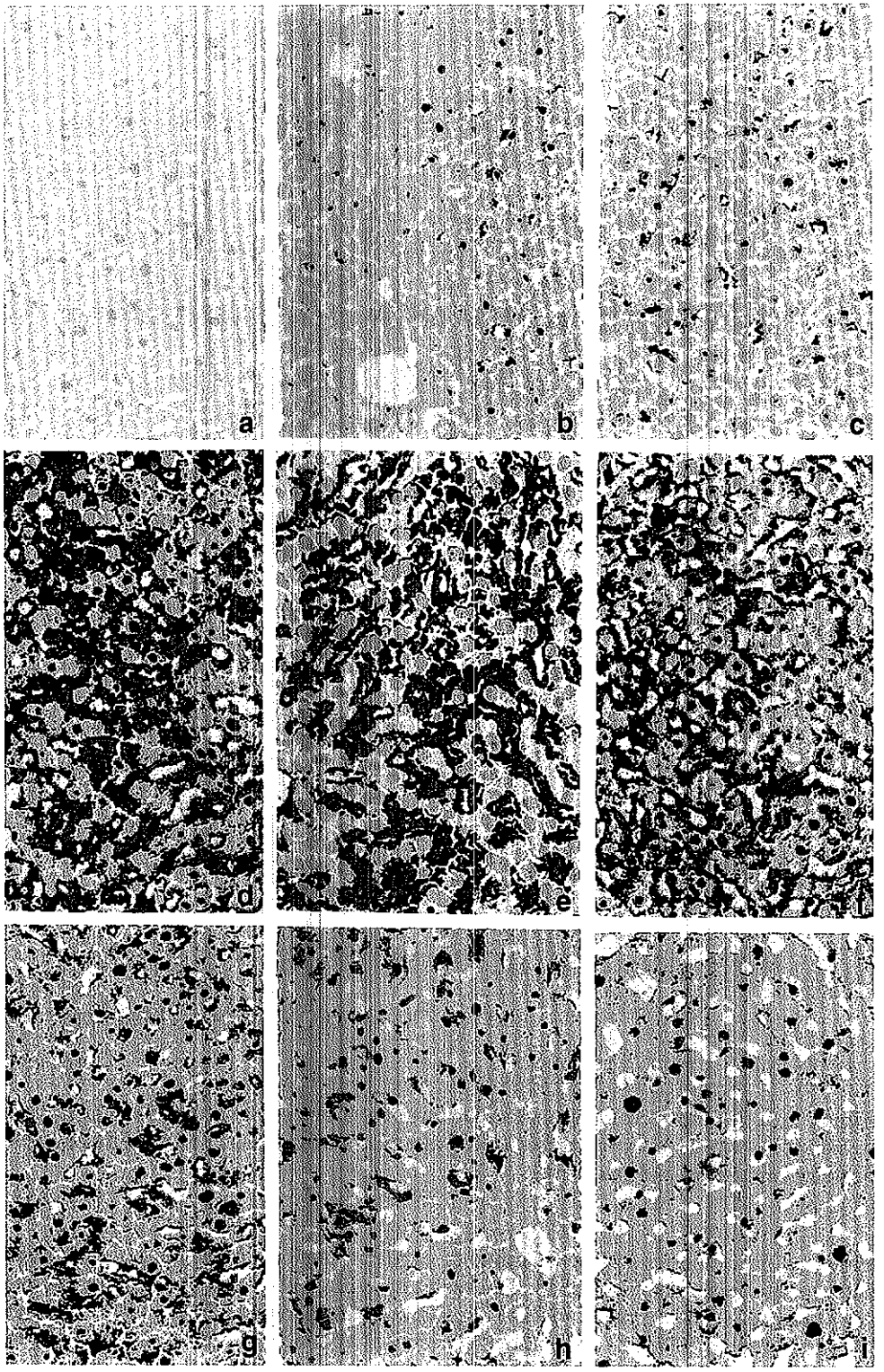
Figure 1.-Immunoprecipitable alglucerase activity in mouse liver (a) and spleen (b) after intravenous administration (60 IU/kg/body weight) of unmodified human placental glucocerebrosidase and alglucerase in absence or presence of mannan. Activity is expressed in nmoles MU per mg protein per hour.



0	15	15	120	120	720	720	1440	1440	min.
–	–	+	–	+	–	+	–	+	mannan

Figure 2.-Immunoprecipitable molecular forms of alglucerase in mouse liver (a) and spleen (b) as revealed by immunoblotting after intravenous injection of alglucerase in absence or presence of mannan.

Figure 3.-Immunoperoxidase staining of cryostat sections from mouse liver after intravenous injection of (a) PBS; (b,c) human unmodified placental glucocerebrosidase; (d-f) alglucerase *minus* mannan; (g-i) alglucerase *plus* mannan. Tissues were frozen at 15 min (a,b,d,g), 120 min (c,e,h) and 1440 min (f,i) after administration. Sections were counterstained with heamatoxyline. 300X


a
b
c
d
e
f
g
h
i

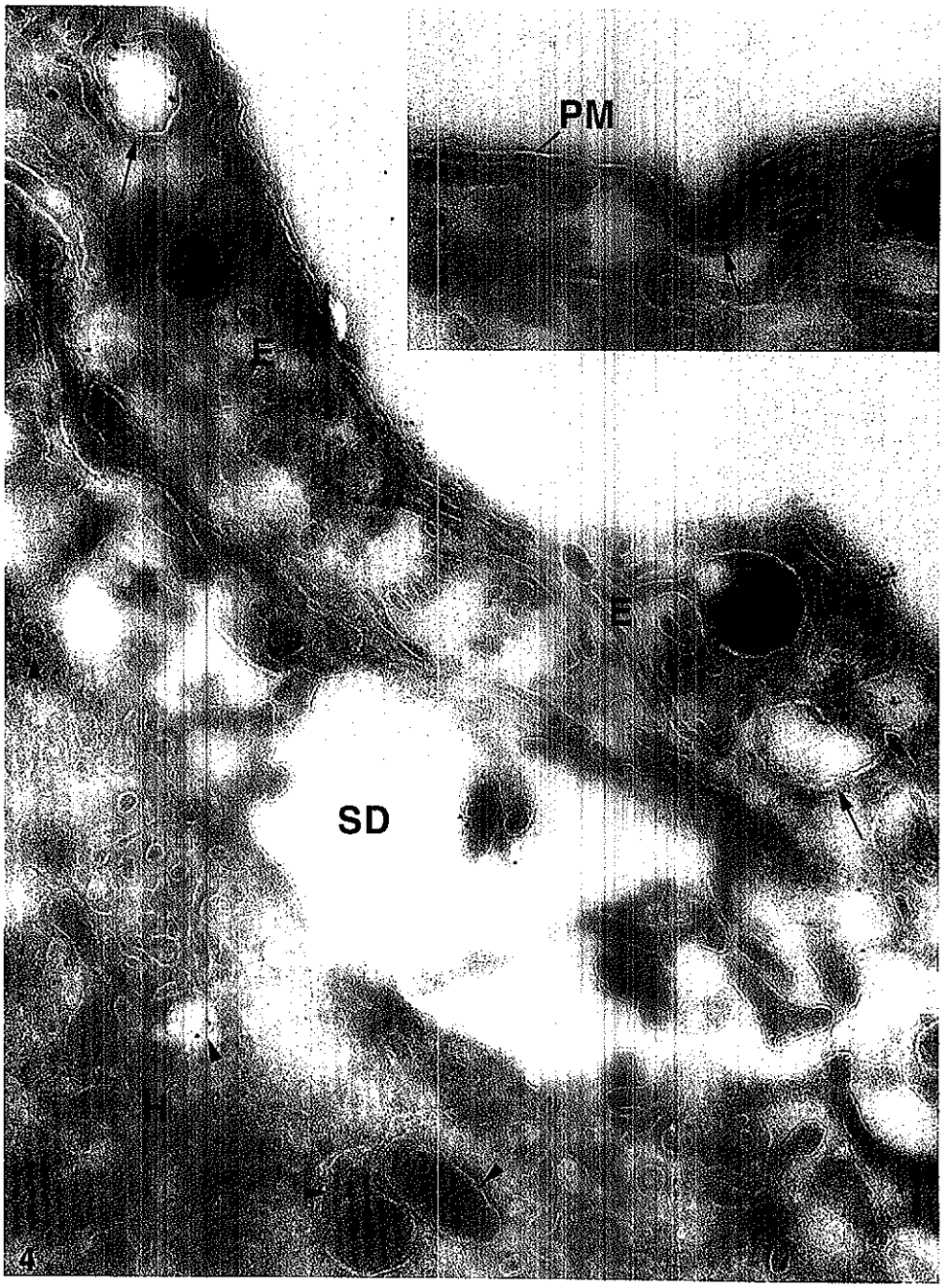


Figure 4.-Subcellular distribution of alglucerase in an endothelial cell (E) and a hepatocyte (H) 120 min after alglucerase injection. Alglucerase in endothelial cells is present in plasmalemmal vesicles and larger vacuoles (arrows) and at the plasmamembrane (PM), often in coated pits (inset, 78,000X). The hepatocyte shows a very weak labelling of the endosomal system (arrow heads). SD, space of Disse. 46,500X

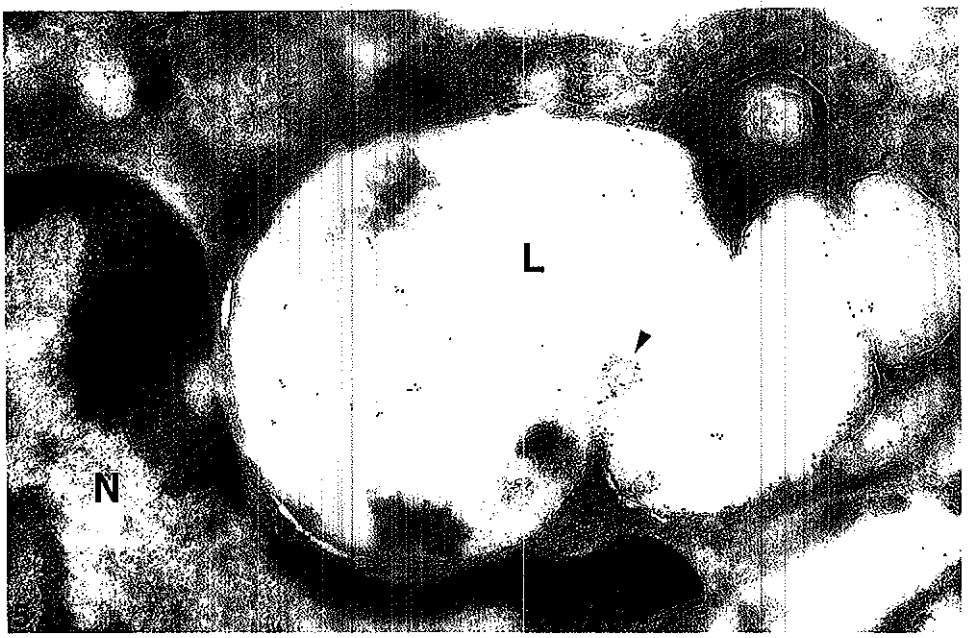


Figure 5.-Subcellular distribution of alglucerase in a Kupffer cell 120 min after alglucerase injection. The enzyme is present in large lucent lysosomes (L). Note that alglucerase is predominantly located at the limiting membrane of the lysosome or associated with intralysosomal membranous material (arrow head). N, nucleus. 29,000X

tissue at 15 minutes after injection. The alglucerase activity was distinguished from the mouse endogenous glucocerebrosidase activity by immunoprecipitation with monoclonal antibody 8E4 that identifies only human enzyme under the described conditions. The highest activity in liver and spleen was recorded at 15 minutes after injection (Fig. 1). The half-life of the enzyme in liver and in spleen is 7 and 5 hours, respectively. As shown in figure 1 the uptake is inhibited when mannan is added to the injected sample. The inhibitory effect is especially evident at the first time point after injection (±80% at 15 minutes). Administration of unmodified human placental glucocerebrosidase does result in a increase of enzyme activity. The results of immunoblotting studies performed to characterize the molecular size of administered glucocerebrosidase in liver and spleen are illustrated in figure 2. Alglucerase Western analyses show appearance of cross-reactive material (CRM) of ±60 kD following alglucerase injection, which is decreased in intensity when mannan was infused simultaneously.

Distribution of Alglucerase in liver

The cellular localization in liver was studied in thick cryostat sections using the indirect immunoperoxidase technique. Cross reactivity of the antibodies with mouse (endogenous) glucocerebrosidase was negligible (Fig. 3a). Only a very weak labelling of Kupffer cells was observed at 15 and 120 minutes after intravenous administration of unmodified human placental enzyme (Fig. 3b and 3c resp.). In contrast, injection of alglucerase resulted in a prominent labelling along both the lining of the sinusoids and in the Kupffer cells. The labelling pattern remained the same for 24 hours after injection, but the intensity decreased with time (Figs. 3d-3f). The inhibitory effect of mannan is shown in figures 3g-3i at the corresponding time points of figures 3d-3f.

The subcellular localization of administered glucocerebrosidase was studied using the indirect immunogold method on ultrathin frozen sections. The localization of alglucerase in endothelial cells and hepatocytes is illustrated in figure 4. In endothelial cells, small plasmalemmal vesicles and larger vacuoles are labelled (Fig. 4).

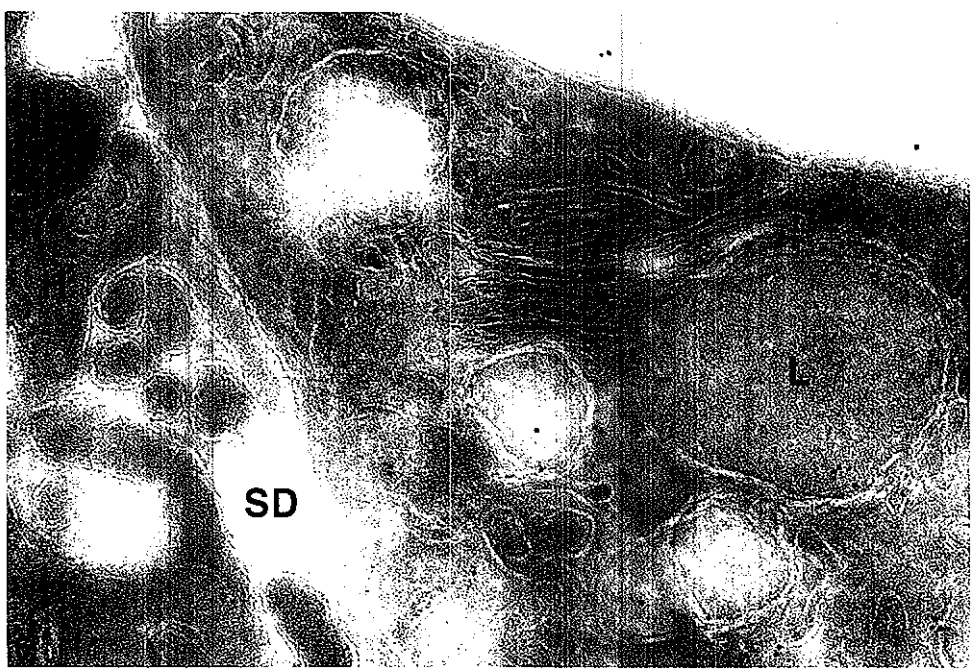


Figure 6.-Subcellular localization of alglucerase in a Kupffer cell 120 min after alglucerase injection. This high magnification shows alglucerase at the limiting membrane of a lysosome (L). SD, space of Disse; H, hepatocyte. 70,000X

The labelling at the plasma membrane is often found in coated pits (Fig. 4, inset). In hepatocytes there is only sporadic labelling of the endosomal apparatus at the baso-lateral side (Fig. 4). In Kupffer cells the alglucerase is mainly located in large lucent vacuoles and is often associated with the limiting membrane or with membranous material inside the vacuoles (Fig. 5). Smaller vacuoles with a more dense character are also observed with gold particles along the limiting membrane (Fig. 6).

DISCUSSION

The current success of enzyme replacement therapy in Gaucher disease has been attributed to the more efficient delivery of glucocerebrosidase to the lysosomes of tissue macrophages (Brady & Barton, 1994a; Brady & Barton, 1994b). When the initial clinical trials with purified unmodified human placental glucocerebrosidase did not result in clinical improvement it was suggested that modification of the carbohydrate moieties might increase the therapeutic effect of the enzyme as a result of more efficient targeting to the Gaucher cells. Indeed, quantitative data on rats showed uptake of intravenously injected native glucocerebrosidase by both non-parenchymal cells and hepatocytes, while more effective targeting to non-parenchymal cells was obtained by modifying the carbohydrate chains to expose terminal mannose residues (Furbish *et al.*, 1978; Furbish *et al.*, 1981; Morrone *et al.*, 1981).
Our present results on the (subcellular) localization of intravenously administered unmodified glucocerebrosidase and alglucerase in mouse liver confirm the previously observed biochemical findings. Following injection of unmodified human glucocerebrosidase, only minimal activity was measured in liver homogenates and very weak staining in sections was obtained, while alglucerase infusion resulted in a significant increase of human glucocerebrosidase activity in liver as demonstrated by enzyme assay, immunoblotting and immunohistochemistry. However, Kupffer cells were not the only target cell for alglucerase. At the light-microscopic level there was significant uptake of alglucerase by endothelial cells along the lining of the sinusoids, that at least in part appeared to be receptor mediated because of the presence of label in coated pits, as revealed by electronmicroscopy. The labelling of large vacuoles is suggestive of subsequent transport of alglucerase to the endosomal/lysosomal system. Furthermore, similar to the normal in vivo situation in both Kupffer and endothelial cells alglucerase is predominantly associated with the limiting membrane of endosomes or lysosomes.

The results of our enzyme uptake inhibition experiments using mannan suggest a mannose receptor mediated uptake of most of the administered alglucerase in Kupffer and endothelial cells. Since hepatic endothelial cells have the classical mannose receptor as evidenced by the capacity to bind mannose-BSA, it is conceivable that this mannose receptor is responsible for uptake of the mannose terminated enzyme in endothelial cells (Hubbard *et al.*, 1979). However, a second mannose receptor distinct from the classical one, as described by Sato and Beutler cannot be excluded from having a role in uptake of alglucerase (Sato & Beutler, 1993). Sinusoidal endothelial cells are the most active of the non-parenchymal cells in binding and internalization of mannose-terminated ligands (Hubbard *et al.*, 1979). In addition to this, the fact that approximately two-third of the non-parenchymal liver cells are endothelial cells suggests that most of alglucerase taken up by liver tissue is targeted to endothelial cells, rather than Kupffer cells.
It is as yet unknown to what extent the alglucerase uptake by endothelial cells contributes to the therapeutic effect. One can speculate about the possibility that alglucerase mediates the uptake and subsequent degradation of serum glucocerebroside by binding to both the substrate and the mannose receptor on the endothelial cells. A mouse model with mild Gaucher disease would be the ideal means to investigate this important issue and facilitate optimalization of enzyme replacement therapy for Gaucher disease.

ACKNOWLEDGEMENTS

The authors wish to thank Mr. J. Brandenburg for animal care, Mrs. J. Lokker for secretarial assistance and Dr. H. Aerts (University of Amsterdam) for stimulating discussions.

REFERENCES

Barneveld, R.A., Tegelaers, F.P., Ginns, E.I., Visser, P., Laanen, E.A., Brady, R.O., Galjaard, H., Barranger, J.A., Reuser, A.J.J. & Tager, J.M. (1983) Monoclonal antibodies against human beta-glucocerebrosidase. Eur. J. Biochem. 134, 585-589.

Barneveld, R.A., Keijzer, W., Tegelaers, F.P., Ginns, E.I., Geurts van Kessel, A., Brady, R.O., Barranger, J.A., Tager, J.M., Galjaard, H., Westerveld, A. & Reuser, A.J.J. (1983) Assignment of the gene coding for human ß-glucocerebrosidase to the region q21-q31 of chromosome 1 using monoclonal antibodies. Hum. Genet. 64, 227-231.

Barranger, J.A. & Ginns, E.I. (1989) Glucosylceramide lipidoses: Gaucher disease. In: The Metabolic Basis of Inherited Disease (C.R. Scriver et al. eds.) pp. 1677-1698. McGraw-Hill, New York.

Barton, N.W., Brady, R.O., Dambrosia, J.M. & DiBisceglie, A.M. (1991) Replacement therapy for inherited enzyme deficiency-macrophage-targeted glucocerebrosidase for Gaucher disease. N. Engl. J. Med. 324, 1464-1470.

Beutler, E., Dale, G.L. & Kuhl, W. (1980) Replacement therapy in Gaucher disease. In Enzyme Therapy in Genetic Diseases (R.J. Desnick ed.), Vol. 2 pp. 369-381. Alan R. Liss., New York.

Brady, R.O. & Barton, N.W. (1994a) Enzyme replacement therapy for Gaucher disease: Critical investigations beyond demonstration of clinical efficacy. Bioch. Med. and Metab. Biol. 52, 1-9.

Brady, R.O. (1984) Enzyme replacement in the sphingolipidoses. In: Molecular Basis of Lysosomal Storage Disorders (J.A. Barranger and R.O. Brady eds.) pp. 461-478. Academic Press, New York.

Brady, R.O. & Barton, N.W. (1994b) Development of effective enzyme therapy for metabolic storage disorders. International Pediatrics 9, 175-180.

Figueroa, M.L., Rosenbloom, B.E., Kay, A.G., Garver, P.G., Thurston, D.W., Koziol, J.A., Gelbart, T. & Beutler, E. (1992) A less costly regimen of alglucerase to treat Gaucher's disease. N. Engl. J. Med. 327, 1632-1636.

Furbish, F.S., Steer, C.J., Krett, N.L. & Barranger, J.A. (1981) Uptake and distributi on of placental glucocerebrosidase in rat hepatic cells and effect of sequential deglycosylation. Biochim. Biophys. Acta 673, 425-434.

Furbish, F.S., Steer, C.J., Barranger, J.A., Jones, E.A. & Brady, R.O. (1978) The uptake of native and desialylated glucocerebrosidase by rat hepatocytes and Kupffer cells. Biochem. Biophys. Res. Commun. 81, 1047-1053.

Grabowski, G.A. (1993)Gaucher disease. Enzymology, genetics and treatment. In: Advances in Human Genetics (H. Harris and K. Hirschhorn eds.) pp. 377-441. Plenum Press, New York.

Hoogeveen, A.T., Willemsen, R., Meyer, N., DeRooij, K.E., Roos, R.A.C., VanOm men, G-J.B. & Galjaard, H. (1993) Characterization and localization of the Huntington disease gene product. Hum. Molec. Genet. 12, 2069-2073.

Hubbard, A.L., Wilson, G., Ashwell, G. & Stukenbrok, H. (1979) An electron microscope autoradiographic study of the carbohydrate recognition systems in rat liver. I. Distribution of ^{125}I-ligands among the liver cell types. J.Cell Biol. 83, 47-64.

Morrone, S., Pentchev, P.G., Baynes, J. & Thorpe, S. (1981) Studies in vivo of the tissue uptake, cellular distribution and catabolic turnover of exogenous glucocerebrosidase in rat. Biochem. J. 194, 733-742.

Pontow, S.E., Kery, V. & Stahl, P.D. (1992) Mannose receptor. Int. Rev. of Cytology 137B, 221-244.

Sato, Y. & Beutler, E. (1993) Binding, internalization, and degradation of mannose-terminated glucocerebrosidase by macrophages. J. Clin. Invest. 91, 1909-1917.

Willemsen, R., VanDongen, J.M., Aerts, J.M.F.G., Schram, A.W., Tager, J.M., Goud smit, R. & Reuser, A.J.J. (1988) An immunoelectronmicroscopic study of glucocerebrosidase in type 1 Gaucher's disease spleen. Ultrastr. Pathol. 12, 471-478.

Willemsen, R., VanDongen, J.M., Ginns, E.I., Sips, H.J., Schram, A.W., Tager, J.M.,

Barranger, J.A. & Reuser, A.J.J. (1987) Ultrastructural localization of glucocerebrosidase in cultured Gaucher's disease fibroblasts by immunocytochemistry. J. Neurol. 234, 44-51.

Zimran, A., Elstein, D., Kannai, R., Zevin, S., Hadas-Halpern, I., Levy-Lahad, E. & Cohen, Y. (1994) Low-dose enzyme replacement therapy for Gaucher disease: effcts of age, sex, genotype and clinical features on reponse to treatment. Am. J. Med. 97, 3-13.

Stellingen behorende bij het proefschrift

GAUCHER DISEASE

An immunoelectron microscopic and biochemical study

I

Immunoelectronenmicroscopische lokalisatie van normale en gemuteerde eiwitten levert een essentiële bijdrage aan het inzicht in de pathogenese van erfelijke ziekten.

Dit proefschrift

II

Gebrek aan informatie over de subcellulaire pathologie van genetisch gemodificeerde muizen kan leiden tot onjuiste conclusies.

Dit proefschrift

III

De effectiviteit van de behandeling van Gaucher patienten met Ceredase™ kan wellicht verhoogd worden door een meer efficiente cellulaire "targeting" van het toegediende glucocerebrosidase.

Dit proefschrift

IV

De prevalentie van de type 1 vorm van de ziekte van Gaucher moet worden bepaald aan de hand van het phenotype in plaats van het genotype.

Horowitz et al., Hum Mutation (1994):1-11.

V

Bij de in vitro bestudering van de klinische heterogeniteit binnen een lysosomale stapelingsziekte verdient het de voorkeur een celtype te kiezen waarbij in vivo daadwerkelijk stapeling optreedt.

Willemsen et al., Ultrastr Pathol (1993):515-527.

VI

In de discussie over de behandeling met hoge-dosis versus lage-dosis Ceredase™ van type 1 Gaucher patienten zouden voorstanders van het hoge-dosis behandelingsschema moeten aantonen dat dit een beter resultaat geeft.

VII

Wetenschappelijk onderzoek naar de vermeende positieve bijdrage van homeopathie aan de reguliere gezondheidszorg zou zich in analogie met de reguliere geneesmiddelen ook moeten richten op de eventuele toxische effecten van homeopathische geneesmiddelen.

VIII

De fysiologische betekenis van het fructaanmetabolisme in planten kan alleen worden begrepen door vergelijking van isogene planten die alleen verschillen door de aan- of afwezigheid van fructaan-stofwisseling.

Hendry et al., New Phytol (1993):3-14.
Pilon-Smits et al., Plant Physiol (1995):125-130.

IX

Het aanbieden van steeds goedkopere ziektekostenverzekeringen door verzekeraars waarbij steeds meer risicogroepen worden uitgesloten bedreigt de solidariteitsgedachte binnen de gezondheidszorg.

X

Het typeert de huidige Nederlandse politiek dat in de discussie of twee gedetineerden in één cel mogen worden opgesloten een grote betrokkenheid van de politici waargenomen kan worden, terwijl deze totaal afwezig is als het gaat om de vraag of psychiatrische patienten een kamer met vier tot zes lotgenoten moeten delen.

XI

IJzel en ZOAB = Zeer Ongeluk(kig) Asfalt Beton.

XII

De verzorging en de opvoeding van kinderen is ook een carrière.

Rotterdam, 27 september 1995 Rob Willemsen