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Gaucher Disease: Heterologous Expression of Two Alleles Associated with Neuronopathic Phenotypes

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Summary

To investigate the molecular basis for the distinct neuronopathic phenotypes of Gaucher disease, acid β-glucosidases expressed from mutant DNAs in Gaucher disease type 2 (acute) and type 3 (subacute) patients were characterized in fibroblasts and with the baculovirus expression system in insect cells. Expression of the mutant DNA encoding a proline-for-leucine substitution at amino acid 444 (L444P) resulted in a catalytically defective, unstable acid β-glucosidase in either fibroblasts from L444P/L444P homozygotes or in insect cells. This mutation was found to be homoallelic in subacute neuronopathic (type 3) Gaucher disease. In comparison, expression of the mutant cDNA encoding an arginine-for-proline substitution at amino acid 415 (P415R) resulted in an inactive and unstable protein in insect cells. This allele was found only in a type 2 patient with the L444P/P415R genotype. The substantial variation in the type 3 phenotype (L444P homozygotes) suggests the complex nature of the molecular basis of phenotypic variation in Gaucher disease. Yet, the association of neuronopathic phenotypes with alleles producing severely compromised (L444P) or functionally null (P415R) enzymes indicates that the effective level of residual activity at the lysosome is likely to be a major determinant of the severity of Gaucher disease.

Introduction

Gaucher disease is the most prevalent lysosomal storage disease and results from inherited defects of the activity of acid β-glucosidase (E.C.3.2.1.45; N-acylsphingosyl-l-O-β-D-glucoside:glucohydrolase) (Desnick et al. 1982). This membrane-associated lysosomal enzyme cleaves the β-glucosidic linkage of glucosylceramide (Brady et al. 1965; Mueller and Rosenberg 1977), the major accumulated substrate in the tissues of patients with Gaucher disease. Three major variants of this autosomal recessive disorder have been delineated on the basis of the absence (type 1) or presence and severity (type 2 [acute] and type 3 [subacute]) of neuronopathic manifestations. However, marked heterogeneity of the clinical manifestations has been observed within and among types 1 and 3. Although numerous genetic lesions have been identified (see below), the molecular basis of the phenotypic variation has not been clearly defined.

The complete sequences of the human acid β-glucosidase structural gene and its pseudogene (Horowitz et al. 1989) have been reported. Results of sequencing studies have indicated that the majority of molecular defects in Gaucher disease are point mutations, and, to date, over 16 alleles containing missense mutations have been identified in the acid β-glucosidase gene from Gaucher disease patients (Graves et al. 1988; Tsuji et al. 1988; Theophilus et al. 1989*a*, 1989*b*; Wigderson et al. 1989; Beutler and Gelbart 1990; Hong et al. 1990; Kolodny et al. 1990; Latham et al. 1990, 1991; Zimran et al. 1990*b*; for review, see Grabowski et al. 1990) It is important that four alleles with multiple authentic point mutations have been identified and that several of these apparently arose from genetic rearrangement with the highly homologous pseudogene (Hong et al. 1990, Latham et al.

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1990, 1991, Zimran et al. 1990*b*). The majority of missense mutations are from rare or private alleles, i.e., restricted to a single or few families. However, two mutations, resulting in either the substitution of serine for asparagine at amino acid 370 (N370S) or the substitution of a proline for leucine at amino acid 444 (L444P), account for ~70% of all alleles present in Gaucher patients (Tsuji et al. 1988; Theophilus et al. 1989*b*; Zimran et al. 1989). The presence of N370S in the homoallelic state has been associated with milder phenotypes and nonneuronopathic disease (Theophilus et al. 1989*b*; Zimran et al. 1989); and, it is important to note, no affected patients with at least one allele bearing this mutation have had neuronopathic manifestations (Tsuji et al. 1988; Theophilus et al. 1989*b*; Zimran et al. 1989). Expression of the N370S cDNA resulted in a kinetically altered, stable, and catalytically active enzyme, thereby demonstrating its causal association with Gaucher disease (Grace et al. 1990). These results were later confirmed by Ohashi et al. (1991). Grace et al. (1990) also indicated that the presence of this partially active allele (N370S) is sufficient to confer the nonneuronopathic phenotype even if the heteroallele expresses an inactive protein.

In comparison, the L444P mutation has been detected in patients with all three major types of Gaucher disease. Homozygosity for the allele encoding this mutation has been shown to be the most frequent, if not exclusive, genotype in the type 3 (subacute neuronopathic) Norrbottnian Gaucher disease population (Dahl et al. 1990; Latham et al. 1990). Paradoxically, type 2 Gaucher disease patients also were apparently homozygous for the L444P allele (Tsuji et al. 1987; Theophilus et al. 1990*a*). This apparent lack of phenotype/genotype correlation was resolved by complete sequence analyses of alleles (Latham et al. 1990) from such type 2 and type 3 patients. The type 3 patients were shown to be truly homozygous for the L444P mutation. However, the type 2 (acute neuronopathic) patients had two different acid β-glucosidase alleles: one with only the L444P mutation and the other with the L444P mutation and additional authentic missense mutations. This result suggested that some "type 2" alleles might express proteins which would not contribute to the residual acid β-glucosidase activity in cells and, thus, would result in a lower level of enzyme activity than found in type 3 Gaucher disease. To test this and to assess the individual contributions of each allele, we used site-directed mutagenesis and expression of mutagenized cDNAs encoding acid β-glucosidases to investigate the functional defects which result from the L444P substitution as well as from another mutation (P415R) associated with the type 2 phenotype. These findings provide insight into the molecular basis for the distinct phenotypes of type 2 and type 3 Gaucher disease.

Material and Methods

Material

The following were from commercial sources: Triton X-100 (Sigma, St. Louis); sodium taurocholate (Cal Biochem, La Jolla, CA); 4-methylumbelliferyl-β-D-glucopyranoside (4MU-Glc) (Genzyme, Boston); Sequenase™ and Sequenase™ kit (USB, Cleveland); Vectastain ABC Kit (Vector Labs, Burlingame, CA); restriction endonucleases (New England Biolabs, Beverly, MA); oligonucleotide-directed in vitro mutagenesis system, multiprime DNA labeling system and [^{32}P]-nucleotides (Amersham, Arlington Heights, IL); β-cyanoethyl phosphoramidites, CPG synthesis columns, and reagent kits for the model 380B DNA synthesizer (Applied Biosystems, Foster City, CA); low-melting-point agarose (BRL, Gaithersburg, MD); RPMI 1640 medium, Grace's medium, and FCS (Gibco, Grand Island, NY); TC lactalbumin hydrolysate and TC yeastolate (Difco Laboratories, Detroit); gentamicin sulfate (Elkin-Sinn, Cherry Hill, NJ), Fungizone™ (Squibb, Princeton, NJ); and *Spodoptera frugiperda* (*Sf9* cloned cells, Crl 1711 [American Type Culture Collection, Rockville, MD]).

Fluorescent glucosylceramide (12-[N-methyl-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)]dodecanoyl-ceramide β-glucose) was prepared and purified as described elsewhere (Grabowski et al. 1984). Bromo-conduritol B epoxide (Br-CBE) and deoxynojirimycin (1,5-dideoxy-5-amino-D-glucose; DNM) were gifts from Dr. Gunter Legler. Wild-type baculovirus, *Autographica californica* nuclear polyhedrosis virus (AcMNPV, family Baculoviridae [Matthews 1982]), and the plasmid pAc610 (Summers and Smith 1987), which contains a polylinker with unique *Eco*RI and *Sac*I restriction sites, were supplied by Dr. Max Summers. Anti-human acid β-glucosidase monoclonal antibody 1 (MCAB 1) has been described elsewhere (Fabbro et al. 1987).

Cell Culture

Human fibroblasts were taken from affected and normal individuals after informed consent and/or as-

sent of minors was obtained. These cell lines were established and maintained as described elsewhere (Fabbro et al. 1987). *Sf9* cells were grown in Grace's medium containing 10% heat-inactivated FCS, modified with lactalbumin hydrolysate (3.3 mg/ml), yeastolate (3.3 mg/ml), and antibiotics (gentamicin [50 μg/ml] and Fungizone™ [2.5 μg/ml]) (Summers and Smith 1987).

Recombinant DNA methods

The T→C point mutation at base 1448 in the cDNA (base numbering is from the most 5′ in-frame ATG of the cDNA, as in the study by Graves et al. [1988]) was introduced into the normal acid β-glucosidase cDNA by oligonucleotide-directed site-specific mutagenesis in M13mp19 by using the phosphorothioate DNA selection procedure (Taylor et al. 1985*a*, 1985*b*). This mutation results in a Leu^{444}→Pro substitution (L444P) in the mature protein, with amino acid 1 being the N-terminal alanine. The oligonucleotide for mutagenesis was synthesized so that the base mismatch was centered in a 21-mer. Following mutagenesis and purification of a mutant clone, the entire coding sequence of each mutagenized cDNA was determined by the dideoxy method (Sanger et al. 1977) as adapted for double-stranded DNA (Chen and Seeburg 1985). The *Eco*RI-*Sac*I fragment of the mutant cDNA was cloned into pAc610 (Grace et al. 1990). The *Eco*RI-*Sac*I fragment of the acid β-glucosidase cDNA contains the entire coding sequence as well as 93 nucleotides of 5′ and 304 nucleotides of 3′ untranslated sequence. The cDNA containing the C→G transversion at base 1361 (base numbering of the cDNA is as in the study by Graves et al. [1988]), which encodes the Pro^{415}→Arg substitution (P415R), was described by Wigderson et al. (1989). An *Eco*RI cDNA fragment was cloned into pAc610 in the sense orientation.

Construction and Purification of Recombinant Baculovirus

Recombinant baculovirus containing the normal or mutant cDNAs was obtained by homologous recombination following calcium phosphate–mediated transfection (Summers and Smith 1987). Infection of *Sf9* cells with wild-type or recombinant AcMNPV virus, determination of viral titers, and calcium phosphate–mediated transfection were done as described elsewhere (Grace et al. 1990). Recombinant viruses containing the acid β-glucosidase cDNAs were purified by plaque hybridization (Summers and Smith 1987) as described elsewhere (Grace et al. 1990). The levels of acid β-glucosidase activity and protein expression from independent recombinant viral isolates for each cDNA expressed were monitored by enzyme assay and immunoblots 4 d after infection. For these studies, titers were increased until high level expression was obtained (Grabowski et al. 1989).

Immunoelectroblotting and Immunotitrations

Immunoelectroblotting was conducted essentially as described using the anti-human acid β-glucosidase monoclonal antibody, MCAB 1 (Fabbro et al. 1987). In brief, *Sf9* cells, infected with wild-type or pure recombinant AcMNPV, were harvested into 0.9% NaCl by vigorous striking of the flasks. Cells were washed three times by centrifugation (525 *g* for 10 min) and resuspension in 0.9% NaCl. Pellets were stored at −20°C until use. Acid β-glucosidase was solubilized by ultrasonic irradiation, at 4°C, of the washed pellets in 0.1% Triton X-100 and 0.1% sodium taurocholate by using a cup sonicator (Branson Cell Disruptor 200, 80 W, pulse times 30 s, 20 s, and 20 s [Grabowski et al. 1985]). The sonicates were clarified by centrifugation (875 *g* for 20 min), and the resulting supernatants were used in the immunoelectroblotting studies (Fabbro et al. 1987). Immunoblots were conducted with the IgG monoclonal antibody, MCAB 1 (Grabowski et al. 1985; Fabbro et al. 1987). Extracts of *Sf9* cells infected with wild-type virus gave no detectable signal on immunoblots.

The relative amount of cross-reacting immunologic material per unit glucosylceramide-cleaving activity (CRIM-specific activity) for normal and mutant acid β-glucosidases expressed in *Sf9* cells were estimated as described elsewhere (Grace et al. 1990), by using signal intensities for the intact 63-kDa enzyme species only. The CRIM-specific activities for the acid β-glucosidases from fibroblasts were determined by immunotitrations as described by Grabowski et al. (1985).

Enzyme Assays

Hydrolysis of glucosylceramide was determined using fluorescent glucosylceramide (Dinur et al. 1984; Osiecki-Newman et al. 1987). The final reaction mixtures (0.2 ml) contained phosphate/citrate pH 5.5, 4 mM β-mercaptoethanol, and 1.25 mM EDTA as well as 0.25% Triton X-100 and 0.25% sodium taurocholate. Enzyme inhibitors (Br-CBE and DNM) were added as concentrated solutions in water. Assays were initiated by the addition of enzyme. IC_{50} values, the concentration of inhibitor which results in 50% loss of initial enzyme activity, were determined as described elsewhere (Osiecki-Newman et al. 1987). The amount

of enzyme from the *Sf9* cell sonicates was adjusted to ensure that <5% of the substrate was hydrolyzed during the reaction. Reactions were terminated after 0.5–2 h at 37°C.

Results

In *Sf9* cells, cDNAs encoding the normal, L444P, or P415R mutant acid β-glucosidases expressed glycosylated enzyme species which were detectable by immunoblotting. As shown in figure 1, *Sf9* cells infected with wild-type baculovirus had no detectable protein bands when MCAB 1 was used (fig. 1, lane A). Recombinant baculovirus containing the normal acid β-glucosidase cDNA (fig. 1, lane B) overexpressed the enzyme with a single detectable protein band (M_r ~63 kDa). In comparison, *Sf9* cells infected with recombinant baculovirus containing the L444P) (fig. 1, lane C) and P415R (fig. 1, lane D) cDNAs overexpressed acid β-glucosidases with intense bands at M_r ~63 kDa; but several additional smaller, and very similar, bands representing specific degradation products were detected in the L444P and the P415R acid β-glucosidase lanes. The same results were obtained from other viral isolates containing these two mutations and at different multiplicities of infection. These results demonstrate that these degradation products are not an artifact of the insect cell expression system but are due to an increased proteolytic susceptibility of these mutant proteins. The absence of such degradation products for the normal enzyme (fig. 1, lane B) as well as with several other mutations (T38K, R120Q, N370S, and D358E; Grace et al. 1990) indicates that the protein fragments from L444P and P415R were due to the intrinsic instability of the L444P and P415R gene products.

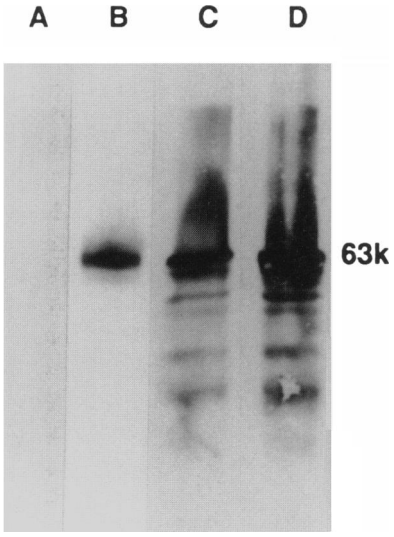

Figure 1 Immunoblot of mutant acid β-glucosidases expressed in *Sf9* cells. Shown are crude cell extracts from *Sf9* cells infected with wild-type virus (lane A; 61 μg cell protein), from *Sf9* cells expressing normal enzyme (lane B; 29 μg cellular protein), expressing acid β-glucosidase containing L444P mutation (lane C; 37 μg cellular protein), and expressing acid β-glucosidase containing P415R mutation (lane D; 33 μg cellular protein).

These results were consistent with previous immunoblotting (Grabowski et al. 1985; Fabbro et al. 1987) and pulse-chase processing studies (Bergmann and Grabowski 1989) of fibroblasts from neuronopathic (type 3 or type 2) patients whose genotypes were L444P/L444P or L444P/P415R (Gm1260). In those studies, detectable steady-state levels of CRIM were present only transiently; that is, the normal half-life for acid β-glucosidase in fibroblasts was ~60 h, whereas that for the detectable acid β-glucosidases in the L444P/L444P or L444P/L415R cells was ~4 h (Bergmann and Grabowski 1989). These findings demonstrate that, if both mutant proteins were present in the latter cells, their interaction (if any) did not significantly influence the half-life of the residual enzyme. Similar results have been obtained with other type 2 and type 3 fibroblasts, but for the majority of these analyses the genotypes of the mutant acid β-glucosidases are not known (Ginns et al. 1982; Beutler and Kuhl 1986; Tager et al. 1986). Since the transit time for acid β-glucosidase to the lysosome is ~2–3 h (Erickson et al. 1985; Bergmann and Grabowski 1989) and since leupeptin, a peptide protease inhibitor, increased the enzyme activity in such fibroblasts (Tager et al. 1986), it is likely that some of the mutant acid β-glucosidases reach the lysosomes but that rapid proteolysis occurs there. Although, the steady-state amount of mutant acid β-glucosidase in the lysosome, i.e., the effective enzyme level, is difficult to determine, it is probably small, since no lysosomal signals were observed on immunolocalization by electron-microscopic analyses of type 2 fibroblasts (Willemsen et al. 1987).

To determine whether CRIM expressed from the L444P or P415R cDNAs was catalytically active, assays were conducted using glucosylceramide and 4MU-Glc substrates (table 1). With several different recombinant viral stocks and multiplicities of infections, easily detectable levels of activity toward either substrate were observed for the L444P acid β-glucosidase. Although substantial acid β-glucosidase protein was synthesized, the P415R mutation resulted in an

Table 1

Characterization of Acid β-Glucosidases Expressed from Normal or Mutant Alleles of Defined Genotype

Source	Specific Activity (nmol/h/mg)		IC_{50} (μM)		Protein Stability
	4MU-Glc	Glucosylceramide	Br-CBE	DNM	
Sf9 cells:					
Normal cDNA	1,500–2,000	1,700–2,200	385	165	Stable
L444P	40–100	60–132	240	135	Unstable
P415R	0	0	NA	NA	Unstable
N370S[a]	40–48	60–90	1150	500	Stable
R120Q[a]	0	0	NA	NA	Stable
Fibroblasts:					
Normal ($n = 6$)	200–400	250–500	340	151	Stable
L444P/L444P, type 3 ($n = 3$)	13–26	14–24	486	167	Unstable
L444P/complex A[b], type 2 ($n = 2$)	9–14	13–17	515	189	Unstable
L444P/P415R, type 2 ($n = 1$)	14	12	525	155	Unstable
N370S/N370S[c], type 1 ($n = 2$)	34–68	52–89	900	370	Stable
N370S/R120Q[c], type 1 ($n = 1$)	24–45	25–65	850	450	Stable

[a] Data from Grace et al. (1990).
[b] Complex A refers to an allele containing three authentic point mutations: L444P, A456P, and V460V (Latham et al. 1990).

enzyme which was devoid of catalytic activity (table 1). These results were consistent with the presence of residual enzyme activity in fibroblasts of all patients with the L444P homozygous (type 3, Norrbottnian) or L444P/P415R (type 2, GM1260) genotypes (table 1). To determine the effect of the L444P mutation on the catalytic rate constant, activities were normalized to the amount of CRIM present in either *Sf9* for the intact 63-kDa species or cultured fibroblasts from affected patients (table 2). Compared with the normal CRIM specific activity, at least 12–16-fold more acid β-glucosidase protein was required per unit of activity with the L444P enzyme expressed in *Sf9* cells or fibroblasts. Consequently, in either the natural or heterologous cellular environment the major effects that this mutation had on the enzyme's instability to proteolysis were compounded by a large decrease in catalytic rate constant of the enzyme. This latter finding also was consistent with localization of an active-site nucleophile (Asp^{443}) to this region (Dinur et al. 1986*a*, 1986*b*).

Table 2

CRIM Specific Activity of Normal and Mutant Acid β-Glucosidase

Source	CRIM Specific Activity[a] (relative to normal)	1/CRIM Specific Activity[a]
Sf9 cells:		
Normal cDNA	1.00	1
L444P cDNA	.08	12.5
P415R cDNA	0	NA
N370S cDNA[b]	.25	4
R120Q cDNA[b]	0	NA
Fibroblasts:		
Normal	1.00	1.0
L444P/L444P	.06	16.7
L444P/P415R	.05	20.0
N370S/N370S[b]	.18	5.6
N370S/R120Q[b]	.11	9.1

[a] Units are nmol/h/mg CRIM.
[b] Data from Grace et al. (1990).

In comparison, this major perturbation of active-site function was not evident from the mutant's normal recognition of the potent inhibitors, Br-CBE and deoxynojirimycin, as reflected by IC_{50} values (table 1). These results were very similar to those obtained with the residual acid β-glucosidase activity in fibroblast extracts from patients homozygous for L444P (table 1). The heteroallelic presence of L444P and P415R in fibroblast extracts (i.e., GM1260) did not alter these values. Although the native intracellular subunit interaction of acid β-glucosidase is not clear (Maret et al. 1983, 1989; Choy et al. 1986), the inhibitor results indicate that the presence of inactive enzyme from the P415R allele does not interact with the enzyme from the active L444P allele in a manner detectable by these methods. Since homozygotes for the P415R allele are not available, the stability of this allele cannot be determined directly from human fibroblast cells. To obtain an indirect assessment, CRIM-specific activities in L444P homozygotes and in the L444P/P415R patient were compared. If the inactive P415R acid β-glucosidase had stability similar to that of L444P enzyme, the CRIM-specific activity would be ~50% decreased compared with that from L444P homozygotes or at least 25–30-fold less than the normal. However, compared with L444P homozygotes, the CRIM-specific activity was only slightly decreased in extracts from fibroblasts with the L444P/P415R genotype. This result indicates that in human fibroblasts the inactive P415R acid β-glucosidase is more unstable than that expressed from the L444P allele.

In comparison, the results obtained with the N370S and R120Q alleles identified in Gaucher disease type 1 (nonneuronopathic) patients (Grace et al. 1990) demonstrated that the N370S acid β-glucosidase was stable and had perturbed active-site functions in *Sf9* cells and fibroblasts from affected patients, whereas that from the R120Q allele was stable but completely inactive in the same cells. The stability of the R120Q acid β-glucosidase in fibroblasts was evident from the fact that CRIM-specific activity in fibroblasts from the patient with the N370S/R120Q genotype was nearly 50% decreased, compared with that in fibroblasts from N370S homozygous patients (table 1; Grace et al. 1990).

Discussion

Expression of normal or mutant proteins in natural or heterologous systems presents numerous difficulties for interpretation (for review, see Schimmel 1990). However, to determine (*a*) the effects that a variety of mutations (natural or created) have on enzyme function and (*b*) their relationship to causality of diseases, systems to express isolated alleles are required. This is particularly relevant to Gaucher disease, since numerous mutations of the acid β-glucosidase locus have been described and since many affected patients are heteroallelic at this locus (Grabowski et al. 1990). Furthermore, cells from all Gaucher disease patients except one (Grabowski et al. 1985) have residual enzyme activity and protein, and all mammalian tissues and cell lines have substantial acid β-glucosidase activity. Indeed, we chose the baculovirus expression system because of the absence of endogenous glucosylceramide-cleaving activity. Consequently, mutant enzymes with extremely low activity can be detected in the absence of any endogenous activity and without potentially interfering interactions with endogenous homologous protein or peptide sequences (Schimmel 1990). Thus, some previous studies concluded that no enzyme activity was expressed from certain mutant cDNAs in COS (Tsuji et al. 1988) or NIH 3T3 (Hong et al. 1990) cells which have high endogenous levels of acid β-glucosidase and in which the endogenous enzyme had to be eliminated using purification techniques which could have selected against or altered the mutant enzymes' properties (Hong et al. 1990; Ohashi et al. 1991).

The present and previous investigations also provide insight into the pathobiology of Gaucher disease. To date, all Gaucher disease patients found to have at least one allele encoding N370S (Tsuji et al. 1988; Theophilus et al. 1989*b*; Zimran et al. 1989; Firon et al. 1990) have been nonneuronopathic (type 1). Consequently, this mutant acid β-glucosidase must provide sufficient residual activity to maintain the nonneuronopathic phenotype even in the presence of a totally inactive allele, i.e., R120Q (Grace et al. 1990). Furthermore, homozygosity for N370S alleles has been associated with milder type 1 phenotypes (Theophilus et al. 1989*b*; Zimran et al. 1989). In comparison, when clinically well-defined populations have been used, homozygosity for the L444P allele has been associated either with neuronopathic variants (type 2 and type 3) (Tsuji et al. 1987; Dahl et al. 1990; Latham et al. 1990) or with very severe, lethal disease in childhood (Theophilus et al. 1989*b*). However, the apparently homozygous Gaucher disease type 2 patients actually were heteroallelic for a L444P allele and another allele, a complex allele (complex A), which contained multiple missense mutations, including

L444P, in exon 10 (Latham et al. 1990): that is, the genotypes of the type 2 patients and type 3 patients were actually different. The findings that multiple authentic missense mutations occur on single acid β-glucosidase alleles (Hong et al. 1990; Latham et al. 1990, 1991; Zimran et al. 1990*b*), that substantial variation of the type 3 phenotype exists in the Swedish population (L444P homozygotes), and that there is a potential association of apparent L444P homozygosity with type 1 disease (Masuno et al. 1990) indicate the complex nature of the molecular basis of phenotypic variation in Gaucher disease. However, the finding that type 2 disease has been associated with a severely compromised allele, L444P, in conjunction with functionally null alleles (P415R and complex A; Grabowski et al. 1990; Hong et al. 1990), indicates that the effective level of residual activity is likely to be a major, but not necessarily exclusive, determinant of the severity of the Gaucher disease phenotype.

Implicit in this hypothesis is the assumption that various thresholds of residual acid β-glucosidase activity levels exist in humans, as has been proposed in a more general manner by Conzelmann and Sandhoff (1984). One threshold prevents the development of symptomatic accumulation of acid β-glucosidase's major substrate, glucosylceramide. Obviously, this level is found both in completely normal individuals and in carriers for Gaucher disease. However, recent molecular studies to determine the carrier frequency of the N370S allele in an unselected Ashkenazi Jewish population (Zimran et al. 1990*a*) demonstrated that ~1/11 individuals in this population are heterozygous for this allele. In addition, homozygosity for the N370S was found in two asymptomatic enzyme-deficient people (Zimran et al. 1990*a*), and family studies of Gaucher disease patients also have led to the serendipitous discovery of enzyme-deficient patients in the sixth to ninth decades of life. Several of these asymptomatic enzyme-deficient patients are N370S homozygotes (G. A. Grabowski, unpublished observation). Thus, although the homozygous presence of N370S clearly is causal to the enzyme deficiency (Grace et al. 1990), apparently it is a permissive factor with regard to the development of symptomatic Gaucher disease. The conclusion that other factors or genetic loci may be required for the development of the Gaucher disease type 1 phenotype is supported by (1) the fact that patients homozygous for the N370S allele can have varying severity of phenotype, ranging from asymptomatic to mild to severe (Theophilus et al. 1989*b;* Zimran et al. 1989) and (2) the presence of "Gaucher-like" cells and glucosylceramide accumulation in individuals without Gaucher disease and whose substrate load to acid β-glucosidase is massively increased by myelogenous leukemia (Leibovitz-Ben-Gershon et al. 1982).

Another threshold of acid β-glucosidase activity is that which leads to the development of the severe and/or neuronopathic Gaucher disease phenotypes. This threshold may be bounded by both that found in type 1 patients with the N370S/"inactive allele" genotype and that found in the type 3 patients with homozygosity for L444P. The relative ethnic homogeneity of the Norrbottnian population of type 3 Gaucher disease patients provides a genetic background to assess the type 3 phenotype variation due to L444P homozygosity. Characterization of this population of patients indicated that all develop mental retardation, have severe visceral and/or body disease, and have shortened life spans (Svennerholm et al. 1982). The severity and age at onset vary significantly among Norrbottnian type 3 patients (Dreborg et al. 1980). This may explain reports in the literature that homozygosity for L444P has been found associated with very severely affected patients who were apparently free of neuronopathic manifestations (Theophilus et al. 1989*b*). It is possible that, had these young patients survived longer, neurological manifestations could have developed. However, greater insight into the molecular determinants of this threshold should be provided by thorough characterization of the mutant acid β-glucosidase genes from (*a*) other type 3 variants, in the juvenile-age group, with horizontal gaze palsy and myoclonic seizures (Winkelman et al. 1983; Latham et al 1990 [patient MSM 74]; Yu et al. 1990; Patterson et al. 1991) and (*b*) either adult patients who develop the onset of Gaucher disease–related mental deterioration late in life or apparently nonneuronopathic teenage patients (Masuno et al. 1990).

The distinctions between type 2 and type 3 are much more clear than those for type 1 and type 3 and may be absolute, since type 2 disease has the onset of neuronopathic involvement in the first year of life and since death occurs by ~2 years of age. Unlike Gaucher disease type 3, type 2 disease is rare and very stereotyped. In this regard the distinction between these two forms of Gaucher disease is similar to that between the infantile and later-onset forms of Tay-Sachs disease, Krabbe disease, and metachromatic leukodystrophy. For example, in Tay-Sachs disease, the infantile form has no residual β-hexosaminidase A activity (Meyerowitz and Proia 1988), whereas the mutation in the

later-onset form of G_{M2}-gangliosidosis results in low (2%–5% of normal) levels of this enzymatic activity (d'Azzo et al. 1984). Continued delineation of the genotype/phenotype correlations in Gaucher disease will provide for more accurate pre- and postnatal diagnosis and will increase accuracy in predicting the course of this disease. Both the advent of effective enzyme-replacement therapy (Barton et al. 1990) and the prospect for other molecular therapies of Gaucher disease necessitate the continued refinement of our understanding of the molecular basis of the severity of Gaucher disease, the most prevalent lysosomal storage disease.

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