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Mutations in the Arylsulfatase A Pseudodeficiency Allele Causing Metachromatic Leukodystrophy

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Summary

We identified a patient suffering from late infantile metachromatic leukodystrophy who genetically seemed to be homozygous for the mutations signifying the arylsulfatase A pseudodeficiency allele. Homozygosity for the pseudodeficiency allele is associated with low arylsulfatase A activity but does not cause a disease. Analysis of the arylsulfatase A gene in this patient revealed a C→T transition in exon 2, causing a Ser 96→Phe substitution in addition to the sequence alterations causing arylsulfatase A pseudodeficiency. Although this mutation was found only in 1 of 78 metachromatic leukodystrophy patients tested, five more patients were identified who seemed hetero- or homozygous for the pseudodeficiency allele. The existence of nonfunctional arylsulfatase A alleles derived from the pseudodeficiency allele calls for caution when the diagnosis of arylsulfatase A pseudodeficiency is based solely on the identification of the mutations characterizing the pseudodeficiency allele.

Introduction

Metachromatic leukodystrophy (MLD) is a lysosomal storage disease caused by the deficiency of arylsulfatase A (ARSA). This enzyme initiates the degradation of cerebroside sulfate, a polar membrane lipid which is mainly found in the myelin sheaths of the nervous system. Affected patients suffer almost exclusively from neurologic symptoms such as spastic tetraparesis, ataxia, optic atrophy, and dementia (for review, see Kolodny 1989). On the basis of the age at onset, three different clinical forms can be distinguished: late infantile, juvenile, and adult. The clinical heterogeneity can be explained by different levels of residual enzyme activity: complete loss of activity causes the most severe, late-infantile form, whereas low residual activity mitigates the course of the disease to the milder, although still lethal, late-onset forms (Polten et al. 1991). ARSA alleles causing MLD are rare (frequency 0.5%), predicting a 1:40,000 incidence of MLD in newborns (Gustavson and Hagberg 1971). However, there is a very frequent ARSA allele (frequency 7.3%–15%) which is associated with a substantial ARSA deficiency (Herz and Bach 1984; Hohenschutz et al 1989). Individuals homozygous for this allele have low residual ARSA activity but are clinically healthy. This condition has therefore been called ARSA pseudodeficiency (ARSA-PD). The low ARSA activity is obviously sufficient to sustain a normal cerebroside sulfate catabolism.

Because homozygous ARSA-PD is frequent (1%–2% of the population), it is not uncommon that patients with ARSA-PD and neurologic symptoms of unknown origin are misdiagnosed as having MLD (Kappler et al. 1991). MLD and ARSA-PD cannot be distinguished on the basis of enzyme activity determinations using artificial substrates. More sophisticated assays measuring in vivo the degradation of radioactively labeled cerebroside sulfate in cultured fibroblasts allow the distinction of MLD and ARSA-PD (Kihara et al. 1980). Alternatively, the ARSA-PD allele can be detected directly, on the basis of knowledge of the underlying sequence alterations (Gieselmann 1991).

The ARSA-PD allele is characterized by two mutations; one mutation leads to the loss of a N-glycosyla-

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tion site and explains the smaller size of ARSA polypeptides in pseudodeficient individuals, and the second mutation causes the loss of the first polyadenylation signal downstream of the stop codon. Whereas the first mutation is a polymorphism that does not affect the activity or stability of the enzyme, the latter causes the loss of about 90% of ARSA mRNA, which explains the loss of 90% of ARSA cross-reacting material and enzyme activity (Gieselmann et al. 1989). So far, both mutations have always been found together, and no alleles carrying only one of the two mutations have been identified.

Mutations causing MLD should occur in the ARSA-PD allele at the same frequency as they do in the normal allele. Given the high frequency of the ARSA-PD allele, we reasoned that we should be able to detect ARSA-PD alleles carrying MLD mutations if we screened an appropriate number of MLD patients for the presence of the ARSA-PD allele by using allele-specific oligonucleotide hybridization.

Material and Methods

Sequence Analysis of the ARSA Gene

The ARSA gene was amplified in two overlapping fragments (C and D in fig. 1, *top*) from genomic DNA isolated from cultured human fibroblasts. The conditions for the PCR have been described, as has the sequence of the oligonucleotides used as primers (Gieselmann et al. 1989; Polten et al. 1991). The amplified fragments were digested with restriction enzymes according to the restriction site present in the PCR primers and were subcloned into M13mp18 (fragments C and D) and mp19 (fragment C) by standard techniques (Sambrook et al. 1989). Sequencing was carried out by the dideoxy chain-termination method (Tabor 1987). All of the expressed gene was sequenced, except for exon 4, 14 bp at the 3′ end of exon 2, and 13 bp at the end of exon 5.

Detection of the ARSA Pseudodeficiency Allele

The ARSA-PD allele was detected by allele-specific amplification. This assay has been described in detail elsewhere (Gieselmann 1991). A brief description is given in the Results section below.

Allele-specific Oligonucleotide Hybridization

Fragments A, C, or D (see fig. 1, *top*) were amplified as described (Polten et al. 1991), denatured, and blotted onto Hybond N filters. The DNA was UV cross-linked to the filters and hybridized to [^{32}P]labeled allele-specific oligonucleotides. Conditions for radioactive labeling and hybridization have been described (Gieselmann et al. 1989). The sequences of the allele-specific oligonucleotides used to detect the ARSA-PD allele have been published (Gieselmann et al. 1989), as have the washing temperatures used to give allele-specific signals. The sequence of the oligonucleotides used in this study to detect the mutation described were as follows: normal allele, 5′ CCC CCC GGG AGC TGG GCA 3′ (63°C); mutant allele, 5′ CCC CCC GGA AGC TGG GCA 3′ (63°C). The filters were washed for 10 min at the temperatures indicated, to give allele-specific signals.

In Vitro Mutagenesis and Transfection of BHK Cells

The mutation was introduced into the ARSA-PD cDNA (Gieselmann et al. 1989) as described (Nakamaye and Eckstein 1986). Cell culture conditions, transfection, and determination of ARSA activity have been described elsewhere (Stein et al. 1989).

Results

Frequency of the Two Mutations Signifying the ARSA-PD Allele in DNA from MLD Patients

DNA was prepared from cultured fibroblasts of 78 patients suffering from MLD. Fragment C (see fig. 1, *top*) was amplified by PCR, denatured, and blotted onto nylon filters. These filters were hybridized with allele-specific [^{32}P] oligonucleotides. The oligonucleotides detect sequences which code for an asparagine or a serine at residue 350. The Asn 350 exchange to serine is one of the mutations characterizing the ARSA-PD allele (Gieselmann et al. 1989) (see fig. 1, *top*). Among the 78 patients, three were identified as homozygous and three as heterozygous for this mutation. The presence of the ARSA-PD allele in these six patients was verified by an allele-specific amplification assay which detects both mutations in the ARSA-PD allele (Gieselmann 1991) (data not shown). All six patients were referred to us as affected with the late-infantile form of MLD. The ARSA genes of one of the patients homozygous for the ARSA-PD allele were analyzed in detail, because her clinical symptoms had been documented in detail earlier (Tonnesen et al. 1983).

Analysis of the ARSA Gene

As shown in figure 1, the ARSA-genes of the patient were amplified in two overlapping fragments, C and

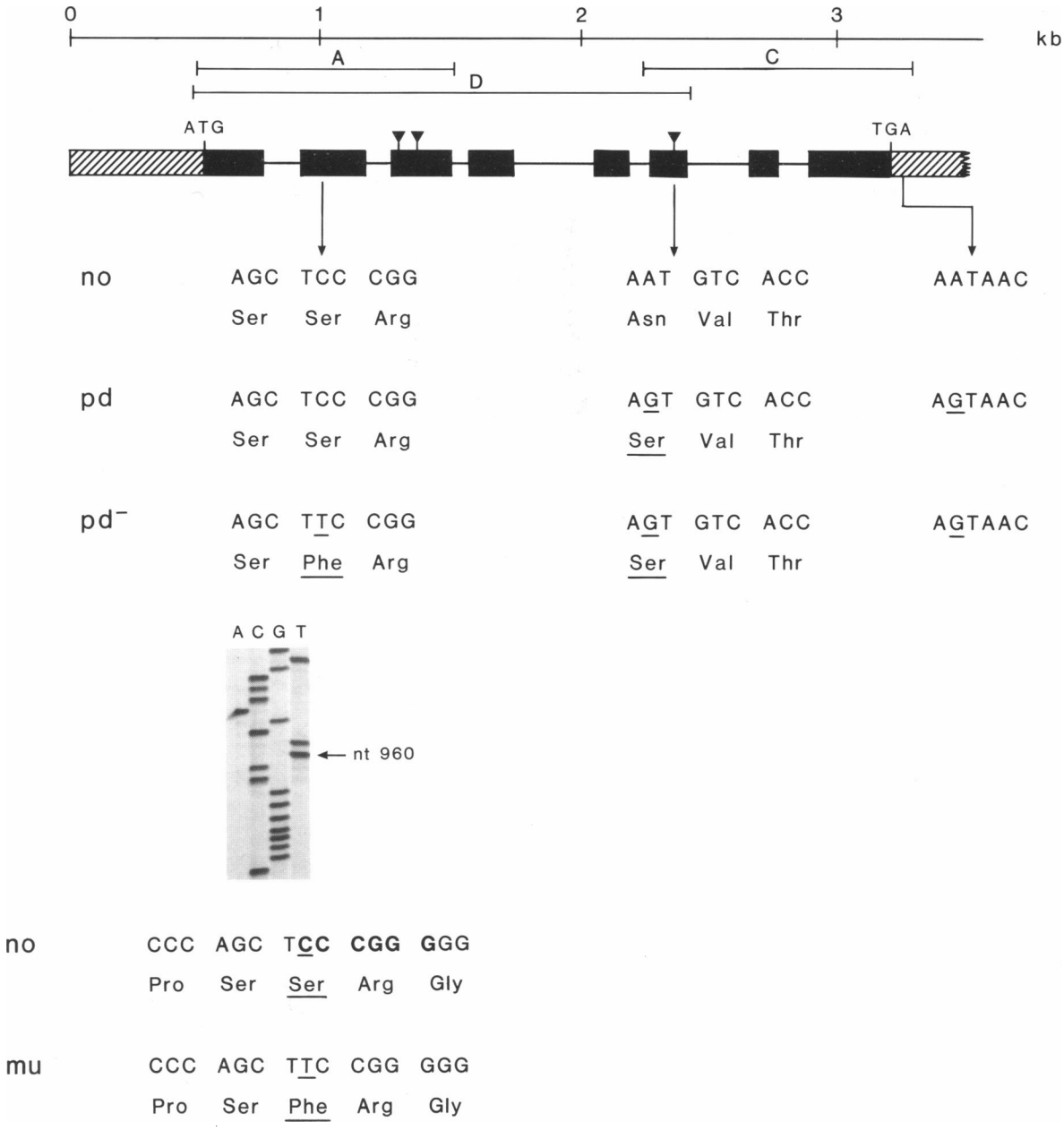

Figure 1 *Top,* Schematic presentation of sequence alterations in ARSA gene. At the top a scale in kilobases (kb) is shown. The structure of the gene is shown schematically: bars represent exons; lines represent introns; black parts represent coding regions; and hatched parts represent 5′ or 3′ untranslated regions. ATG and TGA = initiation and termination codons, respectively. Triangles indicate potential N-glycosylation sites. A, C, and D indicate the fragments that were amplified for allele-specific oligonucleotide hybridization or sequencing. no = nucleotide and amino acid sequence of the normal ARSA allele in the regions which are of interest in this study. pd = sequence alterations occurring in ARSA-PD allele. A A→G transition causes the substitution of Asn 350 by Ser and leads to the loss of a potential N-glycosylation site. Another A→G transition at nucleotide 2725 in the genomic sequence causes the loss of the polyadenylation signal AATAAC. Alterations are underlined. pd⁻ = sequence alterations found in nonfunctional ARSA allele described here. *Bottom,* Sequencing gel showing mutation in exon 2 at nt 960. The normal sequence has been published elsewhere (Kreysing et al. 1990). The normal (no) as well as the mutated (mu) sequence is shown below. The *Sma*I restriction site destroyed by the mutation is shown in boldface letters in the normal sequence.

D (Polten et al. 1991), which were subsequently subcloned into M13mp18/19 and sequenced. Besides the two sequence alterations characteristic of the ARSA-PD allele, one other mutation was found. In exon 2 a C→T transition at nucleotide 960 changes Ser 96 to Phe (see fig. 1). This mutation leads to the loss of a *Sma*I restriction site. The patient was homozygous for the loss of the *Sma*I site (see fig. 2).

Inheritance of the ARSA-PD Allele and the SmaI *Mutation in the Family of the Patient*

DNA was isolated from cultured fibroblasts of the patient, her mother, her father, and her sister. The DNA was analyzed for the presence of the ARSA-PD allele by using an allele-specific amplification assay (Gieselmann 1991). The allele-specific oligonucleotides used as primers in this assay are designed such that the PCR amplification yields a 995-bp fragment only when the sequence of the primers matches the sequence of the genomic DNA. Thus for every individual to be tested two PCRs have to be performed: one for the detection of the ARSA-PD allele and one for the detection of the non-ARSA-PD alleles. Another pair of primers present in the same reaction coamplifies a control fragment (774 bp) which is amplified independently of the mutations. Figure 3 shows the results of the DNA analysis of the patient and her family members when this assay is used. The healthy father is homozygous for the ARSA-PD allele. His daughter affected with MLD appears to have the same genotype, while the mother is heterozygous for the ARSA-PD allele.

To reveal the inheritance of the *Sma*I mutation caused by the C→T transversion in exon 2, we amplified fragment A (see fig. 1, *top*) from the DNA of the patient, his family members, and a control individual. Digestion of the fragment with the restriction enzyme *Sma*I shows that the patient is homozygous for the mutation deleting the *Sma*I restriction site at nucleotide 960, whereas the father and the mother are heterozygous (see fig. 2). The pedigree deduced from these data is shown in figure 4. The mother and sister of the patient are heterozygous for a normal ARSA allele (no) and a MLD allele which arose from the introduction of the Ser 96→Phe substitution into the ARSA-PD allele

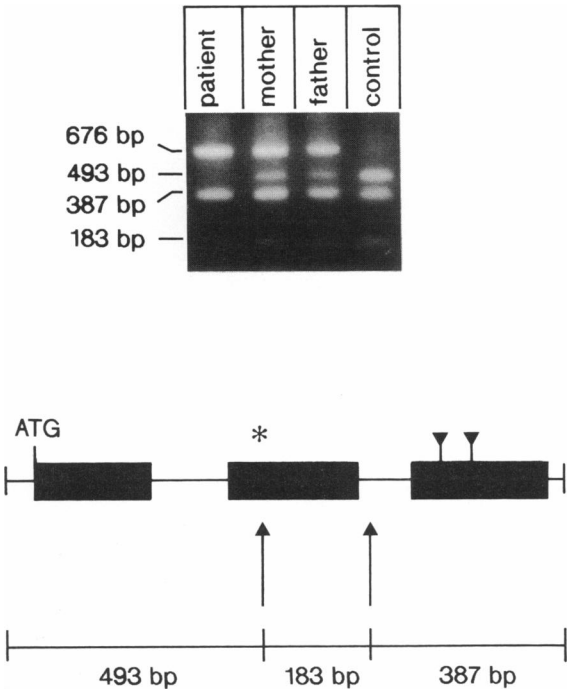

Figure 2 Inheritance of *Sma*I mutation in patient's family. Fragment A (see fig. 1, *top*) was amplified from genomic DNA isolated from the individual indicated at the top and digested with *Sma*I, and the digest was resolved on a 1.5% agarose gel. The sizes of the fragments are given in basepairs (bp). A schematic presentation of the *Sma*I site distribution in fragment A is shown below the gels. ATG indicates the initiation codon; exons are shown as blackened bars; and potential glycosylation sites are shown as black triangles. Arrows indicate the location of *Sma*I restriction sites; asterisks indicate the site that is lost in the PD^- allele. Sizes of fragments generated by a restriction digest of a normal allele are shown below.

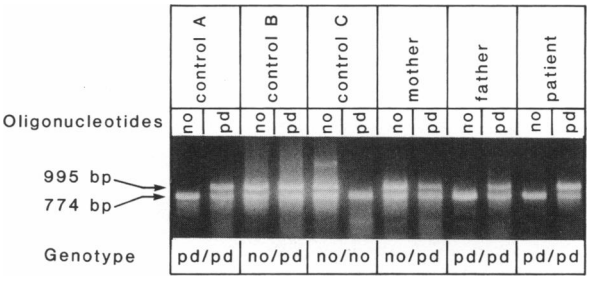

Figure 3 Inheritance of ARSA-PD allele in patient's family. The mutations characterizing the ARSA-PD allele were detected by allele-specific PCR (Gieselmann 1991). Top line indicates the individuals from whom the DNA was isolated. Controls A–C are individuals with a known genotype. For each individual, two PCRs have to be performed, one with oligonucleotides specific for the normal allele (no) and one with oligonucleotides specific for the ARSA-PD allele (pd). The 995-bp fragment is allele specific and is amplified only when the corresponding allele is present in the DNA. The 774-bp fragment is amplified independently and serves as internal control fragment for the amplification reaction. The apparent genotypes of the individuals are shown below (pd = pseudodeficiency allele; no = normal allele).

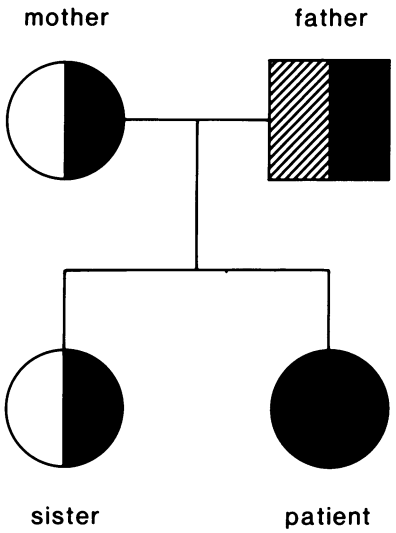

Figure 4 Pedigree of patient's family. The ARSA genotype of the patient, her mother, and her father, from the experiments in figs. 2 and 3, are shown. The genotype of the sister has been determined in a separate experiment (not shown in figs. 2 and 3). Unblackened parts indicate the normal ARSA allele; the hatched part indicates the pseudodeficiency allele; and the blackened parts indicate the nonfunctional ARSA PD$^-$ allele.

(designated as PD$^-$ in fig. 4). The patient is homozygous for the ARSA-PD$^-$ allele, whereas the father is a compound heterozygote for the ARSA-PD$^-$ allele and the normal ARSA-PD allele.

Frequency of the Allele among MLD Patients

We have amplified fragment A (see fig. 1, *top*) from 78 MLD patients with different clinical phenotypes and searched for the presence of the Ser 96→Phe substitution by using allele-specific oligonucleotide hybridization. No other MLD patient carrying the Ser 96→Phe substitution was found.

Effect That the Ser 96→Phe Substitution Has on ARSA Enzyme Activity

An expression vector (pBEH; Artelt et al. 1988) carrying the ARSA-PD cDNA (Gieselmann et al. 1989) was transiently transfected into BHK cells. About 3.5 times more ARSA activity was found in the cell extracts compared with cells transfected with the vector without insert.

The C→T transversion found in exon 2 was introduced into the ARSA-PD cDNA, and the mutated cDNA was cloned into the expression vector pBEH. Cells transfected with this plasmid had enzyme activities identical to those of control cells, while transfection with a plasmid containing an ARSA cDNA with a mutation (Pro 426 to Leu) known to be associated with low ARSA residual activity (Polten et al. 1991) resulted consistently in a slight increase of ARSA activity (1.1 times the control value). This suggests that the Ser→Phe substitution leads to a complete loss of enzyme activity (see fig. 5).

Discussion

Two genetically distinct forms of ARSA deficiency are known: one is rare and associated with the fatal disease MLD, and the other is frequent and benign. The latter has been designated as ARSA-PD. The mutations causing ARSA-PD can be considered as nonsilent polymorphisms which cause reductions in synthesis and size of ARSA polypeptides, without metabolic or clinical consequences. For diagnostic purposes it is

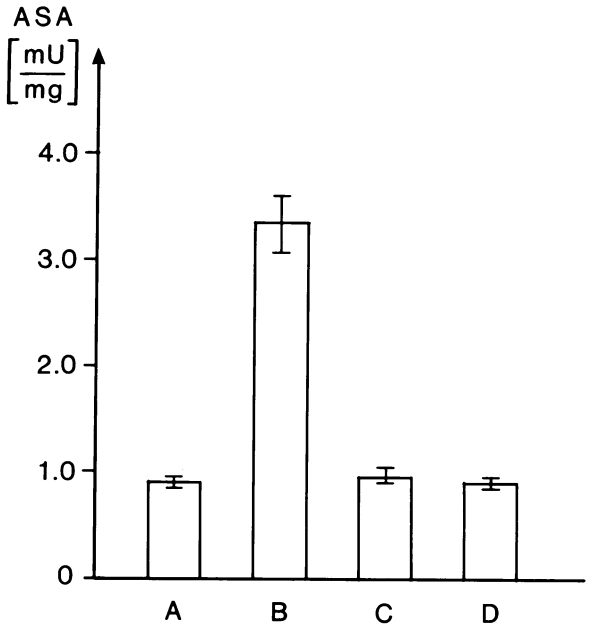

Figure 5 Effects that Ser 96→Phe substitution has on ARSA activity. Normal and mutant (generated by site-directed mutagenesis) ARSA-cDNAs were subcloned into the expression vector pBEH, and BHK cells were transfected transiently. Bars show the ARSA activity (measured in cell extracts) 48 h after transfection. The range of three independent transfections is shown. The BHK cells were transfected with the following pBEH vectors: A, vector without insert; B, vector with ARSA-PD cDNA insert; C, vector with an ARSA cDNA insert carrying a mutation (Pro 426→Leu 426) associated with late-onset MLD (Polten et al. 1991); and D, vector with the ARSA-PD cDNA insert carrying the Ser 96→Phe substitution described here.

important to distinguish MLD and ARSA-PD alleles. In families in which both types of alleles exist, a precise genetic counseling is only possible when ARSA-PD allele carriers can be differentiated from MLD carriers.

Both alleles can be differentiated by allele-specific oligonucleotide hybridization (Gieselmann et al. 1989) and allele-specific amplification (Gieselmann 1991). These assays detect the two mutations of the ARSA-PD allele; however, they cannot exclude the existence of additional mutations in the ARSA-PD allele. Here we show that mutations can occur in the PD allele, which render it nonfunctional. The mutations were found in patients with the severe, late-infantile form of MLD, and one of the mutations was characterized as a Ser 96→Phe substitution. As ARSA-PD must be an old polymorphism, MLD mutations in this allele must have arisen later than the PD mutations.

The existence of MLD mutations in the ARSA-PD allele calls for caution in the diagnosis of ARSA-PD by tests detecting the mutations of the ARSA-PD allele. As will be discussed below, the frequency of MLD mutations in the normal and the PD allele seem to be similar, so that 1:200 PD alleles will carry a MLD mutation. Therefore the chances that an individual that appears to be homozygous for the PD allele will suffer from MLD are 1:40,000. When low (about 0%–15% of normal) ARSA activity is associated with heterozygosity for the PD allele, it can be assumed that the non-PD allele is an MLD allele. In 1:200 cases the PD allele will carry additional mutations rendering it nonfunctional, and what appears to be a pseudodeficient individual by genotype assays will be affected with MLD. In all cases where a nonfunctional ARSA-PD (PD^-) allele is suspected, a cerebroside loading test should be performed (Kihara et al. 1980). This test, although cumbersome and requiring a noncommercially available radioactive substrate, clearly differentiates between nonfunctional and functional ARSA alleles, irrespective of their combination with the ARSA-PD mutations.

Mutations causing MLD occur in PD alleles at a frequency similar to that at which they occur in normal alleles. We identified nine PD alleles among 78 MLD patients, giving an allele frequency of about 6%. This is similar to the 7%–15% frequency of the PD allele in the general population (Herz and Bach 1984; Hohenschutz et al. 1989). Our data, however, may overestimate the frequency of MLD mutations in the PD allele, because for three of the six patients the available data are insufficient to exclude the possibility that their neurologic symptoms are unrelated to MLD and that they are homozygous for the normal ARSA-PD allele.

We have recently described four different ARSA genotypes which are associated with different levels of low (from 0% to about 10%) ARSA residual activity. This range covers the whole clinical variability of MLD, from the severe, late-infantile type of the disease to the apparently healthy individual (Polten et al. 1991). The latter type was represented by compound heterozygotes of the PD and an MLD allele. The residual ARSA activity encoded by a single PD allele is therefore sufficient to support a normal life.

It can be anticipated that mutations in the normal ARSA allele that lower the ARSA activity but are without clinical consequences can cause MLD if they occur in the ARSA-PD allele. In the latter the decrease in synthesis rate caused by the loss of the polyadenylation signal in the ARSA-PD allele may lower the residual activity below the critical threshold.

By analogy, mutations which cause the milder, late-onset forms of MLD when they occur in the normal ARSA allele may lead to the severe, late-infantile form when they occur in the PD allele. Mutations whose effects are aggravated by the reduced synthesis rate of the PD allele are likely to be found in late-onset MLD patients who genetically appear to be carriers of at least one PD allele.

Acknowledgments

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