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BIOCHEMICAL AND GENETIC STUDIES ON G_{M2}-GANGLIOSIDOSIS

Tay-Sachs disease and variants

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A meus Pais

e Carlos

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❑ Ribeiro MG, Pinto RA, Dos Santos MR, Maia M, Sá Miranda MC (1991) Biochemical characterization of ß-hexosaminidase in different biological specimens from eleven patients with G_{M2}-gangliosidosis B1 variant. *J Inher Metab Dis* 14:715-720.

❑ Ribeiro MG, Pinto R, Oliveira P, Sá Miranda MC (1993) Identification of G_{M2}-gangliosidosis B1 variant carriers. *J Inher Metab Dis* 16:1003-1011.

❑ Ribeiro MG, Pinto R, Sá Miranda MC, Suzuki K (1995) Tay-Sachs disease: intron 7 splice junction mutation in two Portuguese patients. *Biochim Biophys Acta* 1270:44-51.

❑ Ribeiro MG, Sonim T, Pinto RA, Fontes A, Ribeiro H, Pinto E, Palmeira MM, Sá Miranda MC. Clinical, enzymatic and molecular characterization of a Portuguese family with a chronic forms of G_{M2}-gangliosidosis B1 variant. *J Clin Genet* In press.

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No cumprimento do Decreto-Lei 388/70, esclarece-se serem da nossa responsabilidade a execução das experiências que estiveram na base dos resultados apresentados neste trabalho (excepto quando referido em contrário) assim como a sua interpretação e discussão.

Sumário

As gangliosidoses G_{M2} são um grupo de doenças hereditárias de transmissão autossómica recessiva caracterizadas pela acumulação intralisossomal do gangliósido G_{M2} e derivados, nas células neuronais. Estas doenças metabólicas são causadas por defeitos nos diferentes genes que codificam as proteínas envolvidas na degradação *in vivo* do gangliósido G_{M2}, as subunidades α e β da hexosaminidase (EC 3.2.1.50), que constituiem a hexosaminidase A, e a proteína activadora G_{M2}. A deficiente actividade da hexosaminidase A (heterodímero $\alpha\beta$) causada por mutações no gene da subunidade α origina a variante B (a forma infantil clássica é designada por Doença de Tay-Sachs) ou a variante B1. A variante B1 é caracterizada pela presença de hexosaminidase A com uma especificidade enzimática alterada em relação aos substractos, devido a uma deficiência no local catalítico da subunidade α; o local catalítico da subunidade β não está no entanto alterado. Os estudos apresentados nesta tese foram efectuados com o objectivo de contribuir para a identificação das causas genéticas e dos mecanismos moleculares que originam a deficiência da subunidade α. Nesse sentido, foram efectuados estudos bioquímicos e genéticos em diferentes amostras biológicas de 23 doentes Portugueses não relacionados com a doença de Tay-Sachs e variantes.

A caracterização da actividade enzimática da hexosaminidase utilizando substractos sintéticos mostrou que 78% (18/23) dos doentes apresentavam a variante B1. Nestes doentes, a actividade da hexosaminidase A contra o substracto sulfatado (hidrolizado pelo local catalítico da subunidade α) era deficiente, enquanto que a actividade enzimática contra o substracto neutro (hidrolizado pelo local catalítico da subunidade β) era inferior ao controlo.

A caracterização do genótipo destes doentes revelou uma elevada heterogeneidade, tendo sido identificadas nove mutações diferentes em 46 alelos. Sete mutações eram causais da variante B, duas mutações de "splicing", G->C +1IVS-7 e G->A +1IVS-9, uma inserção, TATC1278, uma delecção, C1334, e as mutações pontuais G1363A (Gly455Arg), C1495T (Arg499Cys) e G1496A (Arg499His). Duas mutações associadas à variante B1 foram adicionalmente identificadas: G533A (Arg178His) e G755A (Arg252His). Quatro destas mutações não tinham ainda sido descritas (G755A, +1IVS-7, ΔC1334 e G1363A). A transição G533A apresenta uma frequência elevada, tendo sido identificada em 70% (32/46) dos alelos. A caracterização enzimática, genética e clínica permitiu estabelecer uma correlação genótipo-fenótipo, nomeadamente quanto à mutação Arg252His que foi o primeiro alelo B1 descrito a ser associado a uma forma crónica da gangliosidose G_{M2}.

As mutações identificadas no estado homozigótico, G->C +1IVS-7 e G533A, foram particularmente estudadas. No caso da mutação +1IVS-7 foi observado que o processamento do RNA era ineficiente e anormal. Estudos de "pulse-chase" em fibroblastos de doentes homozigóticos para a mutação G533A (Arg178His) mostraram que as cadeias α e β são processadas, originando formas moleculares semelhantes às observadas em células controlo. A quantidade de cadeias α maduras (forma lisosomal) estava no entanto diminuída em relação às células controlo. Os resultados obtidos sugerem que a substituição Arg178His poderá afectar o "assembly" das subunidades α e β da hexosaminidase A.

A variante B1, uma doença mundialmente rara e geográficamente dispersa, é particularmente prevalente na população Portuguesa. Com o objectivo de diminuir a incidência desta patologia, através da identificação de portadores e aconselhamento genético, e de determinar a frequência de portadores, foi desenvolvido um método enzimático altamente específico e sensível para a detecção de portadores do alelo Arg178His.

O trabalho apresentado nesta tese contribui para o conhecimento da epidemiologia genética da doença de Tay-Sachs e variantes da gangliosidose G_{M2} e poderá ser particularmente útil em estudos bioquímicos relativos à caracterização do domínio catalítico da subunidade α da hexosaminidase A.

Summary

G_{M2}-gangliosidoses comprise a group of autossomal recessively inherited disorders characterised by intralysosomal accumulation of ganglioside G_{M2} and related lipids, particularly in neuronal cells. These metabolic diseases result from defects in either of the three genes coding the proteins involved in the *in vivo* degradation of ganglioside G_{M2}: the α- and β-subunit of hexosaminidase (EC 3.2..1.50), which associate to form the β-hexosaminidase A, and the G_{M2} activator protein. The deficient activity of hexosaminidase A (αβ heterodimer) due to genetic defects in the α-subunit leads to B (the classical infantile is named Tay-Sachs disease) or B1 variant. The latter variant is characterised by the presence of a hexosaminidase A with an altered substrate specificity due to a catalytically defective α-subunit; however, the catalytic site located on the β-subunit is not affected. The goal of this thesis was to explore the genetic causes and molecular mechanisms leading to α-subunit deficiency. With this purpose, biological materials from 23 unrelated Portuguese patients with Tay-Sachs disease and variants were used to perform biochemical and genetic studies.

The enzymatic characterisation of hexosaminidase using synthetic substrates showed that 78% (18/23) of the patients were affected with the B1 variant. In these patients the activity of hexosaminidase A against the sulphated substrate (hydrolysed by the α-subunit catalytic site) was deficient, whereas that observed against the unsulphated one (hydrolysed by the β-subunit catalytic site) was lower than control.

Genotype analysis showed a wide genetic heterogeneity for mutations causing either B or B1 variant. Nine different mutations were identified in 46 alleles. Seven mutations were causal to B variant: two splicing mutations, G->C +1IVS-7 and G->A +1IVS-9, one insertion TATC1278, one deletion C1334, and point mutations, G1363A (Gly455Arg), C1495T (Arg499Cys) and G1496A (Arg499His). Two point mutations were associated with B1 variant: G533A (Arg178His) and G755A (Arg252His). Among these nine mutations four were novel (+1IVS-7, G755A, ΔC1334 and G1363A). The most frequent mutation in the α-chain gene was the transition G533A, accounting for 70% (32/46) of total alleles. Enzymatic, genetic and clinical data combined with that reported in the literature allowed the establishment of genotype-phenotype correlation, as in the case of Arg252His that was the first B1 alelle to be associated with a chronic form of G_{M2}-gangliosidosis.

The mutations identified in homozygous state, G->C +1IVS-7 and G533A, were further studied. The splicing mutation +1IVS-7 was found to cause an inefficient and abnormal processing of the transcript. Pulse-chase labelling studies in fibroblasts from patients homozygous for G533A (Arg178His) indicated that α and β chains of mutant hexosaminidase A were processed to molecular forms indistinguishable from those observed in control cells. However, the amount of mature α-chains (the lysosomal form) was decreased in comparison to control cells. The overall data suggest that the substitution Arg178His might cause a defective assembly of α and β of hexosaminidase.

The B1 variant, a world widely rare disease, is prevalent among Portuguese population. With the purpose to reduce the incidence of the disease by carrier identification and genetic counselling, and determine the frequency of B1 variant carriers in the Portuguese population, an enzymatic assay with high specificity and sensitivity for the detection of Arg178His carriers was developed.

Overall the work presented in this thesis contributes to the knowledge of genetic epidemiology of Tay-Sachs disease and variants of G_{M2}-gangliosidosis as well as it will help in future biochemical studies concerned with the characterisation of the catalytic domain of the α-subunit of hexosaminidase A.

Sommaire

Les gangliosidoses G_{M2} sont des maladies héréditaires de transmission autossomique et recessive, caracterisées par l'accumulation intralysosomal du ganglioside G_{M2} et de ses derivés dans les cellules neuronales. Ces maladies métaboliques sont dues à des alterations dans chacun des trois genes qui codifient les proteines responsables par la dégradation "in vivo" du ganglioside G_{M2}: les sous-unitées α et β de l´hexosaminidase (EC 3.2.1.50) dont l'association origine l´hexosaminidase A et la proteine activatrice du ganglioside G_{M2}. Le déficit dans l'activitée de l´hexosaminidase A (heterodimère $\alpha\beta$) due à des mutations dans le gène de la sous-unitée α est à l'origine soit de la variante B (dont la forme classique infantile est designée par maladie de Tay-Sachs) soit de la variante B1. La variante B1 se caractérise par la presence d'une hexosaminidase A presentant une differente specificitée enzimatique contre les substrats, dû à la presence d'une sous-unitée α catalytiquement déficiente ; le site catalytique de la sous-unité β n'étant pas modifiée. Le travail presenté dans cette thèse à été develloppé dans le sense de contribuer pour l'identification des mécanismes génétiques et moleculaires qui sont à l'origine du déficit de la sous-unitée α. Dans ce but des études biochimiques et génétiques ont été effectués dans des differents materiaux biologiques appartenant à 23 malades d'origine portugaise non-apparentés atteints de la maladie de Tay-Sachs ou variantes.

La caractérisation de l'activité enzimatique de l'hexosaminidase contre des substrats synthetiques, a montré que 78% (18/23) des malades presentaint la variante B1. Dans ces malades l'activité de hexosaminidase A contre le substrat sulfaté (hydrolisé par le site catalytique present dans la sous-unité α) était déficiente; par contre, l'activité enzimatique contre le substrat neutre (hydrolisé par le site catalytique present dans la sous-unité β) presentait une activitée inférieure à celle du control.

L'analyse du génotype de ces malades a montré une importante hétérogeneité. Neuf mutations differentes ont été identifiées dans les 46 alleles. Sept de ces mutations étaient causales pour la variante B: deux mutations d'épissage G->C +1IVS-7, et G->A +1IVS-9, une insertion TATC1278, une delection ΔC1334, et les mutations ponctuelles G1363A (Gly455Arg), C1495T (Arg499Cys) et G1496A (Arj499His). Deux autres mutations ponctuelles sont associées à la variante B1: G533A (Arg178His) et G755A (Arg252His). Quatre de ces neuf mutations n'étaient pas encore décrites dans la littérature: G755A, +1IVS-7, ΔC1334 et G1363A. La mutation G533A est la plus fréquente des mutations, correspondant à 70% (32/46)

des alleles. La caractérisation enzimatique, génétique et clinique, a permit d'établir des correlations génotype/phénotype, notamment en ce qui concerne la mutation Arg252His, le premier allele B1 décrite, associé à la forme chronique de la Gangliosidose G_{M2}.

En ce qui concerne les mutations identifiées à l'état homozygote, G->C +1IVS-7 et G533A, une étude plus approfondie a été éffectuée. Dans le cas de la mutation +1IVS-7 on a put observer que l'épissage de l'ARN était anormal et inefficace. Des études de "pulse-chase" dans des fibroblastes des malades homozygotes pour la mutation G533A (Arg178His) ont montré que les sous-unitées α et β de l'HexA étaient normalmment maturées originant des formes moleculaires similaires à celles presentes dans les cellules control. Malgré ça la quantité de chaines α correspondant à la forme mature (intralysosomal) était diminuée par rapport à celles presentes dans des cellules controle. Les résultats obtenues suggerent que la substituition Arg178His peut être à l'origine d'une déficiente assemblage des sous-unitées α et β.

La variante B1, une maladie considerée rare au niveau mondial et géographiquement disperse est particulièrement prévalente dans la population Portugaise. Dans le but de réduire l'incidence de cette maladie à travers de l'identification des porteurs et du conseil génétique, ci-bien que de déterminer la fréquence des heterozygotes, une méthode enzimatique hautement spécifique et sensible pour identifier les porteurs de la mutation Arg178His, a été mise au point.

Les resultats presentés dans cette thèse ont contribué pour une meilleure connaissance de l'épidemiologie génétique de la Maladie de Tay-Sachs et ses variantes, en plus ce travail peut aussi être utile dans des études biochimiques futurs, concernant la characterization du domaine catalytique de la sous-unitée α de l'hexosaminidase A.

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CHAPTER 1

INTRODUCTION

HISTORICAL REVIEW

The history of human genetic lysosomal storage disorders began in 1881 with the clinical description of what is now known as Tay-Sachs disease. Tay (1881) observed a cherry-red spot in the retina of a 1-year-old child with mental and physical retardation. Sachs (1887) noted the distended cytoplasm of neurons. He then concluded that the disease, that was named 'familial amaurotic idiocy', was heritable and prevalent in Jews (Sachs, 1896). In late 1930 a novel group of glycosphingolipids was detected in the brain of these patients (Klenk, 1939). These sialic-acid containing glycolipids were designated by gangliosides due to their high concentration in normal ganglion cells. The main neuronal strorage compound, the ganglioside G_{M2}, was identified by Svennerholm (1962) and the structure of related lipids then characterised (Ledden and Salsman, 1965; Makita and Yamanaka, 1963). The term *G_{M2}-gangliosidoses* was then introduced for disorders characterised by a primary accumulation of ganglioside G_{M2} due to a block in its catabolism (Suzuki and Chen, 1967). The accumulation of this ganglioside implicated a defect in the enzyme responsible for the removal of terminal β-linked N-acetylgalactosamine residue. In late 1960 the defective enzyme, the hexosaminidase A, was identified (Hultberg, 1969; OKada and O'Brien, 1969; Sandhoff, 1969), opening the way to biochemical diagnosis of the disease, and subsequently to carrier screening and prenatal diagnosis in at-risk pregnancies. One year earlier a combined deficiency of two hexosaminidase isoenzymes, the hexosaminidase A and B, was reported in a patient with additional storage of globoside in visceral organs (Sandhoff et al., 1968). Studies on the structure of isoenzymes and their substrate specificity were then carried out. In 1978, the deficiency of another protein, the G_{M2} activator, was reported in an atypical case of G_{M2}-gangliosidosis with storage of ganglioside G_{M2} but without deficiency in either hexosaminidase A or hexosaminidase B (Conzelmann and Sandhoff, 1978). The role of activator protein for *in vivo* degradation of ganglioside G_{M2} was then elucidated (Conzelmann and Sandhoff, 1979). Based on the hexosaminidase isoenzyme remaining in the tissues of affected patients, the G_{M2}-gangliosidoses were classified into three variants: variant B (intially named as Tay-Sachs disease), due to deficiency of hexosaminidase A; variant 0 (initially named as Sandhoff disease), due to deficiency of both hexosaminidase A and B; and variant AB, due to G_{M2} activator deficiency. The purification of isoenzymes in 1974-80 and the production of antibodies led to the study of the biosynthesis, assembly, intracellular transport and post-translational modification in the 1980.

The cDNA clones encoding both subunits were isolated and characterised in 1985, (Myerowitz et al., 1985; O'Dowd et al., 1985) allowing the identification of genetic defects underlying Tay-Sachs disease in Jewish population in 1988 (Arpaia et al., 1988; Myerowitz et al., 1988, Myerowitz and Costigan, 1988). Current research encompassing the fields of enzymology, cell biology, and molecular biology is linking genotypes with the clinical phenotype of patients with Tay-Sachs and related diseases with the purpose to go further into the pathophysiology of G_{M2}-gangliosidosis.

A general overview of the literature on the genes and proteins involved in degradation of ganglioside G_{M2} and their inherited deficiencies is presented below.

1.1. OVERVIEW OF THE LITERATURE

1.1.1. LYSOSOMES AND STORAGE DISEASES

❑ Function of lysosomes

Lysosomes are membrane-bound organelles that are part of the vaccuolar system of the cell. These organelles are responsible for the degradation of a variety of macromolecules derived from biosynthetic or endocytic pathways (rev. by Kornfeld and Mellman, 1989). Lysosomes were discovered in 1955 by de Duve (de Duve et al., 1955) due to the characteristic distribution of acid phosphatase activity during biochemical fractionation of cell extracts. Lysosomes contain over 40 enzymes, including proteases, glycosidases, lipases, nucleases, phosphatases and sulphatases, each responsible for hydrolyzing a well-defined chemical linkage, as well as several proteinaceous cofactors. These cofactors are proteins necessary to the function or stability of specific hydrolases and include activator proteins (O'Brien and Kishimoto, 1991) and protecting proteins (Galjjaard et al., 1984). Hydrolases and cofactors are glycoproteins with N-oligosaccharides attached to their polypeptide chain. The lysosomal enzymes have in common a high degree of specificity and an acid pH optimum between 3.5 and 5.0 (Barrett and Heath, 1977). The acid environment within lysosomal lumen is established by an ATP-dependent proton pump at the lysosomal membrane (rev. by Reeves, 1984). The lysosomal membrane is protected from a premature digestion by a glicocalix formed by the extended carbohydrate moiety of the LIMPs (lysosomal integral membrane proteins) and LAMPs (lysosomal associated membrane proteins) constituting the lysosomal membrane (Carlsson and Fukuda, 1990).

❑ Biosynthesis and targeting of lysosomal proteins

The early events in biosynthesis of lysosomal proteins are common to secretory and plasma membrane glycoproteins. They are synthesized on membrane-bound ribosomes and cotranslationally translocated into the lumen of the endoplasmic reticulum (ER). Concomitantly with the translocation to the rough endoplasmic reticulum (RER), the signal peptide is removed and N-glycosylation occurs through the dolicholphosphate pathway. During the transport through RER, the precursor is folded, the oligosaccharide chain ($Glc_3Man_9GlcNAc_2$) undergoes a first trimming of modification i.e., removal of glucose, and oligomerization may occur if it is required for a functional protein. In Golgi apparatus further post-translational modification occur. Two different pathways are known for targeting proteins to lysosomes, as described below. In lysosomes the glycoproteins are subjected to further proteolytic and oligosaccharide processing (rev. by Goldberg et al., 1984; Hasilik and von Figura, 1984).

Mannose-6-phosphate dependent pathway

The discovery by Wiesmann et al. (1971) that cultured fibroblasts from patients with the inherited metabolic disorder Mucolipidosis II (or I-cell disease) secrete large amounts of soluble lysosomal enzymes into the culture medium whereas the intracellular level of these enzymes is abnormally low, was on the basis of the elucidation of the mannose-6-phosphate dependent pathway.

In Golgi apparatus, mannose residues of soluble lysosomal proteins are phosphorylated. The latter is acomplished in two-step reaction: N-acetylglucosamine-1-phosphate binding to mannose residues and removal of N-acetylglucosamine. The enzymes responsible for these reactions are N-acetylglucosamine-phosphotransferase (the enzyme defective in I-cell disease) and N-acetylglucosamine-1-phosphodiester-α-N-acetylglucosaminidase, respectively (rev. in Goldberg et al., 1984; von Figura and Hasilik, 1986). The arising mannose-6-phosphate acts as a recognition marker for the mannose-6-phosphate receptor (MPR). The specificity of phosphotransferase for targeting soluble lysosomal enzymes is likely to be determined by a conformational domain common to these proteins (Lang et al., 1984; Reitman and Kornfeld, 1981, Baranski et al., 1990).

Two MPR's have been identified, a large (275kDa) receptor, that binds to ligand independently from the presence of divalent cations (CI-MPR) and a small (46 kDa) receptor, that requires divalent cations, particularly Mn^{2+}. It has

now been generally accepted that both MPR's bind mannose-6-phospate containing lysosomal protein in the trans-Golgi network (TGN), where the majority of receptor molecules are localised, the complexes being clustered in clathrin coated vesicles that arise by budding from the TGN. The coated vesicles lose their coat and fuse with acidified endosomal vesicles. The acidic environment of these vesicles causes the dissociation of complexes, the receptors being recycled to plasma membrane or TGN, while enzymes remain in the lumen of endosome. The enzymes are then together with endocytosed proteins transported to lysosomes (Kornfeld and Mellman, 1989; Ludwing et al., 1991).

Mannose-6-phosphate independent pathway

Lysosomal membrane proteins and soluble proteins from leukocytes, kidney, liver, spleen and brain are not sorted in the TGN by the MPR's (rev. by von Figura and Hasilik, 1986). The exact nature of the alternative pathway is not known. It might include transport to the plasma membrane via the constitutive secretory pathway followed by internalization through the endosomal system (Braun et al., 1989) or a direct pathway from the TGN to lysosomes via endosomes (Green et al., 1987). Another possibility is the existence of other receptors, like mannose or asyaloglycoprotein receptors, to capture secreted lysosomal enzymes and target them via receptor-mediated endocytosis to lysosomes (Owada and Neufeld, 1982). It is dificult, however, to envisage how such a mechanism could operate selectively, i.e. not result in transfer of non-lysosomal proteins to lysosomes. It might be possible that recycling receptors act by a mechanism similar to the one of phosphotransferase (Gabel et al., 1984).

❑ Glycosphingolipids

Glycosphingolipids (GSLs) are typical components of the outer leaflet of animal plasma membranes consisting of a hydrophobic ceramide (N-acylsphingosine) and a hydrophilic oligosaccharide chain extended into the extracellular space (Thompson and Tillack, 1985). Gangliosides are GSLs containing one or more N-acetylneuraminic acid (sialic acid) moieties (Svennerholm, 1962; Svennerholm, 1963). Gangliosides are found in highest concentration in the nervous system, particularly in the gray matter of the brain (rev. by Svennerholm, 1980). At the cellular level, gangliosides seem to be higher concentrated in neurons, specially in regions of nerve ending and dendrites (Leeden, 1978), than in glial cells (Roberts et al., 1975) and myelin

(Leeden, 1980). During development the total content and pattern of brain gangliosides change in parallel with neuronal differentiation, synaptogenesis and myelination (Suzuki, 1965).

Function

The function of GSLs in membranes is not clearly established yet. The current view is that GSLs display dual activities, one modulating functional membrane proteins and regulating transmembrane signaling, and the other mediating cell-cell and cell-environment interactions (rev. by Hakomori, 1990). The lyso-sphingolipids (sphingolipids devoid of the fatty acid moiety) have been suggested to function as an intracellular second messenger due to their inhibitory effect on the activity of protein kinase C (rev. by Hannun and Bell, 1989). In addition, the binding capacity of gangliosides for Ca^{2+} (Berh and Lehn, 1973) may indirectly modulate the transduction by decreasing the intracellular concentration of calcium. As gangliosides are specially abundant in nerve endings (Leeden, 1978), it has been suggested that they can play a role in the transmission of nerve impulses across synapses (Rahmann et al., 1982). Individual gangliosides have been identified as binding sites on the cell surface for viruses and bacteria, and as receptores of bacterial toxins, hormones and transmitters, as well as involved in immunological recognition (antigens of blood groups, tumoral antigens lymphocyte markers) (rev. by Svennerholm, 1984).

Biosynthesis, transport and catabolism

The ganglioside biosynthesis starts on the membranes of the ER and continues on the Golgi apparatus. The exact mechanism by which GSLs are transported from site of synthesis (ER or Golgi) to the sites of residence (plasma membrane - PM) and degradation (lysosomes) have not been clearly defined yet. It is generally assumed that GSL formation is coupled with a vesicular membrane flow from the site of synthesis to the PM (Schwarzmann and Sandhoff, 1990); GSLs derived from the PM are end up in intra-endosomal vesicles which are delivered, by sucessive processes of membrane fission and fusion, along the endocytic pathway directly into the lumen of the lysosomes (van Echten and Sandhoff, 1993) However, the involvement of glycolipid binding and/or transfer proteins in the transport of GSL to plasma membrane is not presently excluded (Tiemeyer et al., 1989).

The conversion of oligosialogangliosides to monosialogangliosides is thought to occur in cellular membrane because a ganglioside hydrolyzing sialidase, particularly active in neuronal and glial cells, was identified in plasma membrane (Tettamanti et al., 1972). Monosialogangliosides are degraded in the lysosomes by the sequential action of exohydrolases (Fig. 1). Some of these enzymes, such as the β-hexosaminidase A, are found to need the assistence of small glycoprotein cofactors, the so-called "sphingolipid activator proteins", to attack their lipid substrates (rev. by Furst and Sandhoff, 1992). Products of lysosomal GSL degradation, such as monosaccharides, fatty acids and ceramide are thought to leave the lysosomes either for re-use in biosynthetic pathways or for final degradation and energy production.

Lysosomal storage disorders

Metabolic defects on each step of sphingolipid degradation result in a lysosomal storage disorder characterised by the accumulation of the respective lipid substrates. Such a defect may be caused by either a mutation in the gene of a lysosomal hydrolase or in rare cases also by a mutation in the gene coding for the respective sphingolipid activator protein. The hydrolysis of ganglioside G_{M2} in ganglioside G_{M3} requires three glycoproteins: the α- and β-subunits of β -hexosaminidase A and the G_{M2} activator protein. The deficiency in any of these proteins leads to the accumulation of ganglioside G_{M2}, resulting in a severe neurodegerative disorder known as G_{M2}-gangliosidosis. Tay-Sachs disease and Sandhoff disease are the prototype of the infantile forms of the α- and β-subunity deficiencies, respectively.

At the present time more than 30 different lysosomal storage disorders have been reported, the majority of them inherited in an autosomal recessive manner. The incidence of these diseases 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 Tay-Sachs disease is much higher (1 in 4,000) (Reuser et al., 1994).

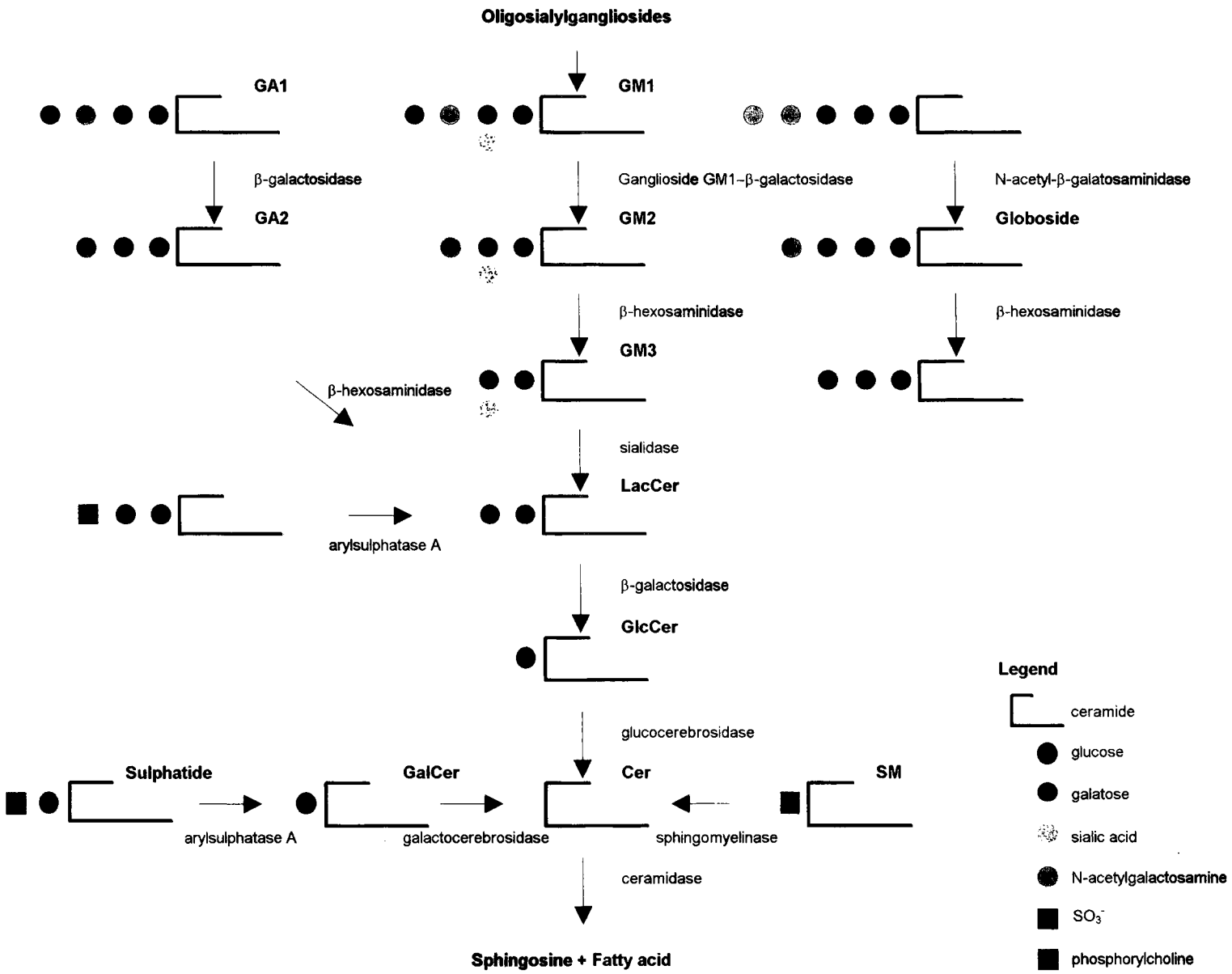


Fig. 1: Intralysosomal catabolism of sphingolipids (from Sandhoff, 1985).

1.1.2 THE β-HEXOSAMINIDASE

❑ Structure and physicochemical properties

The ß-hexosaminidase or simply hexosaminidase (EC 3.2.1.52) is a soluble lysosomal hydrolase with specificity to terminal residues of β-acetylgalactosamine and β-acetylglucosamine (Walker et al., 1961; Woollen et al., 1961) from all glycolipids and glycoproteins that occur in human cells. The lysosomal location of this enzyme was demonstrated by subcellular fractionation (Conchie and Hay, 1963; Sellinger et al., 1960).

The main isoenzymes are the hexosaminidase A (Hex A) and hexosaminidase B (Hex B) (Robinson ans Stirling, 1965). Both isoenzymes are dimeric glycoproteins and their oligosaccharide composition is similar, with the exception of sialic acid that is only present in Hex A (Freeze et al., 1979). The isoelectric points of Hex A and Hex B are 5.4 and 7.2, respectively (Sandhoff et al., 1968). At a temperature of 55°C, the Hex A is thermolabile whereas Hex B is thermostable (Dance et al., 1970). The A and B isoenzymes isolated from human tissues have a molecular mass of 120±20 kDa, as measured by gel filtration or gradient sedimentation (Beutler et al., 1976; Geiger and Arnon, 1976; Lee and Yoshida, 1976; Srivastava and Beutler, 1974; Tallman et al., 1974). A third isoenzyme is the hexosaminidase S (Hex S) that has a molecular mass of 130 to 150 kDa (Geiger et al., 1977; Potier et al., 1979). The hexosaminidase is made up of α and β subunits which can associate noncovalently in different ways, αβ, ββ or αα, to give rise to the Hex A, Hex B, or Hex S, respectively (Beutler et al., 1975; Beutler et al., 1976; Geiger and Arnon, 1976; Ikonne et al., 1975; Tallman, 1974). In lysosomes, each of the subunits are internally nicked, the resulting fragments remaining attached to each other by disulfide bonds[1]. For simplicity, the subunits of hexosaminidase isoenzymes will be considered as αβ, ββ and αα, where α and β are the unnicked precursors.

The minor hexosaminidase isoenzymes are I_1 and I_2 (Prince and Dance, 1972) and P (Geiger et al., 1978). The hexosaminidase I and P have isoelectric points and electrophoretic mobilities between those of Hex A and Hex B. The I (I_1) and P forms from serum appear to be differently glycosylated forms of Hex B, whereas the I form (I_2) isolated from liver and placenta appears to correspond to intermediate forms of Hex A processing (Dewji et al., 1986).

[1]This proteolytic modification is often called maturation.

The hexosaminidase C is a β-N-acetylglucosaminidase with a neutral optimum pH and located in the cytosol (Braidman et al., 1974a; Braidman et al., 1974b; Poenaru and Dreyfus, 1973). This enzyme is particularly abundant in brain and embrionic tissues (Beutler and Kuhl, 1977). Its biological role is not known and so far, the deficiency of this enzyme has not been associated with any genetic disorder.

❑ Genes: structure, transcription and translation

The genes coding to the human hexosaminidase α-subunit (HEXA) and β-subunit (HEXB) are located at chromosome 15q23-q24 (Takeda et al., 1990) and 5q13 (Fox et al., 1984), respectively. The HEXA and HEXB genes are 35 kb and 45 kb long, respectively, and comprise 14 exons and 13 introns (Proia, 1988). With the exception of intron 1 of each gene, all the others interrupt the coding regions at analogous positions (Proia, 1988). In both genes, the coding region is about 1600 bases long. For human β-hexosaminidase α- and β-subunits the identity in amino acid sequences encoded by exons 1 and 2, and those specified by the remaining exons, is 26% and 63%, respectively (Korneluk et al., 1986; Myerowitz et al., 1985). Because of the similarity in gene organization, sequence and function, HEXA and HEXB genes are believed to have arisen via duplication event of a common ancestor (Proia et al., 1988). The hexosaminidase gene is apparently ancient since the genes in the lower eukaryote *Dictyostelium discoideum* (Graham et al., 1988), and in the bacteria *Vibrio vulnificus* (Sommerville and Colwell, 1993), *Vibrio harvey* (Soto-Gil and Zyskind, 1989) and *Porphyromonas gingivalis* (Lovatt and Roberts, 1994), form a phylogenetically coherent group with the mouse and human α and β subunits genes. The mouse α- and β-subunit have 84% and 75% of identity with the sequences of the human α and β-subunits, respectively (Bapat et al., 1988; Beccari et al., 1992). Unlike mammals, bacteria and slime mold have a single gene. The duplication event leading to the appearance of two hexosaminidase genes in mammals may have been related to an increase in the diversity of substrates (Proia et al., 1988). The point in evolution at which the duplication event occurred is not known.

The promotor region of HEXA gene is GC-rich and contains possible TATA and CAAT box motifs. However, the promoter remains to be confirmed in expression experiments. Two mRNA species of 2.1 and 2.6 kb long (the 2.1 kb species is more abundant) have been detected and may differ in the use of a 3'

polyadenylation site. The mRNA codes a prepro-α chain of 529 a.a. (Proia and Soravia, 1987).

The promoter region of HEXB gene is GC-rich, contains several GC-box like sequences with potential SP1-binding sites. A 500-bp fragment has strong promoter activity in COS cells (Neote et al., 1988), although it has not been shown whether any of the recognizable elements contribute to promoter activity. Regions with no TATA box, and with a very high GC content with SP1-binding sites are characteristic of promoters of housekeeping genes, thus corroborating the expression of lysosomal proteins in every type of cells (except in the mature erythrocyte). The mRNA of 2.2 kb codes a prepro-β chain of 556 a.a. (Neote et al., 1988; Proia, 1988).

❑ Transport and post-translational processing

The biosynthesis and post-translational processing of the α- and β-subunits of Hex A are represented in Fig. 2.

I-Signal peptide sequence

The polypeptides are synthesized on membrane-bound ribosomes and cotranslationally translocated to the lumen of ER, where the leader sequence of prepro-α (22 amino acids) and prepro-β (42 amino acids) is removed (Little et al., 1988; Quon et al., 1989; Stirling et al., 1988). The β-signal peptide is exceptionally long and comprises three in-frame AUG before the cleavage site. While all three AUGs can function as initiation codons *in vitro* (Sonderfeld-Fresko and Proia, 1988), only the first was found to be used in intact cells (Neote et al., 1990). These data predict a pro-β chain of 514 amino acid residues (~ 58.8 kDa) and a pro-α chain of 507 residues (~ 58.3 kDa).

II-Glycosylation of the nascent polypeptide

The amino acid sequence of α- and β-chains (deduced from the corresponding cDNA sequence) predicts 3 (Asn 115,157 and 295) and 5 (Asn 84, 142, 190, 327 and 497) glycosylation sites, respectively. Expression in COS cells of α- and β-cDNAs mutagenized at each potencial glycosylation sites showed that in the pro-α chain all sites are glycosylated, but Asn295 is preferentially glycosylated (Weitz and Proia, 1992). In the pro-β chain the first four sites are glycosylated, although the second is not consistently so; those more externally located (Asn84 and Asn327) are preferentially glycosylated (Sonderfeld-Fresko and Proia, 1989).

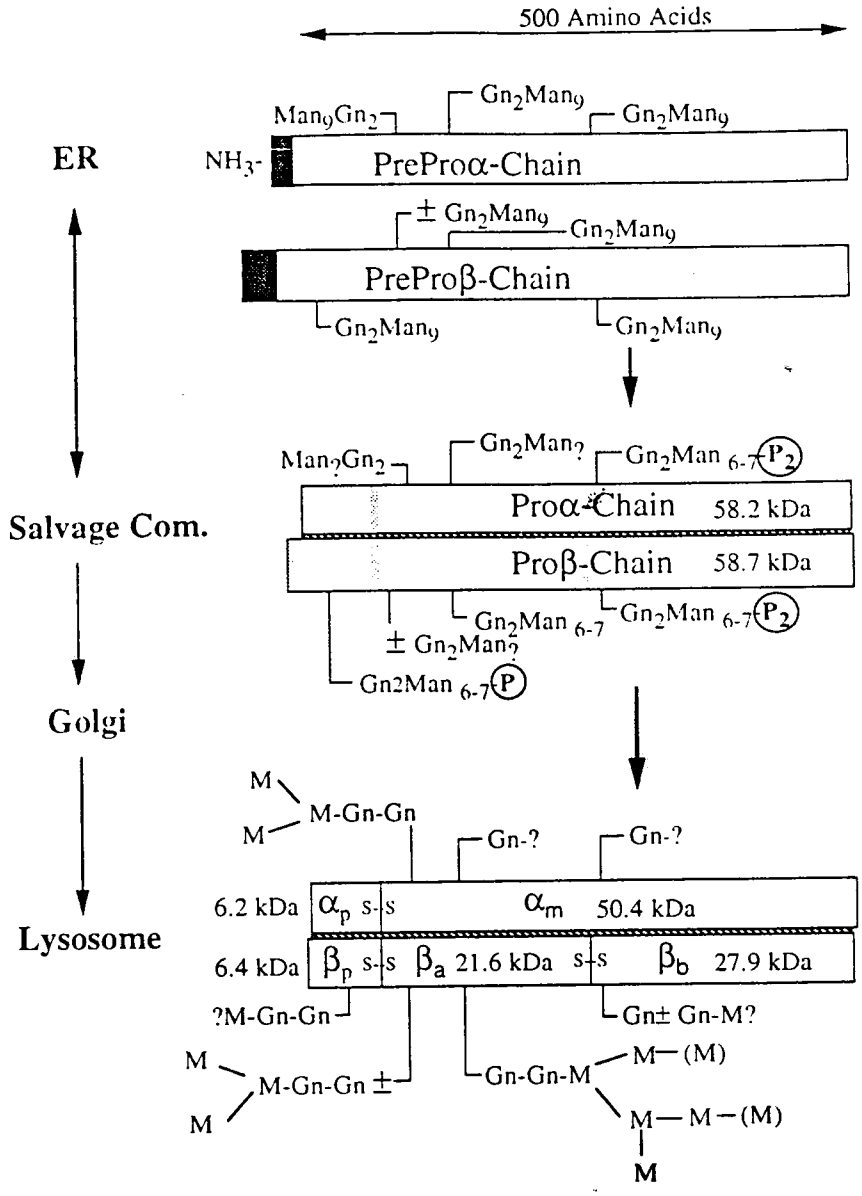


Fig. 2: Biosynthesis and post-translational processing of hexosaminidase A. The cellular compartments in which the processing takes place are indicated on the left. Sections of the polypeptide chains that are removed in succeeding processing steps are indicated by shaded rectangles. Attachment sites for the oligosaccharides and their structures are shown. Those structures that have not been determined (O'Dowd et al., 1988) are indicated by "?". Oligosaccharides that are phosphorylated are identified by "P or P_2". P_2 indicates the preferential site of phosphorylation (Sonderfeld-Fresko and Proia, 1989; Weitz and Proia, 1992). Differential glycosylation or processing is indicated by "±" (Sonderfeld-Fresko and Proia, 1989). Molecular mass is given in kDa. These were calculated from the deduced amino acid sequences and do not contain the mass contributed by the oligosaccharide (s). The mature polypeptides are held together in the mature subunits by disulfide bonds, shown as S-S; however, the placement of these bonds has not been experimentally determined (from Mahuran, 1995).

O'Dowd et al. (1988) identified the carbohydrate attachment sites on the mature α- and β-chains isolated from human placenta by HPLC. Only one glycosylation site, at Asn115, was identified in the α-chain, with no evidence of carbohydrate attached to other sites. In β-chains evidence was found for asparagine-linked oligosaccharides at glycosylation sites 142, 190 and 327, although the majority corresponded to degraded structures, but not at Asn497; no data concerning site 84 was presented because the amino-terminal peptide of the mature form was not detected.

The apparent discrepancy between the results obtained by these authors can be explained by the difference on the degree of processing of the polypeptides. In the expression studies, the subunits were analyzed just a few hours after their synthesis, in precursor form, and therefore in pre-lysosomal organelles, whereas in the other study the subunits were isolated from a tissue where they would have been exposed to the degradative environment of the lysosomes for a considerable period of time. Therefore it is likely that other oligosaccharides rather than those identified are present in placental Hex A but they are nearly completely degraded in the lysosol.

The oligosaccharide chains at sites β: 84 and 327 (Sonderfeld-Fresko and Proia, 1989) and at position α: 295 (Weitz and Proia, 1992) were found to be preferred for phosphorylation by the phosphotransferase. The absence of the two preferentially phosphorylated oligosaccharides in β-chain results in greatly reduced amounts of the mature lysosomal form of Hex B, which is preferentially secreted (Sonderfeld-Fresko and Proia, 1989). The absence of all oligosaccharides in α-chain results in a catalytically inactive enzyme, through the formation of an insoluble, innappropriately disulfide-bonded complex that is trapped in the lumen of the endoplasmic reticulum (Weitz and Proia, 1992). Therefore, the glycosylation appears to be essential for properly folding of the α and β-subunits and exit from ER.

Considering that the spatial positioning between the phosphotransferase recognition domain and the target oligosaccharide is critical for efficient phosphorylation (Lang et al., 1984) and the fact that the α- and β-subunits have related structures and the same preferred phosphorylated oligosacharides (Sonderfeld-Fresko and Proia, 1989; Weitz and Proia, 1992), the phosphotransferase recognition domains are probably similar in structure and position in both subunits. Until tertiary structure information for hexosaminidase, and other soluble lysosomal enzymes, is available the identification of

phosphotransferase recognition domain on hexosaminidase subunits may be difficult.

III-Dimerization of hexosaminidase subunits

Subunit association is an important post-translational event to give catalytically active enzymes since neither the α monomer (Proia et al., 1984) nor the β monomer (Sonderfeld-Fresko and Proia, 1988) is catalytically active. The α- and β-subunits are synthesized in cultured fibroblasts in approximately equal amounts, as judged by early incorporation of a radiolabel amino acid (Hasilik and Neufeld, 1980a; Proia et al., 1984), but they dimerize at different rates. Pulse-chase experiments have shown that the pro-α chain remains in a monomer state for at least twice as long as the pro-β chain (half-life 5.5 versus 2.5) (Proia et al., 1984; Sonderfeld-Fresko and Proia, 1988). The rate-limiting step in the association of α-subunits is not known; it may be folding, disulfide bonding, or some yet unidentified post-translational modification. The site of ββ dimer formation is thought to be the ER, in part because of the rapid rate at which it occurs in intact cells. The site of αβ association is not clear; it has been attributed to the Golgi because it occurs after incorporation of ^{32}P to form the mannose-6-phosphate recognition marker (Proia et al., 1984). However, the available data do not allow unambiguous assignment of αβ dimerization to the Golgi nor to the ER. The structural characteristics of polypeptides that determine the dimerization site are not known.

IV-Proteolytic processing

Once the active pro-Hex A (αβ) and pro-Hex B (ββ) isoenzymes enter the lysosome, a number of proteolytic (and glycosidic) processing events occur (see Fig. 2). The structure of the mature α-subunit can be described as $\alpha_p\alpha_m$, where α_p is the N-terminal fragment of pro-α chain containing ≈ 52 amino acids (the C-terminus of α_p is not well defined) and α_m is most of the remainder, with ≈ 440 amino acids (Hubbes et al., 1989; Little et al., 1988). The structure of mature β-subunit can be described as $\beta_p\beta_a\beta_b$; the larger fragments have different isoelectric points, and were therefore designated as β_a (acidic) and β_b (basic) (Mahuran et al., 1988). The N-terminal β_p peptide contains ≈ 58 residues (C-terminus is not well defined); the β_b chain contains 190 residues; and the β_a contains 242 residues (Hubbes et al., 1989; Quon et al., 1989; Mahuran et al., 1988). The mature α and β subunits are therefore composed of two ($\alpha_p\alpha_m$) and three ($\beta_p\beta_a\beta_b$) polypeptide chains held together by disulfide bonds. The proteinase responsible for the maturation of β-hexosaminidase has not yet been

isolated. In serum (Zuhlsdorf et al., 1983) and in fibroblast media (Hasilik et al., 1980a) the isoenzymes are composed of subunits in pro-form and are catalytically active. This observation suggests that proteolytic modifications are not required for catalytic activity, resulting probably from the exposure of isoenzymes to the degradative environment of lysosomes.

❑ Enzyme kinetics and substrate specificity

In vivo, the activator protein is essential for the hydrolysis of ganglioside G_{M2} and glycolipid G_{A2} by Hex A but not by Hex B, as can be concluded from the occurrence of one variant of G_{M2}-gangliosidosis[2] is in which the Hex A (and Hex S) is defective but not Hex B.

In vitro, in the absence of activator protein the terminal β-linked GalNAc residue from G_{M2} and G_{A2} is not hydrolised by Hex A and Hex B. In the presence of activator, ganglioside G_{M2}, glycolipid G_{A2} and globoside are hydrolised by Hex A (Conzelmann and Sandhoff, 1979) but not by Hex S (Kytzia et al., 1984); Hex B possesses minute but still detectable activity (about 3% of that of Hex A) against glycolipid G_{A2} in the presence of activator (Conzelmann and Sandhoff, 1979). In the presence of detergents, such as taurodeoxycholate, both isoenzymes A and B were found to hydrolise terminal β-linked GalNAc residue from ganglioside G_{M2} and glycolipid G_{A2}; however, the activity of Hex B against G_{A2} was higher than that of Hex A (Erzberger et al., 1980; Harzer et al., 1983; O'Brien et al., 1977). G_{M2}-ganglioside derivatives in which the carboxyl group of the sialic acid is methylated or reduced to an alcohol cannot be degraded by Hex A in the presence of activator protein but are hydrolyzed in the presence of taurodeoxycholate (Li et al., 1984). These findings suggest that the carboxyl group of sialic acid is involved in the interaction with Hex A and/or G_{M2} activator. In addition, the action of detergents seems to be different from that of activator protein.

The kinetic constants K_m and V_{max} of Hex A towards ganglioside G_{M2}/activator complex was estimated to be 1.9 μM and 0.2 μmol/min/mg protein, respectively; the enzymatic activity is optimum at pH 4.2 (Conzelmann and Sandhoff, 1979).

The hexosaminidase isoenzymes, as most lysosomal enzymes, hydrolise several substrates, including synthetic water-soluble substrates. The most sensitive and commonly used substrate for determining hexosaminidase activity is a β-GlcNAc derivative of the fluorogenic compound 4-methylumbelliferone

[2] This variant is commonly named as Tay-Sachs disease

(4MU-GlcNAc). It is recognized by both Hex A and Hex B and does not require the G_{M2} activator (Kytzia and Sandhoff, 1985). A related compound, the sulphated derivative of 4MU-GlcNAc (4MU-GlcNAcS), specifically hydrolyzed by Hex A and Hex S, has also been developed (Kresse et al., 1981; Ludolph et al., 1981).

The interaction of Hex A with the natural and synthetic substrates is schematically represented in Fig. 3.

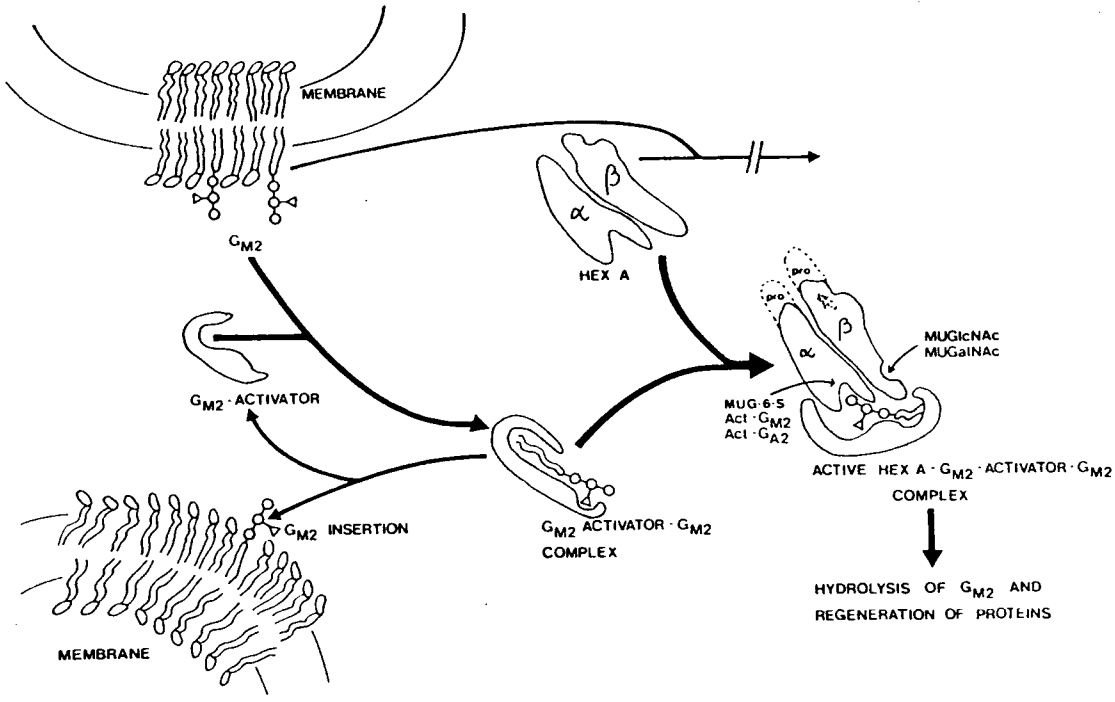


Fig. 3: Interaction of Hex A with the ganglioside/activator complex and synthetic substrates (from Sandhoff et al., 1989).

The data obtained through competition experiments on the hydrolysis of both natural and synthetic substrates by Hex A (Kytzia and Sandhoff, 1985) suggests that the enzyme has two catalytic sites, one located on the α-subunit and the other on the β-subunit. Ganglioside G_{M2}, in the presence of the G_{M2} activator protein, and the substrate 4MU-GlcNAcS are hydrolised by the catalytic site

located on the α-subunit, whereas 4MU-GlcNAc is degraded predominantly by the catalytic site on the β-subunit. These studies also showed that Hex A not only recognizes the ganglioside portion of the ganglioside/activator complex but additionally binds to the activator protein itself. Since 4MU-GlcNAcS degradation by Hex S is inhibited by the activator protein to almost exactly the same extent as that of Hex A, it is possible that the binding of activator is a property of the α rather than the β-subunit (Kytzia and Sandhoff, 1985); however, α-subunits seems to require the assistance of β-subunits to attack the ganglioside/activator complex since Hex S, composed of only α-subunits, is inactive towards the natural substrate (Kytzia et al., 1984).

The kinetic constants of human Hex A and Hex B against 4MU-GlcNAc have been found to be very similar, with K_m / V_{max} values of 0.90 mM / $1.8x10^{-4}$ nmol/h/mg and 0.91mM / $4.4x10^{-4}$ nmol/h/mg of protein, respectively (Kytzia and Sandhoff, 1985). The pH optimum for both isoenzymes is 4.4 (Conzelmann and Sandhoff, 1978; Wenger et al., 1972; Srivastava et al., 1974). The K_m and V_{max} values of Hex S are 2.8 mM and $\geq 1.7x10^{-5}$ nmol/h/mg of protein, respectively (Kytzia and Sandhoff, 1985). This enzyme cleaves the 4MU-GlcNAc optimally at pH 4.8 to 5.0 (Geiger et al., 1977).

The kinetic constants of human Hex A and Hex B against the synthetic substrate 4MU-GlcNAcS are different. Hex A and Hex S have very similar K_m values (0.31 and 0.33mM, respectively), which are about 10-fold lower than that of Hex B (3.4 mM). The V_{max} values of Hex A, Hex B and Hex S are $2.5x10^{-5}$, $1.2x10^{-6}$ and $\geq 5.6x10^{-6}$ nmol/h/mg of protein, respectively. The specific activity of Hex S towards 4MU-GlcNAcS was about one-fifth of that of Hex A, perhaps reflecting the inherent instability of this isoenzyme (Kytzia and Sandhoff, 1985). The pH optimum for Hex A with synthetic sulphated substrate is 3.8 (Kresse et al., 1981).

According to this data, the catalytic site on the α-subunit, common to Hex A and Hex S, acts on negatively charged substrates (the ganglioside G_{M2} is exclusively hydrolyzed by Hex A) whereas the catalytic site on the β-subunit hydrolises neutral synthetic substrates. Since Hex S (αα) is active against the 4MU-GlcNAc and Hex B (ββ) hydrolyzes the 4MU-GlcNAcS, the specificity of active sites is not absolute but preferential.

1.1.3 THE G_{M2} ACTIVATOR PROTEIN

❑ Structure and physicochemical properties

The subcellular location of the G_{M2} activator protein has been studied in cultured human fibroblasts. The activator was clearly shown to be associated with lysosomal fraction (Barnerjee et al., 1984; Burg et al., 1985). This protein has an isoelectric point of 4.8 (Conzelmann and Sandhoff, 1979; Li et al., 1981a). It presents an unusual stability to heat (up to 60°C) and proteinases like trypsin and chymotrypsin (Conzelmann and Sandhoff, 1979; Li et al., 1981a), probably due to the formation of disulfide bridges between the eight cysteine residues present in the protein (Furst et al., 1990). The molecular mass obtained by gel filtration (23.5 or 25 kDa) (Conzelmann and Sandhoff, 1979; Li et al., 1981a) is similar to the value obtained by denaturing SDS-gel electrophoresis (21.5 kDa) (Conzelmann and Sandhoff, 1979) and the value calculated for the polypeptide chain without carbohydrate (17.6kDa) (Furst et al., 1990), indicating a monomeric structure of the protein.

A high concentration of G_{M2} activator was found in human kidney, serum, and urine. Since ganglioside turnover is most rapid in brain, one would expect this tissue also to contain a high amount of activator protein. In fact, the activator level was higher in brain than in most other tissues (liver, spleen and placenta), but still much lower (about one-eighth) than in kidney (Banerjee et al. (1984). The low molecular mass of this protein, which is therefore, probably filtered through the kidneys may explain the high content of activator found in urine. The biological function of the activator in human fluids is not known.

❑ Gene: structure, transcription and translation

The gene coding to the human activator protein (GM2A) has been mapped to chromosome 5q32-q33 (Heng et al., 1993; Swallow et al., 1993). This small gene whose sequence at 5' end remains incomplete is of at least 16 kb, and comprises 4 exons and 3 introns. The promoter of this gene has not been characterised. The mRNA of 2.4 kb codes a preproprotein of 193 a.a. (Klima et al., 1991; Xie et al., 1991). A pseudogene, GM2AP, has been mapped to chromosome 3 (Swallow et al., 1993; Xie et al., 1992).

❑ Transport and post-translational processing

The signal peptide sequence of this protein has been predicted, on the basis of consensus cleavage sites, to be 23 amino acids long (Klima et al., 1991; Xie et al., 1991). It has two possible translation initiation sites, both with weak consensus initiation nucleotide sequence. Using the predicted peptide cleavage site, the size of the resulting pro-G_{M2} activator would be 170 amino acids (≈18.5 kDa). The activator contains a single oligosaccharide at Asn62, the only potential glycosylation site. Further proteolytic processing includes removal of 8 amino acids from the amino end of pro-polypeptide. The mature protein contains 162 amino acids (Furst et al., 1990). Processing studies of activator protein in human skin fibroblasts showed that it is targeted through the mannose-6-phosphate pathway. The precursor form, of 24 kDa, was observed only in fibroblast media whereas the mature form, of 21-22.5 kDa, was detected only in cells, suggesting a rapid processing of the protein (Burg et al., 1985).

❑ Function and mechanism of action

The mechanism of action of activator is not completely understood, although it has been studied in some detail. Meier et al. (1991) has recently proposed a model that combines the findings made by several other investigators in the past. This model is based on the existence on the activator of an oligosaccharide-binding domain, which recognizes β-GalNAc and sialic acid residues, and a hydrophobic domain that anchors the ganglioside molecule by interaction with its ceramide portion. The activator extracts a G_{M2} molecule from the membrane and forms a stable, water-soluble 1:1 protein-lipid complex. Hex A binds to this complex, splits the G_{M2} molecule to yield G_{M3} and releases the activator-G_{M3} complex (see Fig. 3). In G_{M2} and G_{A2} molecules the distance between GalNAc and sialic acid residues, and ceramide is such that these molecules fit exactly into the respective domains of activator, allowing a correct positioning of GalNAc residue into the catalytic site of the α-subunit. The β-subunit can also contribute for a correct positioning into active site through recognition or binding of activator/ganglioside G_{M2} complex. In the absence of activator protein, the β-GalNAc linkage of substrate is not acessible to the active site due to steric hindrance caused by the hydrophilic segment of adjacent membrane lipids. Therefore, the function of the G_{M2} activator is to overcome the steric hindrance that prevents the water-soluble Hex A from binding and hydrolyzing the target β-GalNAc residue on the terminus of the membrane-bound gangliosides. Additionally, Hex A cleaves all substrates of membrane

surface presenting the target β-GalNAc extended far enough into the lysosol and accessible to enzyme without assistance of an activator protein (Meier et al., 1991). Therefore two mechanisms seems to be possible (rev. by Furst and Sandhoff, 1992): the activator protein binds one molecule of sphingolipid and lifts it just a few Å out of the membrane to make the substrate accessible to the catalytic site of the enzyme, or the activator-lipid complex leaves the membrane and the enzyme reaction takes place in the lysosol. However, there is no definitive knowledge of whether the G_{M2} activator, *in vivo*, acts according to one of these modes or to both of them.

1.1.4 THE G_{M2} GANGLIOSIDOSES

The G_{M2}-gangliosidoses are a group of recessively inherited disorders caused by intralysosomal accumulation of ganglioside G_{M2} and related glycolipids, particularly in neuronal cells. Sandhoff et al. (1971) proposed a classification based on the catalytically active hexosaminidase isoenzymes remaining in the tissues and fluids of affected patients: variant B, due to deficiency of Hex A; variant 0 (zero), due to deficiency of both Hex A and Hex B; and variant AB, due to G_{M2} activator deficiency. More recently, the three forms of the disease were classified as hexosaminidase α-subunit defects, hexosaminidase β-subunit defects, and G_{M2} activator defects, respectively (Sandhoff et al., 1989). Gravel et al. (1995) proposed a nomenclature that uses clinical designations for the various forms of the disease and gene designations to describe the mutations. Accordingly, Tay-Sachs disease and variants refer to G_{M2} gangliosidosis due to HEXA mutations; Sandhoff disease and variants refers to G_{M2} gangliosidosis due to HEXB mutations; and G_{M2} activator deficiency refers to to G_{M2} gangliosidosis due to GM2A mutations.

-Tay-Sachs disease and variants (McKusick 272800). Defects on the α-subunit block the formation of Hex A (αβ) and Hex S (αα), but not of Hex B (ββ) (Bartholomew and Rattazi, 1974; Srivastava and Beutler, 1973; Srivastava and Beutler, 1974). Two enzymatic variants are known, the variant B and B1. Variant B is characterised by normal or elevated levels of Hex B and complete absence of Hex A activity (Hultberg, 1969; Okada and O'Brien, 1969; Sandhoff, 1969). In B1 variant, the mutant α-subunit is able to associate with the β-subunit (Sonderfeld-Fresko et al., 1985a); the resulting dimer, αβ, behaves like normal Hex A in some aspects such as isolelectric point and catalytic activity toward the neutral synthetic substrates, but it is inactive towards the physiological substrate

G_{M2} ganglioside and the sulphated synthetic substrates (Kytzia et al., 1983; Li et al., 1981b).

-Sandhoff disease and variants.(McKusick 268800). Defects on the β-subunit cause the deficient activity of both Hex A and Hex B (Sandhoff et al., 1968). The small residual hexosaminidase activity observed in the cells of these patients is due to the presence of Hex S (αα); this activity is probably physiologically irrelevant since the Hex S is inactive against ganglioside G_{M2} (Kytzia et al., 1984).

-G_{M2} activator deficiency (McKusick 272750). This variant is caused by the deficiency of G_{M2} activator protein, not affecting the activity of Hex A and Hex B (Conzelmann and Sandhoff, 1978; Hechtman et al., 1982; Hirabayashi et al., 1983).

❑ Clinical and genetic heterogeneity

Each of the variants referred to above, except the G_{M2} activator deficiency, comprises a series of allelic mutations with great variability in clinical expression.

Clinical and neuropathological presentation

The clinical presentation of each variant vary widely, ranging from infantile to late-onset forms. Infantile forms, with an onset between 3 and 5 months of age, are characterised by a rapid progression of the disease that leads to seizures, loss of intellectual and cognitive abilities and eventually to a vegetative state, with death occuring usually before age 4. Tay-Sachs and Sandhoff disease are the prototype of the infantile form of the α and β-subunit deficiency, respectively. Later-onset forms present a slower progression of neurological signs. The later the disease is manifested, the more variable its symptomatology.

The different variants of G_{M2}-gangliosidosis were initially subclassified according to the age of onset of symptoms into infantile, juvenile and adults forms (Sandhoff et al., 1989). However, differentiation between juvenile and adult variants of the disease was often difficult as many patients presenting the disease in adulthood report symptoms dating back to early childhood. Therefore clinical designations based on the dominance of the encephalopathy rather than the age of onset were adopted (Gravel et al., 1995). They include infantile acute G_{M2} gangliosidosis, which corresponds to the classical infantile form; subacute G_{M2} gangliosidosis, which includes late-infantile and juvenile-onset forms showing a clinical course usually fatal in childhood or early adulthood; and

chronic G_{M2} gangliosidosis, which includes some juvenile and adult-onset forms usually compatible with longer survival.

The neurological features of patients with Tay-Sachs disease and variants, Sandhoff disease and variants, and activator deficiency (all cases reported so far present the infantile form of the disease) are indistinguishable, even from other types of gangliosidoses. A characteristic finding is the macular degeneration (the so-called cherry-red spot), observed under ophthalmoscopic examination, due to lipid storage in ganglia cells surrounding the retina. In Sandhoff patients an involvement of nonneurologic tissues causing organomegaly, skeletal abnormalities, storage cells in bone marrow and oligosaccharidurie is observed due to accumulation of other glycoconjugates (rev. by Sandhoff et al., 1989).

The histopathology is very similar among the three infantile types of G_{M2}-gangliosidoses. The most pronounced histopathological change, often observed in other sphingolipidosis, is the presence of neurons swollen with storage material throughout the central nervous system. The storage material shows strong acid phosphatase activity and consists of numerous concentrically arranged lamellar structures (membranous cytoplasmic bodies, or MCBs) which may fill the entire cytoplasm of a neuronal cell body (rev. by Sandhoff, 1991).

Pathophysiology of G_{M2}-gangliosidosis

The molecular mechanisms that lead to the neuronal dysfunction are not yet understood. Several pathogenic factors may be not mutually exclusive but the exact contribution of each one is not known. Studies of Sonderfeld et al. (1985b) suggest that lysosomal storage material may to some extent be recycled and reach other cellular compartments, such as Golgi and plasma membrane, by vesicle flow. An altered content in the pattern of membrane gangliosides might interfere with the fluidity and transport properties of the membrane and probably also with the establishment of proper connections. The formation of meganeurites and increase of synaptic spines on neurons have been reported by Purpura and Suzuki (1976), which suggest the formation of misconnections in the nervous system, whose extension might determine the clinical course of the disease. Another pathogenic mechanism consists in the accumulation of lysosphingolipids (compounds structurally derived from sphingosine). Lyso-G_{M2} and lyso-G_{A2} have been identified in the brain of patients with Tay-Sachs and Sandhoff disease (Kobayashi et al., 1992; Neuenhofer et al., 1986; Rosengreen et al., 1987). In cultured cells, these

compounds have lytic and cytotoxic properties (Neuenhofer et al., 1986). However, there is no evidence that the toxicity of these compounds is the immediate cause of cell death. In addition, lysosphingolipids are known to be strong inhibitors of protein kinase C activity (Hannun and Bell, 1987; Oishi et al., 1988). The protein kinase C is found in highest concentration in the mammalian nervous system, where it appears to participate in transduction of neurotransmitter and other signals, receptor regulation and neuronal differentiation (Hannun and Bell, 1987). It is therefore possible that the accumulation of ganglioside G_{M2} (and related lipids) lead to the formation of lyso-G_{M2} which would interfere with signal transduction in nerve cells.

Genetic heterogeneity

Multiplicity of mutant alleles had long been suspected from the clinical variability and from differences in the properties of defective enzyme. In the last ten years, as technology for characterizing mutations was improved, an extensive heterogeneity has become evident. Genetic analysis has revealed considerable heterogeneity among allelic variants, even among those with identical clinical presentation. With respect to α-chain, β-chain and G_{M2} activator defects, up to 54, 12 and 2 mutations have been identified, respectively (rev. by Gravel et al., 1995). These mutations include partial gene deletions, small deletions or insertions, and single nucleotide substitutions. Attention will be given to mutations causal to the α-subunity deficiency.

The mutations so far reported in the HEXA gene are shown in Fig. 4. The majority of them give rise to an infantile phenotype (known as Tay-Sachs disease, TSD). Besides those often clustered in particular ethnic groups (Ashkenazi Jews, Moroccan Jews, French Canadians and Japanese), that account for a significant fraction of patients with TSD, a very large number of rare mutations account for the remainder. Particular mutations affecting transcription, translation or post-translational modifications are detailed in Appendix III. The missense mutations clustered in the exon 5 and 13 have provided useful biochemical informations and therefore they are referred below.

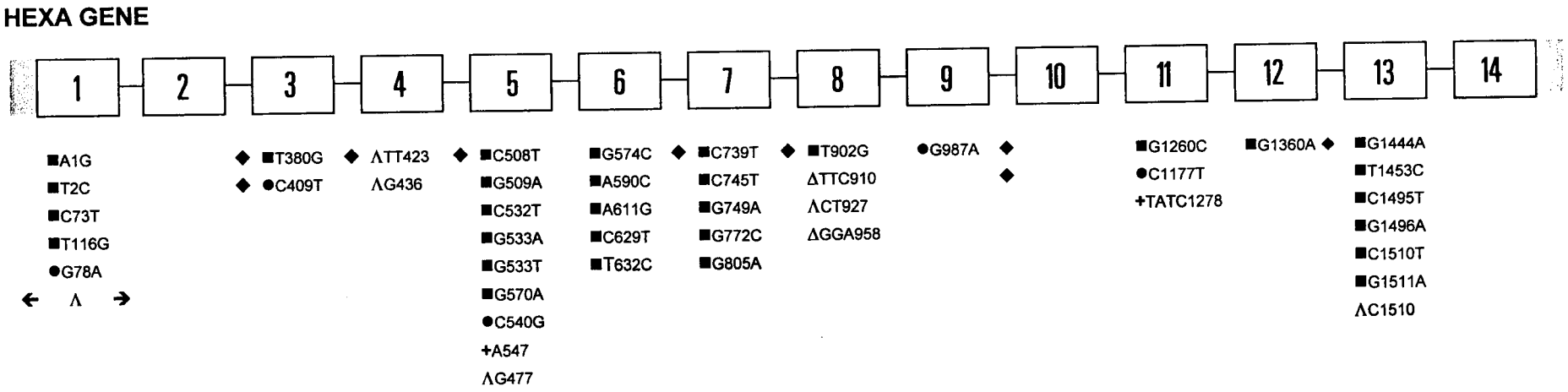


Fig. 4: Schematic representation of mutations described in HEXA gene. The mutations are identified according to the cDNA nomenclature. The exons are identified by numbers; Simbols: ■ missense, ● nonsense, ◆ splicing, + insertion, Λ frameshift deletion, Δ in-frame deletion.

The mutations in exon 5 represent about 20% of the total missense mutations, from which 10% occur in Arg178 codon: G533A (Ohno and Suzuki, 1988a), C532T (Arg178Cys) (Tanaka et al., 1990) and G533T (Arg178Leu) (Triggs-Raine et al., 1991). The introduction of Arg178His mutation in the normal cDNA and expression of mutagenized cDNA in COS cells resulted in the formation of Hex S devoid of catalytic activity against the synthetic sulphated substrate (Tanaka et al., 1990). Brown et al. (1989) reproduced the Arg178His mutation in the homologous site in the β-subunit (Arg211His) by site-directed mutagenesis and showed that the resulting Hex B, produced by expression in COS cells, was catalytically inactive. A less deleterious mutation β:Arg211Lys has been found to produce a Hex B with a markedly reduced catalytic activity against 4MU-GlcNAc (400-fold reduction in V_{max} but normal K_m) (Brown et al., 1991). According to these findings, the Arg178 residue is likely to participate in the catalytic site of the α-subunit but not in substrate binding; another segment of the molecule might therefore determine the different substrate specificity of the α- and β-subunits. Another two α-subunit mutations resulting in B1 phenotype have been described in exons other than exon 5: G574C (Val192Leu) in exon 6 (Ainsworth and Coulter-Mackie, 1992), and G772C (Asp258His) in exon 7 (Fernandes et al., 1992b). These two mutations may also inactivate the catalytic site on the α-subunit. Therefore three different Hex A sites, Arg178, Val192 and Asp258, are candidates for participation in or near the catalytic domain of the α-subunit. Two of them, Arg178 and Asp258, occur at sites conserved among the α- and β-subunit of human hexosaminidase, and the hexosaminidase from *Dictyostelium discoideum* (Brown et al., 1989).

The mutations G1444A (Glu482Lys) (Nakano et al., 1988; Proia and Neufeld, 1982), G1496A (Arg499His) (Paw et al., 1990a), C1510T (Arg504His) (Boustany et al., 1991; Paw et al., 1990a) and G1511A (Arg504Cys) (Paw et al., 1991) in exon 13, representing about 20% of total missense mutations, affect subunit folding and dimerization, thus suggesting that the peptide segment near the C-terminus is important to correct folding of α-precursor and subunit assembly. However, there is no direct evidence for a sequence near the C-terminus specifically involved in the intracellular transport.

Two pseudo-deficiency alleles have been described in HEXA gene: C739T (Arg247Trp) (Triggs-Raine et al., 1992) and C745T (Arg249Trp) (Cao et al., 1993). These mutant alleles result in the formation of Hex A catalytically active against ganglioside G_{M2} but inactive against the synthetic substrates (4MU-GlcNAc and 4MU-GlcNAcS).

❑ Diagnosis

Biochemical diagnosis

The identification of patients with G_{M2}-gangliosidosis is usually done through the determination of enzymatic activity rather than the analysis of accumulated sphingolipids.

I-Enzymatic assays

The most specific method for determining Hex A activity employs the physiological substrate, ganglioside G_{M2}, in the presence of G_{M2} activator protein (Conzelmann et al., 1983). The use of detergents (Erzberger et al., 1980; Harzer, 1983; O'Brien et al., 1977) for this purpose is not advisable since they are likely to change the substrate specificities of isoenzymes, thus leading to incorrect results. Because the natural-substrate test in the presence of activator protein is a time-consuming and expensive assay, it is generally replaced by the water-soluble synthetic substrate assay employing β-GalNAc derivatives.

The β-subunit deficiency, leading to absence or near-absence of total hexosaminidase activity, can be demonstrated with the neutral synthetic substrates (Kytzia et al., 1984).

The diagnosis of α-subunit deficiency requires the demonstration of deficient Hex A activity coupled with normal or even elevated activity of the Hex B isoenzyme. This can be done by using synthetic substrates (Bayleran et al., 1984; Ben-Joseph et al., 1985; Fuchs et al., 1983; Inui and Wenger, 1984; Li et al., 1983) and by separating hexosaminidase isoenzymes and determining their activity against neutral synthetic substrates. The isoenzymes are usually separated by ion-exchange-chromatography (Dance et al., 1970; Nakagawa et al., 1977; Prince and Dance, 1972; Young et al., 1970), isolelectric focusing (Christomanou et al., 1977; Hayase and Kritchevsky, 1973) or electrophoresis on cellulose acetate gel (Klibansky, 1971). While electrophoretic separation allows only a qualitative assay, the other techniques allow quantitative determination of each isoenzyme. The thermostability assay, based on thermal inactivation of Hex A in conditions in which Hex B is thermostable (at 55°C hex A is inactivated for 3 min incubation) (Conzelmann et al., 1978), has been used in the of α-subunit deficiency (O'Brien et al., 1970). This rapid and relatively accurate method has also been employed in large-scale carrier screening (Kaback, 1972; O'Brien et al., 1970).

The G_{M2} activator deficiency is normally demonstrated by an enzyme-linked immunosorbent assay (Barnerjee et al., 1984) or through the normalisation of Hex A activity against ganglioside G_{M2} in the presence of the exogenous activator protein (Kytzia et al., 1983).

II-Analysis of storage compounds

Glycolipids are the major storage compounds in G_{M2}-gangliosidoses, accumulating specially in neuronal cells. Glycolipids identified in brain of patients affected with these diseases are presented in Appendix IV. The accumulation of the major neuronal stroge compound, the ganglioside G_{M2}, starts at an early embryonic age (Conzelmann et al., 1985; Lemos et al., 1995; Navon et al., 1986). The accumulation of G_{A2} is less pronounced in Tay-Sachs than in Sandhoff disease, probably due to a reduced but significative activity of Hex B (about 3% of Hex A activity) against G_{A2} in the presence of activator (Conzelmann and Sandhoff, 1979). The process of lipid accumulation affects primarily the gray-matter and is accompanied by a decrease of gangliosides G_{M1}, G_{D1a}, G_{D1b} and G_{T1b}. In infantile forms the amount of lipids characteristic of myelin (cerebrosides and sulfatides) is decreased. In subacute and chronic forms, the extent of lipid accumulation is much less pronounced and spread throughout the nervous system than in the infantile form. The accumulation of globoside in visceral organs (liver, kidney and spleen) has only been described in patients with Sandhoff disease. (rev. by Sandhoff et al., 1989).

Since extraneuronal tissues possess very low levels of gangliosides, lipid analysis for diagnostic purposes are limited to nervous tissue, and their applicability restricted to postmortem examinations. Therefore, glycolipid analysis is usually performed by studying the metabolism in cultured skin fibroblasts of exogenously added radiolabelled ganglioside G_{M2} (Raghavan et al., 1985; Sonderfeld-Fresko et al., 1985b). Gangliosides dissolved in a culture medium are internalised and metabolised as endogenous glycolipids (Schwarzmann et al., 1983). This assay is particularly useful when conventional enzyme assays are inconclusive, as in G_{M2} activator deficiency.

Significant tissue accumulation and urinary excretion of glycoprotein-derived oligosaccharides containing terminal β-glycosidically linked N-acetyl-glucosaminyl residues, originated by the combined action of proteases, β-endo-N-acetylglucosaminidase, sialidase and β-galactosidase, have so far been found in patients with Sandhoff disease, in which both Hex A and Hex B are deficient (Sandhoff et al., 1989). It is therefore evident that these water-soluble substrates can also be hydrolysed by Hex B. In contrast to the storage of

oligosaccharides derived from glycoproteins, accumulation of glycosaminoglycans has never been clearly documented.

Genetic diagnosis

DNA-based assays have been used in molecular of α-subunit deficiency, as a tool to identify the mutations associated with different clinical phenotypes and to define (either pre- or postnatally) the state of heterozygotes, homozygotes or compound heterozygotes in a family in which known mutation(s) are segregating.

Although Tay-Sachs carrier frequency is low among non-Jewish populations high frequency of carriers is found among relatives of patients. Therefore, the most frequent use of carrier testing is for genetic counselling. The test with highest preditive value (maximum sensibility and sensitivity) of carrier state was shown to result from the combination of both DNA and enzyme-based assays (Fernandes et al., 1992a; Triggs-Raine et al., 1990). In the Ashkenazi Jewish population, the mutations +TATC1278, G->C +1IVS-12 and G805A (Gly269Ser) represent about 75-79%, 15% and 4% of alleles of carriers (as defined enzimatically by conventional assay) (Grebner and Tomczak, 1991; Landels et al., 1991b; Paw et al., 1990b; Triggs-Raine et al., 1990), whereas in non-Jewish population they represent 16-20%, 0% and 3-5%, respectively (Paw et al., 1990b). Therefore, these mutations account for about 94-98% of alleles in Ashkenazi Jewish carriers and for only 19-25% in non-Jewish population. While in certain ethnic groups where a few mutations account for the α-subunit deficiency (such as the Ashkenazi Jewish population) the identification of carriers is reliable through DNA-based assays only, in populations where a high molecular heterogeneity is found the use of this methodology as first approach is not feasible. Thus, it is generally considered that for initial carrier population screening an enzyme-based test is regarded as optimal, since it identifies the vast majority of mutant genotypes, while DNA tests are limited by their unique specificity for each particular mutation. Mutation analysis is then essential to define the heterozygous state at genetic level.

❑ Genotype-phenotype correlation

One can expect that the more drastic the enzyme deficiency, the more severe the disorder, and conversely, the higher the residual activity of the mutant enzyme, the greater the protection against disease. Conzelmann and Sandhoff (1991) have provided a formal kinetic treatment of this concept. Their model proposes a critical threshold of activity, above which the enzyme is

capable of keeping with substrate influx in and below which it cannot keep up and accumulation of undegraded substrate occurs in lysosomes. The critical threshold and the rate of accumulation can differ between cells (e.g. between subsets of neurons), or between the same cells at different times in development, depending on the rate of influx of the substrate into lysosome. Small differences in the residual activity below a critical threshold could significantly affect the severity of clinical and pathological features of the disease, but differences in levels of enzymatic activity above the threshold would have no effect. Experimental verification of this model showed a good inverse correlation of residual activity of Hex A and arylsulfatase A with the severity of G_{M2}-gangliosidosis and metachromatic leukodystrophy, respectively.

More recently, studies on clinical heterogeneity have focused on the allelic diversity, by analysis of the genotype of patients. As consequence of the multiplicity of rare mutant alleles, the majority of TSD patients identified to date were compound heterozygotes. In general, mutations that affect the synthesis or stability of mRNA, which results in complete absence of enzyme activity, are associated with early onset symptoms and a severe clinical course. Mutations compatible with the production of stable normal-sized mRNA and a residual catalytic activity, are associated with later onset and slower progression of the disease. Correlation between genotype and the clinical phenotype have been particularly important for the prognosis of these diseases and counselling of carriers.

❑ Genetic epidemiology of Tay-Sachs disease

Tay-Sachs disease is prevalent among Ashkenazi Jewish population which were isolated for several centuries due to cultural reasons. Ashkenazi Jews communities have been therefore subjected to detailed genetic analysis.

In 1960, the incidence of Tay-Sachs disease was determined in North American Ashkenazi Jewish population and non-Jewish population through data obtained from death certificate information. In Jewish population, the incidence was about 0.0025 (1 in 4000 births). By Hardy-Weinberg analysis, the gene frequency was estimated to be 0.016 (1 in 63) and that of carriers to be 0.031 (1 in 32). In non-Jews, the disease incidence was observed to be 100 times less, corresponding to a tenfold lower carrier frequency population (1 in 316) (rev. by Sandhoff et al., 1989).

Since 1970, large-scale carrier screening programs employing enzymatic assays (Kaback et al., 1970) have been applied to the major populations centers of Ashkenazi Jews throughout North America and Israel with the purpose to reduce the incidence of the disease. Based on this data, the frequency of enzyme-defined carriers has been estimated to be 0.032 (1 em 31) in Jews, about five times higher than the one observed in non-Jews (Kaback et al., 1993). The comparison of Tay-Sachs carrier frequency among Jews originated from different european countries suggested that the gene of Tay-Sachs disease in this ethnic group occurred after 70 A.D. (the second diaspora of the Jews from Palestine) and before 1100 A.D. in areas of central and eastern Europe (Petersen et al., 1983).

The relatively high frequencies of Tay-Sachs gene defects in the Ashkenazi Jewish population, and the persistence of the same defects in non-Jewish populations of diverse geographical origin has been attributed to founder effects with genetic drift or to some selective environmental factor that confers biologic advantage to heterozygotes (rev. by Bach et al., 1992; Jorde, 1992). These factors may not be mutually exclusive. The hypothesis of heterozygote advantage has arisen from the observation that many lysosomal storage disorders, genetically distinct from the TSD but presenting a recessive pattern of inheritance, occur at a high frequency in Ashkenazi Jewish population. The environmental factor has been suggested to be pulmonary disease, particularly tuberculosis that have flagelated for many centuries the populations from eastern and central Europe where the Ashkenazim lived, in relation to which the heterozygous would present an increased fitness. The geographic distribution of Tay-Sachs gene frequencies (Petersen et al., 1983) appears to support this argument. However this argument is, by its nature, circumstantial and difficult to verify. The use of animal models may help to test such hypothesis.

❑ Prospects for therapy

In general, lysosomal storage disorders are considered good candidates for replacement therapy since a small increase in the residual activity of the enzyme is probably sufficient to prevent storage, and subsequently the occurrence of cell damage and clinical abnormalities (Conzelmann and Sandhoff, 1991). However, no effective therapy for G_{M2}-gangliosidoses, or for other lysosomal storage diseases with central nervous system involvement, have been implemented so far. The treatment is restricted to supportive care

and appropriate management of intervening problems (nutrition and hydratation, infectious disease and control of seizures when they occur).

The treatment of these disorders involves replacing of the deficient enzyme with a normal one from an exogenous source. The major methods of enzyme replacement are the direct replacement of purified enzyme, tissue or organ transplantation, and insertion of a normally functioning gene.

Several attemps have been made with enzyme replacement therapy for patients with infantile G_{M2}-gangliosidosis, by intravenous (Johnson et al., 1973) or intrathecal (von Specht et al., 199) administration of purified human Hex A. The results were disappointing, since in both cases the enzyme was not apparently transferred to the central nervous system (CNS).

Other alternatives have been pursued, including bone marrow transplantation. Bone marrow transplantation was attempted based on the hypothesis that cells derived from hematopoietic progenitors of the donor (circulating leukocytes and tissue macrophages) can donate lysosomal enzyme to the deficient cells in all tissues of the host, either through secretion or direct cell-cell interaction. Clinical and biochemical benefit has been observed in patients with lysosomal storage diseases involving peripheral organs (Krivit and Paul, 1986). Beneficial effects on neuronal pathology have been found in the twitcher mouse, which has a galactocerebrosidase deficiency analogous to Krabbe disease in the human, and the blood-brain barrier is believed to have been crossed by donor-derived macrophages (Hoogerbrugge et al., 1988). On the other hand, bone marrow transplantation had no effect on the progression of neurological disease in dogs with G_{M1}-gangliosidosis (O'Brien et al., 1990). Thus, it remained unclear if storage disorders involving the CNS could benefit significantly from this form of therapy. Firstly, bone marrow transplatation has high mortality and morbidity, although this can be partially overcome through a genetically modified autologous transplant; secondly, there is no experimental evidence regarding the extent to which different types of cells can enter the CNS and whether, once having transversed the blood-brain barrier, they can provide adequate enzyme replacement for neuronal cells.

In order to circumvent the blood-brain barrier some advances have been made using either viral vectors carrying Hex A cDNA (Akli et al., 1995) or fusion proteins (the protein of interest is fused to a protein or peptide that has a high affinity for neuronal cells). In cultured rat neuronal cells the Hex A coupled to fragment C of tetanus toxin was found to be taken up more rapidly than the unmodified Hex A (Dobrenis et al., 1992). In a feline model of Sandhoff disease,

the cells exposed to NGF-Hex A conjugate presented no immunodetectable ganglioside G_{M2}, while the unmodified Hex A had minimal effect (Friden et al., 1993). In the near future, the reversible disruption of the blood-brain barrier by hypertonic mannitol (rev. by Neuwelt et al., 1995) might be a promising approach to transfer to CNS high molecular weight compounds, such as the Hex A, and viruses that otherwise would not transverse the blood-brain barrier.

Mouse models for Tay-Sachs (Yamanaka et al., 1994a) and Sandhoff disease (Sango et al., 1995) have been recently created by disruption of the corresponding genes in embryonic stem cells. Unlike the two human disorders, the two mouse models show very different phenotypes. Although exhibiting biochemical and pathological features of the disease, the Tay-Sachs model showed no neurological abnormalities whereas Sandhoff model was severely affected. The phenotypic difference between the two mouse models was the result of differences in the ganglioside catabolic pathway in mice and humans. Whereas in humans, the ganglioside G_{M2} is almost exclusively degraded by Hex A to form G_{M3}, mice, in addition, convert G_{M2} in G_{A2} by the action of sialidase(s). The higher sialidase specificity in mice may allow the degradation, albeit indirectly, by Hex B (this isoenzyme has a low activity gainst G_{A2} in the presence of the activator protein), thus explaining the unexpectedly mild phenotype of Tay-Sachs model, missing only Hex A (Sango et al., 1995). These animal models are undoubtedly invaluable to device strategies for the introduction of functional genes and enzymes in CNS and also for studies on the pathogenic mechanism(s) of neuronal dysfunction in gangliosidosis.

1.2. CLINICAL CHARACTERISTICS OF PORTUGUESE PATIENTS

In the present work, 30 Portuguese patients with Tay-Sachs disease and variants, belonging to 23 unrelated families, were studied. The relevant clinical features on these patients are summarised in Table 1. The clinical phenotype was widely variable, comprising infantile (Tay-Sachs disease) and late-onset forms. Patients affected with late-onset forms presented the subacute or chronic phenotypes as the disease was compatible with survival into childhood /adolescence, or adulthood, respectively. The subacute phenotype was the one more often observed, contrasting with that reported in the majority of patients described so far (rev. in Introduction, Section 1.1.4, Clinical and genetic heterogeneity). Among patients with the subacute phenotype, a group of fourteen patients (PG) with a juvenile onset presented a homogeneous clinical picture (M. Maia, personal communication).

TABLE 1: CLINICAL DATA ON 30 PORTUGUESE PATIENTS WITH TAY-SACHS DISEASE AND VARIANTS.

PATIENTS	SEX	INITIAL SYMPTOM	AGE AT ONSET	PRESENT AGE OR AGE AT DEATH	CLINICAL PHENOTYPE
P1 (SM)	F	Dementia	6 months (I)	18 months (deceased)	Acute
P2 (SF)	F	Dementia	6 months (I)	NA (deceased)	Acute
P3 (SG)	F	Motor weakness	9 months (I)	NA (deceased)	Acute
P4 (AA)	M	NA	3 years (J)	20 years	Chronic
P5 (CM)	M	Gait disturbance and Language delay	3-4 years (J)	25 years	Chronic
P6 (DO)	F	Gait disturbance	14 months (LI)	5½ years (deceased)	Subacute
P7 (VM)	F	Ataxia	24 months (LI)	5 years (deceased)	Subacute
P8 (DN)	M	Gait disturbance	5 years (J)	11 years	Subacute
PG	12 F; 7M	Gait disturbance (13 cases)	3-7 years (J)	8-14 years	Subacute
(n=19)[a]		or Language delay (6 cases)			
P9 (JS)[b]	M	Behavioural alterations	11 years (J)	32 years	Chronic
(AS)[b]	M	Behavioural alterations	7 years (J)	15 years	Chronic
(PS)[b]	M	Behavioural alterations	5 years (J)	24 years	Chronic

The identification of the patients is in accordance to their designation throughout the text. [a]These patients belong to 14 unrelated families. [b]Siblings . I, Infantile onset; LI, Late-infantile onset; J, Juvenile onset. NA, Not available.

AIMS OF THE INVESTIGATION DESCRIBED IN THIS THESIS

The experimental work described in this thesis concerns the study of a human lysosomal enzyme, the β-hexosaminidase. A sample of 23 unrelated Portuguese patients affected with Tay-Sachs disease and variants was used to perform studies at the gene and protein levels. The main aims of the work reported here were:

(i) To contribute to extend the knowledge on genotype-phenotype correlation and molecular mechanisms leading to the α-subunit deficiency.

(ii) To establish the distribution of α-chain gene mutations in the Portuguese population.

To accomplish these aims, biological material from patients was used to characterise the deficiency. The enzymatic activity of hexosaminidase was determined in different biological samples, such as tissues (brain), cells (leukocytes, fibroblasts and lymphoblasts) and serum (Chapter 2). The mutations associated with both variants, B and B1, were then characterised (Chapter 3). The effect of some mutations on protein maturation was studied (Chapter 4).

(iii) To evaluate the possibility of B1 variant carrier identification through an enzymatic-based assay, with the purpose to determine carrier frequency as well as to screen populations at risk and by genetic counselling reduce carrier frequency (Chapter 5).

CHAPTER 2

ENZYMATIC ACTIVITY OF HEXOSAMINIDASE IN TAY-SACHS DISEASE AND VARIANTS

2.1. INTRODUCTION

The B and B1 variants of G_{M2}-gangliosidosis are caused by defects on the α-subunit of Hex A (rev. in Introduction, Section 1.1.4., The G_{M2}-gangliosidoses).

The diagnosis of these disorders is based on the determination of Hex A activity, usually in leukocytes, serum or skin fibroblasts, against fluorogenic or chromogenic synthetic substrates. The separation of hexosaminidase isoenzymes is usually accomplished by ion-exchange chromatography or, most commonly by a more rapid and still relatively accurate method, the thermal inactivation assay. The diagnosis of B variant is established through the absence of Hex A activity against both neutral and sulphated substrates. The diagnosis of B1 variant is demonstrated by the presence of a catalytically active Hex A against the unsulphated substrate but inactive towards the sulphated substrate (rev. in Introduction, Section 1.1.4, Diagnosis, Enzymatic assay). Further enzymatic assays, such as the determination of Hex A activity against the natural substrate, the ganglioside G_{M2}, in the presence of the activator protein, may be useful for discrimination between patients with different clinical variants. In B variant patients, the Hex A activity against ganglioside G_{M2}, in the presence of activator protein, under specific experimental conditions (presumably close to those occurring *in vivo*), was found to be inversely correlated with the severity of the disease, 0.4% in infantile, 3.0% in subacute, 3.6% in chronic and 20% in healthy probands with low Hex A activity (Conzelmann et al., 1983). However, the requirement in this natural-substrate assay for appropriately labelling ganglioside G_{M2} as well as the need for fresh G_{M2} activator, usually purified from postmortem human kidney, make this method difficult, time consuming and expensive.

In the beginning of the study reported here, the majority of B1 patients described in the literature presented a late-infantile onset (Charrow et al., 1985; Goldman et al., 1980; Inui et al., 1983; Kytzia et al., 1983; Li et al., 1981b), except one who had a juvenile onset of the disease (Goebel et al., 1989). The molecular defect(s) underlying the disease was unknown. The study of Hex A activity in leukocytes and serum of patient's parents suggested that the majority of patients were genetic compounds. In fact, since this is a recessively inherited disease and genetically heterogeneous, a high number of combinations of compound heterozygous genotypes is likely to occur. In addition, the use of different biological materials and methods led to a heterogeneous enzymological data as reported in the literature that was difficult to combine in terms of correlation between the biochemical and clinical phenotype. The

experimental work described in this chapter was therefore carried out with the purpose to establish the enzymatic differences among Portuguese B and B1 variant patients who presented a heterogeneous clinical phenotype.

2.2. MATERIALS AND METHODS

2.2.1. Commercial materials

4-Methyllumbelliferyl-N-acetylglucosamine (4MU-GlcNAc) was purchased from Sigma and its sulphated derivative (4MU-GlcNAcS) from Moscerdam (The Netherlands). The commercial preparation of the sulphated substrate was purified by chromatographic procedures as described by Beccari et al. (1987). DE-52 cellulose was obtained from Whatman Biosystems, cellogel strips from Chemetron and bovine serum albumine (BSA) from Sigma. Cell culture reagents were from Gibco BRL. All other reagents were from Merck (pro-analysis grade).

2.2.2. Biological materials

Human leukocytes were isolated from peripheral blood by dextran sedimentation (Skoog and Beck, 1956). Human serum was prepared by blood retraction at room temperature. Epstein-Barr Virus (EBV)-transformed human lymphoblast cell lines were established as described (Neitzel, 1986). Human fibroblast cultures were established from forearm punch biopsies (skin fibroblasts). The cells were grown and propagated in RPMI medium supplemented with antibiotics and 5% foetal calf serum at 37°C in 5% CO_2. Brain from a B1 variant patient that died at the age of 14 years was collected after biopsy, and the different lobes immediately separated, frozen in N_2 (l), and stored at -70°C. Cell-pellets were extracted by brief sonication (30 impulses of 40W each during 6s with intervals of 4s, in Sonicator™ W375) in 0.1% (v/v) triton X-100. After centrifugation for 30 min at 15 000 x g the supernantant (reffered to as extract) was assayed for enzyme activities. Serum was diluted 1:10 in NaCl 0.9% immediately before use. Brain tissue was homogenized in Triton X-100 0.1% (v/v), sonicated and centrifuged for 1h at 50 000 x g.

2.2.3. Enzymatic assays

The hexosaminidase activity was determined against the synthetic neutral (4MU-GlcNAc) and sulphated (4MU-GlcNAcS), based on the assay conditions previously described (Inui and Wenger, 1984; Stirling, 1984). Total extracts were incubated with 4MU-GlcNAc (1.7 mM final concentration) in 0.1 M citrate-

phosphate buffer pH 4.4 at 37°C for 15 min or with 4MU-GlcNAcS (1.0 mM final concentration) in 0.2 M citrate-phosphate buffer pH 4.0 at 37°C for 1 h. The reactions were stopped by the addition of 1.0 ml of 1 M of glycine-NaOH pH 10.0 and the fluorescence of the liberated 4-methylumbelliferone was measured in a Aminco SPR spectrofluorometer with excitation and emmision wavelenghts of 366 and 466 nm, respectively, and compared with that of a standard solution of known concentration of 4-methylumbelliferone. Protein was determined according to Lowry et al. (1951) using BSA as standard.

The determination of Hex A activity against the physiological substrate in the presence of activator protein was performed by Dr. E. Conzelmman (Bonn, Germany) as previously described (Conzelmann et al., 1983).

2.2.4. DEAE-cellulose chromatography

Ion-exchange chromatography was performed essentially as described by Nakagawa et al. (1977). Cellular extracts and/or serum corresponding to about 1000 nmol/h of hexosaminidase activity against 4MU-GlcNAc was mixed with equal amount of 10 mM sodium-phosphate buffer pH 6.0. The diluted sample was applied to 1 ml DE-52 column previously equilibrated with 10 mM sodium-phosphate buffer pH 6.0 and 0.6 ml fractions were collected. After elution of Hex B, the remaining isoenzymes were eluted using a linear NaCl gradient (0 to 0.35 M in 12 ml of the column buffer). The enzymatic activities of the eluted fractions were determined against 4MU-GlcNAc and 4MU-GlcNAcS. The activity corresponding to the main peak eluted during NaCl gradient was expressed as the percentage of total 4MU-GlcNAc recovered activity and reffered to as 4MU-GlcNAc % Hex A activity.

2.2.5. Cellulose acetate gel electrophoresis

Cellulose acetate gel electrophoresis of hexosaminidase was performed on Cellogel strips (5.7 cm x 14.0 cm) in 40 mM potassium-phosphate buffer pH 6.0 at 4°C during 1.5 h at 1mA/cm (Poenaru and Dreyfus, 1973). After electrophoresis hexosaminidase activity was revealed by incubation at 37°C with 4MU-GlcNAc, 2.0 mM in 0.1 citrate-phosphate buffer pH 4.75 for 10 min or with 4MU-GlcNAcS, 2.0 mM in 0.1 M citrate-phosphate buffer pH 4.0 for 45 min. The fluorescence was developed with 1 M glycine-NaOH, pH 10.0, visualised at 366 nm and photographed with type 667 Polaroid film.

2.3. RESULTS

The enzymatic study of hexosaminidase was carried out in 30 patients belonging to 23 unrelated families. These patients presented a variable clinical phenotype, comprising the infantile acute (P1 to P3), subacute (P6 to P8 and PG) and chronic (P4, P5 and P9) forms. For patients other than PG, only leukocytes were available.

The activity of hexosaminidase isoenzymes present in leukocytes was initially analysed by electrophoresis on cellulose acetate gel using the neutral (4MU-GlcNAc) and sulphated (4MU-GlcNAcS) synthetic substrates (Fig. 1). As it can be observed, no activity of Hex A against both synthetic substrates was detected in patients P1 and P5 (lanes I1, I2 and II1, II2). A similar electrophoretic pattern was also observed in patients P2 to P4 (data not shown). The mobility of Hex A and Hex B (revealed with 4MU-GlcNAc) is similar in the control (lane Ic) and in the patients PG (lane I7) and P6 to P9 (lanes I3 to I6). With the sulphated substrate, no activity corresponding to Hex A was present in cells from patients PG (lane II7) and P6 to P9 (lanes II3 to II6) in contrast to that observed in the control (lane IIc). For the same amount of applied protein, the Hex A activity in patient PG (lane I7) was clearly higher than that observed in patients P6 to P9 (lane I3 to I6); the Hex B activity present in patient PG (lane I7) seems to be slighty lower than that observed in the patients P6 to P9 (lane I3 to I6). Based on the different activity of Hex A and Hex B against the neutral and sulphated synthetic substrates two groups of patients were distinguished, P1 to P5 with variant B, and PG and P6 to P9 with variant B1.

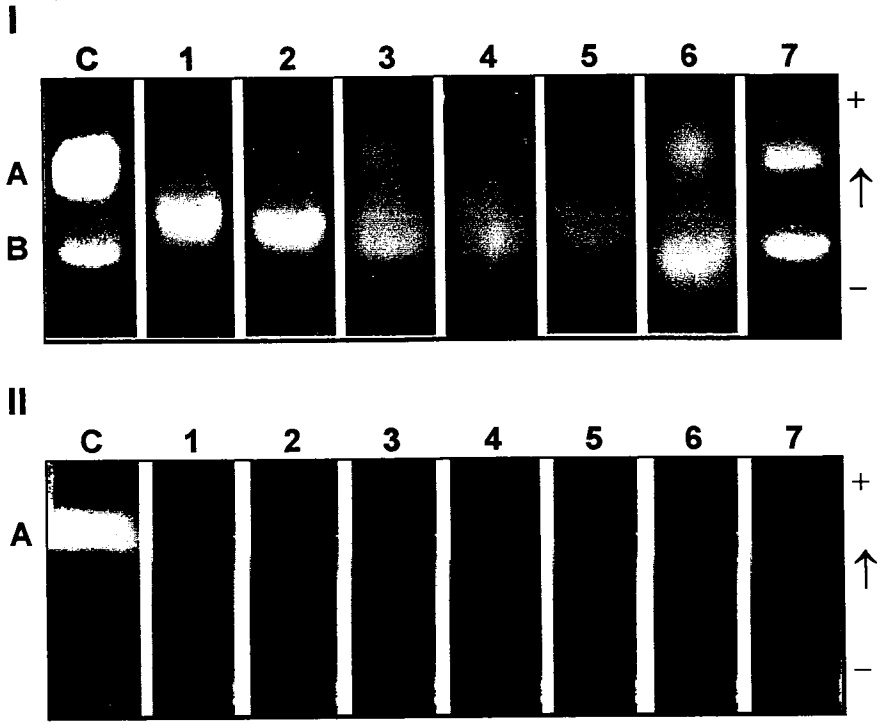


Fig. 1: Cellulose acetate gel electrophoretic profile of hexosaminidase in leukocyte extracts. After electrophoresis hexosaminidase activity was visualised with 4MU-GlcNAc (I) and 4MU-GlcNAcS (II) as described in Materials and methods. Lane c, control; Lane 1, patient P1; lane 2, patient P5; lane 3, patient P6; lane 4, patient P7; lane 5, patient P8; lane 6, patient P9, lane 7, patient PG.

The electrophoretic profile of hexosaminidase observed in brain, fibroblasts, lymphoblasts and serum from B1 patients PG is represented in Fig 2. With exception of brain, in which a reduced Hex A activity is observed, in the other samples the electrophoretic profile is similar to that of control.

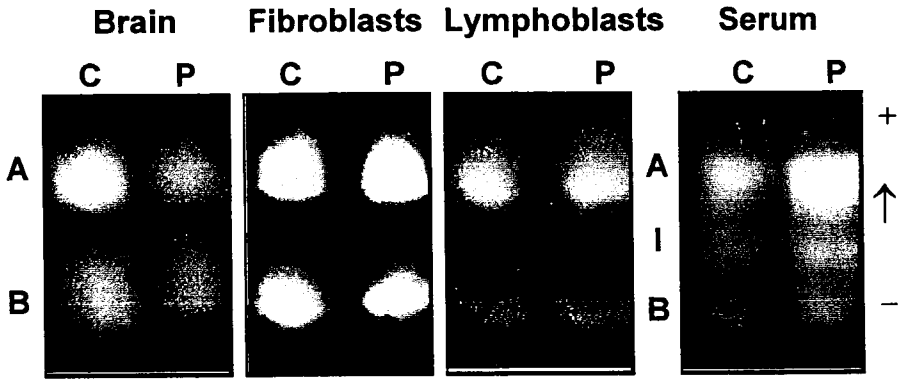


Fig. 2: Cellulose acetate gel electrophoretic profile of hexosaminidase in brain, fibroblasts, lymphoblasts and serum from B1 variant patient PG. After electrophoresis hexosaminidase activity was visualised with 4MU-GlcNAc as described in Materials and methods. Lane C, control; lane P, patient PG.

The electrophoretic profile of hexosaminidase activity in cellulose acetate gel allowed a clear distinction between B and B1 variant patients. However, this qualitative assay cannot replace the ion-exchange chromatography.

The enzymatic activity of hexosaminidase isoenzymes separated by DEAE-cellulose chromatography was therefore determined in leukocyte extracts against the synthetic substrates 4MU-GlcNAc and 4MU-GlcNAcS. The results are shown in Table 1A. The cromatographic profile obtained for a B and B1 variant patient and a control is exemplified in Fig. 3. In leukocytes from all patients, the residual Hex A activity towards 4MU-GlcNAcS was less than 5.5% of the control mean value. The activity of Hex A against 4MU-GlcNAc was undetectable in B variant patients, and corresponded, in B1 variant patients, to about 26.0% in patients P6 to P8, 55.2 % in patient P9 (and siblings) and 60.4% in patients PG.

TABLE 1A: ENZYMATIC ACTIVITY OF HEXOSAMINIDASE IN LEUKOCYTES[a].

	4MU-GlcNAc		4MU-GlcNAcS	
	Total Hex	**% Hex A**	**Hex A**	**β-Gal**
B variant				
P1 (SM)	1200	0	3.49	257
P2 (SF)	1737	0	3.60	148
P3 (SG)	957	0	NA	150
P4 (AA)	1592	0	7.50	193
P5 (CM)	1087	0	14.0	109
mean	1315	0	7.15	171
range	957-1737		3.49-14.0	109-257
B1 variant				
P6 (DO)	1269	24.4	4.00	192
P7 (VM)	664	26.4	4.10	153
P8 (DN)	1022	27.1	3.80	123
P9 (JS)	872	56.3	7.58	239
(AS)*	1058	56.6	8.54	211
(PS)*	785	52.7	5.60	173
PG				
mean	1045	60.4	2.60	220
range	610-1934	42.5-70.3	0.59-7.00	126-310
n=	19	19	19	19
Control				
mean	1523	87.1	255	217
range	967-2294	81.0-91.4	128-319	109-380
n=	54	11	54	54

[a]The hexosaminidase activity was determined in cellular extracts (nmol/h/mg protein) against the substrates 4MU-GlcNAc (Total Hex) and 4MU-GlcNAcS (Hex A); the activity of Hex A (4MU-GlcNAc) obtained after separation of isoenzymes by DEAE-cellulose ion-exchange chromatography was expressed as % of total recovered activity (% Hex A); β-Gal, β-galactosidase, used as reference lysosomal enzyme. NA, not available. *Siblings of patient P9.

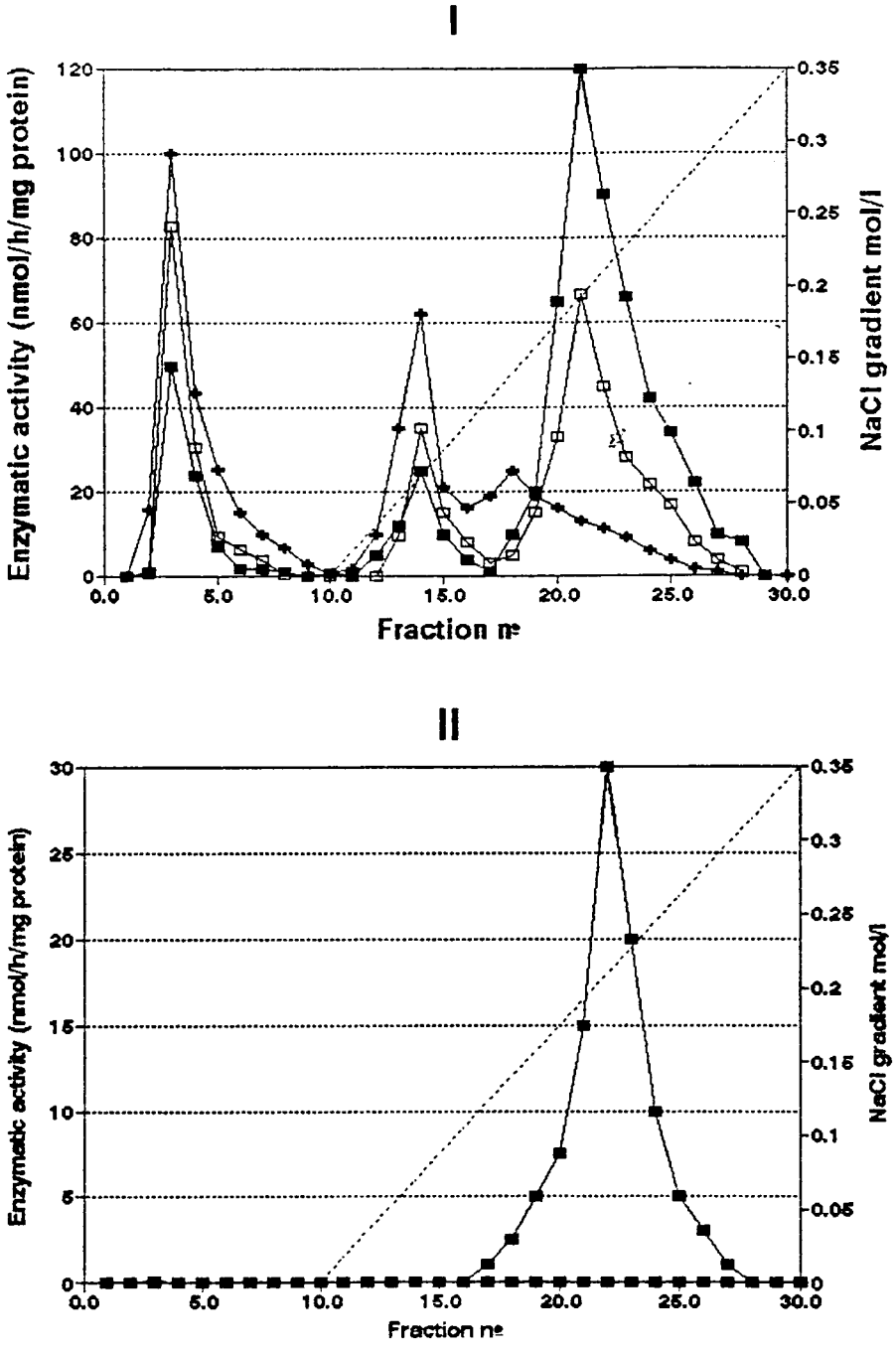


Fig 3: Ion-exchange chromatographic profile of hexosaminidase present in leukocytes. The enzymatic activities were determined against 4MU-GlcNAc (I) and 4MU-GlcNAcS (II). ■, control; +, B variant patient (P1); □, B1 variant patient (PG).

The group of B1 variant patients designated here as PG was further studied by determining the hexosaminidase activity in other biological samples (brain, lymphoblasts, fibroblasts and serum). The results are shown in Table 1B. In all biological samples analyzed the % Hex A (4MU-GlcNAc) present in patients PG was lower than the corresponding control mean value.

In fibroblasts from two patients PG, the activity of Hex A against ganglioside G_{M2}, in the presence of the activator protein, corresponded to 1.5 and 3.3% of control enzyme activity (data obtained by E. Conzelmann).

TABLE 1B: ENZYMATIC ACTIVITY OF HEXOSAMINIDASE IN FIBROBLAST, LYMPHOBLASTS, BRAIN AND SERUM[a].

	4MU-GlcNAc		4MU-GlcNAcS	
	Total Hex	**% Hex A**	**Hex A**	**β-Gal**
Fibroblasts				
B1 variant (PG)				
mean	5182	50.3	10.1	440
range	2625-8265	39.7-62.2	4.40-17.0	281-898
n=	10	10	10	10
Control				
mean	7545	55.8	527	689
range	4421-12353	50.0-62.0	250-784	299-1271
n=	15	8	15	15
Lymphoblasts				
B1 variant (PG19)				
mean	806	77.9	1.86	98.8
range	635-963	72.8-85.3	1.30-2.30	69.9-151
n=	10	10	10	10
Control				
mean	1091	84.3	156	96.1
range	679-1800	80.0-88.0	98.0-31.0	41.0-183
n=	24	8	24	24
Brain				
B1 variant (PG)	1073	58.5	1.70	68.1
Control	906	76.1	55.7	66.4
Serum				
B1 variant (PG)				
mean	749	57.3	1.35	16.1
range	592-1022	49.0-65.2	0.70-2.70	6.60-28.0
n=	9	9	9	9
Control				
mean	943	75.6	110	16.9
range	401-1918	68.9-79.0	65.0-172	5.00-55.0
n=	17	4	17	17

[a]The activity of hexosaminidase was determined in cellular extracts (nmol/h/mg protein) and serum (nmol/h/ml) against the substrates 4MU-GlcNAc (Total Hex) and 4MU-GlcNAcS (Hex A); the activity of Hex A (4MU-GlcNAc) obtained after separation of isoenzymes by DEAE-cellulose ion-exchange chromatography was expressed as % of total recovered activity (% Hex A); β-Gal, β-galactosidase, used as reference lysosomal enzyme.

2.4. CONCLUSION

The hexosaminidase activity was determined in 30 patients (from 23 unrelated families) clinically affected with Tay-Sachs disease and variants. Based on the activity of Hex A against the neutral and sulphated synthetic substrates, they were grouped into B variant patients (cases P1 to P5) and B1 variant patients (cases P6 to P9 and cases PG).

The deficient activity of Hex A against both synthetic substrates observed in leukocytes from B variant patients can be explained by the complete absence of enzyme protein, or by the presence of a residual amount of heterodimer $\alpha\beta$ whose activity against any of these synthetic substrates is undetectable under the enzymatic assays used. The B variant patients studied presented either an infantile or chronic phenotype. Although the *in vitro* activity of Hex A was undetectable in leukocytes from these patients, the effect of their α-chain gene defects on the *in vivo* Hex A activity against G_{M2}-ganglioside must be different to explain the different severity of the disease, thus predicting a high molecular heterogeneity for B variant-causing mutations.

In B1 variant the deficient activity of Hex A (heterodimer $\alpha\beta$) against 4MU-GlcNAcS is caused by genetic defects in the active site on the α-subunit, which do not affect the catalytic site for the hydrolysis of 4MU-GlcNAc, located on the β-subunit (Kytzia and Sandhoff, 1985). Consequently, Hex A is unable to hydrolyze the ganglioside G_{M2} and the sulphated synthetic substrates but is active towards the neutral substrates (Kytzia et al., 1983). In leukocytes from all B1 variant patients studied, no activity of Hex A against 4MU-GlcNAcS was observed, in contrast to that obtained with 4MU-GlcNAc. However, in all patients the activity of Hex A against the unsulphated substrate was lower than the control mean value, particularly in patients P6 to P8 who presented about one-half of the activity observed in patients P9 (and siblings) and PG (25% versus 55.2 and 60.4% of total hexosaminidase activity, respectively). Considering that they all must carry at least one B1-type allele, the different activity of Hex A against the unsulphated substrate can be explained by the presence of different mutations in the second allele. If the mutation produces no mature α-subunit or leads to the production of a reduced amount of $\alpha\beta$ heterodimer, such as in the B variant, the detectable enzymatic activity will be that generated by one B1 allele; however, if the mutation is compatible with the synthesis of a mature protein with the enzymological characteristics of B1 variant, also contributing with residual activity against the synthetic unsulphated substrate, the Hex A activity is expected to be correspondingly higher. Thus,

patients P6 to P8 are likely to carry a B variant mutation in the second allele whereas patients P9 (and siblings) and PG will probably carry two B1 mutations, similar or not, in the respective alleles. The effect of such mutations on the *in vivo* Hex A activity against the physiological substrate must be different to explain the clinical heterogeneity observed among these patients. From the homogeneous clinical phenotype observed among the group of fourteen unrelated patients PG, it was predicted that they all share the same genetic defect(s).

As an attemp to understand the clinical heterogeneity and the enzymological behaviour of Hex A against both synthetic substrates, the genotype of B and B1 variant patients was then characterised.

CHAPTER 3

IDENTIFICATION OF THE GENETIC DEFECTS UNDERLYING B AND B1 VARIANT

3.1. INTRODUCTION

Mutation may be defined as the process by which a structural change occurs in the genetic material (DNA). It represents the driving force behind evolution, the origin of genetic variations and the ultimate cause of hereditary disease. Point mutations and deletions are the most frequent gene lesions in the human genome. The remainder comprise insertions, duplications, inversions and rearrangements (rev. by Cooper and Schmidtke, 1991). Point mutations in promotor regions, splicing junctions, within introns and in polyadenylation sites may interfere with any stage in the pathway of expression from gene to protein product. Point mutations in coding regions may have drastic effects on protein structure, function or stability, or even on RNA splicing if it is close to an intron-exon splice junction. The frequency of mutational lesions may be strongly influenced by the surrounding DNA sequence. At CpG dinucleotides the mutation rate is about 8.5 times higher than that for the average dinucleotides. Over 90% of single-base pair substitutions observed within CpG of coding regions human genetic disease were C->T or G->A transitions, which occur through methylation-mediated deamination of 5-methylcytosine (this is the most common form of DNA modification in eukaryotic genomes and between 70-90% of 5mC occurs in CpG) (Cooper and Youssoufian, 1988).

Recombinant DNA technology has made possible the study of genetic disease at the level of the primary lesion and the *in vitro* expression of both mutant and normal alleles at the mRNA and protein levels. The most immediate practical effect from recombinant DNA technology in medical genetics has been in diagnosis, improving it. The methodology of mutation detection and analysis include cloning and sequencing, Southern and Northern blotting and PCR-based methods (deletion screening, mRNA/PCR-cDNA, chemical mismatch method, denaturing or temperature gradient gel electrophoresis, heteroduplex analysis, single-strand conformation polymorphism analysis, and direct sequencing of PCR-amplified DNA). Once the position of a mutation is known, one should be able to detect it quickly, simply and reliably. Although PCR/direct sequencing may be used, there are a number of alternatives such as restriction fragment lenght polymorphism analysis, dot-blotting and detection by using allele-specific oligonucleotides, competitive oligonucletide priming during PCR amplification or single nucleotide primer extension (rev. by Cooper and Krawczak, 1993).

A wide genetic heterogeneity is associated with the hexosaminidase α-subunit deficiency. To date, up to 54 different mutations have been reported, most of them being point mutations in either the coding sequence or the splicing junctions of HEXA gene (rev. in Introduction, Section 1.1.4., Genetic heterogeneity).

High frequencies of specific mutations associated with the B variant have been found in particular populations:
(i) In Ashkenazim two HEXA mutations were found to be associated with TSD, +TATC1278 (Myerowitz and Costigan, 1988) and a G-to-C alteration at +1IVS-12 (Arpaia et al., 1988; Myerowitz, 1988), which accounts for about 90% of mutant alleles. The major mutant allele is the frameshift mutation, accounting for about 75-79% of alleles examined. So far, the splicing mutation has been identified only in patients with Ashkenazi Jewish origin. A third mutation, the transition G805A, translating into Gly269Ser substitution, has been found in essentially all patients identified to date with late-onset (adult or chronic) G_{M2}-gangliosidosis (Navon and Proia, 1989; Paw et al., 1989).
(ii) In French-Canadian patients, the most frequent mutation is the 7.6-kb deletion at the 5' of the HEXA gene, that have probably arisen from recombination between two Alu I sequences (Myerowitz and Hogikyan, 1986; Myerowitz and Hogikyan, 1987).
(iii) Among Moroccan Jewish patients a three-base deletion, ΔTTC910-912 in exon 8, resulting in the loss of Phe304 ou 305, is often observed (Drucker et al., 1992; Navon and Proia, 1991).
(iv) Among non-Jewish patients of Celtic (English/Scotish/Irish) or French origin the G->A +1IVS-9 transition is particularly frequent (Akerman et al., 1992; Akli et al., 1991; Landels et al., 1992; Landels et al., 1993).
(v) Among the Japanese the transversion G->T at -1IVS-5 accounted for 80% of the mutant alleles examined (Tanaka et al., 1993; Tanaka et al., 1994).
Besides these ethnically restricted forms, the majority of mutations were found in multiple cases of different ethnic origin or confined to just one family.

In B1 variant, five different mutations have been reported, four of them found in sporadic cases, C532T (Tanaka et al., 1990), G533T (Triggs-Raine et al., 1991), G574C (Ainsworth and Coulter-Mackie, 1992) and G772C (Fernandes et al., 1992b). The other mutation, the transition G533A, translating into arginine-to-histidine substitution at residue 178 (Ohno and Suzuki, 1988a) is panethnically distributed and occurs at low frequency (Tanaka et al., 1990).

In order to establish the distribution of mutations and to better understand the clinical spectrum and the enzymatic profiles observed among Portuguese patients with B and B1 variant, we carried out genotype studies next presented.

3.2. MATERIALS AND METHODS

3.2.1. Commercial materials

MaeII restriction enzyme was purchased from Boehringer Mannheim Biochemica, AluI and NlaIII restriction enzymes and T4 polynucleotide kinase were purchased from New England Biolabs. [γ-^{32}P]ATP (>5000 Ci/mmol), [α-^{33}P]dATP (>1000 Ci/mmol), [α-^{35}S]dATP (>1000 Ci/mmol) and [α-^{32}P]dCTP (>3000 Ci/mmol) from Amersham. The full-lenght normal β-hexosaminidase α-subunit cDNA was kindly provided by Prof. K. Suzuki (Chapel Hill, USA). Sources for non-standard materials will be indicated below when appropriate.

3.2.2. PCR amplification of genomic DNA

Genomic DNA was isolated from lymphoblasts or buffy coats of nucleated cells obtained from anticoagulant blood according to standard procedures (Maniatis et al., 1982; Miller et al., 1988). The exons and splicing junctions were amplified from 0.1 to 0.3 μg DNA, in a 100 μl volume containing 0.2 mM of each nucleotide, 1.0 μM primers, 2.5 units of Taq polymerase (Perkin-Elmer Cetus) in the buffer recommended by Cetus. Primers for each of the 14 HEXA exons are represented in Table 1. With exception of exon 12 upstream primer and both exon 13 upstream and downstream primers, all the others were those as described by Triggs-Raine et al. (1991).

TABLE 1: OLIGONUCLEOTIDES FOR AMPLIFICATION OF HEXA GENE.

Exon and primer sequence[a]	Annealing temperature (Td-10) (°C)	Fragment size (bp)[b]
1:		
CTCACCTGACCAGGGTCTCAGGT	64	502
CTCCTGATTGAACCGTAGTCCTA	58	
2:		
GCTTCTAATGGCAGGTTGGCCGCA	66	174
GCTCTTCTAAGACAGGGAACAGGA	62	
3:		
GAATATCTGGTCTATAATCTGAG	52	226
TGAGCAGGGACTGGGTTACTGCA	62	
4:		
GCTCTGCTACATTGAGAACCTTCC	62	203
CTAGGATTCTCAATATTGGGATCC	58	
5:		
ATCTCCCTGTGCCCCCATAGTAA	60	229
TGCTCCATCACCCTAGAACTCTTA	60	
6:		
TGAAACCGGAGAGACTGTGATG	56	211
GCCACAGCCAGATTCAGACATTG	60	
7:		
GCTAGCTTTCAGGAAGTGTGAACC	62	221
TAACAAGCAGAGTCCCTCTCTGGT	56	
8:		
ATGTATTTGTGACACTCATATGGG	56	269
AGTAAGCAACTGATCAGGCCACAG	62	
9:		
CAGGCATTAGGCTTTCAGGATGTT	60	230
CAAGCAGGGCCTGACTCGGTATG	64	
10:		
GTCTAGAACCCATCTGAGCTAAG	58	237
AAGCCCAATCCAAACCAGGAGGA	60	
11:		
CCAGGAATCTCCTCAGCTTTGTGT	62	276
AGCCTCCTTTGGTTAGCAAGC	54	
12:		
AGTTACCCCACCATCACCAGACTG	64	215
GTGTGGCGAGAGGATATTCCA	54	
13:		
TTTTCCTCCAGGCCCAGAGCA	56	122
GCTTACCTCAGCAACTCACAG	54	
14:		
TGACTGGTGTGAAAAGTGTTGCTG	60	273
CCTTTCTCTCCAAGCACAGG	52	

[a]Sequences of 14 primer pairs used to amplify exons, given 5' to 3'. [b]Expected size of amplified fragment. All fragments were amplified using 30 cycles of amplification, each consisting of 30s denaturation at 94°C, 30s annealing at 60°C and 90s extension at 72° C, excepting for the fragments encompassing the exon 7 and 13 that were amplified using 30s denaturation at 94°C, 30s annealing at 55°C and 90s extension at 72°C.

3.2.3. SSCP analysis

Two methods were used:

(A) Isotopic method. In the PCR reaction the concentration of dCTP reaction was reduced to 0.1 mM and 10μCi of [α-^{32}P]dCTP was added. The labelled PCR product was diluted 20 fold in 1% SDS, 20 mM EDTA. A 5μl aliquot of the dilution was mixed with 15μl of formamide loading dye (95% formamide, 10mM EDTA, 0.05% bromophenol blue and 0.05% xylene-cyanol).

(B) Non-isotopic-method. 10 μl of PCR product was mixed with 5 μl of 1% SDS, 20 mM EDTA (pH 8.0) and 10 μl of formamide loading dye (95% formamide, 20mM EDTA, 0.05% bromophenol blue and 0.05% xylene-cyanol).

The samples where denatured by heating at 96°C for 15 minutes, cooled on ice and loaded onto a 0.75mm x 28.0 cm x 14.0 cm 1xHydrolink MDE gel (Bioprobe Systems) / 0.6xTBE. The conformational polymorphisms were visualised by silver staining according to the method described by Budowle et al. (1991). The samples in which mutations could not be detected were also analysed on 0.5xMDE gel and the electrophoresis carried out at room temperature or at 4°C. When an exon was predicted to contain a mutation by mobility shift in a SSCP gel, the corresponding segment of genomic DNA was subjected to asymmetric amplification and subsequently sequenced.

3.2.4. Direct sequencing of PCR products

Two methods were used to sequence different samples:

(A) Isotopic. A portion (2μl) of the first amplification reaction was subjected to asymmetric amplification using only one primer. The asymetric PCR products were separated from primers, nucleotides and salts using QIAGEN kit (Germany) according to the manufacturer's procedure. Another procedure used was as follows: 1.0 to 2.0 μg of genomic DNA was subjected to amplification in a 100 μl volume containing 0.2 mM of each nucleotide, 0.020 μM of sense primer and 0.1 μM of antisense primer or vice-versa, and 2.5 units of Taq polymerase (Perkin-Elmer Cetus) in the buffer recommended by Cetus, using the temperature profile of symmetric PCR. The asymmetric product was further purified by precipitation with 0.5 volumes of 10 M ammonium acetate and 2.5 volumes of ethanol.

Sequencing was performed using Sequenase (version 2.0) kit (US Biochemical) with either [α-^{33}P]dATP or [α-^{35}S]dATP according to the procedure recommended by the manufacturer.

(B) Non-isotopic. 100 µl of symmetric amplification was dry until about 10 µl that was applied onto a 2% Nusieve (LMP) agarose gel. The fragment corresponding to the desired size was cut out from the gel, diluted one fold in water and melted at 65°C. 10 µl were amplified using 8 pmol of upstream or downstream oligonucleotide and fluorescent dinucleotide terminators, in a total volume of 20 µl. The amplification was performed during 26 cycles, each consisting of 10s at 94°C, 5s at annealing temperature used in symmetric amplification and 4 min at 60°C. The amplified fragments were purified by phenol/chloroform extraction, precipitated with 0.5 vol of 3M sodium acetate pH 5.2 and 2.5 vol of ethanol, washed with ethanol 70% and dry under vacuum. 4 µl of formamide solution (95% formamide, 10mM EDTA pH 8.0) was added, the samples were denatured at 95°C for 5 min and put on ice until loading onto polyacrylamide denaturing gel. Sequencing was performed at Prof. Munnich's laboratory (Hôpital Necker-Enfants Malades, Paris) with an automatic DNA sequencer (Applied Biosystems).

Nucleotide numbering is based on the original coding sequence described by Myerowitz et al. (1985).

3.2.5. Restriction enzyme analysis

About 100-200 ng of the amplified PCR fragment was digested overnight in the conditions described below (Table 2). After electrophoresis the DNA bands were visualised by staining with ethidium bromide.

TABLE 2: ANALYSIS OF RESTRICTION FRAGMENT LENGHT POLYMORPHISMS.

PCR Fragment	RE	Restriction sequence	Temperature	Fragment size after digestion (bp)
Exon 7	AluI	5'-AGCT-3'	37°C	216 and 5
Exon 7	NlaIII	5'-CATG-3'	37°C	221
Exon 9	NlaIII	5'-CATG-3	37°C	230
Exon 13	MaeII	5'-ACGT-3'	50°C	85 and 37

RE, restriction enzyme.

3.2.6. Allele-specific oligonucleotide hybridization

The 4-bp insertion in exon 11 and the G->C intron 12 splicing mutation were screened by PCR-ASO probes under the conditions described elsewhere (Tanaka et al., 1990). The primers and allele-specific probes were a kind gift of Prof. Suzuki (Chapel Hill, USA). The stategy is depicted in Fig 1.

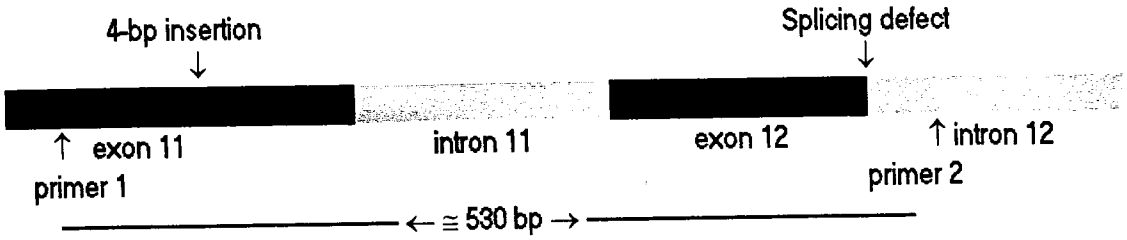


Fig. 1: Strategy for screening for the two classical infantile Ashkenazi Jewish TSD alleles (from Tanaka et al., 1990).

Two primers were used to amplify a segment encompassing both mutations: 5'-GTGTGGCGAGAGGATATT-CCA-3' (forward primer within exon 11) and 5'-AGACCCAATCCCATCTAGCCA-3' (reverse primers approximately 80 nucleotides downstream of the 5' donor site). Two pairs of allele-specific oligonucleotides were used, one pair of normal and mutant sequence for the 4-bp insertion (5'-GAACCGTATATCCTATGGC-3' and 5'-GAACCGTATATC-TATCCTA-3') and one pair of normal and mutant sequences for the splicing-defect mutation (5'-CCAGGCTCTGGTAAGGGTTTT-3' and 5'-CCAGGCTC-TGCTAAGGG-TTTT-3').

The amplified sequence was examined by agarose gel electrophoresis, and the quantity estimated by comparing with a DNA molecular weight marker. Approximately 10 ng of amplified product was applied to GeneScreen Plus membrane (NEN Research Products) and dried. The prehybridization was in 6 x SSC, 5 x Denhardt's solution, 0.05% sodium pyrophosphate, 0.5% SDS and 100 μg heat-denatured salmon-sperm DNA / ml at 37°C for 1h. Hybridization was in the same mixture, except for 2 x Denhardt's solution without SDS and the addition of 10 pmol of probe labelled at the 5' end with [γ-^{32}P]ATP in the presence of polynucleotide kinase. The specific activity of the probes was generally in the range of 1.5-2.0 x 104 cpm/mol. Hybridization was continued

overnight, with gentle shaking, at 37°C for the probes with 4-bp insertion and at 44°C for the probes for the splicing defect. These temperatures were chosen as [Td-(16-20)]°C. The membranes were washed in 6 x SSC, 0.05% sodium pyrophosphate four times for 10 min each at room temperature and then washed twice more for 15 min each in the same solution but at [Td-5]°C. the dried membrane was exposed to X-ray film at -70°C with the Cronex intensifying screen.

3.2.7. RNA preparation, northern blot analysis and cDNA-PCR amplification

Total RNA was isolated from human fibroblast cell lines according to the procedure described by Chomczynski and Sacchi (1987). Integrity of total RNA was checked by electrophoresis, through the estimation of 28S / 18S ratio. About 25 μg of total RNA were electrophoresed in a 1% agarose / formaldehyde denaturing gel and transferred to GeneScreen Plus nylon membrane (NEN Research Products). Hybridization with the full-lenght normal β-hexosaminidase α-subunit cDNA (Myerowitz et al., 1985) labelled with [α-^{32}P]dCTP were performed using the Megaprime™ DNA Labelling System (Amersham) according to the instructions of manufacturer. Blots were exposed to Kodak X-ray film at -70°C using Dupont Cronex lightning Plus intensifying screen. The β-hexosaminidase β-subunit cDNA was used as control probe.

Total RNA was reverse-transcribed into cDNA using GeneAmp RNA PCR Kit (Perkin-Elmer Cetus), according to the manufacturer's procedure with 2 μg of total fibroblast RNA, oligo $d(T)_{16}$ as primer and 1.5 h incubation at 42°C. The amplification of the segment corresponding to the entire coding sequence was performed using 40 pmol of each forward (5'-TCCGAGAGGGGAGACCAG-CGG-3', located 24 bp upstream of the A of the initiation codon) and reverse (5'-TCACCTACAGCCAGCACCCTC-3', located 36 bp downstream of the stop codon) primers and the following amplification profile: initial denaturation at 94° C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min and annealing / extension at 68°C for 5 min.

3.2.8. Southern analysis

The cDNA amplified fragments were electrophoresed in 1.2% agarose gel and transferred to GeneScreen Plus nylon membrane (NEN Research Products). Hybridizations with full-lenght normal β-hexosaminidase α-subunit cDNA (Myerowitz et al., 1985) or normal exon 7 probe (synthesized by PCR using oligonucleotides that exactly matched the ends of the exon) labelled with [α-^{32}P]dCTP were performed using the Megaprime™ DNA Labelling System

(Amersham) according to the instructions of manufacturer. Blots were exposed to Kodak X-ray film at -70°C using DuPont Cronex lightning Plus intensifying screen.

3.2.9. Subcloning and sequencing of PCR products

The mRNA was reverse transcribed as described in the section 3.2.7. of Materials and methods. The cDNA was amplified in two overlapping fragments using the following set of primers, 5' TCCGAGAGGGGAGACCAGCGG 3' and 5' GGCTCAGACCCAGACTAG 3'; 5' GGGTCCTACAACCCTGTCACC 3' and 5' TCACCTACAGCCAGCACCCTC 3', and amplification profile of 45s at 94°C, 45s at 58°C and 2min at 72°C for 30 cycles. The products of PCR amplification were purified by electrophoresis in 1% agarose gel and subsequently recovered using NA-45 cellulose membranes (Schleicher and Schuell). About 100 ng of DNA fragments were phosphorylated with T4 polynucleotide kinase and blunt-end ligated into dephosphorylated SmaI restriction site of pGEM-3Z-f(-). Plasmids with desired insert were purified according to the method described by Hattori and Sakaki (1986). Subclones were sequenced until six mutant clones were identified, by means of the dideoxy chain-termination method (Sanger et al., 1977), using the 17-mer universal primer and T7 DNA polymerase. This work was carried out at Prof. K. Sukuzi laboratory (Chapel Hill, USA).

3.3. RESULTS

The genotype of 23 unrelated patients, 5 with variant B (P1 to P5) and 18 with variant B1 (P6 to P9 and 14 patients designated as PG) was characterised. The amplification products from the majority of patients, representing each of the 14 HEXA exons and their flanking sequences, were screened by SSCP analysis. Whenever possible the DNA from patient's parents were also tested. Mobility shifts suggesting sequence alteration were observed in exons 5, 7, 9, 12 and 13. The results are presented below according to the nature of the mutations identified.

3.3.1. Splicing mutations

❑ **G->C at +1IVS-7** (novel mutation)

The SSCP mobility shifts and sequencing is shown in Fig. 2. The PCR-amplified genomic DNA from patient P1 showed the G->C transversion at +1 position of intron 7. Nucleotide sequence of DNA from parents of B variant patient P2 showed that both the mutant cytosine and the normal guanine at +1IVS-7 were present in mother whereas the father presented the normal

sequence around the junction of exon 7 and intron 7 (data not shown). Thus, the patient 1 was homozygous for the G->C at position +1 and the patient P2 was concluded to be a compound heterozygote with only one allele carrying this mutation.

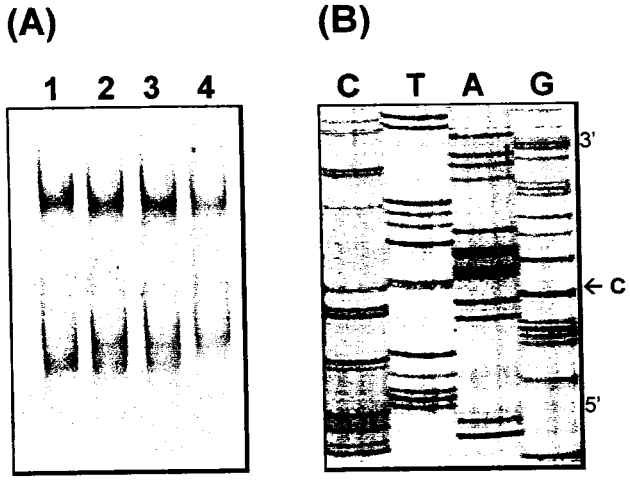


Fig 2: (A) SSCP analysis on 1xMDE gel at room temperature of PCR-fragment encompassing exon 7. Lane 1, control, lane 2, patient P1's father, lane 3, patient P1's mother; lane 4, patient P1. (B) Direct sequencing (sense strand). The relevant portion of the sequence is shown and the nucleotide substitution indicated by an arrow.

The effect of the G->C transversion in 5' donor site of intron 7 on α mRNA was firstly analyzed by Northern blot of total RNA from homozygous patient (Fig. 3A). In contrast to a band of expected size observed in control fibroblasts, a nearly undetectable band of a smaller size than the normal was observed in the patient. Using the appropriate oligonucleotides the entire coding sequence was reverse transcribed and the α chain cDNA amplified by polymerase chain reaction. The length of the amplified product obtained for patient was compared with that of the control after agarose gel electrophoresis, blotting and hybridization using either full-length α chain cDNA or exon 7 as probe (Fig. 3B).

Using the full lenght cDNA as the probe, only the expected band of 1650 bp was seen in the normal control, while multiple bands of varying sizes, mostly smaller than the normal 1650 bp, were present in the patient. When exon 7 was the probe, none of the multiple bands in the patient's fibroblasts were detectable when the film was exposed for the normal lenght of time. When over-exposed, a faint signal was detected for the major, fast migrating band. Thus, most of the amplified fragments lack exon 7. The faster-migrating band detectable in

Northern blotting (Fig. 3A) can be interpreted from its size (1517 bp) and lack exon 7 as an α subunit mRNA lacking exon 7 but otherwise normal.

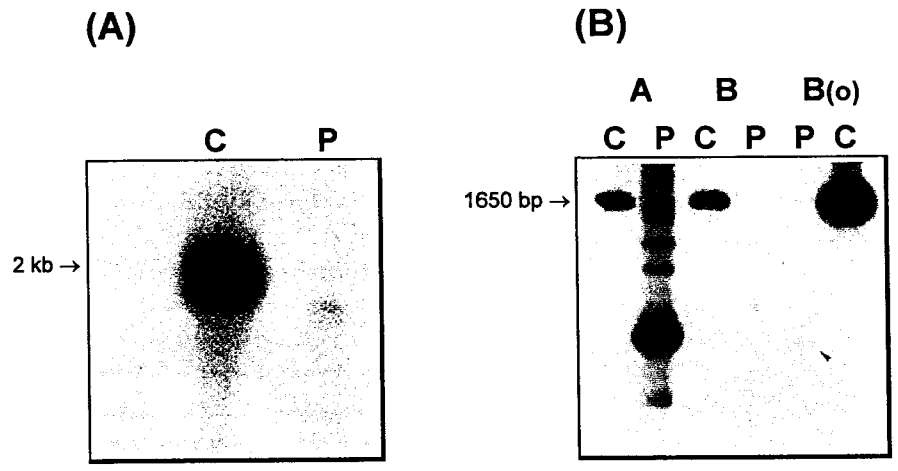


Fig. 3: (A) Northern blot analysis of hexosaminidase α chain mRNA from fibroblasts. The blot was hybridized with full lenght α-subunit cDNA. Lane C, control, 25 μg of total RNA; Lane P, B patient 1, 25 μg of total RNA. **(B)** Analysis of the cDNA amplified fragments. 1/50 of each PCR reaction was loaded on a 1.2% agarose gel, transferred to GeneScreen Plus nylon membrane and hybridized with full-lenght cDNA (A) or exon 7 probe (B). The film was deliberately over-exposed (o) in order to show the cDNA amplified fragments of abnormal sizes in the patient's sample. They were undetectable when the film was properly exposed for the normal sample. The fragment lenght is indicated in base pairs. Lane C, control; Lane P, patient.

The G->C transversion in the 5' end splicing site of intron 7 creates an AluI restriction site as well as it abolishes the restriction site for EcoRII and Scr FI. Since the last two restriction enzymes are used in the diagnosis of mutation G805A (Gly269Ser) (Paw et al., 1989) and G->A +1IVS-7 (Hechtman et al., 1992), the detection of this splicing mutation was specifically carried out through the digestion of the PCR fragment encompassing exon 7 with AluI. Therefore, the G->C transversion can be easily and quickly screened with a restriction assay with AluI endonuclease, whereas unambiguous diagnosis of G->A transitions requires allele-specific oligonucleotide hybridization. The digestion with AluI yields a 216 bp from the normal allele or 176 bp and 40 bp fragments from the mutant allele. The enzyme also produces an undetectable 5 bp fragment from both alleles. As it can be observed in the Fig. 4, the restriction enzyme assay showed in patient P1 both 176 bp and 40 bp fragments (lane 2) and in her parents the three fragments of 216 bp, 176 bp and 40 bp (lane 3 and

4), as expected for heterozygotes. In the mother of patient P2 the three fragments were also present (lane 6), whereas in her father only the 216 bp fragment was detected (lane 5). These results corroborated the sequencing data.

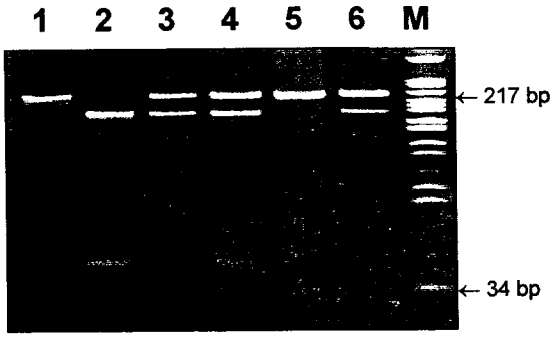


Fig. 4: Detection of G->C transversion at the position +1 of intron 7 of HEXA gene. The PCR-amplified fragment was digested with AluI restriction enzyme and the samples electrophoresed on 10% polyacrylamide gel. Lane M, molecular weight markers (pBr 322 DNA/MspI digest); lane 1, normal control; lane 2, patient P1; lane 3, patient P1's father; lane 4, patient P1's mother; lane 5, patient P2's father; lane 6, patient P2's mother.

❑ G->A +1IVS-9

SSCP mobility shifts and sequencing of patient P5 is presented in Fig. 5. Nucleotide sequence of DNA from patient showed both the mutant adenine and the normal guanine at donor site of intron 9 and therefore the patient was considered heterozygous for this mutation. The G->A transition in the 5' end splicing site of intron 9 creates a NlaIII restriction site. Therefore the mutation was confirmed by restriction enzyme assay. The digestion of PCR-amplified fragment encompassing exon 9 yields 230 bp from the normal allele and 148 bp and 82 bp from the mutant allele. As it can be observed in Fig. 6, the digest of amplified DNA from the patient indicated the presence of a mutation together with the normal allele.

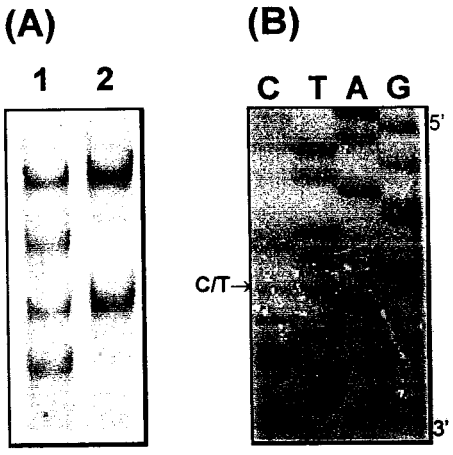


Fig 5: SSCP analysis on 1xMDE gel at room temperature (A) and sequencing (anti-sense strand) (B) of the PCR-fragment encompassing exon 9. The relevant portion of the sequence is shown and the nucleotide substitution indicated by an arrow. Lane 1, patient P5; lane 2, control.

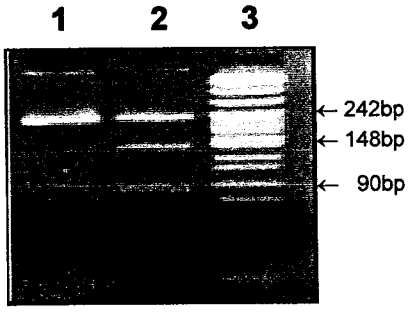


Fig. 6: Confirmation of mutation G->A at position +1IVS-9 by RFLP analysis. The products of the digestion were separated on 3% Nusieve 3:1 agarose gel. Lane 1, control; lane 2, patient P5; lane 3, molecular weight markers (pBR322 / MspI digest).

3.3.2. Frameshift mutations

❑ +TATC in exon 11

The DNA from both parents of patient P2, heterozygote for the splicing mutation G->C +1IVS-7, was tested for the presence of 4-bp insertion in exon 11 and the exon 12 G->C splice site mutation by hybridization with allele specific oligonucleotides (Fig. 7). A positive signal was obtained for the insertion mutation probe in the amplified DNA from the father. The patient was therefore concluded to be heterozygous for +TATC1278 in exon 11.

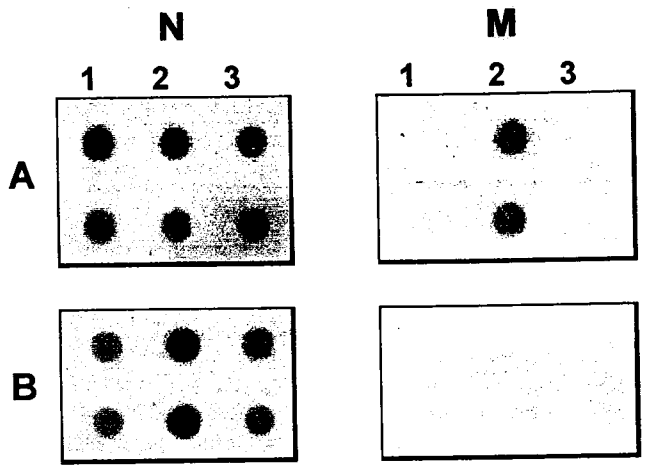


Fig. 7: Analysis of the two infantile Ashkenazi Jewish Tay-Sachs alleles. The region of genomic DNA encompassing both exon 11 4-bp insertion and intron 12 splice site was amplified by PCR and the products blotted onto duplicate in hybridization membranes. The samples were hybridized with allele-specific oligonucleotide probes to detect either the mutated or the corresponding normal sequences. A, 4-bp insertion; B, splicing defect; N, normal probe; M, mutant probe; lane 1, control; lane 2, patient P2's father; lane 3, patient P2's mother.

❑ **ΔC1334 in exon 12** (novel mutation)

SSCP mobility shifts and sequencing of patient P3's parents is represented in Fig. 8. From the patient no biological material for genetic study was available. Nucleotide sequence of DNA showed that the parents were both heterozygous for the deletion C1334. The patient was therefore concluded to be homozygous for that single base deletion in exon 12.

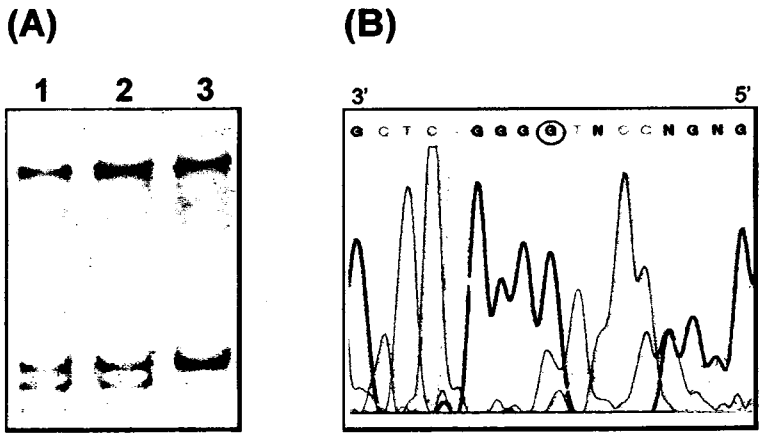


Fig. 8: SSCP analysis on 1xMDE gel at room temperature (A) and sequencing (anti-sense strand) (B) of the PCR-fragment encompassing exon 12. The relevant portion of the sequence is shown and the nucleotide substitution indicated by a circle. Lane 1, patient P3's mother; lane 2, patient P3's father; lane 3, control.

3.3.3. Missense mutations

❑ **G533A in exon 5**

The transition G533A was initially identified in 11 Portuguese B1 patients by Dos Santos et al. (1991), through dot-blot analysis of PCR fragments with allele-specific probes. Ten patients (from the group of patients identified as PG) were homozygotes and one (patient P6) was heterozygote, carrying an unidentified mutation in the second allele. The B1 patients later identified were initially screened for the presence of G533A by ASO hybridization and then by SSCP analysis using suitable controls. Fig. 9 exemplifies the detection of this mutation by allele specific oligonucleotides. The SSCP analysis of PCR-fragments encompassing exon 5 is presented in Fig. 10. According to the mobility shifts observed in the SSCP gel, 9 patients (from the group identified as PG) were concluded to be homozygous and patients P6 to P9 were compound heterozygous for G533A mutation. The parents of all patients were also studied and the SSCP patterns were in accordance with their carrier status (data not shown). Northern blot analysis of RNA isolated from homozygous patient's fibroblasts is shown in Fig.11.

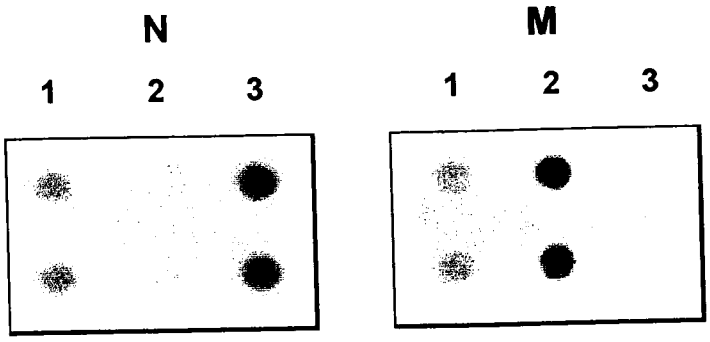


Fig. 9: Detection of G533A mutation by ASO hybridization as described in Materials and methods. Lane 1, heterozygote; lane 2, homozygote; lane 3, wild-type homozygote.

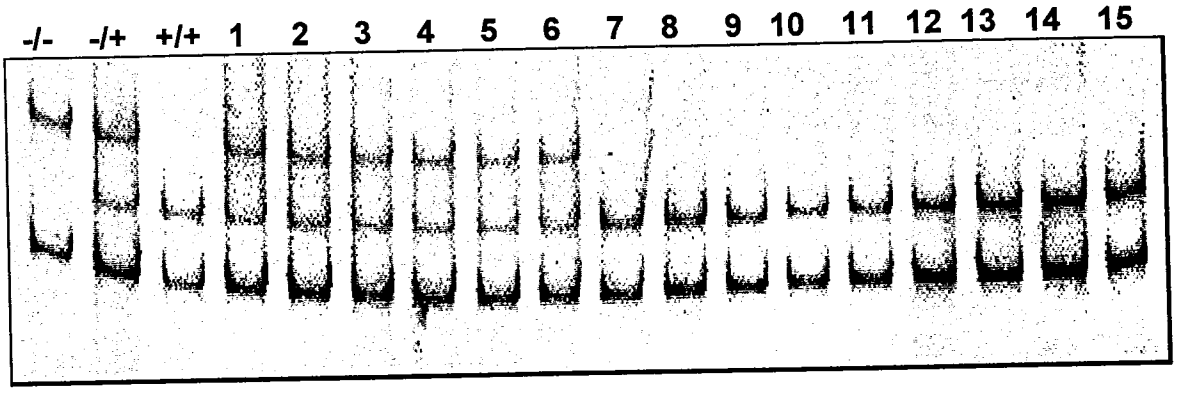


Fig. 10: SSCP analysis on 1xMDE gel at room temperature of the PCR-fragment encompassing exon 5. -/-, negative control for G533A; -/+, positive control heterozygote for G533A; +/+, positive control homozygote for G533A; lane 1 to 6, patients P6 to P9 plus two sibs of patient P9; lane 7 to 15, 9 patients from the group PG.

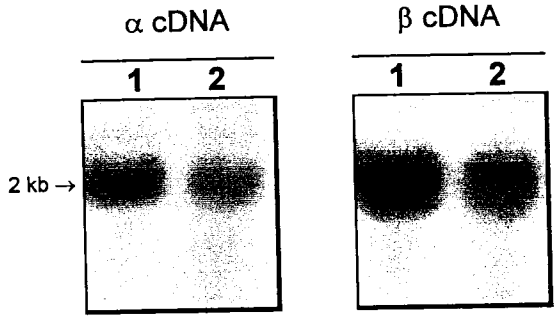


Fig. 11: Northern blot analysis of hexosaminidase α-chain mRNA from fibroblasts. The blot was hybridized with full-lenght α-subunit and β-subunit cDNA. Lane 1, patient homozygous for G533A, 25μg of total RNA; lane 2, control; 25μg of total RNA.

❑ **G755A in exon 7** (novel mutation)

SSCP mobility shifts observed in PCR-fragments of patient P9 (and his siblings) and sequencing are presented in Fig. 12. Sequencing showed the presence of both guanine and adenine at position 755 of HEXA coding sequence. The transition G755A in exon 7 creates a NlaIII restriction site. Therefore the mutation was confirmed by restriction enzyme assay. The digestion of PCR-amplified fragment encompassing exon 7 yields 221 bp from the normal allele and 133 bp and 88 bp from the mutant allele. Digest of amplified DNA from patient indicated the presence of mutation in coupling with the normal allele (Fig. 13), thus corroborating the PCR-SSCP and sequencing data.

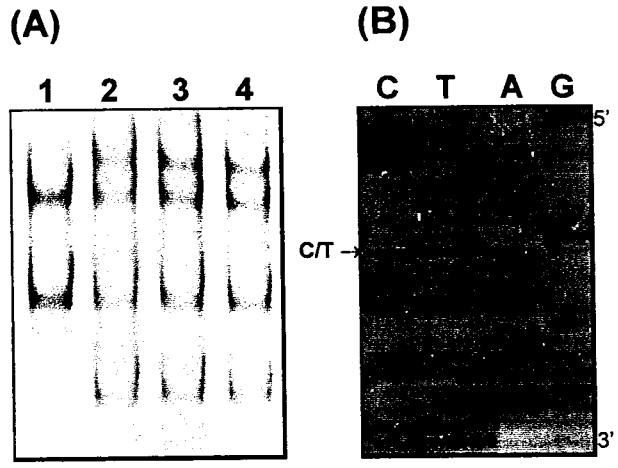


Fig. 12: SSCP analysis on 1xMDE gel at room temperature (A) and sequencing (anti-sense strand) (B) of the PCR-fragment encompassing exon 7. Lane 1, control; lane 2, patient P9; lane 3 and 4, patient P9's siblings.

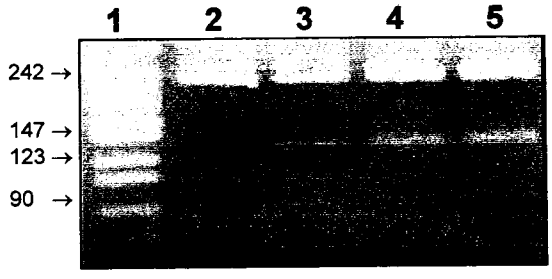


Fig. 13: Confirmation of G755A mutation by restriction enzyme analysis of amplification product with NlaIII. PCR products were digested with the restriction enzyme and subjected to agarose gel electrophoresis (3% Nusieve 3:1 agarose gel). Lane 1, pBR322 / MspI digest; Lane 2, control, lane 3, patient P9; lane 4 and 5, patient P9's siblings.

❑ **G1363A in exon 12** (novel mutation)

SSCP mobility shifts observed in PCR-fragments of patient P6 and sequencing of the corresponding amplified fragment is presented in Fig. 14. Sequencing revealed the presence of both guanine and adenine at position G1363 of normal gene.

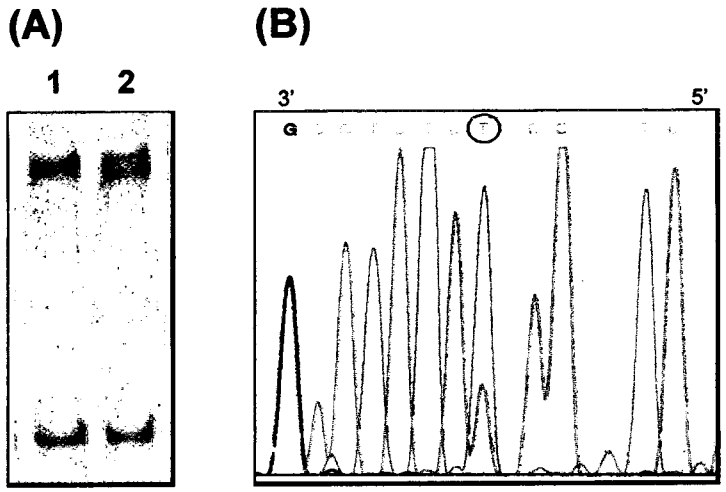


Fig. 14: SSCP analysis on 1xMDE gel at room temperature (A) and sequencing (anti-sense strand) (B) of the PCR-fragment encompassing exon 12. Lane 1, control; lane 2, patient P6.

❑ **C1495T and G1496A in exon 13**

Patient P4 was one of the first cases to be diagnosed with a late-onset form of the disease. The starting material was RNA since the presence of at least one mRNA producing allele was expected from the patient's clinical phenotype. The RNA was reverse transcribed in two overlapping fragments and the cDNA amplified fragment subcloned into pGEM-3Z-f(-) and sequenced directly in the plasmid DNA. Only one mutation was found, the transition G1496A in exon 13 (Fig. 15). SSCP and RFLP analysis were then developed to screen this mutation in other patients later diagnosed.

SSCP mobility shifts observed in PCR-fragments of patients P4, P5, P7 and P8 are shown in Fig. 16. Sequencing profile representing each mutation is presented in Fig. 17. Sequencing revealed the presence of C1495-to-T transversion in patient P7 and of G1496-to-A transition in patients P5, and P8.

The mutations G1495T and G1496A in exon 13 abolish the MaeII restriction site. The identification of these mutations was confirmed by restriction enzyme assay (Fig. 18). Digests of amplified genomic DNA from patients indicated the presence of corresponding mutation in coupling with a normal allele in each case. These mutations cause the destruction of a restriction site and therefore they can not be differentiate by MaeII restriction assay. In contrast, the SSCP analysis based on the principle that under nondenaturing conditions the electrophoretic mobility of single strand DNA depends not only on size but also DNA sequence (Orita et al., 1989a; Orita et al., 1989b), resulted in a characteristic band pattern for each C1495T and G1496A mutation.

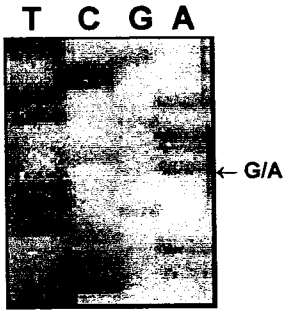


Fig. 15: Nucleotide sequence analysis of cDNA-PCR subcloned fragments.

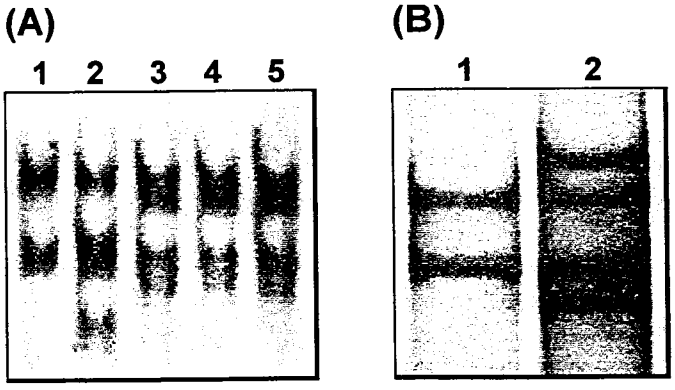


Fig. 16: SSCP analysis in 0.5x MDE gel (A) and 1x MDE gel (B) at room temperature of the PCR-fragment encompassing exon 13. Lane 1, control, lane 2, patient P7; lane 3, patient P8; lane 4, patient P4, lane 5, patient P5.

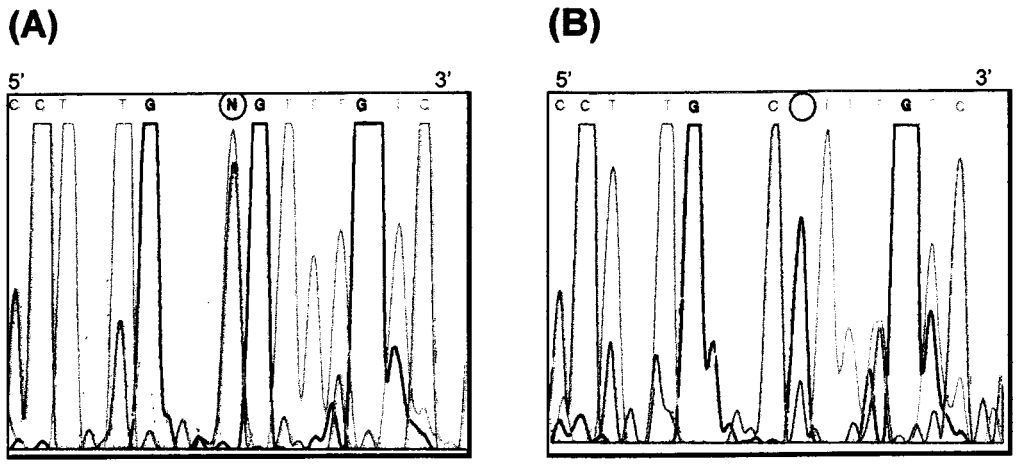


Fig. 17: Sequencing (sense strand) of the PCR-fragment encompassing exon 13. (A) Sequencing profile of patient P7; (B) Sequencing profile of patients P5 and P8.

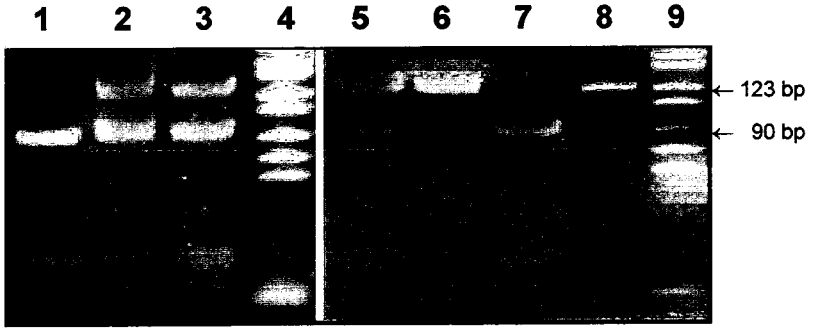


Fig. 18: Confirmation of C1495T and G1496A mutations by restriction enzyme analysis of amplification product with MaeII. PCR products were digested with the restriction enzyme and subjected to polyacrylamide (12%) gel electrophoresis. Lane 1, control; lane 2, patient P4; lane 3, patient P5; lane 4, pBR322 / MspI digest; lane 5, patient P7, Lane 6, patient P8; lane 7, control; lane 8, control non-digested; lane 9, pBR322 / MspI digest.

The distribution of the mutations found in 23 unrelated patients with Tay-Sachs disease and variants is shown in Table 3. Nine mutations were found in 46 alleles, four of which identified for the first time (G755A, G->C +1IVS-7, Δ C1334 and G1363A).

TABLE 3: FREQUENCY OF HEXA MUTATIONS IN 23 UNRELATED PORTUGUESE PATIENTS.

NUCLEOTIDE CHANGE	EXON	CODON CHANGE	DETECTION ASSAY	ALLELES FREQUENCY, %
G533A	5	Arg178His	ASO, SSCP	69.6
G755A[a]	7	Arg252His	SSCP, NlaIII (+)	2.2
G->C[a]	+1IVS-7	Splicing mutation	SSCP, AluI (+)	6.5
G->A	+1IVS-9	Splicing mutation	SSCP, NlaIII (+)	2.2
+TATC1278[b]	11	Frameshift mutation	ASO	2.2
ΔC1334[a]	12	Frameshift mutation	SSCP	4.4
G1363A[a]	12	Gly455Arg	SSCP	2.2
C1495T	13	Arg499Cys	SSCP, MaeII (-)	2.2
G1496A	13	Arg499His	SSCP, Mae II (-)	6.5

-, abolition of a restriction site; +, creation of a restriction site. [a] Novel mutations. [b] This mutation was also screened by heteroduplex analysis and RFLP, Dde (+).

The genotype of patients is presented in Table 4.

TABLE 4: GENOTYPE OF PORTUGUESE PATIENTS.

Patient	Allele 1	Allele 2
Variant B		
P1	+1IVS-7 (G->C)	+1IVS-7 (G->C)
P2	+1IVS-7 (G->C)	+TATC1278
P3	ΔC1334	ΔC1334
P4	Arg499His	?
P5	+1IVS-9 (G->A)	Arg499His
Variant B1		
P6	Arg178His	Gly455Arg
P7	Arg178His	Arg499Cys
P8	Arg178His	Arg499His
P9	Arg178His	Arg252His
PG (n=14)	Arg178His	Arg178His

3.4. CONCLUSION

SSCP analysis was used to screen all mutations in the exons, intron-exon junctions, and immediately adjacent intron sequences of the HEXA gene, except for the frameshift mutation +TATC in exon 11. The DNA for analysis was generated by amplification of the relevant sequences in 14 independent PCR reactions, each corresponding to an exon and its flanking sequences. The fragments displaying an altered SSCP patterns were subjected to direct sequencing. No reproducible band-pattern alterations were found that were not associated with a sequence change. The strategy used allowed the detection of 45 out of 46 alleles, representing 98% of the total. The unability to detect one mutation could be either due to the inherent limitation of SSCP analysis or to the fact that the screening strategy did not include the interior regions of introns or promotor region. In general, the mutation detection rate using SSCP analysis is 60-95%, depending on the length and sequence of the DNA analyzed and the electrophoretic conditions employed (Glavac and Dean, 1993; Hayashi and Yandell, 1993). The SSCP analysis was restricted to the examination of exons and surrounding sequences because most disease-causing mutations have been found within coding sequences or splicing junctions of the genes. Among human disease genes, one of the best studied is the globin cluster. In β-globin, 75% of mutations have been localized in the exons or nearby sequences (Kazazian, 1990).

A high molecular heterogeneity was found for B variant alleles, as expected for a non-Jewish population. Seven different mutations were identified in 13 alleles: two splicing mutations, G->C +1IVS-7 and G->A +1IVS-9, two frameshift mutations, +TATC1278 and ΔC1334, and three missense mutations, G1363A (Gly455Arg), C1495T (Arg499Cys) and G1496A (Arg499His). The frequency of mutations G->C +1IVS-7 and G1496A corresponded each one to about 23% (3/13) of total B variant alleles, a slightly higher value than the one observed for all the other B variant mutations (7.7 or 15.4%). The frequency of any of these mutations in Portuguese population must be low, as the occurrence of B variant is rare. In contrast, only two mutations, G533A (Arg178His) and G755A (Arg252His), were associated with the B1 phenotype, one of which, the transition G533A, was particularly frequent, accounting for 97% (32/33) of B1 alleles.

CHAPTER 4

STUDY OF HEXOSAMINIDASE α- AND β-CHAINS IN B AND B1 VARIANT

4.1. INTRODUCTION

The α- and β-subunits of the lysosomal enzyme hexosaminidase can associate noncovalently in different ways, αβ, ββ or αα, to give rise to the A, B or S isoenzymes, respectively. The α- and β-subunits undergo a sucession of post-translational modifications before functional activity is expressed in lysosomes. Two events occur before the newly synthesized proteins are allowed to pass out of the ER: glycosylation of protein and folding to near its native conformation. The site where oligomerization occurs, in ER or Golgi, is not clearly established; regarding to the α-subunit, the oligomerization is not necessary for exit from ER since it can be found in monomeric form in fibroblast media (Proia et al., 1988). In lysosomes, the precursor subunits of isoenzymes are proteolytically processed and the oligosaccharides extensively degraded (rev. in Introduction, Section 1.1.2., Transport and post-translational processing).

In certain cell types, sorting of hexosaminidase from the secretory pathway requires the phosphorylation of one or more Asn-linked oligosaccharides. Pulse chase experiments in cultured fibroblasts (Proia et al., 1984) showed that (i) after 1-h pulse the intracellular α- and β-chains were exclusively in precursor form and largely unassociated (the half-life of intracellular precursors was about 5.5h and 2.5h, respectively); (ii) the α-chains were phosphorylated still in the monomer state, and subsequently became associated with β-chains (iii) the mature α-subunit was found to predominate after 4-h chase period and therefore the dimerization is likely to occur after that time. However, this system is not completely efficient and a small fraction of the newly synthesized enzymes (usually less than 20% of the total), escapes from segregation into lysosomes and is secreted to the medium in precursor forms; the secreted enzymes can be subjected to receptor-mediated endocytosis and delivery to lysosomes (Hasilik and Neufeld, 1980a). Although it has been widely demonstrated that in cultured fibroblasts the presence of the mannose-6-phosphate is necessary for targeting of newly synthesized enzymes to lysosomes, alternative mechanisms are likely to occur in other cell types such as leukocytes and neuronal cells (rev. in Introduction, Section 1.1.1., Biosynthesis and targeting of lysosomal proteins).

In human cultured skin fibroblasts, the polypeptide chains of hexosaminidase are synthesized as precursors of M_r 67 kDa (α_p) and M_r 63 and 61 kDa (β_p), which are processed proteolytically to the size of mature (lysosomal) forms (α_m, M_r 54 kDa; β_m, 29 kDa plus smaller fragments) (Hasilik and Neulfeld, 1980a;

Hasilik and Neulfeld, 1980b). The complex nature of hexosaminidase, as assessed by SDS-PAGE, is caused by (i) differential carbohydrate attachment in the ER; (ii) differential phosphorylation and processing of the oligosaccharides in the Golgi; and/or (iii) glycosidic as well as proteolytic processing in the lysosome.

In a previous section it was shown that the activity of Hex A against unsulphated substrate (hydrolysed by the active site located on the β-subunit of the dimer αβ) was clearly decreased in leukocytes and brain from B1 patients homozygous for the Arg178His allele as well as in fibroblasts. The effect of this mutation on the amount of intralysosomal αβ dimer is now presented and compared with other B1 variant mutations as well as B variant mutations.

4.2. MATERIALS AND METHODS

4.2.1. Commercial materials

Bovine serum albumine free of hexosaminidase activity, leupeptin, protamine sulphate, anti-human fibronectin goat serum, Protein-A *Staphyloccocus aureus* suspension and sodium dodecyl sulphate (No. 5750, containing other alkyl sulphates) were obtained from Sigma. Pre-stained SDS molecular weight protein markers (26,600-180,000 Da) were obtained from Sigma. [4,5-^{3}H] leucine (65Ci/mmol), Amplify™ and [^{14}C]methylated protein standards (14,300-200,000 Da) were purchased from Amersham. The goat anti-hexosaminidase A antiserum raised in goats against Hex A purified from human placenta (Hasilik and Neufeld, 1980a) was kindly provided by Dr. R. Proia (Bethesda, USA). Anti-goat IgG peroxidase was purchased from Bio-Rad. Cell culture reagents were from Gibco BRL. All other reagents were from Merck.

4.2.2. Biological materials

Fibroblasts cultures were available from B patients P1 and P4 and from B1 patients PG and P6 to P9. The cells were expanded to near confluency in Dulbecco's minimal essential medium suplemmented with L-glutamine, antibiotics and 5% of foetal calf serum at 37°C in 5% CO_2.

4.2.3. Cell labelling

The confluent fibroblast cultures in 75-cm^2 flasks were carefully rinsed twice with 5ml of Dulbecco's metabolic labelling medium free of leucine and incubated with 5ml of this medium for 2h, to allow the depletion of the intracelular leucine pool. The labelling medium, 4.0 ml, was leucine deficient and was

supplemented with 0.1ml (0.5mCi) of L-[4,5-^{3}H]leucine and 5% of dialysed fetal calf serum. The chase was performed by adding unlabelled leucine to each flask to give twice the nominal concentration of this amino acid in Dulbecco's labelling medium, without removing the radioactive medium. Sterile conditions were maintained throughout the experimental procedure. The cell monolayers were rinsed trypsinized, washed with cold 0.9% NaCl and frozen at -70°C.

4.2.4. Extraction of cellular protein and immunoprecipitation

The extraction of cellular protein and immunoprecipitation with anti-Hex A antiserum was essentially as previously described (Hasilik and Neufeld, 1980a). The extraction was performed by incubation with 0.6ml of Tris/NaCl (10mM Tris-HCl, 150 mM NaCl, 0.02% NaN_3, pH 7.4) containing 1% Nonidet P-40 and 10mg/ml of bovine serum albumine. The cell extracts were incubated with 6µl of 3% protamine sulphate for 1h at 4°C and clarified by centrifugation at 50 000xg. To remove fibronectin and other labelled components that might bind nonspecifically to the immune precipitates, cell extracts were preabsorbed with 5µl of antifibronectin for 30 min at 4°C, followed by incubation with 100µl of *S. aureus* suspension (10% w/v), previously treated as described elsewhere (Lemansky et al., 1985) for 15 min at 4°C. The insoluble material was removed by centrifugation, and the supernatant fluids treated twice, with 100µl and 50µl, of the bacterial suspension only. The preabsorbed cell extracts were mixed with 2µl of antiserum and incubated for 16h at 4°C. 25µl of the *S. aureus* suspension were added and after a 30-min incubation at 4°C the immune complexes were collected by centrifugation and washed four times with 0.5ml of 10mM Tris-HCl, 600mM NaCl, 0.1%SDS, 0.05% Nonidet P-40, pH 8.6. The pellets were resuspended in 0.5ml of Tris/NaCl and transferred to clean tubes, centrifuged, suspended in 55µl of gel sample buffer (125mM Tris-HCl, pH 6.8, 1% SDS, 10% glycerol) and heated for 5min at 100°C. After removal of the insoluble material by centrifugation, 10µl of 100mM dithiothreitol were added to the supernatants and the samples were heated for an additional 5 min at 100°C.

4.2.5. Polyacrylamide gel electrophoresis

The polyacrylamide gel electrophoresis was performed by the method of Laemmli (1970) with minor modifications (Hasilik and Neufeld, 1980a). Electrophoresis was performed on gels 14cm long, 1.5mm thick and 20 cm wide. The gels and electrode buffers were prepared as described by Laemmli (1970), except that the ratio of acrylamide to methylene-bisacrylamide was changed from 30:0.8 to 30:0.312. Total monomer concentrations in the separating and the stacking gel were 12.8 and 5.8%, respectively. The

electrophoresis was carried out at 100V and 150V as the bromophenol marker moved through the upper and lower gels, respectively. The radioactive bands were visualised by fluorography by soaking the gel in Amplify for 2h at room temperature, drying and exposing the gel to a Kodak X-Omat AR film at -70°C for 20 to 60 days.

4.2.6. Immunoblotting

The proteins were transferred from the gel to a nitrocellulose membrane (Schleicher & Schuel) using a semi-dry blot (Multiphor II, Pharmacia LKB). The membrane was incubated with PBS/0.05% Tween-20/0.5% BSA for 1h at room temperature and then with anti-Hex A antiserum, previously diluted 1:1500 in the blocking solution, for overnight at room temperature. The membrane was washed twice with PBS/0.05% Tween-20 (PBS-Tween) and incubated for 2h with rabbit anti-goat IgG horseradish peroxidase diluted 1:1500 in the washing solution. Polypeptide bands were visualised using a HPR color development reagent.

4.3. RESULTS

Fibroblasts from B variant patients P1 and P4 and from B1 variant patients PG and P6 to P9 were used to study the intracellular hexosaminidase α and β chains by Western blot analysis and pulse-chase experiments. The hexosaminidase present in patient and control cells was immunoprecipitated with anti-Hex A antiserum which precipitates all α- and β-polypeptides, whether in precursor or mature form (Hasilik and Neufeld, 1980a). In order to investigate whether the antiserum precipitated all isoenzymes of hexosaminidase under the experimental conditions used, the activity of the enzyme against the synthetic unsulphated substrate (4MU-GlcNAc) in the supernatant was measured before and after immunoprecipitation; only about 5% of the activity remained in the supernatant after immunoprecipitation and was no significant differences between various cell lines in this respect.

4.3.1. Patients with B variant

The hexosaminidase chains present in patient P1, homozygous for the mutation G->C +1IVS-7, and in patient P4, a compound heterozygote for the Arg499His allele carrying an unidentified mutation in the second allele, was studied by pulse-chase labelling experiments. Fibroblasts from patient P1 and P4 were labelled for 22-h or labelled for 2h followed by 20-h chase period,

immunoprecipitated with anti-Hex A antiserum, subjected to SDS-polyacrylamide gel electrophoresis and fluorography. The results are shown in Fig. 1. In patient P1, the precursor or mature α-chains were not observed after 2-h pulse and 20-h chase; in contrast, the mature β-chains were clearly detected after 20-h chase period. After 22-h pulse, no precursor or mature α-chains were observed; in contrast, two bands approximately of 63kDa and 61KDa, corresponding presumably to precursor forms of β-subunit, and mature β-chains were clearly detected. In patient P4 cells the α-precursor was observed after 2-h pulse but was not proteolytically processed to the mature form and disappeared during an overnight chase period.

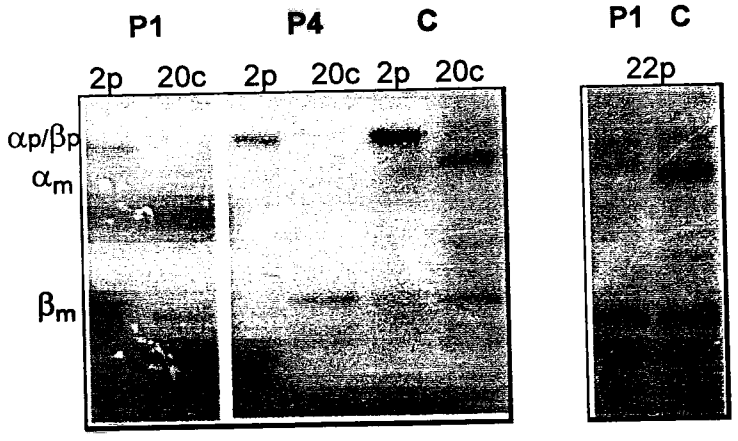


Fig. 1: Biosynthesis and maturation of hexosaminidase in fibroblasts from B variant patients. Cells were washed, preincubated with leucine-free media, labelled with 0.5 mCi of [^{3}H]leucine/flask for 20-h or for 2h, and either harvested immediately (pulse), or incubated for 20h-chase period after addition of unlabelled leucine. Antiserum anti-Hex A was used to immunoprecipitate the cell extracts. p,pulse; c, chase; P1, patient P1, homozygote for +1IVS-7; P4, patient P4, compound heterozygote for the Arg499His allele, carrying an unidentified mutation in the other allele; C, control.

4.3.2. Patients with B1 variant

4.3.2.1. Homozygous patients for the Arg178His allele

The hexosaminidase present in fibroblasts and brain of patients PG and controls was immunoprecipitated with anti-Hex A antiserum, subjected to SDS-polyacrylamide gel electrophoresis, electroblotting and immunodetection with

anti-Hex A antiserum (Fig. 2). The α and β chains of hexosaminidase, whether in precursor or mature form, present in 3 additional patients and controls, were quantified by densitometric scanning of the nitrocellulose sheet (Table 1). In patient cells, the ratio between the mature 54 kDa α-subunit (the lysosomal processed form) and the mature β-subunit was found to be about one half of that obtained in control cells; the amount of β-subunit was slightly increased, probably as result of β-subunit self-association.

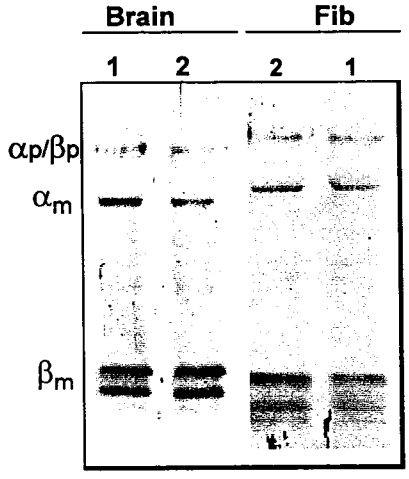


Fig. 2: Immunoblot of hexosaminidase of brain and fibroblasts from Arg178His homozygous patient. The cell extracts were subjected to SDS-PAGE and immunoblotted as described in Materials and methods. Antiserum anti-HexA was used to immunoprecipitate the cell extracts. Fib, fibroblasts; Lane 1, control; Lane 2, Arg178His homozygous patient.

TABLE 1: DENSITOMETRIC SCANNING OF IMMUNOBLOTS OF HEX CHAINS FROM ARG178HIS HOMOZYGOUS PATIENTS.

Sample	% α chain[a]	% β chain[a]	α / β[b]
Brain			
Lane 1 (Control)	23.7	64.6	0.367
Lane 2 (PG)	13.3	82.0	0.162
Fibroblasts			
Lanes 1 (Control)			
mean	23.7	64.6	0.370
range	20.4-27.9	56.2-72.0	0.307-0.456
n=	4	4	4
Lane 2 (PG)			
mean	15.4	77.1	0.202
range	13.1-16.5	69.0-81.7	0.161-0.233
n=	4	4	4

Densitograms of immunoblots and estimation of areas, corresponding to different hexosaminidase chains, were carried out by a Transydine densitometer. [a]The area of α and β chains was reffered as % of area corresponding to all precursor and mature chains. [b]Area of mature α chain relative to that of mature β chains. Lane 1, control; lane 2, Arg178His homozygote B1 patient.

Since RNA instability was ruled out (see Chaper 3), to explain a lower amount of α-chains we next investigated the biosynthesis and processing of newly synthesized α-chains. To that end, cultured fibroblasts from B1 variant patients homozygous for the Arg178His allele were labelled with tritiated leucine for 20-h, or for 2-h and either harvested immediately (pulse) or incubated for two times of chase (5 and 20-h). The results are shown in Fig. 3. The bands were quantified by densitometric scanning of the autoradiogram and the results are shown in Table 2. We were unable to separate the α and β chain precursors and therefore their quantification was not carried out.

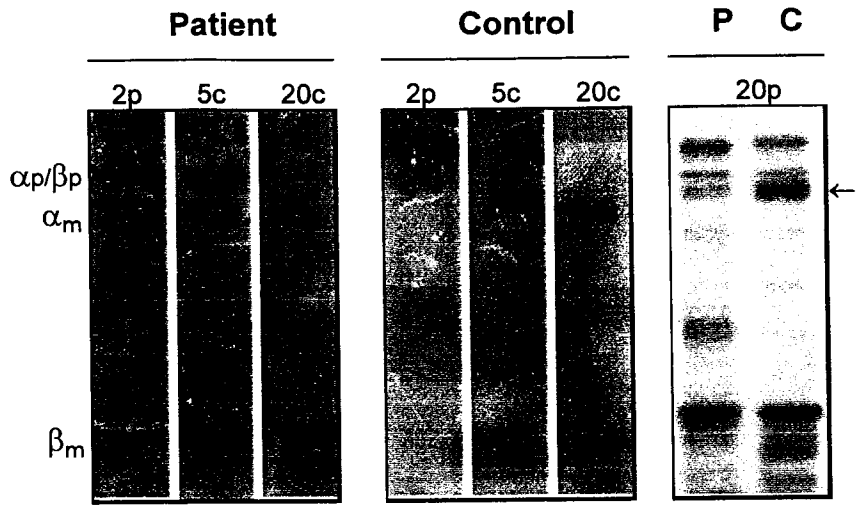


Fig. 3: Biosynthesis and maturation of hexosaminidase in fibroblasts from Arg178His homozygous patients. Cells were washed, preincubated in leucine-free media, labelled with 0.5 mCi of [^{3}H]leucine / flask for 20-h or for 2h, and either harvested immediately (pulse), or incubated for 5h and 20h-chase period after addition of unlabelled leucine. Antiserum anti-Hex A was used to immunoprecipitate the cell extracts. P, patient; C, control; p, pulse; c, chase.

TABLE 1: DENSITOMETRIC SCANNING OF PULSE-CHASE AUTORADIOGRAMS FROM ARG178HIS HOMOZYGOUS PATIENTS.

Sample	% α chain[a]	% β chain[a]	α / β[b]
Patient			
2h-pulse	0	0	0
5h-chase	10.0	57.0	0.175
20h-chase	17.0	68.0	0.250
20h-pulse	19.4	71.3	0.270
Control			
2h-pulse	0	0	0
5h-chase	13.0	41.0	0.320
20h-chase	30.0	52.0	0.580
20h-pulse	34.0	60.4	0.560

Densitograms of autoradiograms and estimation of areas, corresponding to different hexosaminidase chains, were carried out by a Transydine densitometer. [a]The area of α and β chains was reffered as % of area corresponding to precursor and mature chains. [b]Area of mature α chain relative to that of mature β chains.

As it can be observed in Table 1, the amount of mature α-chains was lower in patient cells than in the control. The intensity of a band of aproximately 61 kDa, corresponding probably to a precursor form of β-chain, was higher in the patient than control cells. In two other patients homozygous for the Arg178His allele a similar reduction in the amount of biosynthetically labelled mature α-chains was also observed (data not shown). Since the proteolysis of hexosaminidase chains is apparently inhibited by lysosomal thiol proteinases (Frish and Neufeld, 1981), the effect of leupeptin on the stability of mutant α-chains was evaluated. As illustrated in Fig. 4, at concentration of 50μg/ml leupeptin, the amount of mature α-subunit in patient was still lower than the one observed in control cells.

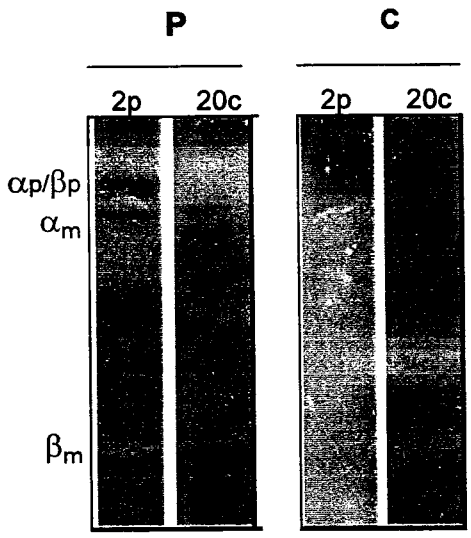


Fig. 4: Biosynthesis and maturation of hexosaminidase in fibroblasts from Arg178His homozygous patient. Cells were washed, preincubated in leucine-free medium, labelled for 2h with 0.5 mCi of [^{3}H]leucine / flask, and either harvested immediately (pulse), or incubated for 20h-chase period after addition of unlabelled leucine. Leupeptin (50μg/ml) was added to preincubation, pulse and chase media. Antiserum anti-Hex A was used to immunoprecipitate the cell extracts. P, patient; C, control; p, pulse; c, chase.

4.3.2.2. Compound heterozygous patients for the Arg178His allele and other mutations

The hexosaminidase present in fibroblasts of patients P6, P7, P8 and P9, carrying the mutation Gly252Arg, Arg499Cys, Arg499His and Arg252His in the other allele, respectively, was immunoprecipitated with anti-Hex A antiserum,

subjected to SDS-polyacrylamide gel electrophoresis, electroblotting and immunodetection with anti-Hex A antiserum (Fig. 5). Quantification of α and β chains of hexosaminidase by densitometric scanning of the nitrocelulose sheet is shown in Table 3.

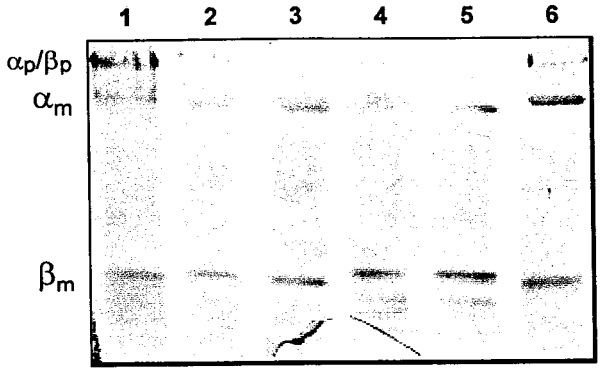


Fig. 5: Immunoblot of hexosaminidase of fibroblasts from B1 variant compound heterozygotes. The cell extracts were subjected to SDS-PAGE and immunoblotted as described in Materials and methods. Antiserum anti-HexA was used to immunoprecipitate the cell extracts. Lane 1, patient homozygous for +1IVS-7 that was included for comparative purpose; lane 2, B1 variant patient P6 (Arg178His / Gly455Arg); lane 3, B1 variant patient P7 (Arg178His / Arg499Cys); lane 4, B1 variant patient P8 (Arg178His / Arg499His); lane 5, B1 variant patient P9 (Arg178His / Arg252His); lanes 6, control.

TABLE 3: DENSITOMETRIC SCANNING OF IMMUNOBLOTS OF FIBROBLASTS FROM B1 VARIANT ARG178HIS COMPOUND HETEROZYGOTES.

Sample	**% α chain[a]**	**% β chain[a]**	**α / β[b]**
Lane 2 (Arg178His/Gly455Arg)	6.90	85.9	0.080
Lane 3 (Arg178His/Arg499Cys)	9.20	87.8	0.105
Lane 4 (Arg178His/Arg499His)	11.1	88.4	0.125
Lane 5 (Arg178His/Arg252His)	16.6	80.8	0.205
Lane 6 (control)			
mean	23.7	64.6	0.370
range	20.4-27.9	56.2-72.0	0.307-0.456
n=	4	4	4

Densitograms of immunoblots and estimation of areas, corresponding to different hexosaminidase chains, were carried out by a Transydine densitometer. [a]The area of α and β chains was reffered as % of area corresponding to precursor and mature chains. [b]Area of mature α chain relative to that of mature β chains. For each patient the procedure was repeated at least twice and the mean value is shown.

In cells from patients P6, P7 and P8 the ratio value for mature chains (α/β) was about one-fourth of that observed in control cells, whereas in patient P9 it corresponded to about one half of the control value and was similar to the one observed in cells from Arg178His homozygous patients.

4.4. CONCLUSION

We have studied the effect of some mutations identified in B and B1 variant patients on the intralysosomal hexosaminidase α-chains.

For both B variant patients P1 and P4 no mature α-subunit (the lysosomal processed form) was observed in cultured fibroblasts. Patient P1 was homozygous for the splicing mutation +1IVS-7 and patient P4 was heterozygous for the Arg499His allele, carrying an yet unidentified mutation in the other allele. In case P1, we know from the Northern blotting result (Chapter 2) that the α subunit mRNA was nearly undetectable and therefore the lack of immunological detectable α-subunit is most likely due to instability of the abnormally spliced RNA. The mutation Arg499His, present in the case P2, was previously described by other authors (Paw et al., 1990a). This mutation in heterozygosity with a null allele (+TATC1278) was found to produce a mutant protein that is neither maturated or secreted to the fibroblasts medium. However, we do not know if the complete absence of mature α-subunit in patient P4 is due to the presence in the second allele of a protein non-producing mutation or to a mutant protein immunologically unrecognizable by the antiserum raised against the normal enzyme protein. Anyway, one can predict that mutations producing an immunologically unrecognizable protein should be rare.

Pulse chase labelling studies performed in fibroblasts from the Arg178His homozygous B1 variant patients showed that the mutant α chain is processed to molecular forms indistinguishable from those observed in control cells. However, the amount of mature α-subunit was reduced in comparison to control cells. The intralysosomal instability of mutant α-chains with respect to leupeptin sensitive proteases was further ruled out. Since the limited proteolysis of hexosaminidase precursors seems to be catalyzed by lysosomal thiol proteinases (Frish and Neufeld, 1981), the reduced amount of mature α-chains might result from a defective transport of dimeric enzyme to lysosomes.

The study of hexosaminidase chains by Western blot technique indicated that in both fibroblasts and brain extracts the amount of mature α-subunit was lower than in the corresponding controls.

By Western blot we found that in fibroblasts from B1 variant patients heterozygous for the Arg178His substitution and carrying the mutations Gly455Arg, Arg499Cys and Arg499His in the second allele, the ratio value for mature chains (α/β) was about one-half of that observed in the Arg178His homozygous patients. Therefore, these mutant alleles are likely to give rise to undetectable mature α-subunits. In contrast, the amount of mature α-subunits generated by both Arg252His and Arg178His alleles was similar to that resulted from two Arg178His alleles. These observations are therefore in accordance with the enzymatic data observed in cellular extracts in that a correlation between the amount of mature α-subunit and the activity of Hex A against the unsulphated substrate was found.

CHAPTER 5

IDENTIFICATION OF B1 VARIANT CARRIERS AND DEVELOPMENT OF AN ENZYME IMMUNOBINDING ASSAY

5.1. INTRODUCTION

Carrier identification is of extreme importance in genetic disorders with high incidence in particular ethnic groups, such as Tay-Sachs disease in the Ashkenazi Jews where the carrier frequency is estimated to be 1 in 32. The enzymatic screening of Tay-Sachs carriers in Ashkenazi Jewish population has been made for several decades by using a simple and inexpensive automated assay (Kaback et al., 1993). This assay is based on the determination of hexosaminidase activity against synthetic substrates after differential thermal inactivation of isoenzymes. In large-scale carrier screening, the biological sample commonly used is the serum. However, since the activity of hexosaminidase in this specimen can be widely variable depending on several factors, such as pregnancy (Stirling, 1972) or certain pathological conditions (Hultberg and Isaksson, 1983; Tucker et al., 1980), leukocytes are also used, particularly when the results are inconclusive. Whatever is the biological sample used, some overlap between the enzymatic activities measured in carriers and normal individuals is observed (Ben-Yoseph et al., 1985; Landels et al., 1991; Triggs-Raine et al., 1990).

Recently, the combination of classical measurements of enzyme activity levels with screening of the most common known mutations in the Ashkenazi population was found to result in a test with a high predictive value of carrier state (Fernandes et al., 1992a; Triggs-Raine et al., 1990). Therefore, one can predict that the combination of DNA-based assay for specific gene lesions found in any particular population with the conventional enzymatic assay will allow to clarify the state of an individual whose enzymatic activity fall in the inconclusive range of values. In addition, this approach is also useful to distinguish between carriers of mutations associated with different clinical variants (infantile, subacute and chronic).

Regarding to B1 variant, a rare and panethnically distributed disorder, the identification of carriers has been usually confined to the family of the affected child (Charrow et al., 1985; Gray et al., 1990) but even so it poses some problems for relatives other than parents.

In Portugal, B1 variant is the most frequent form of G_{M2}-gangliosidosis and the second more frequent lysosomal disease. In our experience, mainly in the north of the country ($5.4x10^6$ inhabitants), 25 patients, out of 18 families apparently non related, have been diagnosed in the last 5 years. Although its incidence is unknown, the number of unrelated patients and the fact that the

majority of them are homozygous for the same mutation (the Arg178His allele) suggest a high frequency of B1 carriers in Portuguese population. In order to determine the frequency of B1 variant carriers and to prevent this severe neurological disease through genetic counselling, the possibility of B1 variant carriers identification was evaluated. Two experimental approaches could be independently used in carrier detection, the genetic or enzymatic assay. Two B1 mutations, one of which accounted for 97% of mutant alleles, were identified among Portuguese B1 patients (see Chapter 3). Although the low heterogeneity of B1 mutations allows carrier screening through a DNA-based test, the genetic assay alone was not considered the best approach to apply in a large-scale carrier screening because: (1) it tests only the known most common B1 mutations, G533A and G755A, and therefore the number of carriers for other mutations might be underestimated; (2) the screening of HEXA exons and boundaries, even using the SSCP analysis as the first approach, is a high cost and time-consuming assay. The possibility of using an enzymatic assay which would detect the great majority of B1 mutant phenotypes was then evaluated, by using different biological samples (leukocytes, serum and urine) as source of enzyme.

5.2. MATERIALS AND METHODS

5.2.1. Enzymatic activity of hexosaminidase in leukocytes and serum

The activity of hexosaminidase in obligate B and B1 carriers and controls against the synthetic substrates 4MU-GlcNAc (4MUG) and 4MU-GlcNAcS (4MUGS) was determined as described in the section of Materials and methods of Chapter 2.

5.2.2. Enzymatic activity of hexosaminidase in urine

5.2.2.1. B1 variant family members and controls

Individuals from seven patient families were studied. Five of these families comprise 6 patients (from the group reffered to as PG) all homozygous for the Arg178His allele. In the other two, patients P6 and P7 were compound heterozygous for the Arg178His allele carrying the substitutions Gly455Arg and Arg499Cys in the second allele, respectively (see Chapter 3). The sample studied comprised 99 members of the 7 families described above (including 14 obligate carriers for variant B1 and 2 obligate carriers for variant B) and 127 unrelated healthy control subjects. The B1 variant family members and controls

were of both sexes and respectively with ages ranging from 9 to 73 years and from 4 to 68 years; in both populations the urine pH ranged from 5.3 to 7.6.

5.2.2.2. Preparation of urine samples

First morning urines were stored at 4°C and transported to the laboratory within 5 hours after voiding. Two procedures were used to concentrate urine samples:

A. Standard procedure (APS): pH was adjusted to 5.2 with 1 mol/L citric acid and the urines (50 ml) were then concentrated by ammonium sulphate precipitation (Aerts et al., 1991). The enzyme is stable in concentrated urine for several days at 0°C. The pellets were ressuspended by sonication in about 1 ml of 50 mmol/L phosphate buffer, pH 6.0. After dialysis against the same buffer, the samples were cleared by centrifugation for 1h at 100 000xg and the supernatants (concentrated about 25 times), referred to as total extracts, used for measuring lysosomal enzyme activities and for enzyme immunobinding assay.

B. Simplified procedure (CENT-10): We have further investigated an alternative procedure to make this step easier and faster. The urines were ultracentrifuged for 1h at 100 000xg and 4 ml of the supernatant dialysed and concentrated about 10 times using the microconcentrator centricon-10. The concentrated supernatants, referred to as total extracts, were used for measuring lysosomal enzyme activities and for enzyme immunobinding assay.

5.2.2.3. Enzymatic activity of hexosaminidase in total extracts

The hexosaminidase activity (nmol/h/ml urine) was determined in duplicate against 4MUGS and 4MUG, as described in the section of Materials and methods of Chapter 2, the result being expressed as the ratio of these two enzymatic activities (4MUGS/4MUG). The 95 % confidence range for this value calculated from the two duplicate readings was $\pm$ 0.015.

5.2.2.4. Enzyme immunobinding assay

The Hex A activity bound to the monoclonal antibody 7E4 (IB Hex A), specific for Hex A, was determined as described previously (Hultberg and Isaksson, 1989) with minor modifications. The plate microtiter wells were coated with 150 µl/well of RAM (6.8 mg/ml, Janssen, Belgium), previously diluted 1/5000 in PBS, and incubated for 1.5h at 37°C. After washing the plate with PBS containing 0.05% Tween-20 (PBS-Tween), the monoclonal antibody 7E4 (50 µg/ml, MonoCarb, Sweeden), 125 µl/well, previously diluted 1/5000 in PBS, was added to each well and the plate incubated for 2.5h at 37°C. After washing with PBS-

Tween, 6 aliquots of 100 µl of the sample, previously diluted in 0.2 mol/L phosphate buffer pH 7.5, were added to the wells of the same plate which was incubated for 1h at 37°C. After washing with PBS-Tween, two assay mixtures, 100µl of 1.5 mmol/L 4MUGS in 0.1 mol/L citrate-phosphate buffer pH 4.0 and 100 µl of 2.0 mmol/L 4MUG in 0.1 mol/L citrate-phosphate buffer pH 4.4, were added in triplicate to those six wells of the plate, which was incubated for 2h at 37°C. After the addition of 100 µl of 1 mol/L glycine-NaOH buffer, pH 10.0, the fluorescence of each well was read in a Titertek fluoroskan fluorimeter at λ_{exc}=355nm and λ_{emi}=480nm. A two degree regression obtained with 4-methylumbelliferone (4-MU) standards (0-5 nmol/well) was used to determine the amount of 4-MU released in the assays. The 95% confidence range for the ratio of immunobound Hex A activities (IB Hex A 4MUGS/4MUG) calculated from both triplicate readings was ± 0.010.

5.2.3. DNA analysis

DNA was extracted from buccal cells according to the standard procedure (Mayall and Williams, 1990). An approximately 800-bp fragment encompassing exon 5 of the ß-hexosaminidase α-chain gene was amplified by PCR with appropriate primers, using DMSO to a final concentration of 10%. PCR products were screened for the presence of G533A by dot-blot analysis (Tanaka et al., 1990).

5.2.4. Statistical methods

The sample distributions have been tested for normality at the significance level of α=0.05, using the appropriate tests (Chi-square and Kolmogorov-Smirnov type), according to the sample size (≥ 30 or < 30 respectively) (Conover, 1980).

5.3. RESULTS

5.3.1. Enzymatic activity of hexosaminidase in leukocytes and serum

The activity of hexosaminidase was determined in leukocyte extracts and in serum of obligate carriers for B variant (parents of patients with the infantile phenotype) and B1 variant (parents of Arg178His homozygous patients) and controls. The results are shown in Table 1. As it can be observed, on the basis of enzyme activity alone the discrimination between B and B1 carriers, and controls, was problematic, particularly in leukocytes. Although within a family the identification of carriers for each variant was often quite clear (data not shown), a reliable identification of carriers in the general population appeared not possible due to overlap between enzyme activities measured for carriers and normal individuals.

Hexosaminidase is present in considerable amounts in human urine (Marinkovic and Marinkovik, 1978) which can be easily obtained in sufficient quantities for assay of lysosomal enzyme from patients and control persons without causing any disconfort to the individuals. Therefore, we next evaluated the possibility of using urine as biological sample.

5.3.2. Enzymatic activity of hexosaminidase in urine

The direct determination of hexosaminidase activity in urine against both synthetic substrates did not allow the discrimation between obligate carriers for B and B1 variant and normal subjects (data not shown). The overlap between carriers and controls was mainly due to the large variation within control population. We next evaluated the specificity and sensitivity of two different enzymatic assays in the detection of B1 variant carriers in urine by:

i) Determining Hex activity against the sulphated substrate (4MUGS) and neutral substrate (4MUG) in total extract, the result being expressed as the enzymatic activities ratio (4MUGS/4MUG);

ii) Determination of immunobound Hex A activities against 4MUGS and 4MUG, expressed as the ratio of both enzymatic activities (IB Hex A 4MUGS/4MUG).

TABLE 1: ACTIVITY OF HEXOSAMINIDASE IN B AND B1 VARIANT OBLIGATE CARRIERS[a].

	NS		SS	Hex A (SS) /	Hex A (SS) /
	Hex Total	% Hex A	Hex A	Hex Total	Hex A (NS)
Leukocytes					
B variant					
mean	1392	57.7	134	0.105	0.163
range	628-2983	47.8-63.5	77.0-173	0.068-0.133	0.100-0.223
n=	9	7	9	9	7
B1 variant[b]					
mean	1415	71.2	119	0.092	0.114
range	726-2983	57.2-85.0	61.0-189	0.047-0.155	0.068-0.187
n=	25	22	25	25	22
Control					
mean	1523	87.1	255	0.145	0.175
range	967-2294	81.0-91.4	128-319	0.062-0.180	0.120-0.258
n=	54	11	54	54	11
Serum					
B variant					
mean	702	40.7	46.3	0.067	0.166
range	379-949	31.3-52.0	28.0-58.3	0.058-0.078	0.142-0.218
n=	5	5	5	5	5
B1 variant[b]					
mean	743	56.2	55.5	0.076	0.129
range	568-901	49.0-64.0	43.0-66.9	0.064-0.098	0.106-0.149
n=	10	6	10	10	6
Control					
mean	943	75.6	110	0.096	0.180
range	401-1918	68.9-79.0	65.0-172	0.070-0.115	0.165-0.230
n=	17	4	17	17	4

[a]The enzymatic activity of hexosaminidase was determined in leukocyte extracts (nmol/h/mh protein) and in serum (nmol/h/ml) against the synthetic substrates 4MU-GlcNAc (Total Hex) and 4MU-GlcNAcS (Hex A); the Hex A activity (4MU-GlcNAc) recovered after isoenzyme separation by ion-exchange chromatography was expressed as % of total recovered activity (% Hex A). The activity of β-galactosidase, used as reference lysosomal enzyme, was determined in all experiments performed.[b]Heterozygous for G533A mutation. NS, 4MU-GlcNAc; SS, 4MU-GlcNAcS.

Table 2 compares the enzymatic activities ratio values (4MUGS/4MUG) obtained in B and B1 variant obligate carriers and controls urine samples using the total extract and the IB Hex A. The frequency distributions of these values are shown in Fig. 1A and 1B, respectively. In control ratio values, the standard deviation (percentage of the respective mean value) was considerably higher for the total extract (23%) than for IB Hex A (8.7%). In urines from B1 variant obligate carriers the mean ratio values for total extract and IB Hex A were respectively 50 % and 57 % of the corresponding control mean values. A clear distinction between controls and B1 variant carriers can be observed when using the IB Hex A activities ratio value (Fig. 1B), contrasting with the overlap observed for 4MUGS/4MUG ratio values obtained in total extracts (Fig. 1A). The two B variant obligate carriers presented in the total extracts a 4MUGS/4MUG ratio value within the corresponding B1 variant obligate carriers' range, whereas the IB Hex A 4MUGS/4MUG values clearly overlapped with the respective control range.

TABLE 2: HEXOSAMINIDASE ENZYMATIC ACTIVITIES RATIOS (4MUGS/4MUG) DETERMINED IN URINES FROM B AND B1 VARIANT OBLIGATE CARRIERS AND CONTROLS.

	Total extract			IB Hex A		
	B	**B1**	**Control**	**B**	**B1**	**Control**
n=	2	14	127	2	14	127
mean=	0.126	0.108	0.222	0.230	0.151	0.263
SD=	0.007	0.021	0.052	0.012	0.015	0.023
Min=	0.121	0.076	0.081	0.218	0.128	0.210
Max=	0.131	0.144	0.384	0.243	0.181	0.323

For details see Materials and Methods.
B = B variant obligate carriers; B1 = B1 variant obligate carriers.

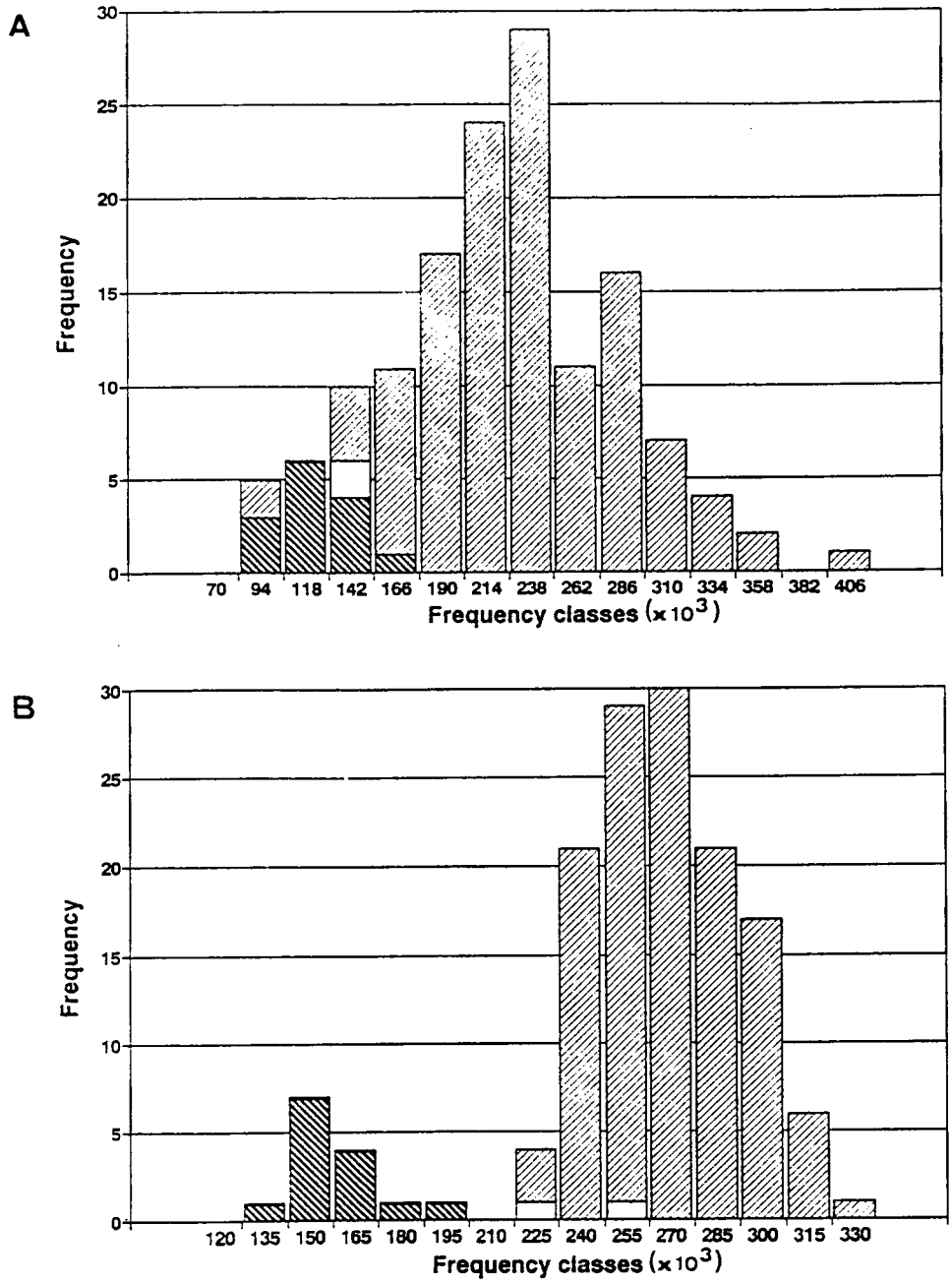


Fig 1. Frequency distributions of ß-hexosaminidase activities ratio values (4MUGS/4MUG) obtained in obligate carriers and control urines using the total extract **(A)** and the IB Hex A **(B)**. Open, B variant obligate carriers; heavy hatching, B1 variant obligate carriers, light hatching, controls.

5.3.3. Applicability of the enzyme immunobinding assay in mass carrier screening

On the basis of the results described above the Hex A immunoassay was applied in the identification of carriers in 83 members of seven families of B1 variant patients. Among these families, all patients were homozygous for the Arg178His allele, except two who carried the substitutions Gly455Arg and Arg499Cys in the second allele.

The frequency distribution of the IB Hex A 4MUGS/4MUG obtained for all family members studied (including the obligate carriers) is shown in Fig. 2. DNA analysis showed that the 53 individuals presenting a ratio of enzymatic activities < 0.195 were carriers for the Arg178His allele, which was not found in any one of the 46 individuals presenting a ratio value > 0.195. Any IB Hex A (4MUGS/4MUG) value falling in the range 0.175-0.215, defined by considering the experimental error from the analysis, was double checked.

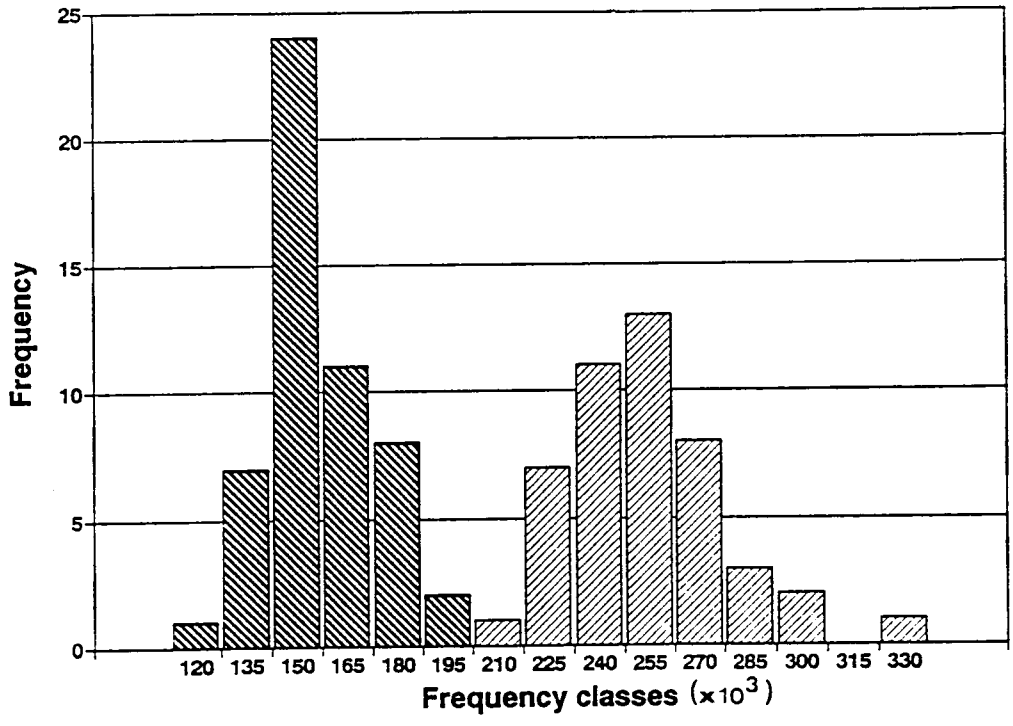


Fig. 2. Frequency distributions of IB Hex A (4MUGS/4MUG) values determined in urines from B1 variant family members. Heavy hatching, Arg178His carriers; light hatching; Arg178His non-carriers.

Fig. 3 shows the expected probability density functions (based on the observed means and standard deviations) for all samples of B1 variant carriers and controls studied. Considering the cutoff value of 0.195 the probability of misclassifying a carrier as a control is 0.16 % (1.6 in 1000 times) and a control as a carrier is 0.41% (4.1 in 1000 times).

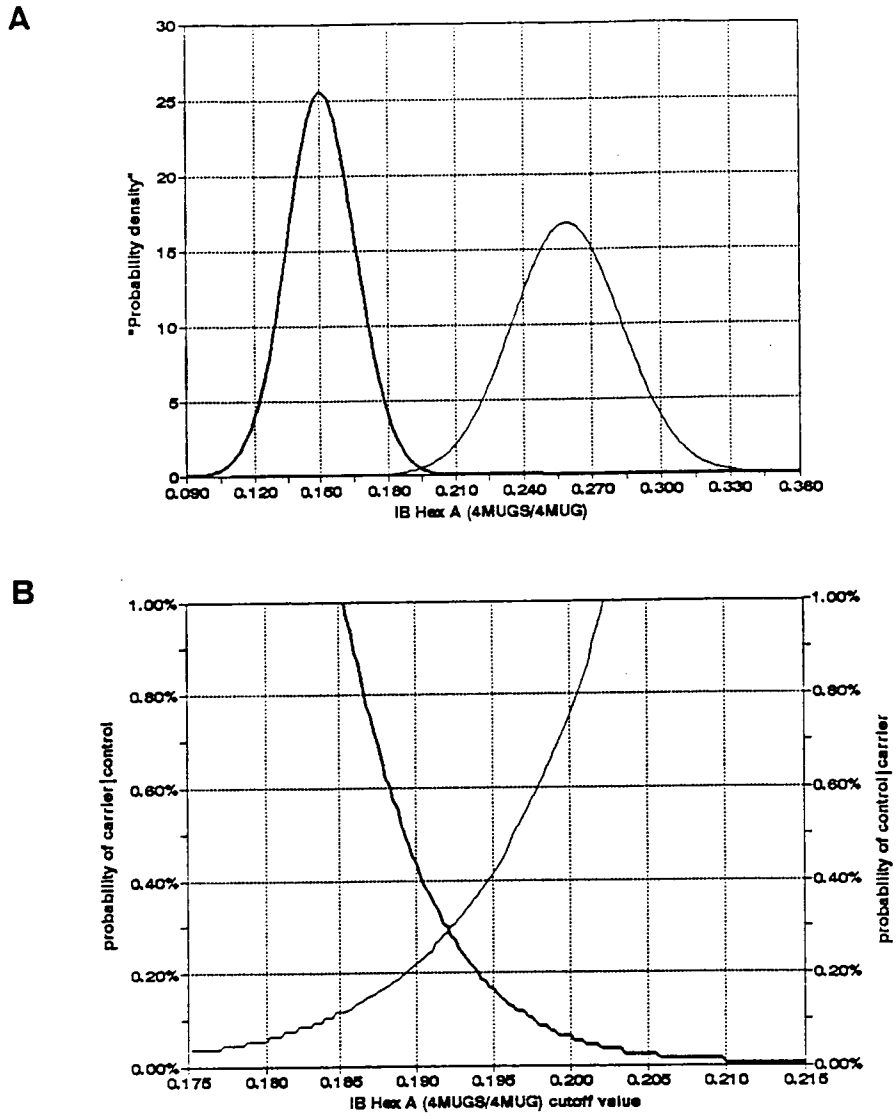


Fig. 3. (A) Probability density functions for B1 variant carriers (heavy line) and controls (light line). **(B)** Probability of misclassifying a carrier as a control (heavy line) and a control as a carrier (light line) as a function of cutoff values.

The two different sample preparation methods (APS and CENT-10) were compared using 30 control urines samples. The results are summarised in Table 3. The standard deviations of the 4MUGS/4MUG ratio values obtained in total extract were about 29% (APS) and 24% (CENT-10), whereas those of the IB Hex A were about 10% of the respective mean values in both procedures. The comparison of means and standard deviations obtained for IB Hex A (4MUGS/4MUG) using APS and CENT-10 showed that to a significance level of 0.05 the observed difference in the parameters is not statistically significant.

TABLE 3: COMPARISON OF THE TWO SAMPLE PREPARATION METHODS (APS AND CENT-10) IN A SAMPLE OF 30 CONTROL URINES.

	APS (4MUGS/4MUG)		CENT-10 (4MUGS/4MUG)	
	Total extract	**IB Hex A**	**Total extract**	**IB Hex A**
n=	28	30	28	30
mean=	0.229	0.263	0.191	0.255
SD=	0.066	0.023	0.047	0.027
Min=	0.141	0.232	0.122	0.214
Max=	0.337	0.313	0.303	0.312

For details see Materials and Methods.

5.4. CONCLUSION

G_{M2}-gangliosidosis B1 variant was found to occur at an exceptionally high frequency in Portugal. Therefore a reliable, practical and inexpensive method for the detection of B1 carriers should be useful to screen the patient families as well as the populations from areas where a high frequency of the disease is expected. In the present study two enzymatic assays using urine as biological sample were compared.

One of these assays was based on the determination of activity of an identical amount of immunobound Hex A against 4MUGS and 4MUG. The ratio of these two enzymatic activities was independent of the amount of Hex A present in urine samples. Under these conditions, the catalytic activity of B1 variant Hex A was similar to that of control Hex A if determined against 4MUG and it was extensively impaired when determined against 4MUGS. Therefore the IB Hex A (4MUGS/4MUG) value for a B1 variant carrier was about one half of the corresponding control value. The IB Hex A (4MUGS/4MUG) value obtained for the obligate carriers, expressed as percentage of control mean value, was higher than that expected probably due to the presence of trace amounts of the neutral substrate in the sulphated substrate preparation. The two B variant obligate carriers studied were not detected using this assay, as expected for any carrier presenting a catalytically normal Hex A activity against both synthetic substrates.

The other enzymatic assay was based on the determination of Hex A activity (4MUGS) and total hexosaminidase activity (4MUG). The ratio value, 4MUGS/4MUG, was expected to be decreased in urines from B and B1 variant carriers. The results obtained in urine from B and B1 variant obligate carriers and controls showed that no cutoff value could be established in order to obtain a test with good sensitivity and specificity for the detection of carriers of both variants. However, for the detection of B variant carriers a larger number of cases need to be studied in order to ascertain the test reliability (specificity and sensitivity).

All individuals studied were from B1 families at risk for the Arg178His allele. The Hex A immunoassay, under the conditions proposed, allowed the detection of all Arg178His carriers present in 83 members from seven B1 variant families. From the probability density functions established for all samples studied (family members and controls) one can predict a sensitivity of 0.996 and a specificity of 0.994, which corrrespond to the probabilities of misdiagnosis a carrier as a control and a control as a carrier of 0.16% and 0.41%, respectively. One can expect 5% of the results to fall within the range 0.175-0.215. For ratio values between 0.175 and 0.215, DNA analysis will reduce the probability of misdiagnosis a carrier as a control and a control as a carrier to 0.005% and 0.03%, respectively.

The reliability of the Hex A immunoassay in the detection of B1 variant carriers, together with its simplicity, low cost and the attractive possibility of automation, makes it the suitable method for carrier screening in populations at risk as well as for the assessment of carrier frequency, particularly if the simplified procedure of urine sample preparation is used.

CHAPTER 6

DISCUSSION AND FUTURE PROSPECTS

6.1 DISCUSSION

The aim of the experimental work described in this thesis was to obtain information regarding Hex A activity, post-translational modifications and molecular defects underlying α-subunit deficiency. To that end, biological material from 23 unrelated Portuguese patients with Tay-Sachs disease and variants was used to perform biochemical and genetic studies. In this chapter the results will be discussed and related to the findings described by other investigators.

❑ Enzymatic studies

The α-subunit deficiency was characterised at enzymatic level using water soluble synthetic substrates that are β-GalNAc derivatives. The competence of the α-catalytic site in Hex A for hydrolyzing ganglioside G_{M2} was evaluated by using the sulphated derivative. Based on the activity of Hex A against 4MU-GlcNAc and 4MU-GlcNAcS in leukocytes, two groups of patients were distinguished, 5 with B variant and 18 with B1 variant. Using the sulphated substrate the activity of Hex A was less than 5.5% of control mean value in all patients. With the unsulphated substrate the activity of Hex A was almost undetectable in B variant patients and ranged from 25 to 60% of total hexosaminidase activity in B1 variant patients. These results can be explained considering that the α- and β-subunits have distinct catalytic sites with different substrate specificities (Kytzia and Sandhoff, 1985). The ganglioside G_{M2} and the sulphated synthetic substrate are hydrolyzed almost exclusively by the catalytic site located on the α-subunit of Hex A, whereas the neutral synthetic substrate appears to be hydrolyzed only by the catalytic site located on the β-subunit, common to Hex A and Hex B. In B variant, the deficient activity of Hex A against both synthetic substrates was therefore attributed to the complete absence of Hex A or to the presence of a residual amount of functional enzyme with undetectable activity against the synthetic substrates. In B1 variant, the presence of Hex A with activity against the neutral substrate but inactive towards the sulphated substrate was probably due to the inactivation of the catalytic site on the α-subunit of Hex A.

Based on clinical data and B1 variant enzymatic profiles, a wide genetic heterogeneity can be predicted for mutations associated with either B or B1 variant. In order to establish a correlation between α-chain gene defects and

enzymatic and clinical phenotypes, the genotype of these patients was characterised.

❑ Genetic heterogeneity of α-chain gene mutations

B1 variant accounted for 78.0% (18/23) of the cases with α-subunit deficiency. Five mutations were identified in 36 alleles: G533A, G755A, G1363A, C1495T and G1496A. The transition G533A was present in at least one allele of each patient. Fourteen patients were homozygous for the mutation G533A, the other four being compound heterozygous carrying different mutations in the second allele.

Among five B variant patients, five mutations were identified in 10 alleles: two splicing mutations, +1IVS-7 (G->C) and +1IVS-9 (G->A), two frameshift mutations, the insertion TATC1278 and the deletion C1334, and one point mutation, G1496A. The mutations +1IVS-7 and ΔC1334 were found in homozygous state. No biological material from the patient homozygous for the deletion was available for further studies.

Four novel mutations were identified: G755A, +1IVS-7, ΔC1334 and G1363A. These mutations were not identified in 50 normal chromosomes, thus supporting the view that these are disease-causing mutations.

Five mutations (G533A, G755A, +1IVS-9, C1495T and G1496A), representing 85% of the total, occurred at CpG dinucleotide, thus confirming that CpG is a "hot-spot" for mutations causing human genetic diseases through methylation-mediated deamination (CG to TG or CG to CA).

The transition G533A was the most frequent mutation, accounting for 70% (32/46) of total alleles. The frequency of any other mutation was quite low, varying between 2.2 (1/46) and 6.5% (3/46) of total alleles. The mutations found in homozygous state were further characterised by studying the α mRNA and post-translational processing.

❑ Transversion G->C in 5' donor site of intron 7

This mutation was found to cause an inefficient and abnormal processing of the transcript. Since the first GT at the 5' donor site of introns are known to be obligatory (Shapiro and Senapathy, 1987), the transversion was expected to result in splicing failure at the junction due to the decrease of the donor site efficiency.

By Northern analysis of total RNA present in fibroblasts from a homozygous patient, the hexosaminidase α mRNA was observed as a nearly undectetable fast migrating band.

Through amplification of α chain transcripts by RT-PCR several fragments of smaller size than the normal transcript hybridized with full-length α cDNA. The high percentage of abnormal spliced molecules might result from an altered balance between normal spliced molecules and those resulting from alternative splicing. Moreover, the smaller fragments are expected to be over-represented after amplification, with respect to longer fragments. The mRNA quantification by cDNA-PCR is only possible in the exponential phase of amplification, generally before the 20^{th} cycle of amplification, and at 35^{th} cycle the amount of amplified material should depend poorly on the initial amount of material to be amplified. Therefore, the data should only be considered as qualitative and not quantitative. The longest fragment observed was identified as corresponding to α mRNA lacking exon 7. The skip of the exon immediately preceding a mutation at the 5' donor site has been reported in the Ashkenazi intron 12 splicing mutation (Ohno and Suzuki, 1988b; Ohno and Suzuki, 1988c). Further studies will be needed to evaluate if the transcripts lacking other segments beside exon 7 might result from the presence of another Tay-Sachs disease mutation or from normally occurring miss-splicing, as described for +1IVS-12 G->C (Ohno and Suzuki, 1988c), although they were not detected in control normal fibroblasts studied.

The sequence of mRNAs laking just exon 7 is in frame and it would encode a protein shortened of 43 amino acids (those encoded by exon 7). However, the labelled mutated α-subunit was undetectable in fibroblasts from homozygous patient. We do know from Northern blotting that the α-subunit mRNA is nearly undetectable. The lack of immunological detectable α-subunit, therefore, is most likely due to instability of the abnormally spliced mRNA and not due to a mutant protein immunologically unrecognizable by the antiserum raised against the normal enzyme protein.

❑ Transition G533A translating into Arg178His substitution

In patients homozygous for the Arg178His allele the activity of Hex A against the unsulphated substrate was reduced in comparison to control in different cell types (leukocytes, fibroblasts, lymphoblasts and brain). According to our results and those reported by other authors (Akli et al., 1991), this mutation does not seem to affect the α mRNA stability. Metabolic labelling studies performed by Sonderfeld-Freko et al. (1985a) showed that in fibroblasts from a compound heterozygote patient carrying the mutation +TATC1278 in the second allele (Ohno and Suzuki, 1988a; Tanaka et al., 1991), both precursor and mature forms of the α-subunit were present but at lower level as compared to normal cells. This result can be interpreted considering that the amount of α-subunits resulted from only one protein-producing allele.

In order to investigate if the mutation Arg178His affects the formation of αβ dimer, which would explain the decreased activity of Hex A against the unsulphated substrate observed in Arg178His homozygous patients (see Chapter 4), the study of hexosaminidase polypeptides was carried out.

The study of hexosaminidase α and β chains was performed by Western blot analysis and pulse-chase labelling experiments using an antiserum raised against human Hex A (Hasilik and Neufeld, 1980a). This antiserum immunoprecipitates all α and β chains, whether in precursor or mature form (Hasilik and Neufeld, 1980a). In Arg178His homozygous patients, the Western blot of hexosaminidase from fibroblasts and brain showed a band co-migrating with control α-chain, corresponding to about 54 kDa (the lysosomal processed form). Quantification of polypeptide bands by densitometric scanning of the blots showed that in both cell extracts the ratio of mature chains (α/β) was about 50% of the control mean value. This could possibly be explained by the mutant α-chains being more rapidly degraded by lysosomal proteinases. Since the enzyme(s) responsible for limited proteolysis appears to be a lysosomal thiol proteinase (Frish and Neufeld, 1981), the problem of intralysosomal instability of mutant protein was adressed by comparative pulse-chase labelling studies in the absence and presence of leupeptin.

Pulse-chase labelling in fibroblasts from Arg178His homozygous patients indicated that the α and β chains of hexosaminidase were processed to molecular forms indistinguishable from those observed in control cells. However, the amount of mature α-chains was present at lower level as compared to normal cells, even in the presence of leupeptin.

The observation that the amount of mature α-chains was reduced in both fibroblasts and brain extracts suggests that the Arg178His substitution affects the transport of dimers to lysosome, independently of the pathway, MPR dependent or independent, used by these cells for targeting β-hexosaminidase.

Overal the data suggest that Arg178His might interfere with the transport of dimeric enzyme to lysosomes. We do not know if it affects the dimerisation with the β-subunit directly, by affecting the binding site or indirectly, by preventing some pos-translational modification that precedes association such as glycosylation. The residue Arg178 is 21 amino acids way from one, that preferentially glycosylated, of the three glycosylation sites of the α-subunit (Weitz and Proia, 1992). It might be possible that Arg178His mutation interfere with the correct folding of α polypeptide and the assembly of subunits by affecting the glycosylation at Asn157 (in this case the αβ dimers would probably be retained in the endoplasmic reticulum and rapidly degraded). It is therefore possible that the mutation Arg178His leads to a more general change in protein structure, as previously predicted by computer analysis (Ohno and Suzuki, 1988a; Suzuki and Vanier, 1991), than a local effect on the active site on the α-subunit. These observations leave open the question whether Arg178 residue is actually a part of the substrate binding/catalytic site. However, because this change interferes with the ability of protein to be sucessfully transported into lysosome, the residue must at least be in close proximity to such a critical site. Further work is need to determine the effect of more conservative changes in and around this region.

❑ Molecular mechanisms of α-subunit deficiency

Table 1 summarizes the genetic and biochemical data described in this study. The genetic causes of the α-subunit deficiency and the molecular mechanisms involved are described below:

B1 variant

(i) Transition G533A translating into Arg178His substitution:

This mutation presumably leads to a conformational alteration that affects the α-catalytic site and interferes with the ability of protein to form dimers and be efficiently transported to lysosomes.

(vi) Transition G755A translating into Arg252His substitution:

This mutation is likely to cause a catalytically defective α-subunit. The effect of the Arg252His on the α-subunit maturation is apparently similar to that produced by the Arg178His allele.

B variant mutations

(i) Transversion G->C in 5' donor site of intron 7:

This mutation causes an inefficient and abnormal processing of the transcript, leading to loss of exon 7.

(ii) Transition G->A at 5' donor site of intron 9:

This splicing mutation generates no detectable normally spliced RNA by RT-PCR (Akerman et al., 1992). Therefore this allele is expected to generate no α polypeptide.

(iii) Insertion of TATC at nucleotide 1278:

This mutation shifts the reading frame and introduces a premature termination codon that causes mRNA instability (Boles and Proia, 1995), generating a profound deficiency of α mRNA in patient's fibroblasts (Myerowitz and Costigan, 1988). Transcription is normal (Paw and Neufeld, 1988). The presence of this mutation in heterozygosity with +1IVS-7 (G->C) is also expected to result in a functional abnormality.

(iv) Deletion of the nucleotide C1334:

This single base deletion shifts the reading frame and introduces a termination codon 21 bases downstream from the deletion site. This mutation is likely to be deleterious by causing mRNA instability as reported for the frameshift mutation +TATC1278 (Boles and Proia, 1995), or by affecting the transport, stability or activity of enzyme protein. The mutant protein, if present, was devoid of catalytic activity against both synthetic substrates.

(v) Transition G1363A translating into Gly455Arg:

This mutation was identified in heterozygosity with the Arg178His allele. In fibroblasts, the activity of Hex A against the unsulphated substrate and the amount of mature α subunit was substantially reduced in comparison to that generated by two Arg178His alleles. These findings suggest that the mutation G1363A does not give rise to a detectable mature α subunit. It is plausible that a change from non-polar amino acid (Gly) to a positively charged amino acid (Arg) may lead to an increased lability.

(vi) Transition C1495T translating into Arg499Cys:

The presence of an additional cysteine residue might result in inappropriate disulfide bridge in the protein with the consequent disruption of the normal three-dimensional structure. This mutation was found in heterozygosity with the Arg178His allele. In patient's fibroblasts the Hex A activity and the amount of mature α-subunit was decreased in relation to Arg178His homozygous patients. These observations might result from interference of the mutation with the stability of the protein, either in the lysosome or in the endoplasmic reticulum (due to the failure of the protein to be properly fold), or with the ability to form dimers.

(vii) Transition G1496A translating into Arg499His:

This mutation was expected to produce no detectable mature α-subunits since other authors have shown (Paw et al., 1990a) that it results in a polypeptide that is neither maturated or secreted to extracellular medium and fails to associate with the β-subunit. These findings were atributted to the unability of mutant protein to fold properly, becoming trapped in the endoplasmic reticulum.

The mutations Arg178His, Arg252His, Gly455Arg, Arg499His and Arg499Cys occur at conserved residues in the human β-subunit (Proia, 1988), in the mouse hexosaminidase α- and β-subunits (Yamanaka et al., 1994b), and in the hexosaminidase polypeptide of *Dictyostelium discoideum* (Graham et al., 1988). Since these residues have been particularly well conserved during evolution, they might be part of functional domains that are essential for folding, assembly and catalytic activity.

TABLE 1: GENETIC HETEROGENEITY AND MOLECULAR MECHANISMS OF THE α-SUBUNIT DEFICIENCY IN PORTUGUESE PATIENTS.

		Protein			Leukocytes β-Hexosaminidase activity		
					4-MU-GlcNAc		4MU-GlcNAcS
Genotype	**α mRNA**	Precursor α-chains	Folding,assembly or/and transport	% α/β chains[a]	Total	% Hex A[b]	Hex A
+1IVS-7 / +1IVS-7	Absent	Absent			1 200	0	3.49
+1IVS-7 / +TATC1278	Absent	Absent			1 737	0	3.60
ΔC1334 / ΔC1334	Absent ?				957	0	NA
Arg499His / ?		Present	Affected	Absent	1674	0	9.40
Arg499His / +1IVS-9	Probably reduced	Present	Affected	Absent	1087	0	14.1
Arg178His / Gly455Arg		Present	Affected	21.6	1300	24.4	4.00
Arg178His / Arg499Cys		Present	Affected	28.4	664	26.4	4.10
Arg178His / Arg499His		Present	Affected	33.8	1 022	27.1	3.80
Arg178His / Arg178His	Normal	Present	Affected	54.6	1045	60.8	2.60
					(610-1934)	(42.5-76.3)	(0.59-7.0)
					n=19	n=19	n=19
Arg178His / Arg252His		Present	Affected	55.4	873	56.3	7.60

[a] Ratio of mature α and β chains (α/β) as percentage of the control mean value; [b] Activity of Hex A expressed as % of total hexosaminidase activity recovered after ion-exchange chromatography.

❑ Function/structure relationship

To date the majority of natural mutations characterised at the molecular level have proven to involve changes that prevent the translation of a protein product. While these types of mutations are of little help in deducing protein structure/function relationships, they have confirmed that the severe infantile phenotype of the α-subunit deficiency results from total lack of residual activity towards ganglioside G_{M2}. On the other hand some mutations have provided useful biochemical informations. The mutation Arg178His occurs at putative catalytic site of the α-subunit, as assessed by *in vitro* expression studies (Brown et al., 1989, Tanaka et al., 1990). Secondary structure prediction provided evidence that the substitution Arg178His disrupts the three-dimensional configuration of the α-subunit (Ohno and Suzuki, 1988a; Suzuki and Vanier, 1991). The Arg499His (Paw et al., 1990a) and Arg504His (Paw et al., 1990a, Boustany et al., 1991), encoded in exon 13 of HEXA gene, were found to interfere with dimerization. These mutations provided starting points for the identification and characterisation of the protein domains that are involved in catalytic site for the hydrolysis of ganglioside G_{M2} and subunit association. Regarding to this aspect, the mutation Arg252His must be added to the group of identified mutations that are candidates for participation in or near the α-catalytic site of Hex A. To date, with the exception of Asp258His (Fernandes et al., 1992b) which also occurs in the segment encoded by exon 7, and of Val126Leu (Ainsworth and Coulter-Mackie, 1992), all the other B1 mutations described in the literature occurred at codon 178 (exon 5), in which arginine is replaced by histidine (Ohno and Suzuki, 1988a), cysteine (Tanaka et al., 1990) or leucine (Triggs-Raine et al., 1991). The residue Arg252 is 74 amino acids far apart of the Arg178 in the coding sequence. Although the similarity on the nature of the replaced amino acid the Arg178His and Arg252His alleles might have different consequences on the disruption of the terciary structure of the protein.

❑ Genotype-clinical phenotype correlation

The clinical variability observed among patients with B and B1 variant was found to be associated with a wide genetic heterogeneity (Table 2). Regarding to correlation between the genotype and the clinical phenotype some conclusions can be taken:

(i) The patients with +1IVS-7 G->C in homozygous status or combined with the null allele +TATC1278 had the infantile acute phenotype.

(ii) The homozygosity for the mutation ΔC1334 generated an infantile clinical phenotype.

(iii) The B1 patients homozygous for the Arg178His allele presented a subacute clinical phenotype with a juvenile onset of the disease, and were considered the prototype of juvenile forms of G_{M2}-gangliosidosis (Suzuki and Vanier, 1991).

(iv) The clinical phenotype of Arg178His compound heterozygous was variable, depending on the nature of the defect present in the second allele.

Assuming that the clinical phenotype depends on the residual catalytic activity of intralysosomal Hex A against G_{M2} ganglioside *in vivo* (Conzelmann and Sandhoff, 1991) it can be predicted that:

- In the homozygotes, the total residual activity of mutant Hex A against ganglioside G_{M2} generated by two Arg178His alleles is presumably sufficient to produce a juvenile-onset form of the disease.

- In compound heterozygotes, when the second allele contributes with no activity at all, the disease will present an earlier onset and more rapid progression but will not be as severe as in patients in whom neither allele generates residual activity, such as in classical infantile TSD; if the second allele carries any mutation that can also contribute with catalytic activity against the natural substrate, the clinical phenotype is expected to be correspondingly milder. Accordingly, the mutant enzyme with either Gly455Arg or Arg499Cys is expected to have less residual activity against G_{M2} ganglioside *in vivo*, than the enzyme expressed from the Arg178His allele, whereas that generated by the Arg499His allele or Arg252His is presumably sufficient to produce a milder phenotype. Considering that a mutant enzyme with higher residual activity will override that with less activity, patients who present at least one Arg499His or Arg252His allele would have a chronic phenotype, irrespective to the nature of mutation in the other allele.

TABLE 2: CORRELATION BETWEEN GENOTYPE AND CLINICAL PHENOTYPE IN PATIENTS WITH HEX α-CHAIN GENE DEFECTS

Genotype	Initial Symptom	Age at Onset	Present Age or Age at Death	Clinical Phenotype
+1IVS-7 / +1IVS-7	Dementia	6 months (I)	18 months (deceased)	Infantile acute
+1IVS-7 / +$TATC_{1278}$	Dementia	6 months (I)	NA (deceased)	Infantile acute
ΔC1334/ ΔC1334	Motor weakness	9 months (I)	NA (deceased)	Infantile acute
Arg178His/Gly455Arg	Gait disturbance	14 months (LI)	5 ½ years (deceased)	Subacute
Arg178His/Arg499Cys	Ataxia	24 months (LI)	5 years (deceased)	Subacute
Arg178His/Arg499His	Gait disturbance	5 years (J)	11 years	Subacute
Arg178His / Arg178His	Gait disturbance (13 cases) or Language delay (6 cases)	3-7 years (J)	8-14 years	Subacute
Arg499His / ?	NA	3 years (J)	20 years	Chronic
Arg499His / +1IVS-9	Gait disturbance and Language delay	3-4 years (J)	25 years	Chronic
Arg178His / Arg252His	Behavioural alterations	11 years (J)	32 years	Chronic

I, Infantile onset; LI, Late-infantile onset; J, Juvenile onset. NA, Not available.

The accumulation of genetic and clinical data was of particular relevance for genetic counselling since it can be used to predict the clinical manifestation in compound heterozygous and to define more accurately the carrier state in the families at risk.

❑ B1 variant carrier frequency

The exceptionally large number of unrelated Portuguese B1 variant patients was unexpected for this presumably very rare disease. Considering that the majority of patients are homozygous for a single gene defect and that consanguinity was found in only 3 of the 14 families, a high frequency for the Arg178His allele in the Portuguese population can be predicted. We know from the experience of other investigators (Fernandes et al., 1992b; Triggs-Raine et al. 1990) and our own experience that the best methodolgy to apply in a large-scale population screening results from the combination of an enzymatic and a DNA-based assay. Since the conventional enzymatic assay is known to lead to false results, the use of an assay based on the determination of activity of an identical amount of immunobound Hex A against the neutral and sulphated synthetic substrates was considered. The statistical evaluation of the data showed that if the most common B1 mutation (the Arg178His allele) was studied when the results falls in the higher misclassifying risk range, a probability of 0.005% and 0.03% of misclassifying a carrier as a control and a control as a carrier, respectively, could be calculated. The reliability of this assay, together with the simplicity, low cost and the attractive possibility of automation, make it the suitable enzymatic method for the assessment of carrier frequency.

❑ Arg178His allele: a Portuguese origin?

Considering that there is no positive record of relatedness among families, that 14 unrelated patients are homozygotes for the Arg178His allele and that only four are compound heterozygotes for that mutation, it appears that the mutation has been present in this population for some time. Moreover, the great majority of B1 patients described in the literature carry at least one Arg178His allele and have family from Portuguese background (Akli et al., 1991; Akli et al., 1993a). In keeping with the hypothesis of a common ancestral origin, the diverse migratory routes of the Portuguese could perhaps account for the wide geographical and ethnic distribution of the mutation. The presence of Arg178His

allele in at least some of the described cases, (i) a Spanish case (Goebel et al., 1989), (ii) the Puerto Rican patient (a large number of Portuguese emigrated to Puerto Rican around 1700) (Ohno and Suzuki, 1988a), (iii) the English/French/Azorean patient (whose maternal and paternal grandparents are from Azores) (Tanaka et al., 1990), and (iv) the French patients (Akli et al., 1993a) could be explained on this basis. The elaboration of variant haplotypes is therefore necessary to study the spread of the Arg178His mutation. Given the relative paucity of restriction fragment length polymorphisms, with a population frequency higher than 1%, identified in HEXA gene (Grinshpun et al., 1995; Kaplan et al., 1993), short tandem repeat analysis at HEXA locus could contribute toward testing this hypothesis. Until such studies are undertaken, it is difficult to prove that in all instances the allele might have been inherited from a single ancestor. Anyway, this is considered a more plausible explanation for its wide distribution than ascribing the finding to numerous independent G533A mutations.

5.2 FUTURE PROSPECTS

The introduction of specific nucleotide changes by *in vitro* mutagenesis and to assay the effect of substitutions through expression of mutant constructs in cells, such as monkey COS cells, CHO or insect cells, will give functional evidence for the implication of novel mutations in the α-subunit deficiency. These experiments are specially important regarding that some mutations were found in heterozygous state and not always combined with a known null allele. Furthermore, if the nucleotide changes are introduced near known codons involved in functional domains, it will be possible to fully ascertain the limits and function of each domain. The characterisation of additional naturally occurring mutations will certainly help to localize other potentially critical domains, such as the binding domain of Hex A to activator protein. A true understanding of the biological consequences of mutations on the transport and function of Hex A, as well as the complete definition of functional domains, will be only provided by analysis of the three-dimentional structure of protein and of active physiologic complex Hex A/ganglioside G_{M2}/activator protein. To date this information is only available for cathepsin A (d'Azzo et al., 1995) that has been purified using a baculovirus expression system. The limiting step in structural characterisation of lysosomal proteins has been the difficulty to obtain a protein preparation suitable for crystallization since the enzymes purified from tissues tend to be nicked and partially deglycosylated, as result of their residence in the

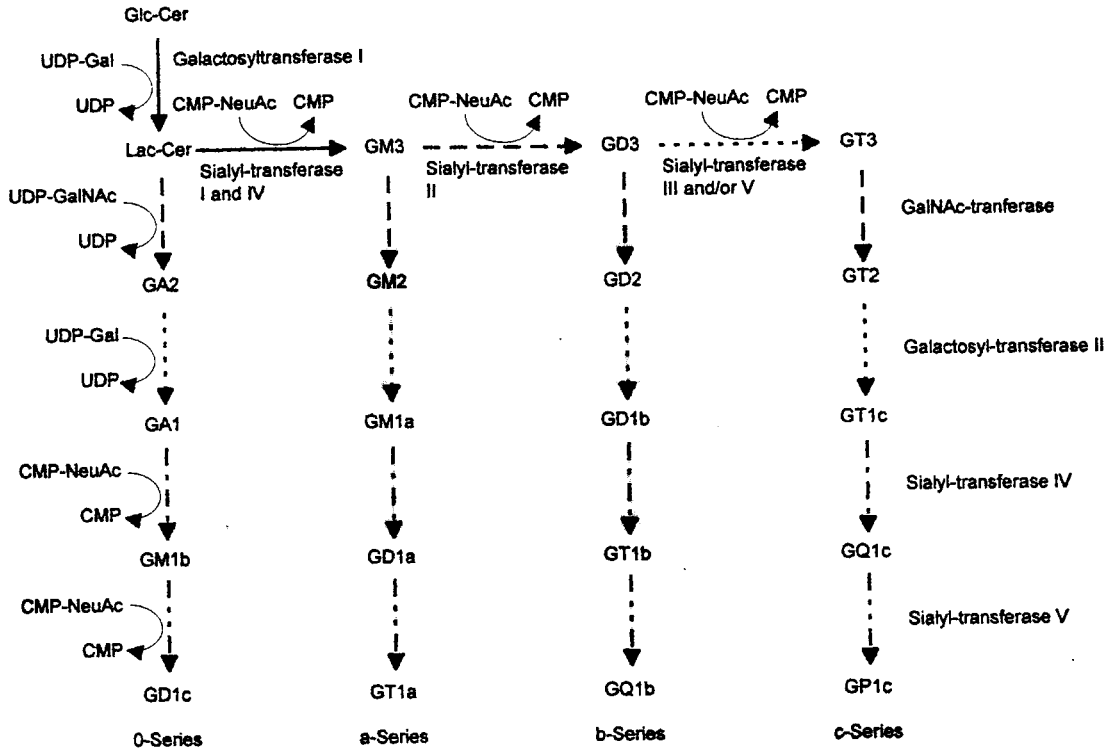
degradative organelles, and thus too heterogeneous. This problem will be soon overcome by using overexpressing cell lines that can secrete large quantities of glycoproteins in intact form and in quantities sufficient for structural studies.

The ability to produce large quantities of Hex A by recombinant DNA technology should also encourage new attempts at enzyme replacement therapy, for which the later-onset forms, such as B1 variant, are better candidates than the infantile Tay-Sachs disease. This approach can be expected to proceed in parallel with the development of gene replacement via viral vectors. The animal models created for Tay-Sachs disease (Yamanaka et al., 1994a) and Sandhoff disease (Sango et al., 1995) will be certainly used in the future to device novel therapeutic strategies and to study the pathophysiology of these disorders.

Another problem to be solved is the molecular basis for the transport of hexosaminidase to lysosomes in some type of cells such as leukocytes and neuronal cells. Since transfection of cDNA coding for α- and β-subunit result in Hex A expression (Brown and Mahuran, 1993), application of this technique in combination with site directed mutagenesis to cultured neuronal cells might be a promising approach.

APPENDIX I

GANGLIOSIDE BIOSYNTHESIS



Ganglioside biosynthesis (from van Echten and Sandhoff, 1994).

APPENDIX II

NUCLEOTIDE (cDNA) AND AMINO ACID SEQUENCES OF THE α-CHAIN OF HUMAN HEXOSAMINIDASE

1 **ATG** ACA AGC TCC AGG CTT TGG TTT TCG CTG CTG CTG GCG GCA GCG TTC GCA GGA CGG GCG ACG GCC CTC TGG CCC TGG CCT CAG

85 AAC TTC CAA ACC TCC GAC CAG CGC TAC GTC CTT TAC CCG AAC AAC TTT CAA TTC CAG TAC GAT GTC AGC TCG GCC GCG CAG CCC

169 GGC TGC TCA GTC CTC GAC GAG GCC TTC CAG CGC TAT CGT GAC CTG CTT TTC GGT TCC GGG TCT TGG CCC CGT CCT TAC CTC ACA

253 GGG AAA CGG CAT ACA CTG GAG AAG AAT GTG TTG GTT GTC TCT GTA GTC ACA CCT GGA TGT AAC CAG CTT CCT ACT TTG GAG TCA

337 GTG GAG AAT TAT ACC CTG ACC ATA AAT GAT GAC CAG TGT TTA CTC CTC TCT GAG ACT GTC TGG GGA GCT CTC CGA GGT CTG GAG

421 ACT TTT AGC CAG CTT GTT TGG AAA TCT GCT GAG GGC ACA TTC TTT ATC AAC AAG ACT GAG ATT GAG GAC TTT CCC CGC TTT CCT

505 CAC CGG GGC TTG CTG TTG GAT ACA TCT CGC CAT TAC CTG CCA CTC TCT AGC ATC CTG GAC ACT CTG GAT GTC ATG GCG TAC AAT

589 AAA TTG AAC GTG TTC CAC TGG CAT CTG GTA GAT GAT CCT TCC TTC CCA TAT GAG AGC TTC ACT TTT CCA GAG CTC ATG AGA AAG

673 GGG TCC TAC AAC CCT GTC ACC CAC ATC TAC ACA GCA CAG GAT GTG AAG GAG GTC ATT GAA TAC GCA CGG CTC CGG GGT ATC CGT

757 GTG CTT GCA GAG TTT GAC ACT CCT GGC CAC ACT TTG TCC TGG GGA CCA GGT ATC CCT GGA TTA CTG ACT CCT TGC TAC TCT GGG

841 TCT GAG CCC TCT GGC ACC TTT GGA CCA GTG AAT CCC AGT CTC AAT AAT ACC TAT GAG TTC ATG AGC ACA TTC TTC TTA GAA GTC

925 AGC TCT GTC TTC CCA GAT TTT TAT CTT CAT CTT GGA GGA GAT GAG GTT GAT TTC ACC TGC TGG AAG TCC AAC CCA GAG ATC CAG

1009 GAC TTT ATG AGG AAG AAA GGC TTC GGT GAG GAC TTC AAG CAG CTG GAG TCC TTC TAC ATC CAG ACG CTG CTG GAC ATC GTC TCT

1093 TCT TAT GGC AAG GGC TAT GTG GTG TGG CAG GAG GTG TTT GAT AAT AAA GTA AAG ATT CAG CCA GAC ACA ATC ATA CAG GTG TGG

1177 CGA GAG GAT ATT CCA GTG AAC TAT ATG AAG GAG CTG GAA CTG GTC ACC AAG GCC GGC TTC CGG GCC CTT CTC TCT GCC CCC TGG

1261 TAC CTG AAC CGT ATA TCC TAT GGC CCT GAC TGG AAG GAT TTC TAC GTA GTG GAA CCC CTG GCA TTT GAA GGT ACC CCT GAG CAG

1345 AAG GCT CTG GTG ATT GGT GGA GAG GCT TGT ATG TGG GGA GAA TAT GTG GAC AAC ACA AAC CTG GTC CCC AGG CTC TGG CCC AGA

1429 GCA GGG GCT GTT GCC GAA AGG CTG TGG AGC AAC AAG TTG ACA TCT GAC CTG ACA TTT GCC TAT GAA CGT TTG TCA CAC TTC CGC

1513 TGT GAG TTG CTG AGG CGA GGT GTC CAG GCC CAA CCC CTC AAT GTA GGC TTC TGT GAG CAG GAG TTT GAA CAG ACC TGA GCC CCA

GGC ACC GAG GAG GGT GCT GGC TGT AGG TGA ATG GTA GTG GAG CCA GGC TTC CAC TGC ATC CTG GCC AGG GGA CGG AGC CCC TTG

CCT TCG TGC CCC TTG CCT GCG TGC CCC TGT GCT TGG AGA GAA AGG GGC CGG TGC TGG CGC TCG CAT TC**A ATA AA**G AGT AAT GTG

GCA TTT TTC TAT **AAT AAA** CAT GGA TTA CCT GTG TTT AAA AAA AAA AGT GTG AAT

Nucleotide (cDNA) and amino acid sequences of human hexosaminidase α-chain (from Myerowitz et al., 1995). The nucleotides are numbered in the 5' to 3' direction , starting with the A of the ATG codon that encodes the initiator methionine; negative numbers refer to 5' untranslated region. Amino acids are numbered so that residue 1 is the initiator methionine. The three possible N-linked glycosylation sites are bolded, and the termination codon is indicated by •••. The polyadenylation signal in the 3' untranslated region is underlined.

1 MET Thr Ser Ser Arg Leu Trp Phe Ser Leu Leu Leu Ala Ala Ala Phe Ala Gly Arg Ala Thr Ala Leu Trp Pro Trp Pro Gln

29 Asn Phe Gln Thr Ser Asp Gln Arg Tyr Val Leu Tyr Pro Asn Asn Phe Gln Phe Gln Tyr Asp Val Ser Ser Ala Ala Gln Pro

57 Gly Cys Ser Val Leu Asp Glu Ala Phe Gln Arg Tyr Arg Asp Leu Leu Phe Gly Ser Gly Ser Trp Pro Arg Pro Tyr Leu Thr

85 Gly Lys Arg His Thr Leu Glu Lys Asn Val Leu Val Val Ser Val Val Thr Pro Gly Cys Asn Gln Leu Pro Thr Leu Glu Ser

113 Val Glu **Asn** Tyr Thr Leu Thr Ile Asn Asp Asp Gln Cys Leu Leu Leu Ser Glu Thr Val Trp Gly Ala Leu Arg Gly Leu Glu

141 Thr Phe Ser Gln Leu Val Thp Lys Ser Ala Glu Gly Thr Phe Phe Ile **Asn** Lys Thr Glu Ile Glu Asp Phe Pro Arg Phe Pro

169 His Arg Gly Leu Leu Leu Asp The Ser Arg His Tyr Leu Pro Leu Ser Ser Ile Leu Asp Thr Leu Asp Val MET Ala Tyr Asn

197 Lys Leu Asn Val Phe His Trp His Leu Val Asp Asp Pro Ser Phe Pro Tyr Glu Ser Phe Thr Phe Pro Glu Leu MET Arg Lys

225 Gly Ser Tyr Asn Pro Val Thr His Ile Tyr Thr Ala Gln Asp Val Lys Glu Val Ile Glu Tyr Ala Arg Leu Arg Gly Ile Arg

253 Val Leu Ala Glu Phe Asp Thr Pro Gly His Thr Leu Ser Trp Gly Pro Gly Ile Pro Gly Leu Leu Thr Pro Cys Tyr Ser Gly

281 Ser Glu Pro Ser Gly Thr Phe Gly Pro Val Asn Pro Ser Leu **Asn** Asn Thr Tyr Glu Phe MET Ser Thr Phe Phe Leu Glu Val

309 Ser Ser Val Phe Pro Asp Phe Tyr Leu His Leu Gly Gly Asp Glu Val Asp Phe Thr Cys Trp Lys Ser Asn Pro Glu Ile Gln

337 Asp Phe MET Arg Lys Lys Gly Phe Gly Glu Asp Phe Lys Gln Leu Glu Ser Phe Tyr Ile Gln Thr Leu Leu Asp Ile Val Ser

365 Ser Tyr Gly Lys Gly Tyr Val Val Trp Gln Glu Val Phe Asp Asn Lys Val Lys Ile Gln Pro Asp Thr Ile Ile Gln Val Trp

393 Arg Glu Asp Ile Pro Val Asn Tyr MET Lys Glu Leu Glu Leu Val Thr Lys Ala Gly Phe Arg Ala Leu Leu Ser Ala Pro Trp

421 Tyr Leu Asn Arg Ile Ser Tyr Gly Pro Asp Trp Lys Asp Phe Tyr Val Val Glu Pro Leu Ala Phe Glu Gly Thr Pro Glu Gln

449 Lys Ala Leu Val Ile Gly Gly Glu Ala Cys MET Trp Gly Glu Tyr Val Asp Asn Thr Asn Leu Val Pro Arg Leu Trp Pro Arg

477 Ala Gly Ala Val Ala Glu Arg Leu Trp Ser Asn Lys Leu Thr Ser Asp Leu Thr Phe Ala Tyr Glu Arg Leu Ser His Phe Arg

505 Cys Glu Leu Leu Arg Arg Gly Val Gln Ala Gln Pro Leu Asn Val Gly Phe Cys Glu Gln Glu Phe Glu Gln Thr •••

APPENDIX III

PARTICULAR MUTATIONS IDENTIFIED IN PATIENTS WITH TAY-SACHS DISEASE AND VARIANTS

MUTATIONS IN HEXOSAMINIDASE α-CHAIN GENE AFFECTING mRNA

Mutation	Location	mRNA	Clinical Phenotype	Origin	References
Δ 7.6 kb	5' to IVS-1	Undetectable	Infantile acute	French Canadians	Myerowitz and Hogikyan, 1986
G509A	Exon 5	Cryptic splicing in exon 5, at site of mutation	Infantile acute	Japanese Moroccan Jews	Nakano et al., 1990 Drucker and Proia, 1992
G->A	+1IVS-9	Abnormal splicing; low level of mRNA with cryptic splices sites	Infantile acute	Diverse; probable Celtic origin	Akerman et al., 1992; Akli et al., 1991; Landels et al., 1993
+TATC1278	Exon 12	Absent, normal transcription; stable mRNA in COS cells	Infantile acute	Diverse; Ashkenazi Jews	Myerowitz and Costigan, 1988; Nishimoto et al., 1988; Paw et al.,1988;
G->C	+1 IVS-12	Trace level of abormal mRNA with retention of intron 12 or exclusion of exon 12	Infantile acute	Ashkenazi Jews	Arpaia et al., 1988; Myerowitz, 1988; Ohno and Suzuki, 1988b; Ohno and Suzuki, 1988c

MUTATIONS IN HEXOSAMINIDASE α-CHAIN GENE AFFECTING PROTEIN

Mutation	Location	Codon change	Protein	Clinical Phenotype	Origin	References
G509A	Exon 5	Arg170Gln	COS transfection: α chain is formed in reduced amount and is unstable	Infantile acute	Japanese Moroccan Jews	Nakano et al., 1990 Drucker and Proia, 1992; Tanaka et al., 1994
C532T	Exon 5	Arg178Cys	B1-type catalytic defect; absence of Hex S activity in COS transfection	Infantile acute	Czechoslovakian	Tanaka et al., 1990
G533T	Exon 5	Arg178His	B1-type catalytic defect; in COS transfection, absence of Hex S activity (normal level of mRNA)	Subacute (homozygotes)	Europeans	Akli et al., 1991; Dos Sant et al., 1991; Kytzia et al., 1983; Ohno and Suzuki, 1988a; Tanaka et al., 199(
G533T	Exon 5	Arg178Leu		Infantile acute	English	Triggs-Raine et al., 1991
C540G	Exon 5	Tyr178Stop		Infantile acute	Moroccan Jews	Drucker and Proia, 1992
G574C	Exon 6	Val192Leu	B1-type catalytic defect	Infantile acute	German/Romanian	Ainsworth and Coulter-Mackie, 1992; Gordon et a 1988;
G749A	Exon 7	Gly250Asp	α-chain unstable and unphosphorylated	Subacute (homozygotes)	Lebanese	Trop et al., 1992
G772C	Exon 7	Asp258His	B1-type catalytic defect	Infantile acute	Scottish-Irish	Fernandes et al., 1992b
G805A	Exon 7	Gly269Ser	Reduced level of α-chain and defective subunit association; in COS transfection, α-chain unstable, soluble and phosphorylated	Chronic (homozygotes)	Diverse; Ashkenazi Jews	d'Azzo et al., 1984; Navon and Proia, 1989; Paw et al 1989
ΔTTC910-912	Exon 8	ΔPhe304 (or 305)	Defective subunit assembly and secretion	Infantile acute	Moroccan Jews; French	Akli et al., 1991; Navon an Proia, 1981

MUTATIONS IN HEXOSAMINIDASE α-CHAIN GENE AFFECTING PROTEIN

Mutation	Location	Codon change	Protein	Clinical Phenotype	Origin	References
G1444A	Exon 13	Glu482Lys	Early defect in processing	Infantile acute (homozygote)	Italian, Chinese	Akalin et al., 1992; Proia and Neufeld, 1982; Nakano et al., 1988
T1453C	Exon 13	Trp485Arg	COS cells transfection gives no detetable mature α-chain or Hex S activity	Infantile acute	Chinese	Akalin et al., 1992
C1495T	Exon 13	Arg499Cys		? (Chronic phenotype with G805A in the second allele)	Slavic/Irish/English/Polish	Mules et al., 1992
G1496A	Exon 13	Arg499His	Early defect in processing, α-chain degraded	Subacute (second allele +TATC1278)	Scotish/Irish Jewish	Paw et al., 1990a
ΔC1510	Exon 13		Truncated α-chain; early defect in processing, α-chain degraded (apparent normal level of mRNA)	Infantile acute (homozygote)	Italian	Lau and Neufeld, 1989; Zozaeem et al., 1987
C1510T	Exon 13	Arg504Cys	In COS transfection fails to to form dimers, the monomer is secreted	Infantile acute (homozygote)	French, algerian, German	Akli et al., 1991; Paw et al., 1991
G1511A	Exon 13	Arg504His	Defective dimerization but α-chain is phosphorylated	Subacute (homozygote)	Assyrian, Lebanese, Armenian, Yugoslavian	Akli et al., 1993b; Boustany et al., 1991; Paw et al., 1990a

APPENDIX IV

STRUCTURE OF GLYCOLIPIDS STORED IN G_{M2}-GANGLIOSIDOSES

GLYCOLIPID	STRUCTURE[a]
G_{M2}	GalNAc(β1->4)NeuAc(α2->3)Gal(β1->4)Glc(β1->1')Cer
G_{A2}	GalNAc(β1->4)Gal(β1->4)Glc(β1->1')Cer
G_{D2}	GalNAc(β1->4)[NeuAc(α2->8)NeuAc(α2->3)]Gal(β1->4)Glc(β1->1')
Globoside	GalNAc(β1->3)Gal(α1->4)Gal(β1->4)Glc(β1->1')Cer
Liso-G_{M2}	GalNAc(β1->4)[NeuAc(α2->3)]Gal(β1->4)Glc(β1->1')Esfingosina
G_{D1a}-GalNAc	GalNAc(β1->4)[NeuAc(α2->3)]Gal(β1->3)GalNAc(β1->4)[NeuAc (α2->3)]Gal(β1->4)Glc(β1->1')Cer
G_{M1b}-GalNAc	GalNAc(β1->4)[NeuAc(α2->3)]Gal(β1->3)GalNAc(β1->4)Gal(β1->4)Glc (β1->1')Cer
G_{M1a}-GalNAc	GalNAc(β1->4)Gal(β1->3)GalNAc(β1->4)[NeuAc(α2->3)]Gal(β1->4)Glc (β1->1')Cer
G_{M3}*	NeuAc(α2->3)Gal(β1->4)Glc(β1->1')Cer
G_{D3}*	NeuAc(α2->8)NeuAc(α2->3)Gal(β1->4)Glc(β1->1')Cer

[a]From Sandhoff et al., 1989. *The gangliosides G_{M3} and G_{D3} are not substrates of Hex A; its accumulation in the G_{M2}-gangliosidoses results probably from a secondary alteration of the disease (Sandhoff et al., 1989).

APPENDIX V

ABBREVIATIONS AND NOMENCLATURE

bp	base pair
BSA	Bovine serum albumine
CNS	Central nervous system
DNA	Desoxiribonucleic acid
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
gDNA	Genomic DNA
GlcNAc	N-acetilglucosamine
GLS	Glycosphingolipid
GM2A	G_{M2} activator gene
Hex A	Hexosaminidase A
HEXA	Hexosaminidase α-chain gene
Hex B	Hexosaminidase B
HEXB	Hexosaminidase β-chain gene
Hex C	Hexosaminidase C
Hex S	Hexosaminidase S
kb	Kilo base pairs
kDa	Kilo dalton
K_m	Michaelis-Menten constant
MCB	Membranous cytoplasmic bodies
MPR	Mannose-6-phosphate receptor
PM	Plasma membrane
NGF	Nerve growth factor
pI	Isoelectric point
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RAM	Rabbit anti-(mouse IgG) antibodies
RER	Rough endoplasmic reticulum
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
SSCP	Single-strand conformational polymorphism
TSD	Tay-Sachs disease
V_{max}	Initial maximum velocity
4MU-GlcNAc	4-methyllumbellyferil-β-D-acetilglucosamine
4MU-GlcNAcS	4-methylumbellyferil-β-D-acetilglucosamine-6-sulphate

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