CLINICAL, BIOCHEMICAL AND GENETIC HETEROGENEITY IN LYSOSOMAL STORAGE DISEASES

PROEFSCHRIFT

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aan Mariette
aan mijn ouders

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ἐὰν μὴ ἔλπηται ἀνέλπιστον οὐκ ἐξευρήσει,

ἀνεξερεύνητον ἐὸν καὶ ἄπορον.

HERACLITUS

CHAPTER I

GENERAL INTRODUCTION

The history of lysosomal storage diseases dates back to the end of the last century when the first clinical reports appeared of patients suffering from these genetic, metabolic disorders (Tay, 1881; Gaucher, 1882; Sachs, 1887; Fabry, 1898). About seventy years would pass before the term "lysosome" was introduced by De Duve and co-workers (1955, 1965) to indicate a membrane-surrounded cytoplasmic particle containing acid hydrolases that was isolated by centrifugal cell fractionation. Later, the lysosomes were cytochemically demonstrated by Novikoff (1961) using an appropriate staining reaction for acid phosphatase and some other acid hydrolases. In the period that followed an impressive amount of work has been carried out on the structure and function of lysosomes (see for review Dingle and Fell, 1969-1975) and at present a few dozen of different acid hydrolases is known to be localized within this organelle. The main function of the lysosomal enzymes is the degradation of material that is enclosed in secondary lysosomes by fusion of the primary lysosome with hetero- and autophagosomes or by other processes (Fig. 1).

The first genetic defect in one of these lysosomal enzymes was discovered by Hers (1963) who described that glycogenosis type II, Pompe's disease, was due to a deficiency of acid α-glucosidase. The lysosomal character of this glycogen storage disease was confirmed by cell fractionation studies, indicating acid α-glucosidase as a lysosomal enzyme and morphological studies which demonstrated the deposit of glycogen within the lysosomes (Lejeune et al., 1963; Baudhuin et al., 1964).

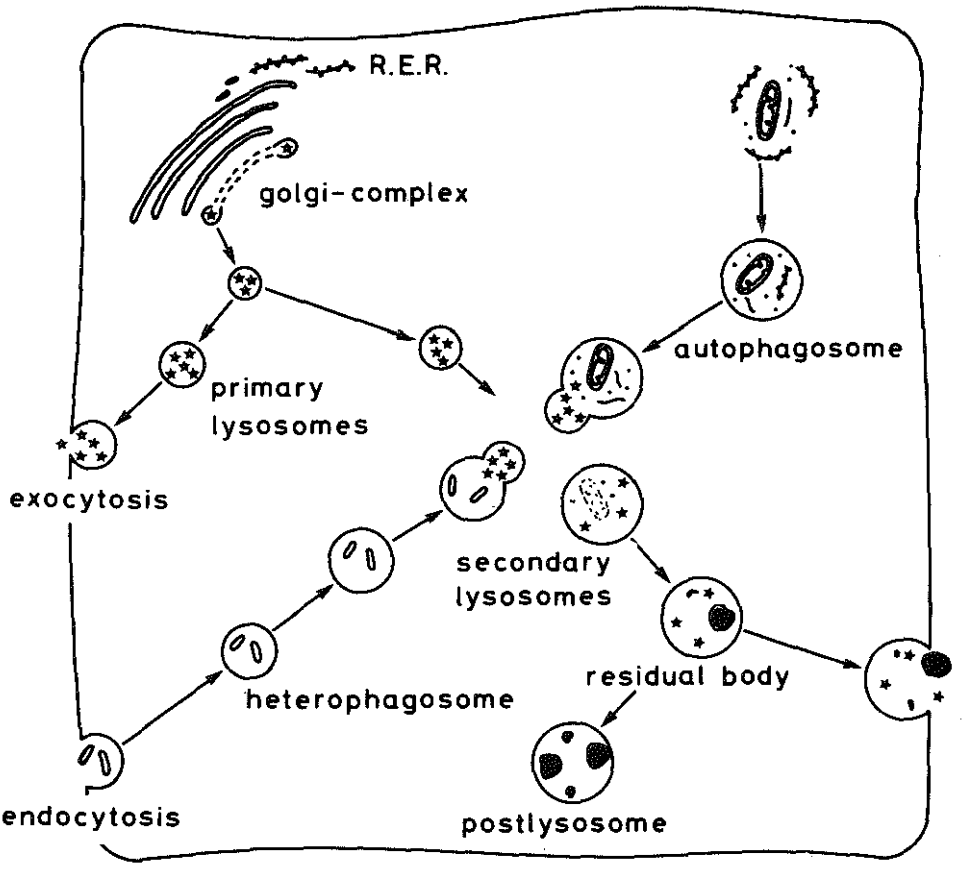

Fig. 1 Schematic representation of the lysosomal system and its function.

During the last decade many other metabolic disorders have been recognized as lysosomal storage diseases and the responsible enzymatic defects were resolved for most of them. Genetic defects have now been found for enzymes involved in the degradation of carbohydrates (Hers and De Barsy, 1973), mucopolysaccharides (Neufeld et al., 1975; Cantz and Gehler, 1976), lipids (Brady, 1972, 1976) and various other metabolites. In most instances the enzyme deficiencies were discovered after the accumulating substances had been analyzed and the main catalytic activity

of the particular enzymes had been determined. Once the enzyme defects were known the diagnosis of many of these diseases has been facilitated by the development of artificial substrates providing simple procedures to assay the enzyme activities (Leaback, 1976).

The diagnosis has further been facilitated by the fact that quite a number of lysosomal enzyme deficiencies can be demonstrated in leucocytes and cultured skin fibroblasts (see for review Nadler, 1972; Nitowsky, 1972). The expression of the enzymes in cultured cells has opened the way to prenatal diagnosis of these diseases (see for review Milunsky, 1973; Galjaard et al., 1975) and has allowed to use cultured cells as model system to study biochemical and genetic aspects of lysosomal storage diseases. For instance the cell culture system has widely been used to study the mucopolysaccharide storage diseases.

Danes and Bearn (1965) described that the accumulation of acid mucopolysaccharides (glycosaminoglycans) occurred in cultured skin fibroblasts from patients with certain types of mucopolysaccharidosis. Subsequently Fratantoni et al. (1968) demonstrated that the intracellular accumulation of mucopolysaccharides was due to an impaired degradation and that the accumulation could be corrected by co-cultivation of cells from the patient with cells from a control individual or from a patient with another type of mucopolysaccharidosis (Fratantoni et al., 1969). In the years to follow these "corrective factors" were purified and identified as lysosomal enzymes involved in the degradation of mucopolysaccharides. The specific enzyme deficiencies have now been resolved for each of the clinical forms of mucopolysaccharidoses (McKusick, 1975; Neufeld et al., 1975; Dorfman and Matalon, 1976).

The cell culture system has also provided the means to study the genetic background of metabolic disorders which is especially of interest in those cases where apparently the same metabolic defect leads to different clinical and pathological manifestations. For example, the technique of somatic cell hybridization followed by an analysis of heterokaryons was first used by De Weerd-Kastelein et al. (1972) to investigate the genetic background of clinical heterogeneity in the skin disease xeroderma pigmentosum. Subsequently this method was used by Thomas et al. (1974) and Galjaard et al. (1974a), who demonstrated independently that two different forms of the lysosomal storage disease G_{M2}-gangliosidosis were based on a separate gene defect.

During the past ten years much has been learned about lysosomal storage diseases. The diagnosis has been extended from the exact description of the clinical symptoms to the morphological and biochemical characterization of the accumulated products, the demonstration of the enzyme or isoenzyme deficiency and sometimes even to the identification of the type of gene mutation. Compared with the development in diagnosis less is known about the pathogenesis of lysosomal storage diseases. The consequence of a lysosomal enzyme deficiency will be that cellular and extracellular constituents that cannot be degraded accumulate in residual bodies which after loss of their lysosomal enzymes are called postlysosomes (Daems et al., 1972). Since most cells are not able to discharge their residual bodies in the extracellular fluid, the accumulation of undigestable material in the lysosomal system continues gradually and this may at last severely interfere with the function of the cells of which many will degenerate. The clinical expression of the enzyme defect will of course largely depend on the type and number of cells that are affected and the particular function of the deficient enzyme in the

metabolism of these cells. However, also the clinical manifestation of a distinct lysosomal enzyme deficiency may vary extremely within the same metabolic disorder and the reason for this clinical heterogeneity is not yet sufficiently understood. Finally there is much to be learned about the correlation between the degree of enzyme deficiency, the disturbance of certain metabolic pathways and the resulting impairment of cellular functions.

The present thesis deals with the study of some of these problems related to the clinical, biochemical and genetic heterogeneity in G_{M2}-gangliosidosis, G_{M1}-gangliosidosis and glycogenosis type II.

CHAPTER II

CLINICAL, BIOCHEMICAL AND GENETIC ASPECTS OF G_{M2}-GANGLIOSIDOSIS, G_{M1}-GANGLIOSIDOSIS AND GLYCOGENOSIS TYPE II

G_{M2}-gangliosidosis

(Tay-Sachs disease, Sandhoff's disease)

G_{M2}-gangliosidoses are autosomal recessive diseases characterized by the lysosomal accumulation of G_{M2}-ganglioside due to a deficiency of N-acetyl-β-hexosaminidase (EC.3.2.1.30). The vast majority of cases concerns type 1 G_{M2}-gangliosidosis or Tay-Sachs disease, a smaller proportion of patients are suffering from type 2 G_{M2}-gangliosidosis, known as Sandhoff's disease.

The clinical manifestation of the disease is rather similar in both types. The first clinical symptoms become apparent between the third and sixth month after birth, the children are apathetic, the psychomotoric development is retarded and the muscular tonus is low. After one year a rapid psychomotor deterioration is noticed and spasticity develops. Ophtalmologic investigation reveals the typical "cherry red spot" as a result of macular degeneration, blindness and deafness occur and the patients expire between one and a half year and five years, usually from recurrent infections (see for review O'Brien, 1973; Sandhoff and Harzer, 1973; Galjaard and Reuser, 1977).

The most stricking pathological manifestations occur in the brain. Ganglion cells of the cerebral cortex and Purkinje cells are packed with vacuoles, that contain concentric layers of lipid, representing deposits of G_{M2}- and asialo

G_{M2}-ganglioside. A considerable number of ganglion cells is lost and many axons are demyelinated. Lipid inclusions are only occasionally found in liver and spleen. In Sandhoff's disease an additional accumulation of globoside was demonstrated in the visceral organs (Sandhoff et al., 1968). There is also evidence that the β-hexosaminidase deficiency affects the degradation of certain glycoproteins (Stricker and Montreuil, 1971), glycosaminoglycans (Cantz and Kresse, 1974) and oligosaccharides (Tsay and Dawson, 1976).

A third form of G_{M2}-gangliosidosis has been distinguished as a juvenile form of Tay-Sachs disease, which is in comparison with the other two forms characterized by a later onset and a longer survival of the patients. In the first years of life the accumulation of G_{M2}- and asialo G_{M2}-ganglioside seems to be less prominent than in the infantile form of Tay-Sachs disease, but at a more advanced age a progressive neurological deterioration develops and death occurs between 5 and 15 years (Bernheimer and Seitelberg, 1968,;Suzuki et al., 1970). In additon to these three main forms of G_{M2}-gangliosidosis, patients have been reported incidentally with an atypical expression of G_{M2}-gangliosidosis (Sandhoff et al., 1971; Spence et al., 1974; Navon et al., 1973; Dreyfus et al., 1975).

In 1966 it was suggested by Svennerholm that G_{M2}-gangliosidosis would be due to a hexosaminidase deficiency. Subsequently Robinson and Stirling (1968) identified two isoenzymes of β-hexosaminidase, A and B, and Okada and O'Brien (1969) discovered that Tay-Sachs disease was caused by a deficiency of hexosaminidase A which enzyme is able to hydrolyze G_{M2}- and asialo G_{M2}-ganglioside (Sandhoff et al., 1971). In Sandhoff's disease, originally presented as a variant of Tay-Sachs disease, a deficiency of both iso-

enzymes was demonstrated (Sandhoff et al., 1968).
The activity of β-D-N-acetylhexosaminidase A and B (Hex A and Hex B) is expressed in most human tissues as well as in cultured skin fibroblasts and amniotic fluid cells, which latter allows the prenatal detection of the disease. The prenatal diagnosis has especially contributed to the prevention of the disease, when combined with a population screening program for heterozygous carriers among Askenazy jews (Kaback et al., 1974). The enzyme assay can be carried out with the artificial substrates p-nitrophenyl-β-D-N-acetylglucosaminide (or-galactosaminide) and 4-methyl-umbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside (or galactopyranoside). Furthermore, Hex A releases the terminal N-acetylgalactosamine from G_{M2}- and asialo G_{M2}-ganglioside and globoside. Hex B would not be active towards G_{M2}-ganglioside (Sandhoff and Wässle, 1971) but this has been disputed by Tallman and co-workers (1974a).

The juvenile form of Tay-Sachs disease seems to reflect a partial deficiency of Hex A (O'Brien, 1969). The other clinical variants that have been described, may result from a partial deficiency of Hex A and Hex B (Spence et al., 1974) or from a specific loss of activity towards the natural substrate (Sandhoff et al., 1971). On the other hand healthy individuals have been described with an enzyme deficiency towards the artificial substrate (Navon et al., 1973, 1976; Dreyfus et al., 1975).

Several components of β-hexosaminidase activity exist in addition to Hex A and Hex B which are called Hex C (Hooghwinkel et al., 1972), Hex S (Ikonne et al., 1975), I_1 and I_2 (Ellis et al., 1975; Price and Dance, 1972) and Hex P (Swallow et al., 1974). Hex A and Hex B have been purified from human liver and placenta and were found to have a

molecular weight of 1-1,5 x 10^5, and to be composed of subunits with a molecular weight of about 25 x 10^3 (Srivastava et al., 1974a,b; Tallman et al., 1974; Lee and Yoshida, 1976). The interrelation between the various isoenzymes and the exact nature of the enzyme defects in various types of G_{M2}-gangliosidosis have been extensively studied by biochemical, immunological and cell genetic analyses. The results of these studies will be discussed in more detail in chapter III.

G_{M1}-gangliosidosis

(Landing's disease, pseudo-Hurler disease, generalized gangliosidosis)

G_{M1}-gangliosidoses are autosomal recessive disorders characterized by the storage of G_{M1}-gangliosides and glycosaminoglycans due to a deficiency of acid β-galactosidase (EC.3.2.1.23). Patients with distinct clinical forms of the disease have been described (see for reviews Van Hoof, 1973; O'Brien, 1975; Galjaard and Reuser, 1977).

Patients with the infantile (type 1) form of G_{M1}-gangliosidosis develop symptoms shortly after birth. The main clinical features are severe psychomotor retardation, bone deformities and hepatosplenomegaly. The disease is rapidly progressive and patients die mostly in the second year of life. The juvenile (type 2) form of G_{M1}-gangliosidosis is milder. The first symptoms appear between six and twenty months after birth, the visceral organs are not or much less involved and bone deformities are minimal (Derry et al., 1968; Wolfe et al., 1970; Lowden et al., 1974).

Progressive mental and motoric retardation are the main clinical symptoms and in general these patients die between their sixth and tenth year of life.

Pinsky et al. (1974) and more recently Andria et al. (1977) described patients with some of the characteristic features of G_{M1}-gangliosidosis but without signs of psychomotor retardation. Finally a number of patients with an adult form of G_{M1}-gangliosidosis has been reported who showed late onset of neurological abnormalities and slow psychomotor regression whereas in some instances skin lesions like angiokeratomata were observed (Loonen et al., 1974; Yamamoto et al., 1974; Wenger,et al., 1974; Orii et al.,1975)

The pathological manifestations comprise of lysosomal inclusions which are either of lamellar membranous nature or they are granular or amorphous. These inclusions are mostly seen in neuronal cells, histiocytes and parenchymal cells of liver and spleen. The accumulation of G_{M1}-ganglioside is most pronounced in brain and accounts for 60 to 80% of the total amount of gangliosides. The amount of G_{M1}-ganglioside in visceral organs varies but the content of keratansulphate like mucopolysaccharide is always increased (Suzuki et al., 1971).

In 1968 Okada and O'Brien and also Van Hoof and Hers demonstrated that a deficiency of the lysosomal enzyme β-galactosidase is responsible for G_{M1}-gangliosidosis. Later, a partial or total deficiency of acid β-galactosidase was found in all forms of the disease both using the natural substrate or one of the artificial substrates p-nitrophenyl -β-D-galactoside or 4-methylumbelliferyl-β-D-galactopyranoside. The enzyme has been demonstrated in brain, kidney, liver and spleen and it can also be detected in leucocytes,

cultured skin fibroblasts and amniotic fluid cells. The latter has enabled prenatal monitory for the disease (Lowden et al., 1973; Kleijer et al., 1976).

From human liver two main forms of acid β-galactosidase were separated: a low molecular weight form (MW = 7×10^4) which is thought to be a monomer and a high molecular weight form (MW = 7×10^5), which might represent an aggregate of identical monomers (Norden et al., 1974) or may represent the low molecular form of the enzyme bound to lysosomal membrane fragments (Cheetham and Dance, 1976). It has been demonstrated that the enzyme may also occur as dimer and that the low molecular forms can be generated from the high molecular form (Hultberg and Ockerman, 1972; Cheetham and Dance, 1976). In patients with G_{M1}-gangliosidosis all these forms of β-galactosidase are deficient. The purified enzyme exhibits activity towards G_{M1}-ganglioside, asialo fetuin, keratansulphate, oligosaccharides and the artificial substrates mentioned above.

In addition to G_{M1}-β-galactosidase there is another acidic β-galactosidase which is deficient in Krabbe's disease (Suzuki and Suzuki, 1970; Malone, 1970) and a neutral form of β-galactosidase that does not have activity towards G_{M1}-ganglioside (Robinson et al., 1967; Ho and O'Brien, 1969; Ockerman and Hultberg, 1968).

Glycogenosis type II

(Generalized glycogenosis, Pompe's disease)

Glycogenosis type II is an autosomal recessive disorder,

characterized by the lysosomal accumulation of glycogen and the deficiency of acid α-glucosidase (EC.3.2.1.20). It was first recognized by Pompe in 1932 as a generalized glycogen storage disease, fatal in the first years of life and classified as glycogenosis type II by Cori (1957). The enzyme defect was discovered by Hers (1963) and the lysosomal character of the disease was established (Lejeune et al., 1963; Baudhuin et al., 1964). Once the enzyme defect was known clinical variants of acid α-glucosidase deficiency were recognized which differed in time of onset and severity of the symptoms. Infantile, juvenile and adult forms of glycogenosis type II have been reported.

In the classical, infantile, form cardiac enlargement is usually detectable shortly after birth, skeletal muscles are firm but hypotonia is severe and neurological defects might be present. Sometimes patients have a protruding tongue and hepatosplenomegaly may occur although the liver function is mostly undisturbed. In general these patients die in their first year of life because of cardiorespiratory insufficiency (Di Sant'Agnese et al., 1950).

Patients suffering from the adult form of glycogenosis type II have less abundant accumulation of glycogen in their skeletal muscles and cardiac symptoms are not prominent. The acid α-glucosidase deficiency is mainly manifested by progressive muscular weakness, starting in the second or third decade of life (Engel, 1970). Often the muscular weakness is misinterpreted as polymyositis or muscular dystrophy.

The juvenile form is in most respects an intermediate one between the infantile and adult form (Hers, 1965; Hers and Van Hoof, 1968; Hudgson et al., 1968).

Acid α-glucosidase activity has been demonstrated in most tissues including lymphocytes, skin fibroblasts and amniotic fluid cells (Nitowsky and Grunfeld, 1967; Salafsky and Nadler, 1971; Hers and De Barsy, 1973). The latter has enabled prenatal diagnosis in pregnancies at risk (Nadler and Messina, 1969; Galjaard et al., 1973; Niermeijer et al., 1975).

Acid α-glucosidase has been purified from rat and bovine liver (Bruni et al., 1969; Jeffrey et al., 1970a), from rabbit muscle (Palmer, 1971a,b) and from human placenta and liver (De Barsy et al., 1972; Belenky and Rosenfeld, 1975; Koster et al., 1976). The molecular weight of acid α-glucosidase is about 10^5 and the enzyme is thought to consist of different subunits (Belenky and Rosenfeld, 1975). Evidence was presented that the enzyme has two separate catalytic sites, one for oligosaccharides and one for glycogen (Jeffrey et al., 1970b; Palmer, 1971a,b; Koster and Slee, 1977). Acid α-glucosidase activity can be determined with glycogen or maltose as substrate but also with the artificial substrates p-nitrophenyl-α-D-glucoside or 4-methylumbelliferyl-α-D-glucopyranoside. Besides hydrolytic activity the enzyme exhibits glucosyl transferase activity. The main catalytic function in vivo seems to be the degradation of lysosomal glycogen but the enzyme might also be involved in the degradation of glycoproteins or oligosaccharides (Wolfe and Cohen, 1968; Schnabel, 1971). In addition to acid α-glucosidase there exist several forms of α-glucosidase with a pH optimum around 6.5. The activities of these "neutral" α-glucosidases towards glycogen are very low but they exhibit activity towards maltose and the artificial substrates (Ushakova and Lukomskaya, 1976).

CHAPTER III

THE EXPERIMENTAL WORK: INTRODUCTION, DESCRIPTION AND DISCUSSION

Introduction

The lysosomal storage diseases discussed before are genetically determined and must be based on the mutation of a gene involved in the "realization" of the relevant enzyme. The term "enzyme realization", introduced by Paigen (1971) covers the whole set of processes that is required to produce a catalytically active enzyme, including transcription, translation and post translation events. Several genes, each with a particular function, will be involved in this process. The structural gene determines the amino acid sequence of the polypeptide chains and thereby to a great extent the specific catalytic properties of an enzyme. Depending on the nature and the position of the structural mutation the enzyme that is coded by the gene may loose its catalytic activity completely or partially. In case an enzyme acts on several substrates it is also possible that the activity towards only one of the several natural substrates is lost. Other structural mutations may completely block the synthesis of the enzyme. In addition to structural genes there may be regulator genes, which control the rate of enzyme synthesis and degradation and the activity of the enzyme. According to Paigen (1971) architectual genes have a function to direct the enzymes to their proper location whereas temporal genes would control the timing of the various events. Also a mutation in one of these latter genes might in principle lead to an enzyme deficiency. Different gene mutations may result in different properties of the "mutant enzyme" and this in turn might have con-

sequences for the clinical and pathological manifestation of the disease.

The most direct approach to investigate whether clinical heterogeneity is based on different gene mutations is to perform complementation analyses as generally used for the genetic characterization of bacterial mutants (see for reviews Fincham, 1966; Ratner and Rodin, 1976). Complementation studies to investigate genetic heterogeneity were first described by De Weerd-Kastelein et al. (1972), who demonstrated that two clinical forms of xeroderma pigmentosum were based on different gene mutations. In the following years a number of other complementation studies including some on lysosomal storage diseases were reported (see for reviews Ruddle and Creagan, 1975; Ringertz and Savage, 1976; Galjaard and Reuser, 1977). In complementation studies cultured fibroblasts from patients with "the same" enzyme deficiency are fused and multinuclear heterokaryons are formed, which carry the genetic information of both mutant cell strains. If the combination of the two genomes in heterokaryons results in a restoration of the metabolic defect, this is called complementation. When complementation occurs, it is a proof that the enzyme deficiency in both patients from whom the cells were derived, is caused by two different gene mutations. When restoration of the enzyme activity results from the cooperation of two different genes, it is called intergenic complementation. When both mutations are located in the same gene and complementation occurs, it is called interallelic or intragenic complementation.

A second approach to identify different gene mutations is to characterize the product that is coded for by the mutant gene. An excellent example of this approach is the study of

variant forms of human haemoglobins which indicated the existence of a large number of mutations in the genetic code for the polypeptide chains that form the haemoglobins (see for review Harris, 1975). The biochemical or immunological characteristics of the mutant gene product might at the same time be informative about its disturbed metabolic function and about the clinical expression of the genetic defect.

The third strategy that can be followed is that of gene localization, where human fibroblasts or leucocytes are fused with cultured fibroblasts from Chinese-hamster or mouse origin. After nuclear fusion has occurred proliferating human-rodent hybrid cells (synkaryons) can be isolated using selective culture media. In such proliferating hybrids a preferential loss of human chromosomes occurs. Correlation of the human chromosome content and the expression of human gene products in various hybrid clones enables the localization of genes (see for review Ruddle and Creagan, 1976) and information may be obtained about the number of genes that code for a particular enzyme.

In our experimental work on the relation between clinical, biochemical and genetic heterogeneity of some lysosomal storage diseases, all three approaches, as mentioned above, have been used. The results will be discussed separately for G_{M2}-gangliosidosis, G_{M1}-gangliosidosis and glycogenosis type II.

Experimental studies on G_{M2}-gangliosidosis

At the beginning of our investigations the enzyme defects responsible for type 1 G_{M2}-gangliosidosis (Tay-Sachs dis-

ease) and for the type 2 variant (Sandhoff's disease) had been elucidated (Okada and O'Brien, 1969; Sandhoff et al., 1968).

The purpose of the experiments described in Publication I was to investigate whether different gene mutations were responsible for the deficiency of hexosaminidase A (Hex A) in Tay-Sachs disease and for the absence of Hex A and Hex B in Sandhoff's disease. Cultured skin fibroblasts from patients with these two variant forms of G_{M2}-gangliosidosis were hybridized using inactivated Sendai virus and the Hex A activity was measured in the heterokaryons 2-7 days after cell fusion. The activity of Hex A was determined by selective heat inactivation of the enzyme and by electrophoretic separation of Hex A and Hex B. Both methods revealed the restoration of Hex A activity in the heterokaryons. A similar result was obtained independently by Thomas et al. (1974) and later by Ropers et al. (1975) and Rattazzi et al. (1976).

The occurrence of genetic complementation meant a definite proof for the existence of two different gene mutations in Tay-Sachs and Sandhoff's disease. We ascribed the restoration of Hex A activity to intergenic complementation, which would fit the hypothesis that Hex A and Hex B share a common subunit, whereas in addition Hex A would have a specific subunit (Robinson and Carroll, 1972; Ropers and Schwantes, 1973; Srivastava and Beutler, 1973). This theory explained Sandhoff's disease on the basis of a defect in the common subunit and Tay-Sachs disease by a defect in the specific Hex A subunit. The complementation studies, however, did not rule out the original hypothesis by Robinson and Stirling (1968) that Hex B would be a precursor of Hex A explaining Tay-Sachs disease by a defect in the

conversion of Hex B to Hex A and Sandhoff's disease by a deficiency of Hex B, thereby also resulting in a deficiency of Hex A (Tateson and Bain, 1971; Goldstone et al.. 1971).

To further investigate the relationship between Hex A and Hex B we have performed gene localization studies on man-Chinese hamster hybrids, which are described in Publication II.

The results of previous gene localization studies concerning the isoenzymes of hexosaminidase had been somewhat confusing. A gene coding for Hex A had been assigned to chromosome 15 and a gene coding for Hex B to chromosome 5, but there was disagreement about the question whether the expression of Hex A was dependent on that of Hex B (Van Someren and Beyersbergen van Henegouwen, 1973; Lalley et al., 1974; Gilbert et al., 1974; Van Cong et al., 1975). The immunological characterization of hexosaminidase isoenzymes occurring in different clones of man-Chinese hamster hybrids (see Publication II) provided the explanation for this discrepancy by showing that a band of activity formerly identified as Hex A and expressed in the absence of Hex B, in fact did not represent Hex A but a heteropolymeric molecule consisting of subunits of Chinese hamster hexosaminidase and α-subunits specific for human Hex A. The gene coding for the specific α-subunit was localized on human chromosome 15, the gene coding for the β-subunit on chromosome 5. Other groups of workers also using man-rodent hybrids, came to similar conclusions although with slightly different methods (Van Cong et al., 1975; Lalley and Shows, 1976; Chern et al., 1976; Swallow et al., 1977).

In the meantime biochemical and immunological studies showed

that Hex A and Hex B indeed consist of several subunits and that Hex A is a heterpolymeric molecule containing α-and βsubunits whereas Hex B is a homopolymeric molecule only containing β-subunits (Srivastava and Beutler, 1973; Srivastava et al., 1974a,b; Bartholomew and Rattazzi, 1974).

A third isoenzyme of β-hexosaminidase was discovered by Hooghwinkel and co-workers (1972) and was called Hex C. The biochemical and immunological properties of this isoenzyme turned out to be quite different from those of Hex A and Hex B: the enzyme is not lysosomal, has a neutral pH optimum and does not cross react with anti Hex A or anti Hex B antisera (Braidman et al., 1974; Penton et al., 1975). There was, however, a discrepancy between these biochemical data and cell genetic data. The latter suggested a relation between Hex A and Hex C because of a simultaneous absence of Hex A and Hex C in Tay-Sachs disease (Van Cong et al., 1975; Ropers and Schwantes,1973). In addition gene localization studies suggested that Hex C was composed of α-subunits which are also present in Hex A (Van Cong et al,1975).

The detection of Hex S as the major component of hexosaminidase activity in tissues from patients with Sandhoff's disease has helped to resolve this discrepancy. It was demonstrated immunologically that Hex S comprises α subunits and that in some electrophoretic systems, the mobility of Hex C and Hex S is nearly identical (Ikonne et al., 1975; Beutler et al., 1975).

The purpose of studies described in Publication III was to investigate the expression of Hex C and Hex S in cultured skin fibroblasts from patients with Tay-Sachs disease and control individuals. To enable these studies a method was deviced to identify Hex C and Hex S separately. The proce-

dure described in Publication III is based on the specific activity of Hex C and Hex S towards 4-methylumbelliferyl-β-D-glucosaminide and 4-methylumbelliferyl-β-D-galactosaminide substrate as well as on their different pH optima and immunological characteristics. It was demonstrated that in control fibroblasts both Hex C and Hex S are present whereas in fibroblasts from patients with Tay-Sachs disease Hex C is expressed but Hex S is absent. Using a different method Swallow et al. (1976) also demonstrated Hex C activity in several tissues from patients with Tay-Sachs disease. These findings fit the hypothesis that Tay-Sachs disease is caused by a mutation in the α subunit. It was concluded that Hex C and Hex S might have been confused in previous studies.

Studies on the dissociation and recombination of subunits as performed by Beutler and Kuhle (1975) provided definite proof for the proposed subunit structure of the hexosaminidases. They showed that Hex A could be generated from Hex B and Hex S after dissociation of these latter isoenzymes and recombination of the subunits. In a similar way Hex B and Hex S could be formed after dissociation of Hex A. The subunit structures for the isoenzymes of β-hexosaminidase according to Beutler et al. (1976) are as follows: Hex A: $\alpha_2\beta_2$; Hex B: β_4; Hex S: α_4. The molecular structure of Hex C is still unknown.

Experimental studies on G_{M1}-gangliosidosis

Soon after the metabolic defect in G_{M1}-gangliosidosis had been elucidated and the infantile and juvenile forms of the disease were recognized, attempts were made to explain the differences in clinical expression of β-galactosidase deficiency.

Three isoenzymes of β-galactosidase were initially separated from human liver, two lysosomal enzymes and one with a neutral pH optimum (Ockerman and Hultberg, 1968; Van Hoof and Hers, 1968; Ho and O'Brien, 1969). It was originally thought that all three isoenzymes were deficient in the infantile, type 1, form of the disease, but only the two acidic isoenzymes would be deficient in the juvenile, type 2, form (O'Brien, 1969). However, this finding was not confirmed by others and at present the general opinion is that there is no relation between the activity of the neutral isoenzyme and the pathogenesis of G_{M1}-gangliosidosis (Suzuki et al., 1971; Van Hoof, 1973; O'Brien, 1975).

According to O'Brien (1975) the later onset and slower progression of type 2 G_{M1}-gangliosidosis might be correllated with a somewhat higher level of residual β-galactosidase activity in this type. Also a number of young adult patients with G_{M1}-gangliosidosis were described and a residual β-galactosidase activity of 10-20% was found in various tissues (Goldberg et al., 1971; Yamamoto et al., 1974; Loonen et al., 1974; Wenger et al., 1974; Orii et al, 1975). The same was, however, true for skin fibroblasts from a seven year old girl (Pinsky et al., 1974) and a two year old boy (Andria et al., 1977), who both showed an atypical form of G_{M1}-gangliosidosis without severe mental retardation.

The purpose of the experimental work described in Publication IV was to investigate the genetic background of this clinical and biochemical heterogeneity. Cultured skin fibroblasts from patients with the infantile type 1 and juvenile type 2 form of G_{M1}-gangliosidosis and fibroblasts derived from the patient described by Pinsky (type 3) and from an adult patient (type 4) discovered in our own

centre (Loonen et al., 1974), were fused with each other. Two days later the β-galactosidase activity was analyzed using both the natural substrate G_{M1}-ganglioside and the artificial 4-methylumbelliferyl substrate, for which similar results were obtained. No restoration of β-galactosidase activity was observed after fusion of type 1 with type 2 cells nor after fusion of type 3 with type 4 cells. However, complementation was found when type 1 or type 2 cells were fused with type 3 or type 4 cells. From these results we concluded that at least two different gene mutations are involved in the different clinical forms of G_{M1}-gangliosidosis. A complementation analysis performed on single binuclear cells after fusion of type 1 with type 4 cells demonstrated that β-galactosidase activity was restored to the level of mononuclear control cells.

The mechanism of complementation still remains obscure. To explain the increase of β-galactosidase activity in heterokaryons on the basis of intergenic complementation we have to postulate that at least two genes are involved in the production of active β-galactosidase. However, studies by Norden et al. (1974) on β-galactosidase purified from human liver, suggest that the enzyme contains only one polypeptide chain. Also in gene localization studies on man-Chinese hamster hybrids only one gene locus coding for β-galactosidase was found which is located on chromosome 22 (De Wit et al., 1977). The possibility remains that other genes as postulated by Paigen, are involved in the expression of β-galactosidase, which are yet to be demonstrated.

The increase of β-galactosidase activity in heterokaryons after certain fusion combinations might also be the result of intragenic complementation. This would imply that two

variants that complement each other are based on structural alterations of the same polypeptide, but at different sites. Such allelic mutations might have different metabolic and therefore clinical consequences. After cell fusion interaction of the two abnormal polypeptide chains might in principle lead to a restoration of normal activity of the enzyme. This type of complementation is known to occur in micro organisms (see for review Fincham, 1966; Ratner and Rodin, 1976). More experimental work is required to elucidate the exact mechanism of complementation.

The nature of the gene mutation responsible for the decrease of β-galactosidase activity was studied in detail in fibroblasts from a two year old patient with an atypical form of G_{M1}-gangliosidosis without apparent psychomotor retardation, which was discovered recently by Andria. The results of these studies are described in Publication V. A residual β-galactosidase activity of 10-20% was found in cultured fibroblasts from this patient whereas activities less than 1% are usually observed in patients with the infantile or juvenile form of the disease. The enzyme kinetic properties of the mutant enzyme as determined with 4-methylumbelliferyl substrate were normal but immunological studies pointed to a structurally altered enzyme. On the basis of genetic complementation studies this patient could be classified in the same group as the adult, type 4, patient (Loonen et al., 1974; Galjaard et al., 1975) and the type 3 patient described by Pinsky et al. (1974). The genetic defect in this patient was thus different from that in type 1 or type 2 G_{M1}-gangliosidosis. It should be awaited whether the clinical course of this patient who is still very young will be similar to that of Pinsky's patient and an adult variant. In the interpretation of the analytical data it should be realized that the activity of β-galactosidase for

natural substrates and under in vivo conditions might be quite different from that in the test tube. Yet, it is the in vivo activity which determines the clinical manifestations.

Experimental studies on glycogenosis type II

In the infantile form of glycogenosis type II the acid α-glucosidase deficiency is expressed by the lysosomal storage of glycogen in nearly all tissues. Patients with this form of the disease usually die in their first years of life because of cardiorespiratory failure. In patients with the adult form of glycogenosis type II storage of glycogen seems to be restricted to the skeletal muscles, the heart is not involved and patients reach the adult age and some of them even become older than sixty years. Various explanations have been given for the different clinical expression of the acid α-glucosidase defect but none of these seemed very satisfactory.

It has been suggested that a compensatory mechanism exists in the adult form preventing the extensive accumulation of glycogen in the lysosomes (Hudgson et al.,1968) but no experimental data have yet been presented to support this idea. The group of Engel (1973) suggested that neutral α-glucosidase would play a role in the pathogenesis of glycogenosis type II. They reported that in cardiac muscle, skeletal muscle and liver from patients with the infantile form of the disease not only the acid α-glucosidase activity was deficient but also the activity of the neutral enzyme (Angelini et al., 1972). Other groups however, found a normal activity of the neutral enzyme in the heart and skeletal muscle from patients with the infantile form of glycogenosis

type II (Illingworth and Brown, 1965; De Barsy and Hers, 1975; Mehler and Di Mauro, 1977). It is difficult to envisage an important role of the neutral α-glucosidase in the clinical expression of glycogenosis II since the activity of this enzyme towards glycogen is very low (Rosenfeld, 1975; Ushakova and Lukomskaya, 1976)
Another possible explanation for the different degree of cardiac involvement in the adult and infantile form could be the existence of a specific isoenzyme in heart muscle which would have a normal activity in the adult form but which is deficient in the infantile form. However, there is no evidence that such a tissue specific isoenzyme exists (Koster et al., 1976).

The purpose of the experimental work described in Publication VI was to investigate whether different levels of residual acid α-glucosidase activity in the various forms of the disease could account for the clinical heterogeneity. Therefore the acid and neutral α-glucosidase activity was measured in cultured skin fibroblasts derived from 7 patients with the infantile form and 5 patients with the adult or juvenile form of glycogenosis type II. In all instances a residual acid α-glucosidase activity of 10-20% was found in fibroblasts from patients with the late onset form , whereas the activity in patients with the infantile form of the disease was always less than 1%. An inverse correlation was thus observed between the level of residual acid α-glucosidase activity in the fibroblasts and the clinical severity of the disease. The activity of neutral α-glucosidase was similar in cells from all types of patients and in controls. If our results on fibroblasts would be representative for the enzyme activity in skeletal muscle this would explain why the accumulation of glycogen in skeletal muscle from adult patients is less than in case of the infantile form. The presently available data

from the literature on this point are however conflicting and can not easily be compared.

Our immunological studies, described in Publication VI, showed the absence of cross reacting inactive acid α-glucosidase in fibroblasts from patients with the infantile form of glycogenosis type II which is in agreement with data on liver and fibroblasts from these patients reported by others (De Barsy et al., 1972; Brown et al., 1975; Koster et al., 1976). In cells from the various patients with the adult forms we observed a decrease in the number of acid α-glucosidase molecules which was proportional with the hydrolytic activity. Since the activity per enzyme molecule was similar as in controls we suggest that the gene mutation involved in these adult forms of glycogenosis type II affects the steady state level of the enzyme. It may either be a mutation which decreases the rate of synthesis of the enzyme or a mutation which enhances its breakdown.

To investigate whether different gene mutations would be involved in the infantile and adult form of glycogenosis type II complementation analyses were performed after somatic cell hybridization. Fibroblasts from clinical variants were fused in different combinations but in none of the experiments restoration of acid α-glucosidase activity was found (Publication VI). In order to rule out the possibility that a partial restoration of acid α-glucosidase activity in multinuclear heterokaryons would be masked by "dilution" with non fused parental cells we have also performed complementation analyses in single binuclear cells, half of which will contain the genome of both patients. In order to be able to perform such studies a microchemical method had to be developed which allows the determination of acid α-glucosidase activity in a single cultured cell. Publication VII describes the details

of such a micro method and it also gives an example of its application in somatic cell hybridization studies. Again after fusion of fibroblasts from the infantile and adult type no evidence for genetic complementation was found. Unlike the observations made in G_{M2}-gangliosidosis and G_{M1}-gangliosidosis complementation studies did not provide evidence that different gene mutations would be responsible for the clinical forms of glycogenosis type II, but judging from the various levels of residual activities the existence of different mutations is likely.

Experimental studies on the intercellular exchange of lysosomal enzymes

In the beginning of this chapter it was mentioned that the function of architectual genes might be to direct enzymes to their correct location. This might also be true for lysosomal enzymes. Mucolipidosis II, or I-cell disease might represent an example of a lysosomal storage disease where such a mutation is involved. It has been described by Leroy and Spranger (1970) that cultured fibroblasts from patients suffering from this disease are deficient in several lysosomal enzymes whereas the activity of these enzymes in the culture medium is increased. Hickman and Neufeld (1972) showed that the cellular uptake of these lysosomal enzymes is less efficient than is the case for enzymes secreted by normal cells. They postulated that in general lysosomal enzymes have to be secreted and subsequently to be taken up via specific recognition sites before they reach their correct location in the lysosomes. The genetic defect in I-cell disease would affect the recognition site on some lysosomal enzymes resulting in an insufficient uptake and hence a deficient intra

cellular activity.

Such a mechanism could in principle be responsible for other genetic enzyme deficiencies and the purpose of the experiments described in Publication VIII was to investigate whether the hypothesis of Hickman and Neufeld had a more general validity. For this purpose we have co-cultivated skin fibroblasts from control individuals with those from patients with G_{M2}-gangliosidosis, G_{M1}-gangliosidosis and glycogenosis type II. Methods were developed to assay the activities of the lysosomal enzymes concerned in single cultured cells Galjaard et al., 1974b; and Publications IV and VII). After 2-10 days co-cultivation in confluent cultures single cells were isolated and the enzyme activity was determined in individual control and mutant cells; these could be identified because they were prelabeled with latex or carbon particles respectively. The results described in Publication VIII indicated that during co-cultivation β-hexosaminidase was transferred from control cells to enzyme deficient Sandhoff cells. No such transfer was found for acid α-glucosidase or β-galactosidase. On the basis of these results it was concluded that the Hickman and Neufeld hypothesis might be valid for some of the lysosomal enzymes but that it does not seem to have a general validity.

CHAPTER IV

GENERAL DISCUSSION

Clinical heterogeneity among patients with the "same" genetic metabolic disease may be the result of genetic heterogeneity i.e. different mutations in the genes coding for the enzyme concerned (see for review McKusick, 1973 and 1975). Different mutations may lead to different degrees of functional impairment of the enzyme and the clinical expression of this will depend on the tissue distibution of the enzyme, its particular function in the metabolic processes in different cell types and also on the degree of residual activity towards its natural substrates. Finally we should be aware that a number of secondary genetic and metabolic factors may influence the expression of a particular enzyme deficiency.

The studies on variant human haemoglobins have contributed significantly to our insight in the nature of gene mutations and their effect. These studies may serve as a model for the understanding of heterogeneity in genetic enzyme deficiencies. At present more than 130 variant forms of haemoglobins have been detected of which more than 90% is based on single amino acid substitutions (see for review Stamatoyannopoulos, 1972). Many other protein variants are known to be the result of different gene mutations and there is no reason to suppose that lysosomal enzymes are not subject to mutations. The investigation of mutant lysosomal enzymes however, is more difficult than in the case of haemoglobin which is present in relatively large quantities in erythrocytes which can easily be isolated. Also the pathological and clinical consequences of a defective lysosomal enzyme are more difficult

to predict. Haemoglobin has one well defined function performed in one cell type, but lysosomal enzymes are widely distributed among the various tissues and they are often involved in the degradation of several substances.

A genetic defect of a lysosomal enzyme may be differently expressed in various tissues. An example of this is found in G_{M1}-gangliosidosis where the β-galactosidase deficiency results in the accumulation of G_{M1}-ganglioside and asialo G_{M1}-ganglioside in neuronal tissues whereas glycosaminoglycans, glycoproteins and oligosaccharides are stored in the visceral organs. The nature of the organ involvement in this case is thus determined by the tissue specific distribution of the natural substrate of the enzyme.

Another example is G_{M2}-gangliosidosis where in the Sandhoff variant globoside accumulates in the visceral organs of the patients because of a deficiency of both isoenzymes of N-acetyl-β-hexosaminidase (Hex A and Hex B) whereas G_{M2}- and asialo G_{M2}-ganglioside accumulate predominantly in the neuronal tissues. In the Tay-Sachs variant where only isoenzyme A is deficient the main pathological finding is the accumulation of gangliosides in neuronal cells. Here the different expression of the disease is due to a different isoenzyme deficiency but also to the tissue distribution of the natural substrates of these isoenzymes.

Finally specific organ involvement can also be caused by the tissue distribution of the defective enzyme. For example in glycogenosis type I (Von Gierke disease) the accumulation of glycogen as a result of glucose-6-phosphatase deficiency is primarily restricted to the liver where this enzyme is normally located (Cori and Cori, 1952). In glycogenosis type V (McArdle disease) glycogen storage due to a specific mus-

cle phosphorylase deficiency will primarily occur in the skeletal muscles (Schmid et al., 1959).

Also the level of residual activity of a defective enzyme might account for clinical heterogeneity. This seems to be the case in glycogenosis type II where there is no indication that tissue specific isoenzymes would account for the different expression of acid α-glucosidase deficiency in the adult and infantile form. Our experiments described in Publication VI suggest that the different levels of residual activity in both forms of the disease would be the primary basis of the clinical heterogeneity, a possibility considered earlier by Zellweger and coworkers (1965). The higher residual acid α-glucosidase activity in the adult form might account for the lower glycogen content of the skeletal muscles compared with that in patients with the infantile form of glycogenosis type II. Such an inverse correlation between residual enzyme activity and severity of clinical symptoms has also been described for variant forms of glucose-6-phosphate-dehydrogenase deficiency (Yoshida, 1973) and different types of Gaucher disease (Brady et al., 1966). Also in G_{M1}-gangliosidosis differences in the expression of β-galactosidase deficiency might be caused by different levels of residual activity of the mutant enzyme. In tissues from patients with the infantile or juvenile form of the disease the activity of β-galactosidase is always less than 1%. In cells from adult patients with β-galactosidase deficiency activities between 5 and 20% of the control value were found (Yamamoto et al., 1974; Orii et al., 1975; Wenger et al., 1974; Loonen et al., 1974; Galjaard et al., 1975; Suzuki et al., 1977). Comparably high residual activities were also detected in cells from some younger patients with a mild form of the disease without severe psychomotor retardation (Pinsky et al., 1974; Reuser et al. in Publication V; O'Brien

et al., 1976). These latter patients are however to young to allow definite conclusions and the further course of their clinical manifestations must be awaited. Also in Tay-Sachs disease patients with the juvenile form seem to have some residual activity of β-hexosaminidase A whereas patients with the infantile form of the disease have no detectable Hex A activity (O'Brien, 1973).

For a correct interpretation of the relationship between clinical symptoms and residual enzyme activity one should be aware of possible differences between the conditions of an in vitro enzyme assay and the in vivo situation. One also has to realize that the activity of the mutant enzyme does not have to be reduced to the same extent for all its different substrates. In our studies on different variants of G_{M1}-gangliosidosis (see Publication IV) similar activities of β-galactosidase were found towards the natural substrate G_{M1}-ganglioside and the artificial methylumbelliferyl substrate. The same is true for acid α-glucosidase in fibroblasts from patients with different clinical variants of glycogenosis type II (see Publication VI) where similar activities were found towards glycogen, maltose and methylumbelliferyl substrate. On the other hand healthy individuals were described with a profound β-hexosaminidase deficiency towards the artificial substrate whereas the activity for the natural substrate was higher (Tallman et al., 1974; Dreyfus et al., 1975; Navon et al., 1976). A reverse situation has also been reported in a patient where β-hexosaminidase A and B were active towards the artificial substrate and presumably inactive towards G_{M2}-ganglioside (Sandhoff et al., 1971).

At present it seems too early to generalize about the relationship between the residual lysosomal enzyme activity and the severity of clinical manifestations. In metachromatic leucodystrophy for instance which is another lysosomal storage disease such relation seems not to exist (see for review Austin, 1973). Although some aspects of clinical heterogeneity in G_{M2}-gangliosidosis, G_{M1}-gangliosidosis and glycogenosis type II can be explained on the basis of different levels of residual enzyme activity we have no idea about the minimum level of activity that is required for a normal development. A further complication is that the activity in cultured skin fibroblasts does not necessarily reflect the situation in other cell types like neurons and liver cells. On the other hand the cell culture system has provided an extremely useful model for studies on the correction of a metabolic defect.

Correction of mucopolysaccharide storage in cultured fibroblasts from patients with different types of mucopolysaccharisosis could be realized by co-cultivation with cells of a different genotype (see for review Neufeld, 1974). Such metabolic correction appeared to be based on the uptake by deficient cells of the necessary enzyme which had been secreted by the other cell type. Subsequently, it was shown that addition of purified α-iduronidase to cells from a patient with Hurler's disease (Bach et al., 1972) and of N-acetyl-α-hexosaminidase to cells of a patient with Sanfilippo B disease (O'Brien et al., 1973) resulted in correction of the intra cellular storage of mucopolysaccharides. Interestingly in this situation an intra cellular activity of only 10 % of the control value was enough for metabolic correction. Of course these results can not directly be extrapolated to the in vivo situation but they may suggest that in certain circumstances even low levels of enzyme

activity may be sufficient to prevent serious physical and mental damage in the patient.

As described in chapter III the enzyme defects in the clinical variants of G_{M2}-gangliosidosis, G_{M1}-gangliosidosis and glycogenosis type II can be caused by various gene mutations any of which may result in a different impairment of the in vivo function of the particular enzymes. Like in haemoglobinopaties, in the majority of cases the mutation will be in the structural gene but a mutation in other genes can not always be excluded. The occurrence of a multiple lysosomal enzyme deficiency as in I-cell disease for instance indicates that the lysosomal enzyme deficiency might also be caused by the mutation of a gene responsible for the correct location of the enzyme. However, for reasons mentioned in Publication VIII it is unlikely that the enzyme deficiencies in any of the three lysosomal storage diseases described in this thesis is due to such a gene mutation.

Complementation analyses and studies concerning the expression of human (iso)enzymes in man-rodent somatic cell hybrids seem promising ways to investigate whether different gene mutations are involved in the "same" enzyme deficiency. Biochemical and immunological studies on the mutant enzyme might further help to elucidate the nature of the genetic defect. Studies concerning the recognition and uptake of lysosomal enzymes may give a better understanding of cell biological processes. The combined use of these approaches as described in this thesis has provided more insight in the relationship between clinical, biochemical and genetic heterogeneity in G_{M2}-gangliosidosis, G_{M1}-gangliosidosis and glycogenosis type II. These fundamental investigations may in future be essential as a basis for therapeutic approaches.

They may well reveal that therapy is only feasible for some lysosomal storage diseases and that different clinical forms of the same disease require a different treatment.

SUMMARY

The main function of lysosomes is the degradation of biological material from the intracellular or extracellular space. The degradation of these substances is performed by hydrolytic enzymes which are localized in these cell organelles. If one of these enzymes is absent or when its function is disturbed, a lysosomal storage of one or several substances may occur. As a result of this the cell might be damaged and may loose its function as well as the organ comprising these cells. At present a few dozen of inherited metabolic diseases are known to be due to a lysosomal enzyme defect. The severity of the physical and mental handicaps of the patient suffering from these diseases varies depending on the nature of the organs involved and the degree of functional impairment of the cells. However, it becomes more and more clear that also within one particular metabolic disease considerable variation in clinical expression may occur. Some patients suffering from a certain lysosomal enzyme deficiency already show severe symptoms shortly after birth and a progressive course of the disease leads to early death. In other patients the first clinical signs become apparent at a more advanced age and some of these patients may survive into late adulthood.

The purpose of the experiments described in this thesis was to get a better insight in the relationship between genetic, biochemical and clinical heterogeneity in three lysosomal storage diseases i.e. G_{M2}-gangliosidosis, G_{M1}-gangliosidosis and glycogenosis type II. The investigations were performed on cultured skin fibroblasts of patients suffering from these diseases.

Three different approaches were used:

- Complementation analyses were performed to answer the question whether different gene mutations are involved in the deficiency of the same enzyme in various clinical forms of the disease. In these studies fibroblasts derived from different patients are fused and subsequently it is examined whether the enzyme defect is restored by the cooperation of both genomes.

- Gene localization studies were performed to obtain information about the number of genes involved in the production of the deficient enzyme concerned, and about the molecular structure of the enzyme. In these studies human fibroblasts and Chinese hamster fibroblasts are fused and proliferating hybrids are selected. These hybrids preferentially loose human chromosomes which allows a correlation between the loss of human genes and the expression of specific human (iso)enzymes.

- Biochemical and immunological studies were performed to characterize the mutant enzyme in order to get information about the nature of the gene mutation and its effect on the function of the enzyme.

The most important findings, which are described in detail in the Publications I - VIII, are summarized below.

G_{M2}-gangliosidosis

Several clinical and biochemical variants of G_{M2}-gangliosidosis have been described. Tay-Sachs disease the variant that occurs most frequently, is due to a deficiency of the

isoenzyme A of β-D-N-acetyl hexosaminidase. In addition there is another variant called Sandhoff's disease in which both the isoenzyme A (Hex A) and the isoenzyme B (Hex B) are deficient. Complementation analyses using fibroblasts from these two types of patients demonstrated that Tay-Sachs disease and Sandhoff's disease are based on mutations in two different genes (Publication I). Further, gene localization studies in man-Chinese hamster cell hybrids have shown that two different genes are coding for Hex A which are localized on chromosome 5 and 15 respectively. Also it appeared that the expression of Hex A was dependent on that of Hex B which is coded by a gene localized on chromosome 5. It turned out that conflicting results in different studies concerning the interrelationship between Hex A and Hex B were based on the mis-interpretation of an isoenzyme expressed in these hybrids. As is shown in Publication II this isoenzyme represents a heteropolymeric molecule consisting of subunits of human Hex A and subunits from Chinese hamster hexosaminidase. The characterization of this hybrid molecule and a better identification of Hex C and Hex S, which are two other hexosaminidase isoenzymes (Publication III) in combination with the results of other groups have finally elucidated the nature of the mutation in the two most frequent variants of G_{M2}-gangliosidosis. Tay-Sachs disease is caused by a mutation in the α-subunit which is part of Hex A and Hex S. The Hex A en Hex B deficiency in Sandhoff's disease is caused by a mutation in the β-subunit which forms Hex B but which is also part of Hex A.

G_{M1}-gangliosidosis

Also in G_{M1}-gangliosidosis which is due to a deficiency of the lysosomal enzyme β-galactosidase, several clinical variants occur. Complementation analyses as described in Publication IV show that at least two gene mutations are involved in these variants. In addition it was found that in the infantile and juvenile forms of the disease, indicated as type 1 and type 2 respectively, less than 1% of β-galactosidase activity is present in cultured skin fibroblasts. In cells from a patient with the adult form (type 4) and of a younger patient (type 3), without severe mental retardation, 10-20% residual activity was demonstrated for both an artificial substrate and the natural substrate G_{M1}-ganglioside. Publication V describes a two year old boy with a β-galactosidase deficiency and who so far had a normal psychomotor development. In view of the residual enzyme activity in the fibroblasts (10-20%) and the fact that complementation occurred when fibroblasts from this patient were fused with those from type 1 or type 2 patients it could be concluded that this patient does not represent the type 1 or type 2 variant of G_{M1}-gangliosidosis. Immunological studies on the β-galactosidase in fibroblasts from the patient described in Publication V showed a decrease in the hydrolytic activity per enzyme molecule which points to a structural mutation. Until now it is not clear whether the different clinical variants of G_{M1}-gangliosidosis are based on different mutations in the same gene or in separate genes.

Glycogenosis type II

Generalized glycogenosis type II or Pompe's disease is the form that occurs most frequently. It is due to a deficiency of the lysosomal enzyme acid α-glucosidase which results in accumulation of glycogen in heart, skeletal muscles, liver and other organs leading to early death. In addition patients have been described with an acid α-glucosidase deficiency where the disease is less severe and less progressive. In this adult form the cardiac involvement is minimal and hypotonia of skeletal muscles is the most prominent feature. The patients may reach a high advanced age. Publication VI describes that in cultured fibroblasts from all adult patients tested a residual acid α-glucosidase activity of 10-20% was found towards an artificial substrate as well as towards maltose, and the natural substrate glycogen. An inverse correlation was found between the level of the residual acid α-glucosidase activity in the fibroblasts on the one hand and the extent of glycogen accumulation in skeletal muscles and the severity of the clinical manifestations on the other hand. In none of the cases a residual acid α-glucosidase activity was found in cultured fibroblasts from patients with the infantile form of glycogenosis type II. Biochemical and immunological analysis of the acid α-glucosidase in the fibroblasts from adult patients showed that the enzyme had normal physicochemical and kinetic properties and that it was present in reduced amounts (Publication VI). These facts point to a mutation in the adult form that either results in a decreased rate of enzyme synthesis or in an increased rate of its degradation. Complementation analyses in multikaryons or individual binuclear hybrid cells did not provide evidence for genetic heterogeneity (Publications VI

and VII). The exact nature of the gene mutations leading to a different degree of enzyme deficiency in the adult and infantile form of glycogenosis type II is still unknown but it seems likely that different gene mutations are involved.

Exchange of lysosomal enzymes

According to a hypothesis by Hickman and Neufeld (1972) lysosomal enzymes have to be secreted by the cell and subsequently are taken up via specific recognition sites at the cell surface, before they reach their lysosomal location. In Publication VIII we have investigated whether such a defective uptake mechanism could account for the enzyme deficiencies in G_{M2}-gangliosidosis, G_{M1}-gangliosidosis or glycogenosis type II. We have studied the intercellular exchange of β-D-N-acetyl hexosaminidase, β-galactosidase and acid α-glucosidase by enzyme analyses in single fibroblasts isolated after co-cultivation of control fibroblasts and enzyme deficient cells. No transfer of β-galactosidase or acid α-glucosidase from control to enzym deficient cells was found but for β-hexosaminidase a transfer from control to enzyme deficient cells from a patient with Sandhoff's disease was demonstrated. From these results it was concluded that the hypothesis mentioned above does not seem to be valid for all lysosomal enzymes. Further studies on the secretion, recognition and uptake of lysosomal enzymes are not only necessary for a better understanding of the exact nature of the different genetic metabolic diseases but they also may provide a basis for future treatment.

SAMENVATTING

Lysosomen spelen een belangrijke rol bij de afbraak van biologisch materiaal afkomstig uit het intra- en extra cellulaire milieu. De afbraak vindt plaats door middel van hydrolytische enzymen die zich in deze celorganellen bevinden. Bij afwezigheid of het niet voldoende functioneren van een van deze enzymen kan lysosomale stapeling van een of meerdere producten ontstaan. De cellen waarin stapeling optreedt kunnen hun functie verliezen, evenals het orgaan waarvan deze cellen deel uit maken. Momenteel zijn enkele tientallen erfelijke stofwisselingsziekten bekend waarvan is aangetoond dat ze op een lysosomaal enzym defect berusten. De ernst van de klinische symptomen bij patienten die aan dit soort ziekten lijden varieert afhankelijk van de aard van de betrokken organen en van de mate van het functieverlies van de daarin aanwezige cellen. Het wordt echter voor steeds meer erfelijke stofwisselingsziekten duidelijk dat ook binnen één bepaalde ziekte nog grote klinische heterogeniteit kan bestaan. Bij eenzelfde erfelijke enzym deficientie vertonen sommige patienten al kort na de geboorte ernstige, progressief verlopende ziekteverschijnselen waaraan zij vroeg overlijden, terwijl bij andere patienten pas op latere leeftijd merkbare afwijkingen optreden met een milder verloop waardoor deze patienten soms een hogere volwassen leeftijd bereiken.

Doel van het in dit proefschrift beschreven onderzoek is geweest meer inzicht te krijgen in de relaties tussen genetische, biochemische en klinische heterogeniteit bij een drietal lysosomale stapelingsziekten met name: G_{M2}-gangliosidosis, G_{M1}-gangliosidosis en glycogenosis type II.

Bij het onderzoek werd gebruik gemaakt van gekweekte huidfibroblasten van patienten met deze ziekten.

Er werden drie methoden van onderzoek toegepast:

- Met behulp van complementatie analysen werd getracht de vraag te beantwoorden of er één of meerdere genmutaties betrokken zijn bij een deficiëntie van hetzelfde enzym in verschillende klinische varianten. Hierbij worden fibroblasten afkomstig van verschillende patienten met elkaar gefuseerd en wordt onderzocht of door het samenwerken van beide genomen het enzymdefect wordt opgeheven.

- Met behulp van genlocalisatie studies werd getracht te bepalen hoeveel genen betrokken zijn bij de productie van het betreffende (deficiënte) enzym en werd de moleculaire structuur van het enzym onderzocht. Hierbij wordt het verlies van menselijke chromosomen in hybride cellen, verkregen door fusie van menselijke en Chinese hamster fibroblasten, gecorreleerd met het verlies van expressie van menselijke (iso)enzymen.

- Met behulp van biochemische en immunologische methoden werd getracht het gemuteerde enzym te karakteriseren teneinde een indruk te krijgen van de aard van de genmutatie en de invloed hiervan op het functioneren van het enzym.

De belangrijkste conclusies van het experimentele werk dat beschreven wordt in Publicaties I tot en met VIII worden onderstaand samengevat.

G_{M2}-gangliosidosis

Er zijn meerdere klinische en biochemische varianten van G_{M2}-gangliosidosis beschreven. De meest voorkomende vorm is de ziekte van Tay-Sachs, die berust op een deficiëntie van het isoenzym A van β-D-N-acetyl-hexosaminidase. Daarnaast bestaat er de ziekte van Sandhoff waarbij zowel het isoenzym β-hexosaminidase A (Hex A) als het isoenzym β-hexosaminidase B (Hex B) deficiënt zijn. Complementatie analysen met fibroblasten van deze typen patienten toonden aan dat deze twee biochemische varianten berusten op mutaties in twee verschillende genen (Publicatie I).
Vervolgens kon met behulp van genlocalisatie studies in mens-Chinese hamster celhybriden worden aangetoond dat hexosaminidase A gecodeerd wordt door twee verschillende genen welke respectievelijk op de chromosomen 5 en 15 liggen. Verder bleek de expressie van Hex A afhankelijk te zijn van de aanwezigheid van Hex B, dat gecodeerd wordt door het gen gelegen op chromosoom 5. De discrepantie welke er in verschillende genlocalisatie studies bestond met betrekking tot de onderlinge relatie tussen Hex A en Hex B bleek veroorzaakt door een verschillende interpretatie van een heteropolymeer molecule dat in deze mens-Chinese hamster celhybriden wordt gevormd. Nadere karakterisering van dit enzym door middel van immunologische methoden (Publicatie II) en een betere identificatie van Hex C en Hex S, twee andere isoenzymen van β-D-N-acetyl-hexosaminidase (Publicatie III) hebben tenslotte in combinatie met de resultaten van andere groepen geleid tot de opheldering van de aard van de mutaties in de twee meest voorkomende varianten van G_{M2}-gangliosidosis. De ziekte van Tay-Sachs blijkt te rusten op een mutatie in de α-subunit die onderdeel uitmaakt van Hex A en Hex S, terwijl de ziekte van

Sandhoff het gevolg is van een mutatie in de β-subunit waaruit Hex B bestaat maar die ook een onderdeel vormt van Hex A, waardoor zowel Hex A als Hex B deficiënt zijn.

G_{M1}-gangliosidosis

Ook van de ziekte G_{M1}-gangliosidosis die veroorzaakt wordt door een deficiëntie van het lysosomale enzym β-galactosidase zijn meerdere klinische varianten bekend. Complementatie analysen beschreven in Publicatie IV toonden aan dat er bij deze varianten tenminste twee verschillende genmutaties zijn betrokken. Tevens werd gevonden dat bij de infantiele en juveniele vormen, respectievelijk type 1 en type 2 minder dan 1% β-galactosidase activiteit aanwezig is in gekweekte fibroblasten. In cellen van patienten met een volwassen vorm (type 4) of een vorm zonder duidelijke mentale retardatie (type 3) werd steeds 10-20% restactiviteit gevonden, zowel voor een artificieel substraat als voor het natuurlijke substraat G_{M1}-ganglioside. In Publicatie V wordt een tweejarig jongetje met een β-galactosidase deficiëntie beschreven waarbij tot nu toe de psychomotore ontwikkeling normaal verloopt. Zowel op grond van de gevonden restactiviteit van β-galactosidase (10-20%) als het optreden van complementatie na fusie van fibroblasten van deze patient met die van type 1 en 2 patienten kon worden vastgesteld dat het hier geen infantiele of juveniele vorm van G_{M1}-gangliosidosis betreft. Immunologische studies van de β-galactosidase in fibroblasten van deze in Publicatie V beschreven patient toonden voorts aan dat de hydrolytische activiteit per enzym molecuul verlaagd was hetgeen zou duiden op een structurele mutatie. Tot nu toe is het niet duidelijk of de diverse klinische varianten berusten op verschillende mutaties van één gen dan wel van meerdere

genen coderend voor β-galactosidase.

Glycogenosis type II

De meest voorkomende vorm van glycogenosis type II is de gegeneraliseerde ziekte van Pompe waarbij ten gevolge van een deficiëntie van het lysosomale enzym zure α-glucosidase stapeling van glycogeen optreedt in skeletspieren, hart, lever en andere organen waardoor de patienten vroeg overlijden. Er zijn echter ook patienten beschreven met een zure α-glucosidase deficiëntie waarbij de ziekte een veel minder ernstig verloop vertoont en waarbij de patienten zonder merkbare hartafwijkingen een hoog volwassen leeftijd kunnen bereiken met als voornaamste symptoom spierzwakte. In Publicatie VI wordt beschreven dat in gekweekte huidfibroblasten van alle onderzochte patienten met deze volwassen vorm van glycogenosis II een residuele zure α-glucosidase activiteit van 10-20% aanwezig is. Dit geldt zowel voor een artificieel substraat, voor maltose als voor het natuurlijk substraat glycogeen.
Verder werd er een omgekeerde relatie gevonden tussen de hoogte van de restactiviteit in de fibroblasten enerzijds en de mate van glycogeenstapeling in skeletspieren en de ernst van de klinische verschijnselen anderzijds. In celmateriaal van patienten met de infantiele vorm werd nooit een restactiviteit gevonden hoger dan 1%. Biochemische en immunologische analyse van de zure α-glucosidase in fibroblasten van patienten met de volwassen vorm toonde aan dat de kinetische en physische eigenschappen van het enzym normaal waren maar dat de totale hoeveelheid enzym verlaagd was (Publicatie VI). Dit wijst erop dat de mutatie in de volwassen vorm leidt tot een verminderde synthese van het lysosomale α-glucosidase of tot een versnelde

afbraak van het enzym. Uit complementatie analysen in multikaryons en individuele binucleaire hybride cellen werden geen aanwijzingen verkregen voor genetische heterogeniteit (Publicatie VI en VII). De precieze aard van de genmutaties is nog niet bekend maar het lijkt waarschijnlijk dat verschillende genmutaties leiden tot een verschillende mate van enzym deficiëntie in de volwassen en infantiele vorm van glycogenosis type II.

Uitwisseling van lysosomale enzymen

Naar aanleiding van een hypothese van Hickman en Neufeld (1972) dat lysosomale enzymen eerst door de cel worden uitgescheiden en vervolgens weer worden opgenomen via een specifiek herkenningsmechanisme aan het celoppervlak voordat zij het lysosoom bereiken werd in Publicatie VIII nagegaan of dergelijke afwijkingen in het opname mechanisme ook een rol spelen bij G_{M2}-gangliosidosis, G_{M1}-gangliosidosis en glycogenosis type II. Door middel van enzym analyse in individuele fibroblasten na samenkweken van controle cellen en cellen met een enzymdeficiëntie werd onderzocht of er intercellulair transport van de betreffende enzymen plaatsvond. Voor α-glucosidase en β-galactosidase werd geen overdracht vanuit normale cellen naar deficiënte cellen gevonden doch voor β-N-acetyl-hexosaminidase werd wel opname door cellen van een patient met de ziekte van Sandhoff geconstateerd. Hieruit werd geconcludeerd dat de bovengenoemde hypothese in ieder geval niet voor alle lysosomale enzymen geldt. Verdere studies op het terrein van de afgifte, herkenning en opname van lysosomale enzymen zijn noodzakelijk, niet alleen om inzicht te verkrijgen in de precieze aard van de verschillende erfelijke stapelingsziekten maar ook om als basis te dienen van een eventuele therapie.

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CURRICULUM VITAE

Achttien jaar na mijn geboorte te Amsterdam ben ik, na door het behalen van het eindexamen der middelbare school daartoe de rechten verworven te hebben, scheikunde gaan studeren aan de Gemeentelijke Universiteit van Amsterdam. Het candidaatsexamen werd afgelegd in 1969 en het doctoraalexamen in 1973 met als hoofdvak biochemie en als bijvakken physiologie van de mens en de plant.
Gedurende enige tijd ben ik als student-assistent verbonden geweest aan het Fysiologisch Laboratorium van de Universiteit van Amsterdam.
Kort na het beeindigen van mijn studie ben ik in februari 1973 aan de afdeling Celbiologie en Genetica van de Erasmus Universiteit te Rotterdam begonnen met het in dit proefschrift beschreven onderzoek.

PUBLICATIONS I – VIII

PUBLICATION I

Tay-Sachs and Sandhoff's disease:

Intergenic complementation after somatic cell hybridization

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Summary. Cultivated skin fibroblasts from patients with Tay-Sachs and Sandhoff's disease were fused and the isoenzymes of *N*-acetyl-β-D-hexosaminidase were investigated after 2 and 7 days of subsequent cultivation. Enzyme analyses after heat inactivation showed a clear increase in the thermolabile component of hexosaminidase when compared with assays on fusion of each of the parental cell strains. Electrophoretic studies revealed that in cell homogenates prepared at various time intervals after cell fusion of Tay-Sachs with Sandhoff's fibroblasts, all three hexosaminidase isoenzymes were present, including hexosaminidase A which lacks in both parental cell strains. These results show that genetic complementation has occurred, which indicates that two different gene mutations are involved in these variants of GM2-gangliosidosis. The relevance of the data obtained for the elucidation of the molecular properties of the (iso)enzymes involved is discussed.

In various normal human tissues [5, 10, 11] and in cultured skin fibroblasts [13] three isoenzymes of *N*-acetyl-β-D-hexosaminidase have been observed which differ in temperature stability, specificity towards natural substrates, electrophoretic mobility and immunological properties [1, 9, 17]. In patients with GM2 gangliosidosis type 1 (Tay-Sachs disease) hexosaminidase A is deficient [10], whereas in the type 2 variant (Sandhoff's disease) both hexosaminidase A and B are deficient [14]. Several hypotheses about the relationship between the different isoenzymes have been postulated in order to understand the isoenzyme deficiencies [1, 4, 12, 13, 15, 16, 17]. Complementation analysis in heterokaryons obtained after cell fusion of fibroblast strains from different patients with a metabolic disorder proved to be a useful tool for the demonstration of different gene mutations [19].

The present paper deals with the assay of hexosaminidase isoenzymes in cultured fibroblasts from normal individuals, from patients with Tay-Sachs and Sandhoff's disease and in homokaryons and heterokaryons after somatic cell hybridization of these cell strains. Enzyme assays after heat inactivation and electrophoretic studies showed the first example of complementation after hybridization of cells from two variants of a lysosomal storage disease indicating that wo different gene mutations are involved.

Methods and Materials

Subcultures of skin fibroblasts from a normal individual and from patients with Tay-Sachs and Sandhoff's disease were cultured for 2–7 days in small falcon flasks in Ham F10 medium with 10% fetal calf serum. Cell hybridization was carried out between 2×10^6 cells of each parental cell strain using inactivated Sendai virus (1 ml 1 000 HAU/ml added to 1 ml cell suspension). The mixture was kept 4 min at 4°C and then incubated for 20 min at 37°C under continuous stirring. Cell fusion was followed by 2 or 7 days' subsequent cultivation with a cell density such that contact inhibition was reached within 1–2 days. Enzyme assays were carried out after harvesting by trypsinization, rinsing in saline and disruption by sonication. Protein was determined according to Lowry [8] using microvolumes [2]. The overall hexosaminidase activity was measured according to a modified procedure as described by Kaback [6]. To enable multiple assays on a small amount of cell material incubation of 1 μl cell homogenate and 2 μl bovine serum albumin (0.2% w/v) was performed during 1 h at 37°C with 2 μl mM 4-methylumbelliferyl-2, acetamido-2, deoxy-β-D-glucopyranoside at pH 4.4 using microprocedures described earlier [2]. Fluorescence readings of 4-methylumbelliferone were made in a Perkin-Elmer fluorometer (exc. 365 nm, 448 nm) after addition of 500 μl 0.5 M carbonate buffer pH 10.7. Heat inactivation was carried out after addition of 1 μl cell homogenate to 2 μl bovine serum albumin (0.2% w/v) during 2 h at 50°C.

Electrophoresis was performed on Phoroslide cellulose acetate strips (Millipore Corp.), 2–4 μl cell homogenate ($7 \times 10^3 - 12 \times 10^3$ cells corresponding to 2–4 μg protein) was applied to each strip and run in 0.05 M phosphate buffer pH 6.0 during 45 min at 20°C (3 mA/strip, 150 V). The isoenzyme pattern was photographed at 365 nm after 30 min incubation with 5 mM methyl-umbelliferyl substrate in citrate/phosphate buffer pH 4.5 followed by spraying with carbonate buffer (pH 10.7).

Biochemical assays were performed in triplo on cell homogenates from cultured fibroblasts from a normal individual, a patient with Tay-Sachs disease and a patient with Sandhoff's disease and also in homogenates 2 days after cell fusion of these cell

strains with themselves (parental fusion) and after fusion of Tay-Sachs with Sandhoff fibroblasts. The whole experiment was repeated twice and in addition the cell fusion between Tay-Sachs and Sandhoff fibroblasts was also analysed after 7 days' subsequent cultivation. In one experiment equal numbers (about $1–10^6$ cells) of Tay-Sachs and Sandhoff fibroblasts were co-cultivated during 2 days under conditions as described above.

Results

Heat inactivation studies after cell hybridization. The overall hexosaminidase activities and the ratio of the thermolabile (mainly hex. A) and thermostabile (hex. B) isoenzymes were determined in cultured fibroblasts from a normal individual and from patients with Tay-Sachs and Sandhoff's disease. Similar values were obtained in different experiments on the same cell strain. These data were compared with those obtained after cell fusion of each cell strain with itself (parental fusion) followed by 2 days' subsequent cultivation. For none of the cell strains was any effect of parental fusion observed on the total hexosaminidase activity and the ratio hex. A : hex. B. Fig. 1 summarizes the mean values obtained in three experiments. Both in non-fused control fibroblasts and at 2 days' cultivation after parental fusion, the ratio of hex. A : hex. B activity is about 1 : 1. After parental fusion of Sandhoff fibroblasts hardly any hexosaminidase activity is detected (about 1 % of the control value) whereas in parental fusions of Tay-Sachs fibroblasts the overall activity is higher than in control cells. In the former, heat inactivation results in a decrease of only 14 % of the activity.

Two days after cell fusion of Tay-Sachs with Sandhoff fibroblasts (last 2 columns of fig. 1) the overall activity is about the average of that of both parental cell strains. A longer cultivation period in contact inhibition (7 days) after cell fusion results in an increase of the overall activity which has also been observed in control cell strains. However,

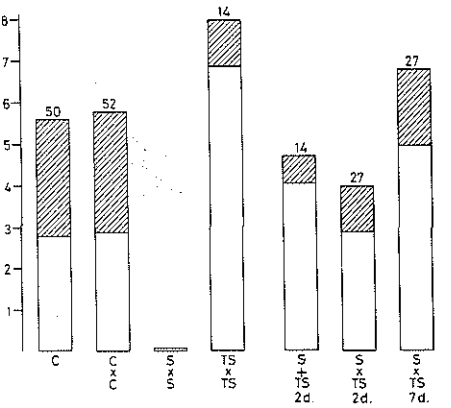

Fig. 1. Ordinate: N-acetyl-β-D-hexosaminidase activity $\times 10^{-6}$ mole/h/mg protein. Comparison of the overall hex. activity and of the ratio hex. A and B in different cell fusions. *C*, normal fibroblast culture; $C \times C$, parental fusion of control fibroblasts; $S \times S$, parental fusion of Sandhoff fibroblasts, $TS \times TS$, parental fusion of Tay-Sachs fibroblasts; $S + TS$, co-cultivation for 2 days of Sandhoff and Tay-Sachs fibroblasts; $S \times TS$, cell fusion between Sandhoff and Tay-Sachs fibroblasts followed by 2 days and 7 days subsequent cultivation. □, percentage thermostabile component (mostly hex. B); ▩, thermolabile component (mostly hex. A). The mean activities of 3 experiments (at least 6 analyses per experiment) are presented and the standard deviation (S.D./*M*) was 4–10 %. The last experiment (7 days after $S \times TS$ fusion only represents 1 experiment (6 analyses).

at both time intervals after fusion of Tay-Sachs with Sandhoff fibroblasts the percentage of the heat-labile component of hexosaminidase has increased to 27 % of the total activity.

This increase, which is probably due to the formation of hexosaminidase A, absent in both parental cell strains, must be the result of genetic interaction in those heterokaryons which contain the genomes of both parental cell strains. As is shown in fig. 1 (5th column) 2 days co-cultivation of Sandhoff and Tay-Sachs fibroblasts, does not result in an increase of thermolabile hexosaminidase.

Electrophoretic studies after cell hybridization. The nature of the thermolabile component

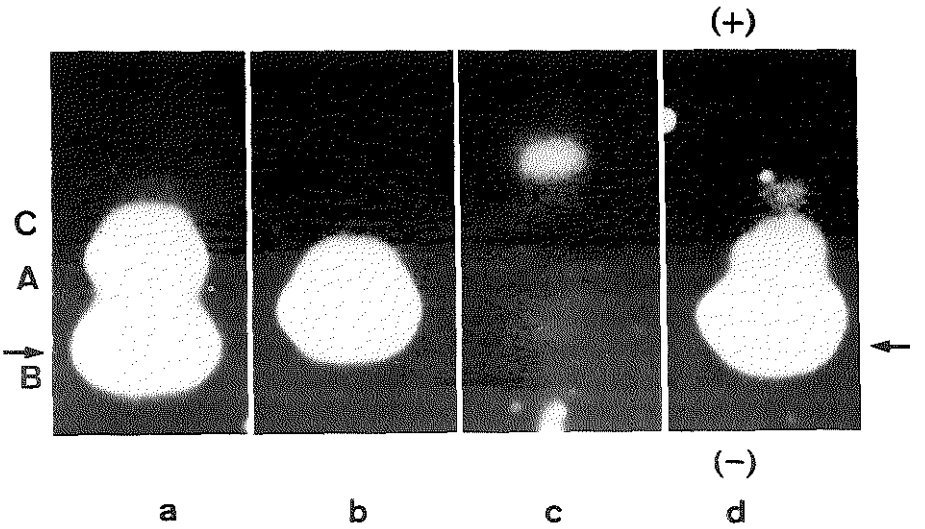

Fig. 2. Cellulose acetate electrophoresis of *N*-acetyl-β-D hexosaminidase isoenzymes after cell hybridization of cultured fibroblasts. Equal amounts (about 3×10^{-3} mg protein) of cell homogenate (obtained after 2 days' cultivation after cell fusion) were applied to each strip and run for 45 min in phosphate buffer at pH 6.0. Incubation with 4-methyl-umbelliferyl. 2, acetamido—2, deoxy-β-D-glucopyranoside in citrate/phosphate buffer pH 4.5 was carried out during 30 min at 20°C. (*a*), parental fusion of control fibroblasts; (*b*), parental fusion of Tay-Sachs fibroblasts; (*c*), parental fusion of Sandhoff fibroblasts; (*d*), cell fusion between Sandhoff × Tay-Sachs fibroblasts. Reproducible results were obtained in three independent experiments and in repeated runs of the same cell homogenate.

resulting from cell fusions between Sandhoff and Tay-Sachs fibroblasts was further investigated by electrophoresis. Equal amounts of cell homogenates of the various cell fusion combinations were applied to different strips and analysed under similar conditions. Fig. 2*a* shows that after parental fusion of control fibroblasts three isoenzymes are detectable. Hex. B is migrating towards the cathode, hex. A migrates towards the anode and hex. C is the fastest moving band towards the anode. The activities of hex. A and B are about the same whereas hex. C is hardly visible, even in fresh cell homogenates. After storage at −20°C the C-band is no longer visible. Parental fusion of Tay-Sachs fibroblasts results only in strong activity of the B-band (fig. 2*b*). After parental fusion of Sandhoff fibroblasts hardly any activity could be detected at the localization of the B- and A-band but the C-band was clearly visible (fig. 2*c*).

However, at 2 and 7 days after cell hybridization of Tay-Sachs with Sandhoff fibroblasts all three isoenzymes were observed (fig. 2*d*). These results indicate that in heterokaryons containing both the genome of Tay-Sachs and Sandhoff fibroblasts hex. A activity is present even if this isoenzyme lacks in both parental cell strains. Co-cultivation during 2 days of these two cell strains, without prior cell fusion does not result in the formation of hex. A (fig. 3*b*), whereas it does occur when cell fusion was carried out (fig. 3*a*).

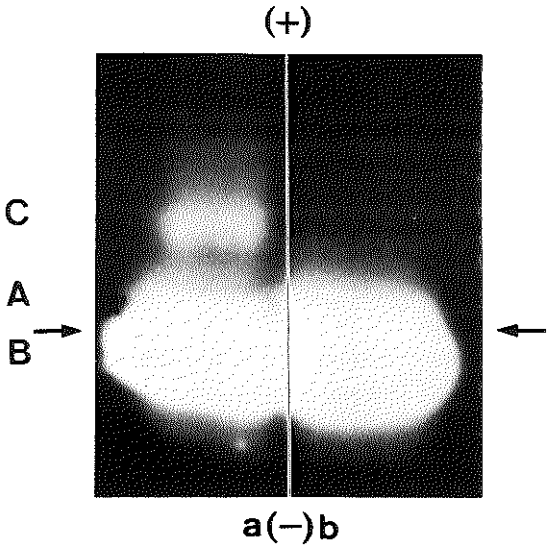

Fig. 3. Comparison of *N*-acetyl-β-D-hexosaminidase isoenzymes after 2 days co-cultivation of Tay-Sachs and Sandhoff fibroblasts 3 (*b*) and after cell fusion of these cell strains followed by 2 days cultivation (*a*). Assay conditions were the same as mentioned in fig. 2.

After heat inactivation of Tay-Sachs with Sandhoff fusions electrophoresis did not show any hex. A or hex. C whereas the B band remained present. These electrophoretic studies indicate that the increase of the thermolabile component occurring after cell fusion of Tay-Sachs with Sandhoff fibroblasts (fig. 1) is due to the formation of hexosaminidase A as a result of genetic complementation.

Discussion

The data illustrated in fig. 1 show that for each cell strain tested the parental cell hybridization procedure does not affect the total hexosaminidase activity nor the isoenzyme pattern. The ratio of the thermolabile and the thermostabile components observed in control fibroblasts (about 1:1) is in agreement with findings by other investigators [7]. The reduction of 14 % of the total activity by heat inactivation of Tay-Sachs fibroblasts is most probably due to an inactivation of hex. C and a partial inactivation of hex. B. It does not seem to be the result of inactivation of a residual activity of hex. A as Beutler & Srivastava [1] could not show any cross reacting material in cells from Tay-Sachs patients with specific hex. A antisera. The fact that we did not observe any hex. A and hex. C activity at low pH in Tay-Sachs fibroblasts, as also shown by others [5, 13] might suggest the presence of a common polypeptide subunit in these two isoenzymes.

Biochemical and immunological studies suggest that hex. A and hex. B have a common subunit [1, 17] and the deficiency of hex. A and B in Sandhoff's disease could be explained by a genetic defect in this polypeptide subunit. The hex. A deficiency in Tay-Sachs disease might then be based on a defect in another sub-unit, specific for hex. A and possibly also occurring in hex. C.

Our data indicate that genetic complementation for hex. A occurs in heterokaryons containing the genomes of Sandhoff and Tay-Sachs fibroblasts each of which cell strains lacks hex. A activity (fig. 2). Such a genetic complementation fits in the hypothesis assuming that the genetic information for the common subunit in hex. A and B is provided by the nucleus of Tay-Sachs fibroblasts whereas the genetic information for the specific hex. A (and hex. C ?) subunit is present in the nucleus of Sandhoff fibroblasts. It is interesting to note that in the heterokaryons recognition occurs of different polypeptide subunits coded for by two different nuclei to form the hex. A isoenzyme which must also be transported into the lysosomes as is evident by its low pH optimum. These processes were shown to be completed within 2 days after cell hybridization and did not change the following 5 days as judged by the percentage of heat-labile component (fig. 1).

Our experiments do not permit a definite

conclusion about the exact molecular properties of the hexosaminidase isoenzymes. Gene localization studies with interspecies cell hybrids as first reported by van Someren et al. [16] and confirmed by others [4] demonstrated independent segregation of the gene loci for hex. A and B which makes it unlikely that hex. A is a conversion product of hex. B [12, 15]. Furthermore, immunological [1, 17] and biochemical studies [18] are at variance with this latter hypothesis. The absence at low pH of hex. C activity and increased activity of hex. B in Tay-Sachs fibroblasts (fig. 2), the immunological experiments [1, 17], the studies in interspecies hybrids [4, 16] and our complementation studies could be explained by either of the following two models

Hex. B = $\beta\beta$; Hex. A = $\beta\gamma$; Hex. C = $\gamma\gamma$

requiring two gene loci [13], or:

Hex. B = $\beta\gamma$; Hex. A = $\beta\alpha$; Hex. C = $\alpha\gamma$

requiring three gene loci. A disadvantage of the latter hypothesis is that one also would expect mutations in the γ polypeptide chain leading to deficiencies of hex. B and C, but this has never been observed. Further investigations of interspecies hybrids with special emphasis on the segregation behaviour of hex. C might give a definite answer.

Our present studies indicate that the biochemical analysis of heterokaryons containing the genetic information of two different mutant human cell strains is a useful tool for the elucidation of the genetic background of different phenotypic variants of inborn errors of metabolism. As there is no selective system for the isolation of human heterokaryons the use of micromethods enabling the assay of some lysosomal enzymes in single binuclear heterokaryons [3] may further contribute to such complementation studies.

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PUBLICATION II

Characterization of β-D-*N*-Acetylhexosaminidase Isoenzymes in Man-Chinese Hamster Somatic Cell Hybrids

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INTRODUCTION

Several forms of β-D-*N*-acetylhexosaminidase (E.C.3.2.1.30) have been demonstrated in different human tissues [1–3]. Four isoenzymes, characterized by immunological and biochemical techniques [4–8], have been designated β-D-*N*-acetylhexosaminidase A, B, C and S, further to be referred to as hex A, hex B, hex C, and hex S.

Deficient hex A activity and an increased activity of hex B is found in G_{M2} gangliosidosis type 1 or Tay-Sachs disease, whereas G_{M2} gangliosidosis type 2 or Sandhoff disease is associated with a deficiency of both hex A and hex B [9–12].

Biochemical, immunological, and genetical studies suggest a structural relationship between hex A and hex B [1, 5, 10, 13–19]. Robinson and Carroll [19] and Desnick et al. [10] suggested that both isoenzymes are composed of multiple subunits, one of which is common to both forms. Two subunit models have been suggested: (1) the three locus model [10]: hex A = $(\alpha\beta)_n$, hex B = $(\beta\gamma)_n$; and (2) the two locus model: hex A = $(\alpha\beta)_n$, hex B = $(\beta\beta)_n$. Tay-Sachs disease can be explained by a defective α subunit specific for hex A and Sandhoff disease, by a defective common β subunit. Recently, strong evidence in favor of the two locus model has been presented by Beutler and Kuhl [20] in in vitro enzyme hybridization studies.

Studies of the segregation of the hexosaminidase markers in man-rodent somatic cell hybrids can discriminate between the different subunit theories. The results however are contradictory. Lalley et al. [21] found that hex A was never expressed in these hybrids in the absence of hex B, which fits the two locus model. Gilbert et al. [15] and van Someren and Beyersbergen van Henegouwen [14], however, reported an independent loss of hex A and hex B in cell hybrids, which favors the three locus model.

These conflicting results may be due to a misinterpretation of the electrophoretic patterns obtained from hybrid lines, if one relies solely on electrophoretic mobility. The formation of heteropolymeric molecules, especially forms with nearly the same electrophoretic mobility as hex A, might confuse the interpretation of the segregation

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results. Therefore, a further characterization of the hexosaminidase isoenzymes in hybrid lines is needed. It is possible to obtain information about the presence of possible heteropolymeric molecules in hybrid cell lines by using specific antisera against human and Chinese hamster hexosaminidase.

In the present study, a series of man-Chinese hamster hybrids were investigated using an anti-Chinese hamster hexosaminidase serum, a specific anti-human hex A serum, and an anti-human hex B serum. It was found that a hex A-like enzyme present in the hybrid lines consists of both Chinese hamster and specific human hex A moieties. The expression of human hex A was found to be dependent on the presence of hex B, and it was shown that a gene, coding for a specific hex A subunit is syntenic with the mannosephosphate isomerase (*MPI*) and pyruvate kinase (*PK-3*) loci, assigned to chromosome 15.

MATERIALS AND METHODS

Purification of Chinese Hamster Hexosaminidase

Chinese hamster hexosaminidase was purified 290 times from pooled homogenates of liver and kidney. The 20,000 *g* supernatant was applied on a ConA sepharose column, equilibrated with 10 mM sodium-phosphate buffer, pH 7.0, containing 0.1 mM $CaCl_2$, 0.1 mM $MnSO_4$, and 0.5 M NaCl. Hexosaminidase was eluted with 1 M α-methylglucoside in the same buffer solution; pooled fractions were dialyzed and applied to a second affinity column. *N*-acetylglucosamine, bound to CN-Br activated sepharose as described by Lotan et al. [22] was used as affinity ligand. Hexosaminidase was eluted with 10 mM *N*-acetylglucosamine and 0.2 M NaCl in 10 mM sodium-phosphate buffer, pH 6.0. Pooled fractions, containing hexosaminidase were lyophilized and used to raise antisera.

Antisera

Antisera against purified human hex A, hex B, and Chinese hamster hexosaminidase were raised in New Zealand white rabbits by a slight modification of the method described by Carroll and Robinson [23]. Specific human hex A antiserum was prepared by absorption of anti-hex A serum with human liver hex B, according to Bartholomew and Rattazzi [24].

Cell Lines

Normal fibroblasts, hypoxanthine-guanine phosphoribosyltransferase (HPRT) deficient fibroblasts, or normal leukocytes were used as human parental cell lines in the fusion procedure. The Chinese hamster parental cell lines used were thymidine kinase (TK) or HPRT deficient fibroblasts. Details of the fusion procedure and the isolation and propagation of the hybrid cell lines have been described previously [25]. Preparation of cell lysates was carried out as described by Meera Khan [26]. The human controls used were a tetraploid epithelial kidney cell line (T-cell) [27] and a normal fibroblast cell strain.

Electrophoresis

Electrophoresis of hexosaminidase, MPI, and PK-3 was carried out on cellulose acetate gel (Cellogel, Chemetron, Milan, Italy). Hexosaminidase electrophoresis was performed in 0.05 M potassium-phosphate buffer, pH 6.6, for 1 hr at 4°C. The staining procedure for hexosaminidase was carried out according to Okada and O'Brien [11]. The electrophoresis and staining procedures of MPI and PK-3 have been published by van Someren et al. [28].

Immunoprecipitation

Lysates were incubated with antiserum overnight at 0°C. After incubation, the treated lysates were centrifuged at 35,000 *g* for 20 min. The supernatant was used for electrophoresis.

Electrophoresis of lysates of normal human fibroblasts reveals three bands of activity: hex B, hex A and hex C (fig. 1*a*, lane 1). In lysates of Chinese hamster fibroblasts, two bands of activity, described as hex 1 and hex 2 (fig. 1*b*, lane 1), were found.

The specificity of the antisera against Chinese hamster hexosaminidase and against human hex A and hex B was tested with lysates of human and Chinese hamster fibroblasts. The resulting electrophoretic patterns are illustrated in figure 1*a* and 1*b*, lanes 2, 3, and 4. With the concentrations used, the antiserum against Chinese hamster hex 1 does not cross-react with human hex A, hex B, and hex C (fig. 1*a*, lane 2) nor with

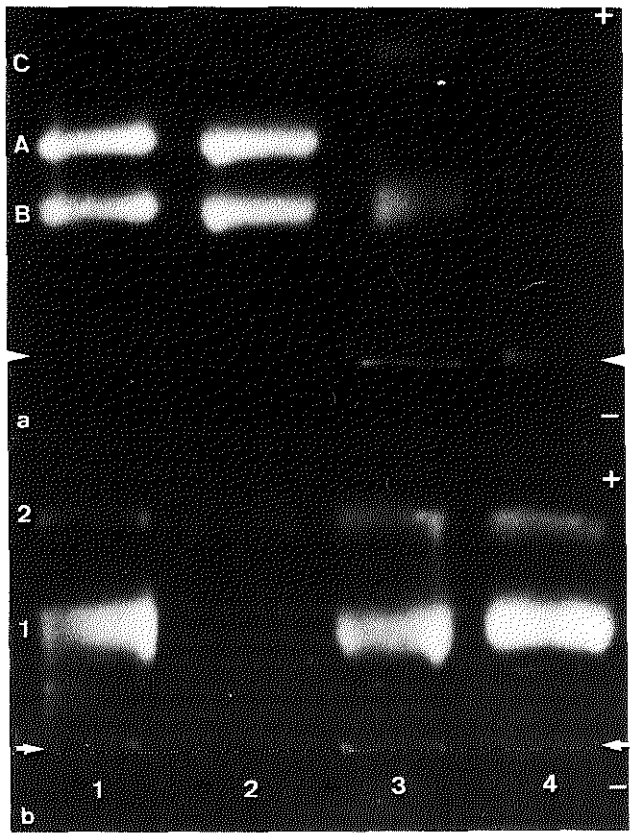

FIG. 1.—Electrophoretic patterns of human (*a*) and Chinese hamster (*b*) fibroblast lysates. *Lane 1*, A, B, C refer to hex A, hex B, and hex C; 1 and 2 to Chinese hamster hex 1 and hex 2: *lane 2*, after treatment with anti-Chinese hamster hexosaminidase serum; *lane 3*, after treatment with specific anti-human hex A serum; *lane 4*, after treatment with anti-human hex B serum. The amount of lysate used in the various lanes depended on the antibody concentrations of the antisera, explaining differences in staining intensity.

the fastmoving Chinese hamster hex 2 band. The specific anti-human hex A serum does not remove hex B and hex C, whereas hex A disappeared (fig. 1*a*, lane 3). The anti-human hex B serum reacts with both hex A and hex B but not with hex C (fig. 1*a*, lane 4). No reaction of specific anti-human hex A and anti-human hex B sera with the Chinese hamster hexosaminidases was observed (fig. 1*b*, lane 3 and 4). These results were confirmed with double immunodiffusion in agarose gels [29] and by antigen-antibody titrations in various combinations of antigens and antisera.

Four types of hybrid lines could be distinguished by electrophoresis of different man-Chinese hamster cell hybrids (fig. 2). They are indicated as hex A′+/hex B+, hex A′+/hex B−, hex A′−/hex B+, hex A′−/hex B−. The band of activity with a mobility comparable to hex A is called hex A′.

Lysates prepared from these four different types of hybrid clones were treated with the various antisera, and the resulting isoenzyme patterns of hexosaminidase were studied by electrophoresis. The results obtained with a hex A′+/hex B− hybrid are shown in figure 3. After treatment with anti-Chinese hamster hex 1 serum, both the Chinese hamster hex 1 and the hex A′ band disappear. The specific anti-human hex A serum removes the hex A′ activity, whereas no reaction with Chinese hamster hexosaminidase was observed. Anti-human hex B serum does not change the electrophoretic pattern. Nine hex A′+/hex B− hybrid clones from five independent fusion experiments were tested in this way; they invariably showed the same immunological characteristics.

Eight hex A′+/hex B+ hybrid cell lines from independent fusion experiments were also studied with antisera (fig. 4). After treatment of the lysates with anti-Chinese hamster hex 1 serum, the hex 1 band disappears along with part of the activity of the hex A′ band (fig. 4, lane 3). Various concentrations of the anti-Chinese hamster hex 1 serum were used, but a complete removal of the activity on the hex A′ position could not be attained. The activity of hex B does not seem to be affected. With specific anti-human hex A serum, all the activity is removed at the hex A′ position. whereas hex B and

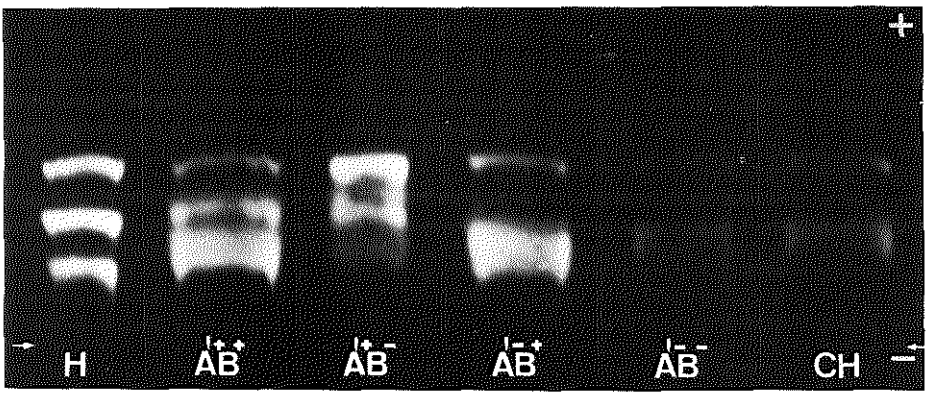

FIG. 2.—Hexosaminidase electrophoretic patterns. *From right to left*: H = human T-cell (Electrophoretic mobility of hex isoenzymes of T-cell and human fibroblast are comparable; the only difference is the intensity of the fastest moving band.) A′+/B+ = hybrid clone with activities at hex A′ and hex B position; A′+/B− = hybrid clone with activity at hex A′ position; A′−/B+ = hybrid clone with activity at hex B position; A′−/B− = hybrid clone having lost the activities at hex A′ and hex B position.

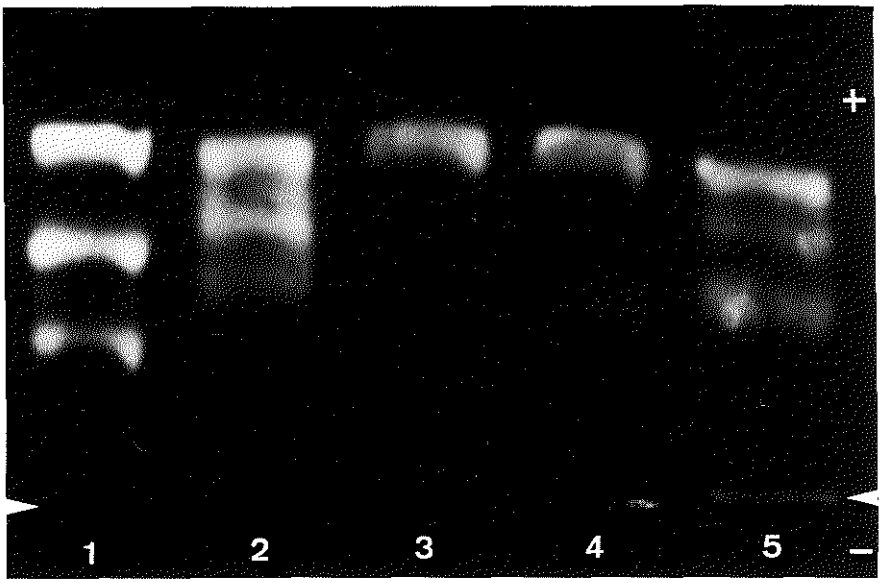

FIG. 3.—Electrophoretic patterns of a hex A′+/hex B− hybrid, following treatment with different antisera. *Lane 1*, human T-cell; *lane 2*, hybrid without antiserum treatment; *lane 3*, hybrid following treatment with the anti-Chinese hamster hexosaminidase serum; *lane 4*, hybrid following treatment with the specific anti-human hex A serum; *lane 5*, hybrid following treatment with the anti-human hex B serum.

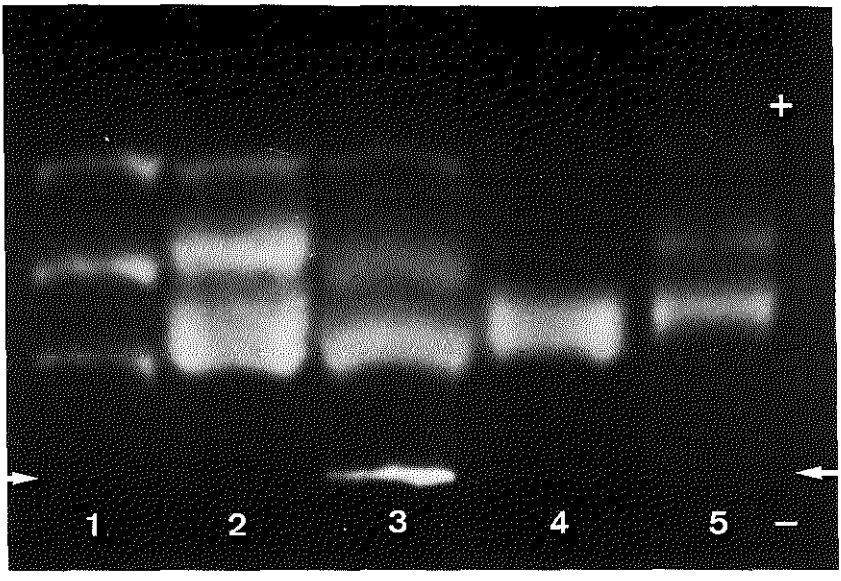

FIG. 4.—Electrophoretic patterns of a hex A′+/hex B+ hybrid, following treatment with different antisera. *Lane 1*, human T-cell; *lane 2*, hybrid without antiserum treatment; *lane 3*, hybrid following treatment with the anti-Chinese hamster hexosaminidase serum; *lane 4*, hybrid following treatment with the specific anti-hex A serum; *lane 5*, hybrid following treatment with the anti-hex B serum.

Chinese hamster hex 1 activities remain unchanged. When treated with anti-human hex B serum, part of the activity at the hex A′ position remains, and a normal Chinese hamster activity was detected. Also in this case, various dilutions of the antiserum were tested, and always residual activity at the hex A′ position remained present. Figure 5 illustrates the typical electrophoretic patterns of a hex A′−/hex B+ hybrid after incubation with the three antisera. Seven clones were tested. Hex B could be removed only by the anti-human hex B serum. The Chinese hamster hex 1 band cross-reacts with the anti-Chinese hamster hex 1 serum. In hex A′−/hex B− hybrids, changes in the electrophoretic pattern were observed only after treatment with the anti-Chinese hamster hex 1 serum, which removes the Chinese hamster hex 1 band.

A relationship between the absence and the presence of the hex A′ band, MPI and PK-3 was established, as shown by the data from several fusion experiments (table 1). No syntenic relationship was found with 24 other enzyme markers tested.

DISCUSSION

The discrepancies in the literature between data obtained in various experiments with man-rodent somatic cell hybrids [14, 15, 21] may be due to different interpretations of the hexosaminidase electrophoretic patterns from hybrid cell lines with particular regard to the activity at the human hex A position. The characterization of the isoenzymes in the hybrid cell lines can be greatly improved by the use of anti-Chinese hamster and anti-human hexosaminidase sera, as seen in the present study.

In hex A′+/hex B− hybrid clones, the hex A′ band was shown to interact with both

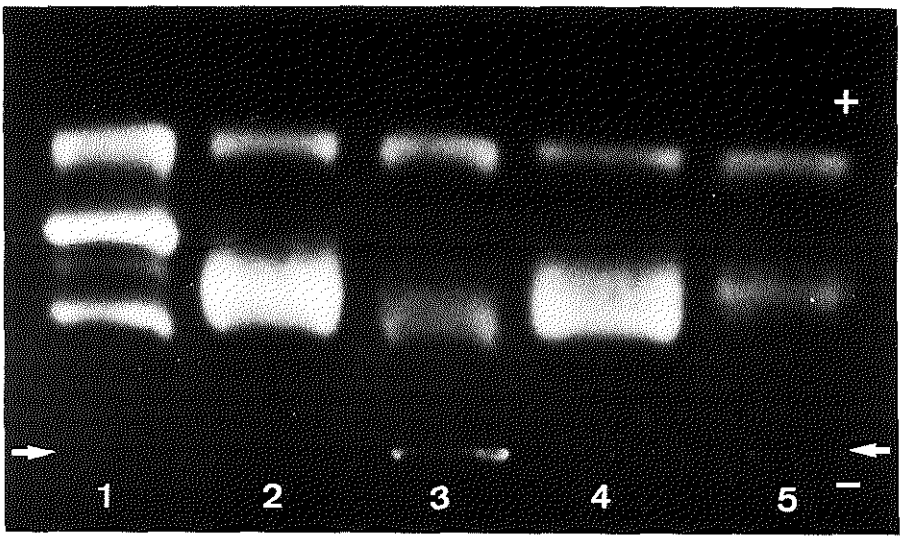

FIG. 5.—Electrophoretic patterns of a hex A′−/hex B+ hybrid, following treatment with different antisera. *Lane 1*, human T-cell. In fresh homogenates an additional band of activity can be found between hex A and hex B. This band corresponds to the intermediate forms 1_1, 1_2, as seen by DEAE cellulose chromatography [34]. *Lane 2*, hybrid without antiserum treatment; *lane 3*, hybrid following treatment with the anti-Chinese hamster hexosaminidase serum; *lane 4*, hybrid following treatment with the specific anti-hex A serum; *lane 5*, hybrid following treatment with the anti-hex B serum.

TABLE 1

ABSENCE AND PRESENCE OF HEX A′, MPI, AND PK-3 IN PRIMARY AND SECONDARY HYBRID LINES

	MPI		PK-3	
	+	−	+	−
Hex A′:				
+	30	3	27	3
−	0	28	0	35
PK-3:				
+	33	0	...	...
−	0	29	...	...

the anti-Chinese hamster hex 1 and the specific anti-human hex A sera, whereas no cross-reaction with the anti-human hex B serum was found. This finding indicates, that in these hybrids the isoenzyme at the hex A′ position is not a normal human hex A, but a heteropolymeric molecule, containing both specific human hex A and Chinese hamster hexosaminidase moieties. The formation of heteropolymeric hexosaminidase molecules in man-Chinese hamster hybrids has been suggested before by Ropers and Schwantes [17], who supposed a heteropolymeric molecule at the hex A position to consist of Chinese hamster and human hex C subunits. In their theory, hex B and hex C are homopolymers, $(\beta\beta)_n$ and $(\alpha\alpha)_n$, respectively, whereas hex A consists of B− and C subunits $(\alpha\beta)_n$. Van Cong et al. [30] described a band of activity comparable with hex A′, which they called "hex A fast." A relationship was demonstrated between the presence and absence of "hex A fast," MPI, and hex C. Following the subunit model of Ropers [16], "hex A fast" was explained as a heteropolymeric molecule with α subunits from human hex C and β-like subunits from Chinese hamster hexosaminidase. Recently it has been shown that at the hex C position two isoenzymes (hex C and hex S) can be detected [3, 20]. In view of these studies, it is likely that the fastest moving hex band described by Van Cong is identical with hex S. Our electrophoresis system does not discriminate between hex S and hex Ç.

The residual activity in hex A′+/hex B+ hybrid clones that could be detected at the hex A′ position after treatment with the anti-Chinese hamster hex 1 or anti-human hex B serum indicates the presence of two different isoenzymes at the hex A′ position. Since no activity is left after treatment with the specific anti-human hex A serum, a heteropolymeric molecule is thought to be present, similar to the heteropolymeric molecule found in hex A′+/hex B− hybrid clones at the hex A′ position, which consists of human hex A and Chinese hamster hex 1 moieties. The other isoenzyme at the hex A′ position cross-reacts with specific anti-human hex A and anti-human B sera and appears to represent a normal human hex A molecule.

From these studies, we conclude that normal human hex A is present only in the presence of hex B, which is in agreement with the results of Lalley et al. [21]. A heteropolymeric isoenzyme can be present independently of human hex B. The results of our present study fits into the two locus subunit model. According to this theory, the heteropolymeric isoenzyme at the hex A′ position has been shown to possess α subunits from human hex A and β-like subunits from Chinese hamster hex 1. The formation of

other heteropolymeric molecules, in particular between the human β subunit and an α-like subunit from the Chinese hamster cannot be excluded. The resolution of our electrophoresis system in the hex 1 and the hex B region is, however, insufficient to distinguish possible heteropolymeric molecules.

Evidence for the syntenic relationship between *hex A*, *MPI*, and *PK-3* [14, 27, 31, 32] and the assignment of *MPI* to chromosome 15 [33] puts this synteny group on chromosome 15. Our results show a relationship between the absence or presence of the isoenzymes on the hex A′ position, and that of MPI and PK-3. With respect to the two locus subunit model, the gene coding for the α subunit of hex A is localized on chromosome 15. Previously, *hex B* has been assigned to chromosome 5 by Gilbert et al. [14]. According to this model, it should be the gene, coding for the β chain which was localized on chromosome 5.

Studies of the expression of hexosaminidases in man-Chinese hamster hybrid cell lines can be a tool for understanding the molecular structure of hexosaminidase, provided that the characterization of human, Chinese hamster, and possible heteropolymeric components does not rely solely on electrophoretic mobilities.

SUMMARY

A series of man-Chinese hamster hybrids were investigated with the use of an anti-Chinese hamster hexosaminidase serum, a specific anti-human hex A serum and an anti-human hex B serum. The expression of human hex A was found to be dependent on the presence of hex B. A heteropolymeric molecule is formed independently of hex B, which consists of Chinese hamster and specific hex A moieties. It has an electrophoretic mobility nearly identical to hex A. A relationship between the absence and presence of the heteropolymeric molecule, mannosephosphate isomerase (MPI), and pyruvate kinase (PK-3), assigned to chromosome 15, was established. With respect to the two locus subunit model, the gene coding for the α subunit, specific for hex A, has been localized on chromosome 15.

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PUBLICATION III

CHARACTERIZATION OF β-D-*N*-ACETYLHEXOSAMINIDASES C AND S IN FIBROBLASTS FROM CONTROL INDIVIDUALS AND PATIENTS WITH TAY-SACHS DISEASE

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1. Introduction

Among the different isoenzymes of β-D-*N*-acetylhexoaminidase that occur in human tissues most attention has been paid to the major forms called Hex A and Hex B. A deficiency of Hex A was shown to be responsible for the metabolic defect in GM_2-gangliosidosis type B (Tay-Sachs disease) [1]. In Sandhoff-Jatzkewitz disease, another form of this lysosomal storage disease, both Hex A and Hex B are deficient [2]. It has been suggested that the simultaneous absence of Hex A $(\alpha\beta)_n$ and Hex B $(\beta\beta)_n$ activity is caused by a defect in the common β-subunit [3]. A third isoenzyme called Hex C was first described by Hooghwinkel et al [4]. In contrast to Hex A and Hex B it failed to hydrolyse 4-methylumbelliferyl-β-D-galactosaminide. Subsequent studies revealed other differences between Hex C and the lysosomal isoenzymes Hex A and Hex B such as a neutral pH-optimum, extralysosomal localization and different immunological properties [5–9]. Recently another isoenzyme of β-hexosaminidase has been characterized and was called Hex S [10–12]. It is the major component of β-hexosaminidase activity in Sandhoff-Jatzkewitz disease and is absent in tissues from patients with Tay-Sachs disease. At acid pH Hex S exhibits activity towards 4-methylbelliferyl-β-D-glucosaminide and 4-methylumbelliferyl-β-D-galactosaminide. From immunological and biochemical studies it was concluded that Hex S shares the α-subunit with Hex A but not the β-subunit [11–13].

It has long been a question whether there is a structural relationship between Hex C and Hex A. Ropers et al. [14] suggested a common subunit structure for Hex A and Hex C. This was based on studies that claimed a deficiency of Hex C in tissues from patients with Tay-Sachs disease [4,14]. The isoenzyme pattern of hexosaminidase in man–rodent hybrids seemed to support this hypothesis [15,16]. Other reports however described the presence of Hex C in Tay-Sachs disease [5–9]. To find a reason for this discrepancy we have investigated the electrophoretic pattern of β-hexosaminidase in fibroblasts from control subjects and patients with Tay-Sachs disease using different staining conditions. With reference to the recent characterization of Hex S special attention was given to the distinction of Hex C and Hex S.

Our findings suggest that there is no evidence for a structural relationship between Hex C and Hex A, and the results rather favor a different interpretation of the studies with man–rodent hybrids.

2. Materials and methods

Skin fibroblasts from three different control individuals and six unrelated patients with Tay-Sachs disease were cultured in Falcon Flasks in Ham's F10 medium supplemented with 15% fetal calf serum and antibiotics as described before [17]. Homogenates of 3×10^6 cells per 100 μl were prepared by sonication in distilled water. Electrophoresis was performed on cellulose acetate gels (Cellogel, Chemetron, Italy) in 50 mM potassium phosphate, pH 6.8, at 4°C, for 90 min at 200 V. 10 μl Cell homogenate was used per

lane and the gels were stained with either 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-galactopyranoside (MU-galactosaminide 1.5 mM) or 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside (MU-glucosaminide 1.5 mM) in phosphate (0.2 M) citrate (0.1 M) buffer, pH 4.0 or pH 7.0 (1 h 37°C). Photographs were taken after exposure of the gels to ammonia vapor. Preparation of antiserum against purified human liver Hex A has previously been described [18]. 10 μl cell homogenate was incubated overnight at 4°C with 10 μl antiserum. The 40 000 × *g* supernatant was applied to the gels.

3. Results

The isoenzyme pattern of β-hexosaminidase was studied using different staining conditions. When 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopuranoside (MU-glucosaminide) is used as substrate, at either pH 4 or pH 7, a band of activity shows up anodal to Hex A both in fibroblasts from control subjects and patients with Tay-Sachs disease (fig.1). We have indicated this band as C/S because activity of Hex S as well as Hex C might be expected at this position using MU-glucosaminide as substrate [11]. At pH 4 this band shows up more strongly in control fibroblasts than in those from the patient. It was further noticed that in fibroblasts from the patient the band exhibits a higher activity at pH 7 than at pH 4. This was presumably because only the neutral isoenzyme Hex C is present in fibroblasts from the patient with Tay-Sachs disease.

When 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-galactopyranoside (MU-galactosaminide) is used as substrate instead of MU-glucosaminide the activity of all isoenzymes is lower, but the most anodal band still shows up in normal fibroblasts both at acid and neutral pH (fig.2). At pH 4 the band is indicated as Hex S, the acid isoenzyme, because it does not show up under these conditions in fibroblasts from the patient. At pH 7 the neutral isoenzyme Hex C contributes to the activity of this band. This is indicated by the weak band of activity at the C/S position at this pH in fibroblasts from patients with Tay-Sachs disease.

To establish that the activity at the C/S position only represents Hex S when the gels are stained at pH 4 with MU-galactosaminide we treated the homogenates with anti-Hex A antiserum before electrophoresis. Figure 3 shows the isoenzyme pattern in fibroblasts from control individuals and from patients with Tay-Sachs disease both with and without antiserum treatment. The band of activity that shows in control fibroblasts at the C/S position using these staining conditions is removed completely by the antiserum. This indicates that the activity was due to Hex S only. The activity that is left at the Hex A position and the origin after antiserum treatment might be caused by unprecipitated immune complexes.

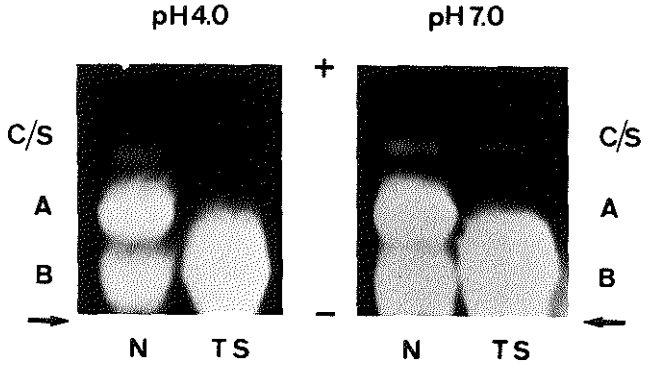

Fig.1. Isoenzyme pattern of β-hexosaminidase in fibroblasts using MU-glucosaminide as substrate. N = control subject. TS = patient with Tay-Sachs disease.

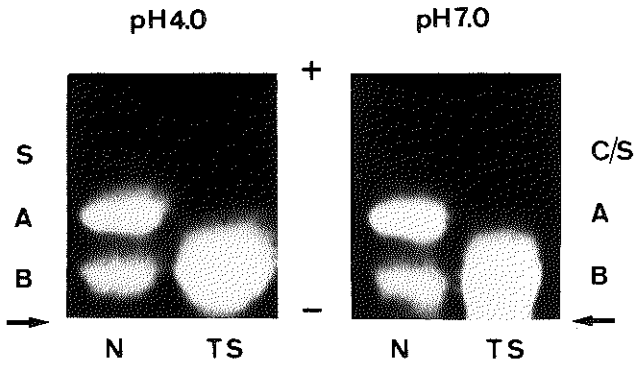

Fig.2. Isoenzyme pattern of β-hexosaminidase in fibroblasts using MU-galactosaminide as substrate. N = control subject. TS = patient with Tay-Sachs disease.

As described above there is no activity at the C/S position in Tay-Sachs fibroblasts under these conditions. Antiserum treatment results in loss of Hex B activity. Hex C can be demonstrated in normal fibroplasts and those from the patient after precipitation of Hex S using MU-glucosaminide at pH 7 (fig.4). With this substrate Hex C activity was also found at pH 4 after antiserum treatment although it was much less.

Three different control fibroblasts strains and six cell strains from unrelated patients with Tay-Sachs disease were investigated in this way and similar patterns were observed. In all three control strains Hex C and Hex S activity could be demonstrated, whereas in none of the fibroblasts strains from the patients was Hex S activity detected. Hex C activity could always be demonstrated.

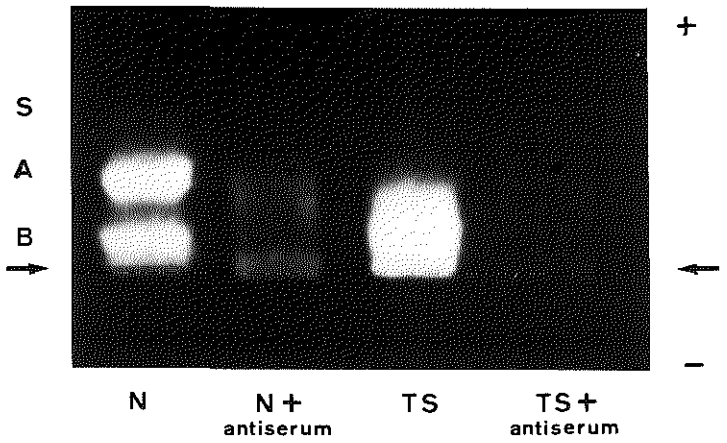

Fig.3. Effect of anti Hex A antiserum on the pattern of β-hexosaminidase in fibroblasts. Gels were stained with MU-galactosaminide at pH 4.0. N = control subject. TS = patient with Tay-Sachs disease.

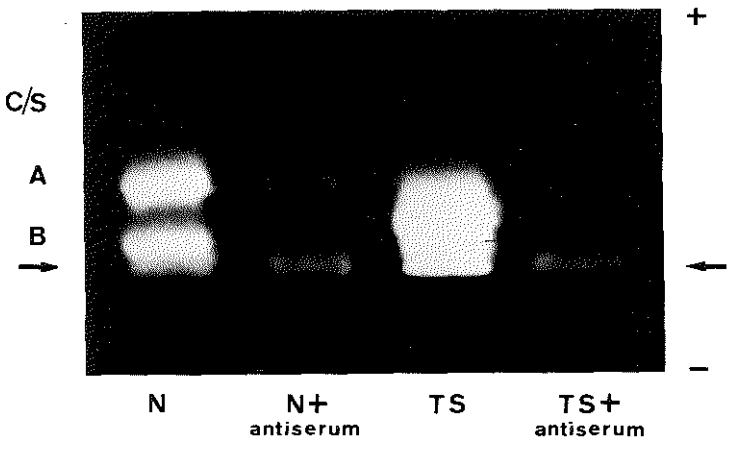

Fig.4. Effect of anti-Hex A antiserum on the pattern of β-hexosaminidase in fibroblasts. Gels were stained with MU-glucosaminide at pH 7.0. N = control subject. TS = patient with Tay-Sachs disease.

4. Discussion

Hex C activity has been demonstrated by several authors in tissues from patients with Tay-Sachs disease [5–9,20]. Others however did not succeed in detecting Hex C in Tay-Sachs disease [4,14,15,16,19]. These conflicting results seem to be explained by the recent characterization of Hex S by Ikonne et al. [11] and Beutler et al. [12] and by the fact that Hex S and Hex C are difficult to seperate in many electrophoretic systems. Also, in the system we used Hex S and Hex C have the same mobility.

Using MU-galactosaminide as substrate at pH 4 conditions were found for the specific staining of Hex S. With MU- glucosaminide no proper distinction could be made between Hex S and Hex C, but Hex C could be demonstrated with this substrate after precipitation of Hex S with anti-Hex A antiserum. At low pH the band of activity at the C/S position might be missed in tissues from patients with Tay-Sachs disease because it only represents the neutral isoenzyme Hex C. In tissues from control subjects Hex S contributes to the activity of this band which makes it easier to detect at low pH.

Regarding this we suggest a different interpretation of results obtained by Van Cong et al. [15,16] with man–rodent hybrids. An isoenzyme was detected that was not present in either of the parental strains. It was called 'Hex A fast' and was thought to be composed of human α-subunits and rodent β-like subunits. It was demonstrated that the formation of this heteropolymeric molecule was dependent on the presence of Hex C. However, taking into account the substrate and the low pH that was used to stain the gels, it may well be that Hex S was scored instead of Hex C. The results would then have to be interpreted as synteny of Hex S and Hex A. This would be compatible with the proposed $(\alpha\alpha)_n$ subunit structure of Hex S [11,12] and the different immunological and biochemical properties of Hex C [5–9,20] in comparison with the other isoenzymes of β-hexosaminidase.

We have recently identified a hexosaminidase isoenzyme in man–chinese hamster hybrids as a heteropolymeric molecule [18]. It has the immunological properties of the α-subunit of human Hex A and chinese hamster hexosaminidase and it segregates with markers of human chromosome 15. One therefore might expect that Hex S is localized on this chromosome unless Hex S has subunits different from α. The present data suggest another locus for Hex C, but this has not been confirmed by gene localization studies. The method described in this

paper and the procedures described by Swallow et al. [20] to discriminate between Hex S and Hex C might facilitate these studies.

Acknowledgements

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PUBLICATION IV

Genetic heterogeneity in GM1-gangliosidosis

GM1-GANGLIOSIDOSIS is an inherited lysosomal storage disease which is due to a deficiency of the acid hydrolase GM1-β-galactosidase[1]. During the past few years several clinical variants have been described[2-6] that differ in time of onset of symptoms, involvement of visceral organs or skeletal tissue and in the degree of neuronal and mental deterioration. Some of these variants have been related to different properties of the deficient β-galactosidases[6,7] but the significance of the experimental data[8] has been questioned. Several investigators have speculated on the genetic-background of the different variants[2,6,9] but no experimental evidence has been provided to support the hypotheses.

Using somatic cell hybridisation techniques[10-12] we have investigated whether different gene mutations are involved in clinical variants of GM1-gangliosidosis. Fibroblasts from a patient with the infantile type 1 and the juvenile type 2 (refs 1-3), and from the type 3 patient without mental retardation described by Pinsky *et al.*[5] were fused with cells from an adult (type 4) patient reported by our group[6]. Two days after hybridisation the occurrence of genetic complementation was tested by β-galactosidase assays with both synthetic and natural substrates. These experiments showed that the gene mutation in types 1 and 2 is different from that in types 3 and 4. Enzyme assays performed in single heterokaryons containing the genomes of type 1 and type 4 cells showed that genetic complementation results in complete restoration of normal enzyme activity.

Normal skin fibroblasts and cells from patients with the different types of GM1-gangliosidosis were grown in Ham F10 medium supplemented with 10% foetal calf serum and antibiotics. In homogenates of fibroblasts from patients with the type 1 or type 2 variant, β-galactosidase activity was less than 1% of control values when measured with the 4-methylumbelliferyl (MU) substrate. Homogenates of fibroblasts from patients with type 3 or type 4 GM1-gangliosidosis had a residual activity of 15-20%. In type 4 cells a 5% and 8% residual activity with the natural substrates GM1-ganglioside and asialofetuin was found by Dr J. S. O'Brien (Department of Neurosciences, University of California, La Jolla).

Cells from the adult type 4 patient were hybridised with those from the other three variants and each cell strain was also fused with itself (parental fusion) as a control. The results of β-galactosidase assays with synthetic substrate are shown in Fig. 1. Levels of activity in unfused cells were similar to those in the parental fusions, which indicated that somatic cell hybridisation did not affect enzyme activity. The β-galactosidase activities in fusions of type 1 × type 2 and of type 3 × type 4 cells remain unchanged relative to the average level expected in a mixture of equal numbers of parental cells. Fusion of type 1 × type 4 cells, however, resulted in a six- to sevenfold increase in enzyme activity. Also there was a markedly increased enzyme activity in fusions of type 4 × type 2 cells.

Similar results were obtained when the β-galactosidase activity was measured with the natural substrate GM1-ganglioside (Table 1). Conditions for cell cultivation, fusion, collection and protein determination were identical to those described in Fig. 1. Whereas no enzyme activity was detected after type 1 × type 2 fusion a marked increase in GM1-β-galactosidase activity was observed after hybridisation of type 4 × type 1 cells. An additional fusion experiment of type 4 × type 1 cells was carried out with inactivated serum in the culture medium. This will inhibit proliferation of the unfused parental cells and thus the proportion of heterokaryons will be higher than if normal serum is used. This is reflected in the higher β-galactosidase activities in the last type 4 × type 1 fusion shown in Table 1.

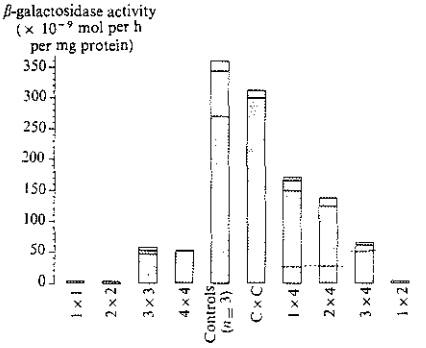

Fig. 1 β-Galactosidase assays on cell homogenates after fusion of cultured fibroblasts from patients with different variants of GM1-gangliosidosis. 2×10^5 cells from each strain were hybridised with inactivated Sendai virus[13] under such conditions[14] that 50-80% of the nuclei were present in multinucleates. Two days after fusion cells were trypsinised, rinsed in saline and disrupted by sonication (2 × 30 s), and after protein analysis[14] the β-galactosidase activity was assayed with 1 mM 4-methylumbelliferyl-β-D-galactopyranoside, *p*H 4.2, as described previously[15]. ————, Independent experiments with the same cell strain; as controls three different normal strains were used; — — —, expected enzyme activity if no complementation occurs, that is the mean enzyme activity as a result of mixing equal numbers of both parental cells.

These results showed that genetic complementation had occurred in the fusion between type 4 cells and type 1 or type 2 cells. Thus the adult form of GM1 gangliosidosis (type 4 variant) is caused by a different gene mutation than is present in the types 1 or 2 variants. Since there was no complementation after fusion of type 4 × type 3 cells these variants might be identical or determined by allelic mutations, as is the case for types 1 and 2 GM1-gangliosidosis[12].

Although complementation could be detected by enzyme analyses in homogenates of heterokaryons it is not possible to evaluate the degree of restoration of activity since the proportions of nuclei from each parental cell present in the multinucleates are unknown. To solve this problem microchemical techniques were developed which facilitated quantitative enzyme assays in individual binuclear heterokaryons[15,16]. Fibroblasts from the adult patient with type 4 GM1-gangliosidosis were hybridised with those from a type 1 variant under such conditions that about 8% of the hybrid cells are binucleate[15]. After fusion cells were grown for 24 h in a Falcon flask, they were then trypsinised and 2×10^4–4×10^4 cells were

Table 1 β-Galactosidase assays using natural substrate after somatic cell hybridisation of different GM1- gangliosidosis variants

	Enzyme activity (× 10^{-9} mol per h per mg protein)	
	GM1-β-galactosidase	4-MU-β-galactosidase
Type 1 × type 1	3.5	1.8
Type 2 × type 2	4.3	4.0
Type 4 × type 4	10.5	13.4
Type 1 × type 2	< 1	< 1
Type 1 × type 4	44.1	38.6
Type 1 × type 4*	99.8	75.3
Control	193.8	246.7

*After fusion, cells were grown on medium with 10% foetal calf serum that had been treated with alkali (*p*H 10.7) for 3 h at 37 °C to destroy enzyme activity in the medium. Conditions of cell culture, hybridisation and collection were similar to those described earlier. GM1-galactoside-β-galactosidase activity was measured according to Ho *et al.*[16].

seeded in a dish with a thin plastic bottom. After 24–48 h of subsequent cultivation these dishes were frozen and freeze-dried immediately and small pieces of plastic each containing one binuclear cell or one non-fused mononucleate cell were dissected under microscopic control. β-Galactosidase assays in individual cells were carried out with synthetic substrate in submicrolitre volumes and fluorescence measurements were made in a microspectrofluorometer as described earlier[15,18].

The distribution of β-galactosidase activity in individual unfused control fibroblasts is shown in Fig. 2*a*. Results from three different cell strains were pooled, but also in cells from each cell strain there was a considerable variation of activity in the range 4×10^{-14}–40×10^{-14} mol h^{-1}. In considering these data it should be realised that the enzyme activity is expressed per cell and that the total cellular dry mass and protein content in individual fibroblasts also show marked variations[18,19].

The mean β-galactosidase activity in control cell strains varied from 19×10^{-14} to 26×10^{-14} mol h^{-1}. Earlier experiments[17] showed that the level of activity in parental fusions of control fibroblasts was approximately twice that of mononucleates. No β-galactosidase activity could be detected in

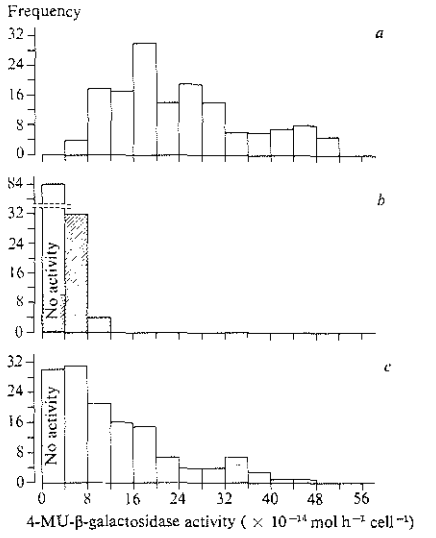

Fig. 2 β-Galactosidase assays in individual heterokaryons after fusion of type 4 × type 1 variants of GM1-gangliosidosis. β-Galactosidase activity was measured by incubating individual cells in 0.2 μl 1 mM 4-methylumbelliferyl-β-D-galactopyranoside in 0.1 M-acetate buffer (*p*H 4.2) containing 0.02% (w/v) bovine albumin. Incubation was carried out during 2 h at 37 °C under paraffin oil using Lowry's "oil well technique"[15,18]; after addition of 1 μl 0.5 M carbonate buffer (*p*H 10.7) the fluorescence was measured in 1-mm glass capillaries with a Leitz microspectrofluorometer[15,18] (objective 2.5 pl). *a*, Distribution of enzyme activities in non fused mononuclear control fibroblasts as measured in three different cell strains; *b*, enzyme activities in individual binuclear cells after parental fusion of type 1 × 1 (open columns) and type 4 × 4 (hatched columns) cells each measured in two independent experiments; *c*, enzyme activities in binuclear heterokaryons after fusion of type 4 × 1 cells as measured in three independent experiments.

individual binuclear cells after parental fusions of type 1 cells while in parental fusions of type 4 cells mean activities of 3×10^{-14} and 5×10^{-14} mol h^{-1} were found in two experiments; this is 10–20% of the activity in parental fusions of control fibroblasts (Fig. 2*b*).

After fusion of type 4 × type 1 cells in 21% of the binucleates no β-galactosidase activity could be detected; these probably represent fusions of type 1 × type 1 cells. In 27% of the binuclear cells the enzyme activity was similar to that in parental fusions of type 4 cells. But in 52% of the binuclear heterokaryons β-galactosidase activity was in the range 8×10^{-14} to 40×10^{-14} mol h^{-1} (Fig. 2*c*). This is greater than in either of the parental cells before or after fusion and the range of activity is similar to that in mononuclear control fibroblasts. Thus genetic complementation after fusion of type 4 × type 1 cells results in restoration of β-galactosidase activity to normal control levels.

The mechanism responsible for the genetic complementation observed after fusion of type 4 cells with type 1 or type 2 cells is not yet understood. In human liver two forms of GM1-ganglioside-β-galactosidases (A and B) have been identified[16,20] both of which are deficient in GM1-gangliosidosis[20]. It has been proposed that the A form of β-galactosidase consists of a single polypeptide chain[21] and that the B form is a multimeric aggregate of the A monomer[9]. On this basis the results of our hybridisation studies would indicate the occurrence of intragenic complementation. In a recent review, O'Brien[9] does not exclude the possibility that the B form of β-galactosidase also contains amino acid sequences which are not common to the A form. In that case the complementation in type 4 × type 1 or 2 heterokaryons could still be intergenic. A third possibility is that the gene mutation in type 4 GM1-gangliosidosis involves a regulator whereas the mutations in type 1 and type 2 are structural mutations. The latter is supported by immunological studies[9,22] which revealed the presence of cross-reactive material in cells from all patients tested with type 1 and type 2 GM1-gangliosidosis. Preliminary analyses of the deficient β-galactosidase in type 4 cells and cocultivation studies are suggestive for a regulator mutation in the adult type 4 GM1-gangliosidosis. Further investigations on the immunological and biochemical properties of the deficient enzyme and of the restored enzyme activity after genetic complementation are required to prove this hypothesis.

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PUBLICATION V

BIOCHEMICAL, IMMUNOLOGICAL AND CELL GENETIC STUDIES IN A TWO YEAR OLD PATIENT WITH AN ATYPICAL FORM OF G_{M1}-GANGLIOSIDOSIS

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SUMMARY

Cultured skin fibroblasts from a two year old boy with an atypical form of G_{M1}-gangliosidosis have been studied. With the artificial substrate 4-methylumbelliferyl-β-D-galactopyranoside, 10-20% residual β-galactosidase activity was found in fibroblasts from this patient. Most of the residual enzyme activity was in the monomeric A form, very little in the multimeric B form. The Km value, pH profile and heat lability of the mutant enzyme were comparable with that of β-galactosidase from control fibroblasts. Immunological studies showed crossreaction of the mutant enzyme with an antiserum raised against human liver β-galactosidase, but the catalytic activity per unit antigenic activity was about three times lower than normal, which suggests a structural mutation of the enzyme. It was demonstrated by somatic cell hybridization that the gene mutation in the present patient is different from that in patients with type 1 or type 2 G_{M1}-gangliosidosis. No genetic complementation was found after fusion with cells from two other clinical forms, designated type 3 and adult type 4 G_{M1}-gangliosidosis.

INTRODUCTION

G_{M1}-gangliosidosis is an autosomal recessive disorder, characterized by the lysosomal storage of G_{M1}-ganglioside and of proteoglycans due to a deficiency of G_{M1} β-galactodase activity (acid-β-galactosidase EC 3.2.1.23) (Okada and O'Brien, 1968, O'Brien, 1972).

There is a considerable clinical heterogeneity among patients with β-galactosidase deficiency. Patients with the infantile form of G_{M1}-gangliosidosis (type 1) develop symptoms shortly after birth. There is a rapid progressive psychomotor deterioration, severe bone deformities, pronounced hepatosplenomegaly and the patients usually die by two years of age. In patients with the juvenile type 2 form the symptoms appear later and progression is slower, hepatosplenomegaly is absent, bone abnormalities are minimal and the patients may survive to 10 years of age (Wolfe et al., 1970; O'Brien et al., 1972b). In addition to these two types several patients have been reported with a different clinical manifestation of β-galactosidase deficiency. Among these are children without severe mental retardation (Pinsky et al., 1974; Wenger et al., 1974; O'Brien et al., 1976) and patients where symptoms became manifest in early adulthood (Yamamoto et al., 1974; Loonen et al., 1974; Orii et al., 1975).

In earlier studies we were able to classify some of these clinical variants in genetically distinct groups by complementation analysis in somatic cell hybrids (Galjaard et al., 1975). It was found that the gene mutation in type 1 and type 2 G_{M1}-gangliosidosis was different from that in the variants type 3 described by Pinsky et al. (1974) and the adult type 4 reported by

Loonen et al. (1974).

The present report deals with biochemical, immunological and cell genetic studies of a two year old patient with only 10-20% of the normal β-galactosidase activity in leucocytes and cultured skin fibroblasts. The study was undertaken to investigate the nature of the mutant enzyme and to extend our knowledge about the relation between clinical and genetic heterogeneity in G_{M1} gangliosidosis.

MATERIALS AND METHODS

The patient concerned is the son of healthy, non consanguineous parents from South Italy who developed normally after uneventful pregnancy and delivery. At 14 months of age he was admitted to the hospital because of bronchopneumonia. A mucopolysaccharidosis was suspected because of peculiar facies with depressed nasal bridge, low set ears, thick eyebrows and small epicanthal folds. Further examinations revealed hepatosplenomegaly, dorsolumbar kyphosis and bilateral hydrocele. Psychomotor development appeared normal and no ophthalmologic abnormalities were found. An extensive clinical report will be published elsewhere by Andria et al. On the last admission, at 28 months of age the psychomotor development was still undisturbed.

Fibroblasts obtained from skin biopsy were cultured in Ham's F10 medium supplemented with 15% fetal calf serum and antibiotics. Confluent cultures were trypsinized and cell homogenates were made in 0,9% NaCl by sonication. Enzyme activities were measured with 4-methylumbelliferyl (MU) substrates (Koch Light) using the following conditions.

α-glucosidase: 2.2 mM MU-α-glucopyranoside in 0.2 M acetate pH 4.4; β-galactosidase: 1 mM MU-β-galactopyranoside (MUGal) in 0.1 M acetate buffer pH 4.4 containing 0.1 M NaCl;
β-N-acetylhexosaminidase: 5 mM MU-β-glucosaminide in phosphate (20 mM)-citrate (12 mM) pH 4.4, α-mannosidase:

4 mM MU-α-mannoside in 0.2 M acetate buffer pH 4.4; β-glucuronidase: 2 mM MU-α-glucuronide in 0.1 M acetate buffer pH 4.4;
α-galactosidase: 1.5 mM MU-α-galactopyranoside in 0.05 M acetate buffer pH 4.5; β-glucosidase: 5 mM MU-β-glucopyranoside in 0.2 M acetate buffer pH 4.4.

In each assay 10 µl cell homogenate was incubated with 20 µl substrate for 1 hour at 37°C. The reaction was stopped by adding 500 µl 0.5 M carbonate buffer pH 10.5 and the fluorescence of liberated methylumbelliferone was measured. For gel filtration a microcolumn of sephadex G 150 superfine was used (15 cm x 0,5 cm). The column was equilibrated and eluted with 10 mM sodium phosphate pH 6.5 containing 5 mM NaCl, as described by Ho and O'Brien (1971). Fractions of 150 µl were collected.

For immunological studies an antiserum was used, that was raised against partial purified human liver β-galactosidase. Details of the preparation procedures have been described by J. de Wit et al (1977). An aliquot of 15 µl cell homogenate was incubated overnight at 4°C with an equal volume of serial dilutions of the antiserum. Immune-complexes were removed by centrifugation at 40,000 x g for 30 minutes. The activity of unprecipitated enzyme was measured in the supernatant using MUGal substrate.

Complementation studies were performed by fusion of a million of cells from each parental strain and subsequent assay of β-galactosidase activity as described before by Galjaard et al. (1974).

RESULTS

The activities of several lysosomal hydrolases in fibroblasts from this patient were compared with those of control strains (Table 1). The activity of β-galactosidase measured with 4-methylumbelliferyl-β-D-galactopyranoside (MUGal) was found to be only 10-20% of the control value, whereas the other activities were in the normal range, β-glucosidase and α-mannosidase activity being increased.

TABLE 1. LYSOSOMAL ENZYME ACTIVITIES IN FIBROBLASTS FROM OUR PATIENT Z.R. AND CONTROLS

ENZYME	CONTROLS	PATIENT Z.R.
β-hexosaminidase	4000 - 12,000 (n = 23)	7,600
β-galactosidase	500 - 1,200 (n = 20)	180
α-galactosidase	20 (n = 1)	25
β-glucosidase	27 - 80 (n = 11)	140
α-glucosidase	30 - 150 (n = 26)	70
β-glucuronidase	104 - 230 (n = 7)	200
α-mannosidase	41 - 140 (n = 16)	180

Activities are expressed in moles MU x 10^{-7} per hour per mg protein. n = number of strains tested.

The reduction of β-galactosidase activity was not due to the presence of an inhibitor since a mixture of cell homogenates from fibroblasts derived from our patient and from a control individual exhibited an activity which was the average value of the two parental strains.

The high residual activity in the patient's fibroblasts allowed us to examine the physico-chemical and immunological characteristics of the mutant enzyme. As shown in Fig. 1, no differences were detected in the pH profile of the

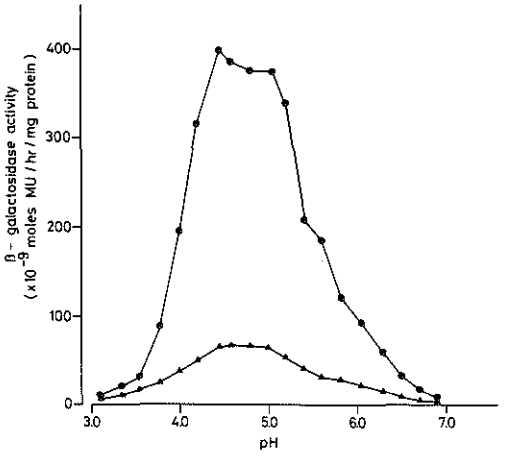

Fig. 1 pH profile of β-galactosidase in human fibroblasts. ▲—▲ patient, ●—● control

mutant enzyme compared to that of the control fibroblasts except that the activity of the former was decreased over the whole pH range. Also the Km value of the mutant enzyme for MUGal was similar to that of β-galactosidase in normal cells i.e. 0.49 mM.

The heat lability of the normal and the mutant enzyme was studied at 41°C, using 0.1 M acetate pH 4.4, containing 0.1 M NaCl as incubation mixture. The protein concentration of the fibroblast homogenate from the patient was brought to 1 mgr/ml by dilution with buffer. The homogenate of control cells was diluted until equal β-galactosidase activity per ml was obtained. Subsequently the protein concentration was brought to 1 mgr/ml by addition of a concentrated homogenate of fibroblasts from a type 1 patient of which the β-galactosidase activity was neglectable. Fig. 2 shows that the heat inactivation curves for the normal and the mutant enzyme are similar.

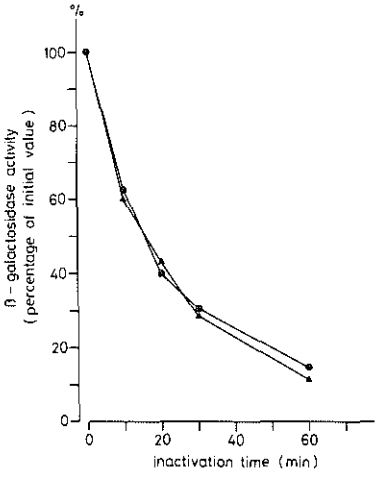

Fig. 2 Heat lability of β-galactosidase in human fibroblasts. ▲—▲ patient, ●—● control

Gel filtration on sephadex G 150 was carried out to study the isoenzyme pattern of the mutant enzyme. For this purpose the cell homogenates were prepared by freezing and thawing instead of sonication, since a loss of the high molecular form was sometimes noticed after sonication. Cell debris was removed by centrifugation and the supernatant was applied to the column. As illustrated in Fig. 3 most of the mutant enzyme elutes with the low molecular weight form of β-galactosidase from a control strain and very little activity comes of the column together with the high molecular weight form.

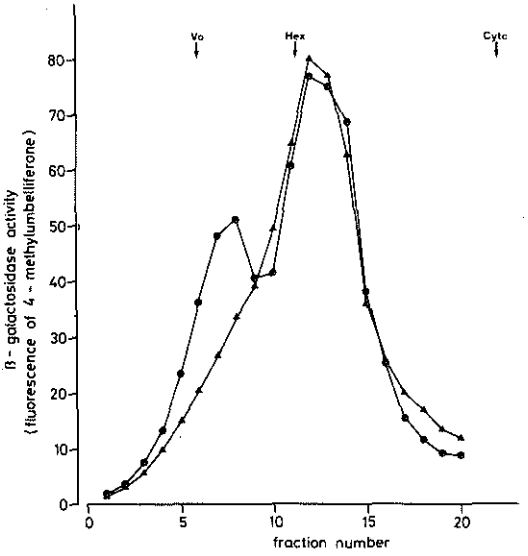

Fig. 3 Gel filtration of β-galactosidase in human fibroblasts. ▲—▲ patient, ●—● control, V_o = void volume. Hex: position of peak of β-hexosaminidase (MW 10^5 - 1.5 x 10^5). Cyt c: position of cytochrome c (MW 12 x 10^3)

The immunological characteristics of the normal and the mutant β-galactosidase were compared by immunotitration with anti β-galactosidase antiserum. Fixed amounts of cell homogenate were incubated with increasing amounts of

antiserum at 4°C overnight. The unprecipitated activity in the 40,000 g supernatant was measured with MUGal as substrate. Fig. 4 shows that under these circumstances β-galactosidase from control fibroblasts is precipitated three times as effective as β-galactosidase from the patient. This indicates that either the specific catalytic activity or the antigenic activity per molecule of the mutant enzyme is less than normal.

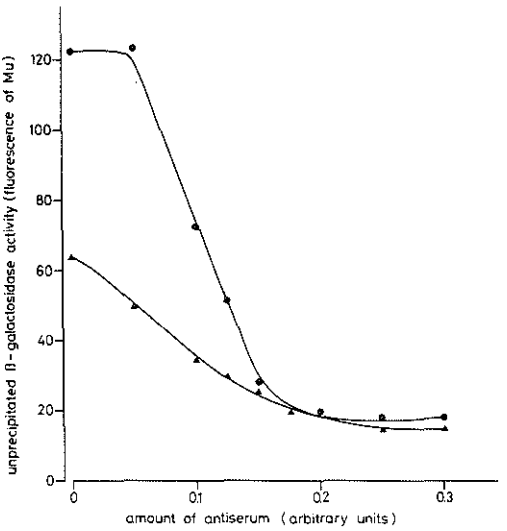

Fig. 4 Immuno precipitation of β-galactosidase in human fibroblasts by an antiserum raised against human liver β-galactosidase. ▲—▲ patient, ●—● control

The nature of the gene mutation in our patient was further investigated by somatic cell hybridization studies. Fibroblasts from this patient were fused with equal numbers of fibroblasts derived from patients with different clinical types of G_{M1}-gangliosidosis. Fig. 5 shows that fusion of fibroblasts from our patient with those from an infantile type 1 or a juvenile type 2 patient results in an increase of β-galactosidase activity. No complementation occurs after fusion with fibroblasts from an adult type 4

patient nor with cells from the type 3 patient. The β-galactosidase activity after fusion of type 1 and type 4 fibroblasts, as published before (Galjaard et al., 1975) is given as a reference for a positive complementation result.

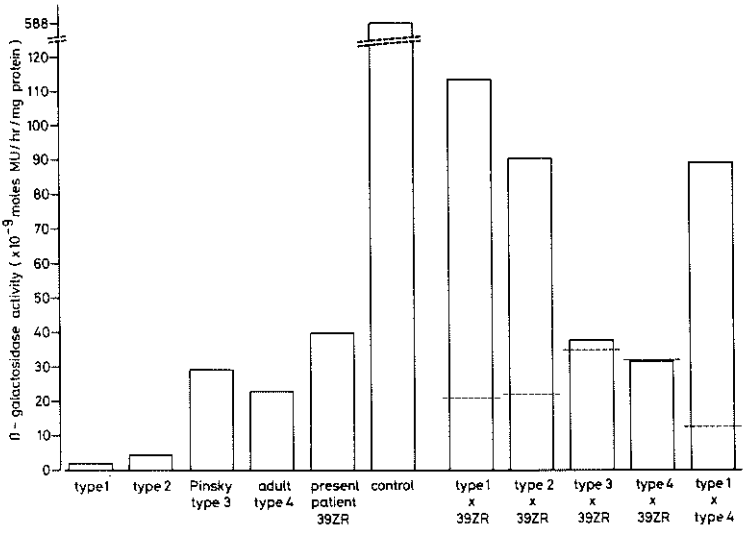

Fig. 5 Genetic complementation studies after fusion of fibroblasts from patients with different clinical forms of G_{M1}-gangliosidosis. Columns 1-6 show the mean activity of 3-5 analyses of mononuclear fibroblasts. Columns 7-11 show the mean activity of multikaryons after cell fusion. The dotted line indicates the mean activity of the two fusion partners before fusion, i.e. the activity to be expected if no complementation would occur after cell fusion.

DISCUSSION

The enzymatic studies described in this paper are suggestive for the diagnosis G_{M1}-gangliosidosis (Andria et al., 1977). Normal or increased activities were found for all lysosomal enzymes tested except for β-galactosidase, the activity of which was highly reduced. The decreased enzyme activity was not due to inhibition by accumulated products as described for the liver β-galactosidase in cases of mucopolysaccharidosis (Ho and Fluharty, 1975) since a mixture of mutant fibroblasts and control cells gave average values of β-galactosidase activity. It was recently demonstrated by O'Brien et al. (1976) that the clinical manifestations might depend on different residual activities of β-galactosidase for various natural substrates. Yet, in earlier studies on different mutant cell strains and control fibroblasts a good agreement between assays with the artificial substrate 4-methylumbelliferyl-β-D-galactopyranoside and the natural substrate G_{M1}-ganglioside was found (Galjaard et al., 1975). We therefore like to conclude that the decrease of β-galactosidase activity as measured with methylumbelliferyl substrate is correlated with a genetic defect of the enzyme that is normally involved in the degradation of G_{M1}-ganglioside.

The activity of β-galactosidase in fibroblasts varies with cell culture conditions (Heukels and Niermeijer, 1976). It is therefore difficult to make a proper estimation of the level of residual activity unless strictly standardized conditions are used and cultures are followed over a longer period. The β-galactosidase activity in fibroblasts from our patient varied from 7 to 16 percent in different experiments. This activity is significantly higher than that found in patients with type 1 or type 2

G_{M1}-gangliosidosis (Galjaard et al., 1975; Norden and O'Brien, 1975). Although there are no sufficient data to support a clear correlation between the degree of enzyme deficiency and the severity of the clinical manifestations, it seems that in a number of cases where the residual activity is relatively high the symptoms are milder and the patients survive longer (Pinsky et al., 1974; Loonen et al., 1974; Wenger et al., 1974, Yamamoto et al., 1974; Orii et al., 1975).

The patient described here is too young to allow conclusions about the further clinical course, but the absence of psychomotor retardation and severe bone deformities as well as the relatively high residual β-galactosidase activity are atypical for G_{M1}-gangliosidosis with such early onset of symptoms.

The results obtained by Galjaard et al. (1975) indicate that two different mutations are involved in the various clinical forms of G_{M1}-gangliosidosis. Our complementation studies described in the present paper indicate that our patient should not be classified as type 1 or type 2 since the gene mutation is not the same as in these forms. The fact that fusion of fibroblasts from our patient with cells from Pinsky's type 3 and the adult type 4 patient does not result in an increase of β-galactosidase activity, could mean that the mutations in these three variants are located in the same gene. It should be awaited whether the two year old boy described in this paper and Pinsky's patient who is now 7 years old, will develop a similar clinical course as the patient with the adult form of G_{M1}-gangliosidosis. (O'Brien, 1975; Galjaard et al., 1975; Galjaard and Reuser, 1977).

The molecular basis of complementation in G_{M1}-gangliosidosis is still unknown. To discriminate between inter-allelic and intergenic complementation, it will be a great help to know the molecular structure of G_{M1}-β-galactosidase. If the conclusion of Norden et al. (1974) is correct that β-galactosidase exists both as a monomer and as an aggregate of identical monomers the increase of β-galactosidase activity in heterokaryons after fusion must be explained by intragenic complementation. This would require polypeptide chains with structural alterations at different sites able to interact in such a way that the hydrolytic activity is restored. When β-galactosidase consists of more than one different subunit the situation might be comparable with that in β-N-acetyl-hexosaminidase deficiency where the appearance of hexosaminidase A after fusion of fibroblasts from patients with Tay-Sachs and Sandhoff's disease most likely results from intergenic complementation (Galjaard et al., 1974; Thomas et al., 1974).

The immunological studies on the fibroblasts from the patient described in this paper provide evidence for a structural mutation because the activity of the mutant enzyme per unit antigenic activity was reduced to 30% whereas the specific β-galactosidase activity in the cell homogenate is 10-20%. These combined data might indicate that the mutant enzyme is more rapidly degraded than the normal enzyme. This has also been described for structural altered β-galactosidase in E. Coli mutants by Zipser et al. (1976). Using the artificial substrate we did not detect any abnormal physico-chemical properties of the mutant enzyme such as described for another variant of G_{M1}-gangliosidosis by Norden and O'Brien (1975). However, similar to these studies we found that nearly

all residual activity consisted of the low molecular (A) form of β-galactosidase (Fig. 3). This might indicate that the structural mutation interferes with the aggregation of monomers to decamers (Norden and O'Brien, 1975). Further studies are in progress to elucidate the mechanism of genetic complementation in order to obtain further insight in the clinical, biochemical and genetic heterogeneity in G_{M1}-gangliosidosis.

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PUBLICATION VI

BIOCHEMICAL, IMMUNOLOGICAL AND CELL GENETIC STUDIES IN GLYCOGENOSIS TYPE II

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ABSTRACT

Fibroblasts from patients with the adult, juvenile and infantile form of glycogenosis type II (M. Pompe) were cultured under standardized conditions and the activity of acid α-glucosidase (EC 3.2.1.20) towards glycogen, maltose and 4-methylumbelliferyl-α-D-glucopyranoside was measured. Also the amount of glycogen in muscle biopsies and in cultured fibroblasts from patients was determined. Residual enzyme activities varying from 7-22% were detected in fibroblasts from patients with the adult form but not in cells from patients with the infantile form of glycogenosis II. An inverse correlation was found between the severity of the clinical manifestation and the degree of residual enzyme activity in the fibroblasts. The kinetic and electrophoretic properties of acid α-glucosidase in fibroblasts from the adult patients and from control individuals were similar. Immunological studies demonstrated that the decrease of acid α-glucosidase activity in all clinical forms of glycogenosis II was attended with a proportional reduction of the total number of enzyme molecules. The results suggest that acid α-glucosidase deficiency is caused by a mutation that affects the production or degradation of the enzyme rather than its catalytic activity. Complementation studies were carried out by fusing fibroblasts from patients with the adult, juvenile and infantile form of glycogenosis II, but conventional assays on multi heterokaryons nor enzyme assays on single binuclear heterokaryons gave any evidence for genetic heterogeneity among these forms.

INTRODUCTION

Glycogenosis type II (Pompe's disease) is an autosomal recessive inherited disorder, characterized by the lysosomal accumulation of glycogen (1,2). The impaired glycogen degradation is due to a deficiency of acid α-glucosidase (EC 3.2.1.20) (3). Several clinical forms of glycogenosis II have been recognized which differ in age of onset, organ involvement and progression of the disease (4-6). In the infantile form (generalized glycogenosis II) nearly all tissues are affected and symptoms become apparent shortly after birth. Hepatosplenomegaly and muscular weakness are present and cardiac failure caused by the extensive accumulation of glycogen usually results in death within the first year of life. In the juvenile form there is no or minimal cardiac involvement, difficulty in walking is generally the first symptom and progressive weakness of the skeletal muscles is the main clinical feature. In patients with the adult form symptoms do not appear until the second or third decade of life and the myopathy, usually beginning in the lower limbs, develops with different degrees of progression. Patients over sixty years have been described (6-8).

During the past decade several investigators have tried to explain how these different clinical forms can result from apparently the same enzyme deficiency. No clear answer could be given but it seems that glycogen accumulation in skeletal muscles of patients with the adult form is less pronounced than in cases of the infantile type (6,8,9). Recently Mehler and Di Mauro (10) found residual acid α-glucosidase activity in muscles from patients with the adult or juvenile form of glycogenosis II but not in the infantile form.

The present paper deals with a study of the level of residual acid α-glucosidase activity in cultured fibroblasts derived from patients with the adult, juvenile and infantile form of glycogenosis II. The kinetic, electrophoretic and immunological properties of the residual α-glucosidase in adult and juvenile cases were studied. Somatic cell hybridization experiments were carried out to investigate whether different gene mutations were responsible for the clinical heterogeneity in glycogenosis II.

MATERIALS AND METHODS

Seven patients with the infantile form of glycogenosis II (code 6-12) and five patients with the juvenile or adult form (code 1-5) have been studied (Table 1).

Cell cultivation and hybridization

Subcultures of fibroblast strains of about the same passage derived from controls and patients were grown in Ham's F10 medium supplemented with 15% fetal calf serum and antibiotics. Ten days after the cultures became confluent, they were trypsinized and 4 x 10^6 cells of each strain were equally devided over four Falcon flasks (25 cm^2). Each following week one of these flasks was harvested and cell homogenates were prepared by sonication in distilled water for measurements of the α-glucosidase activity and the glycogen content. During cultivation the medium was changed three times a week including the day before harvesting. Separate cultures were kept confluent for 15 days and were then used for enzyme kinetic, electrophoretic and immunological studies.

Cell hybridization was carried out by fusing a million cells from each parental strain using inactivated Sendai virus (11). After 2-8 days of subsequent cultivation cell homogenates were prepared and the acid α-glucosidase activity of the mixed population of non fused mononuclear cells and of multiheterokaryons was measured.

Enzyme assays

Acid α-glucosidase assays with 2,2 mM 4-methylumbelliferyl-α-D-glucopyranoside (MUGlu) (Koch-Light) were carried out as described before by incubating 10 µl cell homogenate and 20 µl substrate for 1 hour at 37°C (13). Assays on single binuclear cultured cells were performed by incubating the cells in 0.08 µl of MUGlu substrate and subsequent measurement of the concentration of methylumbelliferone by microscope fluorometry (12). The acid α-glucosidase activity in 10 µl cell homogenate was also measured with maltose (50 µl of 10 mg/ml, Baker) or glycogen (50 µl of 50 mg/ml, GIBCO) as substrate, both dissolved in potassiumphosphate (0,2 M)-citrate (0,1 M) buffer pH 4.4. After incubation and heating for two minutes at 100°C the amount of liberated glucose was determined by adding 200 µl glucose reagent according to the procedure of Lloyd and Whelan, as modified by Koster et al. (14). pH profiles were made by mixing 10 µl cell homogenate, 50 µl substrate, dissolved in water and 10 µl of phosphate-citrate buffer of proper pH. Electrophoresis was carried out at 4°C with cellulose-acetate gels (Cellogel, Chemetron, Italy) in 50 mM potassium phosphate pH 6.8 for two hours, 200V.

Glycogen determination

The amount of glycogen was determined according to a modified procedure of Huijing (15). The cell homogenates were heated for 2 minutes at 100°C. After cooling, a 50 µl aliquot was incubated with 50 µl of a mixture of α-amylase (Diastase Hog Pancreas, Sigma 50 µg/ml) and α-glucosidase (15018 EGBC, Boehringer 1.25 g/ml) in sodium acetate pH 6.0 for one hour. The samples were then heated for 2 minutes at 100°C and the amount of liberated glucose was determined as described above.

Immunological methods

An antiserum against human liver acid α-glucosidase was prepared as described before (16). The IgG fraction was obtained by ammoniumsulphate precipitation and used for the present studies. Crude cell homogenates were diluted with phosphate buffered (10mM pH 6.8) saline (0.9% NaCl) to an α-glucosidase activity of 20-50 n moles MU/hr/ml and 15 µl aliquots were then incubated overnight at 4°C with 15 µl of serially diluted antiserum. The immunecomplexes were spun down at 40,000 x g for 30' and the activity of unprecipitated α-glucosidase was measured in the supernatant using MUGlu substrate.

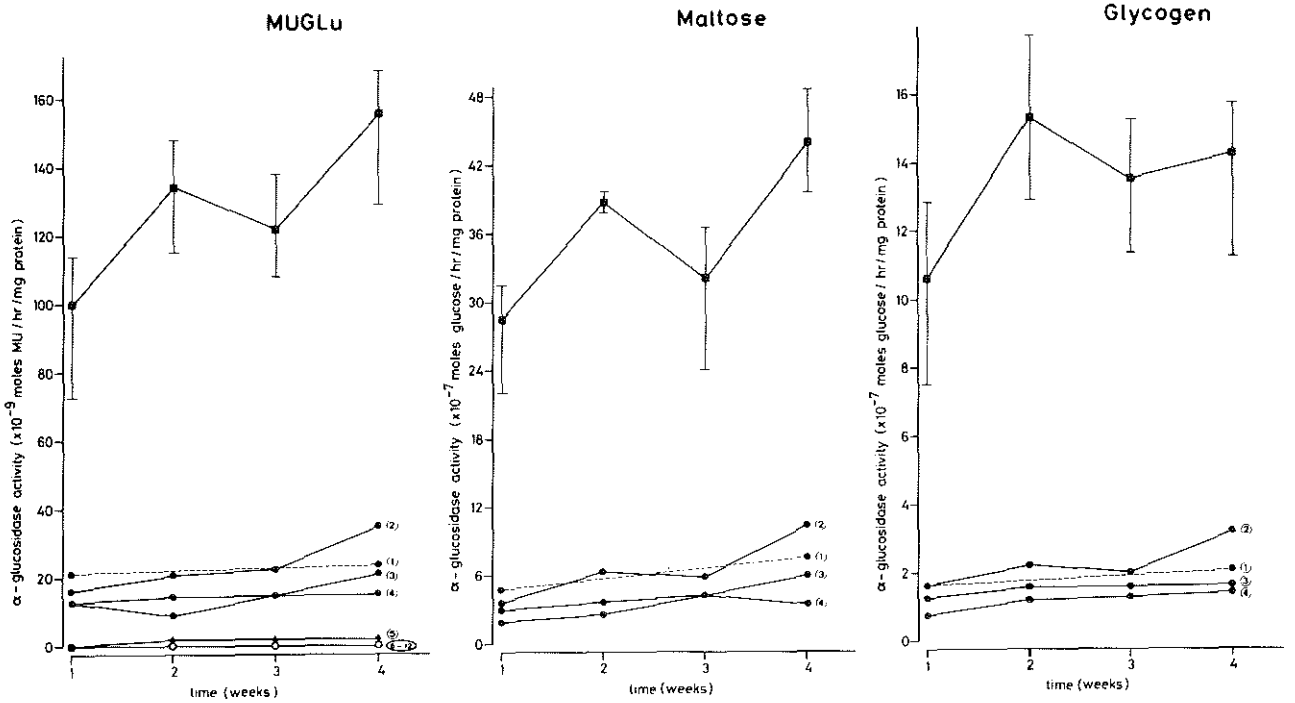

Fig. 1 Acid α-glucosidase activity in fibroblasts after different periods of cultivation. Substrates used were: MUGlu (left), maltose (middle) and glycogen (right). ■—■ mean activity of 4 control strains with indication of extreme values, ●—● adult patients (cases 1-4), ▲—▲ juvenile patient (case 5), O—O infantile patients (6-12)

RESULTS

Cell cultivation conditions were standardized to enable the comparison of acid α-glucosidase activity in fibroblasts from control individuals and from patients with different clinical forms of glycogenosis II. As illustrated in Fig. 1 the specific activity of acid α-glucosidase for each of the substrates increased in fibroblasts from controls and from patients with the late onset form of glycogenosis II during the four weeks period of cultivation. During this period about a doubling of the amount of protein per flask was found. The acid α-glucosidase activity in fibroblasts from patients of the adult type (cases 1 to 4) ranges from 7 to 22 percent of the mean control value, independent of the substrate used (Fig. 1 and Table 1). The activity in the juvenile form

TABLE 1. CLINICAL AND LABORATORY DATA OF PATIENTS WITH VARIOUS FORMS OF GLYCOGENOSIS TYPE II

CODE	TYPE	AGE OF ONSET	AGE	ACTIVITY IN FIBROBLASTS* ACID α-GLUCODIDASE	NEUTRAL α-GLUCOSIDASE	MUSCLE** GLYCOGEN
1	adult	± 35 yr	56 yr	2.1	74	-
2	adult	± 35 yr	48 yr	3.2	62	-
3	adult	± 14 yr	32 yr†	1.6	77	107
4	adult	± 11 yr	26 yr	1.4	59	622
5	juvenile	unknown	18 yr†	<0.2	61	-
6-12	infantile	3 mnths	< 2 yr†	<0.2	66 (n=7)	1300 (n=7)
Controls				14	69 (n=4)	32-79 (n=13)

* Mean activities after 4 weeks cultivation; acid α-glucosidase measured with glycogen as substrate at pH 4.4, expressed as: x 10^{-7} moles glucose/hr/mg protein; neutral α-glucosidase measured with MUGlu as substrate at pH 6.5, expressed as: x 10^{-9} moles MU/hr/mg protein.

** Amount expressed in µg glycogen/mg protein

(case 5) was below the level of detection in the assays with maltose or glycogen but 1 to 2 percent residual activity was found with MUGlu as substrate which method is more sensitive. In cells from all seven patients with the infantile form of glycogenosis II (cases 6 to 12) the activity was less than one percent for all three substrates.

In conclusion, the residual activity of acid α-glucosidase at all periods of cultivation is higher in fibroblasts from patients who reached a more advanced age and the specific activities for maltose, glycogen and MUGlu are always reduced to the same extent.

The mean glycogen content of fibroblasts from patients with glycogenosis II was 0,5 mg glycogen/mg protein, that of normal fibroblasts 0,25 mg/mg. No significant differences in glycogen content were observed among cells from different types of patients. The accumulation of glycogen in skeletal muscle, however, was less in patients with a late onset of symptoms than in patients with the infantile type of glycogenosis II (Table 1).

A pH profile was made of the α-glucosidase activity in the various cell strains, using three different substrates. Fig. 2 shows that the activity of the neutral enzyme is highest towards MUGlu substrate: with maltose the activity is much less and with glycogen its activity is not detectable. The pH profiles further demonstrate that the genetic defect in glycogenosis II only involves the acid α-glucosidase. The activity of neutral α-glucosidase was similar in fibroblasts from the different types of patients and from control individuals (see also Table 1). By comparing the different pH profiles it is concluded that the activity measured at pH 4,4 in fibroblasts from adult patients must be due to residual activity of acid α-glucosidase.

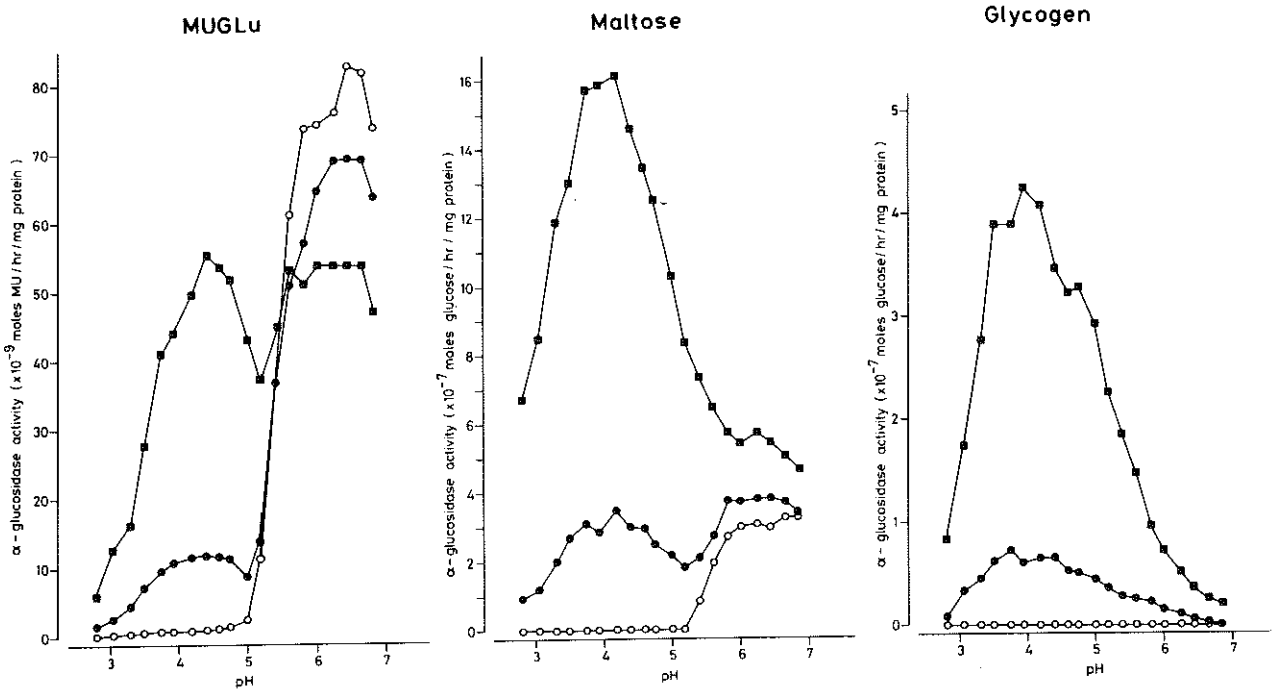

Fig. 2 pH profile of α-glucosidase activity with different substrates. MUGlu (left), maltose (middle) and glycogen (right).
■—■ control, ●—● adult patient, O—O infantile patient

The Km values of acid α-glucosidase did not vary among the different adult patients: for MUGlu the mean Km was 1,1 mM for maltose 11.9 mM and for glycogen 29.7 mg/ml. These values are comparable with those of acid α-glucosidase in control cells.

Also heat inactivation studies did not reveal a different behaviour of the normal and the "mutant" enzyme (Fig. 3).

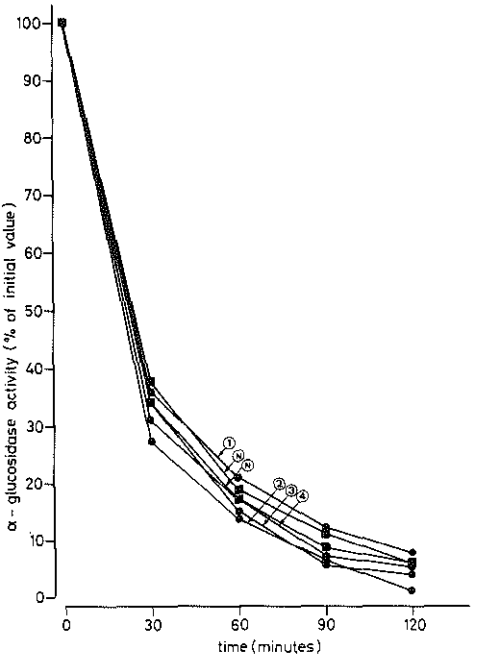

Fig. 3 Heat inactivation of acid α-glucosidase. Experimental conditions: 56°C, 0,2 M sodium acetate pH 4.4, protein concentration in each of the samples 1 mg/ml.
■—■ controls (N), ●—● adult patients. The numbers refer to the cases listed in Table 1.

The electrophoretic pattern of the mutant enzyme in fibroblasts from the adult patients cannot be distinguished from normal acid α-glucosidase (Fig. 4). No band of activity was detected in a cell homogenate from the infantile type.

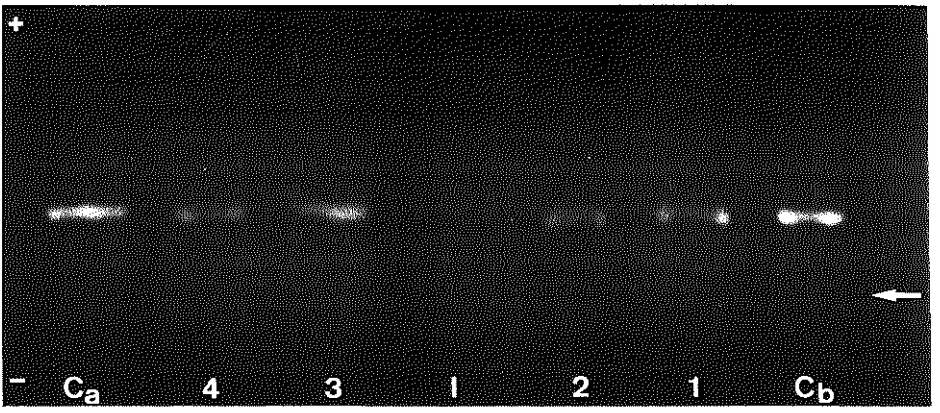

Fig. 4 Cellulose acetate gel electrophoresis of acid α-glucosidase. Ca and Cb = control strains; I = infantile form of glycogenosis type II; 1-4 = adult forms of glycogenosis type II.

The nature of the residual acid α-glucosidase activity in adult patients with glycogenosis II was further investigated immunologically with an antiserum raised against acid α-glucosidase from human liver. This antiserum does not crossreact with the neutral enzyme. Increasing amounts of antiserum were added to a fixed amount of a homogenate of cultured fibroblasts from patients with the adult and juvenile form and from controls. Starting with a comparable enzyme activity the mutant enzyme in all cases precipitates equally well as the normal enzyme (Fig. 5). However, in this situation the protein concentration of the crude homogenate of mutant cells is higher than that of control cells. To equalize both the enzyme activity and the protein concentration of the cell homogenate from control and patients, cell homogenate from a patient with the infantile form was added to that of the control. Under these circumstances normal acid α-glucosi-

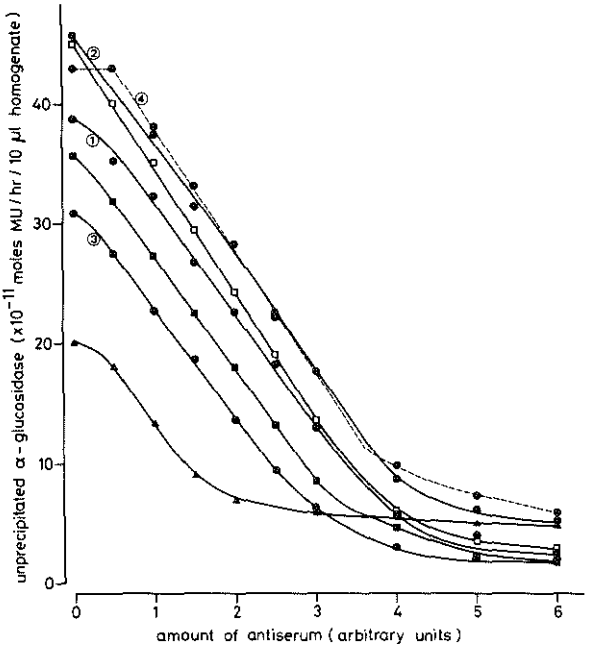

Fig. 5 Immuno precipitation of acid α-glucosidase. Increasing amounts of IgG were added to a fixed amount of crude cell homogenate. ■—■ control fibroblast (protein concentration 1.6 mg/ml). ⊡—⊡ mixture of control cells and cells from a patient with the infantile form of glycogenosis type II (protein conc. 6.1 mg/ml). ●—● adult patients (protein conc. nr.1: 2.6 mg/ml, nr.2: 5.7 mg/ml nr.3: 7.5 mg/ml, nr.4: 10.6 mg/ml). ▲—▲ juvenile patient (protein conc. 15 mg/ml). The numbers refer to the cases listed in Table 1.

dase precipitates only slightly better than the mutant enzyme. The immunological data indicate that the decrease in acid α-glucosidase activity is caused by a reduction of the number of enzyme molecules rather than by a reduced catalytic activity of each molecule. In cultured fibroblasts from patients with the infantile form of glycogenosis II no crossreactive acid α-glucosidase was found.

To investigate whether different gene mutations were involved in the various clinical forms of glycogenosis II complementation studies were performed after somatic cell hybridization. Fibroblasts from patients of the adult, juvenile and infantile type were fused in different combinations using inactivated Sendai virus. The activity of acidα-glucosidase was measured after 2 to 8 days of subsequent cultivation after cell fusion. More than 20 combinations were tested and the activity in each case was measured with MUGlu, maltose and glycogen as substrate. In none of these complementation experiments any increase of acid α-glucosidase activity was observed.

DISCUSSION

The present study shows that there is a significant difference in the level of residual acid α-glucosidase activity in cultured fibroblasts from patients with the adult form and those with the infantile form of glycogenosis II. In cells from the infantile form the activity always remained below 1% of the control values despite prolonged cultivation. In contrast, the activity in fibroblasts from patients with the adult form varied between 7 and 22%. The activity of acid α-glucosidase in the juvenile form (case 5) was very close to that of the infantile cases but no overlap with this latter category was found. It seems as if this inverse correlation between the level of residual activity and the severity of the disease even holds within the group of adult patients. The activity measured at pH 4.4 is not due to the neutral enzyme as was demonstrated by the pH profile as well as by the fact that the activity at this pH was removed by the antiserum raised against purified acid α-glucosidase.

Normal activities of neutral α-glucosidase were found in fibroblasts from all types of patients. It therefore seems unlikely that the neutral enzyme plays a role in the clinical expression of glycogenosis II as was suggested by some investigators (6,17,18).
As far as the characteristics of the residual acid α-glucosidase are concerned, no differences could be detected between the physico-chemical properties of the enzyme in fibroblasts from adult patients with glycogenosis II and controls. Electrophoresis of the mutant enzyme showed one band with the same mobility as normal acid α-glucosidase. The activity of the mutant enzyme for maltose and glycogen was reduced to the same extent. These combined data indicate that the mutation in the late onset form does not directly interfere with the confirmation of the different catalytic centres (19, 20, 23).

Our immunological studies are in support of this view because the amount of cross reacting material was decreased proportionally with the enzyme activity and a normal hydrolytic activity per molecule of enzyme was found. These data suggest that the acid α-glucosidase deficiency is caused by a decreased rate of synthesis of the enzyme or by a structural alteration resulting in an increased rate of degradation. The infantile form of glycogenosis II might represent an extreme example of this situation since hardly any enzymatic activity could be detected in fibroblasts nor any cross reacting immunological material. The latter has also been observed by others (21-23).

The different levels of residual activity in fibroblasts from the adult, juvenile and infantile forms might in principle be the result of different allelic mutations. Our somatic cell hybridization studies did not provide any

evidence for genetic heterogeneity. No increase in acid α-glucosidase activity was found after fusion of cells from different clinical variants nor in biochemical analyses on cell homogenates containing non fused mononuclear cells and multi heterokaryons neither in microchemical assays on single binuclear cells (12). In other lysosomal storage diseases complementation analysis has proven to be useful in demonstrating different gene mutations (11,24,25).

If the finding of residual α-glucosidase activity in fibroblasts from all patients with the adult type of glycogenosis II tested might be extrapolated to the activity in skeletal muscle, this would explain why accumulation of glycogen in this tissue is less severe in the adult form than in the infantile form (Table 1)(6,8-10). More investigations on the α-glucosidase activity in muscle biopsies from patients with late onset forms are required to allow definite conclusions. Cardiac involvement seems to be a quantitative rather than a qualitative difference between the adult and infantile form since patients have been described where the heart was "slightly" involved or where cardiac symptoms gradually developed with increasing age (7,26,27). In fibroblasts the glycogen accumulation was found to be similar in various types of glycogenosis II despite the different levels of acid α-glucosidase activity. However, the extent of glycogen storage in vivo, in various tissues, will not only depend on acid α-glucosidase activity but also on carbohydrate supply, turnover of extra lysosomal glycogen, hormonal control and rate of cell renewal (28). The cultured fibroblast seems in this respect not a suitable model for studying glycogen storage but it provides a proper tool for the biochemical and genetic analysis of the normal and the mutant enzyme.

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PUBLICATION VII

METHODS FOR ANALYSIS OF ACID α-1,4-GLUCOSIDASE ACTIVITY IN SINGLE (HYBRID) CELLS[1]

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Two methods are described which allow the quantitative assay of the lysosomal enzyme α-1,4-glucosidase in single fibroblasts. In the first procedure the substrate was maltose, and liberated glucose was measured with an enzymatic cycling procedure for reduced nicotinamide adenine dinucleotide phosphate. Single cultured fibroblasts were found to have enzyme activities in the range of 0.5–10 × 10^{-13} moles glucose/hr. In the second procedure the artificial substrate 4-methylumbelliferyl-α-D-glucopyranoside was used. It is hydrolyzed in a single step reaction to the fluorescent product 4-methylumbelliferone (MU). By reducing the incubation volume and by measuring the fluorescence in microdroplets with a microscope fluorometer, a sensitivity of 10^{-14} moles MU could be obtained. Activities were found ranging from 0.5–10 × 10^{-14} moles MU/hr/cell. Both procedures for single cell analysis proved to be reliable when compared with conventional assays on cell homogenates. Cocultivation and cell fusion studies were performed to demonstrate that these methods can be used to study the metabolic and genetic interaction between normal and enzyme-deficient fibroblasts derived from patients with glycogenosis II.

The biochemical analysis of lysosomal enzymes in cultured cells has received increasing attention in the last few years. Prenatal diagnosis of several lysosomal storage diseases is possible in early pregnancy by assay of enzyme activities in cultured amniotic fluid cells (24, 26, 28). Enzyme assays in cultured cells have become an important tool in basic studies in cell biology and somatic cell genetics. Complementation analyses after somatic cell hybridization have demonstrated the differences in genetic background of different clinical variants of hereditary metabolic defects (6, 22, 27, 31, 32). The phenomenon of metabolic correction of enzyme-deficient cells in culture by uptake of enzyme secreted by normal cells in cocultivation studies or by uptake of partially purified enzymes from other sources has received increasing attention in connection with enzyme therapy (1, 3, 9, 14, 33).

Conventional biochemical assays on cell homogenates do not provide data on the different populations of cells which may be present in the same culture. Biochemical information from individual cells selected from the mixed population would be of great interest. Analysis of individual cells can be accomplished either by using specific cytochemical staining methods (17, 18, 30) or by increasing the sensitivity of the analytical procedures so that the activities of single cells can be measured (4, 7, 15, 34).

The present paper describes two methods for the quantitative assay of the lysosomal enzyme acid α-1,4-glucosidase in single human fibroblasts. These procedures were developed to investigate the genetic background of different clinical variants of glycogenosis II (2, 12) and to permit studies on the metabolic interaction between normal and enzyme-deficient fibroblasts in culture. Some examples of application in these fields are presented.

MATERIALS AND METHODS

Cell cultivation:

General procedures: Fibroblasts from normal individuals and from patients with glycogenosis II were grown in culture flasks in Ham's F-10 medium supplemented with 15% fetal calf serum and antibiotics (100 μg of streptomycin and 100 units of penicillin/ml). Ten days after the last subculture the cells were trypsinized and cell homogenates were prepared for enzyme assays. A small number of cells was reseeded in Petri dishes with a thin plastic foil bottom (Mylar dishes) at a cell density of 7000 cells/cm^2. After 1 more day of cultivation the medium was poured off, the

[1] This study was supported by the Netherlands Foundation for Fundamental Medical Research (FUNGO) with financial aid from the Netherlands Organization for Pure Scientific Research (Z. W. O.).

dishes were rinsed twice with 0.9% NaCl, quickly frozen in liquid nitrogen and freeze-dried *in vacuo* at −20°C.

Single cells were isolated by microdissection according to the method of Galjaard *et al.* (7).

Cocultivation: Metabolic cooperation was studied by mixing equal numbers of normal fibroblasts and α-glucosidase-deficient fibroblasts from a patient with glycogenosis II. After 10 days of cocultivation the mixed cultures were trypsinized. Homogenates were made and part of the cells was reseeded in low density in Mylar dishes. After 24–30 hr of subsequent cultivation, individual fibroblasts were dissected at random and analyzed for α-glucosidase activity.

Cell fusion: For complementation studies cells were hybridized using inactivated Sendai virus (125 hemagglutinating units/10^6 cells), according to the method of Harris and Watkins (11). Single binuclear cells were isolated 48 hr after fusion as described by Galjaard *et al.* (7).

Biochemical assays:

Maltose: The activity of acid α-1,4-glucosidase in cell homogenates was determined with either 10 *mM* maltose as substrate according to Nitowsky and Grunfeld (29) or 2.2 *mM* 4-methylumbelliferyl-α-D-glucopyranoside as described by Galjaard *et al.* (8). The protein content of the samples was measured following the method of Lowry *et al.* (21).

For the single cell assay with maltose as substrate, enzymatic cycling was carried out with the oil well technique as described by Lowry *et al.* (19, 20). A modification of the procedure of Matschinsky *et al.* (23) was used for the determination of glucose. The procedure is summarized in detail in Table I. The reaction mixture was transferred from the oil well to a 25-μl Drummond glass capillary and reduced nicotinamide adenine dinucleotide phosphate (NADPH) concentration was measured with a Leitz microfluorometer as described by Galjaard *et al.* (7).

4-Methylumbelliferyl-α-D-glucopyranoside: Incubations performed with 4-methylumbelliferyl substrate were carried out on a Teflon foil in microdroplets which were covered with oil (40% hexadecane: 60% paraffin oil) to prevent evaporation. After addition of carbonate buffer, pH 10.7, the fluorescence intensity of 4-methylumbelliferone was measured directly with a Leitz inverted type of microfluorometer with Ploemopak epi-illumination, according to Jongkind *et al.* (15).

Further details of both procedures will be given in Results.

In all single cell assays empty pieces of Mylar were dissected and analyzed in the same way to serve as blanks.

Mean blank values were calculated from about 20 pieces. The enzyme activities were calculated from standard curves for glucose and methylumbelliferone after subtraction of the mean blank. Standard solutions were prepared by weighing.

All purified enzymes, coenzymes and the substrate glucose 6-phosphate were obtained from Boehringer; D-glucose was from BDH. Maltose (maximum, 0.1% w/w glucose) was obtained from Baker. Before use, the glucose content was reduced to 0.003% w/w by treatment with glucose oxidase. Bovine serum albumin and α-ketoglutaric acid were obtained from Sigma; 4-methylumbelliferyl-α-D-glucopyranoside and 4-methylumbelliferone were from Koch Light.

RESULTS

Methodology of acid α-1,4-glucosidase assay in single cultured cells:

Maltose as a substrate: The α-1,4-glucosidase activity of single human fibroblasts varies from 1–10 × 10^{-13} moles glucose/hr when maltose is used as substrate. This amount of glucose is far too low to detect with conventional methods and just below the sensitivity of microfluorometric procedures. Therefore, enzymatic cycling was used to assay the α-glucosidase activity of single cells. Detailed conditions are summarized in Table I. Under the conditions used, the amplification achieved by cycling is about 15,000-fold.

Because of the low enzymatic activity of single fibroblasts, even minor contamination of maltose with glucose causes unreliable assays. The glucose content of commercial maltose was lowered to 0.003% w/w by treatment with glucose oxidase. With an incubation volume of 62 nl, the amount of glucose present as impurity is 0.36 × 10^{-13} moles.

To test the reliability of the method, different amounts of glucose were dissolved in 62 nl acetate buffer containing 10 *mM* maltose. The results in Figure 1A show a linear relationship between the amount of glucose in the sample, ranging from 0.5–20 × 10^{-13} moles, and the fluorescence intensity of the final amount of NADPH. The relatively high blank values were due to glucose impurity in the maltose and possible residual NADP after step four of the assay procedure.

In several experiments single cells were isolated from a culture of normal human fibroblasts and their acid α-glucosidase activity was measured. An example of such an experiment is illustrated in Figure 1B. The fluorescence intensities of the blanks, consisting of empty pieces of plastic foil dissected at random from the culture dishes, are indicated in the figure. The enzyme activities of single fibroblasts, as presented in Figure 1B, were calculated from the

TABLE I

Detailed Conditions of Acid α-1,4-Glucosidase Analysis, Using Maltose as a Substrate, followed by NADPH Cycling[a]

Step	Volume	Buffer	Substrate	Cofactors	Enzymes	Additions	Time	Temperature
	μl							°C
1	0.062	10 *mM* NaAc-HAc, pH 4.3	Maltose, 10 *mM*			BSA,[b] 0.02%	2 hr	37
2							3 min	90
3	0.4	25 *mM* Tris-HCl, pH 7.6		1 *mM* $MgCl_2$ 0.2 *mM* ATP 0.02 *mM* NADP	5 μg HEX 0.35 μg G6PDH/ml	0.5 *mM* Dithiothreitol BSA, 0.04%	45 min	25
4	0.5					0.2 *M* NaOH	20 min	80
5	3.1	0.1 *M* Tris-HCl, pH 8.0	5 *mM* G6P 5 *mM* α-Ketoglutaric acid	0.1 *mM* ADP	0.2 mg GDH 0.02 mg G6PDH/ml	10 *mM* NH_4-Ac BSA, 0.02%	1 hr	37
6							10 min	90
7	5.5	40 *mM* Tris-HCl, pH 7.6		2 *mM* NADP	0.04 mg 6PGDH/ml	0.1 *mM* EDTA	45 min	25

[a] The scheme is based on the procedures described by Matschinsky (23).

[b] BSA, bovine serum albumin; ATP, adenosine triphosphate; G6P, glucose 6-phosphate; ADP, adenosine diphosphate; HEX, hexokinase; EDTA, ethylenediaminetetraacetate; GDH, glutamate dehydrogenase; G6PDH, glucose-6-phosphate dehydrogenase; 6PGDH, 6-phospho-gluconate dehydrogenase.

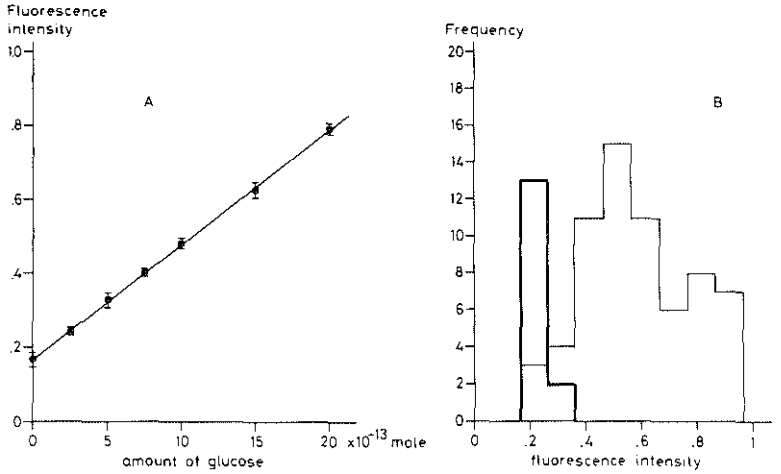

FIG. 1. A, standard curve of glucose. Different amounts of glucose were dissolved in 0.06 μl of the maltose substrate mixture. Fluorescence intensity of NADPH was measured, after enzymatic cycling, in a volume of about 10 μl. Intensities are given in arbitrary units. Each point represents the mean value of five different samples. B, frequency distribution of acid α-1,4-glucosidase activity in single fibroblasts, as measured with maltose as substrate. Fluorescence intensities are given in arbitrary units without subtraction of the mean blank. One unit corresponds with an activity of 16×10^{-13} moles/hr/cell. Mean activity of 65 cells, 5.6×10^{-13} moles glucose/hr/cell. ——, blanks; ——, normal fibroblasts.

glucose standard curve after subtraction of the mean blank value. The mean activity of the cells in this experiment was 5.6×10^{-13} moles glucose/hr/cell. As shown there is a considerable variation in enzyme activity among the individual cells which had been dissected at random from the same dish. Acid α-glucosidase activity in the cell homogenate prepared from the trypsinized cells that were reseeded for single cell analysis was found to be 4.3×10^{-13} moles glucose/hr/cell.

Methylumbelliferyl glycoside as a substrate: Under the conditions used the activity of acid α-1,4-glucosidase is about 10 times lower for this artificial substrate than for maltose. Still a high sensitivity can be obtained because of the strong fluorescence of the reaction product 4-methylumbelliferone (MU). The enzyme activity of a single cultured fibroblast is $1\text{–}10 \times 10^{-14}$ moles MU/hr. This small amount of MU cannot be detected with a conventional fluorometer, but by reducing the final volume the concentration of MU was increased and the fluorescence could be measured accurately in submicroliter volumes with a microscope fluorometer. The best results were obtained by incubating the cells for 2 hr at 37°C in 0.06 μl of 2.2 *mM* methylumbelliferyl substrate in 0.2 *M* acetate buffer, pH 4.3. Albumin was added to prevent surface denaturation at a concentration of 0.02%.

Microdroplets of substrate covered with oil were incubated on sheets of thin plastic foil and the reaction was stopped by the addition of 0.3 μl of 0.5 *M* carbonate buffer, pH 10.7. The fluorescence of the released MU was measured in the same microdroplets using an inverted microscope fluorometer with epi-illumination according to Jongkind *et al.* (15).

A standard curve of different amounts of MU which were dissolved in the reaction mixture is illustrated in Figure 2A. A linear relationship was found between the amount of MU and fluorescence intensity in the range of $1\text{–}20 \times 10^{-14}$ moles MU. The blank value is due to slight fluorescence of the methylumbelliferyl substrate, spontaneous hydrolysis of the substrate and aspecific fluorescence of the albumin added to the reaction mixture. Figure 2B shows the fluorescence intensities of randomly dissected normal human fibroblasts. Again a considerable variation in activity among the indi-

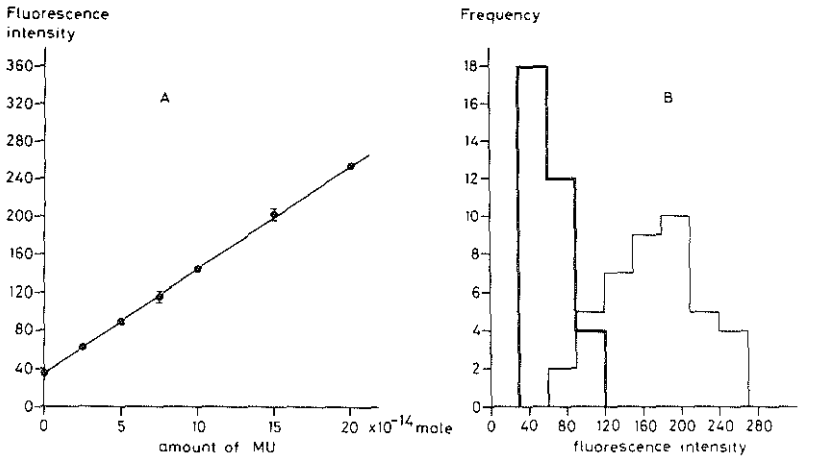

FIG. 2. A, standard curve of methylumbelliferone (MU). Different amounts of MU were dissolved in the 4-methylumbelliferyl substrate mixture. Each point represents the mean value of five different samples. B, frequency distribution of acid α-1,4-glucosidase activity in single fibroblasts, as measured with methylumbelliferyl substrate. Fluorescence intensities are given in arbitrary units without subtraction of the mean blank. Forty units correspond with an activity of 1.83×10^{-14} moles/hr/cell. Mean activity of 45 cells, 4.6×10^{-14} moles MU/hr/cell. —— , blanks; ——, normal fibroblasts.

vidual cells was found. Activities were calculated using the MU standard curve after subtraction of the mean blank (empty pieces of plastic foil). The mean α-glucosidase activity of more than 40 cells was 4.6×10^{-14} moles MU/hr/cell. The enzyme activity calculated from the cell homogenate was 6.4×10^{-14} moles/hr/cell.

Examples of application in metabolic and genetic interaction of cultured fibroblasts:

Metabolic interaction: To study the metabolic interaction between normal and α-glucosidase-deficient cells, confluent cultures were initiated with equal numbers of fibroblasts derived from normal individuals and patients with glycogenosis II and cultivated during different time intervals. Analysis of single cells was performed by harvesting the mixed confluent cultures with trypsin and reseeding on Mylar dishes. Single cells were isolated after 1 day more in culture as described in Materials and Methods. Acid α-glucosidase activity was measured using methylumbelliferyl substrate. Figure 3 illustrates the results of such an experiment in which normal and enzyme-deficient fibroblasts had been cocultivated for 10 days. Enzyme assays were also performed on single cells of both types cultured separately under similar conditions. No enzyme activity could be detected in individual cells from a patient with glycogenosis II (Fig. 3B), as is shown by the fact that the fluorescence intensities of these cells are similar to those of the blanks (Fig. 3A). The α-glucosidase activity of normal fibroblasts shows a considerable variation. After a period of 10 days of cocultivation in confluent growth and 1 day of subsequent cultivation in a Mylar dish, single cells were selected at random from the mixed population. Figure 3C shows that the frequency distribution of enzyme activities was not significantly different from distributions derived from both parental strains. Even after 10 days of cocultivation, both enzyme-deficient cells and cells with normal α-glucosidase activity were present, indicating that no intercellular exchange of acid α-glucosidase had occurred from normal to enzyme-deficient fibroblasts.

Genetic interaction: Enzyme assays on single cultured cells are also of great potential interest in somatic cell hybridization studies. Normal human fibroblasts as well as fibroblasts derived from two patients with clinical variant types of glycogenosis II (infantile and adult) were hybridized by using Sendai virus and binuclear

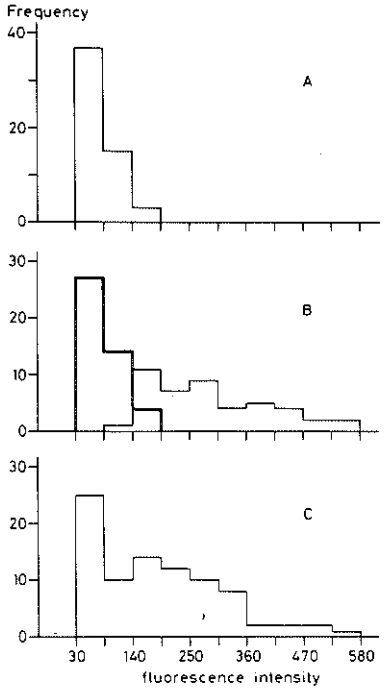

FIG. 3. Cocultivation of normal and enzyme-deficient fibroblasts. Fluorescence intensities are expressed in arbitrary units. A, frequency distribution of blanks consisting of pieces of plastic foil. B, frequency distribution of acid α-1,4-glucosidase activity in single fibroblasts. —, normal human fibroblasts; ——, fibroblasts derived from a patient with glycogenosis II. C, frequency distribution of single human fibroblasts isolated at random from a mixed population of normal and enzyme-deficient cells.

and mononuclear cells were isolated at random from the same Mylar dish. Their acid α-glucosidase activity was measured with methylumbelliferyl substrate (Fig. 4). As a control, normal human fibroblasts were fused and mononuclear nonfused cells as well as binuclear hybrid cells were dissected. The frequency distributions of these two cell types are shown in Figure 4A and B. The α-glucosidase activity per cell is about twice as high in the binuclear cells as in mononuclear cells. Finally, the result of a fusion of fibroblasts derived from patients with the adult and infantile type of glycogenosis II is illustrated in Figure 4C. No significant levels of enzyme activity could be demonstrated in binuclear cells. Thus no genetic complementation occurs after hybridization of these two different cell strains.

DISCUSSION

Both precedures described here make possible the quantitative analysis of the acid α-glucosidase activity in single cultured fibroblasts. From a practical point of view, the

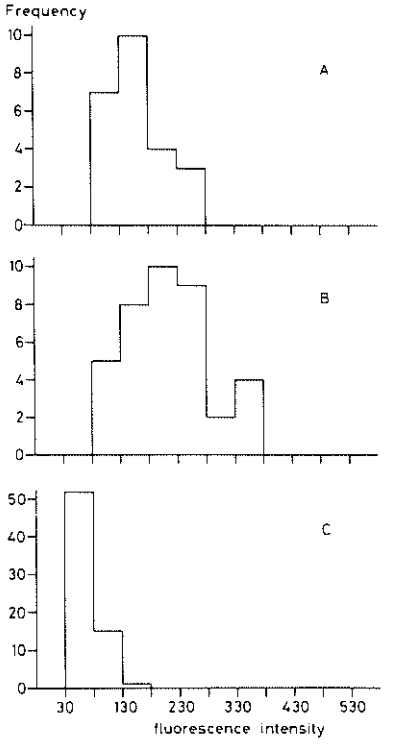

FIG. 4. Frequency distribution of acid α-1,4-glucosidase activity in mono- and binuclear human fibroblasts isolated at random after cell fusion. Fluorescence intensity is given in arbitrary units, without subtraction of the mean blank. Fifty units correspond with an activity of 3×10^{-14} moles/hr/cell. A, mononuclear normal human fibroblasts. Mean activity of 24 cells, 3.3×10^{-14} moles MU/hr/cell. B, binuclear normal human fibroblasts from the same population. Mean activity of 39 cells, 5.8×10^{-14} mole/hr/cell. C, binuclear cells isolated at random after fusion of fibroblasts derived from two patients with different variants of glycogenosis II. No enzyme activity could be detected after subtraction of the mean blank.

two-step procedure with the artificial substrate 4-methylumbelliferyl-α-D-glucopyranoside is advantageous. Especially in experiments where large numbers of cells have to be analyzed, the enzyme assay with maltose as substrate is much more time consuming.

On the other hand, use of the natural substrate is to be preferred in studies on the relationship between a specific enzyme deficiency and the resulting pathologic and clinical manifestations in patients with inherited metabolic disorders. The cycling procedure gives a somewhat better separation between blank values and enzyme activities found in single cells. Theoretically, the sensitivity of the cycling procedure can be further improved by using a higher cycling rate or by double cycling (19). The sensitivity of the methylumbelliferyl method can only be improved by decreasing the incubation volume and the final volume in which the fluorescence intensity is measured (7, 8, 15, 34).

A disadvantage of the method, described by Wudl and Paigen (34), is the lack of visual control during isolation of the cells. The isolation procedure used in the present study allows the selection of binuclear cells in order to investigate the quantitative aspects of genetic complementation.

The relatively large variation in α-glucosidase activity of single cultured fibroblasts observed with both assay procedures may be due in part to differences in total dry weight of the cells. Such differences have been observed by interference microscopic measurements on individual fibroblasts within the same culture (7, 16).

The reliability of both methods described is illustrated by a reasonable correspondence between the mean activities obtained in single cell assays and the activities as measured in cell homogenates of the same cell strain. The cell cultivation conditions for enzyme assay on single cells and cell homogenates are not the same, and it is well known that the activity of various lysosomal enzymes is affected by cell cultivation conditions (10). The enzyme activity of single cells may also vary during the cell cycle (25).

The ability to perform quantitative assays on single cultured fibroblasts allows new experimental approaches to the study of metabolic and genetic interaction between different cell types within the same culture. The finding that no exchange of acid α-1,4-glucosidase occurred after 10 days of cocultivation of normal and enzyme-deficient fibroblasts could not have been established by conventional biochemical assays on homogenates of these mixed cell populations. This result is inconsistent with the hypothesis of Hickman and Neufeld (13) about secretion and subsequent uptake of lysosomal enzymes via specific recognition sites, at least for acid α-1,4-glucosidase. An alternative explanation is that the enzyme deficiency in glycogenosis II is caused by a mutation in a specific cellular membrane component which prevents uptake of the enzyme. Further, the exchange of the lysosomal enzyme β-galactosidase between normal and enzyme-deficient cells in similar cocultivation experiments (9) could not be demonstrated.

The results of α-glucosidase assays on single binuclear cells after hybridization of two different fibroblast strains show the feasibility of this approach in genetic complementation studies. Complementation has been demonstrated after hybridization of fibroblasts from clinically different patients with xeroderma pigmentosum (32), maple syrup urine disease (22) and GM2-gangliosidosis (6, 31). Recently, different gene mutations were found by complementation studies with different clinical variants of GM1-gangliosidosis with the cell hybridization technique (5). All of these complementation studies, except that on xeroderma pigmentosum, were carried out with cell homogenates and therefore did not permit any conclusions about the quantitative aspects of complementation in these hybrid cells. Since there is no selective system for human hybrid cells, techniques for single cell analysis such as described in this paper seem to be the only means to provide such data. These techniques are likely to contribute to other investigations where quantitative biochemical analysis of different cell types within the same culture is required.

ACKNOWLEDGMENTS

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PUBLICATION VIII

INTERCELLULAR EXCHANGE OF LYSOSOMAL ENZYMES: ENZYME ASSAYS IN SINGLE HUMAN FIBROBLASTS AFTER CO-CULTIVATION

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SUMMARY

Intercellular exchange of N-acetyl-β-D-glucosaminidase (EC 3.2.1.30) β-galactosidase (EC 3.2.1.23) and acid α-glucosidase (EC 3.2.1.20) was studied after co-cultivation of normal and enzyme deficient human fibroblasts in confluent cultures. Enzyme activities were measured in single cells using microchemical procedures. After co-cultivation of normal control fibroblasts and those from a patient with Sandhoff's disease an increase of activity of N-acetyl-β-D-glucosaminidase was found in Sandhoff cells, together with a decrease of activity in normal control cells. After co-cultivation of normal fibroblasts and those from patients with glycogenosis II and GM1-gangliosidosis, no indication was found for intercellular transfer of acid α-glucosidase and β-galactosidase respectively. The significance of the results is discussed in respect of the hypothesis of Hickman and Neufeld about secretion and uptake of lysosomal enzymes.

INTRODUCTION

Cultures of human fibroblasts have proven to be a useful model system for the study of lysosomal storage diseases. Correction of the metabolic defect by administration of enzyme preparations to fibroblasts in culture was reported in several studies (1-7). It has been suggested that enzyme replacement might be a feasible therapy in lysosomal storage diseases.

A more fundamental interest in uptake and secretion of lysosomal enzymes was raised in studies with cultured cells from patients with I-cell disease. For several acid hydrolases increased levels of activity were found in the medium of cultured cells from these patients, together with decreased levels of intracellular activities

(8, 9). Hickman and Neufeld suggested that lysosomal enzymes have to be secreted and subsequently are taken up via specific recognition sites in order to reach their lysosomal destination. I-cell disease would be the result of a defective uptake. To investigate the general validity of this hypothesis we have studied the intercellular exchange of three acid hydrolases in mixed confluent cultures of normal and enzyme deficient cells. In this way administration of enzyme preparations was avoided whereas the conditions for transfer of enzyme were optimal by close contact of donor and acceptor cells. The exchange of N-acetyl-β-D-glucosaminidase (further to be referred as hexosaminidase), β-galactosidase and acid α-glucosidase was studied using fibroblasts from patients with deficiencies for these enzymes as acceptor cells. Enzyme activities were measured in single cells after co-cultivation with normal cells.

METHODS

Cell culture and isolation: Cells were cultured in Ham's F10 medium supplemented with 15% fetal calf serum and antibiotics. In addition to fibroblasts from control subjects fibroblasts were cultured from patients with Sandhoff's disease (deficient in both lysosomal forms of hexosaminidase), GM1-gangliosidosis type 1 (deficient in lysosomal β-galactosidase) and glycogenosis II (deficient in lysosomal α-glucosidase). Preceeding co-cultivation cells from different strains were labeled with either latex or carbon particles. Suspensions of latex particles were obtained from Serva (Dow latex, 0.79 μ) and were added at a concentration of 0.02 ml to 2×10^6 cells in 10 ml culture medium. Suspensions of carbon particles were obtained from George T Gurr (Indian Ink, cat no 51400) and were added at a concentration of 0.1 ml. The medium was removed after one day culturing, the cells were washed three times with 0.9% NaCl and trypsinized. Equal numbers of normal and enzyme deficient cells were mixed and confluent cultures were initiated. Growth in confluency was allowed for one to ten days. The cultures were trypsinized after this period of co-cultivation and reseeded in low density in petri dishes with a thin plastic foil (mylar) bottom, to enable single cell analysis. After cultivation for 20 hrs. differently labeled cells were identified with phase contrast microscopy and localized using a micro grid. Cultures were then freeze dried, whereupon preselected cells could be isolated by microdissection as described by Galjaard et al. (11).

Enzyme assays: 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside, 4-methylumbelliferyl-β-D-galactopyranoside and 4-methylumbelliferyl-α-D-

glucopyranoside were used as substrates for the determination of hexosaminidase, β-galactosidase and α-glucosidase activity respectively. Detailed conditions for the assay of β-galactosidase are described by Galjaard et al (12). A method for the single cell analysis of α-glucosidase is described by Reuser et al (13). To assay hexosaminidase activity single fibroblasts were incubated in 0.1 µl phosphate (20 mM)-citrate (10 mM) buffer pH 4.5 containing 5 mM substrate and 0.02%, heat inactivated, bovine serum albumin. The reaction was allowed to proceed for one hour at 37°C and stopped by the addition of 1 µl, 0.5 M carbonate buffer pH 10.7. Fluorescence was measured in glass capilleries as described for β-galactosidase. Enzyme activities were calculated using a standard curve of methylumbelliferone (MU). Empty pieces of plastic foil were dissected to serve as blanks.

RESULTS

Normal control fibroblasts and enzyme deficient cells were labeled with either latex or carbon particles. Control experiments showed that marker material did not influence the enzyme activities measured. In several experiments equal numbers of fibroblasts from a control subject and from a patient with Sandhoff's disease lacking both lysosomal forms of hexosaminidase were mixed and co-cultivated in confluent cultures for various time periods. As a control both cell lines were cultured separately for the same period. In the experiment described, the cells were co-cultivated for ten days as a confluent culture and then reseeded in low density in petri dishes. After a subsequent cultivation for 20 hours the cells were localized, freeze dried and isolated selectively as described in Methods. Hexosaminidase activity was assayed in single cells of both types.

Fig. 1A shows the frequency distribution of hexosaminidase activity in fibroblasts from control subject and patient when cultured separately. Low residual activity was present in Sandhoff cells (mean activity 0.06×10^{-12} moles MU per hr) whereas in normal fibroblasts the mean activity was 4.6×10^{-12} moles MU per hr. After ten days of co-cultivation marked changes were noticed in the distributions as shown in Fig. 1B. The mean activity of Sandhoff cells was increased to 0.7×10^{-12}

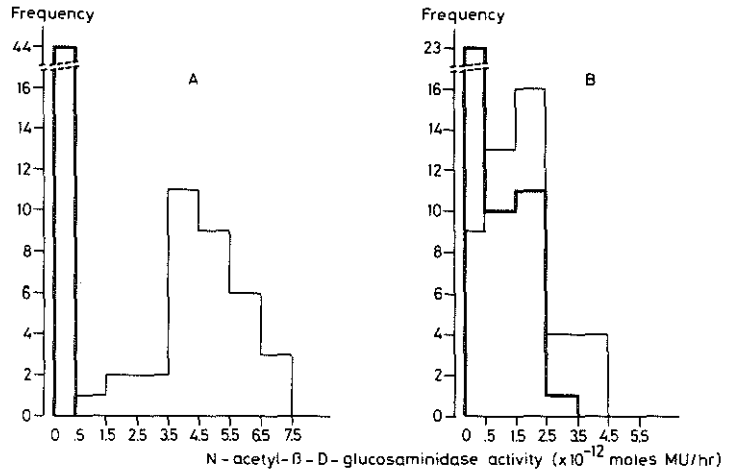

Fig. 1. Frequency distributions of hexosaminidase activity in single human fibroblasts derived from a control subject and from a patient with Sandhoff's disease.
A. Separate cultivation
B. Co-cultivation for 10 days in confluency
— patient — control

moles MU per hr whereas the mean activity of control cells was decreased to 1.7×10^{-12} moles MU per hr. The same effect was found after three days of co-cultivation although less prominent.

In similar experiments exchange of β-galactosidase was studied. Fibroblasts from a patient with GM1-gangliosidosis, lacking lysosomal β-galactosidase, were co-cultivated with normal human fibroblasts in equal numbers for eight days. The frequency distributions of enzyme activity for both types of cells when cultured separately are given in Fig. 2A. Distributions after co-cultivation are shown in Fig. 2B. In neither case β-galactosidase activity could be demonstrated in fibroblasts from the patient. The mean activity of normal cells was 1.36×10^{-13} and 1.38×10^{-13} moles MU per hr. respectively. This indicates that no intercellular exchange of β-galactosidase occurred.

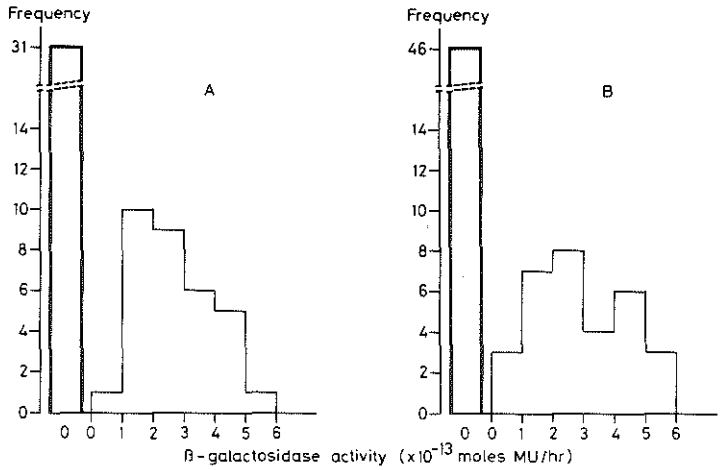

Fig. 2. Frequency distributions of β-galactosidase activity in single human fibroblasts derived from a control subject and from a patient with GM1 gangliosidosis.
A. Separate cultivation
B. Co-cultivation for 8 days in confluency
▬ patient — control

Fibroblasts from a patient with glycogenosis II lacking acid α-glucosidase were used as acceptor cells to study exchange of this enzyme. The experiments were done in a similar way as described above. Cells were co-cultivated for seven days. The frequency distributions of α-glucosidase activity of normal and deficient cells in separate and mixed cultures are shown in Fig. 3A and 3B respectively.

No enzyme activity could be demonstrated in fibroblasts from the patient with glycogenosis II. The mean activity of normal cells was 4.7×10^{-14} moles MU per hr. when cultured alone and 3.9×10^{-14} after co-cultivation.

No transfer of acid α-glucosidase activity from normal to deficient cells was found.

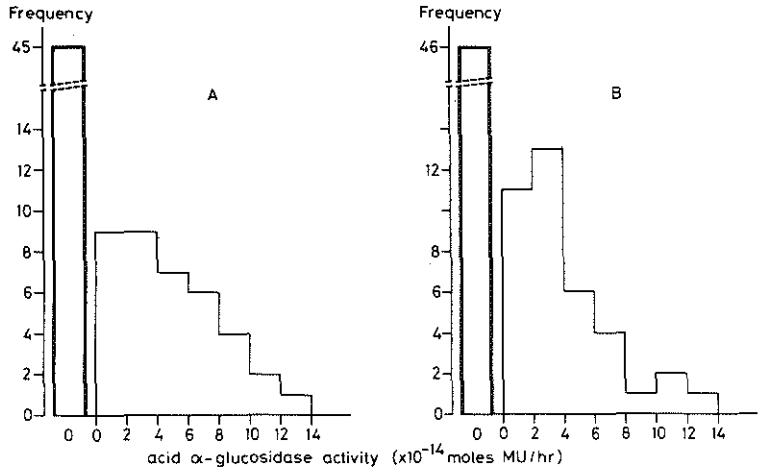

Fig. 3. Frequency distributions of acid α-glucosidase activity in single human fibroblasts, derived from a control subject and from a patient with glycogenosis II.
A. Separate cultivation
B. Co-cultivation for 7 days in confluency
▬ patient — control

DISCUSSION

Selective and bulk pinocytosis are distinguished for uptake of extracellular material. The former process requires specific recognition sites on the cell surface as well as on the molecule that is taken up (14). According to the hypothesis of Hickman and Neufeld (10) secretion followed by selective uptake is essential for packaging of lysosomal enzymes. This implies the possibility of transfer of enzyme from one cell to another. Mutual correction of the metabolic defect in cultured fibroblasts from clinically different patients with mucopolysaccharidosis was demonstrated to be the result of free exchange of lysosomal enzymes (1, 2, 3). The hypothesis of Hickman and Neufeld is mainly based on studies with I-cells and the authors indicate

that the theory might not apply to all lysosomal enzymes. Strong evidence has been presented for the uptake of hexosaminidase via specific recognition sites (15).

We have studied exchange of hexosaminidase, β-galactosidase and α-glucosidase by co-cultivation of normal control and enzyme deficient cells in confluent cultures thereby trying to approach the in vivo situation, avoiding the administration of enzymes in quantities far exceeding the intracellular activity.

The clear transfer of hexosaminidase activity from normal cells to enzyme deficient cells fits in with the Hickman and Neufeld hypothesis. Interestingly the increase in hexosaminidase activity in Sandhoff cells, as a result of co-cultivation, is accompanied by a loss of activity in normal fibroblasts. No compensation seems to occur for the loss of intracellular activity from normal cells.

No transfer of β-galactosidase or α-glucosidase activity could be demonstrated in our experiments. This might indicate that for these enzymes the packaging into lysosomes is different compared to hexosaminidase. In this case the simultaneous lack of β-galactosidase and hexosaminidase in I-cell disease can not be explained by a mutation affecting recognition sites on the enzyme molecules.

A different explanation for our results might be that the enzyme deficiencies in GM1-gangliosidosis and glycogenosis II are caused by a defect in the recognition sites on the cell surface, preventing uptake of β-galactosidase and α-glucosidase respectively. No cross-reacting material could be demonstrated in liver cells of a patient with glycogenosis II (16) which may support this hypothesis. Detection of cross-reacting material in the liver cells of a patient with GM1-gangliosidosis (17) however, points to a structural mutation in the enzyme molecule rather than to a defect in the uptake of β-galactosidase.

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