A new form of Niemann-Pick disease characterised by temperature-labile sphingomyelinase

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SUMMARY A new type (F) of Niemann-Pick disease characterised by childhood onset of splenomegaly, lack of neurological involvement, and diminished sphingomyelinase activity is described. The clinical presentation and heat-labile sphingomyelinase activity of this type F Niemann-Pick disease distinguishes it from other types of Niemann-Pick disease.

Five distinct clinical forms of Niemann-Pick disease have been described: the four subtypes (A to D) delineated by Crocker (1961) and an adult or type E form (Lynn and Terry, 1964). All of these forms of Niemann-Pick disease are characterised by an accumulation of sphingomyelins in visceral and/or cerebral tissues (Brady, 1978). Almost complete absence of sphingomyelinase activities in visceral tissues and cultured fibroblasts has permitted enzymatic pre- and postnatal diagnosis of the infantile or type A form of this disorder (Sloan *et al.*, 1969; Epstein *et al.*, 1971). The noncerebral or type B form is also characterised by diminished levels of sphingomyelinase activities, and is usually less severe than the infantile form (Gal *et al.*, 1975). Enzymatic characterisation of the 3 remaining subtypes of Niemann-Pick disease has produced varying results, with either normal or slightly decreased levels of sphingomyelinase activity. Recently, attempts have been made to distinguish these subtypes by electrophoretic examinations of sphingomyelinase activities (Callahan *et al.*, 1975).

We have previously described a family with 'sea-blue' histiocytosis (Blankenship *et al.*, 1973), hepatosplenomegaly, and decreased sphingomyelinase activities (Golde *et al.*, 1975). In this report, we will describe the temperature-labile sphingomyelinase activities found in fibroblast cultures derived from

members of this family, as well as cultures obtained from a second family.

Materials and methods

CLINICAL HISTORIES

The clinical histories of the L and K families are extensively described elsewhere (Blankenship *et al.*, 1973; Golde *et al.*, 1975; Sawitsky *et al.*, 1978). In brief, individuals had a relatively benign course characterised by the onset of splenomegaly in childhood and an absence of the neurological involvement observed in types A, C, D, and E Niemann-Pick disease.

CELL CULTURES

Punch biopsies (2 to 4 mm) were obtained from the upper arm of members of the L and K families after informed consent had been obtained. Cell cultures from patients with type A and C Niemann-Pick disease were supplied by the Mutant Cell Repository of the Institute for Medical Research, Camden, New Jersey, while the cell cultures from patients with type B Niemann-Pick disease were obtained from Dr Howard Sloan, National Institutes of Health, Bethesda, Maryland.

Skin tissue from the L family was explanted and subcultured in Eagle's Minimal Essential Medium supplemented with glutamine, 10% fetal calf serum

(Flow), and 50 µg/ml aureomycin (Gibco) by techniques described previously (Schneider and Mitsui, 1976). After sufficient outgrowth of fibroblasts from the explant had occurred, the cells were transferred to 75 ml plastic flasks (Falcon). Cells from 4 confluent 75 ml flasks were inoculated into 1875 ml roller bottles (Bellco) to obtain sufficient cells for enzymatic analysis. Cells were removed from the confluent roller bottles by scraping and then washed twice with Dulbecco's phosphate buffered saline.

Establishment and cultivation of cell cultures from the K family were similar to that described above, except that the culture medium was McCoy's 5A supplemented with 100 units/ml penicillin (Gibco) and 100 µg/ml streptomycin (Gibco), and harvesting was performed by trypsinisation.

Cell pellets were either analysed immediately after harvesting or frozen and stored at -20°C for subsequent analysis.

ENZYME ASSAYS

Cell pellets were suspended in 0.5 ml 25 mM citrate-phosphate buffer, pH 6.0, containing 2.0 mg/ml Cutscum and 10 mg/ml sodium taurocholate, then sonicated for 15 seconds in an ice bath. After centrifugation at 50 000 g for 30 minutes, the clear supernatants (containing 10 to 15 mg protein) were used for enzyme assays. Sphingomyelinase was measured with ¹⁴C-sphingomyelin as previously described (Kanfer *et al.*, 1966). Both acid phosphatase and β-glucuronidase were assayed by fluorogenic techniques (Glaser *et al.*, 1975).

In mixing experiments, equal amounts of extract protein were mixed together and the resultant enzyme activities measured.

For heat stability studies, cell extract supernatants were incubated at 50°C for periods ranging from 1 to 60 minutes and then assayed at the standard low temperature (Kanfer *et al.*, 1966).

Results

Examination of the lysosomal enzyme activities of fibroblast extracts from the 3 affected sibs of the L family (LII.2, 3, and 4) showed sphingomyelinase activities which were less than 20% of control levels (Table 1). Levels of acid phosphatase and β-glucuronidase activities in these fibroblasts were comparable to those observed in cells derived from controls. Previous studies of other lysosomal enzymes, including α- and β-galactosidases, N-acetylglucosaminidase, α- and β-fucosidases, and β-mannosidase, also produced normal levels of activities in these affected individuals (Blankenship *et al.*, 1973). Sphingomyelinase activities of fibroblasts derived from the mother of the 3 affected sibs, LI.2, and the son of

Table 1 Activities of lysosomal enzymes in fibroblast cultures derived from members of the L family

	Sphingomyelinase	Acid phosphatase	β-Glucuronidase
LII.2	11.2	157	28.2
LII.3	7.9	145	21.5
LII.4	11.1	121	27.7
LI.2*	15.9	115	19.0
LIII.1†	24.5	143	32.8
Controls(7)	69.3 ± 4.4	140 ± 8.9	24.2 ± 4.5

Enzyme activities in nmol/mg protein per hr

*Mother of LII.2, 3, and 4

†Son of LII.2

1 of these sibs, LIII.1, were 23% and 35% of control values.

Although sphingomyelinase activities of fibroblasts derived from normal donors remain relatively stable after storage at -20°C, a 95% decrease in sphingomyelinase activities was observed in fibroblasts derived from the 3 affected sibs of the L family after storage at this temperature for 4 months. After increasing storage time at -20°C, no differences were found in fibroblast activities of acid phosphatase and β-glucuronidase between controls and members of the L family. Because of this cold lability for sphingomyelinase activities, further studies were performed on fresh, non-frozen samples.

The results of incubating fibroblast extracts derived from an individual with this temperature-labile sphingomyelinase at 50°C are seen in Fig. 1. While sphingomyelinase activities of fibroblast extracts derived from normal individuals remain relatively stable, with half lives ranging from 40 to 90 minutes, sphingomyelinase activities in fibroblast extracts from affected individuals are quite heat-labile, with half lives in the 4 to 8 minute range.

In Table 2, the initial sphingomyelinase activities and their half lives at 50°C are listed for fibroblast cultures obtained from the 2 index families, patients with other forms of Niemann-Pick disease, and from healthy, normal controls. No significant amount of sphingomyelinase activity was detected in fibroblasts derived from a patient with the infantile or type A Niemann-Pick disease and, therefore, no half life determinations could be performed. Though cells derived from patients with the noncerebral or type B Niemann-Pick disease had extremely low levels of sphingomyelinase activity, these activities were relatively heat stable. Sphingomyelinase activities of fibroblasts derived from patients with type C Niemann-Pick disease varied from 6 to 40% of control levels, but were heat stable. Fibroblasts from the 2 affected sibs from the L family (LII.2, LII.4) and their mother (LI.2) all showed a marked reduction in sphingomyelinase activity as well as temperature lability. Sphingomyelinase activities of fibroblast ex-

Fig. 1 Temperature lability of sphingomyelinase activities at 50°C from fibroblast extracts derived from a control individual (—○—), a member of the L family (---●---), and a mixture of equal amounts of extracts from both individuals (□). The triangles and the dotted line are values based on the independent actions of 50% control and type F sphingomyelinase-containing extracts. Note that the observed results of this mixing experiment closely approximate the predicted values, indicating a lack of soluble inhibitors or promoters of sphingomyelinase temperature stability.

Table 2 Sphingomyelinase activities and half lives at 50°C in Niemann-Pick cell lines

Cell line	Classification	Sphingomyelinase activity (nmol/mg protein per hr)	Half life at 50°C (min)
GM 112	Type A	—	—
MM	Type B	0.49	54
AS	Type B	0.67	51
GM 165	Type C	18.3	62
GM 110	Type C	2.62	60
LII.2	Type F	1.18	5.0
LII.4	Type F	1.60	4.0
LI.2*	Type F	4.39	5.2
KII.2	Type F	18.0	13
KII.7	Type F	12.0	11
Controls(6)†	—	46.2	63

*Mother of LII.2 and 4.

†Sphingomyelinase activities varied considerably between experiments; therefore controls were performed for each set of determinations.

tracts from the son of an affected sib (LIII.1) had a half life of 60 minutes.

The sphingomyelinase activity levels of the fibroblasts derived from members of the second family (KII.2 and KII.7) were reduced to 26 to 39% of control levels while the half life of these activities was 11 and 13 minutes at 50°C (compared with 63 minutes for control fibroblasts).

To test for the presence of soluble inhibitors of sphingomyelinase activities in the fibroblast extracts, mixing experiments were performed. When control and affected offspring fibroblast extracts were mixed, the resultant sphingomyelinase activities were 96% of the theoretically predicted values, assuming no inhibition or augmentation of activity. To evaluate the possible presence of inducers of temperature lability or absence of factors necessary for temperature stability, fibroblast extracts from control and affected offspring were mixed in equal parts and a temperature-sensitivity assay performed. The results of this experiment, seen in Fig. 1, show that the resultant decrease in sphingomyelinase activity at 50°C closely approximated the theoretical prediction, indicating that neither temperature-labile inducing factors nor temperature-stabilising factors were responsible for the observed temperature lability of sphingomyelinase activities in families L and K.

The pedigrees of the L and K families with results of the temperature-sensitive experiments performed on their fibroblasts are seen in Fig. 2.

Discussion

During the past 2 decades, the number of sphingolipid disorders has increased from less than 6 to well over 36 disorders (Stanbury *et al.*, 1978). These newly identified disorders were first delineated on a clinical basis and, more recently, by biochemical techniques. Similarly, in Niemann-Pick disease, the first clear delineation of several distinct entities was made on a clinical and pathological description of the affected individuals (Crocker, 1961).

Recently, an attempt has been made to distinguish between the various clinical forms of Niemann-Pick disease by separating sphingomyelinase activities into separate components by isoelectric focusing. These studies have indicated that tissues from patients with type C and E Niemann-Pick disease may lack specific sphingomyelinases (Callahan *et al.*, 1975).

Until biochemical studies were initiated on the 2 families described in this report, these individuals were classified as having 'sea-blue histiocytosis'. This description includes a wide variety of lipid storage diseases which exhibit 'sea-blue' staining histiocytes as well as a primary entity with no known lipid stored (Silverstein *et al.*, 1970; Sawitsky *et al.*, 1972). The common denominator of these disorders is the storage of a ceramide compound which, when stained with Giemsa or Wright dyes, appears sea-blue in colour.

Since the clinical condition associated with these 2 families is mild, no individuals have reached postmortem examination or even splenectomy. Therefore, no visceral tissue is currently available to measure sphingomyelin and other sphingolipid deposition.

Fig. 2 Pedigrees of the L and K families indicating individuals who possess temperature-labile sphingomyelinase activities in their cultured skin fibroblast extracts. Individuals with 'sea-blue histiocytosis', but who have not been tested for sphingomyelinase activities, as well as tested members with heat-stable sphingomyelinase and untested individuals are also indicated.

Unfortunately, fibroblasts, even those from patients with almost complete absence of sphingomyelinase activities, contain only small amounts of sphingomyelin (A. E. Gal, 1978, personal communication). However, the reduced sphingomyelinase activities of fibroblasts derived from members of the L and K families, as well as the normal levels of other lysosomal enzymes in these cells, indicate that these individuals have a form of Niemann-Pick disease. In addition, sphingomyelin deposition in patients with Niemann-Pick disease can appear 'sea-blue' after Wright or Giemsa staining (Brady and King, 1973).

Individuals with this new type of Niemann-Pick disease (type F to follow current nomenclature) can be distinguished from those with the other types of Niemann-Pick disease. The noncerebral nature of this form of Niemann-Pick disease comes closest to resembling the clinical presentation of the type B form. However, despite the overlap in enzyme activities between these entities, the sphingomyelinase activity of fibroblasts from individuals with type B appears to be temperature stable in contrast to the F form. Both the clinical presentation and the level of sphingomyelinase activities distinguish this type F from type A or infantile Niemann-Pick disease. The clinical picture and the temperature lability of this type F Niemann-Pick disease delineates it from type C Niemann-Pick disease. Lastly, though type D fibroblasts were not examined, the clinical presentation is quite distinct from the relatively benign nature of type F.

The pedigree of the L family is most interesting since members of more than 1 generation are involved. All the sphingolipid disorders described to date have been inherited in either an autosomal recessive or sex-linked recessive fashion. The pedigree of family K

would be typical of the former type of inheritance. However, the diminished activity and normal temperature stability of sphingomyelinase activity measured in fibroblasts derived from the offspring of 1 of the affected members of the L family suggest that a more complex situation may exist in this family. Perhaps the most likely explanation is that the father of these affected individuals was a heterozygote for type A Niemann-Pick disease, while the mother was heterozygous for a temperature-labile form of sphingomyelinase (type F allele). All 3 offspring then received both abnormal alleles and, therefore, had lower sphingomyelinase activities than their mother, as well as temperature-labile sphingomyelinase.

Finally, the grandson received the type A allele and, therefore, was heterozygous for type A Niemann-Pick disease like his grandfather. Unfortunately, the untimely death of the father of these individuals and the lack of other available relatives eliminates the possibility of confirming this mode of inheritance.

Differences in sphingomyelinase activities and in temperature lability between individuals in the 2 index families may be the result of the different tissue culture conditions or different harvesting procedures. However, it is also possible that affected members of the K family are heterozygous compounds of the type F allele and the type B or C Niemann-Pick disease allele.

Genetic compounds such as those proposed above have been reported in both mucopolysaccharide (McKusick *et al.*, 1972) and sphingolipid (Tallman *et al.*, 1974) disorders. The delineation of a compound disorder before that of one of its homozygous components is not surprising, since the frequency of a compound composed of a relatively common allele, such as Niemann-Pick type A, and a presumed rare allele, such as that producing temperature-labile

sphingomyelinase activity, would be considerably higher than the frequency of the homozygous rare disorder (homozygous Niemann-Pick type F disease).

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