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New Tools for the Study of Niemann-Pick Disease: Analogues of Natural Substrate and Epstein-Barr Virus-transformed Lymphoid Cell Lines

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ABSTRACT. Acid sphingomyelinase activity was determined in Epstein-Barr virus-transformed lymphoid cell lines (LCL) established from patients affected with Niemann-Pick disease (NPD) using several substrates: sphingomyelin derivatives, radiolabeled [^{14}C]sphingomyelin (SM), fluorescent *N*-(10-(1-pyrene)decanoyl)sphingomyelin (P_{10}-SM) or colored trinitrophenylaminolauryl-sphingomyelin, and the chromogenic non-natural substrate 2-*N*-(hexadecanoyl)amino-4-nitrophenylphosphoryl-choline. LCL from NPD Type A and Type B showed a severe deficiency of acid sphingomyelinase determined using either substrate, whereas LCL from normal subjects had an activity close to that of blood leukocytes. Sphingomyelinase in normal LCL had the same pH optimum (5.0–5.2) and molecular form (pI 5.8) as the enzyme from other sources; identical profiles and activity levels were obtained using the various analogues of sphingomyelin. However, among these derivatives, the assay using P_{10}-SM appeared as the most useful and sensitive for enzymatic diagnosis of NPD. Electron microscopy of NPD LCL demonstrated the lysosomal storage. These results prove the validity of LCL as an experimental model system for NPD. (*Pediatr Res* 19:153–157, 1985)

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Abbreviations

LCL, lymphoid cell lines
NPD, Niemann-Pick disease
SM, sphingomyelin
P_{10}-SM, *N*-(10-(1-pyrene)decanoyl)sphingomyelin

TNPAL-SM, trinitrophenylaminolaurylsphingomyelin
HDA-PC, 2-*N*-(hexadecanoyl)amino-4-nitrophenylphosphorylcholine

Niemann-Pick disease is a heterogeneous group of hereditary diseases characterized by accumulation of sphingomyelin in tissues of affected patients (5). Following clinical, genetic, and biological criteria, several types can be separated: five types following Crocker *et al.* (5, 8) or six types according to Neville *et al.* (26). Only Type A (acute neuropathic form) and Type B (chronic non-neuropathic form) of the Crocker's classification show a severe deficiency of acid sphingomyelinase activity (EC 3.1.4.12) (5). In Type C, contradictory results on sphingomyelinase activity (6, 15, 34) and recent studies on sphingomyelin metabolism (23) suggest that sphingomyelinase deficiency, if it exists, is not the primary defect. In Types D and E, sphingomyelinase activity seems generally to be in the normal range (5, 28); a new Type F characterized by a heat-labile sphingomyelinase has been recently reported by Schneider *et al.* (32).

For the detection of acid sphingomyelinase deficiency, several natural or semisynthetic substrates [radiolabeled (5), fluorescent or colored (11), or a synthetic chromogenic analogue (10, 22)] can be accurately used. In contrast, synthetic chromogenic or fluorogenic phosphodiesters seem not to be reliable for the diagnosis of sphingomyelinase deficiency because of their hydrolysis by several nonspecific phosphodiesterases (17, 21, 25).

Experimental studies can be performed using cell cultures. The diploid fibroblast culture is the most commonly used model system but has several disadvantages: limited lifespan and aging (16); modifications of enzyme activities during senescence (27); and difficulties in producing large quantities of cellular material for biochemical studies. In contrast, Epstein-Barr virus-transformed LCL have several advantages: continuous cultures for long periods; minimal changes in phenotypic expression; and the possibility of obtaining a large amount of cellular material due to a short doubling time and the capacity of these cells to grow in suspension culture (12, 27). Such lines have been used in the study of several lysosomal disorders (24, 29–31, 35).

We report in this paper data concerning two new tools (substrates and culture model system) useful for the study of NPD. Enzymatic and ultrastructural studies permit the evaluation of these first LCL established from NPD Types A and B.

MATERIALS AND METHODS

Chemicals. The different substrates were purchased from: New England Nuclear (Paris) for [^{14}C]methylcholine-SM), specific activity 51 mCi/mmol; Sigma (St Louis, MO) for P_{10}-SM and TNPAL-SM; and Koch-Light (Colnbrook, U. K.) for HDA-PC. Ultrodex and Ampholines 3.5–10.0 were supplied from LKB (Bromma, Sweden). RPMI 1640 and fetal calf serum were from Gibco (Grand Island, NY) and other reagents were from Merck (Darmstadt, F. R. G.).

Long term lymphoid cell lines. These were established from blood lymphocytes of normal or sick subjects after incubation with the B_{95} strain of Epstein-Barr virus as previously reported (30, 31). The different LCL corresponded to: Be, normal adult; C_{49}, normal newborn; E1G, child with NPD Type A, and Par and Alb, NPD Type B. The transformed cells were cultured in RPMI 1640 as described in Ref. 31, collected 3 days after medium change, and stored at −70° C until use. Informed consent was obtained from all human patients.

Determination of molecular forms. Analysis of sphingomyelinase was carried out using preparative electrofocusing as previously reported for hexosaminidase (30).

Enzyme assays (Table 1). Cells were homogenized by sonication (three cycles of 15 s) on Triton X-100, 0.25%. Sphingomyelinase activity was tested using SM according to Wenger (36), using HDA-PC as previously reported (22) and using P_{10}-SM and TNPAL-SM according to Gatt *et al.* (11). The enzymatic reaction with these two latter substrates was linear at least for 2 h and up to 0.5 mg of protein/ml of assay. Protein concentration was determined using the method of Bradford (4) with serum albumin as standard.

Analysis of phospholipids in LCL. Lipids were extracted from the cell samples by the method of Folch *et al.* (9). Phospholipids were separated by thin layer chromatography on Silica Gel G (Merck) with a solvent system of chloroform/methanol/water, 100:42:6 (vol/vol/vol). The content of different phospholipids was determined by phosphorus assay (7).

Electron microscopy. Cellular ultrastructure was studied as previously described (1).

RESULTS

Table 2 shows the sphingomyelinase activities determined with the different analogues of sphingomyelin in normal and NPD LCL. In LCL from normal subjects, there was no difference in activity levels using either substrate. In LCL from NPD Type A or B, sphingomyelinase activity was severely deficient. The three synthetic analogues of sphingomyelin were also reliable for demonstration of the sphingomyelinase deficiency.

These analogues were tested to determine sphingomyelinase activity as a function of pH (Fig. 1); normal LCL showed a pH optimum between 5.0 and 5.2. The enzymatic values obtained at acidic pH were practically superimposable on those by radioassay. At neutral pH (7.5–8.0), slight activity was observed with SM and P_{10}-SM (the sensitivity of the assay using TNPAL-SM was not sufficient to determine it); at this pH, HDA-PC was not hydrolyzed, in agreement with previous results in brain (20).

Table 1. *Assays of sphingomyelinase in LCL*

Assay	SM	P_{10}-SM	TNPAL-SM	HDA-PC
Substrate concentrations (mmol·liter^{-1})				
Sphingomyelin	0.4	0.28	0	0
Labeled substrate	2000 dpm·nmol^{-1}	0.015	0.3	14
Buffer (mol·l^{-1})	0.15	0.25	0.1	0.1
Protein requirement (mg·ml^{-1})	0.2–0.5	0.2–0.5	0.2–0.5	1–4
Final volume (ml)	0.1	0.2	0.2	0.1
Incubation time (min)	90	120	90	120
Extraction system*	C/M/W	I/H/W/SA	I/H/W/SA	EA/I/G
	2:1:0.6	1:1:1:0.015	1:1:1:0.015	5:1:5
Molar extinction coefficient			10 000	10 000
Assay sensitivity (pmol/assay)	20–40	60–100	500–1000	500–1000
References	20, 36	11	11	20, 22

* The measured product of the enzyme reaction was extracted in the upper phase of each biphasic system. Solvents were: C, chloroform; M, methanol; W, water; I, isopropanol; H, heptane; SA, sulfuric acid; EA, ethyl acetate; and G, glycine buffer, pH 10.5 (0.2 mol·liter^{-1}).

Lymphoid cell extracts solubilized in Triton X-100 were subjected to isoelectric focusing (Fig. 2): in cells from normal subjects, only one peak (pI 5.8) was observed using the derivatives of natural substrates (radioactive, fluorescent, or colored) or the non-natural compound, HDA-PC. Again, enzyme activities using either substrate were very similar. In LCL from NPD Types A and B, no peak of sphingomyelinase activity was detected.

Table 2. *Acid sphingomyelinase activity (nmol·h^{-1}·mg^{-1}) in LCL from normal subjects and from NPD patients using natural substrate and various analogues of sphingomyelin**

Cell line	SM	P_{10}-SM	TNPAL-SM	HDA-PC
Normal subjects				
Be (adult)	10.3 (7.5–12.5)	10.5 (8.0–11.0)	8.9 (8.0–10.0)	8.0 (6.5–11.5)
C_{49} (newborn)	8.5 (7.5–10.5)	9.7 (9.0–12.0)	9.5 (7.5–9.8)	10.0 (7.5–13.0)
NPD patients				
E1G (type A)	0.8 (0.0–1.8)	0.4 (0.0–1.2)	0.1 (0.0–0.2)	0.9 (0.0–2.1)
Par (type B)	1.6 (0.0–2.0)	1.1 (0.0–1.8)	0.3 (0.0–0.5)	1.0 (0.0–1.8)
Alb (type B)	1.0 (0.6–1.9)	0.9 (0.0–1.7)	0.2 (0.0–0.4)	0.9 (0.0–2.0)

* Activity is the mean of 3 to 11 determinations on different cell batches.

Total phospholipid content approximated 120 nmol·mg protein^{-1} in normal LCL; these results agree with those reported by Gottfried in cultured lymphoid cells (13). In NPD LCL, total phospholipid amount varied from 210 to 280 nmol·mg protein^{-1}. In contrast to the constant amount of phosphatidylcholine, the amount of sphingomyelin was increased by 3 to 6 times in NPD LCL (Table 3).

Ultrastructural investigations of LCL from NPD showed an abnormal presence of electron-dense intracytoplasmic inclusions (Fig. 3*b*) compared to normal cell lines (Fig. 3*a*). High resolution micrographs demonstrated that the granules were made up of pleiomorphic material. The major component exhibits alternating light and dark bands. This results from the organization of the material into irregular concentric lamellae (Fig. 3*c*). These osmiophilic bodies are similar to those found in noncultured biopsy liver specimens of NPD patients (not shown).

DISCUSSION

The aim of this work was (i) to demonstrate the valididy of NPD LCL as a new experimental model system for enzymatic studies of this disease, and (ii) to compare the reliability of various analogues of sphingomyelin for diagnosing sphingomyelinase deficiency.

The validity of LCL as a model of NPD was proved enzymatically and ultrastructurally. First, sphingomyelinase in normal LCL seems to be not modified by Epstein-Barr virus transfor-

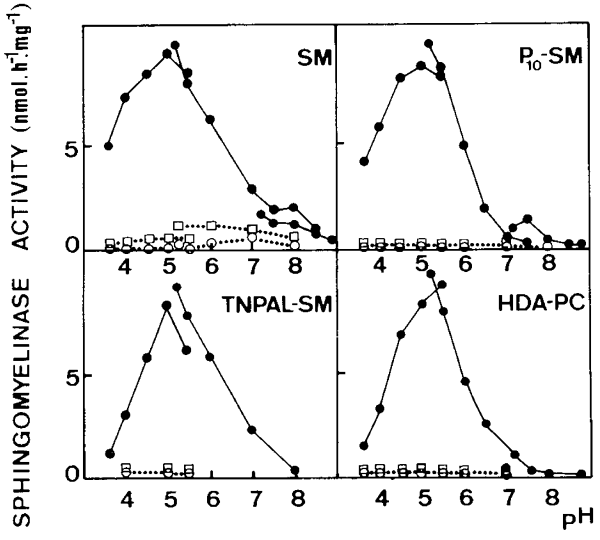

Fig. 1. Effect of pH on sphingomyelinase activity in LCL. Sphingomyelinase activity was tested in LCL from normal subjects (●——●) and from patients affected with NPD Type A (line E1G, O····O) and Type B (line Par, □····□) using the various substrates (for abbreviations, see "Materials and Methods"). Assays were performed as described in the text, using the following buffers (0.1 M): sodium acetate buffer (pH 3.6–5.5), Tris-maleate buffer (pH 5.2–8.5), and Tris-HCl buffer (pH 7.2–9.0).

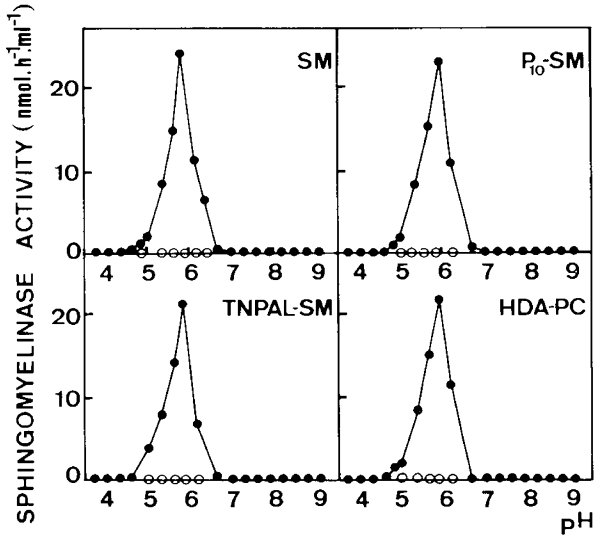

Fig. 2. Electrofocusing profiles of acid sphingomyelinase in LCL from normal subjects and from NPD patients. Sphingomyelinase activity (expressed as nmol/h/ml of fraction) was tested in LCL from normal subjects (line Be, ●——●) and from NPD Type A or Type B patients (lines E1G or Par, O····O). The Triton extracts were prepared from about 500 × 10^6 cells and 15 mg of protein of 220,000 × *g* supernatant fluid were applied on the granulated gel. Assays were performed as described in the text.

Table 3. *Sphingomyelin levels in LCL**

Cell line	Protein† (mg·ml^{-1})	Phosphatidylcholine (nmol phosphorus/mg protein)	Sphingomyelin (nmol phosphorus/mg protein)
Normal subjects			
Be (adult)	19.5	51	17
C_{49} (newborn)	13.6	79	16
NPD patients			
E1G (type A)	6.8	63	55
Par (type B)	4.4	94	90
Alb (type B)	2.1	102	71

* All determinations were done in duplicate.

† The number of cells was respectively 100 × 10^6, 50 × 10^6, and 20 × 10^6 for Be and C_{49}, E1G and Par, and Alb.

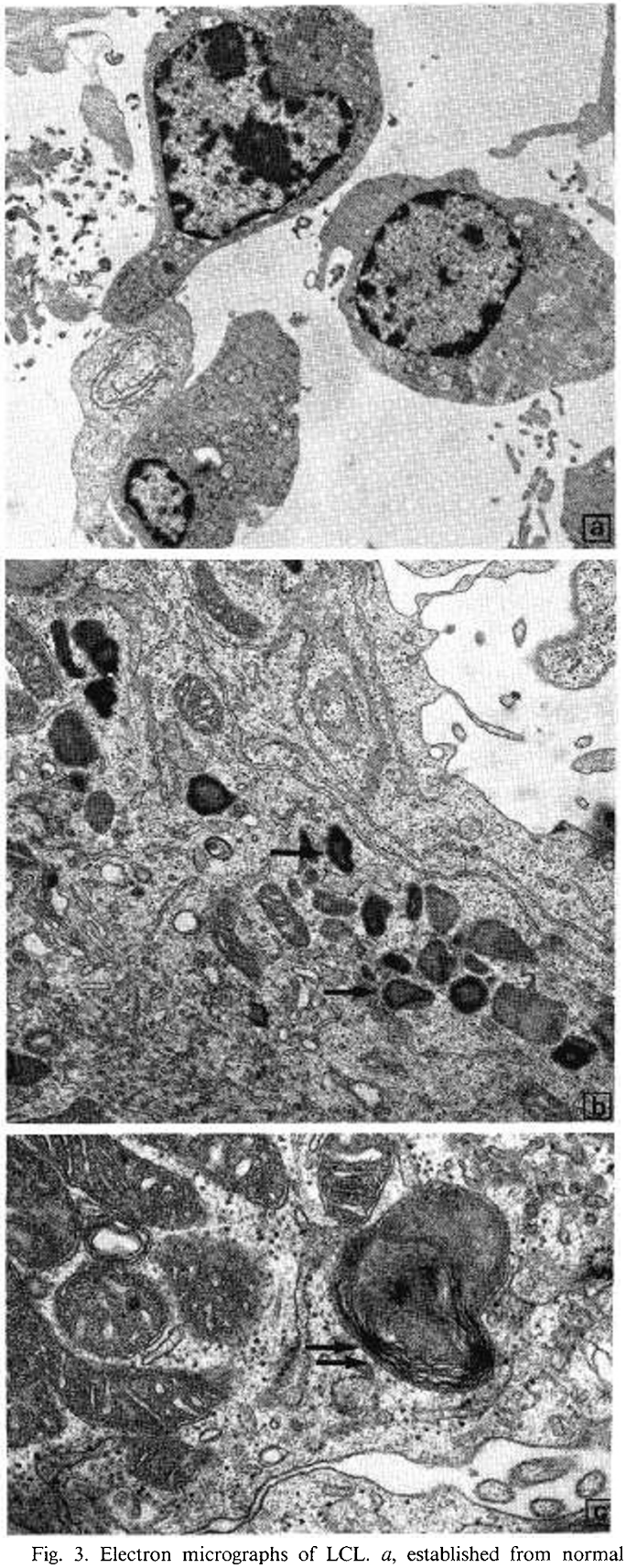

Fig. 3. Electron micrographs of LCL. *a*, established from normal subject (line Be); *b* and *c*, established from NPD patient (line Par). Note numerous irregular whorled cytoplasmic inclusions (→) corresponding to distended lysosomes which contain disordered, dense lipid lamellae (⇉) separated by clear, electron-empty zones. *a*, × 3,500; *b*, × 22,500; *c*, × 45,000).

mation since it shows the same pH optimum as in other cells (3, 14, 37) or tissues (33). Activity levels in normal LCL were slightly higher than in blood leukocytes (3, 14, 21, 37). Moreover, LCL have only one molecular form (pI 5.8): these results agree with those reported by the same procedures on blood leukocytes, lymphocytes, and human brain (19). We did not observe the minor molecular forms described by Callahan *et al.* (6) or Besley (2); these differences could be due to the different tissue origin and different electrofocusing technique (on thin layer in our case, on column for other authors). Secondly, LCL appear to be a useful model for NPD; indeed, LCL from affected patients showed a severe sphingomyelinase deficiency (Table 2; Figs. 1 and 2). This deficiency explains the cytoplasmic storage of abnormal osmiophilic material similar to myelin-like structures at the electron microscopic level (Fig. 3) as reported in tissues (5).

Moreover, the nature of these amphiphilic lipids was specified by the phospholipid analysis that showed a sphingomyelin storage in NPD LCL (Table 3). However, this accumulation is less than in the tissues of patients, probably because of the absence of contribution by exogenous sphingomyelin (in contrast to the macrophage cells in the organism) and a short doubling time of LCL.

Several analogues of sphingomyelin have also been tested on LCL for enzymatic diagnosis of NPD without the use of radioactivity. We compared (Table 1) the assays using the new derivatives of sphingomyelin synthesized by Gatt *et al.* (11) and the assay using the chromogenic analogue HDA-PC; methylumbelliferyl-derived phosphodiesters have been previously shown as nonspecific for sphingomyelinase determination (21).

The linearity of P_{10}-SM and TNPAL-SM hydrolysis as a function of protein concentration was very similar to that reported for fibroblasts and amniotic cells (11). On the other hand, the similarities of the activity levels obtained using the new substrates and using radiolabeled substrate or HDA-PC are consistent with the data of Gatt *et al.* (11). The specificity of these derivatives is demonstrated by the fact that pH optimum curves and electrofocusing profiles are superimposable on those of natural substrate, and by the fact that they are able to show the sphingomyelinase deficiency in NPD LCL. Ranges of enzyme activity in various cell batches were similar when using natural or synthetic substrate. Consequently, these substrates can be used readily for sphingomyelinase assay and NPD diagnosis.

However, we will discuss their advantages and disadvantages in comparison to natural substrate. The radiolabeled substrate is specific but requires a radiometric procedure, and it is subject to decomposition with time (18). The chromogenic compound HDA-PC provides an easy assay, specific for acid sphingomyelinase (20) but a good product extraction system is essential to eliminate possible interference by hemoglobin (22). However, this non-natural chromogenic substrate gives a relatively insensitive assay. The derivatives recently synthesized by Gatt allow a rapid assay of sphingomyelinase using small quantities of cellular material and a convenient fluorometric or spectrometric determination. These analogues are hydrolyzed by both acid and neutral sphingomyelinases (11). However, the fluorometric assay using P_{10}-SM is much more sensitive than that using TNPAL-SM; thus, P_{10}-SM can be used 20-fold-diluted whereas TNPAL-SM must be used pure (thus it is more expensive).

Our present results indicate that the above substrates can be used to determine acid sphingomyelinase activity. However, the fluorescent assay using P_{10}-SM seems to be the most useful, reliable and sensitive assay. In the same way as these substrates constitute a new tool for NPD diagnosis, LCL from NPD represent a new experimental model system for this lysosomal storage disorder. We are now investigating the metabolic features of this model.

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