Functional compartments of sulphatide metabolism in cultured living cells: evidence for the involvement of a novel sulphatide-degrading pathway

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The modes of uptake and degradation of radiolabelled cerebroside sulphate (CS or sulphatide) were investigated in cultured living skin fibroblasts and Epstein–Barr virus-transformed lymphoblastoid cell lines established from control individuals and patients affected with metachromatic leucodystrophy (cerebroside sulphatase deficiency), multiple sulphatase deficiency and low-density-lipoprotein-receptor-negative familial hypercholesterolaemia. In both cell types, CS was taken up through a non-receptor-mediated process. In fibroblasts, CS degradation occurred intralysosomally as was evident from the findings that fibroblasts from metachromatic leucodystrophic patients accumulated the sulphatide and that chloroquine inhibited its degradation by normal cells. In contrast, under

similar conditions of CS availability, lymphoblastoid cell lines from patients with metachromatic leucodystrophy could degrade the incorporated sulphatide exactly as their normal counterparts. This metabolic pathway was also fully active in lymphoblastoid cells from patients with multiple sulphatase deficiency and was not inhibited by chloroquine treatment. These data are consistent with a non-lysosomal type of hydrolysis. In addition to the lysosomal and non-lysosomal compartments, a third compartment was identified in the two cell types which is probably formed by the pool of the sulphatide molecules incorporated into the plasma membrane. This is the first report on the existence of a CS-degrading pathway in intact cells with deficient lysosomal cerebroside sulphatase activity.

INTRODUCTION

The sulphated sphingolipid, galactosyl sulphatide (galactosyl 3-sulphate ceramide or cerebroside sulphate, CS) is the major sulphate-containing lipid in the nervous system, particularly the myelin sheath [1]. Physiologically, as an amphipathic membrane component, sulphatide is implicated in active Na⁺ transport, the opiate receptor, the binding sites of γ -aminobutyrate and 5-hydroxytryptamine, the binding to adhesive glycoproteins and clotting substances and the cytotoxicity of natural killer cells (see [1]).

Desulphation is the first step in the degradation of sulphatide, leading to galactosylceramide. This enzymic hydrolysis is catalysed by the cerebroside sulphate sulphatase (or arylsulphatase A, ASA, EC 3.1.6.8). Lysosomal accumulation of sulphatide is the characteristic feature of metachromatic leucodystrophy (MLD) (McKusick 25010), a term given to a group of inborn autosomal recessive disorders resulting in progressive neurological degeneration, and commonly subdivided into late-infantile, juvenile and adult types [1]. Underlying defects of this disease of myelin metabolism include deficiencies of cerebroside sulphatase activity [2,3] and the sulphatide activator protein called SAP-1 or saposin B [4,5], a factor required for *in vivo* hydrolysis of sulphatide [6,7]. Sulphatide storage is also found in multiple sulphatase deficiency (MSD) (McKusick 27220), an inherited disorder characterized by the deficient activity of several different sulphatases, including the cerebroside sulphatase [1].

Administration of exogenous sulphatide to intact cultured skin fibroblasts has been used to show that cells from patients with MLD accumulate sulphatide [8]. By using a radiolabelled sulphatide, this procedure permitted differentiation into the various MLD clinical subtypes, on the basis of the inverse

correlation between the age of onset of the symptoms and the degree of intracellular accumulation of sulphatide [9]. This sulphatide-loading test has also been employed to distinguish between MLD homozygotes and heterozygotes, patients carrying the pseudo-deficiency allele (ASA pseudodeficiency is a quite common condition observed among healthy individuals with *in vitro* ASA activities similar to those seen in MLD [1]), and patients with the sulphatide activator deficiency [4,10–16]. Uptake and intracellular metabolism of the exogenous sulphatide has been followed by using a sulphatide radiolabelled in the sulphate [4,5,8–11,13], fatty acid [12,17–19] or sphingosine [15,16,20] moieties. Alternatively, various fluorescent derivatives of CS have been employed [21–26].

All these studies were performed on cultured skin fibroblasts and were mainly concerned with diagnosis or characterization of the genetic heterogeneity of leucodystrophies. On the basis of the degradation defect in MLD and on subcellular fractionation observations [16,18,19], these studies supported the conclusion that the hydrolysis of CS in cultured skin fibroblasts exclusively takes place in the lysosome. Up to now, there has been no investigation of *in situ* sulphatide metabolism in normal and mutant cultured cell types other than fibroblasts.

We have previously demonstrated the suitability of Epstein–Barr virus-transformed lymphoblastoid cell lines (LCL) as an experimental model for the study of inborn deficiencies of ASA [27]. LCL derived from patients with MLD or MSD exhibit a severe reduction in *in vitro* cerebroside sulphatase/ASA activity. These permanent cell lines have some advantages over cultured skin fibroblasts: unlimited lifespan, continuous cell culture without senescence, short doubling time and growth in suspension [28]. In the present paper, the metabolism of sulphatide was studied in LCL derived from normal individuals and MLD

Abbreviations used: CS, cerebroside sulphate; ASA, arylsulphatase A; MLD, metachromatic leucodystrophy; MSD, multiple sulphatase deficiency; XLI, X-linked ichthyosis; FH, familial hypercholesterolaemia; LCL, lymphoblastoid cell lines; 4-MUS, 4-methylumbelliferyl sulphate; LDL, low-density lipoprotein.

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patients by using a naturally occurring radiolabelled sulphatide. The modes of uptake and subsequent catabolism of this CS in LCL have been investigated and compared with those observed in cultured fibroblasts. Our data indicate a non-receptor-mediated mode of endocytosis of this lipid and provide convincing evidence for the existence of a novel sulphatide-degrading pathway which is fully active in lysosomal cerebroside sulphatase/ASA-deficient LCL.

MATERIALS AND METHODS

Materials

p-Nitrocatechol sulphate, *N*-hydroxysuccinimide, taurodeoxycholic acid, chloroquine and bichinchonic acid were obtained from Sigma (St. Louis, MO, U.S.A.). DE-52 DEAE-cellulose was purchased from Serva (Heidelberg, Germany). [9,10-³H]Oleic acid (specific radioactivity, 10 Ci/mmol) was supplied by NEN Du Pont (Paris, France). RPMI 1640 medium, fetal calf serum, streptomycin, penicillin, glutamine and trypsin/EDTA were from Seromed (Berlin, Germany) or Gibco; the serum substitutes Ultrosor G and Ultrosor HY were from IBF (Villeneuve-La-Garenne, France). All other chemicals and solvents were from Merck (Darmstadt, Germany) or Prolabo (Paris, France).

Synthesis of radiolabelled CS

Unlabelled sulphatide was isolated from a chloroform/methanol (2:1, v/v) extract of bovine brain, submitted to mild alkaline hydrolysis and passed through a Florisil/Hyflosuperpel column [29]. The purity of the sulphatide was checked by silica-gel t.l.c. using chloroform/methanol/water (100:42:6, by vol.) as developing solvent system. Lipid spots were visualized by the use of iodine vapour and by spraying with orcinol (2 g/l in 3.5 mol/l H₂SO₄). The sulphatide was tritiated by catalytic reduction (Centre d'Etudes Nucléaires de Saclay, Gif-sur-Yvette, France) and repurified by t.l.c. Chemical hydrolysis of the tritiated sulphatide by KOH/butanol [30] showed that more than 97% of the radioactivity resided in the ceramide moiety and that both fatty acid and sphingoid base were radiolabelled. The specific radioactivity of the final compound approximated 200 mCi/mmol.

Alternatively, radioactive sulphatide was prepared by deacylation of bovine brain CS and reacylation with a mixture of unlabelled and [9,10-³H]oleic acids. The sulphatide was deacylated using KOH/butanol and the lyso-sulphatide purified by t.l.c. by the procedure of Dubois et al. [30]. The lyso-sulphatide was then allowed to react with the *N*-hydroxysuccinimide ester of [9,10-³H]oleic acid under the conditions described by Ahmad et al. [31]. The radioactive sulphatide formed was purified by preparative t.l.c. in chloroform/methanol/water (100:42:6, by vol.). Its final specific radioactivity was 2.5 mCi/mmol.

Cell culture

Cultured fibroblasts were obtained after skin biopsies from control subjects and from patients affected with the late-infantile form of MLD (Ale, Faj, Mau) or with the low-density-lipoprotein (LDL)-receptor-negative form of familial hypercholesterolaemia (FH) (GM 486A, Han). Lines Ale and Mau were kindly supplied by Dr. C. Borroni and Dr. R. Gatti, Istituto Giannina Gaslini (Genova, Italy), and line Han by Dr. S. Eisenberg, Department of Medicine B, Hadassah University Hospital (Jerusalem, Israel).

LCL were established by Epstein-Barr virus (B95/8 strain) transformation [32] of blood B lymphocytes obtained from normal individuals and from patients affected with the late-infantile form (line Faj) or the juvenile form of MLD (line GM 1017), MSD (GM 3246 and GM 4916), X-linked ichthyosis (XLI) (Car, Mem) and receptor-negative FH (GM 1459 and GM 1767). The GM cell lines were purchased from the Human Genetic Mutant Cell Repository (Camden, NJ, U.S.A.).

Fibroblasts and LCL were routinely grown at 37 °C in humidified CO₂ in RPMI 1640 medium supplemented with penicillin (100 units/ml), streptomycin (100 µg/ml), glutamine (2 mmol/l) and 10% heat-inactivated fetal calf serum [33]. Fibroblasts were studied between the 8th and 18th passages.

Sulphatide-loading experiments

For the metabolic studies, fibroblasts were treated with trypsin, plated into 35 mm-diameter Petri dishes and grown in a medium containing 10% fetal calf serum. Usually 2 days later, the medium was removed and replaced by RPMI 1640 medium containing 2% Ultrosor G, a serum substitute devoid of lipoproteins. After 2 more days, all the fibroblast cultures being confluent, the experiments were initiated. Similar preincubation conditions were used for the LCL, except that Ultrosor HY was employed.

A chloroform/methanol (2:1, v/v) solution of the radiolabelled sulphatide was evaporated under nitrogen. The residue was dissolved in a small volume of ethanol and mixed with sterile culture medium containing either 10% fetal calf serum or only 2% Ultrosor HY. These media were warmed at 37 °C for about 15 min and added to the cultured cells. The final concentration of sulphatide in the incubation medium was usually about 10⁶ d.p.m./ml, i.e. 2.5 nmol/ml. The final concentration of ethanol did not exceed 0.7% (by vol.). In some experiments, before the addition of radiolabelled CS, native CS was included in the incubation medium and dispersed by sonication (5 min).

After incubation of the cells for various periods at 37 °C, the experiments were terminated by removal of the culture medium and washing the cells three times with PBS containing BSA (2 mg/ml), and then twice with PBS alone. The fibroblasts were harvested by scraping with a rubber policeman and sedimented by low-speed centrifugation (5 min at 300 g). LCL were harvested by low-speed centrifugation and washed as above. The cell pellets were stored at -20 °C.

In pulse-chase experiments, cells were incubated for 20 h with the radiolabelled sulphatide in the presence of fetal calf serum or Ultrosor. After removal of the incubation medium, cells were washed twice and further incubated for 2 and 4 days with the same culture medium as that used for the pulse period but devoid of radioactive sulphatide. At the end of the chase periods, cells were processed as mentioned above.

Lipid extraction and analyses

Cell pellets were suspended in 0.6 ml of distilled water and sonicated in ice for 3 × 15 s (Soniprep MSE sonicator). A portion (50 µl) was taken for protein determination. Another one (50 µl) was counted in a scintillation spectrometer (Packard Tricarb 4530) using Picofluor 15 to determine the amount of total cell-associated radioactivity. The remainder was extracted with 2.5 ml of chloroform/methanol (2:1, v/v) [34], vortex-mixed and centrifuged at 1000 g for 15 min. After evaporation of the lower phase under a stream of nitrogen, the residue was dissolved in a small volume of chloroform/methanol (2:1, v/v), the lipids were resolved by analytical t.l.c. in chloroform/methanol/water

Table 1 Properties of sulphatases in normal and mutant LCL

This information is adapted from previous [27] and unpublished work (M.-C. Tempesta, T. Levade and R. Salvayre). K_m was calculated with 4-MUS as substrate. For heat-lability, the residual activity after preincubation at 55 °C, pH 3.5, for 60 min is expressed as a percentage of the value determined in the absence of heat-inactivation. Residual activity is expressed as a percentage of the values determined in normal cells; the enzyme activities correspond to the range of values determined on three MLD, one MPS VI, three XLI and two MSD cell lines (2–15 separate determinations). pNCS, *p*-nitrocatechol sulphate; DHEAS, dehydroepiandrosterone sulphate; PP, pyrophosphate; MPS VI, mucopolysaccharidosis type VI; ND, not determined.

Properties	Cerebroside sulphatase/ASA	Arylsulphatase B	Steroid sulphatase/arylsulphatase C
Substrates	Radiolabelled CS, pNCS, 4-MUS	pNCS, 4-MUS	Radiolabelled DHEAS, 4-MUS
pH optimum	5.2–5.5	5.5	8.0
Inhibitors	AgNO ₃	PP/NaCl	
Apparent K_m (mM)	4.5	1	ND
Heat-lability	6%	100%	ND
Molecular forms (pI)	4.1, 4.4, 4.8	7.0, 7.5, 8.2, 9.2	
Residual activity in:			
MLD cells	0–10%	73–107%	108%
MPS VI cells	160%	2–3%	85%
XLI cells	ND	ND	0–2%
MSD cells	5–15%	2–7%	2–4%

(100:42:6, by vol.) and the plate was analysed using a Berthold (Tracemaster 20) t.l.c. radiochromatocan. Identification of the radiolabelled metabolites was performed by comparing their R_F values with those of authentic commercial standards.

Other determinations

Protein content was determined by using the Folin–Ciocalteu reagent [35] or bichinchoninic acid solution [36].

Cerebroside sulphatase activity was assayed on dialysed cell suspensions using [³H-*oleoyl*]sulphatide (5700 d.p.m./nmol) exactly as described by Raghavan et al. [37]. After incubation for 2 h, the reaction was stopped by adding 1 ml of methanol and the mixture was passed through a Pasteur pipette column of DEAE-cellulose (DE-52). Alternatively, the sulphatide tritiated by catalytic reduction was employed using similar experimental conditions.

ASA activity in cell homogenates was determined using *p*-nitrocatechol sulphate as substrate after overnight incubation at 4 °C as described by Tempesta et al. [27] and Lee-Vaupel and Conzelmann [38]. After incubation the reaction was stopped by adding 1 ml of NaOH (1 mol/l) and the liberated *p*-nitrocatechol quantified by measuring the absorbance at 515 nm (Philips spectrophotometer).

Other enzyme assays were performed as detailed in [27].

RESULTS

Characteristics of cell lines used in this study

The properties of arylsulphatases in LCL are summarized in Table 1. In normal LCL, ASA accounts for about 20–25 % of the total 4-methylumbelliferyl sulphate (4-MUS) sulphatase activity [27]. The arylsulphatase A and B molecular forms in LCL were shown to have the same pI as those previously described in other tissues or cells [39]. The K_m values of these arylsulphatases for 4-MUS in LCL are also similar to previously reported values. As the ASA activity in LCL (as measured with 4-MUS) is heat-labile, specific conditions employing heat-inactivation and addition of inhibitors have previously been set up for the determination of arylsulphatase A and B in LCL [27]. Under these conditions, LCL from patients with MLD were shown to be deficient in CS/ASA activity (see also Figure 7). This ASA deficiency was unequivocally confirmed by assaying the enzyme

with its natural substrate, i.e. CS, and by electrofocusing techniques for accurate separation of the various arylsulphatase activities. This agrees with the ASA defect reported by Beratis et al. [40]. LCL from patients with MSD were shown to be deficient in the lysosomal sulphatases (cerebroside sulphatase, arylsulphatases A and B) as well as the non-lysosomal arylsulphatase, arylsulphatase C/steroid sulphatase (Table 1). The total *p*-nitrocatechol sulphate or 4-MUS sulphatase activities determined in the absence of inhibitors or heat-inactivation were also deficient in these LCL.

Uptake of CS by cultured cells

To investigate the mode of uptake of CS by LCL, we studied the effect of CS concentration in the culture medium and incubation time on the amount of cell-associated lipid. To examine further the possible role of lipoproteins in CS uptake, cells were incubated in the presence or absence of fetal calf serum. CS uptake was also investigated in cells from patients with FH which lacked the LDL receptor.

Incubation of cultured skin fibroblasts and LCL for 20 h with radiolabelled CS in the presence of 10 % fetal calf serum resulted in a concentration-dependent uptake of this sphingolipid (Figures 1a and 1b). The total cell-associated radioactivity increased almost linearly with the concentration of exogenous sulphatide (at least up to 60 nmol/ml). In addition, there was no difference in the uptake rates between normal and FH cells, suggesting that the CS became associated with the cells via an LDL receptor-independent pathway. As previously noted for fibroblasts [17,19], some differences were observed between different normal cell lines; these were attributed to the rapidity of cell growth.

When cultured cells were incubated with radiolabelled CS in the presence of 2 % Ultrosor, i.e. in a medium devoid of lipoproteins, CS uptake was also linear with the amount added to the medium (Figures 1c and 1d). In all cells tested, the rates of CS uptake were higher (up to 10 and 25 times) in the presence of Ultrosor than in the presence of serum.

Figure 2 illustrates the time-dependent uptake of low concentrations of CS by cultured cells. After a short phase of rapid incorporation, a continuous increase in the cell-associated radioactivity was observed up to about 20 h. Again, a higher uptake in the absence of serum is evident. On the basis of the above data,

Figure 1 Uptake of CS by cultured skin fibroblasts (left) and LCL (right) as a function of CS concentration in the culture medium

After preincubation for 2 days in a medium containing 2% Ultrosor, normal (■, N1; △, N2; ▲, N3) and LDL-receptor-negative FH cells (□) were incubated with RPMI 1640 medium supplemented with radiolabelled CS (10^6 d.p.m./ml, i.e. 2.5 nmol/ml) and various concentrations of bovine brain CS in the presence of 10% fetal calf serum (a and b) or 2% Ultrosor HY (c and d). After 20 h incubation, cells were washed, harvested and the total cell-associated radioactivity determined as described in the Materials and methods section.

Figure 2 Uptake of CS by skin fibroblasts (left) and LCL (right) as a function of incubation time

Normal cells were incubated for the indicated times with radiolabelled CS (2.5 nmol/ml) in the presence of 10% fetal calf serum (○) or 2% Ultrosor HY (●). The total cell-associated radioactivity was determined as described in the Materials and methods section. The protein content was 25–75 μ g and 150–500 μ g for fibroblasts and LCL respectively.

Figure 3 T.l.c. patterns of the lipids extracted from normal (a, c) and MLD-derived (b, d) fibroblasts (left) and LCL (right)

Cells were incubated for 20 h with radiolabelled CS (2.5 nmol/ml) in the presence of 10% fetal calf serum. Next, the cells were washed twice with fresh medium and incubated further for 4 days in medium containing 10% fetal calf serum. The lipids were extracted from the washed cells and separated by t.l.c. using chloroform/methanol/water (100:42:6, by vol.) as developing solvent. The Figure shows representative patterns obtained after radiochromatostaining of the t.l.c. plates. The approximate running distance of the main lipids was: polar lipids, 5–10 cm; CS, 12 cm; galactosylceramide, 14 cm; ceramide and other neutral lipids, 16 cm. Starting position was at 2.5 cm. The various lipids were identified by comparing their R_f values with those of authentic standards. The t.l.c. profile of the administered radiolabelled CS (not shown) was similar to that for fibroblasts from patients with MLD.

subsequent experiments were performed with a CS concentration in the medium of 2.5 nmol/ml and an incubation time of 20 h.

Metabolism of CS in cultured fibroblasts and LCL in the presence of serum

The metabolic fate of the radiolabelled CS taken up by the cells was examined by analysing the lipids on t.l.c and radiochromatostaining. Figure 3 shows the metabolic patterns of the lipids extracted from cells that had been incubated for 20 h with CS, washed and incubated further in a medium devoid of CS for 4 days. Normal skin fibroblasts (Figure 3a) were found to contain some unhydrolysed sulphatide (about 25–30%) and its metabolic derivatives. These metabolites, which originated from the recycling of both the radiolabelled fatty acid and sphingosine moieties, included galactosylceramide, ceramide, glycerophospholipids (mainly phosphatidylcholine and phosphatidylethanolamine), other sphingolipids (mainly sphingomyelin, lactosylceramide and ganglioside GM3) and other neutral lipids (cholesteryl esters and triacylglycerols). These data were compatible with previous reports [17,19,20]. In fibroblasts from a patient with the late-infantile form of MLD, 95% of the sulphatide remained unhydrolysed (Figure 3b).

In normal LCL, CS was metabolized to the same lipids but to a lesser extent than in normal fibroblasts (Figure 3c). Of particular interest was the surprising finding that LCL derived from a patient with MLD, i.e. cells with deficient *in vitro* cerebrosidase sulphatase activity [27], exhibited the same metabolic pattern as their normal counterparts (Figure 3d). Similar data were obtained when using sulphatides labelled exclusively in the fatty acid moiety or in both the sphingosine and the fatty acid moieties

Figure 4 Metabolism of CS by cultured skin fibroblasts (left) and LCL (right) in the presence of fetal calf serum

Cells derived from normal individuals and patients with MLD, MSD and XLI were incubated for 20 h in medium containing 10% fetal calf serum and radiolabelled CS (2.5 nmol/ml). After removal of the radioactive medium, the cells were washed twice with medium supplemented with 10% fetal calf serum and incubated further for the indicated times. After the cells had been washed with PBS, the cellular lipids were extracted, separated by t.l.c. and quantified by radiochromatoscanning as described in the Materials and methods section. For each chase period, the amount of the various radiolabelled lipids is expressed as percentage of total lipids. The data correspond to means $\pm$ S.D. of one to three separate experiments performed on two to three different cell lines of each class. ■, sulphatide; □, galactosylceramide; ○, ceramide + other neutral lipids; ▲, glycerophospholipids + other sphingolipids (sphingomyelin, lactosylceramide and ganglioside GM3).

(results not shown). In an attempt to clarify the normal level of metabolism in LCL from patients with MLD, pulse-chase experiments were conducted to study the time course of CS degradation in normal and MLD-derived LCL as well as in fibroblasts for comparison.

In Figure 4 are shown the kinetics of degradation of CS and appearance of its lipid metabolites in normal and sulphatase-deficient cells pulsed with radiolabelled CS in the presence of serum. In normal skin fibroblasts, the radioactive sulphatide only represented 44% of the total cellular lipids after 20 h pulse and continued to decrease to 17.5% after 4 days chase. This was accompanied by a parallel increase in radioactivity from glycerophospholipids and sphingomyelin; neither galactosylceramide nor ceramide (which represented 8–10% each) accumulated. In fibroblasts from patients with late-infantile MLD, the radiolabelled CS remained almost unhydrolysed (almost 95% of total lipids) throughout the pulse-chase experiment (Figure 4, left). In marked contrast, LCL derived from patients affected with the late-infantile or juvenile forms of MLD could hydrolyse the sulphatide as well as control LCL (Figure 4, right). At the beginning of the chase period, the undegraded CS represented $77 \pm 2\%$ and $67 \pm 10\%$ of the total lipids in MLD-derived and normal LCL respectively. After 4 days chase, these values decreased to $61 \pm 3\%$ and $53 \pm 8\%$. There was a corresponding

increase in the radioactivity of polar lipids, indicating that CS was indeed metabolized by LCL. Very similar results were observed in two other LCL deficient in cerebroside sulphatase activity, which were derived from patients affected with MSD [27] (Figure 4). Even after a 12-day chase, there was no detectable accumulation of CS in cells from MLD patients compared with control LCL (results not shown). It is also noteworthy that cells derived from patients with XLI, which are deficient in non-lysosomal steroid sulphatase activity (M. C. Tempesta, T. Levade, R. Salvayre, unpublished work), could degrade the exogenously supplied CS normally (Figure 4).

Metabolism of CS in cultured fibroblasts and LCL in the absence of serum

To investigate further the influence of the serum proteins and/or lipoproteins present in the incubation medium on the metabolism of CS, pulse-chase experiments were also carried out by incubating the cells in the absence of serum, i.e. in the presence of the serum substitute Ultrosor. Figure 5 compares the rates of production of the CS metabolites in normal and sulphatase-deficient cells with or without serum. First, the data clearly indicate that omission of serum from the incubation medium resulted in a reduction (20–40%) in the relative rates of CS

Figure 5 Effect of the composition of the incubation medium on the metabolism of CS by skin fibroblasts (left) and LCL (right)

Cells derived from normal individuals and patients with MLD, MSD and XLI were incubated for 20 h with radiolabelled CS in the presence of 10% fetal calf serum (▨) or 2% Ultrosor (■). The experiment was then continued as described in the legend to Figure 4. In the experimental set where the cells were pulsed in the presence of Ultrosor, the cells were washed once with medium containing fetal calf serum and once with medium containing Ultrosor, and further incubated for the indicated times in the presence of 2% Ultrosor. For each chase period, the amount of the total radiolabelled lipid products of CS metabolism is expressed as percentage of total lipids. The nature and relative amounts of the various radiolabelled lipid products were similar to those found in the presence of serum (Figure 4). The data correspond to the mean $\pm$ S.D. of different experiments and cell lines as reported in the legend to Figure 4, except for XLI where the data correspond to a single cell line.

hydrolysis by normal fibroblasts and LCL. However, in the absence of serum, fibroblasts from MLD patients again exhibited a strong deficiency in CS metabolism (Figure 5, left); after 4 days chase, the unhydrolysed CS represented $97.7 \pm 2.6\%$ compared with $45.3 \pm 6.4\%$ in normal fibroblasts. In contrast, under similar conditions of administration, LCL from MLD, MSD and normal subjects did not show significant differences in the rates of CS degradation at any time of the chase (Figure 5, right). Therefore the ability of cerebroside sulphatase-deficient LCL (MLD and MSD) to catalyse the hydrolysis of CS was not altered by changing the composition of the incubation medium and by increasing the amount of cell-associated CS (see Figures 1 and 2).

Release of radiolabel into the incubation medium

In an attempt to understand the differences in the metabolic behaviour between fibroblasts and LCL from MLD patients, pulse-chase experiments were also performed to study the possible release of radioactivity into the chase medium. After a 20 h pulse, cells were carefully washed and incubated further in a medium devoid of CS. As shown in Figure 6, during the chase period, a considerable fraction (40–60%) of the initial cell-associated radioactivity was recovered in the chase medium of

both fibroblasts and LCL. Release of radioactivity from the cells occurred during the first 2 days of the chase and did not increase further during the following 2 days. In addition, there was no significant difference in the amount of radioactivity released between normal cells and those from patients with MLD or MSD. Analysis of the composition of the lipids present in the chase medium of fibroblasts and LCL revealed that most (about 80%) of the radioactivity was represented by undegraded CS.

Effect of chloroquine on CS metabolism by cultured cells

In order to obtain more insight into the CS metabolic pathway in LCL, the effect of chloroquine, a well-known inhibitor of lysosomal functions, on CS degradation by fibroblasts and LCL was investigated. Preliminary experiments indicated that cell viability, as assessed by the Trypan Blue-dye-exclusion test, was not dramatically impaired after 20 h of incubation up to a chloroquine concentration of $50 \mu\text{M}$ in fibroblasts and $300 \mu\text{M}$ in LCL. Under viable conditions, the uptake of CS turned out to be insensitive to chloroquine. As shown in Table 2, addition of chloroquine to the incubation medium resulted in a severe reduction in CS hydrolysis by normal skin fibroblasts. This inhibition was evident at $25 \mu\text{M}$ and was almost complete at

Figure 6 Loss of radiolabel from skin fibroblasts (left) and LCL (right) during the chase period

Normal and mutant cells were incubated for 20 h with radiolabelled CS in the presence of 10% fetal calf serum (○) or 2% Ultrosor (●), and then chased as described in the legends to Figures 4 and 5. For each chase period, the total cell-associated radioactivity was determined and is expressed as percentage of the sum of the radioactivities present in the cells and the chase medium.

Table 2 Effect of chloroquine on the *in situ* sulphatide metabolism by cultured skin fibroblasts and LCL

Normal skin fibroblasts and LCL from normal and MLD subjects were incubated for 20 h in the presence of serum with or without chloroquine and with radiolabelled CS as described in the legend to Figure 4, the chloroquine being added to the incubation medium together with the CS. *In situ* degradation rates are expressed as percentage of the values determined in cells incubated without chloroquine. The data correspond to the means of one to five separate experiments. ND, not determined (because of the cytotoxic effect of chloroquine at this concentration).

Chloroquine (μ M)	<i>In situ</i> degradation rates of radiolabelled sulphatide (%)		
	Normal fibroblasts	Normal LCL	MLD LCL
0	100	100	100
25	27	107	99
50	13	100	98
100	ND	77	97
300	ND	99	81

treated with chloroquine [41]. In contrast with that in fibroblasts, no significant inhibitory effect could be detected in LCL derived from either normal or MLD subjects even at concentrations that exceed 6–12 times those used with fibroblasts. These data suggest that the CS-degradation pathway in LCL differs from that in fibroblasts.

In vitro LCL cerebroside sulphatase activities

The experiments described in Figures 3–6 suggest the existence of an alternative pathway for CS degradation, which is apparently not mediated by the lysosomal cerebroside sulphatase. As this pathway results in the same metabolic products as that catalysed by the lysosomal cerebroside sulphatase, we looked at the presence of a CS-degrading activity in LCL homogenates. As shown in Figure 7, when the enzyme activity was measured in the presence of sodium taurodeoxycholate (under classical optimal conditions [37]), only the lysosomal cerebroside sulphatase was detected. This activity was indeed maximal at pH 5.0 in control LCL and, as expected, severely deficient in cells from patients with MLD. Under these conditions, no other activity was clearly detected in the latter cells at any other pH. On omission of the bile acid (Figure 7, inset) or its replacement by 0.1% Triton X-100 (results not shown), no CS-cleaving activity was detected even in normal cell homogenates.

50 μ M. These data are consistent with the intracellular reduction of ASA activity as previously described in cultured fibroblasts

Figure 7 *In vitro* cerebroside sulphatase activity in LCL from a normal individual and a patient with MLD

Cerebroside sulphatase activity as a function of pH was determined in duplicate on dialysed LCL homogenates from a normal individual (■) or a patient with MLD (□) in the presence or absence of detergent. The assay mixture contained radiolabelled sulphatide (2 nmol), 5 mmol/l MnCl_2 , 25 mmol/l sodium acetate buffer (pH 4.0–5.0), Tris/maleate buffer (pH 5.2–7.0), Tris/HCl buffer (pH 7.2–9.0) or glycine/NaOH buffer (pH 9.5–10.0), 3 mg/ml sodium taurodeoxycholate and enzyme (50–100 μg of protein/assay) in a final volume of 0.2 ml. After 3 h incubation at 37 °C, the enzyme activity was determined as described in the Materials and methods section. The inset shows the same assay without the presence of sodium taurodeoxycholate (the axis labels are the same as those of the main Figure).

DISCUSSION

In previous investigations, Epstein–Barr virus-transformed LCLs derived from patients with MLD or MSD were shown to have a severe deficiency in cerebroside sulphatase/ASA activity as determined *in vitro* with both natural and artificial substrates [27,40]. In the present paper, the metabolism of CS by normal and sulphatase-deficient LCL was investigated and compared with that of skin fibroblasts. To this end, the uptake and subsequent degradation of a radiolabelled CS prepared from bovine brain was followed.

Uptake experiments clearly indicated that LCL could take up the sulphatide by the same process as fibroblasts. The finding that the amounts of cell-associated CS in normal and FH cells were similar and increased linearly with its concentration in the incubation medium demonstrates that uptake of CS occurs independently of the LDL receptor and more generally by passive diffusion and/or fluid-phase endocytosis. This contrasts with the mode of uptake of other sphingolipids, e.g. ceramide [42], sphingomyelin [43] and neutral glycosphingolipids [44] which can be associated with plasma lipoproteins and taken up through LDL delivery. The mode of uptake of CS described in this (Figure 2) and other [22,23,26] studies resembles that of other acidic sphingolipids, i.e. gangliosides [45]. Furthermore, as previously described for a fluorescent analogue of sulphatide [22], the amount of cell-associated CS could be modulated by the amount of serum protein in the incubation medium. Incubation of cells in the presence of Ultrosor (a substitute containing ten times less protein than serum) resulted in increased total cell-associated CS, suggesting some kind of competition for sulphatide between the serum proteins and the cell surface. Although uptake studies did not show differences between cultured skin fibroblasts and LCL, comparison of CS-degradation patterns in

the two cell types led to the most striking observation in this paper, i.e. the existence in LCL of an alternative pathway of CS hydrolysis. The fact that CS catabolism, and not simply cell-association, indeed occurred on incubation with LCL was ascertained by the appearance of various metabolic products (see Figures 3–5). Although LCL metabolized the sulphatide less actively than fibroblasts, no significant difference in the degradation rates was observed between normal LCL and cells with a genetic deficiency of cerebroside sulphatase/ASA activity, i.e. cells from patients affected with MLD.

Several lines of evidence support the conclusion that this result was not an artifact. First, the data on the metabolic fate of CS in cultured fibroblasts, namely the clear-cut catabolic defect in cell lines from MLD patients, fully agree with the literature [8–10,12,15–19,21,23]. Second, CS degradation was observed in two different LCL from MLD patients as well as in two cell lines from patients with MSD. Third, even under experimental conditions of maximal degradation (in pulse-chase), normal and sulphatase-deficient LCL could not be distinguished: no significant difference was noted between several normal cell lines and those from patients with MLD or MSD. Finally, the active degradation noted in LCL could not be attributed to a structural modification of the sulphatide that might have occurred during the radiolabelling procedure causing a different pattern of metabolism from that of the natural substrate [43]. Indeed, the radiolabelled CS that accumulated in fibroblasts from patients with MLD did not accumulate in LCL.

The CS-degrading activity present in LCL from patients with MLD is unlikely to be due to elevated residual activity of cerebroside sulphatase/ASA, as these cell lines were established from patients affected with late-infantile or juvenile MLD, i.e. clinical forms with a severe enzyme deficiency [1]. Our previous *in vitro* studies have demonstrated that these LCL completely lack the various molecular forms of ASA [27]. In addition, in the present study we had the opportunity to compare fibroblasts and LCL obtained from the same patient with MLD (lines Faj) which gave very different metabolic data.

The possibility that the hydrolysis of CS seen on incubation with LCL could result from some extracellular (serum) activity can be ruled out because of the following observations: (i) incubation of the radiolabelled CS at 37 °C for several days in culture medium was not accompanied by substrate breakdown; (ii) almost no CS hydrolysis was detected in MLD-derived fibroblasts incubated in the presence of serum; (iii) CS was still actively catabolised in sulphatase-deficient LCL grown in the absence of serum.

Two additional interesting features were noted with respect to the metabolism of CS by both fibroblasts and LCL. Omission of fetal calf serum from the incubation medium resulted in a reduction in rates of CS hydrolysis (Figure 5). This is in agreement with the finding of Inui et al. [19] on skin fibroblasts pulsed with fatty acid-labelled CS. These authors have proposed a role for serum in the translocation of sulphatide from plasma membrane to intracellular sites of degradation. Elsewhere, the high content of undegraded CS in the chase medium of both fibroblasts and LCL suggests that some of the sulphatide bound to the cells (in a trypsin-resistant way) was released into the medium in the initial period of the chase (Figure 6). A comparable finding was obtained by Monti et al. [23] using fibroblasts labelled with a fluorescent sulphatide analogue. In our study, use of a chase medium devoid of serum also indicated that CS release was not significantly enhanced by the presence of serum, suggesting that the exchange process did not require a specific serum factor.

The data in this paper strongly suggest the existence of at least three compartments for the metabolism of CS. These are

Figure 8 Schematic representation of the possible different compartments of CS metabolism in cultured skin fibroblasts (left) and LCL (right)

In normal skin fibroblasts, most (if not all) of the sulphatide which is taken up by the cells is degraded in the lysosomal compartment (pathway II) by the lysosomal cerebroside sulphatase. No degradation occurs in fibroblasts derived from patients with MLD suggesting that there is no other catabolic compartment than the lysosomes. In both normal and MLD fibroblasts, some of the cell (most probably plasma membrane)-associated sulphatide can be released into the extracellular medium (compartment I). In LCL, the cell-associated CS can also be exchanged with the medium. However, there is virtually no hydrolysis in the lysosomes, but considerable degradation of CS is catalysed in a non-lysosomal compartment (compartment III) by a CS-degrading enzyme which is also active in lysosomal cerebroside sulphatase-deficient (MLD) cells. Compartments II and III are functionally defined compartments which do not necessarily refer to distinct subcellular localizations. See the Discussion section for further details.

demonstrated schematically in Figure 8. Compartment I corresponds to the pool of CS that is probably incorporated into the plasma membrane and can release CS molecules into the extracellular medium (Figure 6). This exchange pool probably resides in the plasma membrane, as subcellular fractionation studies performed on skin fibroblasts have already shown that a considerable fraction of the cell-associated CS remained unhydrolysed at the plasma membrane [16,19]. Our results confirmed that such a compartment also exists in LCL. The second compartment involves the translocation of CS from the plasma-membrane compartment (or perhaps directly from exogenous CS molecules) to the site of degradation. A recent observation on oligodendrocytes indicated that a fluorescent derivative of CS was translocated to the Golgi apparatus before its degradation [26]. The assumption that the lysosome is indeed the intracellular site of CS catabolism in cultured skin fibroblasts is based on subcellular fractionation studies [16,19] and on the catabolic defect observed in cells genetically deficient in the lysosomal cerebroside sulphatase, i.e. fibroblasts derived from patients with MLD and MSD [46]. The products of CS catabolism are then translocated to other subcellular compartments for further recycling or may in part be released into the extracellular medium. As fibroblasts from patients with MLD accumulate the sulphatide, one can reasonably speculate that these cells do not possess another cellular pathway of CS degradation; if present, the latter route would be very minor compared with the lysosomal one. In contrast with fibroblasts, LCL almost exclusively metabolized the CS through an apparently non-lysosomal pathway (com-

partment III), as it was fully active in cerebroside sulphatase/ASA-deficient (MLD and MSD) cells. This metabolic pathway was demonstrated in LCL by using the same experimental conditions as those employed for fibroblasts, thereby minimizing the possibility that translocation of CS to different subcellular sites was only due to different modes of administration. On the other hand, the absence of detectable storage of CS in LCL from patients with MLD (Figures 4 and 5) suggests little (or even nil) contribution of the lysosomal cerebroside sulphatase in the degradation of sulphatide. This contrasts with the active participation of lysosomes of LCL in the degradation of sphingomyelin and neutral lipids which accumulate in the LCL of their corresponding lysosomal storage disorders, i.e. Niemann-Pick and Wolman diseases [43,47].

Pathway III implicated a compartment which is functionally distinct from lysosomes. This conclusion was corroborated by the effects of chloroquine. In this study, chloroquine was used as a well-known lysosomal inhibitor because LCL from patients affected with mucopolipidosis II or III (a genetic condition in which most of the lysosomal functions are impaired) were not available and might not express the lysosomal defect in contrast with fibroblasts [48]. Whereas low concentrations (25–50 μ M) of chloroquine strongly inhibited CS hydrolysis in skin fibroblasts as well as the lysosomal hydrolysis of LDL-associated sphingomyelin in both fibroblasts and LCL (T. Levade, unpublished work), CS degradation by LCL was unaffected (Table 2). This supports the conclusion that CS metabolism in fibroblasts and LCL takes place in different functional compartments.

Several hypotheses can be postulated as to the origin of the non-lysosomal pathway present in LCL. The first possibility is that LCL might possess a cerebroside sulphatase activity other than the acidic lysosomal one. Unfortunately, *in vitro* determinations of cerebroside sulphatase activity under different detergent and pH conditions failed to demonstrate the presence of an alternative enzyme to the lysosomal cerebroside sulphatase/ASA which is clearly deficient in cells from patients with MLD (Figure 7). However, it cannot be excluded that a hitherto undescribed cerebroside sulphatase catalyses the degradation of CS in LCL. The second possibility is that lysosomal sulphatases other than ASA could degrade the sulphatide in LCL. This eventuality was eliminated because LCL derived from patients with mucopolysaccharidosis Type VI, which lack arylsulphatase B [27], degraded the sulphatide normally (results not shown), as well as cells from patients with MSD did. In addition, the involvement of the non-lysosomal sulphatase, steroid sulphatase/arylsulphatase C, was excluded on the basis of the normal metabolic pattern obtained in LCL from patients with XLI. Another possible explanation of the appearance of radiolabelled lipid products would be the occurrence of a reaction analogous to the transesterification seen in phospholipid metabolism. Finally, the action of a 'transferase' catalysing the transfer of sulphate from CS to an acceptor molecule (for instance, cerebroside) or the reversal of the synthetic pathway of sulphatide (3'-phosphoadenosine 5'-phosphosulphate + cerebroside → CS + 3'-phosphoadenosine 5'-phosphate [1]), an energy-requiring reaction, could potentially contribute to the non-lysosomal pathway of LCL. However, no such reactions were observed in fibroblasts from patients with MLD. A last alternative explanation, which cannot be rejected at the present time, would be that LCL contain some stabilizing factors that provide enough protection of the residual activity found in cells from patients with MLD to ensure normal CS degradation *in situ* (see theoretical considerations in [16]).

In conclusion, exogenously supplied sulphatide can be metabolized in cultured cells by at least two pathways. The first, active in skin fibroblasts, is lysosomal. The second has been demonstrated in Epstein-Barr virus-transformed LCL deficient in sulphatase activity and is apparently non-lysosomal. Although this study has emphasized the fact that LCL cannot be used like fibroblasts for *in situ* diagnosis of MLD, the use of LCL has permitted the demonstration of a novel pathway for CS metabolism. This study has raised a number of unresolved questions concerning this non-lysosomal pathway. What is the nature of the enzyme reaction (hydrolase or transferase)? What is its subcellular localization? Are other sphingolipid (galactosylceramide and ceramide) hydrolases involved in this pathway? Are other sulphoconjugates similarly degraded? The development of an *in vitro* assay for this new enzyme may help to answer some of these questions. However, attempts to differentiate this enzyme activity from the lysosomal ASA by the use of competitive substrates in kinetic studies have up to now been impossible, as cells from patients with MLD or MSD do not exhibit any cerebroside sulphatase/ASA or total sulphatase activity respectively that is measurable *in vivo* (see Figure 7 and its inset). Elsewhere, the physiological significance of this alternative pathway has to be elucidated as well as its contribution to the metabolism of sulphatide *in vivo* in normal and diseased states. The study of the tissue and cellular distribution of the non-lysosomal activity might be of particular importance in obtaining insight into the physiological roles of sulphatide.

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