Different Pathways of Uptake and Degradation of Sphingomyelin by Lymphoblastoid Cells and the Potential Participation of the Neutral Sphingomyelinase*

The metabolism of sphingomyelin (SPM) was investigated in Epstein-Barr virus-transformed lymphoid cell lines from normal individuals and from patients with Niemann-Pick disease Type A (deficient in the acid, lysosomal sphingomyelinase) and familial hypercholesterolemia (lacking the low density lipoprotein receptor). Cells were incubated with the following radioactive or fluorescent SPMs: [choline-methyl-14C]SPM, [oleoyl-3H]SPM, pyrene-propenoyl-SPM (P3:1-SPM), pyrene-butanoloyl-SPM (P4-SPM), pyrene-docanoyl-SPM (P12-SPM), and pyrene-sulfonilamino-undecanoyl-SPM (PSA11-SPM). Several pathways of uptake and subsequent metabolism of SPM in the lymphoblastoid cells were identified. [choline-methyl-14C]SPM and the P12-analog, administered to the cells in the presence of lipoproteins, were taken up through the apoB/E receptor-dependent pathway of endocytosis and degraded solely by the lysosomal sphingomyelinase. Under similar conditions, the other sphingomyelins, i.e., [oleoyl-3H]SPM, P3:1-SPM, P4-SPM, and PSA11-SPM, were taken up by a low density lipoprotein receptor-independent pathway and degraded mostly by a nonlysosomal sphingomyelinase which also catalyzed their hydrolysis in Niemann-Pick cells. In the absence of serum, all sphingomyelins were taken up by an apoB/E receptor-independent pathway and hydrolyzed by a nonlysosomal sphingomyelinase. Indeed, in vitro assays demonstrated the presence, in lymphoblastoid cells, of the neutral magnesium-activated sphingomyelinase, which was also fully active in the Niemann-Pick cells. In conclusion, our observations are consistent with: (i) the existence in lymphoblastoid cells of several pathways for the uptake and subsequent utilization of SPM; (ii) a major role of lipoproteins for the metabolic routing of the SPM; and (iii) the effect of the structure of the fatty acyl residue of SPM on its possible association with lipoproteins and/or cell membranes.

Sphingomyelin (ceramide phosphocholine or d-erythro-2-N-acyl-sphingosyl-1-phosphocholine, SPM) is a major constituent of most membranes of eukaryotic cells. It is found in relatively high concentrations in the plasma membrane where it plays an important role in controlling the fluidity of this membrane (1, 2). It has been proposed that biosynthesis of SPM occurs in the plasma membrane and/or the Golgi apparatus by direct transfer of phosphocholine from phosphatidylcholine to ceramide (3-11). Its intracellular degradation is achieved by the enzyme sphingomyelinase (E.C. 3.1.4.12) which produces ceramide and phosphorylcholine. Two cellular mammalian enzymes catalyzing this reaction have been described (reviewed in Ref. 12). One of these sphingomyelinases is a lysosomal hydrolase with a pH optimum at about 5.0, and is ubiquitously distributed in all mammalian tissues (12, 13). The activity of this enzyme is deficient in Niemann-Pick disease (NPD), an inherited disorder characterized by accumulation of SPM (12, 14). The second sphingomyelinase has a neutral pH optimum, is magnesium-activated, and found at high activity levels in neural tissues (15, 16). This neutral enzyme is membrane-associated (17, 18) and differs from the lysosomal enzyme since patients with Niemann-Pick disease have normal levels of the neutral activity (12, 19).

The precise pathways of SPM metabolism in vivo are not yet fully elucidated. Several investigators have used cultured skin fibroblasts both for studying the metabolism of SPM in intact living cells and for gaining insight into the nature of the defect in NPD (20-31). Several SPMs, employed in the above studies, were labeled with radioactive (20-27, 30) or fluorescent (25, 28, 29, 31) probes, thus differing in the structure of the fatty acyl moiety. They were incubated with cultured cells utilizing several modes of administration. These studies led to proposals for the existence of several pathways for SPM metabolism, involving the lysosomal (21-28) and, possibly, the neutral magnesium-dependent (23, 25, 29) sphingomyelinases, the transfer of phosphocholine to diacylglycerol (23, 24), CMP (23), or CTP (30) (this transfer being either direct (from SPM to diacylglycerol) or due to the action of a yet unidentified sphingomyelinase (30), as well as a nonmetabolizable pool due to a plasma membrane SPM recycling pathway (29, 31).

In previous investigations, cultured Epstein-Barr virus-transformed lymphoid cell lines, derived from normal individuals and Niemann-Pick disease patients, were used for study.

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§ To whom correspondence should be addressed. Tel: 33-61-32-25-33 (ext. 4272); Fax: 33-61-32-29-53.

1 The abbreviations used are: SPM, sphingomyelin; NPD, Niemann-Pick disease; LDL, low density lipoprotein; PBS, phosphate-buffered saline; P3:1-SPM, N-[3-(1-pyrene)-propenoyl]-sphingomyelin; P4-SPM, N-[4-(1-pyrene)-butanoloyl]-sphingomyelin; P12-SPM, N-[12-(1-pyrene)-docanoyl]-sphingomyelin; PSA11-SPM, N-[11-(1-pyrene)-sulfonilamino-undecanoyl]-sphingomyelin.
ing biochemical and ultrastructural features of the respective cell lines (12, 32-35). In the present study, several radioactive and fluorescent fatty acyl-SPM analogues were synthesized and administered to lymphoblastoid cells derived from normal subjects or patients with NPD Type A. Lymphoblastoid cells from a patient with apoB/E receptor-negative familial hypercholesterolemia were also used to examine the involvement of the LDL receptor-mediated pathway in the uptake and degradation of SPM. Evidence is provided for the existence in lymphoid cell lines of several pathways of uptake and degradation of SPM and for the influence of lipoproteins as well as the structure of the fatty acyl moiety of SPM on its metabolic fate.

EXPERIMENTAL PROCEDURES

Materials—[methyl-3H]Choline (77 Ci/mmol) was purchased from CEA (Gif-sur-Yvette, France); [choline-methyl-14C]Sphingomyelin (54 mCi/mmol) and [9,10-3H]oleic acid (10 Ci/mmol) from New England Nuclear Corp. (Paris, France). 1-Pyrene propionic acid (PSA11), 1-pyrene palmitic acid (P4), 1-pyrene stearic acid, 1-pyrene dodecanic acid (P12), and 11-(1-pyrenesulfonylamidino)-undecanoic acid (PSAI1) were obtained from Molecular Probes (Eugene, OR). Bovine brain sphingomyelin, N-hydroxysuccinimide, bovine serum albumin, and Histopaque-1077 were supplied from Sigma; N,N'-dicyclohexylcarbodiimide and oleic acid from Aldrich (Steinheim, Federal Republic of Germany); 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide from Behring Diagnostics; Silica Gel 60 thin-layer chromatography plates and aluminium sheets from Merck (Darmstadt, Federal Republic of Germany). All solvents and other reagents obtained from Merck or Prolabo (Paris, France) were of analytical grade.

RPMI 1640 medium, penicillin, streptomycin, L-glutamine, and fetal calf serum were purchased from Seromed (Berlin, Federal Republic of Germany). The serum substitute, Ultrasor HY, was from IBF (Villeneuve-la-Garenne, France).

Synthesis of Fatty Acid-labeled Sphingomyelins and Ceramides—The sphingomyelin derivatives, pyrene-propenoyl-SPM (P3:1-SPM), pyrene-butanoxy-SPM (P4-SPM), pyrene-pentanoyl-SPM (P5-SPM), pyrene-dodecanoyl-SPM (P12-SPM), pyrene-sulfonamido-undecanoyl-SPM (PSAI1-SPM), and [oleoyl-3H]SPM were synthesized by replacing the fatty acid of natural SPM by the corresponding fluorescent or radioactive fatty acid analogue. Bovine brain SPM was deacylated by acidic methanolysis (36) and the liberated sphingosyl-phosphorylcholine was purified by preparative thin-layer chromatography and corresponded to 97-99%. The SPM analogues were synthesized by using N-hydroxysuccinimide and N,N'-dicyclohexylcarbodiimide from Behring Diagnostics; Silica Gel 60 thin-layer chromatography and corresponded to 97-99%. The SPM analogues were synthesized by replacing the fatty acid of natural SPM by the corresponding fluorescent or radioactive fatty acid analogue. The final concentration of ethanol did not exceed 0.7% (by volume). The concentration of the total natural sphingomyelin contained in the fetal calf serum was less than 5-11 nmol/ml (24, 47). However, since all the experiments were not performed in the presence of serum, the amount of natural SPM contained in the fetal calf serum (when present) was not considered in the calculation of the amounts of total cell-associated radioactivity or fluorescence. In the experiments with endogenous SPM, the modes of uptake and degradation of the administered labeled SPMs. Using different concentrations of endogenous SPM (by varying the amount of serum in the incubation medium) did not result in any change of the metabolic behavior of the various SPMs. After incubation at 37 °C for 24-48 h, the “pulse” experiments were terminated by centrifuging the cells. After removal of the incubation medium, the cells were washed three times with PBS containing bovine serum albumin (2 mg/ml) and then twice with PBS alone (48). The cell pellets were stored at -20 °C.

In “pulse-chase” type experiments, after 48 h incubation with endogenous SPM, the cells were sedimented by centrifugation, washed in RPMI 1640 medium containing fetal calf serum (10%) and bovine serum albumin (2 mg/ml), and suspended in fresh medium supplemented with 10% fetal calf serum. The cells were distributed in several Petri dishes and incubated further for the indicated times, after which they were processed as described above for pulse experiments.

There was no significant difference in cell recovery and protein content during incubations with the various SPMs, nor was the cell viability altered by incubating the cells with these substrates.

Lipid Extraction and Analyses—Cell pellets were suspended in 0.5 ml of distilled water and sonicated for 3 × 15 s (Sonoprep MSE sonicator). An aliquot was taken for protein determination (49, 50) and the remaining was extracted with 2.5 ml of chloroform/methanol (2:1, by volume), vortex mixed, and centrifuged at 1000 × g for 15 min (24).

When using [choline-methyl-14C]SPM, the upper aqueous phase of the Folch extract (containing the radioactive phosphorylcholine) was
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counted in a scintillation spectrometer (Packard Tricarb 4500, equipped with the disintegrations/min option) using 10 ml of Pico-fluor 15. In the labeling experiments with radioactive choline or when using radiolabeled exogenous SPMs, an aliquot of the lower phase was also counted and the lipid extract was subjected to a mild alkaline hydrolysis (52) as follows: after evaporating the lower phase under a nitrogen stream, the residue was dissolved in 0.25 ml of chloroform, 0.25 ml of 0.5 M methanolic NaOH and incubated for 2 h at 37 °C. After adding 0.85 ml of chloroform, 0.25 ml of 0.5 M methanolic HCl, and 0.43 ml of water, mixing, and centrifuging, the upper phase (containing the choline label released from phosphatidylcholine) was counted. The organic phase of the Folch extract after mild alkaline hydrolysis (containing the sphingomyelin) was evaporated under nitrogen, dissolved in a reduced volume of chloroform/methanol, and counted. Alternatively, for estimating the amounts of radiolabeled phosphatidylcholine and SPM in the extract, the lipids were resolved by analytical thin-layer chromatography in chloroform/methanol/water (100:42:6, by volume) and the plate analyzed using a Berthold LB 2832 radiochromatoscan.

When using the fluorescent derivatives of sphingomyelin, the upper phase of the Folch extract was discarded and the fluorescence intensity of the lipid phase was determined at the appropriate excitation and emission wavelengths (343 and 378 nm for P4, P5 and P12, 351 and 378 nm for PSA11 derivatives, respectively). This organic phase was then evaporated under nitrogen and dissolved in heptane/isopropyl alcohol/water for the separation of fluorescent catabolic SPM products. Briefly, in the experiments with PSA11-, P4-, P5- and P12-SPMs, 1.25 ml of acid Dole's mixture (heptane, isopropyl alcohol, 0.5 M H2SO4, 40:10:1, by volume), 0.75 ml of isooctyl alcohol, and 1.00 ml of water were added to the lipid residue (50). In the experiments with PSA11-SPM, 1.50 ml of heptane, 0.45 ml of isooctyl alcohol, and 0.45 ml of water were added; after mixing and centrifuging, the upper phase was separated and back-washed with 0.35 ml of water (54). The fluorescence intensity of the upper heptane-rich phases, containing fluorescent ceramide (28, 54), was measured. For quantification, the fluorescence intensity of standards of the various fluorescent sphingomyelins and ceramides in the appropriate solvents was recorded. Further identification of the fluorescent metabolites was performed by thin-layer chromatography developed in chloroform/methanol/water (100:42:6, by volume) and chloroform/methanol/acetic acid (96:4:1, by volume).

Measurement of In Vitro Sphingomyelinase Activities—Sphingomyelinase activity was assayed on lymphoblastoid homogenates prepared by sonication (3 × 15 s) of frozen cell pellets suspended in distilled water. Enzyme activity was determined according to previously described procedures, with the following substrates: [choline-methyl-14C]SPM (55); P3:1-, P5-, P12-, and [oleoyl-3H]SPMs (53) and PSA11-SPM (54). Unless otherwise stated, the acid sphingomyelinase was assayed in the presence of sodium acetate buffer (pH 5.0) (250 mmol/liter) and EDTA (5 mmol/liter) and the neutral sphingomyelinase in the presence of Tris maleate buffer (pH 7.0) (250 mmol/liter) and MgCl2 (5 mmol/liter) (54, 55). Hydrolysis of SPM was linear with time for 120 min at 37 °C (33). The specific activity of the neutral magnesium-stimulated sphingomyelinase was calculated by subtracting the activity measured, at pH 7.0, in the presence of EDTA from that in the presence of MgCl2. In the studies of the effect of pH, no considerable buffer effect on the sphingomyelinase activity was noted. For example, at pH 5 there was no difference using sodium acetate or Tris maleate buffers.

Statistics—The Student t test was used in the statistical analysis, with p < 0.05 considered to be a significant difference between two means.

RESULTS

The metabolism of SPM was investigated in normal and Niemann-Pick disease Type A Epstein-Barr virus-trans-
formed lymphoblastoid cells by studying the degradation of endogenous SPM, labeled with tritiated choline, of several radioactive and fluorescent SPMs which were administered to the cells.

Metabolism of Intracellular [methyl-H]Choline-labeled SPM—Preincubation of lymphoblastoid cells for 6 days with [methyl-3H]choline (pulse) resulted in a similar incorporation in the SPM of normal and NPD cells (data not shown). Further incubation for 3 weeks in a medium without [methyl-3H]choline ("chase") led to a considerable decrease (70%) of the radioactivity of either cell type. Similar data were obtained for phosphatidylcholine, although a much greater incorporation (4-7 times) and a higher turnover rate of this phospholipid relative to SPM were observed. This resulted in an increasing ratio of SPM to phosphatidylcholine during a prolonged chase period. No significant difference in the SPM/phosphatidylcholine ratios was observed between the NPD lymphoblastoid cells and their normal counterparts. Since under these experimental conditions of endogenous labeling, the study of SPM metabolism is overshadowed by the considerably greater turnover of phosphatidylcholine, subsequent experiments utilized exogenous SPMs for administration into the cells and following their intracellular metabolism.

Uptake and Metabolism of Exogenous Radioactive and Fluorescent SPMs—The uptake and catabolism of two radioactively labeled and several fluorescent derivatives of SPM were studied in lymphoblastoid cells of normal individuals or Niemann-Pick disease Type A patients. In a previous study of SPM metabolism in cultured skin fibroblasts, we used a pyrene-containing SPM and showed association of this SPM with the fetal calf serum lipoproteins (28). To investigate the potential role of lipoproteins in the uptake of SPM by lymphoid cells, the respective compounds were presented to the cells in the absence or presence of fetal calf serum. Uptake of SPM was also examined in cells derived from a familial hypercholesterolemia patient which lack the apoB/E receptor. Based on the data of cell-association and degradation, two separate metabolic groups of SPM were defined: one group includes the choline-labeled SPM and the P12 analogue (Fig. 2); the second is represented by [oleyl-H]SPM and the fluorescent P3-1, P4-1, P5-1, and PSA11 derivatives (Fig. 3). Metabolism of Exogenous SPMs in the Presence of Serum—As shown in Fig. 2 (A and C), the extents of uptake of the radioactive choline-labeled SPM or P12-SPM by familial hypercholesterolemia lymphoid cells were considerably lower (p < 0.001) than those observed in normal or NPD cells. Thus, in the presence of serum, the control lymphoblastoid cells incorporated 5.5 to 7.5 times more P12-SPM or [choline-methyl-14C]SPM than the corresponding apoB/E receptor-negative cells, suggesting an LDL receptor-mediated pathway for the endocytosis of these SPM molecules. Although the impact of genetic variation between different donors has not been explored, the difference in SPM uptake between familial hypercholesterolemia and normal cells does not seem to be due to such a variation since lymphoblastoid cells from patients with various metabolic diseases also showed uptake values much higher than those of familial hypercholesterolemia cells (data not shown). When the normal and NPD cells were incubated for 48 h with these respective SPMs, more radioactivity or fluorescence intensity was noted in the latter cells (Fig. 2, A and C). This is most probably due to much lesser (p < 0.001) degradation because of the deficient acid sphingomyelinase activity in NPD cells (see below). Indeed, when the degradation of the administered SPMs was followed by measuring the appearance of breakdown products in the cell extracts (i.e. radioactive phosphatidylcholine and water-soluble products (phosphocholine) for [choline-methyl-14C]SPM or fluorescent P12-ceramide for P12-SPM), practically all metabolism was detectable in NPD lymphoblastoid cells (Fig. 2, B and D). Thus, while in normal lymphoid cells 60-65% of the [choline-methyl-14C]SPM was metabolized in 2 days and converted to a mixture of phosphatidylcholine and phosphocholine, only 4.5% was degraded in NPD cells, thereby correlating with the profound acid sphingomyelinase deficiency of these cells (see Fig. 7 and Refs. 32-34). Such a catabolic defect was found in two other NPD cell lines, but not in cells from other lysosomal disorders which do not involve acid sphingomyelinase (data not shown). Similar data were observed using a bovine brain SPM tritiated by catalytic hydrogenation (data not shown) and with P12-SPM. As seen in Fig. 2D, the rates of degradation of the fluorescent SPM in normal cells were lower than those measured with the radiolabeled natural SPM (Fig. 2B). The above experiments also indicated some catabolic impairment in familial hypercholesterolemia lymphoblastoid cells, as previously observed by Mazière et al. (26) on cultured skin fibroblasts. This dysfunction is probably linked or secondary to the LDL receptor mutation inherent in these cells. The possibility of involvement of another dysfunction in the defective uptake of SPM is not excluded. However, it seems very unlikely that an additional mutation (for example) exists in all the fibroblast and lymphoblast lines tested so far by us and Mazière's group.

A different set of data was obtained with other derivatives
phoblastoid cells (Fig. 3). Uptake of these SPMs occurs by a mode which is not dependent on the LDL receptor. Furthermore, considerable hydrolysis of the fluorescent SPMs was observed in the acid sphingomyelinase-deficient (NPD Type A) lymphoblastoid cells, as measured by the appearance of fluorescent ceramide (Fig. 3, B, D, and F). Of considerable interest was the finding (Fig. 3, G and H) that both uptake and degradation of \([\text{octoyl-}^3\text{H}]\) SPM resembled the patterns observed with the second group of SPMs (i.e., P3:1-, P4-, and PSA11-SPMs) rather than the first group, which also included a radioactively labeled SPM. In the experiments of Fig. 3, depending on the SPM analogue and the control cell line, the rates of degradation in NPD cells represented 21-72 and 67-107% of those found in normal and hypercholesterolemia lymphoblastoid cells, respectively. These results are in marked contrast with the data obtained when using the previous group of SPMs, where the rates of degradation in NPD cells did not exceed 7 and 15% for \([\text{choline-methyl-}^{14}\text{C}]\)SPM and P12-SPM, respectively, when compared to the normal cell lines and 18 and 22% when compared to familial hypercholesterolemia cells. This strongly suggests that the catabolism of the second group of SPMs occurs for the most part through a nonlysosomal pathway.

The differences in the metabolic behavior between the two groups of SPM are further emphasized by comparing pulse-chase type experiments. Lymphoblastoid cells were incubated with the various SPM derivatives for 48 h, washed, and then incubated in a full medium devoid of labeled SPM. Fig. 4 reports the fate of these SPMs during the chase period. As illustrated in Fig. 4, A and B, \([\text{choline-methyl-}^{14}\text{C}]\)SPM and P12-SPM were degraded in the normal but not the NPD Type A cells during the chase. The unhydrolyzed SPM in NPD
cells remained constant with time of chase at least up to 3 days. Thus, while at the beginning of chase the NPD lymphoblastoid cells showed values for the undegraded radioactive and fluorescent SPMs exceeding 6- and 1.4-fold, respectively, those in normal cells (as calculated from the data given in the legend to Fig. 4), after 3 days of chase these values increased to 14- and 2-fold, correspondingly. As already observed in Fig. 2D, the rates of hydrolysis of P12-SPM were lower than those of radiolabeled SPM. Also, it is of interest to note that the catabolic rates in normal cells leveled off after 24-48 h.

Using P3:1-SPM and PSA11-SPM, a metabolic pattern very different from the above was obtained (Fig. 4, C and D). With the latter two SPM analogues, little differences between the normal or NPD (as well as familial hypercholesterolemia) cells were observed, the amounts of unhydrolyzed SPM in all cell lines decreasing rapidly to about 20-40% of the values determined at the beginning of the chase period. This again indicates that the SPMs of the second group are degraded by a different pathway, probably by a nonlysosomal sphingomyelinase.°

Metabolism of Exogenous SPMs in the Absence of Serum—To further examine the role of fetal calf serum and particularly of lipoproteins in the mode of uptake of the various SPM derivatives, these were incubated with lymphoblastoid cells in medium without serum and supplemented with Ultroser HY, i.e. a serum substitute devoid of lipoproteins. Under these conditions, [choline-methyl-14C]SPM and P12-SPM were taken up by all lymphoblastoid cell lines, including those of familial hypercholesterolemia (Fig. 5, A and C). As for degradation (Fig. 5, B and D), the extents of hydrolysis of the radiolabeled and P12-SPM were somewhat lower than in the presence of serum (see Fig. 2, B and D). No significant difference in the degradation extents was observed between control and acid sphingomyelinase-deficient NPD Type A cells.

When using P3:1-SPM or PSA11-SPM (Fig. 6), in the presence of the serum substitute, results very similar to those described in Fig. 5 (i.e. for [choline-methyl-14C]SPM and P12-SPM in the absence of serum) as well as those in Fig. 3 (i.e. with P3:1- and PSA11-SPM in the presence of serum) were obtained with respect to uptake and degradation. Similar data were also observed when the experiments were repeated using medium supplemented with 10% lipoprotein-deficient serum (data not shown).

The similarity of the data of Figs. 5 and 6 indicates that, in the absence of full serum, the SPMs of either group get associated to the cells through an LDL receptor-independent pathway and are hydrolyzed by a nonlysosomal process.

In Vitro Sphingomyelinase Activities—The experiments described in Figs. 3-6 indicate the existence of an alternate pathway for SPM degradation, which is not mediated by the lysosomal sphingomyelinase but which results in ceramide as the catabolic product. We therefore expected the presence of a nonlysosomal sphingomyelinase activity in lymphoblastoid cell homogenates. Fig. 7 corroborated this and demonstrated that, in addition to the pH 5.0 lysosomal enzyme, normal lymphoid cells contain a neutral sphingomyelinase activity with a pH optimum close to 7.0. This neutral sphingomyelinase which required magnesium ions and was inhibited by chelating agents such as EDTA (Fig. 7A) was also present in the NPD Type A lymphoblastoid cells that are deficient in the lysosomal acid sphingomyelinase. While the NPD cells exhibited virtually no activity at pH 5.0, that of the neutral enzyme was similar to normal cells (Fig. 7B). Table I shows that all the respective SPMs employed in this study were substrates for the neutral sphingomyelinase in vitro; similar values were observed in extracts of normal, NPD, or familial hypercholesterolemia cells.

In the experiments of Table II, several potential activators and inhibitors of sphingomyelinase activities were tested at pH 5.0 or 7.0. The activity of the neutral enzyme was strongly affected by divalent cations: Ca2+ inhibited it while Mn2+ and Mg2+ stimulated. The observed specificity of ion requirement is very close to that reported for the neutral sphingomyelinase from other tissues (15, 17, 56–59). EDTA completely inhibited the pH 7.0 activity (see also Fig. 7A). Table II and Fig. 7 show that while in the presence of EDTA, NPD cells have no sphingomyelinase activity whatsoever, normal cells still exhibited at pH 7.0 some activity, most probably due to the lysosomal enzyme. Dissimilar to the acid sphingomyelinase, the neutral enzyme was strongly inhibited by Hg22+ and phenylmercuribenzoate but was unaffected by AMP, as previously noted for other enzyme sources (17, 58, 57).

DISCUSSION

In this study, we investigated the metabolism of sphingomyelin in Epstein-Barr virus-transformed lymphoid cell lines established from normal subjects and from patients with sphingomyelin lipidosis (NPD Type A), which are characterized by a severe deficiency of the acid lysosomal sphingomyelinase in vitro activity (32, 34) and by lysosomal accumulation of SPM (33, 35). In initial experiments, the endogenous SPM pool was labeled by incubating the cells with [methyl-3H]choline, but this procedure did not provide clear cut data on the metabolic defect in the NPD lymphoblastoid cells. Similar data were reported by Spence et al. (23) using cultured skin fibroblasts and probably stem from the fact that the major compartment of SPM is not lysosomal but plasma-membrane related (60). Therefore, other experiments utilized exogenous SPM for studying its intracellular catabolism. The use of various SPM analogues and modes of administration has provided evidence for the existence in lymphoblastoid cells of several pathways for uptake and cellular degradation of SPM. In this respect, two groups of SPMs could be defined. The first one included [choline-methyl-14C]SPM and the P12 analogue, whereas the second was represented by [oleoyl-3H]SPM and the fluorescent P3:1, P4, P5, and PSA11 derivatives.

When comparing normal and familial hypercholesterolemia lymphoblastoid cells, uptake of the SPMs of the first group was considerably lower in the latter cells, which lack the apoB/E receptor, suggesting an LDL receptor-mediated mode of uptake of these compounds. Because of the receptor-mediated endocytic pathway it is expected that the [choline-methyl-14C]SPM or P12-SPM will be directed to the lysosomal compartment of the cells (48). Indeed, a lysosomal degradation of these two respective compounds is supported by the nearly complete absence of their hydrolysis in the NPD Type A cells which are deficient in the lysosomal sphingomyelinase activity (Figs. 2 and 4).

A second pathway for the catabolism of SPM was suggested by the study of the uptake and degradation of the second
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**Fig. 5.** Uptake (left) and degradation (right) of [choline-methyl\(^{14}\)C]SPM (A and B) and P12-SPM (C and D) by lymphoblastoid cells in the absence of fetal calf serum. Normal LDL receptor-negative familial hypercholesterolemia (FH) and Niemann-Pick disease Type A (NPD A) lymphoblastoid cells were incubated for about 48 h in the presence of 2% Ultroser HY, a serum substitute, either with [choline-methyl\(^{14}\)C]SPM (121,500 dpm/nmol; 0.66 nmol/ml) or with P12-SPM (about 4 nmol/ml). See legend to Fig. 2 for further details.

**Fig. 6.** Uptake (left) and degradation (right) of P3:1-SPM (A and B) and PSA11-SPM (C and D) by lymphoblastoid cells in the absence of fetal calf serum. Normal LDL receptor-negative familial hypercholesterolemia (FH) and Niemann-Pick disease Type A (NPD A) lymphoblastoid cells were incubated in the presence of 2% Ultroser HY, a serum substitute, for 24 h with 2 nmol/ml of P3:1-SPM or 1.5 nmol/ml of PSA11-SPM. See legend to Fig. 3 for further details.

group of SPM derivatives, i.e. P3:1-, P4-, P5-, PSA11-, and [oleoyl-\(^3\)H]SPMs. In the presence of serum, their association to lymphoid cells was independent of the apoB/E receptor as evident from the finding that there was no reduced uptake by the familial hypercholesterolemia cells (Fig. 3). These SPMs also differed from the first SPM group by their mode of degradation which occurred mostly in a nonlysosomal compartment, a conclusion supported by the fact that even in the NPD cells a substantial breakdown of these substrates was present (Fig. 3). The data of pulse experiments, which compared the catabolic rates in normal and NPD cells (Fig. 3), suggest that up to 75% of SPM degradation proceeded independently of the lysosomal sphingomyelinase. This assumption is further corroborated by the experiments of Fig. 4 which showed that, following a prolonged chase, only a small fraction of the original SPM still remained in the NPD cells.

The extensive metabolism of the SPMs of the second group might be related to differences in the degree of uptake of the two groups. However, when comparing the data obtained using similar incubation times and substrate concentrations, the P4-, P5-, and P12-SPMs exhibited similar extents of uptake. Under these conditions the P12 derivative was incorporated at about the same level as the commercially choline-labeled SPM (data not shown). However, the P3:1- and PSA11-SPM derivatives indeed showed higher levels of uptake. But it seems very unlikely that the degree of incorporation can affect the modes of uptake (LDL receptor-mediated or independent) or degradation (lysosomal or non-lysosomal) because of the following observations. First, using low substrate concentrations (0.5-1.0 nmol/ml of medium) of P3:1-SPM or PSA11-SPM resulted in uptake levels comparable to those of the first SPM group. Under these conditions, metabolic behaviors identical to those reported in Fig. 3 (left and right) were obtained with P3:1-SPM or PSA11-SPM. Second, while the degree of uptake of choline-labeled SPM was similar in the presence or absence of serum (about 150 pmol/mg for the cell-associated radioactivity in normal cells), different patterns of metabolism were observed (Figs. 2 and 5). This was also true for the P12-SPM derivative. Third,
Protein concentration in the assays ranged from 150 to 430 fig. Each determination was performed at least in duplicate. Enzyme (480 and 430 fig of protein/assay for normal and NPD A experiment as cells from two normal (line Bea in experiments 1 and 2, line C49 in experiment 1, line MS-325 in experiments 2 and 3) lines. The mia (FH), and two Niemann-Pick disease Type A (NPD A, line ElG) lines. The activities of the two respective enzymes were determined as described under “Experimental Procedures.”

**Fig. 7. In vitro activities of the acid and neutral sphingomyelinas in lymphoblastoid cells of a normal individual and a patient with Niemann-Pick disease Type A (NPD A).** Sphingomyelinase activity, as a function of pH, was determined in duplicate on homogenates of lymphoid cell lines using PSA11-SPM as substrate. The assay mixture contained 100 nmol/ml fluorescent SPM, 1 mg/ml Triton X-100, 5 mmol/liter of EDTA (Fig. 7A) or MgCl₂ (Fig. 7B), 250 mmol/liter of sodium acetate buffer (pH 3.6-5.5), Tris maleate buffer (pH 5.2-7.0), or Tris-HCl buffer (pH 7.2-8.5) and enzyme (480 and 430 fig of protein/assay for normal and NPD A lymphoid cell homogenates, respectively) in a final volume of 0.2 ml. After 2 h incubation at 37 °C, the enzyme activities were determined as described under “Experimental Procedures.”

**TABLE I**

Activities of the acid and the neutral magnesium-dependent sphingomyelinas in lymphoblastoid cell homogenates

The activities of the two respective enzymes were determined as described under "Experimental Procedures" using lymphoblastoid cells from two normal (line Bea in experiments 1 and 2, line C49 in experiment 3), an LDL receptor-negative familial hypercholesterolemia (FH), and two Niemann-Pick disease Type A (NPD A, line ElG in experiment 1, line MS-325 in experiments 2 and 3) lines. The protein concentration in the assays ranged from 150 to 430 μg. Each determination was performed at least in duplicate.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Substrate</th>
<th>Acid sphingomyelina</th>
<th>Neutral sphingomyelina</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Normal FH</td>
<td>NPD A</td>
</tr>
<tr>
<td>1</td>
<td>[choline-¹⁴C]SPM</td>
<td>2.30</td>
<td>1.85</td>
</tr>
<tr>
<td>2</td>
<td>P3:1-SPM</td>
<td>0.40</td>
<td>0.00</td>
</tr>
<tr>
<td>3</td>
<td>P3:1-SPM</td>
<td>1.43</td>
<td>1.42</td>
</tr>
<tr>
<td>4</td>
<td>P3:1-SPM</td>
<td>0.34</td>
<td>0.30</td>
</tr>
<tr>
<td>5</td>
<td>PSA11-SPM</td>
<td>3.14</td>
<td>3.39</td>
</tr>
<tr>
<td>6</td>
<td>P5-SPM</td>
<td>2.88</td>
<td>2.79</td>
</tr>
<tr>
<td>7</td>
<td>[oleoyl-H]SPM</td>
<td>3.20</td>
<td>3.60</td>
</tr>
<tr>
<td>8</td>
<td>P12-SPM</td>
<td>1.05</td>
<td>1.18</td>
</tr>
<tr>
<td>9</td>
<td>[choline-¹⁴C]SPM</td>
<td>0.62</td>
<td>1.01</td>
</tr>
<tr>
<td>10</td>
<td>PSA11-SPM</td>
<td>2.00</td>
<td>2.07</td>
</tr>
<tr>
<td>11</td>
<td>P5-SPM</td>
<td>2.98</td>
<td>2.84</td>
</tr>
<tr>
<td>12</td>
<td>[oleoyl-H]SPM</td>
<td>3.68</td>
<td>4.05</td>
</tr>
</tbody>
</table>

**TABLE II**

Comparison of various activators and inhibitors of acid (pH 5.0) and neutral (pH 7.0) sphingomyelinases of lymphoblastoid cells

Each determination of enzyme activity was performed in duplicate, using 2-4 normal lymphoid cell lines and using PSA11-SPM as substrate.

<table>
<thead>
<tr>
<th>Effector Concentration</th>
<th>Sphingomyelinase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 5.0</td>
</tr>
<tr>
<td>None</td>
<td>91</td>
</tr>
<tr>
<td>EDTA 5</td>
<td>100*</td>
</tr>
<tr>
<td>MgCl₂ 5</td>
<td>102</td>
</tr>
<tr>
<td>MnCl₂ 5</td>
<td>103</td>
</tr>
<tr>
<td>ZnCl₂ 5</td>
<td>46</td>
</tr>
<tr>
<td>CaCl₂ 5</td>
<td>91</td>
</tr>
<tr>
<td>HgCl₂ 0.12</td>
<td>72</td>
</tr>
<tr>
<td>p-OHMB 5</td>
<td>127</td>
</tr>
<tr>
<td>AMP 2</td>
<td>12</td>
</tr>
</tbody>
</table>

* The activities under these conditions were set at 100%.

† p-OHMB, p-hydroxymercuribenzoate.

‡ Activities measured in the presence of 5 nmol/liter of MgCl₂.

under comparable experimental conditions, choline- and oleic acid-labeled SPMs exhibited different metabolic behaviors although their rates of uptake by normal cells were similar (Figs. 2 and 3).

In normal lymphoblastoid cells, the lysosomal pathway led to the formation of ceramide (as indicated by the appearance of P12-ceramide) and to the release of choline-labeled water-soluble products (phosphocholine) with the labeling of phosphatidylcholine. The latter labeling is probably consecutive to the cleavage of SPM by the lysosomal sphingomyelinase, followed by the incorporation of the released phosphocholine into phosphatidylcholine, as described by Spence et al. (30). The absence of either phosphorylcholine or phosphatidylcholine in NPD Type A cells suggests that, under our experimental conditions, no appreciable direct transfer of phosphocholine from SPM to diacylglycerol (23, 24) occurred and that the lysosomal pathway is the main way of degradation of [choline-methyl-¹⁴C]SPM and P12-SPM delivered to the cells in the presence of serum. It was of interest to note that, in normal lymphoblastoid cells, considerable quantities of radioactive water-soluble products were recovered while in skin fibroblasts they comprised only about 5% of the total radioactivity (22, 26, 27). This indicates that in lymphoid cells the reutilization process of phosphocholine is slower than the rate of its release from SPM. Using P12-SPM as substrate, its hydrolysis rates were lower than those of the corresponding radiolabeled SPM (Figs. 2 and 4). Since these respective two compounds are similarly hydrolyzed by the lysosomal sphingomyelinase in vitro (54), the reasons for the differences observed in the intact cells need yet to be clarified.

The data of this paper suggest at least two separate pathways of SPM uptake and cellular degradation. These, as well as other potential pathways are summarized graphically in Fig. 8. In this figure, we propose three main pathways. The first (Pathway 1) is characterized by endocytotic uptake of SPM through the apoB/E receptor, trafficking to the lysosomes, and degradation by the acid sphingomyelinase. The second pathway is characterized by an LDL receptor-independent cell-association of the SPM (adsorption of lipid or lipid particles to the cell surface, or intercalation in the membrane bilayer), followed by its nonlysosomal hydrolysis (Pathway 2). The third proposed pathway again involves a receptor-
Sphingomyelin Metabolism in Lymphoblastoid Cells

FIG. 8. Schematic representation of the possible different pathways for uptake and degradation of sphingomyelin by lymphoid cell lines. In the presence of serum, the natural SPM and the P12 derivative are internalized through the apoB/E receptor-dependent pathway of endocytosis and degraded by the lysosomal sphingomyelinase (Pathway 1). These two SPMs, when incubated in medium devoid of serum, as well as the other SPMs are taken up independently of the LDL receptor and hydrolyzed mainly through a nonlysosomal pathway (Pathway 2) by the magnesium-dependent neutral sphingomyelinase. A minor part of the catabolism of these compounds takes place in the lysosomes (Pathway 3). Some of the SPM molecules might also enter a futile cycle (Pathway 4) resulting in a nonmetabolizable pool. See "Discussion" for further details.

Independent binding of the SPM to the cell membrane, followed by trafficking into the lysosomes, where it is most likely degraded by the acid sphingomyelinase. The SPMs of the second group (i.e.: P3-1-, P4-, P5-, PSA11-, and [oleoyle-3H] SPMs) are processed by the latter two pathways, Pathway 2 representing the major catabolic route. When the SPMs were incubated with the lymphoblastoid cells in medium devoid of serum or lipoproteins, even the SPMs of the first group were metabolized by Pathways 2 and 3 (Fig. 8). Elsewhere, for the SPMs of the first group administered in the presence of serum, the possible involvement of Pathway 2 to account for the faint but detectable degradation ability of NPD cells cannot be definitely excluded.

These data emphasize the role of lipoproteins both for the mode of uptake and degradation of SPM. Thus, to obtain an LDL receptor-mediated endocytic uptake of the SPMs of the first group, serum must be present. Alternatively, the latter could be replaced by a mixture of LDL and lipoprotein-deficient (human or bovine) serum. This suggests the possible involvement of a factor present in the lipoprotein-free serum required for an efficient association and/or transfer of natural or P12-SMP to LDL. Pathway 1 could also be reproduced for P12-SMP (but not for P3-1-SPM) by administering the sphingolipid together with pure apoE to fibroblasts or lymphoblastoid cells. The (in)ability of the various SPM analogues to integrate (or stick) into the LDL or the plasma membrane, their respective physicochemical state in the culture medium, and the molecular mechanisms of their association with the phospholipid layers represent the major questions raised by the present investigation. Preliminary results from time course kinetic studies of the excimer to monomer ratio of the pyrene-SPM fluorescence emission suggest complex mechanisms for the association of labeled SPM with the lipoproteins that are influenced by the structure of the fatty acyl residue of SPM, and by the presence in the lipoprotein-deficient serum of at least two factors, possibly phospholipid transfer proteins (61), one being heat-stable the other heat-labile. These physicochemical studies require further extensive investigation.

Pathway 2 implicated a nonlysosomal and, most probably a plasma membrane-related sphingomyelinase. Indeed, in vitro determinations of sphingomyelinase activity have demonstrated the presence of the neutral magnesium-dependent enzyme whose activity equaled that of the lysosomal acid sphingomyelinase. Spence et al. (56) have recently described a sphingomyelinase in serum which is stimulated by Zr+ ions. However, its properties differ from those of the neutral activity of the lymphoblastoid cells (Table II). Our data, which fully agree with previous observations on the properties of the neutral sphingomyelinase, suggest that the lymphoblastoid cell enzyme is identical with the plasma membrane-bound neutral sphingomyelinase which predominates in neural tissues (15-18).

A possible involvement of a serum-sphingomyelinase in the in situ nonlysosomal degradation of SPMs is excluded since hydrolysis of SPMs by the intact lymphoblastoid cells also occurred in the absence of serum. Furthermore, in the presence of serum, the NPD cells did not hydrolyze the externally supplied [choline-methyl-14C]SPM or P12-SPM. On the other hand, no detectable acid or neutral sphingomyelinase activity was found in the fetal calf serum we used (which is heat-inactivated), either in the presence of magnesium or zinc ions (data not shown). This result was not surprising since the serum sphingomyelinase described by Spence et al. (56) is heat labile. The action of a "transferase" catalyzing a direct transfer of phosphocholine from SPM to diacylglycerol to form phosphatidylcholine (23, 24), could potentially contribute to the nonlysosomal pathway described in this paper. However, such a possibility is minimized by the observation that, using [choline-methyl-14C]SPM in the presence of serum, very little phosphatidylcholine was produced in NPD cells (Fig. 2B). Finally, it is possible that a hitherto undescribed nonlysosomal sphingomyelinase or the combined action of a phospholipase D and a phosphatase (undetected in vitro) provide some contribution to the nonlysosomal mode of degradation.

In addition to the metabolic pathways depicted above (Fig. 8), a SPM recycling pathway, resulting in a nonmetabolizable compartment might also exist in lymphoblastoid cells (Pathway 4). The presence of such a pathway is indicated by the data of the pulse-chase experiments which showed a plateau in the SPM degradation (Fig. 4). Similar observations have been reported on fibroblasts (24, 27) and would be consistent with the conclusions of Koval and Pagano (29, 31) for a plasma membrane lipid recycling pathway.

The present study also emphasizes the influence of the structure of the fatty acyl residue of SPM on its uptake and utilization by lymphoblastoid cells. The two main metabolic pathways described in this study could be distinguished from

4 T. Levade and S. Gatt, unpublished observations.
6 T. Levade and S. Gatt, manuscript in preparation.
5 S. Gatt, T. Dinur, and V. Agmon, manuscript in preparation.
one another by use of SPM analogues that structurally appear to differ only in the degree of saturation (and possibly the position of the unsaturated group) of the fatty acyl amide group, or proximity of a bulky aromatic group near the amide carbonyl group. This phenomenon might explain some of the discrepancies observed when comparing the data of the studies on SPM metabolism (20–31). Differences in the fatty acyl moieties would affect the transition temperatures of the respective SPMs (2, 39) and might also influence their association with serum lipoproteins and cell membranes. The possible relation of these factors to the data of this paper is currently under investigation. Elsewhere, the difference in the metabolic behaviors between the various SPMs is probably not related to a potential modification of the SPM molecule, due to the deacylation-reacylation procedure, because of the following: (i) various SPMs prepared using the deacylation-reacylation method have already been chemically characterized (37, 39, 62); (ii) the P12-SPM derivative synthesized by this procedure behaved metabolically as the natural choline-labeled SPM; and (iii) we have shown that all the synthesized SPMs were enzymatically hydrolyzed as the natural SPM (see also Ref. 54). Concerning the differences observed between the choline and oleic acid-labeled SPMs, it has been shown that the introduction of one double bond is accompanied by strong modifications, as the decrease (from 45 to 33 °C) of the transition temperature (39). Thus, at 37 °C (e.g. under our experimental conditions) one SPM is above its gel-to-liquid crystalline transition temperature and the other below. This could result in a different physicochemical behavior. Other studies have demonstrated that the metabolic fate of SPM is related to its fatty acid composition and degree of saturation (63). In particular, these authors have reported significant differences in the metabolism of C18:0- and C18:1-SPMs.

In conclusion, exogenous SPMs can be metabolized in lymphoblastoid cells by at least two independent pathways. The first involves those molecules which are transported to the lysosomes (e.g. after LDL receptor-mediated endocytosis) and which are hydrolyzed by the lysosomal sphingomyelinase. The second is extra-lysosomal and most probably is catalyzed and which are hydrolyzed by the lysosomal sphingomyelinase. The first involves those molecules which are transported to lymphoblastoid cells by at least two independent pathways. This could result in a different physicochemical behavior. Other studies have demonstrated that the metabolic fate of SPM is related to its fatty acid composition and degree of saturation (63). In particular, these authors have reported significant differences in the metabolism of C18:0- and C18:1-SPMs.

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