Use of pyrenemethyl laurate for fluorescence-based determination of lipase activity in intact living lymphoblastoid cells and for the diagnosis of acid lipase deficiency

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Pyrenemethyl laurate (PMLes), a fluorogenic substrate for determining *in vitro* lipase activity [Nègre, Salvayre, Dagan and Gatt (1989) *Biochim. Biophys. Acta* **1006**, 84–88], has been administered to cultured lymphoblastoid cells from normal subjects and from a patient affected with Wolman disease, which is characterized by a deficiency of lysosomal acid lipase. The intracellular degradation of PMLes was dependent on the mode of administration of the substrate into the cells, and occurred by two separate pathways involving lysosomal and extra-lysosomal hydrolases. PMLes incorporated into LDL was taken up by normal lymphoblastoid cells through the apolipoprotein-B/E-receptor-mediated pathway and degraded in the lysosomal compartment, as suggested by the degradation block in Wolman cells. In contrast, when PMLes dissolved in 2% dimethyl sulphoxide was added directly to the culture medium, its hydrolysis was similar in lymphoblastoid cells from controls and from patients affected with Wolman disease, neutral lipid storage disease or familial hypercholesterolaemia. This suggested that the administered PMLes was degraded by a non-lysosomal enzyme which is not deficient in Wolman cells. This enzyme also differs from the neutral lipase system which is deficient in lymphoblastoid cells from patients with neutral lipid storage disease. When pyrenemethanol was administered directly to the

cell culture, it was only poorly acylated and was rapidly released into the culture medium. These results and the fluorescence properties of PMLes ('monomeric' emission in a hydrophobic environment and 'excimeric' emission in a hydrophilic environment) and pyrenemethanol ('monomeric' emission in a hydrophilic environment) allowed us to design a 'direct reading' procedure by monitoring (without any lipid extraction) the fluorescence of intact living cells and that of the culture medium during pulse–chase experiments. This method allowed the direct evaluation of the time course of *in situ* degradation of PMLes. In pulse–chase experiments with LDL-PMLes, the fluorescence of normal cells decreased relatively rapidly with time whereas the fluorescence of the culture medium increased concomitantly. With Wolman cells, the cellular fluorescence decreased only very slightly, whereas that of the culture medium remained at the basal level; this demonstrates the catabolic block in intact living cells from patients with Wolman disease. *In vitro* degradation of PMLes indicated the existence of two PMLes-degrading enzymes in lymphoblastoid cell homogenates: one is the acid lipase which is involved in PMLes degradation in the lysosomal compartment (and is deficient in Wolman cells), while the second is a cytoplasmic enzyme (not deficient in Wolman cells).

INTRODUCTION

Wolman disease is a rare autosomal recessive disorder characterized by a severe deficiency in the lysosomal acid lipase, resulting in massive lysosomal storage of neutral lipids, cholesteryl esters and triacylglycerols [1–4]. The neutral lipids, degraded in the lysosomal compartment of normal cells but not of Wolman cells, originate in lipoproteins [5,6] which are taken up through the apolipoprotein (apo) B/E-mediated receptor pathway in lymphoblastoid cells [7] as well as in other cell types [6,8]. This apoB/E-mediated receptor pathway is deficient in cells from patients with familial hypercholesterolaemia (FH) [6–9]. Degradation of endogenously biosynthesized triacylglycerols [10] is blocked in neutral lipid storage disorder (NLSD; also known as multisystemic lipid storage myopathy or Chanarin–Dorfman syndrome) [11–13]. Comparisons between Wolman and NLSD fibroblasts have indicated the existence of separate lysosomal and cytoplasmic catabolic pools [10].

Several fluorescent neutral lipids have been synthesized and

used as substrates for various lipases and cholesterol esterases [14–19]. The fluorescent analogues of the natural substrates, triacylglycerols and cholesteryl esters containing pyrene fatty acids, are suitable for the determination of lysosomal acid lipase and for the *in vitro* diagnosis of Wolman's disease [16,17]. Pyrene fatty acids have been used for the study of the intracellular metabolism of cytoplasmic triacylglycerols [12,20,21]. We have reported recently the synthesis and use of an acyl ester of pyrenemethanol, the pyrenemethyl lauryl ester (PMLes), and its use for continuous kinetic assays without the need for isolating the product (pyrenemethanol) liberated by the lipase activity [22,23].

We report here on studies of the degradation of PMLes in intact living cultured cells from normal subjects and from a patient affected with Wolman disease following its incorporation into low-density lipoprotein (LDL) in order to direct it to lysosomes via the apoB/E receptor pathway [5–8]. The results reported here showed that PMLes can be used for the *in situ* determination of the activity of the lysosomal lipase by reading

Abbreviations used: PMLes, pyrenemethyl laurate; PMAcEs, pyrenemethylacetyl ester; LDL, low-density lipoproteins; FH, familial hypercholesterolaemia; NLSD, neutral lipid storage disorder; PCMB, *p*-chloromercuribenzoic acid; apoB/E, apolipoprotein B/E; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide.

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the fluorescence of intact cells or/and that of the culture medium. This method has been used for determining the residual activity of acid lipase and for diagnosing Wolman disease in intact cells.

MATERIALS AND METHODS

Chemicals

Lauryl chloride, *p*-chloromercuribenzoate (PCMB), diethylparanitrophenylphosphate, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), Trypan Blue, BSA and EDTA were purchased from Sigma (St. Louis, MO, U.S.A.); pyrenemethanol was from Molecular Probes (Eugene, OR, U.S.A.); silica-gel G t.l.c. analytical plates from Merck (Darmstadt, Germany); RPMI 1640, fetal calf serum, glutamine, streptomycin and penicillin from Gibco (Cergy-Pontoise, France); Ultrosor G from IBF (Villeneuve-la-Garenne, France); cell culture flasks and multiwell culture plates from Nunc (distributed by Polyabo-Block, Strasbourg, France); Hydragel from Sebia (Issy-les-Moulineaux, France); Picofluor from Packard (Rungis, France); and the other reagents and organic solvents from Merck or Prolabo (Paris, France). PMLes was synthesized as previously described [22,23].

Cell cultures

Lymphoblastoid cell lines from normal subjects (Lau, Be) and from a patient affected with Wolman's disease (ZR) have been established under previously described conditions [24], in collaboration with Professors C. Borroni and P. Durand (Istituto Gaslini, Genova, Italia) and Dr. G. Lenoir (Centre International de Recherche sur le Cancer, Lyon, France). Lymphoblastoid cell line derived from a subject with FH (GM1459) was obtained from the NIGMS Human Genetics Mutant Cell Repository (Camden, NJ, U.S.A.).

Lymphoblastoid cells were grown in RPMI 1640 containing 10% fetal calf serum. At 24 h before the experiments, this medium was replaced by RPMI 1640 containing 2% Ultrosor HY (a lipoprotein-free serum substitute); this was used during the pulse-chase experiments.

Cell viability was assessed by the Trypan Blue exclusion test, performed as previously indicated [9], or by the MTT test according to Price and McMillan [25].

Preparation of lipoproteins and of reconstituted LDL-PMLes

LDL from the pooled plasma of healthy donors were isolated by sequential flotation according to Havel et al. [26] (using a Beckman ultracentrifuge model L8), dialysed and kept at 4 °C until use, as previously described [9,27].

PMLes was incorporated into LDL according to a procedure derived from that of Roberts et al. [28]. Briefly, a 2% PMLes solution in 2% dimethyl sulphoxide was added to 4 ml of delipidated serum, followed by addition of 2 mg of LDL, and the mixture was gently shaken overnight at 37 °C. LDL-PMLes was re-isolated by ultracentrifugation, dialysed and sterilized by filtration (through a 0.2 µm-pore-size Millipore filter) as previously described [9,27].

Uptake and *in situ* metabolism of LDL-PMLes and free PMLes

LDL-PMLes (3 nmol of PMLes/150 µg of apoB per ml of culture medium) was added to the lipoprotein-free culture medium (RPMI 1640 containing 2% Ultrosor HY) for a 24 h pulse period. At the end of the pulse period (also designated as time zero of the chase), the cells were pelleted by centrifugation,

washed twice with PBS containing 2% BSA and twice with PBS alone, and cultured again in fresh RPMI medium containing 10% fetal calf serum (chase period). At the indicated time of the chase, cells were washed twice in PBS and kept at -20 °C until use.

Alternatively, cells were pulsed with free PMLes (dissolved in dimethyl sulphoxide) and added directly into the culture medium (5 nmol of PMLes/ml of culture medium; 0.1% dimethyl sulphoxide). Pulse-chase was performed under the conditions used with LDL-PMLes.

Lipid extraction and analysis

The extraction of fluorescent lipids was performed as previously described [22,23]. Briefly, the cell pellet, after washing twice with 0.15 M PBS, pH 7.3, was suspended in 1 ml of distilled water and sonicated (four cycles of 10 s; MSE sonicator). An aliquot of the cell homogenate (0.5 ml) was extracted with 0.5 ml of ethanol and 1 ml of heptane. Under these conditions, PMLes partitioned into the upper heptane phase whereas pyrenemethanol partitioned into the lower aqueous/ethanolic phase. The fluorescence of each phase was measured under the conditions for 'monomeric' emission (excitation 340 nm; emission 380 nm) [22,23].

Direct reading of cellular fluorescence

Direct reading experiments were done in Phenol Red-free RPMI 1640 culture medium under the conditions described for Figures 1 and 2. At the indicated time, cells from a 4 ml Petri dish were separated from the culture medium by centrifugation (500 *g* for 10 min) and resuspended in 1 ml of Phenol Red-free RPMI 1640. The total cellular fluorescence and that of the culture medium were determined at an excitation wavelength of 340 nm and an emission wavelength of 380 nm.

Enzymic studies

Enzymic assays for PMLes-hydrolysing enzymes were performed under the previously described conditions [22,23]. The assay mixture contained 20 nmol of PMLes/ml, 0.2 mol/l citrate/phosphate or Tris/HCl buffer (at pH indicated below; under standard conditions, pH 4.0 for the acid lipase and pH 7.0 for the neutral enzymes) and the enzyme solution in a final volume of 1 ml. The kinetics of the enzymic reaction were followed directly in the spectrofluorimeter (excitation 340 nm; emission 380 nm). For converting the fluorescence units into enzymic activity, the fluorescence of a known concentration of pyrenemethanol in water was read under the same conditions and used as a standard. The enzymic assays for pyrenemethyl acetyl ester (PMACes)-hydrolysing enzymes were performed under the same conditions as for PMLes, but in a final volume of 100 µl. Since a direct reading cannot be done when using PMACes, the enzymic reaction was stopped at the end of the incubation time by adding water/ethanol/heptane (1:1:2, by vol.) and the fluorescence of the lower aqueous/ethanolic phase was recorded [22,23]. The activity of neutral lipase was determined using [³H]triolein as previously described [10,29].

Proteins were determined according to Lowry et al. [30].

RESULTS

Uptake of LDL-PMLes via the apoB/E receptor pathway

Uptake of LDL-PMLes was saturable in normal and Wolman cells, while in FH lymphoblastoid cells it was low and linearly related to the LDL concentration (i.e. non-saturable). These data

Figure 1 Uptake of LDL-PMLEs by cultured lymphoblastoid cells

(a) Uptake of LDL-PMLEs (15 nmol of PMLEs/mg of apoB) by cells from a normal subject (●), from a subject with Wolman disease (○) and from a subject with FH (□). Increasing concentrations of LDL-PMLEs were added to the culture medium, and after 12 h the culture medium was removed, cells were washed three times and harvested. Lipids were extracted as indicated in the Materials and methods section and the fluorescence was determined (excitation 340 nm; emission 380 nm) and quantified using a PMLEs standard (in the same solvent). (b) Uptake of LDL-PMLEs (120 μg of apoB/ml) in the absence (–) or in the presence (+), of unlabelled LDL (1 mg of apoB/ml) by lymphoblastoid cells from normal (N), Wolman (W) and FH subjects. (c) Time course of the uptake of a fixed amount of LDL-PMLEs (120 μg of apoB/ml) by lymphoblastoid cells from a normal subject (N) and a subject with Wolman disease (W). Results are means $\pm$ S.E.M. of three separate experiments.

Figure 2 Degradation of cellular PMLEs taken up by lymphoblastoid cells

Cells were from a normal subject (●) and from patients affected with Wolman disease (○) or with FH (□). Degradation was measured after a pulse with LDL-PMLEs (120 μg of apoB/ml for 24 h). Lipids were extracted and partitioned as indicated in the Materials and methods section and the fluorescence was determined (excitation 340 nm; emission 380 nm) and quantified using PMLEs and pyrenemethanol standards (prepared under the same partition procedure) (a) Total cell-associated fluorescent lipids; (b) PMLEs; (c) pyrenemethanol. Results are means $\pm$ S.E.M. of three separate experiments.

are consistent with the lack of apoB/E-receptor-mediated uptake in FH lymphoblastoid cells [7,9] (Figure 1a). When the uptake of LDL-PMLEs was determined in the presence of a large excess of unlabelled LDL (LDL-PMLEs/LDL ratio of 10:1, mol/mol), LDL-PMLEs uptake was inhibited in both normal and Wolman cells (Figure 1b). In contrast, in FH cells the uptake of LDL-PMLEs was not affected by the addition of LDL (Figure 1b). These data are consistent with a competitive effect of excess LDL on the receptor-mediated uptake of LDL-PMLEs, supporting the assumption that PMLEs was genuinely incorporated into LDL. When lymphoblastoid cells (control and Wolman cells) were pulsed with LDL-PMLEs, the level of PMLEs taken up by the cells increased progressively with time (Figure 1c). All these data

taken together suggest that PMLEs, incorporated into LDL, is taken up by normal and Wolman lymphoblastoid cells through the apoB/E receptor pathway and then trafficked to the lysosomal compartment in these cells [7]. This is similar to previous results with fibroblasts [5,6].

Metabolism of LDL-PMLEs in pulse-chase experiments

In pulse-chase experiments, cells were pulsed for 24 h and PMLEs degradation was studied during the subsequent 96 h chase period. During the chase, the cell-associated fluorescence of normal cells decreased progressively (Figure 2a) as a consequence of PMLEs degradation (Figure 2b). Thus 80% of the

Figure 3 Uptake (a) and metabolism (b) of pyrenemethanol in normal lymphoblastoid cells

(a) Cells were incubated in the presence of pyrenemethanol (10 or 30 nmol/ml) for 24 h. The cell-associated fluorescent lipids (▨, pyrenemethanol; ■, pyrenemethanol esters) were extracted and analysed as indicated in the Materials and methods section. (b) Cells were pulsed for 24 h with 30 nmol/ml pyrenemethanol, then chased for 48 h in the standard culture medium. Cell-associated fluorescent lipids (●, total fluorescent lipids; △, PMLs; ▲, pyrenemethanol) were analysed as in (a). The inset shows the comparative residual levels of pyrenemethanol (▨) and PMLs (■) in normal (N) and Wolman (W) cells following a 24 h chase.

PMLs had disappeared after a 48 h chase and all the cellular fluorescence had disappeared completely by 96 h (Figure 2a). Consequently, a relatively low level of pyrenemethanol was detected in the cell extract (Figure 2c). In contrast, in Wolman lymphoblasts the decrease in PMLs was very slow (Figure 2b) and pyrenemethanol was not detectable (Figure 2c). On the basis of the rate of decrease of PMLs, the half-life in Wolman lymphoblasts was estimated to be about 15–20 days, and the *in*

situ residual activity of the lysosomal enzyme involved in the degradation of PMLs was estimated to be about 5% of the activity in normal cells.

Incorporation of pyrenemethanol

Figure 3(a) shows that the fluorescence of cells pulsed with pyrenemethanol increased with increasing concentrations of this compound in the culture medium. Analysis at the end of the pulse period showed that about 20% of the pyrenemethanol in the cells was esterified to form pyrenemethanol acyl esters. After a pulse of normal and Wolman cells with 30 nmol/ml pyrenemethanol, the metabolism of these compounds (pyrenemethanol and pyrenemethyl acyl ester) was monitored during a chase period of 48 h. The fluorescence of the cell-associated lipids decreased rapidly, and had disappeared completely in 1 day not only in normal cells but also in Wolman cells (after a 24 h chase the residual fluorescence was less than 10% of that at the time (0); the fluorescence was recovered in the chase medium. As shown in the inset of Figure 3(b), only pyrenemethanol was found in the culture medium, thus suggesting that pyrenemethanol itself was released, but the pyrenemethanol acyl esters produced were not. These pulse-chase experiments with pyrenemethanol supplement the results of Figure 2, indicating that, once formed in the intact cells, pyrenemethanol is only poorly esterified and can be released into the culture medium.

Direct fluorescence reading and fluorescence microscopy of cells labelled with LDL-PMLs

The results reported above suggested that a direct reading of the cellular fluorescence could be used to evaluate the *in situ* degradation rate of LDL-PMLs in intact living cells, obviating the need for lipid extraction.

During the pulse with LDL-PMLs performed under the conditions of Figure 2, the direct reading of cell suspensions showed that the cellular fluorescence increased approximately

Figure 4 Direct reading of the cellular fluorescence of intact living cells incubated with LDL-PMLs during the pulse (a) and the chase (b), and of the culture medium during the chase (c)

Lymphoblastoid cells were from a normal subject (●) and from patients affected with Wolman disease (○). The experiment was performed under the conditions described for Figures 1 and 2, but the cellular fluorescence was determined on a cell suspension of intact living cells or alternatively in the culture medium following separation of cells and culture medium by centrifugation as indicated in the Materials and methods section. The inset of (b) shows fluorescence microscopy of single lymphoblastoid cells from Wolman disease (top) and normal (bottom) subjects following a 48 h chase.

Figure 5 Pulse-chase experiment with free PMLEs

(a) Lymphoblastoid cells from a normal subject (N), and from subjects with Wolman disease (W), FH and NLS were pulsed with 5 nmol/ml PMLEs (dissolved in dimethyl sulphoxide) for 12 h, then chased in the standard culture medium for an additional 48 h. Lipids were analysed as indicated in the Materials and methods section at the end of the pulse (p) or the chase (c). ■, PMLEs; ▨, pyrenemethanol. (b) Fluorescence microscopy of Wolman cells at the end of the 12 h pulse with free PMLEs (top) and at the end of the 48 h chase period (bottom).

linearly with time in Wolman cells, whereas it tended to a plateau in normal cells (Figure 4a). During the chase, the cellular fluorescence decreased progressively in normal cells (Figure 4b), whereas the fluorescence of the medium increased (Figure 4c). In contrast, in Wolman cells the cellular fluorescence was almost constant during the same chase period (Figure 4b) and no appreciable fluorescence increase was detected in the culture medium (Figure 4c). As shown by fluorescence microscopy imaging, this pulse-chase procedure with LDL-PMLEs also

permitted discrimination between normal and Wolman cells by fluorescence microscopy (inset of Figure 4b).

Uptake and metabolism of free PMLEs

As shown in Figure 5, free PMLEs dissolved in dimethyl sulphoxide and introduced into the culture medium (RPMI containing 2% Ultrosor HY, but lipoprotein-free) was taken up to a similar extent by the four types of cells used here, i.e. normal, Wolman, NLS and FH cells, the latter indicating that the uptake of free PMLEs is independent of the apoB/E receptor pathway. After a 48 h chase, the cell-associated fluorescent lipids decreased progressively with time in the three types of cells. The lack of any inhibition of degradation in Wolman cells suggests that the degradation of free PMLEs taken up by these cells is independent of the lysosomal lipase (which is deficient in the lysosomal compartment of Wolman cells) and occurs in a non-lysosomal compartment. It is noteworthy that in NLS lymphoblastoid cells (deficient in the degradation of cytoplasmic triacylglycerols) the degradation rate of 'free' PMLEs was in the normal range. This suggests that free PMLEs is degraded by a cytoplasmic enzyme different from the neutral lipase system involved in the hydrolysis of cytoplasmic triacylglycerols. This led us to attempt to identify the cellular lipolytic enzymes involved in the degradation of PMLEs by *in vitro* studies.

In vitro studies of lipolytic enzymes involved in the degradation of PMLEs

Lymphoblastoid cell homogenates exhibited two PMLEs-hydrolysing activities, one with an acid pH optimum (pH 4.0) and a second neutral with an optimum at pH 7.0. As reported in Figure 6 and Table 1, the pH 4 enzyme was relatively heat-stable, was activated by taurocholate and inhibited by PCMB and NaCl (Table 1), and was completely absent from Wolman cells. All of these properties suggest that this enzyme is the lysosomal acid lipase as previously discussed [3,14–18]. The neutral (pH 7)

Figure 6 *In vitro* hydrolysis of PMLEs

(a) The reaction mixture contained 20 nmol of PMLEs (dissolved in 50 μ l of dimethyl sulphoxide), enzyme solution, 100 μ g of protein from the homogenates of lymphoblastoid cells from a normal subject (●) or a Wolman disease patient (○) and 0.2 mol/l citrate/phosphate buffer with the pH varying from 3 to 8, in a final volume of 1 ml. After a 30 min incubation at 37 °C, the fluorescence of the reaction mixture was directly read in the spectrofluorimeter, and quantified using a pyrenemethanol standard. (b) Effect of heat treatment on PMLEs-hydrolysing enzymes (continuous lines) and reference enzymes (broken lines) from lymphoblastoid cells. The cell homogenate was incubated at 50 °C for variable periods of time (from 0 to 30 min). Enzyme activities were then determined under the standard conditions for PMLEs assays at pH 4.0 (▲) and 7.0 (△), with radiolabelled [3 H]triolein at pH 7.0 (■) for the neutral lipase, and with pyrenemethyl acetyl ester at pH 7.0 (□) for the neutral carboxylesterase.

Table 1 Properties of enzymes that hydrolyse PMLes in lymphoblastoid cell homogenates

Enzyme activities are expressed as percentages of the control activity, and are means $\pm$ S.E.M. for three separate experiments. n.d., not determined; E600, diethylparanitrophenylphosphate.

Conditions	Substrate and pH optimum ...	Activity (% of control)			
		PMLes-hydrolysing enzymes		Lipase	Carboxylesterase
		PMLes; pH 4	PMLes; pH 7	Triolein; pH 7	PMACes; pH 7
Heating for 20 min at 50 °C		80 $\pm$ 5	72 $\pm$ 8	90 $\pm$ 4	8 $\pm$ 3
Effectors					
Taurocholate (1.5 mmol/l)		250 $\pm$ 30	130 $\pm$ 15	95 $\pm$ 8	40 $\pm$ 4
PCMB (5 mmol/l)		32 $\pm$ 3	92 $\pm$ 5	10 $\pm$ 2	90 $\pm$ 5
E600 (10 μ mol/l)		65 $\pm$ 6	50 $\pm$ 4	55 $\pm$ 4	8 $\pm$ 2
NaCl (200 mmol/l)		50 $\pm$ 5	95 $\pm$ 4	n.d.	96 $\pm$ 4
EDTA (10 mmol/l)		90 $\pm$ 5	98 $\pm$ 4	100 $\pm$ 6	70 $\pm$ 5
Activity in Wolman cells		5 $\pm$ 1	100 $\pm$ 9	100 $\pm$ 6	100 $\pm$ 7

PMLes-hydrolysing enzyme was relatively heat-stable and resistant to the various inhibitors tested, and was present at normal levels in Wolman cells. The properties of this pH 7 hydrolysing enzyme are clearly different from those of the non-specific neutral carboxylesterase (which exhibits a short-chain substrate specificity, is heat labile and is inhibited by taurocholate and by diethylparanitrophenylphosphate, a serine-blocker) and from the neutral lipase which exhibits a long-chain substrate specificity and is strongly inhibited by PCMB (Table 1).

DISCUSSION

The enzymic defects of inherited lysosomal lipid storage disorders are generally investigated by *in vitro* assays for determining enzyme activities under conditions designed to obtain an optimal dispersion of the substrate and a maximal enzyme activity. Such *in vitro* assays permit the effective diagnosis of these diseases. A key problem concerning the pathophysiology of lysosomal storage disorders is to ascertain the relationship between the severity of the catabolic block, as measured *in vitro*, and the clinical expression of the disease. However, such *in vitro* assays probably do not reflect the 'true' residual activity existing in the lysosomal compartment of an intact living cell, because the conditions employed (such as the presence of detergents and a lack of natural activators) are considerably different from those existing in the lysosomal compartment. The investigation of lysosomal disorders could be improved by the enzyme activity being evaluated directly *in situ* in the intact living cell. Such an *in situ* evaluation of a catabolic activity can be approached by the use of fluorescent substrates. The following conditions must be continued: (i) selective trafficking of the substrate to the subcellular compartment concerned (e.g. lysosomes, as here); (ii) minimal re-utilization of the labelled products by cellular biosynthetic pathways leading to re-synthesis of substrate; and (iii) quantitative recording of fluorescence in intact living cells or in the culture medium (should the product formed be released from the cells). PMLes, a synthetic analogue of neutral lipids has been shown to be a substrate which permits *in vitro* determination of acid lipase activity and thereby the enzymic diagnosis of Wolman disease [16,17].

The hydrophobic character of PMLes (R_p on silica gel t.l.c. between those of cholesteryl esters and triacylglycerols) permitted its incorporation into LDL (probably in the core), using the technique of Roberts et al. [28]. We obtained 20 nmol/mg of apoB (i.e. around 10 molecules of PMLes per LDL particle), a

yield similar to that obtained with triacylglycerols or cholesteryl esters. Moreover, we did not detect any major structural or functional alteration of the lipoprotein, nor any appreciable amount of PMLes hydrolysis within the LDL.

Experiments with LDL-PMLes in normal and FH cells indicated uptake through the apoB/E receptor pathway and trafficking to the lysosomal compartment, where it was degraded in normal cells but not in Wolman cells [5–9]. In contrast, PMLes added to the culture medium in the absence of lipoproteins was degraded at a similar rate in Wolman and normal cells, indicating the existence of an alternative catabolic pathway that differs from the lysosomal enzyme. This emphasized the importance of fully incorporating PMLes into the LDL and of purifying the LDL-PMLes for targeting the substrate to the lysosomal compartment. The presence of contaminating free PMLes (not incorporated into LDL) would result in errors in the *in situ* determination of lysosomal catabolism.

As previously shown, PMLes can be used for determining the *in vitro* activity of the acid lipase and for detecting its deficiency in Wolman disease [16,17]. In this paper we show that it can be considered as an analogue of the natural substrates of acid lipase (cholesteryl esters or triacylglycerols) and as a reliable substrate for *in situ* determination of this enzyme activity. An advantage of the use of PMLes for *in situ* studies on intact cultured living cells is the low metabolic re-utilization of the fluorescent product pyrenemethanol. In contrast, the use of substrates containing fluorescent fatty acids could lead to under-evaluation of the degradative activity, since the pyrene fatty acids liberated will be re-utilized and incorporated into cellular lipids [12,21,29]. A second advantage of PMLes is the relatively rapid extrusion of the product pyrenemethanol into the culture medium, compared with the very low release rate of the substrate (i.e. PMLes). This made possible the use of PMLes for determination of the activity of the lysosomal lipase by recording directly (i.e. without any extraction or purification of pyrenemethanol) the fluorescence of the cell suspension or that of the culture medium during pulse-chase experiments. The latter enabled us to evaluate the time course of the intracellular degradation of the substrate by measuring the fluorescence released into the culture medium without a need for cell harvesting.

It is interesting to note that, under the experimental conditions used, the apparent *in situ* hydrolytic rate of LDL-PMLes was relatively low (the half-life of LDL-PMLes was around 24 h) in comparison with the hydrolytic rates of apoB, cholesteryl esters [5,7,31], LDL-triolein and LDL-cholesteryl oleate (apparent half-

life of 8–10 h, A. Nègre-Salvayre, unpublished work). This could be explained by several mechanisms, as follows. (i) PMLes could be hydrolysed by the acid lipase more slowly than are the natural substrates, since the *in vitro* activity of the acid lipase varies depending on the structure of the substrate (for instance, the relative hydrolytic rates of triolein, cholesteryl oleate, methylumbelliferyl palmitate and methylumbelliferyl oleate were 35, 7, 150 and 800 nmol/h per mg of protein respectively) [32]. *In vitro*, the rate of hydrolysis of PMLes by the acid lipase is apparently in the same range as that of cholesteryl oleate [23]. However, we did not test competition between PMLes and cholesteryl oleate. (ii) The lysosomal hydrolysis of PMLes is probably slowed down by competition by natural substrates. For instance, in the reported experiments, reconstituted LDL contained around 20, 3000 and 200 nmol of PMLes, cholesteryl esters and triacylglycerols respectively per mg of apoB (on the basis of the PMLes content and the data of Esterbauer et al. [33]). This large excess of natural substrates in LDL, entering the lysosomes at the same time as PMLes, will probably compete with hydrolysis of PMLes by the acid lipase and thus reduce the rate of this reaction. (iii) The fluorescence of intact cells is the sum of the fluorescence of the substrates (PMLes) and of the hydrolysis product (pyrenemethanol) associated with the cell. On the basis of data of Figure 3(b), the release of pyrenemethanol occurs with an apparent half-life of about 6 h. Therefore this transport time also acts to increase the apparent half-life of the decay of the cellular fluorescence.

Unlike fluorescent triacylglycerols, PMLes cannot be used for determining the activity of the neutral lipase that is defective in NLSD. This is probably due to a restricted substrate specificity of the neutral lipase, which seems to be specific for triacylglycerols, in contrast to the acid lysosomal lipase which exhibits a broader substrate specificity (being able to hydrolyse triacylglycerols, cholesteryl esters and various synthetic analogs) [4,14–17]. The pH 7 PMLes-hydrolysing enzyme is probably different from the pH 7 lipase (which hydrolyses triolein) on the basis of differential inhibition by PCMB and the lack of accumulation of PMLes in NLSD cells; it differs also from the short-chain carboxylesterase on the basis of heat-inactivation and differential inhibition by diethylparanitrophenylphosphate. Therefore the identity of the pH 7 PMLes-hydrolysing enzyme remains unknown, and several lipolytic enzymes with a broad specificity (e.g. phospholipases, cholesteryl esterases or, more generally, carboxylesterases) [15] could hydrolyse PMLes in the non-lysosomal compartment.

In conclusion, PMLes can be taken up and degraded by intact living lymphoblastoid cells by two separate pathways. When PMLes is incorporated into LDL, it is taken up via the apoB/E receptor pathway and degraded by the acid lipase in the lysosomal compartment. When free PMLes is incubated with the cells in lipoprotein-free culture medium, it is taken up via a receptor-independent pathway and degraded in a non-lysosomal compartment. The latter hydrolysis is probably by an enzyme other than the neutral lipase. The present studies demonstrate that LDL-PMLes can be used for estimating intracellular hydrolysis by direct reading of the fluorescence of cells or of the culture medium. This permits the evaluation of acid lipase activity in normal lymphoblastoid cells, and of the degradation block in Wolman cells, using suspension of intact living cells.

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