Processing of lipoproteins in human monocyte-macrophages

Brian J. Van Lenten and Alan M. Fogelman

Division of Cardiology, Department of Medicine, School of Medicine, University of California at Los Angeles, Los Angeles, CA 90024-1679

Abstract Subcellular fractionation of human monocyte-macrophages (HMM) yielded a fraction rich in endosomes, lysosomes, and mitochondria. This pellet was further fractionated in a metrizamide gradient and the subcellular organelles were distributed among seven distinct bands. All of the bands contained lysosomal enzymes in similar amounts. However one band, poor in mitochondria, was markedly enriched in cathepsin D and cholesteryl ester hydrolase activities. A number of different ligands (low density lipoprotein (LDL), malondialdehyde-altered LDL, β-migrating very low density lipoprotein, high density lipoprotein, reductively methylated LDL, mannose-bovine serum albumin, and transferrin) were presented to HMM at a concentration of 20 μg/ml at 4°C. Three minutes after warming the cells at 37°C all ligands except two were found predominantly in the cathepsin D- and cholesteryl ester hydrolase-rich fraction. Unlike the other ligands, LDL had distributed to other more dense fractions and reductively methylated LDL was found mainly in less dense fractions. At a lower concentration, 2 μg/ml, the distribution of LDL was identical to the other ligands. In vitro incubation of the fractions obtained from the gradient suggested that cathepsin D was largely responsible for the hydrolysis of the lipoproteins. We conclude that studies of LDL metabolism in HMM must take into account the different processing of this ligand at commonly used concentrations. —**Van Lenten, B. J., and A. M. Fogelman.** Processing of lipoproteins in human monocyte-macrophages. *J. Lipid Res.* 1990. **31:** 1455–1466.

Supplementary key words subcellular fractions • cholesterol transport

Goldstein et al. (1) have demonstrated that freshly isolated murine macrophages possess distinct binding sites for modified lipoproteins called scavenger receptors. The uptake and hydrolysis of these modified lipoproteins led to the massive accumulation of cellular cholesteryl esters in these cells. However, uptake of LDL did not produce CE accumulation and it was concluded that these murine macrophages did not have a normal low density lipoprotein (LDL) receptor pathway. A year later, our laboratory (2) and Traber and Kayden (3) reported that human monocyte-macrophages (HMM), cultured even in the presence of serum, readily took up and degraded LDL by a pathway described in fibroblasts. In contrast to this high affinity process in fibroblasts, the apparent saturation of receptor activity in cultured HMM occurred between 25 and 50 μg/ml of LDL (2, 3), which could be further suppressed by additional medium lipoprotein (4). From these studies it was concluded that LDL at high concentrations was entering the HMM by a process distinct from fluid endocytosis that could deliver cholesterol capable of regulating intracellular cholesterol metabolism.

In addition to our own laboratory (2, 4–9) a number of other laboratories have studied LDL metabolism in HMM at concentrations of 20 μg/ml or greater (3, 10–22). We demonstrate in this report that LDL at such concentrations behaves differently from a number of other ligands having distinctly different receptor systems. We also show that the protease cathepsin D is largely responsible for the degradation of lipoproteins in HMM.

MATERIALS AND METHODS

Materials

Metrizamide was purchased from Accurate Chemical & Scientific Corp., Westbury, NY. Percoll was purchased from Pharmacia. Bovine serum albumin (BSA), PMSF (phenylmethylsulfonyl-fluoride), leupeptin, pepstatin A, transferrin, and all substrates for the enzyme assays were purchased from Sigma Chemical Co. Mannose-bovine se-

Abbreviations: LDL, low density lipoprotein; acetyl LDL, low density lipoprotein modified by acetic anhydride; HDL, high density lipoprotein; MDA-LDL, malondialdehyde-altered low density lipoprotein; R-CH_3-LDL, reductively methylated low density lipoprotein; FC, free cholesterol; CE, cholesteryl ester; C/P, total cholesterol to protein ratio; BSA, bovine serum albumin; PMSF, phenylmethylsulfonyl fluoride; TCA, trichloroacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid; HMM, human monocyte-macrophages; β-VLDL, β-migrating very low density lipoprotein; apoB, apolipoprotein B; SDS, sodium dodecyl sulfate; ^{125}I-LDL, ^{125}I-labeled LDL; ^{125}I-MDA-LDL, ^{125}I-labeled malondialdehyde-altered LDL.

rum albumin (mannose-BSA) was purchased from E-Y Labs, Inc., San Mateo, CA. Rabbit antisera to the bovine adrenal LDL receptor were kindly supplied by Drs. Janet Boyles and Tom Innerarity, Gladstone Foundation, San Francisco, CA, and Dr. David Russell, Dallas, TX. All other tissue culture supplies and equipment were purchased from sources previously reported (23).

Separation of cells

Normal subjects were recruited from the staff and student body at UCLA and were screened as previously described (23).

Five hundred ml of blood was taken after an overnight fast, and the monocytes were separated from 300 ml of blood by counterflow centrifugation (24) or by a modification of the Recalde method (23).

Cell culture

Human monocyte-macrophages were suspended in 30% autologous serum in Iscove's Modified Dulbecco's Medium supplemented with 2 mM glutamine, penicillin (100 units/ml), streptomycin (100 μg/ml), and Fungizone (0.25 mg/ml) hereafter referred to as medium B. Five- or 10-ml samples of the cell suspension containing 10^6 monocytes/ml were transferred to 60 × 15 mm or 100 × 20 mm plastic tissue culture dishes, or 0.5-ml samples were transferred to 2.0-cm^2 polystyrene wells and incubated at 37°C in a humdified incubator with 5% CO_2. To enhance expression of LDL receptor activity, the cells were transferred to 10% lipoprotein-deficient serum in medium B 20 h before the experiment.

Classification and viability of cells

The cells were classified and their viability was determined as described previously (23). Because of the media changes and the washes prior to beginning each experiment, the cells were 99% monocyte-macrophages before the radioactive lipoproteins were added. More than 95% of the cells were viable at the end of the incubations.

Subcellular fractionation

To study the subcellular distribution of internalized lipoprotein by monocyte-macrophages, cells were fractionated by a modification of the technique of Wattiaux et al. (25). The cells were cultured for 7 to 10 days before the experiment. At the end of the experiment the cells were scraped from the dishes in PBS using a Teflon cell scraper and collected into siliconized glass tubes. After pelleting, the cells were dispersed in 0.25 M sucrose and homogenized to greater than 85% breakage by means of a glass tube fitted with a Teflon pestle set at a rotating speed of 6 on a Fisher Dyna-Mix homogenizer (Fisher Scientific). The homogenate was then centrifuged at 200 *g* for 15 min using a JS 4.2 rotor to remove cellular debris, unbroken cells, and nuclei. The resultant supernatant was centrifuged at 20,000 *g* in a JA-17 rotor for 15 min and the resultant pellet was centrifuged in a metrizamide gradient. The gradient was constructed by gently mixing with a pipette 0.75 ml of the 20,000 *g* pellet in 0.25 M sucrose with 0.75 ml of a stock solution of metrizamide to yield a solution of d 1.234 g/ml. This was sequentially overlaid with metrizamide solutions of d 1.213, 1.170, 1.144, 1.134, 1.122, 1.113, and 1.029 g/ml. The gradient was then centrifuged in an SW 41 rotor at 95,000 *g* for 2 h at 4°C. The resultant bands were clearly visualized and removed from the top of the tube with a Pasteur pipette, leaving a distinctly clear layer above the next denser band. In some experiments the 20,000 *g* pellet from above was resuspended in 0.25 M sucrose and layered over Percoll of density 1.07 g/ml, as described (26). Fractionation was performed by centrifugation at 17,000 rpm for 60 min in a JA-17 rotor. Fractions were collected from the top of the tube and the densities were determined by refractometry.

Preparation of ligands

Human LDL (d 1.019–1.063 g/ml) and HDL (d 1.063–1.210 g/ml) were prepared from sera of normal fasted subjects as described (27). β-VLDL was isolated from cholesterol-fed rabbits (6). LDL and ^{125}I-labeled LDL were modified by malondialdehyde (MDA-LDL and ^{125}I-labeled MDA-LDL, respectively) as described (28), and by reductive methylation (29). [^{3}H]cholesteryl linoleate was incorporated into MDA-LDL or ^{125}I-labeled MDA-LDL according to the technique of Brown, Ho, and Goldstein (30). Thin-layer chromatography was carried out as described (31). All proteins were radioiodinated according to the method of McFarlane (32) as modified by Bilheimer, Eisenberg, and Levy (33).

Assays

Prior to the addition of radioactive lipoproteins, the cells were washed three times with 2 ml (60-mm dishes) or 5 ml (100-mm dishes) of Dulbecco's modified Eagle's medium containing 10 mM HEPES (medium C). Radioactive lipoproteins were added either in the same medium supplemented with 24 mM $NaHCO_3$ and 2 mg/ml glucose (medium D) for the 37°C incubations (conducted in a 5% CO_2 humidified incubator), or in medium C for the 4°C incubations (conducted in room air).

Cells to be used in binding studies were chilled for 30 min at 4°C, the media were removed, and the radioactive lipoproteins were added in medium C at 4°C for 90 min on a rotating platform (60 rpm). At the end of this time the cells were washed 2 times with PBS containing 0.2% BSA and then 4 times with PBS alone. The cells were then either scraped from the dishes or lysed with 0.1 N NaOH. In other experiments, after incubation for 90 min at 4°C the cells were washed 4 times with ice-cold medium C,

transferred to warm medium D, and incubated at 37°C for various periods of time. At the end of the incubation period at 37°C the media were removed, the cells were washed 2 times with PBS containing 0.2% BSA followed by 4 washes with PBS alone, and either scraped from the dishes or lysed with 0.1 N NaOH.

β-Hexosaminadase and β-glucuronidase activities in subcellular fractions were measured according to the method of Li and Li (34) and Lusis, Tomino, and Paigen (35), respectively. Cholesteryl ester hydrolase activity was measured by the method of Coates, Langer, and Cortner (36). Cathepsin D activity was measured as described by Diment, Leech, and Stahl (37).

To study the effects of protease inhibitors on lipoprotein hydrolysis, the cells were fractionated as described above to isolate band 1, which was kept at 4°C. Band 1 was then diluted in 20 mM acetate buffer containing 120 mM KCl, 5 mM NaCl, 5 mM $MgCl_2$, at pH 4.5, and divided into replicate samples. The samples were then incubated at 37°C for 30 min in the presence or absence of 5 μg/ml of leupeptin or pepstatin A, or 1 mM PMSF. Aliquots were then removed and undegraded ligand was precipitated with 50% TCA. Aliquots of the supernatant were then counted in a gamma-counter for the content of ^{125}I. A tube containing the same amount of radioactive lipoprotein but without band 1 was used as a blank and subtracted from the values obtained (≤ 0.02% of added radioactivity from band 1).

The proteolytic degradation of ^{125}I-labeled lipoproteins was measured as described by Goldstein and Brown (38).

The concentrations of protein in whole cell lysates and in subcellular fractions after removal of metrizamide by dialysis were determined by the method of Lowry et al. (39).

Electrophoretic and autoradiography procedures

One-dimensional sodium dodecyl sulfate (SDS) gel electrophoresis was performed according to the method of Laemmli (40). Electrophoresis was carried out using a gradient gel system of 3.5–12% polyacrylamide at 35 v for 16 h at room temperature. Equal amounts of radioactivity from the TCA-soluble supernatants and TCA-precipitates, generated from the in vitro hydrolysis of band 1 as described above, were applied to each sample lane. After electrophoresis the radiolabeled apoB fragments were visualized by autoradiography (41) and their molecular weights were estimated from protein standards run concurrently with the samples on the gel.

Electron microscopy

Subcellular fractions were cross-linked with 2% glutaraldehyde in PBS and postfixed and 1% OsO_4 in PBS, dehydrated with ethanol and embedded (42). Sections approximately 1000 Å thick were stained with uranyl acetate and lead citrate and examined with a JEOL JEM-100CX electron microscope (JEOL, Ltd., Tokyo, Japan).

Scatchard analyses were conducted using the computerized modeling program LIGAND as described by Munson and Rodbard (43).

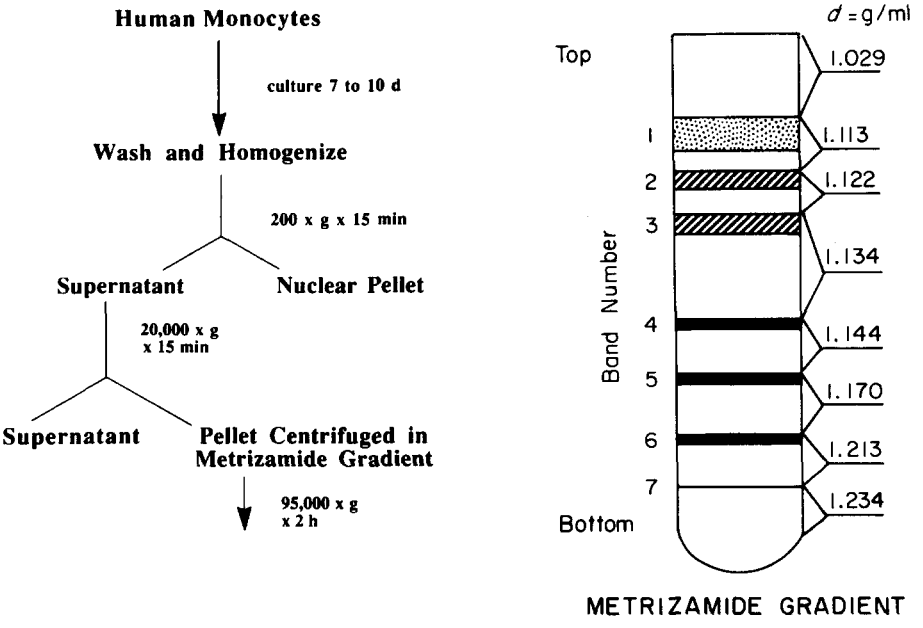
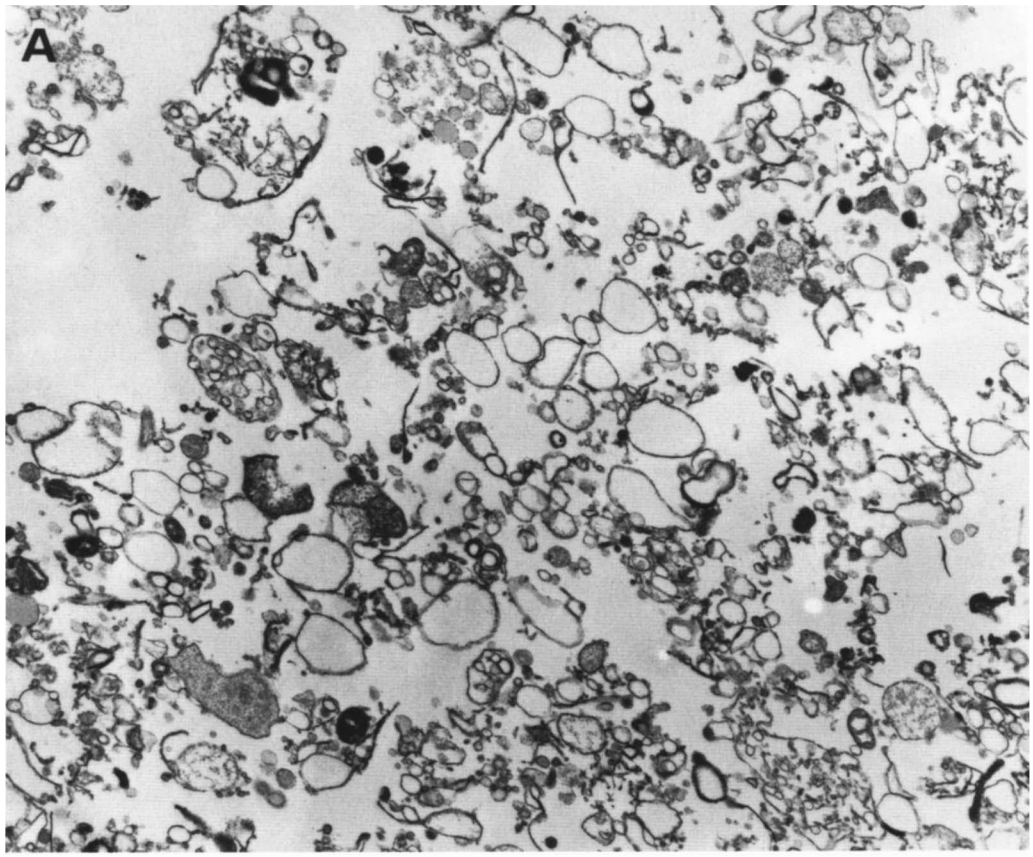
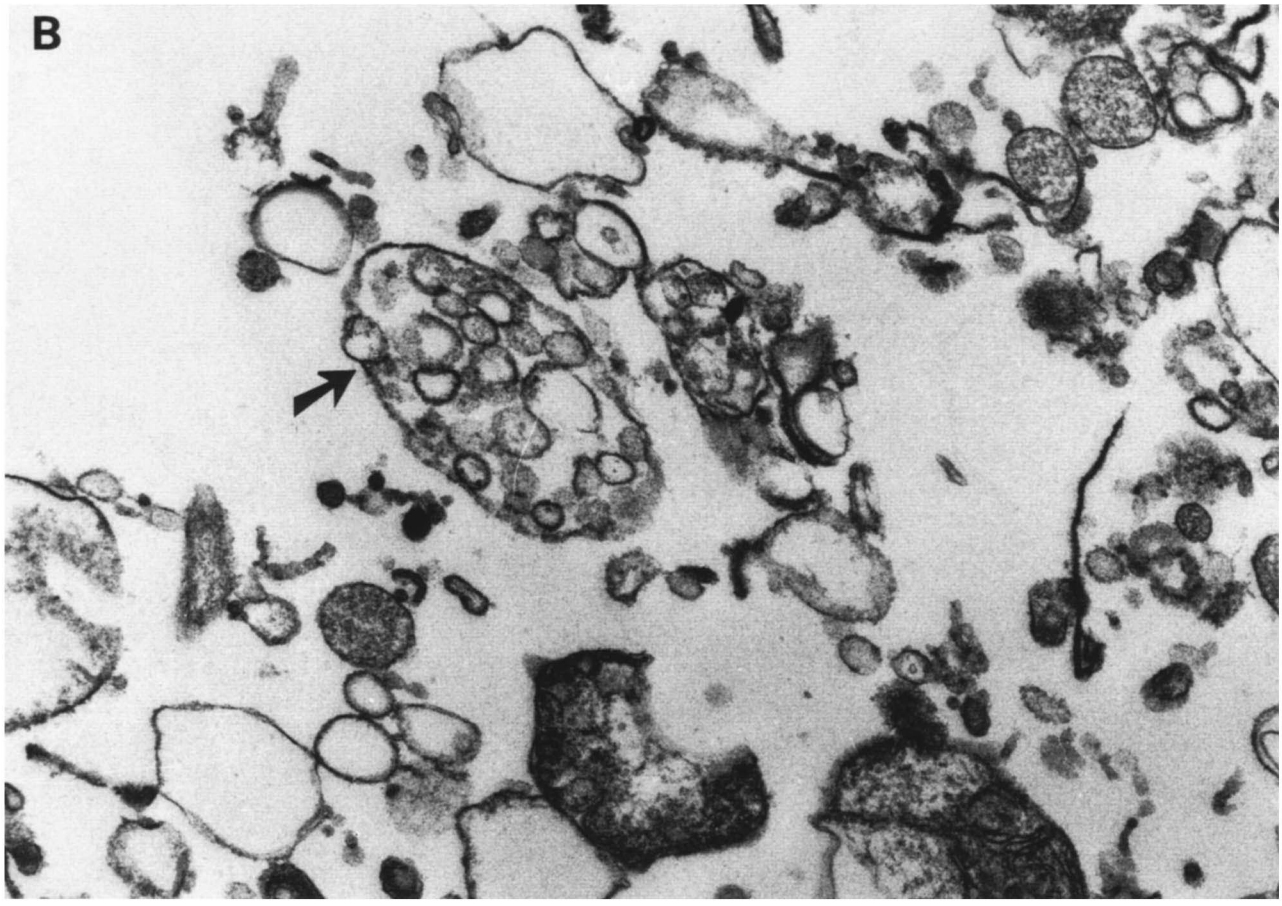


Fig. 1. Protocol for the subcellular fractionation of human monocyte-macrophages (left panel) and the banding pattern obtained after centrifugation of the 20,000 *g* pellet of monocyte-macrophages in a metrizamide gradient (right panel).





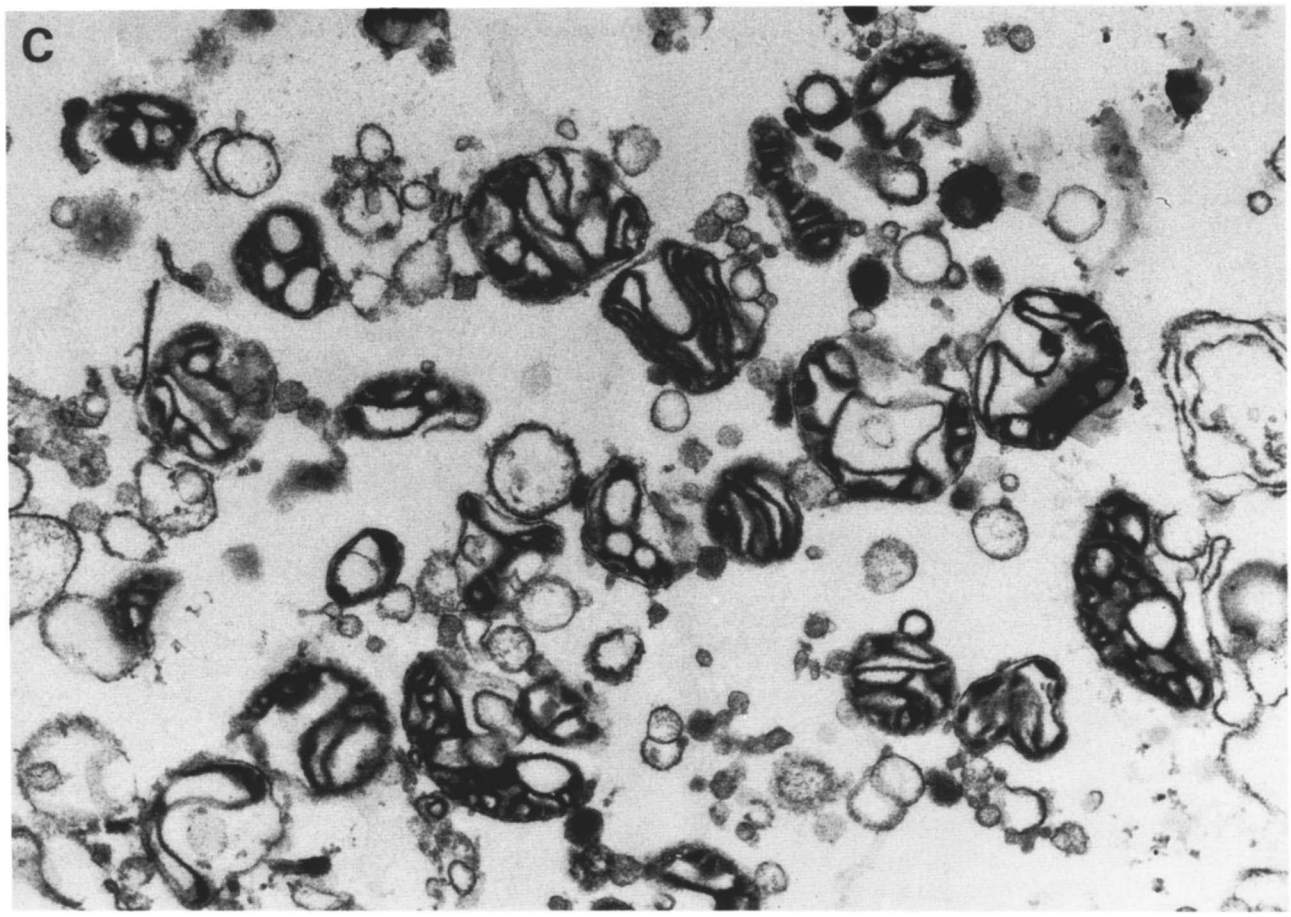


Fig. 2. Electron micrograph of fractions isolated from the 20,000 *g* pellet after centrifugation on the metrizamide gradient; A: band 1 (× 174,000); B: a multivesicular endosome from band 1 (arrow) (× 603,200); C: band 3 (× 348,000).

RESULTS

Subcellular fractionation

Fig. 1 outlines the protocol used in these studies for subcellular fractionation (left panel) and schematically depicts the distribution of the 20,000 *g* pellet in the metrizamide gradient (right panel). Centrifugation in the metrizamide gradient resulted in seven visually distinct bands which were examined by electron microscopy. Band 1 (**Fig. 2, A**) contained subcellular structures many of which resembled multivesicular endosomes (44) (Fig. 2, B). The more dense band 2 was similar in composition to band 1 but contained smaller organelles, whereas band 3 contained many mitochondria (Fig. 2, C).

Distribution of enzyme activities

Fig. 3 shows the distribution of several enzyme activities among the bands isolated from the metrizamide gradient in the current study with HMM. The activities of lysosomal hexosaminadase and glucuronidase were approximately equal in the bands of the gradient. Although 30% of the gradient protein was found in band 1, only 10% of the total activities of these enzymes was found. In bands 2 through 7 the distributions of cathepsin D and cholesteryl ester hydrolase activities were equal to or less than that of the lysosomal hexosaminadase and glucuronidase activities. In contrast, in band 1 the total cathepsin D and cholesteryl ester hydrolase activities were five-fold higher than the lysosomal hexosaminadase and glucuronidase activities. The distribution of these enzymes among the bands of the metrizamide gradient was the same whether or not the cells had been exposed to lipoproteins (data not shown).

Comparison of the subcellular localization of ^{125}I-labeled ligands following internalization

When cells were incubated at 4°C for 90 min with 2 μg/ml of ^{125}I-MDA-LDL, ^{125}I-mannose-BSA, or ^{125}I-LDL, washed extensively, and warmed at 37°C for 3 min, the distribution of radioactivity from all three ligands was similar, almost exclusively localized to a single band (**Fig. 4**). This would suggest that after high affinity binding and uptake, LDL, MDA-LDL, and mannose-BSA distributed similarly.

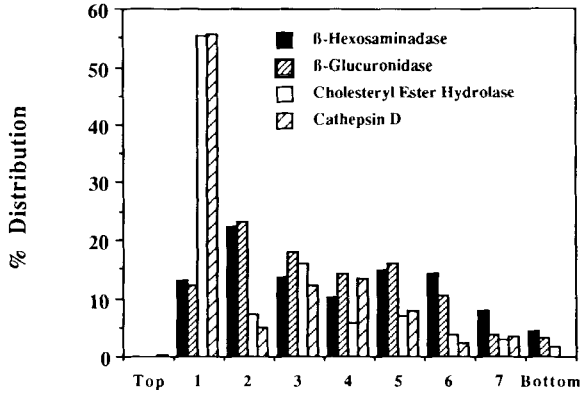


Fig. 3. Distribution of enzyme activities among metrizamide gradient bands (fractions). Cells were prepared as described under Materials and Methods (Subcellular fractionation). The activities of cholesteryl ester hydrolase (□), cathepsin D (▨), β-glucuronidase (▨), and β-hexosaminadase (■) were determined in each of the isolated fractions from the metrizamide gradient. Seventy percent of the total homogenate activity was recovered in the metrizamide gradient. The values are graphed as the percent of total gradient activity present in each fraction. Protein concentrations in fractions 1–7 and in the bottom fraction were 106, 35.2, 40.2, 24.1, 35.3, 14.7, 29.4, and 45 μg/ml, respectively. One hundred percent values for cholesteryl ester hydrolase, cathepsin D, glucuronidase, and hexosaminadase activities were 963 nmol/min per mg protein, 99.4 U/mg protein, 2.2 nmol/min per mg protein, and 24.7 nmol/min per mg protein, respectively.

However, when HMM were incubated with 20 μg/ml of ^{125}I-LDL or ^{125}I-MDA-LDL for 90 min at 4°C and the cells were subsequently warmed for 3 min and fractionated (Fig. 5), the results were quite different. In the cells incubated with ^{125}I-MDA-LDL, the radioactivity was localized to one band. In contrast, ^{125}I-LDL was distributed over bands 1 to 3. The percent of total cell-associated radioactivity recovered in the bands from the metrizamide gradient was similar for cells incubated with ^{125}I-MDA-LDL and ^{125}I-LDL (45.5% and 42.5%, respectively). These results were observed in more than 20 separate experiments. The remaining cell-associated radioactivity in the nuclear pellets and the 20,000 *g* supernatants was also similar for the two ligands, indicating that the distribution of LDL taken up by HMM is influenced by the amount of lipoprotein presented to the cell.

When HMM were incubated with radioactive lipoproteins at 4°C for 90 min and then fractionated without warming to 37°C (**Fig. 6A**), greater than 85% of the radioactivity was found in bands 4–6 for both ^{125}I-MDA-LDL and ^{125}I-LDL, and presumably represented radioactive lipoprotein associated with the plasma membrane. Conventionally, LDL are isolated in an ultracentrifuge at a density of 1.019–1.063 g/ml after 24 h at 265,000 *g*. We added radioactive lipoproteins alone to the bottom of the metrizamide gradient (at a density of 1.234 g/ml) in the same way as was the 20,000 *g* pellet, and then centrifuged at 95,000 *g* for the same 2-h length of time (Fig. 6B). The radioactivity was found in a density range of 1.170–1.234 g/ml, (mainly in bands 6 and 7 rather than in band 1). Although we cannot rule out the possibility that a cellular modification of the ligands had occurred, a more likely explanation would be that after internalization of the radioactive ligands, the buoyant density of the subcellular structures determined the distribution of the ^{125}I-labeled ligands in the metrizamide gradient.

In other experiments (**Fig. 7**), cells were incubated at 4°C for 90 min with 20 μg/ml of ^{125}I-LDL, ^{125}I-MDA-LDL, or ^{125}I-β-VLDL (Fig. 7A), or with 20 μg/ml of ^{125}I-HDL or ^{125}I-transferrin (Fig. 7B). After warming to 37°C

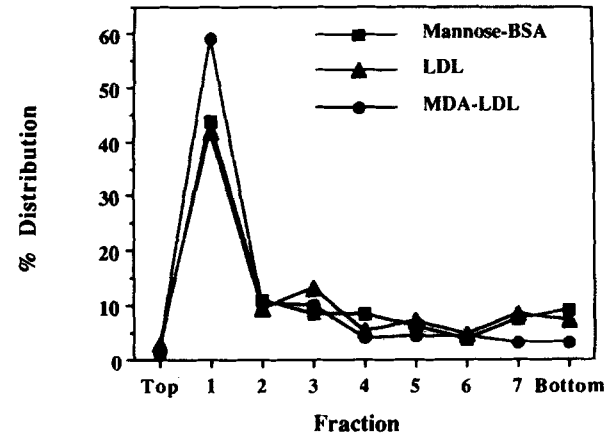


Fig. 4. Distribution of ^{125}I-MDA-LDL, ^{125}I-mannose-BSA, and ^{125}I-LDL among metrizamide gradient fractions. Cells were cultured for 6 days in 30% autologous serum, switched to 10% lipoprotein-deficient serum for 20 h, and then incubated with 2 μg/ml ^{125}I-MDA-LDL, ^{125}I-mannose-BSA, or ^{125}I-LDL for 90 min at 4°C in medium C. The cleis were then washed, incubated at 37°C in medium D for 3 min, and fractionated as described under Materials and Methods.

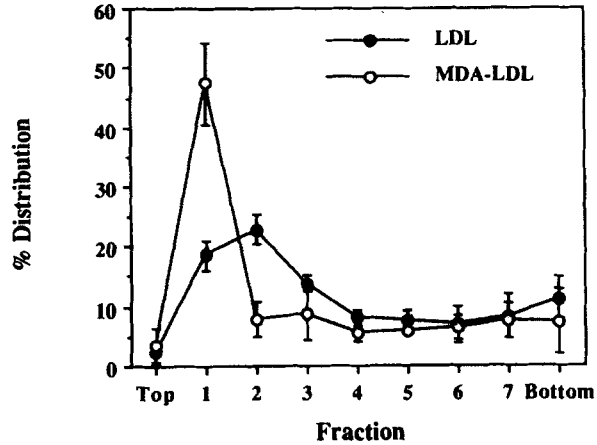


Fig. 5. Distribution of ^{125}I-MDA-LDL and ^{125}I-LDL among metrizamide gradient fractions. Cells were cultured for 6 days in 30% autologous serum, switched to 10% lipoprotein-deficient serum for 20 h, and then incubated in medium C with 20 μg/ml ^{125}I-MDA-LDL (○) or ^{125}I-LDL (●) for 90 min at 4°C followed by incubation in medium D at 37°C for 3 min. The cells were then fractionated as described under Materials and Methods.

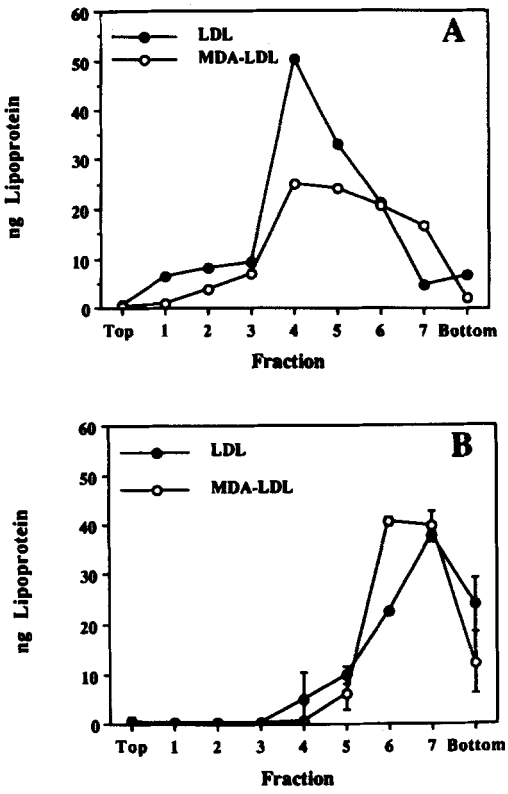


Fig. 6. Distribution of ^{125}I-MDA-LDL and ^{125}I-LDL among metrizamide gradient fractions. (A) Cells were cultured for 6 days in 30% autologous serum, switched to 10% lipoprotein-deficient serum for 20 h, and then incubated in medium C with either 10 μg/ml of ^{125}I-MDA-LDL (○) or 20 μg/ml of ^{125}I-LDL (●) for 90 min at 4°C. The cells were then fractionated as described under Materials and Methods. (B) Distribution of radioactive lipoproteins that were not exposed to the cells. Values shown are the mean ± SD from three separate experiments.

for 3 min, the radioactivity from ^{125}I-HDL, ^{125}I-transferrin, ^{125}I-MDA-LDL, and ^{125}I-β-VDL was localized primarily to band 1 after fractionation, (as was ^{125}I-mannose-BSA at a concentration of 20 μg/ml in experiments not shown), whereas ^{125}I-LDL was equally distributed between bands 1 and 2 (Fig. 7), or was greater in band 2 (Fig. 5). ^{125}I-reductively methylated LDL was found primarily in the top fraction (Fig. 7C).

Specificity of lipoprotein binding and uptake

Scatchard analyses for ^{125}I-MDA-LDL and ^{125}I-LDL revealed two binding sites: a high and a low affinity site. Whereas the K_d values for the low affinity sites were similar for both lipoproteins (65 nM and 45 nM, respectively), the K_d for the high affinity site for MDA-LDL was fivefold less than for LDL (0.225 nM vs. 1.3 nM, respectively). The experiments shown in Fig. 4 were repeated in the presence and absence of excess unlabeled lipoprotein. In data not shown, a 50-fold molar excess of unlabeled lipoprotein virtually eliminated band-associated radioactivity, and antiserum to the bovine adrenal LDL receptor markedly reduced the uptake and degradation of ^{125}I-LDL but not ^{125}I-MDA-LDL. These data strongly suggest that lipoprotein entry into the subcellular fractions occurred primarily by saturable processes.

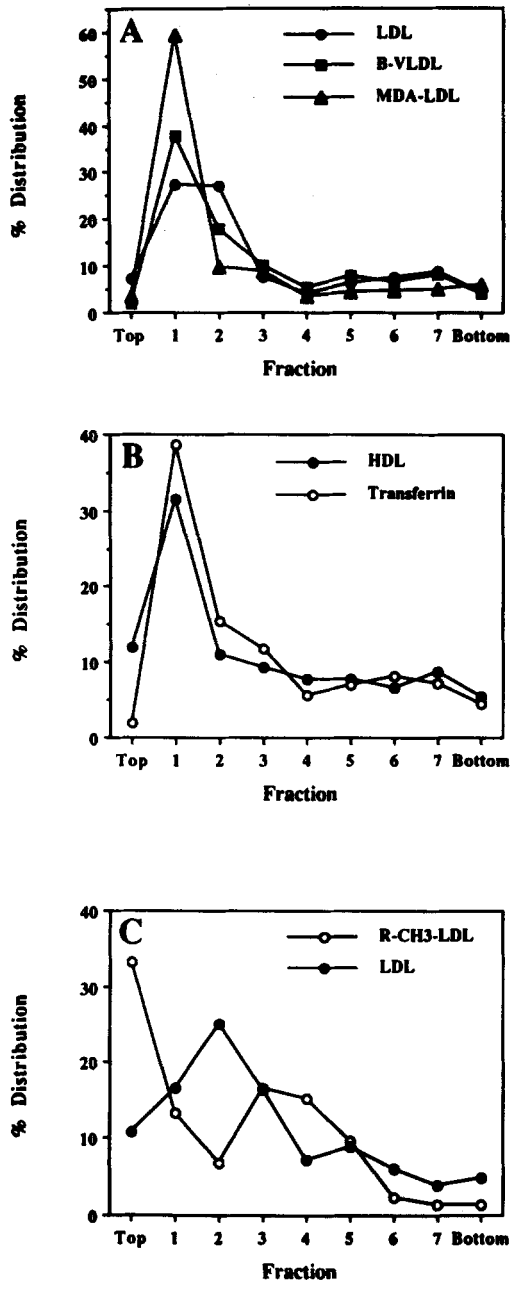


Fig. 7. Distribution of several ligands among metrizamide gradient fractions. Cells were cultured for 6 days in 30% autologous serum, switched to 10% lipoprotein-deficient serum for 20 h, and then incubated in medium C with 20 μg/ml ^{125}I-MDA-LDL (▲), ^{125}I-β-VLDL (■), or ^{125}I-LDL (●) (panel A), ^{125}I-HDL (●) or ^{125}I-transferrin (○) (panel B), or ^{125}I-LDL (●) or ^{125}I-reductively methylated LDL (R-CH_3-LDL) (○) (panel C) for 90 min at 4°C followed by incubation in medium D at 37°C for 3 min. The cells were then fractionated as described under Materials and Methods.

Lipoprotein hydrolysis

To study the hydrolysis of lipoprotein-cholesteryl ester in HMM, the cells were incubated for 90 min at 4°C with 25 μg/ml of ^{125}I-MDA-LDL containing [^{3}H]cholesteryl linoleate (31). The medium was removed, the cells were washed extensively, and incubated at 37°C for 15 min. The cells were then fractionated and the radioactivity in each fraction was determined. Based on the homogenate value of 100%, approximately 70% of the label was in the 200 *g* supernatant (55% in CE). After the 20,000 *g* spin about 68% of the label was pelleted, and 51% was in CE. Gradient isolation showed that band 1 contained about 50% of the label of which almost all was in CE after 15 min of incubation. Thus, band 1, although having contained less than 1% of the total cell protein in this experiment (140 μg), contained more than half of the internalized lipoprotein after 15 min.

By following the ratio of free to esterified cholesterol (FC/CE) and the ratio of total cholesterol to protein (C/P) in band 1 with time, it was apparent from the increase in the FC/CE ratio and the C/P ratio that hydrolysis had occurred (data not shown). To determine whether lipoprotein hydrolysis occurred similarly in bands 1, 2, and 3, the experiment in **Fig. 8** was carried out. HMM were incubated with 20 μg/ml of ^{125}I-LDL at 4°C, warmed at 37°C for 3 min, and fractionated. Although band 2 contained half of the ^{125}I-LDL and band 3 had an amount similar to band 1 (approximately 25% in each) (Fig. 8A), a greater amount of ^{125}I-LDL was hydrolyzed in band 1 (Fig. 8B), suggesting that band 1 contained the major compartment for lipoprotein hydrolysis in these cells.

The hydrolysis of lipoprotein occurred in an acidic compartment similar to the compartment hydrolyzing ligands for the mannose receptor (45). After incubation of the cells with ^{125}I-MDA-LDL, band 1 was isolated and incubated in vitro at various pH values. The optimal pH for lipoprotein hydrolysis (pH 4.5) was identical to that reported for the hydrolysis of ^{125}I-mannose-BSA in rabbit alveolar macrophages (45). The hydrolysis of the lipoprotein under these conditions was linear with respect to time for up to 30 min (r^2 = 0.987).

To establish whether the hydrolysis occurred in a pepstatin A-sensitive compartment, the experiments shown in **Fig. 9** were performed. Cells were incubated with 10 μg/ml of ^{125}I-MDA-LDL or 2 μg/ml ^{125}I-LDL and band 1 was isolated and incubated in vitro with PMSF, leupeptin, or pepstatin A (top panel). PMSF incubated with band 1 had no effect on lipoprotein hydrolysis. Leupeptin inhibited hydrolysis 20–30% compared to control. In contrast, pepstatin A inhibited hydrolysis of both ^{125}I-MDA-LDL and ^{125}I-LDL 60–70%, suggesting that cathepsin D was primarily responsible for lipoprotein hydrolysis in band 1.

TCA-insoluble fragments from band 1 were generated upon hydrolysis of lipoproteins. In the experiment shown in Fig. 9 (bottom panel), HMM were incubated with ^{125}I-MDA-LDL, band 1 was isolated and incubated with or without pepstatin A in vitro as above. The predominant fragments ranged in molecular weight from 40,000 to 80,000 (lane 2). The formation of these hydrolytic products of apoB was inhibited by pepstatin A (lane 4). Since lanes 1 and 3 showed no detectable radioactivity after autoradiography, the TCA-soluble products were presumably of molecular weights smaller than 14,000. Similar results were seen when ^{125}I-LDL was used instead of ^{125}I-MDA-LDL (data not shown).

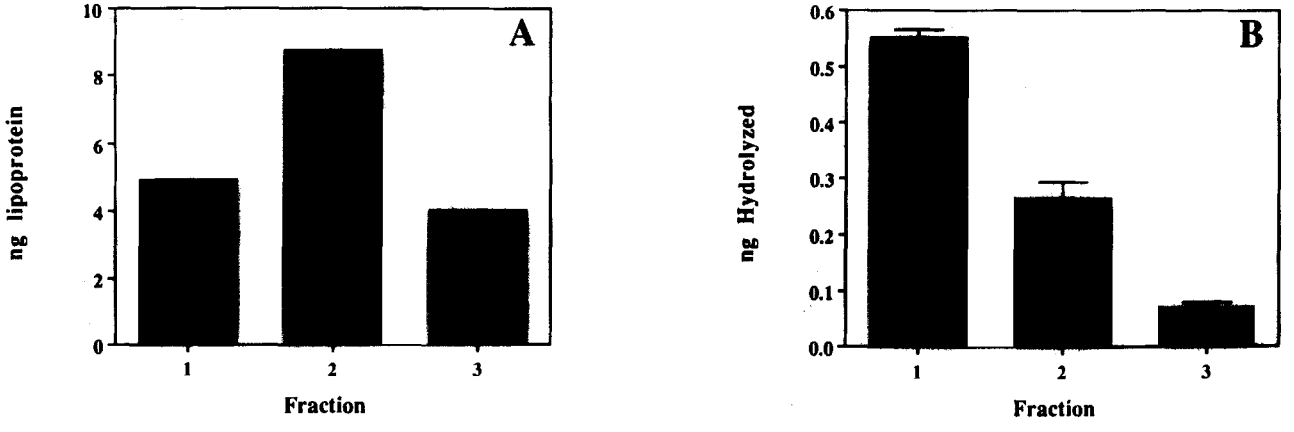


Fig. 8. The hydrolysis of ^{125}I-LDL in metrizamide gradient fractions. Cells were cultured for 6 days in 30% autologous serum, switched to 10% lipoprotein-deficient serum for 20 h, and then incubated in medium C with 20 μg/ml ^{125}I-LDL for 90 min at 4°C. The cells were then fractionated as described under Materials and Methods. After isolation, bands 1–3 were then diluted 20 mM acetate buffer containing 120 mM KCl, 5 mM NaCl, 5 mM $MgCl_2$, at pH 4.5. The samples were then either used for lipoprotein quantitation or incubated at 37°C for 30 min to determine hydrolysis of lipoprotein. From the latter, aliquots were removed and undegraded ligand was precipitated with 50% TCA. Aliquots of the supernatant were then counted in a gamma-counter. A tube containing the same amount of lipoprotein but without band 1 was used as a blank and subtracted from the values obtained (≤ 0.02% of added radioactivity from band 1). Values shown in panel B are the mean ± 1 SD from quadruplicate determinations.

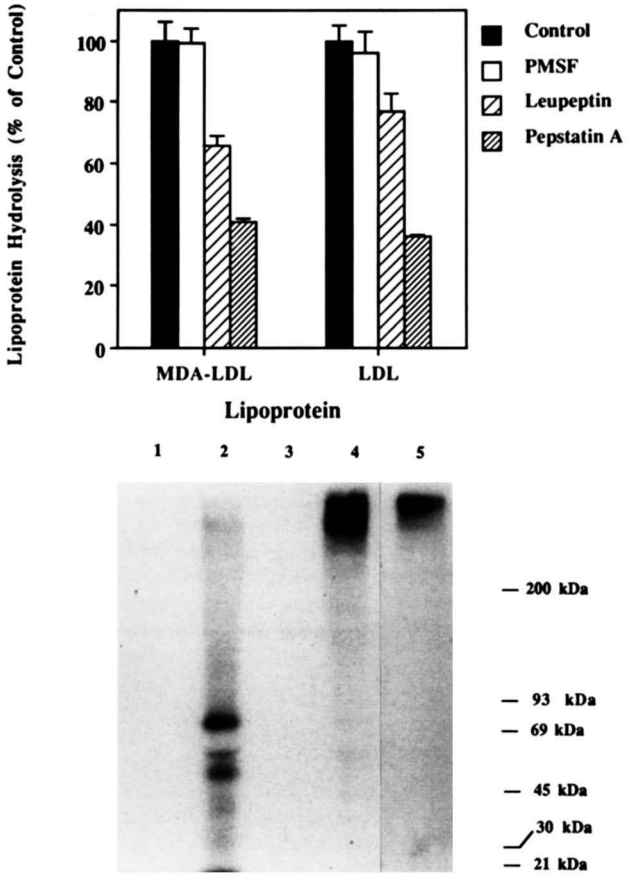


Fig. 9. Effect of protease inhibitors on the in vitro hydrolysis of lipoprotein in band 1. Top: cells were cultured for 6 days in 30% autologous serum, then switched to 10% lipoprotein-deficient serum for 20 h. The cells were then incubated in medium C with 10 μg/ml ^{125}I-MDA-LDL or 2 μg/ml ^{125}I-LDL for 90 min at 4°C, warmed at 37°C for 3 min, and then fractionated as described under Materials and Methods. After isolation, band 1 was then diluted in 20 mM acetate buffer containing 120 mM KCl, 5 mM NaCl, 5 mM $MgCl_2$ at pH 4.5 and divided into replicate samples. The samples were incubated at 37°C for 30 min in the presence or absence of 5 μg/ml of leupeptin or pepstatin A, or 1 mM PMSF, and the reaction was stopped by transfer to ice. Aliquots were then removed and undegraded ligand was precipitated with 50% TCA. Aliquots of the supernatant were then counted in a gamma-counter. A tube containing the same amount of radioactive lipoprotein but without band 1 was used as a blank and subtracted from the values obtained (≤ 0.02% of added radioactivity from band 1). Values shown are the mean ± 1 SD from quadruplicate determinations. Bottom: the treatment of cells was the same as in the top panel except that the samples of band 1 were incubated at 37°C for 30 min in the presence or absence of 5 μg/ml of pepstatin A and the reaction was stopped by transfer to ice. Aliquots were then removed and undegraded ligand was precipitated with 50% TCA. Equal amounts of radioactivity from TCA supernatants and TCA precipitates were applied to SDS gels and autoradiography was carried out as described under Materials and Methods. TCA supernatant in absence of pepstatin A (lane 1); TCA precipitate in the absence of pepstatin A (lane 2); TCA supernatant in presence of pepstatin A (lane 3); TCA precipitate in the presence of pepstatin A (lane 4); ^{125}I-MDA-LDL before incubation with the monocyte-macrophages (lane 5).

DISCUSSION

The prominent feature of the atherosclerotic plaque is the presence of CE-loaded foam cells (46). Although many cultured cells possess high affinity LDL receptors, LDL uptake does not lead to significant CE accumulation (47). In the macrophage, CE accumulation can be induced by LDL if the LDL is first modified by chemical agents such as malondialdehyde to form MDA-LDL, a ligand for the scavenger receptor (2). Different rates of receptor recycling (48) or the existence of different intracellular pathways for the LDL receptor and the scavenger receptor in the HMM could explain, in part, the resistance of HMM to LDL-induced CE accumulation. In HMM we demonstrated that LDL, at concentrations commonly used in many laboratories (3, 11–23), was processed differently from ligands with other types of receptor systems: MDA-LDL, mannose-BSA, transferrin, HDL, and β-VLDL. It is possible that, at high concentrations of LDL, the number of LDL-receptor complexes that can reach the compartment for hydrolysis may be more rate-limited than is the case for the other ligands studied. Using isopycnic subcellular fractionation Nenseter, Wiik, and Berg (49) have shown that the rate of intracellular processing of LDL in rat liver was very slow compared to that of asialoglycoproteins (50). However, HDL and transferrin appeared to traverse similar subcellular pathways in rat macrophages (51). One explanation may be that shortly after internalization all ligands enter a common intracellular pool or pathway. Further processing may then proceed uniquely for individual ligands.

Scatchard analysis described two binding sites for LDL in HMM in our study. A second explanation may be that one class of receptor sites exists that is saturated at low ligand concentrations and may share the same intracellular pathway as that taken by other ligands. Other LDL receptor sites on the HMM, although specific in their recognition of LDL (possibly, a lower affinity site), may direct ligands into a different intracellular pathway. The experiments from Fig. 8 suggest that the efficiency of lipoprotein hydrolysis may be due in part to entry into the subcellular organelles contained in band 1.

The techniques used in the present study for subcellular fractionation do not permit us to precisely identify the subcellular organelles into which the ligands entered. The techniques that permit such conclusions have largely been applied to studies of liver (52) and are not suitable for the study of lipoprotein metabolism in HMM because of the limited amount of cell material and the difficulty in breaking HMM as compared to liver cells. However, our results cannot be attributed to broken organelles since the distribution of free ligands did not overlap the distribution of the ligands after they had been internalized and subjected to subcellular fractionation. Diment et al. (37) used a Percoll gradient to study the intracellular distribution of ligands in rabbit macrophages. They found that 65% of the cathepsin D activity was associated with d 1.1 g/ml, but in the d 1.05 g/ml fraction cathepsin D activity was threefold higher than β-hexosaminadase activity. Our

results from Fig. 3 are consistent with those of Diment et al. (37) since fraction 1 in our study represented a density range of 1.029–1.113 g/ml and contained most of the cathepsin D activity in HMM. Moreover, a greater cathepsin D/β-hexosaminadase activity was observed in our fraction 1. We found that a Percoll gradient did not allow us to separate free radioactive lipoproteins that had never been exposed to cells from radiolabel that was associated with the 20,000 *g* pellet. After application of the 20,000 *g* pellet to the Percoll gradient, the density fraction 1.036 g/ml contained 81–88% of the label. When radioactive lipoproteins alone were added to the Percoll gradient, virtually all of the radioactivity was found in the density range 1.02–1.04 g/ml (data not shown).

Almost a decade ago, it was suggested that cathepsins play an important role in lipoprotein hydrolysis (53). We provide evidence that cathepsin D is largely responsible for lipoprotein hydrolysis in HMM. More recent studies in nonhuman cells have added support for this role. Rankin, Knowles, and Leake (54) have demonstrated suppression of LDL hydrolysis in murine macrophages by inhibitors of cathepsins. Working with growing chicken oocytes, Nimpf, Radosavljevic, and Schneider (55) reported that after the receptor-mediated internalization of VLDL the apoB of the VLDL was proteolytically cleaved by cathepsin D into a characteristic set of peptides that ranged in molecular mass from 40 to 200 kDa. Three prominant bands were seen between 40 and 80 kDa. It should be noted that these authors did not exclude the possibility that some of their apoB was also hydrolyzed to TCA-soluble products. This proteolysis occurred at an acidic pH in yolk platelets (endosomes) and was postulated to prepare the VLDL for storage in the yolk. We have demonstrated in the present studies that, in vitro, cathepsin D hydrolyzed the apoB of MDA-LDL and LDL to both TCA-soluble and TCA-precipitable products. In HMM three prominent fragments of apoB were seen between 40 and 80 kDa (Fig. 9 bottom) similar to those in the growing chick oocyte (55). It is tempting to speculate that these fragments might play some role in intracellular cholesterol transport; however no data as yet support this hypothesis.

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