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Interaction of high density lipoproteins with cholesteryl ester-laden macrophages: biochemical and morphological characterization of cell surface receptor binding, endocytosis and resecretion of high density lipoproteins by macrophages

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Morphological and biochemical experiments were carried out to investigate the interaction of human serum high density lipoproteins (HDL) with mouse peritoneal macrophages. It is demonstrated that resident mouse peritoneal macrophages express HDL receptors. Subsequent to receptor-mediated binding, HDL are internalized and intracellularly transported into endosomes. These endosomes do not fuse with the lysosomal compartment but interact with the margin of intracellular plasma lipid droplets. Macrophages do not degrade, but rather resecrete internalized HDL particles as described for the transferrin-receptor pathway. HDL binding to freshly isolated macrophages is saturable at a concentration of ~320 ng HDL-protein/mg cell protein and a Scatchard plot indicates the presence of some 130 000 – 190 000 receptors/cell with a K_d of $\sim 9 \times 10^{-7}$ M. Binding of HDL on the macrophage surface is significantly enhanced in cholesterol-laden macrophages, whereas the increase in the rate of uptake and secretion is less pronounced. Within the HDL fraction the HDL_2 subclass showed higher binding, uptake and secretion activity as compared with HDL_3. From these experimental data we postulate that cholesterol uptake from macrophages is mediated by HDL particles which interact with these cells *via* a receptor-mediated retroendocytosis pathway.

Key words: macrophages/HDL receptor/cholesteryl ester accumulation/retroendocytosis/gold-labeled lipoproteins

Introduction

The accumulation of cholesterol and cholesteryl esters within arterial cells, notably smooth muscle cells and monocyte-derived macrophages is an outstanding pathological feature of atherosclerosis (Brown and Goldstein, 1983; Ross and Glomset, 1973; Goldstein and Brown, 1977; Stein *et al.*, 1975). The concentration of cholesterol within the cells is determined by the balance between the uptake of lipoprotein-bound cholesterol, intracellular cholesterol synthesis and cholesterol secretion (Goldstein and Brown, 1977; Mahley and Innerarity, 1983; Goldstein *et al.*, 1979a). In both smooth muscle cells and macrophages the uptake of lipoproteins appears to occur primarily by receptor-mediated mechanisms (Goldstein and Brown, 1977; Mahley and Innerarity, 1983; Goldstein *et al.*, 1979b; Brown and Goldstein, 1983). In the smooth muscle cell, as in the fibroblast which expresses the apo B,E-receptor, the cholesterol content of the cell is strongly regulated because the uptake of lipoprotein-bound cholesterol suppresses the expression of new surface receptors and also reduces the rate of cholesterol synthesis by suppressing the activity of hydroxy-methylglutaryl CoA (HMG-CoA) reductase (Brown *et al.*, 1976). These cells do not seem to depend, therefore, on mechanisms of cholesterol efflux, except perhaps in cases of extreme overload, that can be induced by chemically modified low density lipoproteins (Goldstein and Brown, 1977). On the other hand, in macrophages, stimulation of uptake of lipoprotein-bound cholesterol by receptor-mediated mechanisms, such as the uptake of acetyl-LDL or β-VLDL, does not lead to a suppression of receptor expression, and prevention of accumulation of cholesterol depends upon the presence of an effective cholesterol acceptor, such as HDL, in the external medium (Bailey, 1973; Brown *et al.*, 1979; Ho *et al.*, 1980). In the absence of such an acceptor, cholesteryl esters accumulate in the cytoplasm (Brown *et al.*, 1979; Brown and Goldstein, 1983) and undergo a continual cycle of hydrolysis and re-esterification (Brown *et al.*, 1980; Spector *et al.*, 1979). The mechanism of cholesterol secretion by macrophages to acceptors such as HDL is unclear. It has been shown, however, that cholesterol-laden macrophages synthesize and secrete apo E/phospholipid particles (Basu *et al.*, 1981, 1983) and it has been suggested that these, together with free cholesterol, act as a substrate for lecithin cholesterol acyltransferase (LCAT), thus leading to the formation of a cholesteryl ester-enriched HDL particle (HDLc) (Mahley, 1979; Basu *et al.*, 1982). This particle can be taken up rapidly by hepatocytes (Mahley, 1981; Hui *et al.*, 1981) thus mediating 'reversed cholesterol transport' to the liver (Brown and Goldstein, 1983; Basu *et al.*, 1982; Mahley, 1981). Here we report studies of the mechanism of HDL-stimulated secretion of cholesterol from cholesteryl ester-laden macrophages using morphological and biochemical methods. We show that the cells express HDL receptors which are increased by cholesterol ester loading and that HDL particles bound to the receptor are internalized to a non-lysosomal, cytoplasmic compartment and then secreted by retroendocytosis.

Results

The morphology of HDL binding on the macrophage surface was studied with freshly isolated and cholesteryl ester-laden resident macrophages in tissue culture experiments using gold-labeled HDL. The macrophages were laden with cholesteryl ester, using unlabeled acetyl-LDL for 0 h (Figure 1a), 3 h (Figure 1b) and 18 h (Figure 1c) and then chased with gold-labeled HDL for 1 h at 4°C. The macrophages showed an apparent heterogeneity in the distribution of HDL binding sites on their surface as revealed after single treatment of cells with gold-labeled HDL. In freshly-isolated macrophages the HDL-gold complexes appear predominantly in small clusters of 20 – 30 gold particles (Figure 1a), whereas the gold particles on the surface of cholesteryl ester-laden macrophages are organized in large plaques which are more or less randomly distributed over the entire macrophage cell surface (Figure 1b,c). As obvious from the number of bound gold particles, HDL receptor activity is significantly increased when the macrophages are challenged with cholesterol.

Control experiments were carried out by exposing the cells first to the unlabeled lipoprotein for 1 h at 4°C, thoroughly

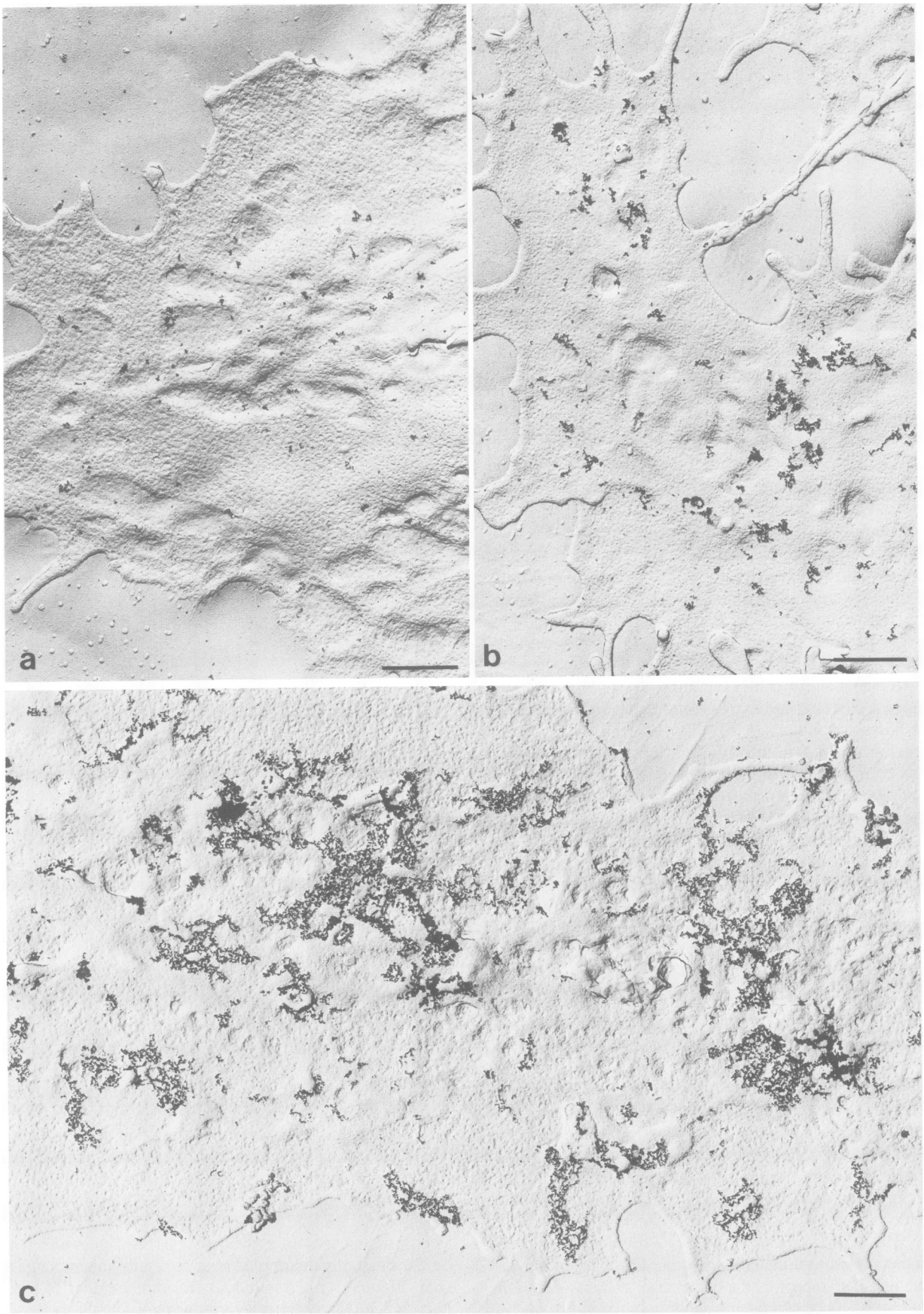

Fig. 1. Cell surface replicas of cultured macrophages laden with cholesterol after treatment with unlabeled acetyl-LDL for 0 h (**a**), 3 h (**b**) and 18 h (**c**) at 37°C. Thereafter the cells were incubated with 16 nm gold-labeled HDL for 1 h at 4°C. The HDL-receptor activity as discerned from the number of gold-labeled HDL particles on the surface of the macrophages is significantly increased subsequent to acetyl-LDL incubation (**a**–**c**). Bar 1 μm.

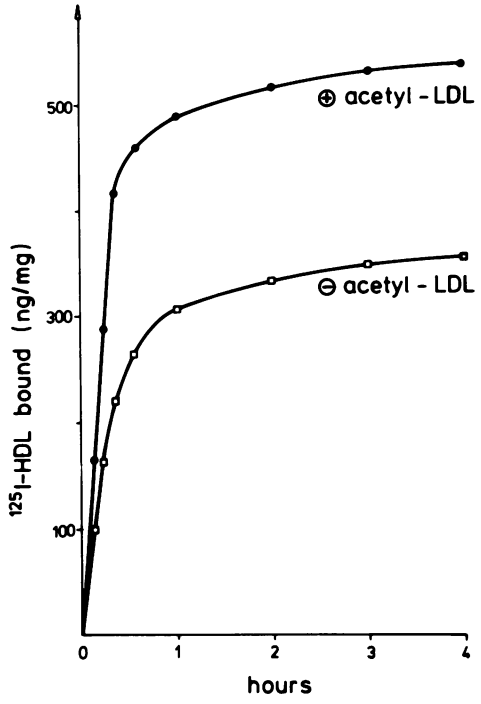

Fig. 2. Binding of [^{125}I]HDL by mouse peritoneal macrophages at 4°C. 1 ml of medium without serum was added to each dish. For at least 1 h the dishes were placed at 4°C. Each dish was then incubated with 400 μg of [^{125}I]HDL (80 c.p.m./ng protein). At the indicated intervals the incubation was stopped by removing the [^{125}I]HDL from duplicate dishes of freshly isolated or cholesterol-laden macrophages. After the standard wash the cells were dissolved by incubating the dishes at 37°C for 1 h with 600 μl of 0.3 N NaOH. The [^{125}I]HDL activity associated with the cells was determined for each dish. The mean cellular protein content was 250 μg/dish. Cholesteryl ester loading with acetyl-LDL was performed for at least 6 h (75 μg/dish).

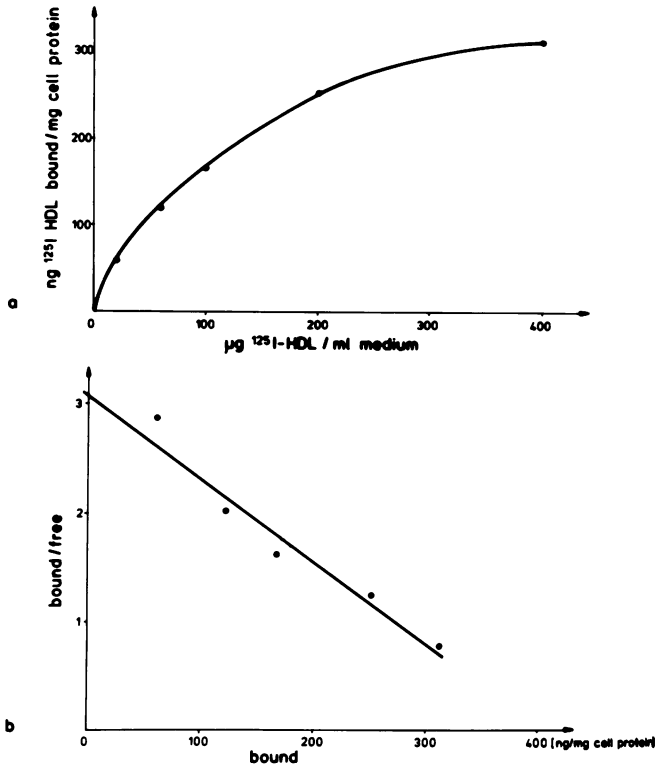

Fig. 3. Rate and equilibrium constants for binding of [^{125}I]HDL for mouse peritoneal macrophages at 4°C. (a) Concentration-dependent binding of human [^{125}I]HDL (80 c.p.m./ng of protein) to freshly-isolated macrophages. The monolayers were incubated for 4 h at 4°C with the iodinated lipoprotein at the concentrations indicated. Each point represents the average of duplicate determinations. The mean cellular protein content was 250 μg/dish. Maximal binding was reached at 320 ng HDL/mg cell protein. Half-maximal binding was reached at a concentration of ~95 μg HDL/ml medium. (b) Scatchard plot for binding of HDL to macrophages. 'Bound/free' is the lipoprotein bound (ng of protein/mg cell protein)/lipoprotein free in the medium (ng of protein/ml medium). Slope = -7.6×10^{-6} ml/ng; $K_d = 9 \times 10^{-7}$ M.

washing them, and then treating them with the same lipoprotein bound to gold for a further hour at 4°C. When the cells were treated first with unlabeled lipoprotein and then with the same lipoprotein labeled with gold (either the 16 nm or 40 nm diameter variety), the cell surface was always completely devoid of gold label. Binding of the unlabeled ligand to its receptor thus prevents subsequent binding of the labeled ligand, confirming that the lipoprotein-gold complexes interact specifically with the lipoprotein receptors and that there are no additional binding sites for the gold itself under the experimental conditions used. Negative staining of lipoprotein-gold complexes shows that each gold particle is completely buried within a covering of lipoprotein molecules (4–6 lipoprotein particles/gold particle). Thus, only the lipoprotein, and not the gold itself, is exposed and available for binding to the receptor at the cell surface.

Similarly, as demonstrated for gold-labeled HDL, binding studies with ^{125}I-labeled HDL (Figure 2) revealed that accumulation of macrophage cholesterol by pre-incubation with unlabeled acetyl-LDL leads to a significant increase in HDL binding (320 ng HDL bound/mg cell protein in freshly-isolated macrophages compared with 510 ng HDL bound/mg cell protein in cholesteryl ester-laden macrophages). As a function of [^{125}I]HDL concentration (Figure 3a) the macrophages bind increasing amounts of [^{125}I]HDL with a maximum of ~320 ng HDL-protein/mg cell protein, and a Scatchard plot indicates the presence of some 130 000–190 000 receptors with a K_d of $\sim 9 \times 10^{-7}$ M (Figure 3b). In competition experiments (not shown here) the pre-incubation with a 5- to 10-fold molar excess of unlabeled HDL completely suppresses [^{125}I]HDL binding to the macrophage surface.

To elucidate possible differences in binding of the major HDL subclasses HDL_2 and HDL_3, cholesteryl ester-laden mouse peritoneal macrophages were incubated with identical concentrations (protein or cholesterol) of ^{125}I-labeled HDL_2 and HDL_3 (isolated by ultracentrifugation from the same donor) using the same experimental protocol as described for total HDL. In these studies, HDL_2 exhibited an ~1.5-fold higher binding capacity compared with HDL_3. The intracellular pathways of acetyl-LDL and HDL were investigated in thin sections of cholesterol-laden macrophages (Figures 4–7). Cholesteryl ester-laden macrophages, when chased with gold-labeled acetyl-LDL for 15 min at 37°C, specifically deposit the acetyl-LDL particles in the lysosomal compartment (Figure 4a). By contrast, gold-labeled HDL, when incubated for 15 min at 37°C with cholesterol-laden macrophages, is taken up by these cells into non-lysosomal endosome compartments (Figure 4b). The different subcellular localization of acetyl-LDL *versus* HDL was further substantiated by acid phosphatase staining of macrophages. In thin sections of macrophages warmed up to 37°C for 15 min, gold particles were found in coated pits and coated vesicles (Figure 4c–e), indicating that the uptake of HDL in macrophages occurs through the process of receptor-mediated endocytosis.

The morphology of the intracellular acetyl-LDL and HDL

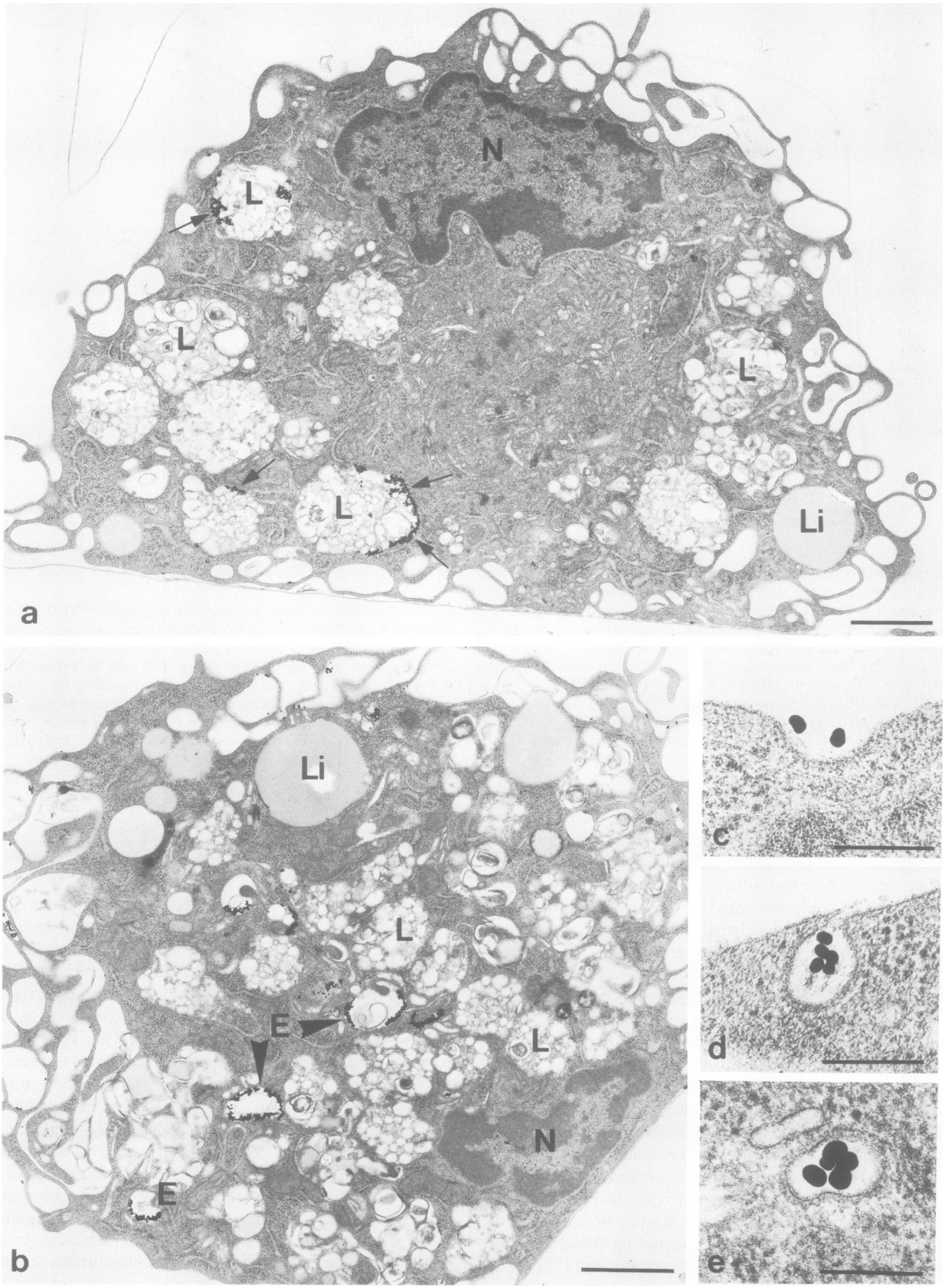

Fig. 4. Intracellular appearance of gold-labeled acetyl-LDL and HDL in cholesterol-laden macrophages (18 h, 37°C). Li = lipid droplet, N = nucleus. (**a**) Cholesterol-laden macrophages were incubated with 16 nm gold-labeled acetyl-LDL for 15 min at 37°C. Gold grains (arrows) are restricted to lysosomal compartments (L). Bar 1 μm. (**b**) Cholesterol-laden macrophages were incubated with 16 nm gold-labeled HDL for 15 min at 37°C. The label is taken up by these cells into non-lysosomal endosome compartments (E). Bar 1 μm. (**c**–**e**) The endocytosis of 40 nm gold-labeled HDL by coated pits (**c**), coated invagination (**d**) and coated vesicle (**e**). Bar 0.2 μm.

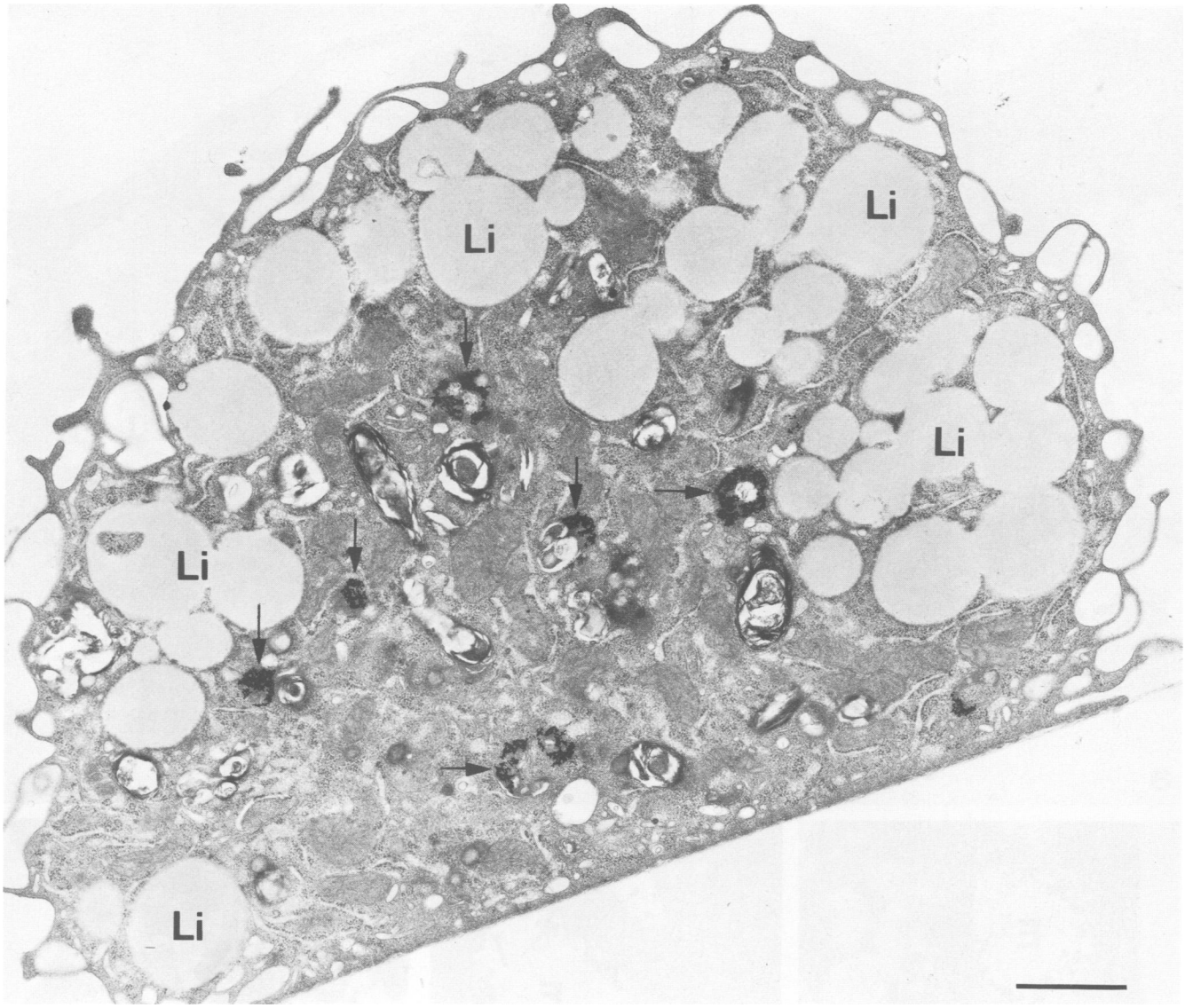

Fig. 5. Macrophage laden with cholesterol for 24 h at 37°C in the presence of unlabeled acetyl-LDL, then incubated with 16 nm gold-labeled acetyl-LDL for 1 h at 37°C. After thorough washings the cells were cultivated for another 3 h at 37°C in lipoprotein-free culture medium. The cell is filled with numerous lipid droplets (Li) and tertiary lysosomes containing gold particles (arrows). Bar 1 μm.

pathways was further investigated in thin sections of cholesterol-laden macrophages sequentially incubated with gold-labeled acetyl-LDL or gold-labeled, iron-saturated transferrin and gold-labeled HDL for various time intervals. (i) Macrophages laden with cholesterol for 24 h at 37°C using unlabeled acetyl-LDL were incubated with 16 nm gold-labeled acetyl-LDL for 1 h at 37°C. After thorough washings the cells were further incubated for 1 – 3 h at 37°C in lipoprotein-free culture medium in order to canalize all gold-labeled acetyl-LDL complexes into lysosomal compartments. Besides 'foamy organelles' representing the lysosomal compartment (as shown after acid phosphatase staining, Shio *et al.*, 1974), cytoplasmic 'lipid droplets' and tertiary lysosomes filled with gold particles can be observed (Figure 5). (ii) Cholesterol-laden macrophages were sequentially incubated for 1 h at 37°C with 16 nm gold-labeled acetyl-LDL and 40 nm gold-labeled HDL (for details see legend in Figure 5). The acetyl-LDL-gold particles were found in the multivesicular vacuoles (lysosomes), whereas the HDL-gold particles were identified in non-lysosomal compartments (Figure 6). HDL-containing vesicles in part were detectable in intimate contact with the margin of cytoplasmic lipid droplets (Figure 6a – d). In numerous sections, fusion of the endocytotic vesicles and the cytoplasmic lipid droplets was observed (Figure 6b,c). (iii) When the cells treated as described in (ii) were chased again with lipoprotein-free culture medium for 3 h at 37°C, both types of gold particles were found at surface regions of the macrophages (Figure 7a,b). Acetyl-LDL and HDL, however, were secreted at separate membrane areas (Figure 7b). (iv) To differentiate further between the AcLDL- and HDL-pathway in macrophages, experiments were performed as described in (ii), but instead of AcLDL, iron-saturated transferrin was used. Cholesterol-laden macrophages were sequentially incubated for 30 min at 37°C with 40 nm gold-labeled HDL and 16 nm gold-labeled iron-saturated transferrin. Both the HDL-gold particles and the transferrin-gold particles were found in the same non-lysosomal compartments (Figure 9a,b).

Uptake and possible degradation of HDL by macrophages was monitored by parallel experiments using [^{125}I]HDL. As shown in Figure 8 left, the cell-associated [^{125}I]HDL radioactivity of freshly-isolated (Figure 8, left □) as well as cholesterol-laden macrophages (Figure 8, left ●) decreases with incubation time.

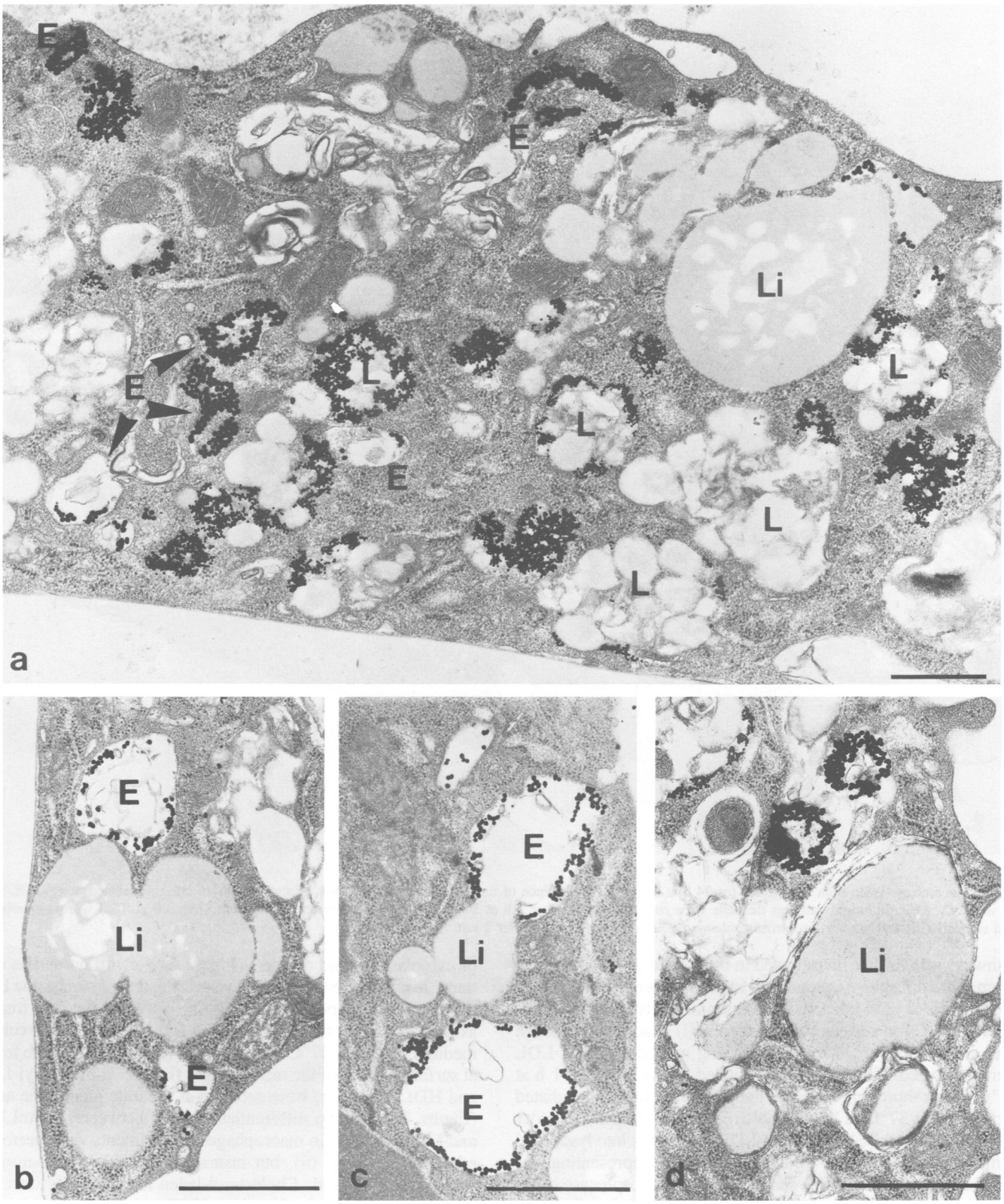

Fig. 6. Thin sections of macrophages after sequential exposure to acetyl-LDL 16-nm gold and HDL 40-nm gold. E = endosome, L = lysosome, Li = lipid droplet. (**a**) Cholesterol-laden macrophage (18 h, 37°C) treated with acetyl-LDL 16 nm gold for 1 h at 37°C, chased in normal culture medium for 1 h at 37°C and further incubated with HDL 40-nm gold. After sequential treatment the deposition of each of both lipoprotein types occurs in different cellular compartments and is almost identical to that obtained with separate incubations of acetyl-LDL and HDL, respectively (see Figures 4 and 5). Bar 1 μm. (**b**–**d**) HDL containing vesicles (E) are in close contact with lipid droplets (Li) (**b**) or have fused with the lipid droplets (**c,d**). Bar 1 μm.

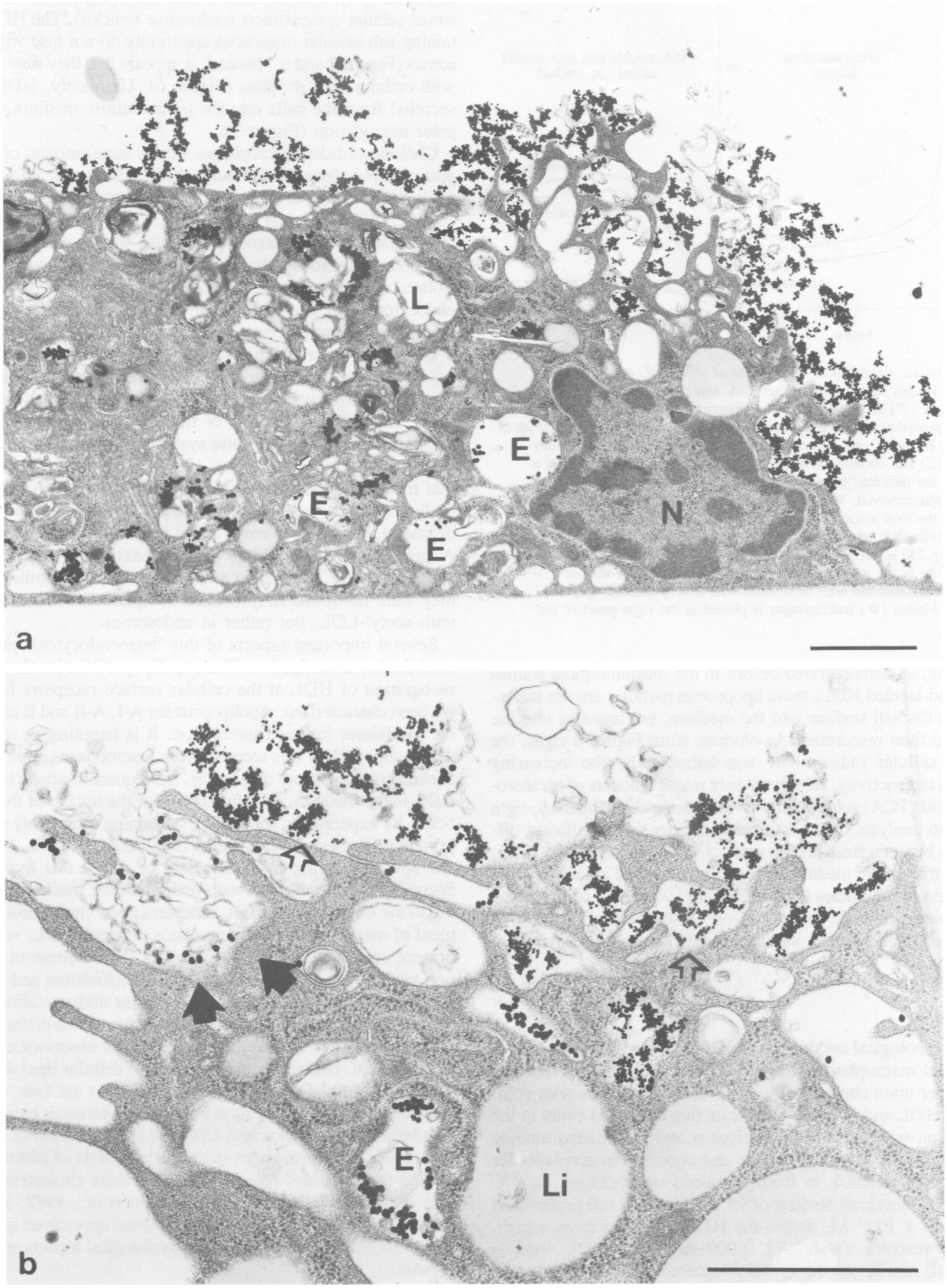

Fig. 7. (**a**) Cholesterol-laden macrophages (acetyl-LDL, 18 h, 37°C) were incubated for 1 h at 37°C with acetyl-LDL, 16-nm gold, chased with normal culture medium for 1 h at 37°C, exposed to HDL 40-nm gold for 1 h at 37°C and chased again for 3 h at 37°C with normal culture medium. Both types of gold particles are found on the surface of the macrophages. Bar 1 μm. (**b**) The small (open arrows) and the large (filled arrows) gold particles are independently secreted on separate membrane areas. Bar 1 μm. E = endosome, L = lysosome, Li = lipid droplet, N = nucleus.

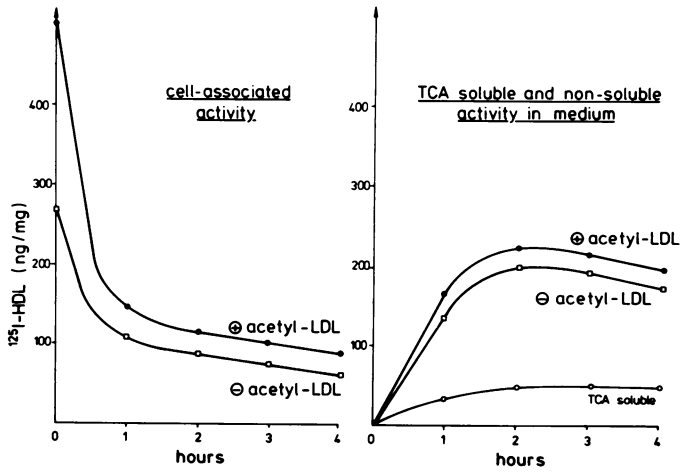

Fig. 8. Uptake, degradation, and release of [^{125}I]HDL at 37°C by cholesterol-laden macrophages. Each dish was first incubated for 1 h at 4°C with 400 μg [^{125}I]HDL (80 c.p.m./ng protein). Dishes were washed by the standard procedure. Then cells were incubated in the presence of 400 μg of unlabeled HDL in 1 ml Dulbecco medium. Duplicate dishes of freshly-isolated (□) and cholesterol laden (●) macrophages were incubated at 37°C for the indicated intervals and rapidly chilled to 4°C. Then the medium was removed. We determined, as described in Materials and methods, the total amount of [^{125}I]HDL activity that remained associated with the cells (**left panel**). The content of ^{125}I-labeled TCA-soluble material (by adding 250 μl of 50% TCA per dish) (right panel, ○) as well as the total activity in the medium was measured. The TCA-non-soluble ^{125}I-activity in the medium after incubation with freshly-isolated (□) and cholesterol-laden (●) macrophages is plotted in the right panel of the figure.

However, as demonstrated before in the morphological studies with gold-labeled HDL, these lipoprotein particles are not released from the cell surface into the medium, but ingested into the cells and then resecreted. As obvious from Figure 8 right, the loss of cellular radioactivity was balanced by the increasing medium radioactivity. However, only minor amounts of trichloroacetic acid (TCA) soluble activity were detectable (Figure 8, right ○) upon analysis of medium radioactivity (no significant differences between fresh and cholesterol-laden macrophages), while the majority of the medium ^{125}I-activity remained associated with the lipoprotein particles (Figure 8, right □: freshly isolated, and ●: cholesterol-laden macrophage), indicate that [^{125}I]HDL is resecreted from the cells and not degraded in the lysosomal compartment.

Discussion

Our morphological and biochemical data demonstrate that mouse peritoneal macrophages exhibit HDL receptors which increase in number upon cholesterol loading of cells. Studies with gold-labeled HDL and [^{125}I]HDL indicate that the initial event in the interaction with these cells involves receptor-mediated binding (Figures 1 and 4c–e). The rate and equilibrium constants for binding of [^{125}I]HDL to freshly-isolated macrophages, at 4°C with a half-maximal binding of 95 ng HDL/mg cell protein and a K_d of 9 x 10^{-7} M, define the HDL binding site as a high-affinity receptor (with ~130 000–190 000 HDL binding sites/cell) similar to the apo B,E-receptor site (Goldstein and Brown, 1977; Mahley and Innerarity, 1983).

Subsequent to binding, HDL is internalized (Figures 4 and 6). In contrast to acetyl-LDL, which is also taken up through receptor-mediated endocytosis by mouse peritoneal macrophages (Goldstein *et al.*, 1983; Robenek *et al.*, 1984), HDL is not degraded by lysosomes, but is exclusively present in a non-lysosomal cellular compartment (endosomic vesicles). The HDL-containing sub-cellular organelles apparently do not fuse with lysosomes (Figure 4 and 6). Instead, it appears that they may interact with cellular lipid droplets (Figure 6). Ultimately, HDL is resecreted from the cells into the tissue culture medium without prior degradation (Figure 7).

Cholesterol-laden macrophages secrete large amounts of apo E/phospholipid discs (Basu *et al.*, 1981, 1982) and HDL in the medium is capable of removing free cholesterol from these cells (Bailey, 1973; Ho *et al.*, 1980). However, the detailed mechanisms of cholesterol removal are still unclear. It appears from our experiments that HDL, when incubated with freshly-isolated and cholesteryl ester-laden macrophages, undergo retro-endocytosis and that cholesterol uptake from macrophages requires specific interaction with cytoplasmic lipid droplets.

Using iron-saturated human transferrin instead of acetyl-LDL we have shown that both transferrin and HDL are transported to endosomal compartments. We have chosen transferrin as a probe since the endocytosis of transferrin provides interesting contrasts to other endocytotic systems (Newman *et al.*, 1982; Octave *et al.*, 1983; Crichton, 1984). In particular, it is known that transferrin dissociates from these receptors, probably in a prelysosomal compartment (Octave *et al.*, 1983), so that it is not degraded, but rather released intact from cells after endocytosis. A similar pathway could be substantiated in our studies of the HDL pathway. Transferrin and HDL were also similar in that they were not found in lysosomal compartments, as is the case with acetyl-LDL, but rather in endosomes.

Several important aspects of this 'retroendocytosis pathway' need further investigation. First, apolipoproteins involved in the recognition of HDL at the cellular surface receptors have not yet been characterized. Apolipoproteins A-I, A-II and E are possible candidates for such interaction. It is tempting to speculate that apolipoprotein E is secreted from macrophages upon cellular cholesterol enrichment and serves, subsequent to interaction with HDL in the medium, as a modulator for the uptake of these particles. By experiments with HDLc containing exclusively apolipoprotein E (Brown and Goldstein, 1983; Mahley, 1979, 1981) we are now attempting to substantiate or refute this hypothesis. Secondly, the subcellular events and molecular mechanisms leading to the expression of HDL receptors upon cholesterol enrichment of macrophages in the presence of acetyl-LDL, remain at present unresolved. In LDL-receptor cells, cholesterol depeletion leads to the expression of receptors (Goldstein and Brown, 1977; Mahley and Innerarity, 1983). Better understanding of this paradox should provide important insight into two different regulating processes. Finally, the morphologic observation of 'fusion' of HDL-containing endosomes and cellular lipid droplets, as well as our observation that lysosomes do not fuse with the endosomal organelles, point to important differences between the cellular metabolism of acetyl-LDL and HDL. In view of the suggested role of macrophages in the pathogenesis of atherosclerosis and the presumed role of HDL in reverse cholesterol transport (Brown and Goldstein, 1983; Basu *et al.*, 1982; Mahley, 1979; Hui *et al.*, 1981) elucidation of these unresolved questions may shed light on important pathophysiological aspects of atherogenesis.

Materials and methods

Materials

Cells were harvested from male NMRI-SPF mice (25–35 g). Fetal calf serum (FCS) was obtained from Gibco Bio-Cult Ltd. (Cat. No. 629). Dulbecco's modified

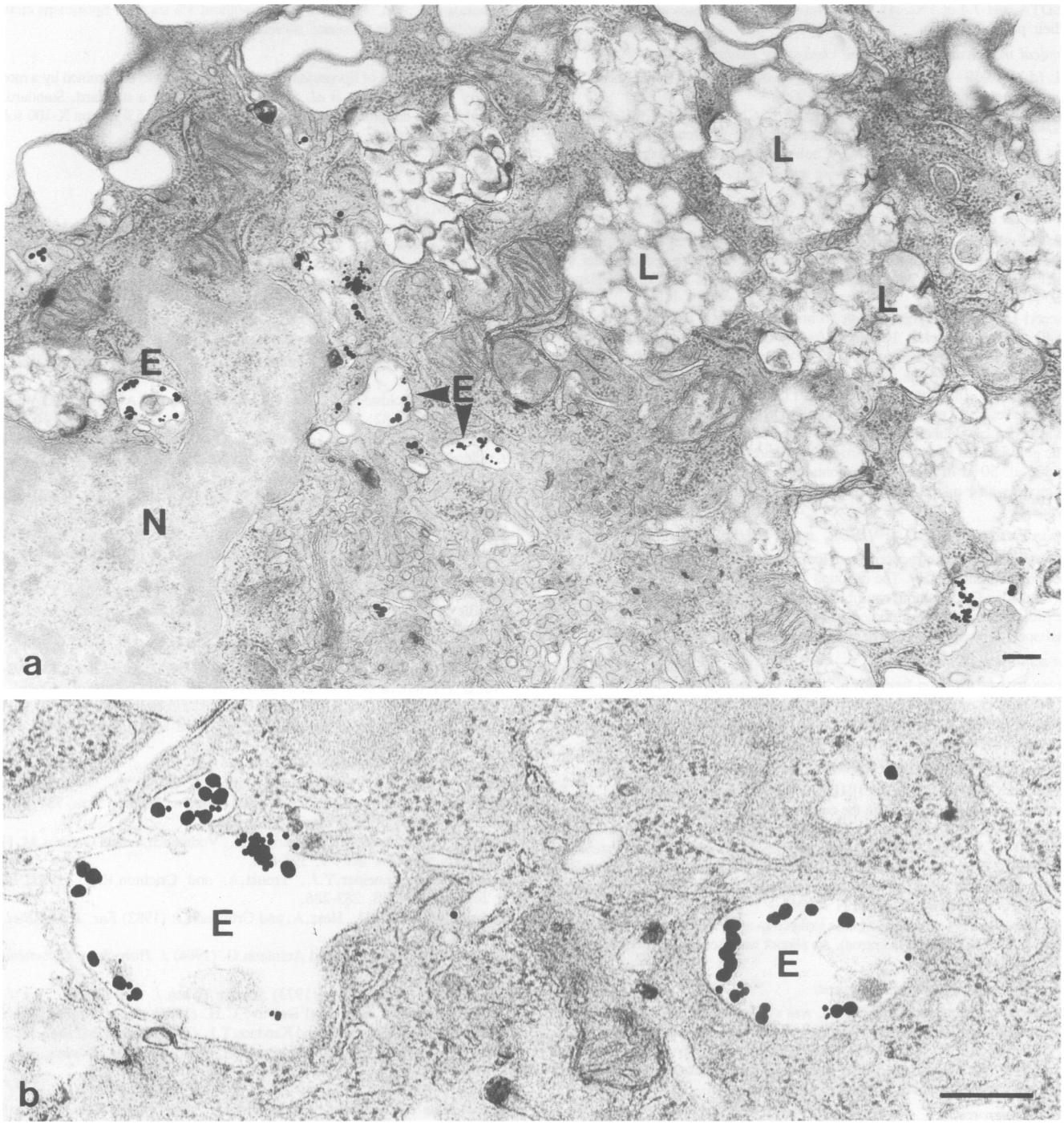

Fig. 9. Thin sections of cholesterol-laden macrophages after sequential exposure (30 min at 37°C) to HDL (40 nm gold) and iron-saturated transferrin (16 nm gold). Bars 0.5 μm. (**a**) The deposition of both types of gold particles occurs in the same endosomal compartments (E). Gold particles are not taken up by these cells into lysosomal compartments (L). N = nucleus. (**b**) Two endosomes (E) containing both types of gold particles and showing fusion with small endosomes.

Eagle medium (DMEM) and phosphate-buffered saline (PBS) were purchased from Flow Lab. (Cat. No. 10-331-25 and 18-610-24). Tissue culture equipment was obtained from Falcon.

Mouse macrophage monolayers

Murine peritoneal macrophages were obtained from unstimulated mice by peritoneal lavage in PBS containing 0.5 U heparin/ml. The peritoneal fluid from 30–50 mice was pooled and the cells were centrifuged (400 *g*, 10 min, room temperature) (Ho *et al.*, 1980), washed once with 20 ml DMEM and resuspended in 12 ml DMEM containing 10% FCS, penicillin (100 U/ml) and streptomycin (100 μg/ml). Aliquots (0.5–1 ml) of the cell suspension corresponding to the number of cells from two mice were dispensed into 35 x 10 mm plastic Petri dishes containing 1 ml DMEM with the additions mentioned above and then incubated in a humidified incubator at 37°C (5% CO_2). On the second day, each dish was extensively washed with 1 ml of DMEM without serum until there were no non-adherent cells visible under the microscope. Each dish contained ~150–250 μg of total cell protein.

Lipoproteins

HDL (d = 1.063–1.21), HDL_2 (d = 1.063–1.125), HDL_3 (d = 1.125–1.21) and LDL (d = 1.019–1.063) were isolated from serum of individual normolipidemic E-3 homozygote volunteers by sequential utlracentrifugation in a Beckman L 8-70 ultracentrifuge using a 50.3 Ti or 70 Ti rotor (Beckman) at 4°C. The lipoprotein fractions were dialyzed against 0.15 M NaCl/5 mM

NaEDTA, pH 7.4 at 4°C. All concentrations of lipoproteins are given in terms of their protein content.

Chemical modification of LDL and cholesterol loading of macrophages

LDL (d = 1.019 – 1.063 g/ml) was acetylated by repeated additions of acetic anhydride as described (Brown *et al.*, 1979).

Acetyl-LDL was dialyzed against 0.15 M NaCl, 0.3 mM NaEDTA, pH 7.4 at 4°C. The modified LDL showed enhanced mobility on electrophoresis in agarose gel at pH 8.6 (Brown *et al.*, 1976). Cholesterol loading was performed by incubating the dishes at 37°C with 75 μg acetyl-LDL/dish for the indicated time intervals.

Preparation of colloidal gold and complexes with lipoproteins

Colloidal gold (16 nm versus 40 nm diameter) was prepared by the method of Frens (1973). Conjugation of LDL with the negatively-charged colloidal gold particles was performed according to a method modified from that of Handley *et al.* (1981).

Acetyl-LDL was conjugated with 16 nm gold particles and HDL with 16 nm and 40 nm gold particles. 100 μg of freshly dialyzed lipoprotein were diluted to 500 μl with distilled water and 5 ml of colloidal gold solution was added in a fast jet. After 5 min the complexes were separated by ultracentrifugation in a Beckman SW 28.1 rotor for 30 min at 11 000 r.p.m. and 4°C. All preparations were routinely examined by negative stain before use. The complex stability was tested by incubation with 0.5 M NaCl solution. The final concentration of the gold-labeled lipoprotein fractions was determined to be ~40 – 60 μg protein/500 μl. 50 μl of this solution containing 4 – 6 μg gold-labeled lipoprotein was mixed with 1 ml of tissue culture medium for each dish. For details see figure legends.

Preparation of [^{125}I]HDL, [^{125}I]HDL$_2$ and [^{125}I]HDL$_3$

HDL and the HDL subfractions were iodinated by the iodine monochloride procedure (McFarlane, 1958). The unbound iodine was removed by chromatography on a Sephadex G-25 column equilibrated with PBS buffer containing 0.5% FCS. The protein fractions were pooled and dialyzed against 0.15 M NaCl for 20 h. Final specific activities were between 60 and 90 c.p.m./ng of protein.

Iron-saturation of transferrin

Human transferrin (iron-free, from Sigma) was iron-saturated by the methods of Galbraith *et al.* (1980) and used in all experiments.

Binding of iron-saturated transferrin to 16-nm gold particles was carried out following the protocol of Harding *et al.* (1983).

Binding experiments with ^{125}I-labeled lipoproteins

Dishes were incubated with [^{125}I]HDL or [^{125}I]HDL subfractions at 4°C. The experiments were performed with the concentrations indicated in the figure legends, according to the methods described by Goldstein and Brown (1977) and Brown *et al.* (1976). After the incubation, the cells were washed five times extensively with PBS/0.2% bovine serum albumin (BSA) and 3 times with PBS without BSA. After this standard wash the cells were released from the dishes by incubation at 37°C for 1 h in 600 μl of 0.3 N NaOH.

All associated ^{125}I radioactivity was assayed in an Automatic Gamma counting System MR 252 C/TP/TM (Kontron). An aliquot was used to determine the cellular protein content.

Uptake and degradation measurements

The proteolytic degradation of [^{125}I]HDL was assayed by the amount of ^{125}I-labeled TCA-soluble (non-iodine) material in the culture medium (Goldstein and Brown, 1977; Brown *et al.*, 1976). Uptake of [^{125}I]HDL was determined after incubation at 37°C for the indicated time intervals. Uptake measurements were performed as described under binding experiments.

Electron microscopy

Thin sectioning. After fixation of the cultured macrophages the cells were post-fixed in 1% OsO_4 for 1 h, washed, dehydrated and embedded in Epon 812. Ultrathin sections were cut on an Ultrotome III (LKB) with diamond knives, stained with uranyl acetate followed by lead citrate and examined in a Phillips EM 201 at 60 kV.

Surface replication. For surface replication, 1 x 1 cm pieces from the bottom of the Petri dish, covered with the macrophages, were cut out using a soldering iron. The fixed cells were dehydrated and critical point dried. Platinum-carbon replicas of the surface of the cell cultures were made in a Balzers BA 300 freeze-etching apparatus (Balzers AG, Liechtenstein), equipped with electron gun evaporator and a quartz crystal thickness monitor. Replicas were obtained by shadowing the cell surface with platinum-carbon at an angle of 38°, followed by carbon at 90°. The replicas were cleaned overnight in household bleach and washed in distilled water. They were picked up on 200 mesh copper grids and examined in a Phillips EM 201 and 60 kV (Robenek *et al.*, 1982).

Acid phosphatase staining. After pre-fixation, the cells were washed and tested for acid phosphatase by the method described by Shio *et al.* (1974). A modified Gomori medium (Barka and Anderson, 1962) with β-glycerophosphate as substrate was used, as pH 5.0 with or without 5% sucrose. Specimens incubated in medium without substrate served as controls.

Other methods

The protein content of lipoprotein fractions and cells was determined by a modified Lowry method (Lowry *et al.*, 1951), using BSA as a standard. Standards and samples were delipidated by addition of 100 μl of a 2.5% Triton X-100 solution prior to photometric measurements.

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