Enhanced Macrophage Uptake of Low Density Lipoprotein after Self-Aggregation

John C. Khoo, Elizabeth Miller, Patricia McLoughlin, and Daniel Steinberg

Incubation of mouse peritoneal macrophages with native human low density lipoprotein (LDL) did not cause any significant storage of intracellular cholesteryl esters. However, when the LDL was subjected to brief (30-second) vortexing, it formed self-aggregates that were rapidly ingested and degraded by macrophages, converting them to cholesteryl ester-rich foam cells. Such aggregates were as potent as acetyl-LDL in stimulating cholesterol esterification in the macrophages. The degradation of LDL aggregates was strongly inhibited by cytochalasin B (85%), whereas degradation of native LDL was only weakly inhibited (23%), suggesting that uptake occurred by phagocytosis rather than pinocytosis. Several lines of evidence suggest that the phagocytic uptake depends, in part, upon the LDL receptor and not the acetyl-LDL receptor: 1) soluble, native LDL and β-VLDL (but not acetyl-LDL) competed for uptake and degradation of LDL aggregates; 2) reductive methylation of LDL before vortexing reduced the effect of the aggregates on degradation and cholesterol esterification; 3) heparin, which inhibits binding of native LDL to its receptor, reduced the degradation of LDL aggregates. These studies show that self-aggregation of LDL markedly enhances its uptake by macrophages, probably by phagocytosis and at least, in part, via the LDL receptor. Aggregates of LDL in the artery wall—either self-aggregates or mixed aggregates including matrix components—may induce foam cell formation and favor the formation of the fatty streak. (Arteriosclerosis 8:348–356, July/August 1988)

Lipid-laden foam cells in the atherosclerotic lesion are derived largely from monocyte-macrophages. However, in vitro studies show that macrophages take up native low density lipoprotein (LDL) at relatively low rates, rates insufficient to cause cholesteryl ester accumulation to the extent found in vivo. Macrophages can accumulate large amounts of cholesteryl esters when incubated with either chemically modified LDL or LDL isolated from human atherosclerotic lesions. LDL is also capable of forming insoluble complexes with dextran sulfate, arterial proteoglycans, heparin-fibronectin-denatured collagen, and granules of mast cells. These insoluble LDL complexes are rapidly taken up by macrophages, but the mechanisms of their uptake and degradation have not been fully elucidated.

During studies of foam cell formation in cell culture, we noted marked differences among different LDL preparations in their ability to induce lipid storage in mouse peritoneal macrophages. While most preparations caused little accumulation of lipid-staining droplets, an occasional preparation caused striking lipid accumulation. The tubes containing these samples generally showed some precipitated material. We then tried to develop a reproducible way to effect the changes apparently caused by storage and found that simply vortexing LDL solutions at room temperature would cause aggregation. The ease with which LDL is denatured at surfaces has been recognized for some time. However, the extent of such denaturation during vortexing turns out to be surprising; vortexing for even 30 seconds caused about 50% of the LDL protein to become precipitable. The studies described below show that these LDL self-aggregates are avidly ingested by mouse peritoneal macrophages. The LDL aggregates were as effective as soluble, native LDL in competing for the degradation of soluble native LDL. In addition, heparin reduced the degradation of both LDL aggregates and native LDL to nearly the same extent. We suggest that the common denominator in this, and the several other LDL complexes that are degraded more rapidly than LDL itself, is simply that they represent aggregates. The self-aggregation of LDL produced by vortexing does not involve the addition of new potential ligands and thus, clearly identifies the aggregation per se as the key variable.

The remarkable ease and extent with which LDL undergoes denaturation during this simple physical handling at room temperature or at 4°C raises the possibility that some analogous process may occur in vivo at body temperature and contribute to foam cell formation during atherogenesis.

**Methods**

**Isolation of Mouse Peritoneal Macrophages**

Primary cultures of mouse peritoneal macrophages were prepared as described previously. Macrophages...
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Figure 1. The formation of LDL aggregates by vortexing. A. 1.0 ml of native LDL (•) or methyl-LDL (△) (0.25 mg protein/ml PBS containing 0.01% EDTA and 40 μM BHT) was vortexed in a 15-ml conical tube on a flat-surfaced Thermomix for the indicated times at room temperature. Turbidity was measured by spectrophotometric absorbance at 680 nm. B. Aliquots of 0.5 ml of 125I-labeled LDL (0.5 mg protein/ml) were vortexed for up to 60 seconds. At each time-point, an aliquot of 0.1 ml was removed for total 125I-LDL per sample, and 0.3 ml was pipetted into 1.5 ml microtubes and centrifuged immediately at 10,000 g for 10 minutes (♦). The percentage of 125I-LDL precipitated was determined by counting the 125I-LDL in the precipitate and the supernatant.

Degradation of 125I-labeled LDL Protein and Incorporation of 14C-oleate Into Cholesteryl Esters by Macrophages

The degradation of 125I-labeled LDL protein and the incorporation of 14C-oleate into cellular cholesteryl 14C-oleate were determined as described by Goldstein et al. To study the degradation of LDL protein, each monolayer of macrophages on a 35-mm dish received 1 ml of RPMI 1640 medium containing 5 mg/ml of LPDS and the indicated concentrations of 125I-labeled LDL protein. After incubation at 37°C for the specified times, the culture medium was removed and the amount of 125I-labeled trichloroacetic acid (TCA)-soluble (noniodide) material was determined. Corrections were made for small amounts of acid-soluble 125I-labeled material present in parallel incubations without cells.

To study the incorporation of 14C-oleate into cholesteryl 14C-oleate, each monolayer of macrophages on a 35-mm dish received 1 ml of RPMI 1640 medium containing 0.2 mM 14C-oleate/albumin (2000 dpm/nmol), 5 mg/ml of LPDS, and the indicated concentrations of nonradioactive LDL protein. After incubation at 37°C for the specified times (usually 16 hours), the monolayer was washed with PBS, and the total lipid was extracted in situ with hexane/isopropanol alcohol (3:2). The cholesteryl 14C-oleate was isolated by silica gel-G thin-layer chromatography by using a solvent system of petroleum ether/diethyl ether/acetic acid (80:20:0.7, vol/vol/vol). The cholesteryl ester band was visualized with iodine vapor, scraped into a vial,
Apo B

Figure 2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of LDL protein after delipidation. Lane 1. LDL precipitate (separated by centrifugation at 10,000 g for 10 minutes from the LDL after vortexing for 30 seconds). Lane 2. Soluble LDL in the supernatant of the vortexed LDL. Lane 3. Native LDL. Lane 4. Standard molecular weight markers shown as M, x 10

Measurement of Cellular Sterol Content

Macrophage monolayers from 60-mm Petri dishes were scraped into 1 ml distilled water and sonicated for 20 seconds. Aliquots (25 µl) were removed for protein determination and the remainder was extracted for total lipids by the method of Folch et al. The mass of free and esterified cholesterol was determined by the fluorometric enzymatic method of Gamble et al.

Oil-Red O Staining

Macrophages (5 x 10⁴ cells) were plated on 12-mm circular coverslips within a 24-well (16-mm diameter) clustered dish. Each coverslip received 0.5 ml of RPMI 1640 medium containing 5 mg/ml of LPDS and the indicated LDL protein. After incubation at 37°C for 24 hours, the medium was replaced with fresh medium of identical composition. At the end of 48 hours, the cells were washed with PBS, fixed in 1% glutaraldehyde, stained with oil-red O, and counter-stained with hematoxylin. The coverslips were mounted face-down on glass microscope slides using pre-warmed, liquified 50%

Other Methods

Lipid peroxidation was determined by quantitation of thiobarbituric acid (TBA)-reactive materials. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of LDL protein was carried out as described by Laemmli.

Results

Preparation and Properties of LDL Aggregates

LDL aggregates were prepared by vortexing LDL in PBS containing 0.01% EDTA and 40 µM BHT at room temperature. As shown in Figure 1A, the fraction of LDL as aggregates increased with the vortexing time. The turbidity produced by LDL aggregates was monitored by
measuring the absorbance at 680 nm. The LDL aggregates formed after vortexing were stable at room temperature for several hours and could not be sedimented by centrifugation at 1000 g for 10 minutes, but they could be sedimented at 10 000 g for 10 minutes. As shown in Figure 1B, approximately 55% of the ¹²⁵I-LDL vortexed for 30 seconds could be recovered in the precipitate. The centrifugation, however, tended to further increase the size of the aggregates, making it difficult to resuspend them in PBS for further study. For this reason, most experiments were carried out with LDL vortexed for 30 seconds and not centrifuged. This was the optimal time to generate aggregates that would induce cholesterol esterification and degradation in macrophages, as discussed further below. Reductive methylation, which modifies the lysyl residues of apoprotein (apo) B,²² did not prevent self-aggregation upon vortexing (Figure 1A), but these aggregates were handled differently by the macrophage. Vortexing at 4°C yielded identical results.

We considered the possibility that vortexing might be causing oxidative modification of the lipoprotein. However, vortexing LDL for 30 seconds under standard conditions (in the presence of 40 μM BHT and 0.01% EDTA) did not generate TBA-reactive material (i.e., there was no significant peroxidation of the LDL), nor was there further degradation of apo B. In addition to the predominant apo B-100 of native LDL, there were also two faint bands of apo B-74 and apo B-26 (Figure 2, Lane 3). The LDL aggregates in the precipitate (Lane 1) and the soluble LDL in the supernate (Lane 2) showed no difference from this pattern after 30 seconds of vortexing.

**Uptake and Metabolism of LDL Aggregates**

Incubation of macrophages with LDL aggregates (200 to 300 μg protein/ml) for 48 hours resulted in a marked accumulation of intracellular cholesteryl esters. There was only a slight increase in cellular free cholesterol (Figure 3). Macrophages incubated with LDL aggregates (200 μg protein/ml) for 48 hours amassed 187 μg of cholesteryl esters per milligram of cell protein, while cells incubated with the same concentration of native LDL (200 μg protein/ml) accumulated only 5 μg of cholesteryl esters per milligram of cell protein. This considerable storage of cholesteryl esters due to uptake of LDL aggregates was borne out by light microscopy of oil-red O-stained cells (Figure 4), showing many positively staining lipid droplets.

As a further index of uptake and degradation of LDL, we measured the incorporation of ¹⁴C-oleate into cellular cholesteryl esters, a process known to correlate with the amounts of cholesterol entering the cell.³⁰ Macrophages incubated 16 hours with native LDL (50 μg protein/ml) incorporated less than 1.2 nmol of ¹⁴C-oleate into cholesteryl ester per milligram of cell protein (Figure 5). In contrast, macrophages incubated 16 hours with LDL aggregates (50 μg protein/ml) incorporated 48 nmol cholesteryl oleate per milligram of cell protein—a 40-fold increase. The rate of cholesteryl esterification induced by the LDL aggregates exhibited an initial lag of approximately 4 hours and then remained constant for at least 16
The incorporation of $^{14}$C-oleate into triacylglycerol was unchanged by vortexing the LDL.

To test whether the stimulation of cholesteryl ester synthesis was due to the uptake of LDL aggregates via a phagocytic process, we added cytochalasin B to the culture media. Cytochalasin B (10 mg/ml) caused an 82% reduction in cholesteryl ester synthesis in macrophages incubated with LDL aggregates (Figure 6A) and an 85% reduction in degradation of LDL aggregates (Figure 6B). In contrast, it produced only a 26.5% reduction in cholesteryl ester synthesis in cells incubated with native LDL and only a 23% reduction in degradation of native LDL. Furthermore, it produced virtually no inhibition of the cholesteryl ester synthesis induced by acetyl-LDL and a 14% reduction in the degradation of acetyl-125I-LDL.

**Effect of Vortexing Time**

Even as little as 5 seconds of vortexing of LDL doubled the rate of its subsequent degradation by thiglycollate-elicited peritoneal macrophages (Figure 7A), and the magnitude of the effect was a function of vortexing time. A maximal effect was observed at 30 seconds, when there was an eightfold increase in degradation rate. The incorporation of $^{14}$C-oleate into cellular cholesteryl oleate induced by LDL also increased markedly with vortexing time (Figure 7B). Aggregates (50 mg protein/ml), formed after vortexing LDL for only 2 seconds, produced a fourfold increase in cholesteryl ester synthesis; the same extent of stimulation required a much higher concentration of native LDL (500 mg protein/ml). After 20 seconds of vortexing, LDL aggregates (50 mg protein/ml) stimulated the synthesis of cholesteryl esters to a level actually slightly higher than that caused by an equal concentration of acetyl-LDL. By 60 seconds of vortexing, the aggregates had become larger, rendering them more difficult to disperse for study of uptake and degradation by macrophages; preparations vortexed for 60 seconds were actually less effective in stimulating cholesterol esterification than prepa-
Figure 7. The effect of vortexing time on LDL as measured by the degradation of \(^{125}\)I-LDL and the formation of cholesteryl esters in macrophages. A. LDL aggregates were prepared by vortexing 1 ml of \(^{125}\)I-LDL (0.5 mg protein/ml) for the indicated times. Each monolayer of thioglycollate-elicited macrophages received 1 ml of RPMI 1640 medium containing 5 mg/ml of LPDS and \(^{125}\)I-LDL (10 \(\mu\)g protein/ml) that had been vortexed for the indicated times. After incubation at 37°C for 16 hours, the amount of \(^{125}\)I-labeled TCA-soluble material in the medium was determined. Each point represents the mean of duplicate determinations. B. Each monolayer of thioglycollate-elicited macrophages received 1 ml of RPMI 1640 medium containing 0.2 mM \(^{14}\)C-oleate/albumin, 5 mg/ml of LPDS, and 50 \(\mu\)g protein/ml of LDL vortexed for the indicated times (○); 50 \(\mu\)g protein/ml of methyl-LDL vortexed for the indicated times (△); 50 \(\mu\)g protein/ml of native LDL (●); or 50 \(\mu\)g protein/ml of acetyl-LDL (▲). For comparison, each monolayer of resident peritoneal macrophages received 1 ml of RPMI 1640 medium, 0.2 mM \(^{14}\)C-oleate/albumin, 5 mg/ml of LPDS, and 50 \(\mu\)g protein/ml of LDL vortexed for the indicated times (〇). After incubation at 37°C for 16 hours, the cellular content of cholesteryl \(^{14}\)C-oleate was determined. Each point represents the mean of duplicate determinations.

Concentration Dependency of Degradation of LDL Aggregates and of Stimulation of Cholesteryl Ester Synthesis

As shown in Figure 8A, degradation of \(^{125}\)I-LDL aggregates formed by vortexing for 30 seconds was 6.6- to 10-fold greater than that of equal concentrations of native LDL, and the process appeared to be saturable. The increase in macrophage cholesteryl ester synthesis induced by the LDL aggregates also appeared to be saturable, with half-maximal stimulation at approximately 17 \(\mu\)g protein/ml medium (Figure 8B). Native LDL gave almost no stimulation in the incorporation of \(^{14}\)C-oleate into cholesteryl esters.

Role of Apoprotein B and LDL Receptor on Macrophages

To investigate whether recognition of apo B by the LDL receptor might play a role in facilitating the phagocytosis of LDL aggregates, the lysyl residues of apo B were derivatized by reductive methylation to block interaction with that receptor. Reductive methylation did not interfere with the formation of aggregates in vortexing. Figure 9 shows a comparison of the degradation of aggregates of native, underivatized \(^{125}\)I-LDL and the degradation of aggregates of previously methylated \(^{125}\)I-LDL. The degradation of aggregates of methyl-LDL was only 34% that of the LDL aggregates prepared in the usual way.

Incubation of macrophages either with a constant concentration of methyl-LDL (50 \(\mu\)g protein/ml) that had been vortexed for varying times (Figure 7B) or with increasing concentrations of methyl-LDL vortexed for a fixed time (30 seconds, Figure 8B) showed that reductive methylation reduced the effect of aggregates on macrophage cholesterol esterification by 36% and 32%, respectively. These data imply that unmodified apo B is required for the optimal uptake and degradation of the LDL aggregates by macrophages and for maximal stimulation of cholesterol esterification.

To test further whether the LDL receptor might be involved in the phagocytic uptake of LDL aggregates, we determined the extent to which LDL aggregates competed for the degradation of native \(^{125}\)I-LDL. As seen in Figure 10, LDL aggregates (prepared by vortexing for 30 seconds) and soluble, native LDL were effective in inhibiting the degradation of native \(^{125}\)I-LDL (74% and 68.5%, respectively) at a 25-fold excess concentration. \(\beta\)-VLDL, which has a higher affinity than native LDL for binding to the LDL receptor, competed even more effectively (90%). In addition, LDL aggregates, soluble native LDL, and \(\beta\)-VLDL were also effective in inhibiting the degradation of \(^{125}\)I-LDL aggregates—by 48%, 31%, and 61%, respectively, at a 25-fold excess concentration (Figure 11). On the other hand, no competition was observed with acetyl-LDL at a concentration 25-fold in excess of that of \(^{125}\)I-LDL aggregates. Reciprocally, unlabeled LDL aggregates and native LDL (concentration 25-fold in excess) did not compete for the degradation of acetyl-\(^{125}\)I-LDL (1 \(\mu\)g protein/ml) at all, while acetyl-LDL (25 \(\mu\)g protein/ml) and...
Figure 8. Concentration dependency on the degradation of LDL aggregates and on the cholesteryl ester formation in macrophages. A. LDL aggregates were prepared by vortexing 1.5 ml of 125I-LDL (0.5 mg protein/ml) for 30 seconds. Each monolayer of macrophages received 1 ml of RPMI 1640 medium containing 5 mg/ml of LPDS and the indicated concentrations of 125I-LDL aggregates (●) or native 125I-LDL (▲). After incubation at 37°C for 16 hours, the amount of 125I-labeled TCA-soluble material in the medium was determined. Each point represents the mean of duplicate determinations. B. The aggregates were prepared by vortexing 2 ml of LDL or methyl-LDL (1 mg protein/ml of PBS) for 30 seconds. Each monolayer of macrophages received 1 ml of RPMI 1640 medium containing 0.2 mM 14C-oleate/albumin, 5 mg protein/ml of LPDS, and the indicated concentrations of LDL aggregates (●), methyl-LDL aggregates (▲), native LDL (◇), or methyl-LDL (▲). After incubation at 37°C for 16 hours, the cellular content of cholesteryl 14C-oleate was determined. Each point represents the mean of duplicate determinations.

Fucoidin (25 μg/ml) competed effectively, by 84% and 93%, respectively (Figure 12). This same unlabeled, vortexed LDL (25 μg protein/ml) used in Figure 12 induced a 35-fold increase in cholesterol esterification over that induced by native LDL (25 μg protein/ml), while the amounts of triacylglycerol synthesized were identical (data not shown).

Heparin forms a soluble complex with LDL and prevents the binding of native LDL to its receptor.32 We, therefore, tested the possibility that heparin might also inhibit the uptake of LDL aggregates via phagocytosis. As shown in Figure 13, heparin at a concentration of 5 mg/ml effectively reduced the degradation of native 125I-LDL and of aggre-
Discussion

Aggregates of LDL generated by vortexing for 2 to 60 seconds at room temperature were avidly taken up and degraded by macrophages, leading to the formation of cholesteryl ester-rich foam cells. The extent of aggregation was a function of vortexing time, and the observed rate of macrophage degradation of the vortexed LDL increased progressively with vortexing time. The extreme sensitivity of LDL to this presumably surface-induced aggregation is demonstrated by the fact that the LDL aggregates formed from 50 μg of native LDL (after only 2 seconds of vortexing) stimulated cholesterol esterification in the macrophage fourfold, an effect equivalent to that induced by 10 times as much native LDL. After 20 seconds of vortexing, the LDL aggregates formed from 50 μg of native LDL stimulated cholesterol esterification to an extent similar to that caused by an equal amount of acetyl-LDL.

Very soon after vortexing was begun, there was a definite opalescence in the LDL preparations and this progressed to frank turbidity. Examination of these preparations under the light microscope demonstrated the appearance of threadlike material, presumably resulting from progressive self-aggregation and polymerization. We, therefore, considered the possibility that at least these large LDL aggregates (and possibly some not that large) might be taken up by phagocytosis. Cytochalasin B is known to inhibit phagocytosis of opsonized sheep erythrocytes by macrophages at a concentration as low as 1 μg/ml, while having no discernible effect on pinocytosis of dinitrophenylated bovine serum albumin, ferritin, and colloidal gold. Cytochalasin B functions to disrupt the contractile microfilament system and cell motility. It causes the retraction of macrophage pseudopodia. Miller and Yin reported that cytochalasin B (10 μg/ml) inhibited the degradation of 125I-LDL by human skin fibroblasts by about 50%, and they attributed this to the disruption of cytoplasmic microfilaments.

In the present studies, we were able to dissect out the relative contributions of receptor-mediated endocytosis (pinocytosis) of LDL and receptor-mediated phagocytosis of LDL aggregates by mouse peritoneal macrophages. The degradation of native 125I-LDL was inhibited only 23% by cytochalasin B (10 μg/ml), whereas the degradation of
The ability of nonradioactive LDL aggregates, native LDL, acetyl-LDL, and fucoidin to compete for the degradation of acetyl-\(^{125}\)I-LDL. Each monolayer of macrophages received 1 ml of RPMI 1640 medium containing 5 mg/ml of LPDS, 1 \(\mu\)g protein/ml of acetyl-\(^{125}\)I-LDL and: I) 25 \(\mu\)g protein/ml of acetyl-LDL, II) 25 \(\mu\)g protein/ml of LDL aggregates, or IV) 25 \(\mu\)g protein/ml of native LDL. After incubation at 37°C for 4 hours, the amount of \(^{125}\)I-labeled TCA-soluble material in the medium was determined.

\(^{125}\)I-LDL aggregates was inhibited by 85%. Similar quantitative differences were observed with respect to the inhibition of the LDL effect on cholesterol esterification by cytochalasin B (Figures 6A and 6B). Aortic smooth muscle cells, which have minimal phagocytic capacity compared to mouse peritoneal macrophages, did not accumulate cholesteryl esters when incubated with LDL aggregates (data not shown). Interestingly, cytochalasin B appeared to have little or no effect on cholesterol esterification induced by acetyl-LDL nor on the degradation of acetyl-LDL (Figures 6A and 6B). This raises the question of whether cytoplasmic microfilaments are at all involved in the scavenger receptor-mediated endocytosis of acetyl-LDL, or whether the microfilaments involved are different in some way from those involved in LDL receptor-mediated endocytosis.

The selective inhibition by cytochalasin B suggests that the LDL aggregates were probably taken up predominantly by phagocytosis. Macrophages phagocytose selectively, presumably because macrophage receptors recognize one or more ligands on the surface of the particle to be ingested (e.g., opsonized red blood cells). Our studies suggest that apo B in the LDL aggregates is being recognized by the macrophage LDL receptor and that this receptor-ligand interaction is essential for the enhanced uptake. Several lines of evidence support this interpretation: 1) reductive methylation, while not hindering LDL self-aggregation, reduced subsequent degradation of labeled LDL aggregates by 66% and reduced the LDL aggregate-induced cholesterol esterification to a similar extent; 2) heparin, which inhibits the binding of LDL to its receptor,\(^{32,37}\) markedly inhibited the degradation of labeled aggregates as much as it inhibited the degradation of native LDL; 3) native LDL and LDL aggregates demonstrated reciprocal competition for uptake and degradation by macrophages. On the other hand, the failure of acetyl-LDL to compete for the degradation of labeled aggregates

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**Figure 12.** The ability of nonradioactive LDL aggregates, native LDL, acetyl-LDL, and fucoidin to compete for the degradation of acetyl-\(^{125}\)I-LDL. Each monolayer of macrophages received 1 ml of RPMI 1640 medium containing 5 mg/ml of LPDS, 1 \(\mu\)g protein/ml of acetyl-\(^{125}\)I-LDL and: I) 25 \(\mu\)g protein/ml of acetyl-LDL, II) 25 \(\mu\)g protein/ml of LDL aggregates, or IV) 25 \(\mu\)g protein/ml of native LDL. After incubation at 37°C for 4 hours, the amount of \(^{125}\)I-labeled TCA-soluble material in the medium was determined.

**Figure 13.** The effect of heparin on the degradation of \(^{125}\)I-LDL aggregates, \(^{125}\)I-LDL, and acetyl-\(^{125}\)I-LDL. \(^{125}\)I-LDL aggregates were prepared by vortexing \(^{125}\)I-LDL (0.5 mg protein/ml) for 30 seconds. Each monolayer received 1 ml of RPMI 1640 medium containing 5 mg/ml of LPDS and: 10 \(\mu\)g protein/ml of \(^{125}\)I-LDL aggregates (A), 10 \(\mu\)g protein/ml of \(^{125}\)I-LDL aggregates (B), or 10 \(\mu\)g protein/ml of acetyl-\(^{125}\)I-LDL (C). The results were expressed as percentages of the degradation of the \(^{125}\)I-protein in the absence of heparin. After incubation at 37°C for 4 hours, the amount of \(^{125}\)I-labeled TCA-soluble material in the medium was determined. The results were expressed as percentages of the degradation of the \(^{125}\)I-protein in the absence of heparin. The 100% values for degradation of \(^{125}\)I-LDL aggregates, \(^{125}\)I-LDL, and acetyl-\(^{125}\)I-LDL were 3.1, 0.49, and 14.3 \(\mu\)g/mg cell protein/4 hours, respectively.
(and vice versa) appears to rule out a role for the acetyl-LDL or scavenger receptor.

We suggest, then, that the striking ability of LDL aggregates to generate foam cells depends on the presence of multiple recognition sites on each particle, recognition sites that appear to represent the same apo B binding site involved in recognition of native LDL by its receptor. A number of other aggregates of LDL that involve complexing of LDL with other macromolecules such as proteoglycans, dextran sulfate, heparin-fibronectin-denatured collagen, and granules of mast cells have been described. In these instances, the second ligand may also be recognized by macrophage receptors and thus more than one receptor may be involved, i.e., the LDL receptor recognizing apo B and another receptor recognizing the second ligand. The enhanced uptake, however, may depend more on the simple fact that there is an aggregate, rather than a particular agent, involved in creating the aggregate. In the present studies, we had only LDL in the system and the interpretation is thus made simpler.

The fact that LDL is peculiarly sensitive to surface denaturation has been known for a long time, but investigators have, perhaps, been insufficiently alert to the implications of that fact. For example, vortexing is commonly used during lodination of LDL by the iodine monochloride method to avoid high local concentrations during addition of the oxidant. If done briefly and at a low temperature, that may be acceptable, but one needs to be alert to the fact that it can modify the metabolic behavior of the LDL. It has been also known for some time that LDL stored in the refrigerator develops a precipitate over time, and investigators generally have not used preparations that were more than a couple of weeks old. The question of whether the material still in solution begins to aggregate and alter its metabolic behavior even before visible precipitates appear needs to be explored.

Finally, these results suggest the possibility that denaturation of LDL and self-aggregation could play a role in atherogenesis. Recent studies by Schwenke and Carew have shown that the lifetime of LDL in developing atherosclerotic lesions can be quite long (7 days in lesion areas relative to 3 hours in nonlesioned areas). The conditions in such developing lesions may favor aggregation—either self-aggregation, as described here, or aggregation with matrix materials of the subendothelial space, as suggested by others. Either or both of these possibilities should be considered. If self-aggregation or heteroaggregation is involved, one might be able to devise methods of interfering with it, giving us still another means of intervening to slow the progression of the atherosclerotic lesion at the artery wall level.

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