Enhanced Uptake and Impaired Intracellular Metabolism of Low Density Lipoprotein Complexed With Anti-Low Density Lipoprotein Antibodies

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We have previously shown that incubation of human macrophages with antigen–antibody complexes prepared with native human low density lipoprotein (LDL) and rabbit anti-LDL antibodies (LDL-ICs) results in an increased intracellular accumulation of cholesteryl esters (CEs) and induces a marked increase in the number of LDL receptors. To determine whether the increased CE accumulation in these cells occurred during incubation of the cells with LDL-ICs or whether it was secondary to the uptake of LDL by overexpressed LDL receptors, we incubated human macrophages with LDL-ICs for 22 hours, followed by incubation with native LDL for another 20 hours. We found that about 90% of the accumulated CEs could be accounted for by the first incubation with LDL-ICs. We then proceeded to show that the CEs accumulated during incubation of cells with LDL-ICs was secondary to enhanced uptake and impaired degradation of the LDL complexed with immunoglobulin G (IgG) (LDL-IC), which led to a marked intracellular accumulation of undegraded LDL (levels 199-fold higher than those obtained when the cells were incubated with the same concentration of native LDL not complexed with IgG). We have also shown that not all CEs accumulated in these cells were derived from accumulation of undegraded LDL and that some of them were derived from the reesterification of free cholesterol released during hydrolysis of LDL. LDL-ICs promoted increased CE accumulation and foam cell formation at concentrations as low as 25 μg/ml. To determine which receptors were involved in the uptake of LDL-ICs, we performed experiments in which the uptake of LDL-ICs was competitively inhibited with heat-aggregated gamma globulin, native LDL, β-very low density lipoprotein, or acetylated LDL. Our results demonstrated that LDL-IC uptake was most effectively inhibited by heat-aggregated gamma globulin, partially inhibited by native LDL or by a monoclonal antibody to the LDL receptor, and not inhibited by acetylated LDL or β-very low density lipoprotein. Thus, we conclude that the majority of LDL-ICs are taken up through Fcγ receptors. Finally, we investigated whether the increase in LDL receptor expression was dependent on the receptor pathway used by the LDL-ICs, and we were able to demonstrate that when macrophages were incubated with LDL-ICs prepared with F(ab′)2 fragments of the anti-LDL antibody, LDL receptor expression was not enhanced. Therefore, we postulate that the uptake of LDL-ICs through Fcγ receptors results in an uncoupling of the normal regulation of the LDL receptor expression. (Arteriosclerosis and Thrombosis 1991;11:1356–1367)

The initial stages of atherogenesis are characterized by the subendothelial accumulation of foam cells.1 Substantial experimental evidence suggests that the majority of foam cells present in atheromatous lesions are derived from monocytes/macrophages2–3 and that the intracellular lipid is derived primarily from plasma low density lipoprotein (LDL).4,5 However, the precise mechanisms responsible for LDL uptake and macrophage transformation into foam cells remain incompletely un-
understood. Therefore, there is considerable interest in studying the factors that influence lipoprotein metabolism in macrophages, in the hope that such studies will contribute to an understanding of the genesis and progression of atherosclerosis.

Numerous studies have shown that intracellular accumulation of lipids in monocyte-derived macrophages can occur when chemically or biologically modified LDLs are taken up through the scavenger receptor.\(^6\)\(^-\)\(^8\)\(^-\)\(^9\) Accumulating evidence from our laboratory and others suggests that monocyte-derived macrophages may have additional receptors, distinct from the scavenger receptor, that participate in the recognition and removal of an overlapping range of modified products,\(^10\) including minimally modified lipoproteins such as glucosylated LDL,\(^11\) and advanced glycation end product–modified LDL,\(^12\) and that the uptake of these lipoproteins by macrophages may also lead to intracellular accumulation of lipids.

Furthermore, in certain conditions lipoproteins or lipoprotein complexes taken up by the LDL receptor may also lead to foam cell formation.\(^13\)\(^-\)\(^14\) The notion that LDL taken up by the classical LDL receptor would not be accumulated intracellularly was first challenged by Tabas et al.,\(^15\) who showed that the J774 murine macrophage–like tumor cell line had increased uptake of native LDL and concomitant cholesteryl ester (CE) accumulation.\(^15\) In addition, it has been shown that the LDL receptor of human macrophages can bind a variety of ligands other than native LDL, such as \(\beta\)-very low density lipoprotein,\(^13\)\(^-\)\(^16\) lipoprotein(a),\(^17\)\(^-\)\(^18\) and chylomicron remnants,\(^19\) as well as complexes of LDL and lipopolysaccharide\(^14\) and LDL–anti-LDL immune complexes (LDL-ICs).\(^20\) Experimentally, several of these lipoproteins and lipoprotein complexes have been shown to lead to macrophage transformation into foam cells, even though binding and uptake occur (at least partially) through the classical LDL receptor. Furthermore, the metabolism of native LDL may be affected by the state of activation of the macrophages. In previous studies we demonstrated increased uptake of native LDL and subsequent CE accumulation in human macrophages stimulated with microbial or microbial-related products.\(^21\)

Recently, we have shown that the stimulation of human macrophages with LDL-ICs leads to a marked increase in native LDL uptake and subsequent intracellular CE accumulation.\(^20\) We have also shown that the increase in native LDL uptake was due to an increase in the expression of LDL receptors.\(^20\)

In the present study we have performed experiments to determine the mechanisms leading to the massive CE accumulation observed in macrophages incubated with LDL-ICs, to define the relative contributions for CE accumulation by the uptake of LDL-ICs and of native LDL, and to investigate whether the uptakes of LDL-ICs by the Fc\(\gamma\) and by the LDL receptor are equally able to stimulate LDL receptor expression and uptake of native LDL.

### Methods

#### Isolation and Characterization of Monocytes/Macrophages

Monocytes were isolated from leukapheresis specimens by countercurrent centrifugal elutriation as previously described.\(^20\)\(^-\)\(^23\) The leukapheresis specimens used for the separation of monocytes were obtained from normal volunteers by use of a Celltrifuge II apparatus. Donors with abnormalities detected by physical or laboratory examinations were excluded as previously described.\(^23\)

The purity of the monocyte preparations obtained by elutriation was confirmed by morphological examination of Wright’s-stained cytospin preparations, by nonspecific esterase staining, and by their ability to ingest latex particles.\(^24\) Viability was determined by Trypan blue dye exclusion. The average purity of the monocytes used in this study, as determined by Wright’s staining, was 93%; by esterase staining, 92%; and by latex ingestion, 93%. The average viability of the cells employed was 99%.

The isolated monocytes were matured by incubation at \(37^\circ\)C for 8 days in a humidified incubator with a 5% CO\(_2\) atmosphere. The medium used to induce maturation was a defined supplemented Iscove’s modified Dulbecco’s medium prepared as previously described\(^21\)\(^-\)\(^25\) and containing 30% (vol/vol) of whole human serum (Whittaker M.A. Bioproducts, Walkersville, Md.). The protein content of the macrophages was, on average, three times higher than that of monocytes; thus, the pooled human serum used was not toxic to the cells. After maturation of monocytes into macrophages, medium without addition of whole human serum and without cholesterol supplementation was used to perform all the experiments.

#### Lipoprotein Isolation, Modification, and Labeling

Blood was collected in EDTA (1 mg/ml) after 12 hours of fasting. LDLs (1.019 <d<1.063 g/ml) were isolated from plasma by sequential ultracentrifugation on an ultracentrifuge (Beckman L5-50, type 50 rotor\(^26\)), washed by ultracentrifugation, dialyzed against a 0.15 M NaCl solution containing 1 mM EDTA (pH 7.4), and stored under N\(_2\) in the dark. They were passed through an Acrodisc filter (0.2-\(\mu\)m pore size) to remove aggregates. An aliquot of LDL was iodinated with iodine-125 by the McFarlane procedure as modified by Brautzler et al.\(^27\) The labeling conditions were adjusted to obtain a specific activity of 100–400 cpm/ng protein. Radioactivity localized in the lipid moiety of the lipoproteins was determined after a “Folch lipid extraction”\(^28\) and constituted less than 4% of the total radioactivity. Acetylation of LDL was performed by use of the protocol described by Basu et al.\(^29\)

#### Preparation of Immune Complexes

Insoluble ICs were prepared with LDL (1 mg/ml) and the immunoglobulin G (IgG) fraction of a rabbit anti-LDL antiserum raised by us\(^30\) at a concentration
of 1 mg/ml. A precipitin curve was prepared by incubating 1-mg aliquots of IgG anti-LDL overnight at 4°C with varying amounts of LDL, ranging from 5 to 1,000 μl. The antigen to antibody ratio yielding the highest amount of precipitate was considered to correspond to the equivalence point and was used for preparation of insoluble ICs. For our particular anti-LDL antibody, equivalence was reached usually at a 1:10 (wt/wt) antigen to antibody ratio. For instance, to prepare 2.5 mg ICs we typically started with 8 mg IgG and 800 μg LDL in eight separate sterile tubes. We then centrifuged (500g, 5 minutes, 4°C) and pooled the precipitates formed, washed the pellet twice with cold sterile phosphate-buffered saline (PBS), and resuspended it in 2–2.5 ml sterile PBS to obtain a final concentration of 1–1.5 mg IC/ml. The protein content of the IC was determined after washing by use of the Bio-Rad protein assay, calibrated with serial dilutions of a preparation of heavily heat-aggregated IgG with known amounts of IgG. Before addition to cell cultures, the IC preparations were sterilized by gamma irradiation.

**Preparation of F(ab′)2 Fragments**

F(ab′)2 fragments were prepared by a procedure that was modified from Stewart and Stanworth. 30 In brief, rabbit IgG (10–20 mg/ml) was dialyzed for 24 hours at 4°C against two changes of 0.1 M sodium acetate buffer, pH 4.5. The IgG concentration was adjusted to 2 mg/ml with 0.1 M sodium acetate buffer, pH 4.5. Pepsin (Sigma Chemical Company, St. Louis, Mo.) digestion was performed at an enzyme to substrate ratio of 1:100 for 4 hours at 37°C. Digestion was terminated by the addition of solid tris(hydroxymethyl)methylamine to raise the pH to 8.0. NaHSO4 (Sigma) was then added to the digest at room temperature under constant-stirring conditions until a concentration of 18% of the saturation level was reached. The digest was then centrifuged at 10,000g for 20 minutes. The supernatant was discarded and the precipitate dissolved in distilled water, followed by dialysis for 24 hours at 4°C against two changes of 0.1 M sodium acetate buffer, pH 7.4. Undigested IgG was removed by affinity chromatography in a Protein A–sepharose column as described by Stingl et al. 31 The final preparation was reacted with anti-whole IgG and anti-light chain antisera to precipitate ICs to be used in our experiments.

**Preparation of Heat-Aggregated Gamma Globulin**

Human gamma globulin (Sigma) at 20 mg/ml in PBS was heated at 63°C for 20 minutes. Protein content was determined by Bio-Rad assay, and sterility was ensured by gamma irradiation.

**CRL-1703 Hybridomas**

CRL-1703, a murine hybridoma developed by Huettinger et al. 32 that secretes IgG, antibody against purified bovine LDL receptor, was obtained from the American Type Culture Collection, Rockville, Md. Monoclonal antibodies to bovine LDL receptors have been shown to cross-react with human LDL receptors and to inhibit the binding of native LDL by as much as 80%. 33 The CRL-1703 cells were seeded at a density of 2x10^6 cells/ml in RPMI-1640 supplemented with 10% fetal bovine serum and 4.5% glucose, as recommended. The hybridoma cultures were grown to approximately 1x10^6 cells/ml, after which time the culture supernatants were harvested.

**Measurement of Total, Free, and Esterified Cholesterol Content in Macrophages**

To perform cholesterol mass studies, the macrophage monolayers after being extensively washed with PBS were extracted with hexane/isopropanol (3:2, vol/vol) as previously described. 26 Free and total cholesterol contents were assayed on a gas chromatograph equipped with an H2 flame ionization detector. A glass column packed with 3% SP-2250 on 80/100 mesh Supelcoport (Supelco, Bellefonte, Pa.) was used for the chromatographic separation, and its temperature was maintained at 250°C during the separation. N2 was used as the gas carrier. For assay of total cholesterol the cellular extracts were evaporated to dryness, and the residue was hydrolyzed by Ishikawa's method 25 as previously described. 26 CE levels were obtained by subtracting free cholesterol from total cholesterol levels. β-Stigmasterol was used as an internal standard.

**Cholesteryl Ester Synthesis**

CE synthesis was measured in the presence or absence of 50 μM chloroquine as previously described by us. 11,21 The chloroquine concentration was determined by dose–response studies, in which cellular viability and inhibition of [125I–LDL-IC degradation were measured. With the concentration used in all subsequent experiments (50 μM), cellular viability was greater than 90% while degradation was inhibited to greater than 80%. The cells were extracted twice for 30 minutes at room temperature with 1 ml hexane/isopropanol (3:2, vol/vol) extraction mixture containing 2.2x10^6 cpm tritiated cholesteryl oleate ([cholesteryl-1,2,6,7(n)-3H] oleate, New England Nuclear, Boston, Mass.) and 20 μg cholesteryl oleate. The [3H]cholesteryl oleate was added as an internal standard to correct for procedural losses.

**Lipoprotein and Low Density Lipoprotein-Immune Complex Binding, Accumulation, and Degradation**

LDL intracellular accumulation and proteolytic degradation were assessed as previously described. 26 After incubation of the [125I–Labeled LDL or [125I–LDL-IC with the cells, the medium was removed and the cells washed twice with 1 ml PBS containing...
was added to the macrophages with or without the medium was used to determine the proteolytic degradation of LDL, as described by Bierman et al.37 Intracellular degradation levels were corrected by subtracting the small amounts of 125I-labeled acid-soluble material obtained due to spontaneous breakdown of LDL when labeled LDL was incubated without cells.

To assay LDL-IC binding, the incubation as well as the washing and harvesting procedures were performed at 4°C instead of 37°C. The incubation period was 3–4 hours. The data that represent specific binding, uptake, and degradation were determined from the difference of the levels measured in the presence and absence of a 25-fold excess of unlabeled ligand. 125I-labeled LDL-IC isotherm saturation binding studies were performed with increasing concentrations of LDL-IC. Curves representing the best fit were determined by unweighted least-squares nonlinear regression analysis. Scatchard analysis was performed on the saturation binding data as previously described.38,39

Competition and Blocking Studies

In one series of experiments 125I-LDL–labeled ICs (50 μg/ml) were incubated at 4°C for 3–4 hours in the presence of medium alone or in medium containing increasing concentrations of heat-aggregated gamma globulin, native LDL, β-very low density lipoprotein, or acetylated LDL. After incubation, the cultures were washed and the cellular binding of 125I-LDL–labeled ICs was determined.

In another set of experiments the macrophages were incubated with medium alone or medium containing either native LDL (100 μg/ml), heat-aggregated gamma globulin (10 mg/ml), or undiluted CRL-1703 hydridoma supernatant for 1 hour at 37°C. After that period unlabeled LDL-ICs (250 μg/ml) were added to each well and the cells incubated for another 22 hours. The cells were washed, and their lipid content was extracted, as previously described, for measurement of total, free, and esterified cholesterol by gas chromatography.

To perform the blocking experiments, macrophages were incubated for 22 hours at 37°C with medium alone or medium containing LDL-ICs (250 μg/ml), F(ab)’2-ICs (250 μg/ml), LDL-1C (250 μg/ml) plus heat-aggregated gamma globulin (10 mg/ml), or LDL-ICs (250 μg/ml) plus LDL (250 μg/ml). After incubation the medium was removed, the cells were extensively washed, and 125I-LDL (10 μg/ml) was added to the macrophages with or without the addition of a 25-fold excess of unlabeled LDL. The cells were then incubated for 20 hours at 37°C and after this period, they were washed and the cellular binding of 125I-LDL was determined.

Statistics

Statistical analysis was performed with the mean paired Student’s t test and the Wilcoxon signed rank test to compare the differences between paired data. For evaluation of differences between means in unpaired data, the Wilcoxon rank sum test and Student’s t test were used. Results are reported as mean±SEM. Significance was assumed for probability values less than 0.05.

Results

In a previous report,20 we presented data showing that human macrophages after being stimulated for 22 hours with LDL-ICs accumulated approximately six times the amount of native LDL and expressed approximately 20-fold more LDL receptors than did unstimulated macrophages. This increased uptake of native LDL was associated with increased intracellular accumulation of cholesterol and CEs and morphological transformation of macrophages into “foam cells.” To determine whether the intracellular accumulation of CEs in LDL-IC-stimulated cells was mainly due to the enhanced uptake of native LDL or to the intracellular accumulation of the LDL taken up by the cell as an IC, we compared the levels of total cholesterol and CEs in macrophages stimulated with LDL-ICs with or without subsequent exposure to native LDL (Figure 1). There was a marked increase in the total cholesterol and CE content of the LDL-IC-stimulated cells when compared with unstimulated cells, independent of their subsequent exposure to native LDL. There was no significant difference in total cholesterol and CE mass in unstimulated human macrophages incubated in the presence or absence of native LDL (data not shown).

In contrast, a small but statistically significant difference (p<0.05) was found both in total cholesterol and CE mass in LDL-IC-stimulated macrophages exposed to native LDL versus those that were incubated with LDL-ICs alone. However, 90–92% of the CE content of macrophages stimulated with LDL-ICs and incubated after stimulation with native LDL was secondary to the uptake of the LDL contained in LDL-ICs. The subsequent uptake of native LDL contributed only 8–10% to the cellular CE mass.

Because the majority of CE accumulated was due to LDL-IC uptake, we decided to investigate whether LDL complexed with IgG (LDL-IC) was taken up by the macrophages to a greater extent than native LDL. Thus, we incubated human macrophages with the same concentration of 125I-LDL (22.6 μg) either by itself or complexed with IgG (LDL-IC) for 5 hours at 37°C. The 125I-LDL preparation used to perform the experiments was also used to prepare the LDL-ICs. The total amount of LDL (cell-associated LDL plus degraded LDL) taken up by macrophages as part of LDL-ICs was 149.9 μg/mg cell protein. In contrast, the uptake of LDL not complexed with IgG was considerably less.
(20.54 μg/mg cell protein). Accumulation (cell-associated LDL) and degradation of LDL complexed with IgG and of LDL by itself were, respectively, 128.1 and 0.64 μg/mg cell protein for accumulation (199-fold difference) and 21.8 and 19.9 μg/mg cell protein for degradation (1.09-fold difference).

Because degradation of LDL taken up as an IC seemed to be impaired, we compared the degradation of similar amounts of LDL complexed with IgG and of native LDL (22.6 μg/ml) at different time points (5, 8, 24, 36, and 48 hours). We found that in the first 5 hours virtually all the native LDL taken up was degraded (96.9±0.14%, mean±SD) and that in contrast, only 14.5±1.4% of the LDL complexed with IgG was degraded. The degradation of the LDL complexed with IgG at 8, 24, 36, and 48 hours was, respectively, 27.8±2.1%, 67.3±8%, 68.7±9.5%, and 72.8±7.1% of the total LDL taken up. Because in these experiments the cells were continuously exposed to 125I-LDL-ICs, we could not accurately determine the rate of degradation of the LDL taken up as part of an LDL-IC at the start of the experiment as a function of time in culture. Thus, we performed a pulse-chase experiment by initially incubating human macrophages for 2 hours at 4°C with 125I-LDL-ICs containing 22.7 μg 125I-LDL. After the first incubation the cells were washed extensively to remove the unbound 125I-LDL-IC, and fresh medium containing the same amount of unlabeled LDL-IC was added to the cells, which were then incubated at 37°C for the periods of time indicated in Figure 2. These studies revealed that after 5, 8, 18, 28, and 48 hours of incubation, the degradation of the 125I-LDL-IC was, respectively, 34.4±0.6%, 53.4±2.3%, 73.2±2.9%, 94.9±1.7%, and 96±0.35% of the total 125I-LDL-IC taken up at the start of the experiment (Figure 2).

To determine the concentration of LDL-IC necessary to induce the transformation of macrophages into foam cells, we incubated human macrophages with progressively increasing levels of LDL-ICs. Figure 3 illustrates the concentration-dependent effect of LDL-ICs on the total and esterified cholesterol mass. CE accumulation increased progressively with increasing concentrations of LDL-IC although the

![Figure 1. Bar graph of cholesterol mass, expressed as micrograms total cholesterol (TC), free cholesterol (FC), and cholesteryl ester (CE) per milligram cell protein in unstimulated (●) and low density lipoprotein-immune complex (LDL-IC) stimulated human monocyte-derived macrophages incubated for 20 hours in the presence (●) or absence (□) of 100 ng/ml native LDL. Macrophages were incubated for 22 hours with either Iscove’s modified Dulbecco’s medium containing LDL-ICs (250 μg/ml) or medium alone. After incubation the medium was removed, the cells were extensively washed, and native LDL (100 μg/ml) was added to half the wells containing LDL-ICs (●) and to half the wells containing medium alone (□). To the other half, medium alone was added. Cells were incubated for another 20 hours and after adequate washing were extracted for determination of TC and FC contents. Values are mean±SEM from eight different experiments with triplicate observations in each experiment. *p<0.05.](http://atvb.ahajournals.org/)

![Figure 2. Line plot of time-course degradation (percent per hour) of iodine-125-labeled low density lipoprotein (LDL)–immunoglobulin G (IgG) (○) by human macrophages. 125I-LDL (22.7 μg/ml) complexed with IgG was added to each well and incubated at 4°C for 2 hours. After this incubation, the cells were washed and fresh medium containing the same amount of unlabeled LDL-IgG was added to each well and incubated at 37°C for the periods of time indicated. After the respective incubation period, LDL accumulation and degradation were determined. Results are expressed as amount of LDL degraded as percentage of total LDL uptake (mean±SEM). SEMs were calculated for all time points but in some were too small to be visualized in the figure. Accumulation (cell-associated LDL) and degradation of LDL complexed with IgG was 24.8 μg/mg cell protein for accumulation and 12.5 μg/mg cell protein for degradation at the end of 5 hours. Percentage of total uptake was calculated as degradation divided by (cell-associated LDL plus degradation) times 100.](http://atvb.ahajournals.org/)
To determine whether the CE accumulation in LDL-IC-stimulated cells was entirely due to accumulation of the intact LDL contained in the IC, we measured acyl coenzyme A: cholesterol acyltransferase (ACAT) activity in these cells in the presence or absence of chloroquine, an inhibitor of lysosomal degradation. The concentration of chloroquine was determined in dose-response cellular viability studies, and a concentration of 50 μM was shown to inhibit 125I-LDL-IC degradation by more than 80%, while cellular viability remained satisfactory (≥90%). As shown in Figure 4, CE synthesis was determined in two sets of human macrophages: one set served as the unstimulated experimental control (A), while the other set was incubated with LDL-ICs (250 μg/ml) (B). Each set was treated similarly with or without 50 μM chloroquine. In the unstimulated macrophages, carbon-14-labeled oleate incorporation into CE did not differ significantly in cultures containing or lacking chloroquine. However, in macrophages stimulated with LDL-ICs, [14C]oleate incorporation into cholesterol was markedly depressed in the presence of chloroquine. As chloroquine was present throughout the assay, such disparity could reflect the effect of chloroquine on LDL-IC uptake. However, experiments in which we measured 125I-LDL-IC binding in the presence or absence of chloroquine failed to show any significant differences (12.5±0.63 versus 11.78±0.49 μg/mg cell protein, respectively). In contrast, there was a marked inhibition of degradation of the LDL-IC in the presence of chloroquine (24.4±4.78 versus 4.06±0.98 μg/mg cell protein, respectively) and an increase in accumulation as expected (63.2±2.8 versus 80.9±4.7 μg/mg cell protein, respectively). These observations suggest that one of the sources of CE accumulated in macrophages incubated with LDL-IC is the free cholesterol released upon lysosomal hydrolysis of LDL-IC, which is subsequently reesterified.

To determine whether LDL-ICs were taken up by macrophages through a receptor-mediated pathway or through a nonspecific mechanism, saturation binding isotherm studies were performed with progressively increasing concentrations of 125I-LDL-labeled ICs with or without a 25-fold excess of unlabeled LDL-IC to determine nonspecific binding. These studies (Figure 5) showed that the binding of LDL-ICs by macrophages was saturable at 17.3±0.9 μg LDL-IC/mg cell protein. Scatchard plot analysis revealed nonlinearity and suggested several independent classes of binding sites, thereby prohibiting meaningful extrapolations of affinity and number of binding sites (data not shown).
The next point that we investigated was whether LDL-ICs were taken up by the macrophages as a consequence of binding to Fcγ, LDL, LDL-related, or scavenger receptors. This point was addressed by competition studies in which the LDL-IC was used as the radioligand and heat-aggregated gamma globulin, native LDL, β-very low density lipoprotein, and acetylated LDL were used as competing ligands. Figure 6 illustrates that acetylated LDL did not inhibit 125I-LDL-IC binding, whereas both native LDL and heat-aggregated gamma globulin inhibited binding of 125I-LDL-IC in a concentration-dependent fashion up to a maximum of approximately 25% for native LDL and 70% for heat-aggregated gamma globulin. The binding of 50 μg/ml LDL-IC in the presence of increasing concentrations of β-very low density lipoprotein (50, 100, 175, and 250 μg/ml) was 89%, 107%, 105%, and 105%, respectively, of the levels obtained when β-very low density lipoprotein was absent. Similar studies with a monoclonal antibody against the LDL receptor (CRL-1703) as well as a monoclonal antibody against rat liver glutathione-S-transferase B, subunit YaYc (a gift from Irene Y. Wang, Department of Biochemistry, Medical University of South Carolina) were also performed. The latter monoclonal antibody was chosen as a negative control because it should not affect either LDL or IC uptake. The cells were incubated for 1 hour at 4°C with either medium containing 15% fetal calf serum, 375 μg/ml native LDL, and 2.5 mg/ml heat-aggregated IgG, or with the supernatants of either the CRL-1703 murine hybridoma (anti-LDL receptor antibody) or of the hybridoma secreting the monoclonal antibody against the subunit YaYc of rat liver glutathione-S-transferase B. The supernatants of both hybridomas were centrifuged immediately before use at 48,000g for 30 minutes to remove any IgG aggregates that might possibly block the Fcγ receptor. After 1 hour, 125I-LDL-IgG complexes containing 15 μg 125I-LDL were added to the cells and incubated for another 3 hours at 4°C. 125I-LDL-ICs bound to the cells in the presence of the monoclonal antibody against the LDL receptor, the monoclonal antibody against rat liver glutathione-S-transferase B, heat-aggregated gamma globulin, and native LDL were, respectively, 72.2±8.7%, 102.9±9.8%, 36.3±2.5%, and 73.8±0.4% of the levels of the LDL-ICs bound to the cells in the presence of medium alone. Therefore, it can be concluded that LDL-ICs bind predominantly to Fcγ receptors and to a small extent to LDL receptors.

Further experiments were performed to confirm that the uptake of LDL-ICs by macrophages was...
Human macrophages were incubated for 1 hour at 37 °C with lipoprotein-immune complex (LDL-IC) uptake on total cholesterol mass (micrograms cholesterol per milligram cell protein) expressed as free cholesterol (■) and cholesteryl ester (▲). Human macrophages were incubated for 1 hour at 37 °C with medium alone or medium containing 100 ng/ml LDLr Ab. Afterward, 250 μg/ml LDL-anti-LDL-ICs were added to cell cultures and was incubated for 22 hours. After incubation, cells were extracted as described, and total and free cholesterol contents were measured by gas chromatography. Values are mean±SEM of four different experiments, with triplicate observations in each experiment. *p<0.05.

FIGURE 7. Bar graph of effect of competition for low density lipoprotein-immune complex (LDL-IC) uptake on total cholesterol mass (micrograms cholesterol per milligram cell protein) expressed as free cholesterol (■) and cholesteryl ester (▲). Human macrophages were incubated for 1 hour at 37 °C with medium alone or medium containing 100 μg/ml LDL, 10 mg/ml heat-aggregated gamma globulin (HAGG), or undiluted CRL-1703 hybridoma supernatant (LDLr Ab). Afterward, 250 μg/ml LDL-anti-LDL-ICs were added to each well, and cells were incubated for 22 hours. After incubation, cells were extracted as described, and total and free cholesterol contents were measured by gas chromatography. Values are mean±SEM of four different experiments, with triplicate observations in each experiment. *p<0.05.

mainly mediated through Fcγ receptors and to a small extent by the LDL receptor. As shown in Figure 7, in cultures preincubated with native LDL or heat-aggregated gamma globulin the CE mass was significantly diminished (from 171±6.3 μg CE/mg cell protein in cultures without competitor to 132.6±9.6 μg CE/mg cell protein in cultures blocked with native LDL and 54.3±7.1 μg CE/mg cell protein in cultures blocked with heat-aggregated gamma globulin). To further confirm the contribution of the LDL receptor to LDL-IC uptake, we performed inhibition studies with a monoclonal antibody to the LDL receptor. Figure 7 illustrates that in macrophage cultures preincubated with anti-LDL receptor antibody, the CE mass (124.6±6.6 μg CE/mg cell protein) was reduced to levels similar to those determined in cultures preincubated with native LDL. Therefore, blocking the LDL receptor with native LDL or anti-receptor antibody inhibits LDL-IC binding and subsequent CE accumulation by approximately 25%. The ability of the monoclonal antibody used in our experiments to block the LDL receptor and prevent the uptake of LDL was tested in human fibroblasts by incubating these cells with 10 μg/ml 125I-LDL in the presence or absence of the anti-LDL receptor monoclonal antibody and determining the degree of inhibition of LDL internalization and degradation. The internalization of LDL by human fibroblasts in the presence of the monoclonal antibody against the LDL receptor was 22% of that observed in the absence of the antibody (538±3.2 ng/mg cell protein). Similarly, the degradation of LDL in the presence of the monoclonal antibody was 24.5% of the level obtained in the absence of antibody (1,584±116 ng/mg cell protein).

Additional experiments to investigate whether the uptake of LDL-IC was mediated predominantly by Fcγ receptors were performed by comparing the binding and degradation of 125I-LDL-IC prepared with intact IgG and with F(ab')2 fragments. The binding of 125I-LDL-F(ab')2-IC (4.84 μg/mg cell protein) was similar to that observed when 125I-LDL-IgG-ICs were incubated in the presence of an excess of heat-aggregated gamma globulin (4.93±0.44 μg/mg cell protein), which represented 14% of the binding of the IC in the absence of heat-aggregated gamma globulin (34.4±3.78 μg/mg cell protein). Similarly, the degradation of 125I-LDL-IC prepared only 17% of the degradation of 125I-LDL-IgG-IC (120.4±3.44 μg/mg cell protein). The amount of 125I-LDL complexed with intact IgG and with F(ab')2 fragments was similar. Using the same preparation of 125I-LDL to prepare both compounds, we found that the specific activity of the two types of IC was, respectively, 79 cpm/ng IC for LDL-IgG-IC and 81.6 cpm/mg IC for the LDL-F(ab')2-IC. In conclusion, all our experiments are in agreement with the interpretation that the LDL-IC complexed with IgG (LDL-IC) was the main source of intracellular CE and that the uptake of LDL-ICs was mainly mediated by Fcγ receptors, although a minor portion of LDL-ICs were internalized after binding to the LDL receptor.

We had previously demonstrated that human macrophages stimulated with LDL-ICs have an increased expression of LDL receptors compared with unstimulated cells. Considering that LDL-ICs can interact with macrophages both through Fcγ receptors and through the LDL receptor, the question arose as to which one of those interactions determined the increased expression of LDL receptor expression. This was investigated by performing two-step experiments, in which we first inhibited LDL-IC uptake with either native LDL or heat-aggregated gamma globulin and then determined the receptor-mediated accumulation of 125I-LDL. Figure 8 shows that LDL-IC-stimulated macrophages accumulated approximately nine times more 125I-LDL than unstimulated cells (283 ng LDL/mg cell protein). When native LDL was used to inhibit LDL-IC interaction with the LDL receptor, subsequent receptor-mediated accumulation of 125I-LDL decreased but remained about fourfold above the unstimulated level. When heat-aggregated gamma globulin was used to prevent the interaction of LDL-IC with Fcγ receptors or when LDL-ICs were prepared with the F(ab')2 fragment of anti-LDL antibodies (which will not interact with Fcγ receptors), 125I-LDL accumulation was not significantly different from that observed in unstimulated macrophages.

To determine whether high expression of LDL receptors was present in cells stimulated by LDL-ICs for extended periods of time and whether it could be observed even in cells with a high content of unester-
Unesterified and esterified cholesterol, we performed experiments by incubating human monocyte-derived macrophages with 250 μg/ml LDL-IC for 3 and 6 days. At the end of the incubation period, the cells were washed to remove unbound LDL-ICs and then incubated for 20 hours with or without addition of a 25-fold excess of unlabelled LDL. Values are mean±SEM for two experiments, with triplicate observations in each experiment. Receptor-mediated accumulation of 125I native LDL represented 85.4–86.6% of total accumulation. *p<0.05.

**FIGURE 8.** Bar graph of effect of competition for low density lipoprotein-immune complex (LDL-IC) uptake on receptor-mediated accumulation of iodine-125-labeled native LDL (10 μg/ml) by human monocyte-derived macrophages. Human macrophages were incubated for 22 hours at 37°C in the presence of medium alone (SFM) or medium containing 250 μg/ml LDL-IC (IC), 250 μg/ml LDL-F(ab')2 (FAB), 250 μg/ml LDL-IC plus 250 μg/ml LDL (LDL), or 250 μg/ml LDL-IC plus 10 mg/ml heat-aggregated gamma globulin (HAGG). Native LDL and HAGG were added to the medium 1 hour before addition of 250 μg/ml LDL-IC. After incubation with LDL-IC, medium was removed, cells were washed, and fresh medium containing 125I native LDL (10 μg/ml) was added, and the cells were incubated for another 20 hours with or without addition of a 25-fold excess of unlabelled LDL. Values are mean±SEM for two experiments, with triplicate observations in each experiment. Receptor-mediated accumulation of 125I native LDL represented 85.4–86.6% of total accumulation. *p<0.05.

TABLE 1. Time-Course Study of Low Density Lipoprotein Accumulation and Cholesterol Mass in Low Density Lipoprotein–Immune Complex–Stimulated Cells

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Incubation period (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td>125I-LDL accumulation</td>
<td></td>
</tr>
<tr>
<td>(Receptor-mediated)*</td>
<td></td>
</tr>
<tr>
<td>LDL-IC-stimulated cells</td>
<td>12.0±4</td>
</tr>
<tr>
<td>Unstimulated cells</td>
<td>0.60±0.01</td>
</tr>
<tr>
<td>Cholesterol mass</td>
<td></td>
</tr>
<tr>
<td>(Unesterified cholesterol)†</td>
<td></td>
</tr>
<tr>
<td>LDL-IC-stimulated cells</td>
<td>99±2.5</td>
</tr>
<tr>
<td>Unstimulated cells</td>
<td>60±2.5</td>
</tr>
<tr>
<td>Cholesterol esters†</td>
<td></td>
</tr>
<tr>
<td>LDL-IC-stimulated cells</td>
<td>193±13</td>
</tr>
<tr>
<td>Unstimulated cells</td>
<td>11±0.93</td>
</tr>
</tbody>
</table>

LDL, low density lipoprotein; LDL-IC, low density lipoprotein–immune complex.

*Micrograms LDL per milligram cell protein.
†Micrograms per milligram cell protein.

amount of 125I native LDL accumulated by the cells stimulated with LDL-IC, as well as the content of unesterified and esterified cholesterol in the same cells, was determined and is shown in Table 1. An increase in the accumulation of native LDL was observed in cells stimulated for 3 and 6 days with LDL-IC regardless of the high content of unesterified and esterified cholesterol present in the stimulated cells.

**Discussion**

Mechanisms leading to intracellular accumulation of cholesterol and foam cell formation are the object of intense investigation, in the hope that understanding these mechanisms will provide important clues about the pathogenesis of atherosclerosis. It is well known that when macrophages are incubated with modified or abnormal lipoproteins, subsequent transformation of the macrophages into foam cells can be observed. However, intracellular CE accumulation can also be due to an increased uptake of native LDL, particularly when the macrophages have been previously activated or when macrophages are incubated with native LDL complexed with IgG (LDL-IC), as we and others have recently reported. We also demonstrated that CE accumulation was associated with a 20-fold increase in LDL receptor number. Neither the accumulation of CE nor the overexpression of LDL receptors was simply due to activation of macrophages by ICs because insoluble ICs prepared with a variety of nonlipoprotein antigens, including keyhole limpet hemocyanin (which has a molecular weight similar to LDL), human IgG, human transferrin, and killed Candida albicans as well as antigenic extracts of C. albicans did not induce the transformation of these cells into foam cells and did not lead to enhanced uptake of native LDL.

At least two mechanisms, not mutually exclusive, could contribute to the increased intracellular CE accumulation observed in LDL-IC–stimulated macrophages. One would involve increased CE accumulation secondary to the enhancement in LDL receptor expression and increased uptake of native LDL in the stimulated cells. The other would involve the intracellular accumulation of LDL complexed with IgG during the stimulation of the cells with LDL-IC, due to an impaired degradation of the LDL contained in the IC.

In studies in which we compared the intracellular accumulation of CE in unstimulated macrophages and in macrophages stimulated with LDL-IC before and after their subsequent incubation with native LDL, we observed a marked difference in total and esterified cholesterol content between unstimulated and LDL-IC–stimulated cells even when native LDL was not added to the cultures after stimulation with LDL-ICs. When native LDL was added to the cultures, a further increase in total cholesterol and CE content was observed but only in the LDL-IC–stimulated cells. The contribution of native LDL uptake to the intracellular accumulation of cholesterol in the
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LDL-IC-stimulated cells was estimated to be between 8–10%.

The major source of intracellular CE appears to be the LDL taken up as part of LDL-IC, as there is a 7.3-fold increase in LDL uptake (accumulation and degradation) when LDL is presented to the macrophage complexed with IgG relative to when it is presented as native LDL. In addition, the degradation of LDL complexed with IgG is impaired, being only 1.09-fold higher than that of native LDL, which leads to a 199-fold increase in the amount of LDL accumulated intracellularly. Comparing the degradation of LDL complexed with IgG with the degradation of native LDL at different time points, we found that the native LDL was virtually taken up and degraded in about 5 hours while the LDL of the LDL-IC, although sufficiently taken up in the same period of time, was only partially degraded. Pulse-chase experiments have shown that in the first 5 hours, 34% of the LDL complexed with IgG was degraded and that at 48 hours, virtually all LDL (96%) had been degraded. Thus, the accumulation of CE in macrophages incubated with LDL-ICs was primarily due to increased uptake and impaired degradation of the LDL contained in the IC.

The enhanced uptake of LDL complexed with IgG by macrophages could be likely explained by the uptake of the IC by Fcγ receptors, which may be more efficient than uptake by the LDL receptor, by allowing the ingestion of large complexes with several aggregated LDL molecules. Also, it is possible that uptake by the Fcγ receptors may also explain the delay in the degradation of LDL, as the increased uptake of large LDL-antibody aggregates may lead to an overload in the lysosomal compartment of the cell. The major participation of Fcγ receptors in the recognition of LDL-ICs was confirmed by competition studies, which showed that the greatest degree of inhibition (75%) of LDL-IC binding was obtained with heat-aggregated gamma globulin, whereas LDL only inhibited LDL-IC binding by 25%. Competition with acetylated LDL or β-very low density lipoprotein did not inhibit the binding of the LDL-IC, ruling out the participation of the scavenger and LDL-related receptors in the uptake of LDL-ICs. The same conclusions were reached when a monoclonal antibody against LDL receptor was found to block 125I-LDL-IC binding in a similar manner. The specificity of the block was confirmed when an irrelevant monoclonal antibody secreted into a medium with similar composition failed to inhibit the uptake of 125I-LDL-IC by the cells. Furthermore, similar conclusions were reached when the effects of preincubation with heat-aggregated gamma globulin, native LDL, or acetylated LDL on CE accumulation were studied. In addition, LDL-ICs prepared with the F(ab′)2 fragment of anti-LDL antibody induced CE accumulation to levels approximately 28% of those seen with complete LDL-ICs. Thus, we can safely conclude that LDL-IC uptake occurs through two receptor pathways—primarily through Fcγ receptors and to a lesser degree through the LDL receptor pathway.

It is interesting to note that phagocytosis of LDL aggregates, which seems to involve the classical LDL receptor, is also followed by CE accumulation and foam cell formation. These observations suggest that the interaction with LDL receptors can be associated with pinocytosis (native LDL) or phagocytosis (aggregated LDL and perhaps some LDL-IC). Therefore, a general rule seems to emerge: while pinocytic internalization is associated with the well-known tight regulation of intracellular cholesterol levels and expression of LDL receptor, phagocytosis is not. Whether this is a reflection of the incorporation into different intracellular pools or of some other mechanism can only be a matter of speculation at this time.

A most paradoxical finding in our experiments is the increased expression of LDL receptors in cells that have a markedly increased level of intracellular cholesterol. This increased expression is maintained for an extended period of time (up to 6 days) regardless of the level of intracellular cholesterol. We considered the possibility that this could be secondary to cell activation and subsequent to the ingestion of LDL-ICs via Fcγ receptors. If so, we should have been able to abrogate the increase when the Fcγ receptors were blocked. Indeed, when the Fcγ receptors were blocked with heat-aggregated gamma globulin, the accumulation of 125I-LDL added subsequently to incubation of the macrophages with LDL-ICs was significantly reduced (84%), but a marked reduction (58%) was also seen when the LDL receptor was blocked with native LDL before addition of the LDL-IC. These results seem to suggest that LDL-IC, taken up via either the LDL receptor or the Fcγ receptor, had the capability of stimulating LDL receptor expression. However, because the addition of native LDL to block LDL receptors may not only block the receptors but also down-regulate their expression, the subsequent incubation of these cells with LDL-ICs was likely to have a less marked effect on the expression of LDL receptors, explaining at least in part the 58% decrease in LDL uptake observed. To circumvent this difficulty, we performed experiments with LDL-F(ab′)2-IC, which will not bind to Fcγ receptors but will bind to the LDL receptor, and demonstrated that the upregulation of the LDL receptor could not be induced with LDL-F(ab′)2-IC. Therefore, it appears as if the interaction of the LDL-IC with Fcγ receptors is responsible for the subsequent increase in the expression of LDL receptors in LDL-IC-stimulated cells. The cellular mechanisms responsible for this increased expression of LDL receptors have yet to be defined.

It is possible that LDL bound to anti-LDL antibody and taken up through Fcγ receptors is incorporated into a cellular cholesterol pool that differs significantly from that used by LDL taken up through the LDL receptor. This could lead to an increased availability of free cholesterol to ACAT and to a decrease in the regulatory free cholesterol pool, with
subsequent increase in LDL receptor expression. Alternatively, macrophage activation may result in cholesterol translocation from the plasma membrane pool to the ACAT substrate pool, depleting membrane cholesterol pools and in effect increasing the cellular requirement for membrane cholesterol. Against this hypothesis is the lack of an increase in LDL receptor expression in cells stimulated by ICs containing antigens other than lipoproteins.

The clinical significance of our findings hinges heavily on the natural occurrence of anti-LDL antibodies and the formation of LDL-ICs in vivo. Anti-LDL antibodies have been described in patients with arteriosclerosis, and LDL isolated from patients with coronary heart disease has been shown to be immunologically different from native LDL. It is also known that the respiratory burst of macrophages is associated with the release of reactive oxygen intermediates and other products that have the potential of modifying LDL. Such modifications, interestingly enough, are associated with increased immunogenicity and production of anti-LDL antibodies. Witzum and coworkers demonstrated that the antibodies to modified lipoproteins were directed against the added radicals and failed to react with native LDL. However, most tests developed for the detection of anti-LDL antibodies in humans have used unmodified LDL as antigen, therefore suggesting that a diversity of antibodies may be formed with different specificities and cross-reactivities. In any case, these anti-LDL antibodies have the potential to react with LDL and form LDL-ICs. In the studies reported in this article, we used insoluble LDL-ICs as models. Insoluble ICs are not likely to exist in the circulation for extended periods of time but could very well be formed in an already altered arterial cell wall when LDL has diffused into the subendothelial space, where it will be likely to react with anti-LDL antibodies. Furthermore, experiments performed in our laboratory have demonstrated that erythrocyte-bound ICs are even more efficient than insoluble ICs in inducing the intracellular CE accumulation and foam-cell formation. Thus, although it is tempting to postulate that the LDL-ICs may play a role in the pathogenesis of arteriosclerosis, it is rather premature to speculate about their clinical relevance. The investigation of a possible connection between the development of arteriosclerosis and the presence of LDL-ICs in an animal model would help to answer this interesting question.

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