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R. L. Griffith
Medical University of South Carolina

G. T. Virella
Medical University of South Carolina

H. C. Stevenson
National Cancer Institute

M. F. Lopes-Virella
Veterans Administration Medical Center

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LOW DENSITY LIPOPROTEIN METABOLISM BY HUMAN MACROPHAGES ACTIVATED WITH LOW DENSITY LIPOPROTEIN IMMUNE COMPLEXES

A Possible Mechanism of Foam Cell Formation

By R. L. GRIFFITH,‡ G. T. VIRELLA,‡ H. C. STEVENSON,§ and M. F. LOPES-VIRELLA*

*From the *Veterans Administration Medical Center; the *Department of Medicine; and the ‡Department of Basic and Clinical Immunology and Microbiology, Medical University of South Carolina, Charleston, South Carolina 29425; and the §National Cancer Institute, Bethesda, Maryland 21701*

Human monocytes are known to be multifunctional cells that combine a variety of functions, including phagocytosis, antigen processing and presentation to immune cells, secretion of a large number of bioactive products with significant roles in the immune and inflammatory reactions, and the ability to kill tumor cells and other abnormal cells by a variety of mechanisms, including antibody-dependent cell-mediated cytotoxicity (1–5). Tissue macrophages are believed to derive from circulating monocytes, although the two types of cells differ by a variety of morphological and functional criteria (6–10). The role of macrophages is not always well understood, since it combines potentially useful properties related to its ability to ingest and process foreign and altered materials with the capacity to secrete large amounts of mediators having the potential to cause inflammatory changes and tissue damage in general (11–14). In atherosclerosis, substantial evidence has been gathered suggesting that the "foam cells" seen in early atherosclerotic plaques are derived from monocytes/macrophages (15–18). The formation and subendothelial accumulation of foam cells are believed to represent a critical event in the onset of atheromatous plaque formation (19).

Some interesting correlations can be drawn between the involvement of macrophages in the pathogenesis of atherosclerosis and increasing evidence suggesting that immunologic mechanisms may influence the development or evolution of this pathologic process. In the early 1970s, it was postulated that immune mechanisms involving circulating immune complexes could contribute to the pathogenesis of atherosclerosis (20). The evidence supporting this role of immune complexes was both experimental (animals undergoing serum sickness and given a lipid-rich diet developed accelerated atherosclerosis [21]) and clinical (patients with IgA myelomas with anti-lipoprotein activity had massive hyperlipemia and accelerated atherosclerosis

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[22]). More recently, it was also shown that immune complexes (IC)[1] involving low density lipoprotein (LDL) induce profound changes on cholesterol metabolism at the cellular level (23). Further support for the involvement of IC in the pathogenesis of atherosclerosis has been recently obtained by Szondy et al. (24), who demonstrated increased levels of IC and anti-LDL antibodies in patients with clinical manifestations of coronary heart disease. The possibility that IC interactions with macrophages may lead to their activation and, therefore, play a pathogenic role in the development of atherosclerosis is extremely challenging.

Until recently, the mechanism proposed to explain how monocyte-derived macrophages could be transformed into foam cells has focused upon the interaction between macrophages and modified LDL or lipoproteins of abnormal composition, such as β-very low density lipoproteins (VLDL). Modified LDL can be taken up in a nonregulated fashion via the scavenger receptor, resulting in the intracellular accumulation of cholesteryl esters (CE) and in the formation of a foam cell. In contrast, it has been observed that cultured macrophages exposed to native LDL (N-LDL) do not accumulate CE due to the stringent regulation of LDL receptors. However, it has been recently shown that in certain conditions, macrophages exposed to native LDL may accumulate CE. In our laboratory, we have shown that human macrophages stimulated with microbial or microbial-related products have an increased uptake of N-LDL and accumulated CE (25). Tabas et al. (26) observed increased uptake of N-LDL and concomitant CE accumulation in J774 cells, a mouse macrophage-like tumor cell line. They postulated that this was due to the enhanced metabolic activity of this tumor cell line (26). Klimov, et al. (27) had shown excessive CE accumulation in mouse peritoneal macrophages exposed to LDL immune complexes compared with control cells. A common denominator for all these observations is the known ability of microbial products and immune complexes to activate macrophages. Therefore, we decided to examine the effect of macrophage activation on N-LDL metabolism. More specifically, we wanted to investigate the effect of LDL-anti-LDL IC on N-LDL and cholesterol metabolism in human macrophages, and determine whether this type of IC can induce the transformation of human macrophages into foam cells contributing to the development of atherosclerosis.

Materials and Methods

Isolation of Monocytes. Monocytes were isolated from leukapheresis specimens by countercurrent centrifugal elutriation as previously described (28, 29). Briefly, leukapheresis specimens were passed over standard Ficoll-Hypaque gradients to produce an unfractionated mononuclear leukocyte suspension. These leukocyte cell preparations were then suspended in elutriation medium (RPMI 1640 [Flow Laboratories, Inc., McLean, VA] with 2 g per 100 ml of clinical grade human albumin [Cutter Laboratories, Berkeley, CA] with a final pH of 7.4) and entered with a Sarns cardiovascular pump (Highland Corp., Highland, IN) into an elutriation chamber and rotor system (model JEG; Beckman Instruments, Inc., Palo Alto, CA) at an initial medium flow rate of 5 ± 0.4 ml/min. The elutriation rotor speed was maintained at 2,020 ± 10 rpm, and the centrifuge temperature was held at 18°C. All medium used was documented to be endotoxin free (<0.1 ng/ml of endotoxin, by limulus assay) and no antibiotics were added to any stage of the monocyte isolation procedure. The leukaphe-

[1] *Abbreviations used in this paper:* CE, cholesteryl esters; *d*, density; HDL, high density lipoproteins; IC, immune complexes; IMDM, Iscove's modified Dulbecco's medium; KHL, keyhole limpet hemocyanin; LDL, low density lipoprotein; N, native; SFM, serum-free medium; VLDL, very low density lipoprotein.

resis specimens used for the separation of monocytes as described above were obtained by leukapheresis of normal volunteers for 2 h on a Celltrifuge II apparatus (Baxter-Travenol Laboratories, Deerfield, IL). Donors with abnormalities on physical or laboratory examinations were excluded as previously described (28).

Monocyte Identification Procedures. The purity of the monocyte preparations obtained by elutriation was confirmed by morphology on Wright's-stained cytocentrifuge preparations, by nonspecific esterase staining, and by the ability to ingest latex particles as previously described (30). Viability was determined by trypan blue dye exclusion. The average purity of the monocytes used in this study by Wright's staining was 93%; by esterase staining, 92%; and by latex ingestion, 93%. The average viability of the cells used was 99%.

Transformation of Monocytes into Macrophages. Monocytes isolated as described above were suspended in a specially formulated serum-free medium (SFM) to a final concentration of 10^6 cells/ml. The medium was prepared using Iscove's modified Dulbecco's medium (IMDM) supplemented with human serum albumin (fatty acid free, 4 mg/ml), cholesterol (>90% pure, 20 μg/ml), L-α-phosphatidylcholine (80 μg/ml), and human transferrin (98% pure, 1 μg/ml), all from Sigma Chemical Co. (St. Louis, MO); human insulin (0.128 U/ml, Eli Lilly and Co., Indianapolis, IN); and ferrous chloride (7×10^{-11} M, Fisher Scientific Co., Fairlawn, NJ) as previously described (31).

1 ml of the above cell suspension was plated in each well of a 24-well Costar plate and incubated at 37°C in a 5% CO_2 incubator for 2–3 h to allow the monocytes to adhere to the plastic. After this time, the medium was removed and replaced with medium containing 30% (vol/vol) of whole human serum (Whittaker M.A., Bioproducts, Walkersville, MD). The cells were further incubated for 6–8 d, and the medium was changed every 3 d. The protein content of the macrophages was on the average three times higher than that of monocytes, thus, the pooled human serum used was not toxic for the cells. After maturation of monocytes into macrophages, SFM containing no cholesterol was used to perform all the experiments.

Lipoprotein Isolation, Modification, and Labeling. Blood was collected in EDTA (1 mg/ml) after 12 h of fasting. VLDL, LDL, and high density lipoproteins (HDL) were isolated from plasma by sequential ultracentrifugation on an ultracentrifuge rotor (L5-50, type 50; Beckman Instruments, Inc.), as previously described (32). VLDL were isolated at plasma density (density [d] <1.006 g/ml) at 50,000 rpm/min for 15 h. If chylomicrons were present, they were removed by centrifuging the plasma at 26,000 g for 1 h before ultracentrifugation. LDL (d > 1.019 < 1.063 g/ml) were isolated after appropriate adjustment of density with KBr, at 50,000 rpm/min for 22 h. HDL d > 1.063 < 1.21) were isolated after appropriate adjustment of density with KBr, at 50,000 rpm/min for 24 h. LDL, HDL, and VLDL preparations were washed by ultracentrifugation, dialyzed against a 0.15 M NaCl solution containing 1 mM of EDTA, pH 7.4, and stored under nitrogen in the dark. They were passed through an Acrodisc filter (0.2-μm pore size; Gelman Sciences, Inc., Ann Arbor, MI) in order to remove aggregates. An aliquot of LDL was iodinated with ^{125}I by the McFarlane procedure as modified by Bratzler et al. (33). The labeling conditions were adjusted in order to obtain a specific activity of 100–400 cpm/ng of protein. Radioactivity localized in the lipid moiety of the lipoproteins was determined after a "Folch lipid extraction" (34) and constituted <4% of the total radioactivity. Acetylation of LDL was performed using the protocol described by Basu et al. (35).

Lipoprotein Accumulation and Degradation. Macrophages were incubated for 22 h with 250 μg of LDL containing immune complexes. After the incubation with IC, the medium was removed, and the cells, after appropriate washing procedure, received 1 ml of fresh IMDM medium containing 10 μg of ^{125}I-labeled native LDL. In half of the culture wells, 250 μg of unlabeled native LDL was appropriately added to correct for nonspecific degradation. In some of the experiments, the IC were left for the duration of the experiment. The medium removed after incubation with the lipoproteins was used to study LDL proteolytic degradation as described by Bierman et al. (36). The proteolytic degradation of ^{125}I-labeled N-LDL by human macrophages was measured by assaying the amount of ^{125}I-TCA-soluble (non-iodide) material formed by the cells and excreted into the culture medium. Corrections were made for the small amounts of ^{125}I-labeled acid-soluble material that was found in parallel incubations without cells.

The amount of ^{125}I-LDL bound and internalized by macrophages, i.e., cellular accumu-

lation, was determined by incubating the cells with ^{125}I-LDl for 20 h at 37°C. After the incubation, the medium was removed, and the cells were washed two times with 1 ml PBS containing 0.2% (wt/vol) BSA followed by two additional washes with PBS without albumin. The cells were dissolved in 1 M NaOH, and an aliquot of the solubilized cells was taken to determine the amount of ^{125}I-LDL radioactivity present in the cell pellet. Another aliquot was used to determine the amount of cellular protein by the method of Lowry et al. (37).

To assay LDL binding, the above protocol was also followed, but the incubation as well as the washing and harvesting procedures were performed at 4°C instead of 37°C. The period of incubation of LDL with the cells was 4 h. Saturation binding analysis of ^{125}I-N-LDL binding to LDL-IC-stimulated macrophages and nonstimulated macrophages incubated with increasing concentrations of ^{125}I-N-LDL was performed. The data represent specific ^{125}I-N-LDL bound, determined from the difference in binding in the presence and absence of a 25-fold excess unlabeled N-LDL at the indicated concentrations, and is plotted against the total ^{125}I-N-LDL concentration. The curves represent the best fit determined by unweighted least-squares nonlinear regression analysis. Scatchard analysis was performed on the saturation binding data as described (38, 39).

Competition Studies. In one series of experiments, ^{125}I-LDL-labeled immune complexes (250 μg/ml) were added to macrophages incubated with SFM alone, SFM containing heat-aggregated gamma globulin (10 mg/ml), SFM containing unlabeled N-LDL (250 μg/ml), or SFM containing unlabeled Ac-LDL (250 μg/ml). The cultures were then incubated at 4°C for 4 h. After that period, the cultures were washed, and the cellular binding of ^{125}I-LDL-labeled IC was determined.

In another set of experiments, macrophages were incubated for 22 h, as described above, with SFM or SFM containing LDL-IC. After the incubation, the medium was removed, the cells were extensively washed, and ^{125}I-N-LDL (10 μg/ml) was added to macrophages incubated with SFM alone, SFM containing unlabeled N-LDL (250 μg/ml), or SFM containing unlabeled Ac-LDL (250 μg/ml). The cells were incubated for 4 h at 4°C, and after this period, they were washed, and the cellular binding of ^{125}I-LDL was determined.

Measurement of Total, Free, and Esterified Cholesterol Content in Macrophages. To perform these experiments, 10^6 monocytes were plated in 24-well culture plates (Costar, Cambridge, MA) and matured into macrophages as described above. After maturation into macrophages, the cells were washed with PBS to remove whole human serum and incubated for 22 h with SFM alone or SFM containing immune complexes. Afterwards, the medium was removed, and the cells were washed with PBS. To control and IC-stimulated dishes, SFM containing 100 μg/ml of native LDL was added. The cells were incubated for 20 h. In some of the experiments, the medium after the 22-h incubation with immune complexes was not removed before the addition of LDL, and the cells were left in the presence of the IC throughout the entire experiment (42 h). Afterwards, medium was removed, and the cells were extensively washed with PBS. The macrophage monolayer was extracted with hexane/isopropanol (3:2) (vol/vol), as previously described (40). Free and total cholesterol were assayed on a gas chromatograph equipped with a hydrogen flame ionization detector. A glass column packed with 3% SP-2250 on 80/100 mesh Supelcoport was used for the chromatographic separation, and its temperature was maintained at 250°C during the separation. N_2 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 (41), as previously described (32). Cholesteryl ester levels were obtained by subtracting free cholesterol from total cholesterol levels. β-Stigmasterol was used as an internal standard.

Immunization and Antibody Isolation. For immunization, a narrow density cut (1.030–1.050 g/ml) of human LDL was isolated. The rabbits received six immunizations over an 8-wk period. For the primary immunization, 1 mg of LDL protein with an equal volume of CFA was injected bilaterally, intramuscularly into all four quadrants. IFA was used for the second immunization, and no adjuvant was used for subsequent immunizations. The plasma for antibody isolation was obtained 7–10 d after the last immunization. Antibody was precipitated from rabbit plasma using caprylic acid and 50% saturated ammonium sulfate (42). The precipitate was dissolved in distilled water, dialyzed against 0.04 M Tris-phosphate buffer,

pH 8.4, and passed through a DEAE Sephacel column (Pharmacia Fine Chemicals, Piscataway, NJ). Absorbed proteins were eluted with a 0.04 M Tris-phosphate, pH 8.4, to 0.5 M Tris-phosphate, pH 3.4, gradient. The IgG content was measured by absorbance at 280 nm. The purity of the preparation was assessed by immunodiffusion and SDS-PAGE.

Preparation of Immune Complexes. Insoluble immune complexes were prepared using LDL (1 mg/ml) and the IgG fraction of a rabbit anti-LDL antiserum (1 mg/ml). 1 ml of antiserum was incubated with varying amounts of LDL (5–1,000 μl) at 4°C overnight. Based on the results of precipitin curves, the ratio of Ag/Ab giving the greatest amount of precipitate was 1:10 (wt/wt). The protein content of the insoluble IC was determined after washing three times with PBS using a protein assay (Bio-Rad Laboratories, Richmond, CA) calibrated with serial dilutions of a preparation of heavily heat-aggregated IgG with known amounts of IgG. The sterility of the IC preparations was ensured by γ irradiation.

Preparation of Heat-aggregated Gamma Globulin. Human gamma globulin (G4386; Sigma Chemical Co.) at 20 mg/ml in PBS was heated at 63°C for 20 min. Protein was determined by a Bio-Rad Laboratories assay and sterility was insured by γ irradiation.

CRL-1703 Hybridoma. The CRL 1703, known also as 9D9, is a murine hybridoma obtained from the American Type Culture Collection, Rockville, MD. It was made by Huettinger et al. (43) against purified bovine LDL receptor and was determined to be of the IgG1 subclass. mAbs to bovine LDL receptors have been shown to crossreact with human LDL receptors (44). The CRL-1703 were seeded at a density of 2×10^5 cells/ml in RPMI 1640 supplemented with 10% FCS and 4.5% glucose, as recommended. The hybridoma cultures were grown to $\sim 10^6$ cells/ml, whereupon the culture supernatants were harvested and used as described in Results.

Oil-Red O Staining. The neutral lipid inclusions in human monocyte-derived macrophages were stained using oil-red O, as previously described (45).

Statistics. Results are reported as mean ± SEM. The nonparametric Wilcoxon signed rank sum test was used for the statistical comparison of LDL degradation and accumulation, and CE mass by stimulated and nonstimulated macrophages. Significance was assumed for p values <0.05.

Results

Human macrophages stimulated for 22 h with LDL-IC (250 μg/ml) and exposed afterwards to ^{125}I-N-LDL for 20 h showed a four- and sixfold increase, respectively, in the degradation and accumulation of ^{125}I-N-LDL over the values observed in nonstimulated cells (Fig. 1) ($p < 0.05$). Experiments were performed either adding ^{125}I-N-LDL to the cells after the removal of the LDL-IC as described above (Fig. 1) or leaving the LDL-IC for 42 h (22 h before and 20 h after the addition of ^{125}I-N-LDL). The stimulation of the uptake and degradation of ^{125}I-N-LDL was further

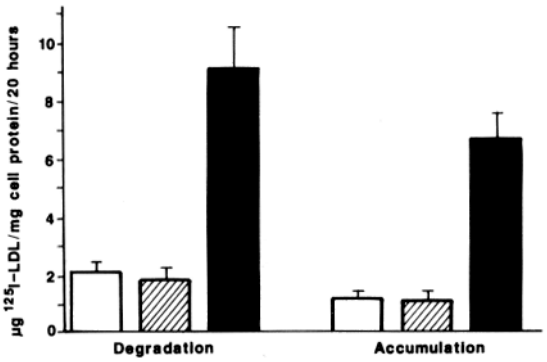

FIGURE 1. Receptor-mediated degradation and accumulation of ^{125}I-N-LDL (10 μg/ml) by human monocyte-derived macrophages after 22 h of stimulation with 250 μg/ml of LDL anti-LDL immune complexes, (■), and 250 μg/ml anti-LDL antibody (▨), as compared with nonstimulated control cells (□). Values are the means ± SEM for 13 experiments with triplicate observations in each experiment.

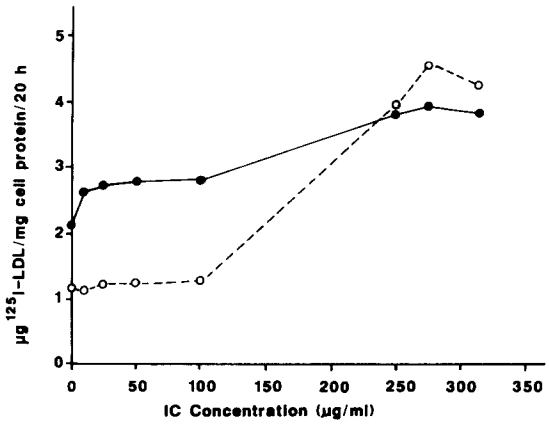

FIGURE 2. Effect of progressively increasing concentrations of LDL anti-LDL immune complexes on receptor-mediated accumulation (○) and degradation (●) of ^{125}I-N-LDL (10 μg/ml) by human macrophages stimulated for 22 h at the indicated concentrations of LDL-anti-LDL immune complexes. Values are a representative example of three experiments with triplicate observations in each experiment. All subsequent experiments used LDL-anti-LDL immune complexes at a concentration of 250 μg/ml.

enhanced when the LDL-IC were left throughout the experiment (data not shown). The optimal LDL-IC concentration (250 μg/ml) and incubation time (22 h) were determined through dose-response studies (Figs. 2 and 3). To exclude the possibility that the heterologous antisera used to prepare the IC were responsible for the effect observed on LDL uptake, we carried out control experiments incubating macrophages with anti-LDL antisera; we did not observe any significant increase in the uptake and degradation of ^{125}I-N-LDL in these control experiments ($p > 0.5$) (Fig. 1).

To determine the mechanism leading to increased uptake of LDL by LDL-IC-stimulated macrophages, saturation binding isotherm studies were performed (Fig. 4, *A* and *B*) using progressive concentrations of ^{125}I-N-LDL with or without a 25-fold excess of N-LDL to determine nonspecific binding. The LDL-IC-stimulated macrophages (Fig. 4 *A*) bound ~10-fold more ^{125}I-N-LDL than the nonstimulated cells (Fig. 4 *B*). Scatchard plot analysis of the specific binding of ^{125}I-N-LDL by macrophages stimulated with LDL-IC (Fig. 5 *A*) indicates a slope of -9.8 ± 0.6 with an extrapolated *x*-intercept of 625 ng. Similar analysis of the data obtained in nonstimulated macrophages reveals and approximate slope of -8.5 ± 0.8 and an extrapolated *x*-intercept of 31 ng. These data suggests the presence of an increased number of LDL receptors in macrophages stimulated with LDL-IC.

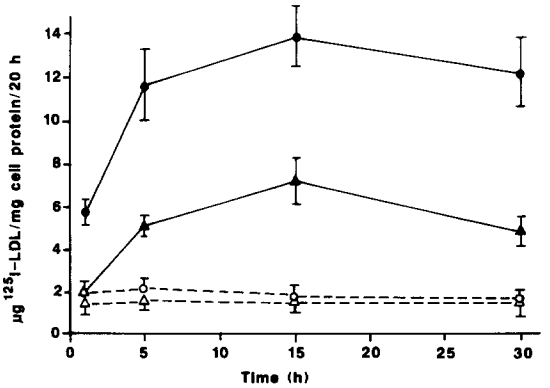

FIGURE 3. Temporal effect of LDL-anti-LDL immune complexes upon receptor-mediated degradation (●, ○) and accumulation (▲, △) of ^{125}I-N-LDL by human macrophages. Cells were stimulated with LDL anti-LDL immune complexes (*closed symbols*) for the indicated time periods compared to nonstimulated controls (*open symbols*). Values are the means ± SEM for three experiments with triplicate observations in each experiment.

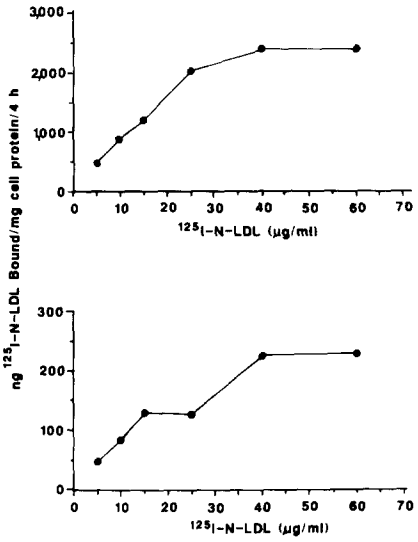

FIGURE 4. (*Top*) Receptor-mediated binding of ^{125}I-N-LDL by macrophages stimulated with LDL anti-LDL immune complexes. Monocyte-derived macrophages were incubated for 22 h with LDL anti-LDL immune complexes. The cells were washed three times with cold PBS and incubated for 4 h at 4°C with increasing concentrations of ^{125}I-N-LDL, with or without addition of a 25-fold excess of unlabeled LDL. (*Bottom*) Receptor-mediated binding of ^{125}I-N-LDL by nonstimulated macrophages. Cells were processed identically as described for the top of the figure. The values represent the mean of triplicate observations of one representative experiment from a total of three experiments.

To rule out the possibility that the increased uptake of ^{125}I-N-LDL observed in our studies was mediated by the binding to Fc receptors of immune complexes formed by the association of anti-LDL antibody released from the LDL-IC used for the cell stimulation and ^{125}I-N-LDL added in the second phase of the experiment, the following experiments were performed.

After appropriate incubation of macrophages with LDL-IC, as described above, 1 h before the 20-h incubation with ^{125}I-N-LDL, we added various concentrations (250–500 μg/ml) of a F(ab')$_2$ goat anti-rabbit IgG to nonstimulated and washed LDL-IC-stimulated macrophages. With this step, we expected to crosslink any residual rabbit anti-LDL antibody remaining in the culture and prevent its binding

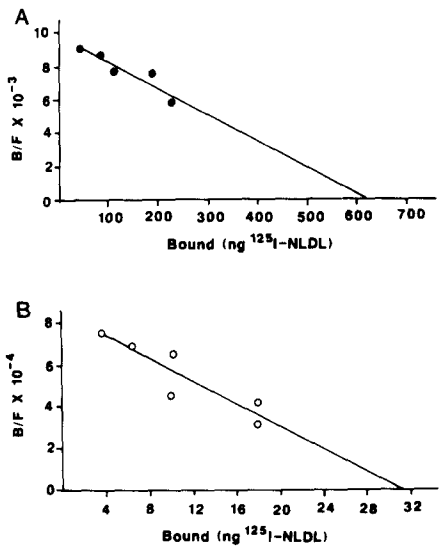

FIGURE 5. (*A*) Scatchard plot analysis of the specific binding of ^{125}I-N-LDL by macrophages stimulated with LDL-anti-LDL immune complexes (●) (slope, −9.8 ± 0.6; x − intercept, 625 ng). (*B*) Scatchard plot analysis of the specific binding of ^{125}I-N-LDL by nonstimulated macrophages (○), (slope, −8.5 ± 0.8; x − intercept, 31 ng). The data for scatchard analysis was obtained from the specific ^{125}I-N-LDL binding in Fig. 4.

to ^{125}I-N-LDL. The concentrations of F(ab')$_2$ goat anti-rabbit IgG were chosen after determining, in preliminary experiments, that they effectively prevented insoluble LDL-IC formation. No significant differences were found in the uptake of ^{125}I-N-LDL between LDL-IC-stimulated macrophages in the presence or absence of the goat anti-rabbit antibody (data not shown). An alternate approach using the anti-LDL receptor antibody obtained from the mouse hybridoma line CRL-1703 was also pursued. Nonstimulated and washed LDL-IC-stimulated macrophages were incubated with 1 ml, 500 μl, or 250 μl of the supernatant from the hybridoma culture containing anti-LDL receptor antibody (undiluted, twofold, and fourfold dilutions). Since the hybridoma supernatants contained 10% FCS, parallel control cultures of stimulated and nonstimulated macrophages were supplemented with 1 ml, 500 μl, and 250 μl of RPMI 1640 medium containing 10% FCS, to rule out possible inhibition or stimulation of ^{125}I-N-LDL accumulation by factors present in the FCS. 1 h later, ^{125}I-N-LDL (10 μg/ml) was added, and the cells were incubated at 4°C for 4 h in a final volume of 1 ml. In the nonstimulated cells, irrespective of the dilution of the hybridoma supernatant, the specific accumulation of ^{125}I-N-LDL was inhibited by ~70% (6.25 ng ^{125}I-N-LDL accumulated per mg cell protein in control cultures vs. 1.91 ng in anti-LDL receptor-treated cells). In the LDL-IC-stimulated macrophages, the inhibition of specific ^{125}I-N-LDL accumulation decreased with progressive dilutions of the hybridoma supernatant (49.7 ng ^{125}I-N-LDL accumulated per mg cell protein in control-stimulated cells vs. 2.0 ng with undiluted hybridoma supernatant, 8.4 ng with a 1:2 dilution, and 11.4 ng ^{125}I-N-LDL accumulated with a 1:4 dilution). The marked inhibition of ^{125}I-N-LDL accumulation by the anti-LDL receptor antibody in the stimulated macrophages strongly supports that the increased uptake of ^{125}I-N-LDL is mediated by the LDL-receptor. Interestingly, with maximum inhibition in both the nonstimulated and LDL-IC-stimulated cells, approximately the same amount of ^{125}I-N-LDL accumulates (~2.0 ng/mg cell protein). The residual accumulation seen in cells incubated with maximal concentrations of the monoclonal anti-LDL receptor antibody could be explained by the fact that this antibody being raised against bovine LDL receptors and crossreactive with human receptors may have a relatively low affinity to the human LDL receptor. Alternatively, a certain proportion of human LDL receptors may not crossreact with the antiserum, although there is no concrete evidence for LDL receptor heterogeneity in humans. It needs to be noted that Beisiegel et al. (44) found that the human LDL receptor in fibroblasts could be maximally inhibited (~80%) with an mAb to bovine LDL receptor, a result strikingly similar to ours.

Another possible mechanism for the increased uptake of ^{125}I-N-LDL observed in our studies was a recognition of LDL-IC as modified LDL. If that occurred, the increased uptake of ^{125}I-N-LDL could be mediated by the uptake through the scavenger receptor of immune complexes formed by the association of anti-LDL antibody released from the LDL-IC used for cell stimulation and the ^{125}I-N-LDL added in the second phase of the experiment. To explore this possibility, we performed the following competition experiments. Human macrophages were incubated at 4°C for 4 h with ^{125}I-LDL-labeled immune complexes (250 μg/ml) in presence of unlabeled N-LDL (250 μg/ml), heat-aggregated gamma globulin (10 mg/ml), or unlabeled Ac-LDL (250 μg/ml). Unlabeled N-LDL and HAGG inhibited the binding of ^{125}I-labeled LDL-IC by ~22% and 70%, respectively. Acetylated LDL did not inhibit ^{125}I-labeled LDL-IC binding.

To exclude the possibility that the ^{125}I-N-LDL added to the stimulated macrophages was modified by the stimulated macrophages, competition studies in presence of unlabeled N-LDL or unlabeled acetylated LDL were performed. The binding of ^{125}I-N-LDL to stimulated macrophages was not inhibited by acetylated LDL. In contrast, it was markedly inhibited (84% inhibition) by unlabeled N-LDL (data not shown).

To determine whether the increased uptake of ^{125}I-N-LDL in macrophages stimulated with LDL-IC was accompanied by intracellular accumulation of lipids, we stained the cells with oil-red and we measured by gas chromatography the total, free, and esterified cholesterol content of the cells. Macrophages incubated with N-LDL after LDL-IC stimulation for 22 h showed a greater number of oil-red-stained inclusions than nonstimulated cells (Figs. 6, *A* and *B*). They also show significantly higher levels of esterified cholesterol (~13 times the values in nonstimulated cells: 185 ± 30 μg CE/mg cell protein vs. 14 ± 8 μg CE/mg cell protein) ($p > 0.05$) (Fig. 7). When the LDL-IC were present throughout the entire assay (42 h), the cholesteryl ester content increased to 16 times the levels observed in nonstimulated cells (232 ± 29 μg CE/mg cell protein vs. 14 ± 8 μg CE/mg cell protein) (Fig. 7).

Previous studies have shown a direct correlation between the size of IC and their ability to be ingested and subsequently alter macrophage function (46–48). We wanted to determine if the enhanced uptake of N-LDL was specific for LDL-IC-stimulated macrophages or if it was just a general property of IC-stimulated macrophages. Therefore, IC were prepared involving a variety of antigens such as IgG, transferrin, keyhole limpet hemocyanin (KLH), and killed *Candida albicans* (both whole candida and cytoplasmic protein extracts). All complexes were used at 250 μg/ml along with the corresponding antibody control. IC prepared with *C. albicans* and KLH increased the uptake and accumulation of LDL by 47% and 23%, respectively. Stimulation of cells with IgG-IC did not alter LDL metabolism. In contrast, stimulation with transferrin-IC depressed LDL metabolism (accumulation was 44% of the value obtained in nonstimulated cells).

Since the effect on LDL metabolism seems to be considerably greater when the cells were stimulated with LDL-IC than when other IC were used, we decided to determine whether IC prepared with other lipoproteins would have a similar effect to that observed with LDL-IC. Thus, IC were prepared with VLDL and HDL, and the cells were stimulated as in the conditions previously described for LDL-IC. LDL-IC-stimulated cells were used as controls. In VLDL-IC- and HDL-IC-stimulated cells, the accumulation of ^{125}I-N-LDL was approximately twofold over control values (Fig. 8). In contrast, in LDL-IC-stimulated cells, the accumulation of ^{125}I-N-LDL was ~12-fold over the control values. When the studies were performed leaving the IC throughout the length of the experiment, a more marked accumulation of ^{125}I-N-LDL was seen, especially in VLDL-IC-stimulated cells (sevenfold increase in ^{125}I-N-LDL uptake over nonstimulated cells) (Fig. 8).

Similarly to what happened in the experiments with LDL-IC, the cells stimulated with VLDL-IC and HDL-IC for either 22 h or longer periods of time (42 h) showed an increase in the total cholesterol mass (two times above control values), with CE composing ~ 43% and 45% of the total cholesterol mass, respectively (Figs. 9 and 10).

Finally, we wanted to determine whether the changes observed in LDL metabolism stimulating macrophages with LDL-IC were also observed when monocytes were submitted to similar stimulation. Fig. 11 illustrates that although monocytes

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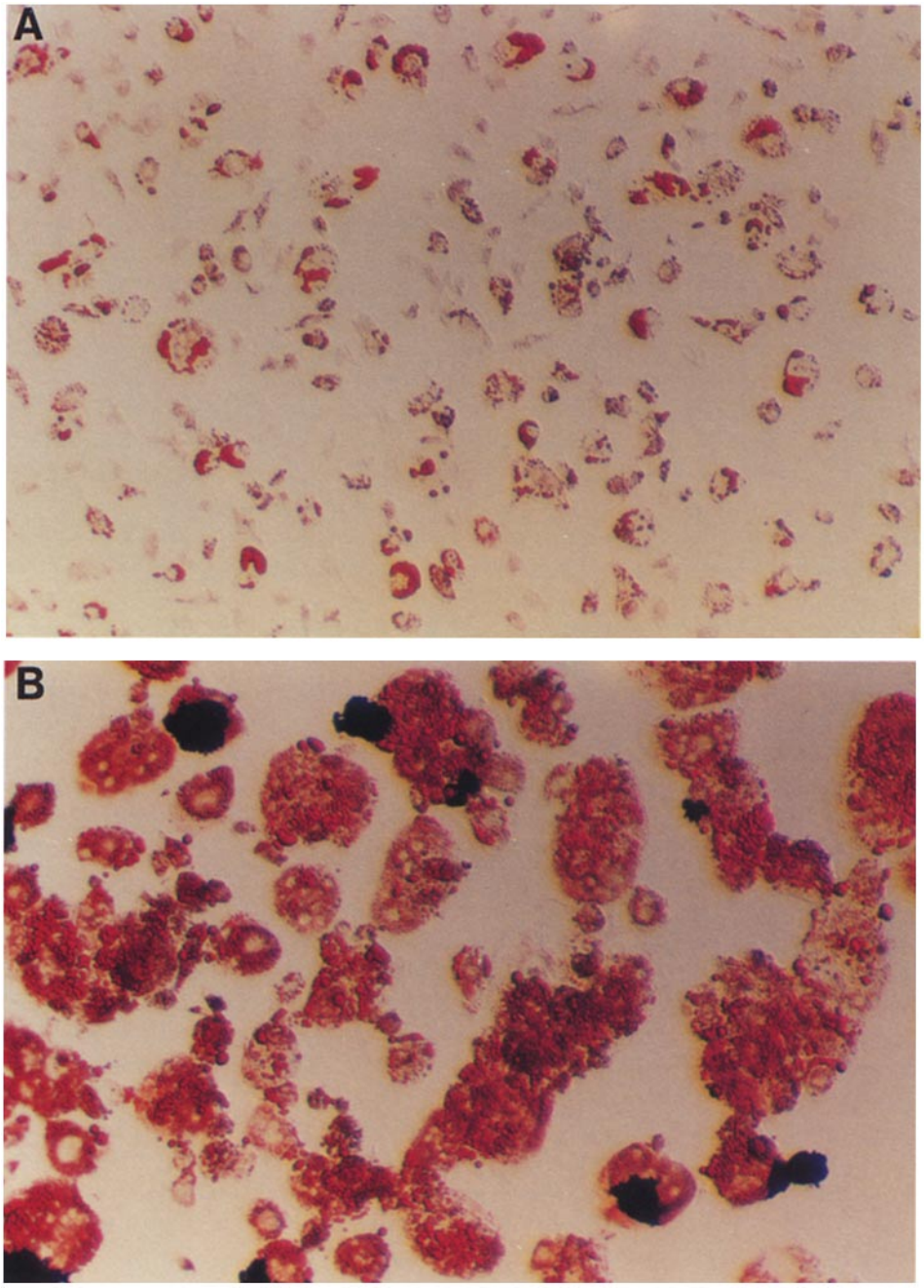

FIGURE 6. Light microscopy photographs of oil-red-O-stained macrophages. (*A*) Nonstimulated macrophages incubated for 20 h with 100 μg/ml of native LDL; (*B*) macrophages stimulated for 22 h with LDL-anti-LDL immune complexes and incubated for 20 h with 100 μg/ml of native LDL. The degree of magnification (×60) was identical in both *A* and *B*.

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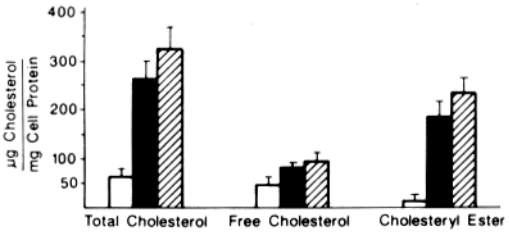

FIGURE 7. Cholesterol mass (µg/mg cell protein) in macrophages stimulated with LDL-anti-LDL immune complexes for 22 h (■) vs. 42 h (▨) as compared with nonstimulated controls (□). After a 22-h incubation with LDL-anti-LDL immune complexes, the cultures were washed to remove free immune complexes, followed by a 20-h incubation with 100 µg/ml of native LDL. To a parallel set of cultures, the native LDL was added for 20 h at the end of the first 22 h of stimulation without removing the LDL anti-LDL immune complexes. The values expressed are the means ± SEM for eight different experiments with triplicate observations in each experiment.

can be stimulated by LDL-IC to increase LDL uptake, the optimal stimulation by LDL-IC occurred after 3 d in culture with 30% WHS. At that time, the cultured monocytes have already acquired macrophage characteristics (49–51).

Discussion

The presence of large numbers of lipid-laden cells, also known as "foam cells" in the early stages of atheroma formation, has attracted the attention of many investigators. Given the early appearance of these cells in the lesion, it is logical to believe that some crucial steps in the pathogenesis of atherosclerosis may be associated with their formation. Most current evidence suggests that the large majority of foam cells in the atherosclerotic lesion are derived from monocytes/macrophages (52–55). The mechanisms leading to the transformation of monocytes/macrophages into foam cells have been the object of detailed studies. One of the best studied mechanisms involves the recognition of modified lipoproteins by the scavenger receptor (51–54). Recently, however, it has become apparent that macrophage activation as a conse-

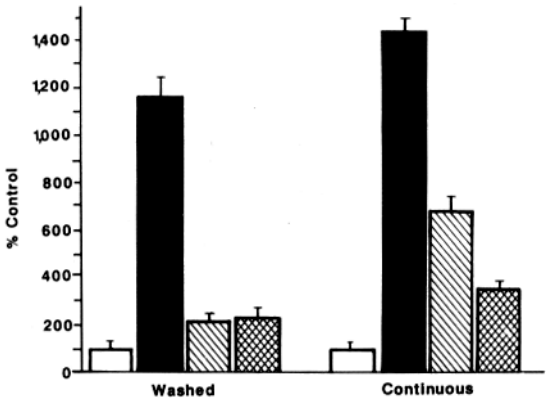

FIGURE 8. Receptor-mediated accumulation of ^{125}I-N-LDL (10 µg/ml) in macrophages stimulated with LDL anti-LDL immune complexes (■), VLDL anti-VLDL immune complexes (▨), and HDL anti-HDL immune complexes (▩) for 22 h before the addition of radiolabelled LDL. The values are expressed as a percentage of the receptor-mediated ^{125}I-N-LDL accumulation in nonstimulated cells (□) (0.620 ng accumulated per mg cell protein). The bars to the left represent the levels of ^{125}I-N-LDL accumulated in cultures washed to remove free immune complexes before the addition of radiolabeled LDL. The bars on the right represent levels of ^{125}I-N-LDL accumulated in cultures exposed to immune complexes throughout the entire length of the experiment. The values are the means ± SEM for three experiments with triplicate observations for each experimental parameter.

FIGURE 9. Cholesterol mass (µg/mg cell protein) in macrophages incubated for 20 h with 100 µg/ml native LDL after stimulation for 22 h with LDL-anti-LDL immune complexes (■), VLDL-anti-VLDL immune complexes (▨), and HDL-anti-HDL immune complexes (▧), compared with non-stimulated controls (□). The cultures were washed at the end of the 22-h stimulation period to remove free immune complexes. The values expressed are the means ± SEM for eight different experiments with triplicate observations in each experiment.

quence of interactions with microbial or immunological products may also result in lipid accumulation by monocytes/macrophages.

The J774 mouse macrophage-like tumor cell line shows excessive CE accumulation when the cells are exposed to N-LDL. It has been postulated that the J774s, due to their tumorigenic nature, may be either in an activated state or resistant to the specific cellular regulatory mechanisms that prevent intracellular cholesterol accumulation (26). Lopes-Virella et al. (25) have shown that human macrophages stimulated by microbial or microbial-related substances when exposed to native LDL had enhanced CE synthesis and accumulation. In contrast to the above studies, some investigators have shown that activation of macrophages may lead to inhibition of lipoprotein metabolism (56, 57). Therefore, various stimuli may affect macrophage lipoprotein metabolism quite differently. This is not surprising, since activation of macrophages is a multistep process requiring an interplay of signals to achieve full effector functions (13) and stimulation by various substances may activate different pathways. Stevenson et al. (58) have recently shown that human monocyte/macrophages can be differentially activated to distinct secretory functions by a variety of well-defined activation signals; this differential functional capability stems from activation signal- mediated gene transcription in some cases and post-transcriptional regulation mechanisms in others (58, 59). It is plausible, therefore, that different activating signals may enhance or repress metabolic processes in different ways.

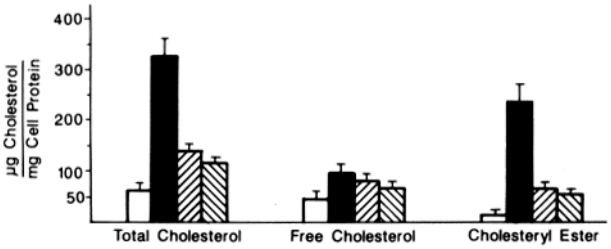

FIGURE 10. Cholesterol mass (µg/mg cell protein) in macrophages incubated with 100 µg/ml of native LDL after stimulation for 42 h with LDL-anti-LDL immune complexes (■), VLDL-anti-VLDL immune complexes (▨), and HDL-anti-HDL immune complex (▧) as compared with nonstimulated controls (□). The native LDL was added to the cultures at the end of the first 22 h stimulation; the immune complexes were not removed. Values expressed are the means ± SEM of eight different experiments with triplicate observations in each experiment.

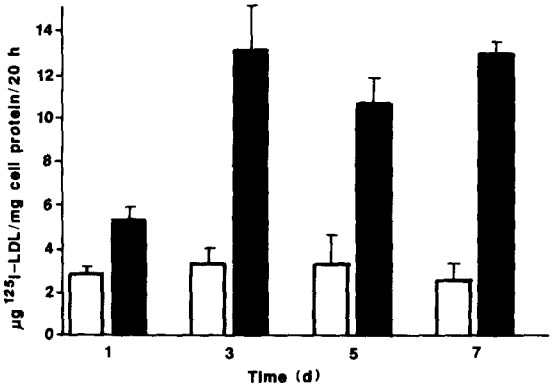

FIGURE 11. Receptor-mediated accumulation of ^{125}I-N-LDL (μg/ml) by nonstimulated (□) and LDL-anti-LDL immune complex stimulated cells (■) during monocyte-to-macrophage maturation. The cells were studied either immediately after isolation (monocytes) or after incubation in 30% WHS for 3, 5, and 7 d (macrophages). Each bar represents the mean ± SEM of three different experiments with triplicate observations in each experiment.

In the present study, we assessed the effect of macrophage activation by immune complexes on lipoprotein metabolism. Since in patients with clinical manifestations of coronary heart disease, increased levels of circulating immune complexes containing LDL have been reported (24, 60), we started by examining the effects of macrophage activation with LDL-anti-LDL IC. Our results show that stimulation of the cells with LDL-IC caused a marked increase in N-LDL uptake and degradation and led to intracellular CE accumulation and foam cell formation. Furthermore, we have shown that the enhanced uptake of LDL was due to an increase in LDL receptor number in stimulated cells, and that the binding affinity was similar in stimulated and nonstimulated cells.

Although the affinity of the LDL antibody is likely to be high since we obtained the antisera from hyperimmune rabbits, we could not rule out the possibility that during the washing procedure, IC bound to the cells could become free, dissociate, and the released antibody could form a new complex with the ^{125}I-N-LDL added to the system. If that happened, the increased uptake of ^{125}I-N-LDL seen after stimulation of the macrophages with LDL-IC could be due to the uptake of the new complexes either through the Fc receptor or through the scavenger receptor. The latter possibility would occur if the LDL-IC were recognized as modified LDL and thus taken up by the scavenger receptor. This hypothesis was excluded, since we have shown that acetylated LDL did not inhibit the binding of ^{125}I-labeled LDL-IC to the macrophages. The likelihood of increased N-LDL uptake through Fc receptors is also minimal since goat anti-rabbit [F(ab')$_2$] IgG did not inhibit ^{125}I-N-LDL uptake in stimulated cells. Furthermore, our experiments showing a marked inhibition of ^{125}I-N-LDL uptake in cells preincubated with an anti-LDL receptor antibody favors the hypothesis that an increase in LDL receptor number occurred in LDL-IC-stimulated macrophages.

Finally, the unlikely possibility that ^{125}I-LDL was modified by products released by the stimulated macrophages and taken up by the scavenger receptor was excluded since acetylated LDL did not inhibit the uptake of ^{125}I-N-LDL in stimulated cells.

A major question of this study was whether the ability of LDL-IC to stimulate macrophages and promote increased uptake and degradation of ^{125}I-N-LDL, along with increased CE accumulation, is specific for a certain type of IC or a general property of IC-stimulated macrophages. We studied the effects of several immune

complexes varying in antigen composition and size. Some of the IC were able to stimulate, although to a lesser degree, the uptake of native LDL by macrophages, but our data indicate that lipoprotein IC are more efficient and have the greatest capacity to increase uptake of N-LDL and CE accumulation. The ability to promote ^{125}I-N-LDL accumulation was enhanced if the ICs were present continuously throughout the culture. This was evident both in LDL-IC and in VLDL-IC-stimulated macrophages. Our results suggest that the type of antigen contained within the IC plays a role in the ICs ability to perturb intracellular regulatory mechanisms of lipoprotein metabolism. Therefore, the alteration of lipoprotein metabolism must be a consequence of mechanisms other than the general activation of macrophages by the interaction of IC with Fc receptors. Recently, Tabas and Boykow (61) have postulated the presence of a short-lived intracellular protein that inhibits ACAT-mediated cholesterol esterification in mouse peritoneal macrophages and that may be missing or inactivated in the J774 macrophages. It is possible that a similar phenomenon may occur in activated human macrophages such that stimulation or inhibition of ACAT-mediated cholesterol esterification may depend on the type of antigen contained in the IC. If the IC was to enhance ACAT activity, the intracellular-free cholesterol levels would decrease and lead to increased LDL receptor expression. Such alterations in macrophage physiology may contribute to CE accumulation and concomitant foam cell formation.

Two possible mechanisms, not mutually exclusive, may contribute to the increased intracellular CE accumulation. One, as mentioned above, involves the increased LDL uptake due to the enhancement in LDL receptor expression induced by LDL-IC cell activation. The other involves the uptake by Fc receptors of the LDL contained in the IC used to stimulate the macrophages with resulting intracellular accumulation. The later mechanism has been described by Klimov et al. (27), who demonstrated that mouse peritoneal macrophages stimulated with LDL-IC had a CE content 60 times the levels obtained in nonstimulated cells. The magnitude of CE accumulation in our studies is considerably less as we found only a 10–15-fold increase in the CE accumulation in stimulated cells vs. nonstimulated cells. This may be due to the length of incubation with the LDL-IC, as the mouse peritoneal macrophages were incubated for 72 h vs. our incubation times of 22–42 h or to differences between the metabolic behavior of human and murine macrophages.

Our studies show conclusively that the formation of foam cells is an active process, associated with several signficant functional changes. Given the wide range of effector functions of macrophages, it is likely that the activation caused by LDL-anti-LDL-IC will be reflected not only in lipid metabolism, but also in other functions. Among the general functions of macrophages whose activation may be significant in the pathogenesis of atherosclerosis are the release of proteases and collagenase, growth factors, and smooth muscle cell mitogens, all of which are particularly relevant. Preliminary data obtained in our laboratory suggests that macrophage-derived growth factor is released in increased amounts by macrophages incubated with LDL-anti-LDL-IC (data not shown).

The significance of our findings hinges heavily on the natural occurrence of anti-LDL antibodies. Such antibodies have been described in patients with atherosclerosis (24, 60) and LDL isolated from patients with CHD has been shown to be immunologically different from N-LDL. It is also known that the respiratory burst

of macrophages is associated with the release of reactive oxygen intermediates and other products (62–64) which have the potential of modifying LDL (65, 66). Such modifications, interestingly enough, are associated with increased immunogenicity and production of autoantibodies (67–69). Therefore, it could be postulated that macrophage activation by infectious or other stimuli could lead to the initial modification of LDL, and subsequently to the formation of antibodies to modified LDL, soluble IC involving either modified or native LDL could be formed (the antibodies to modified LDL would likely be crossreactive with native LDL). The formation of LDL-IC would promote foam cell formation and perpetuate the cycle of macrophage activation LDL modification, playing a significant role in the development and perpetuation of atherosclerotic lesions.

Summary

Human macrophages play a key role in atherogenesis and are believed to be the progenitors of the cholesteryl ester (CE)-laden foam cells present in early atherosclerotic lesions. Several mechanisms by which macrophages accumulate CE have been recently described. One involves a perturbation in LDL metabolism subsequent to macrophage activation. Thus, we decided to study the effect of macrophage activation by immune complexes on N-LDL metabolism. Initially, LDL-containing immune complexes (LDL-IC) were chosen, since increased plasma levels of these IC have been reported in patients with coronary heart disease. Human macrophages stimulated for 22 h with LDL-IC (250 μg/ml) and incubated afterwards for 20 h with 10 μg/ml ^{125}I-N-LDL showed a six- and fourfold increase in the accumulation and degradation, respectively, of ^{125}I-N-LDL over the values observed in nonstimulated cells. Scatchard analysis of ^{125}I-N-LDL-specific binding suggests an increase (20-fold) in the number of LDL receptors in macrophages stimulated with LDL-IC. We studied other immune complexes varying in size and antigen composition. Some of the IC were able to stimulate, although to a lesser degree, the uptake of N-LDL by macrophages. Lipoprotein IC are more efficient and have the greatest capacity to increase N-LDL uptake and CE accumulation. We conclude that human macrophage activation by LDL-IC leads to an increase in LDL receptor activity and promotes in vitro foam cell formation.

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