Lipoproteins of the extravascular space: enhanced macrophage degradation of low density lipoproteins from interstitial inflammatory fluid

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Abstract Current evidence has demonstrated that cholesteryl ester-loaded macrophages are important components of the atherosclerotic lesion. Additional studies have implicated low density lipoproteins (LDL) and circulating monocytes as central to the origin of lipid-laden foam cells found in the arterial wall. This is a result of the finding of accelerated macrophage uptake of LDL chemically modified by reaction with malondialdehyde (MDA-LDL), acetic anhydride (Ac-LDL), or incubation with arterial cells in vitro. In concert with these chemical modifications, we have previously demonstrated selective in vivo modification of LDL isolated from interstitial inflammatory fluid (IF) of the rabbit. Utilizing the polyvinyl sponge implant model, we reported that IF-LDL had an altered chemical composition, electrophoretic mobility, and particle size distribution when compared to LDL isolated from homologous plasma (WP-LDL). In this study reported herein, we examined the metabolism of IF-LDL by resident mouse peritoneal macrophages (MPM) in culture. IF-LDL was degraded substantially faster by MPM, and resulted in a substantial increase in cellular cholesteryl ester when compared to cells incubated with WP-LDL. IF-LDL binding to MPM was inhibited by Ac-LDL derived from WP-LDL, but only minimally by unmodified WP-LDL. Transmission electron microscopy of MPM revealed extensive lipid deposition in cells incubated with Ac-LDL and IF-LDL. These results implicate LDL from interstitial inflammatory fluid as an in vivo modified lipoprotein that can enhance uptake via the acetyl LDL receptor pathway in resident macrophages. —**Raymond, T. L., S. A. Reynolds, and J. A. Swanson.** Lipoproteins of the extravascular space: enhanced macrophage degradation of low density lipoproteins from interstitial inflammatory fluid. *J. Lipid Res.* 1985. **26:** 1356–1362.

Supplementary key words cholesteryl ester • rabbit • modified LDL

Accelerated uptake of low density lipoproteins (LDL) by monocyte/macrophages is thought to contribute to the development of lipid-laden foam cells in the atherosclerotic lesion. Several laboratories have demonstrated massive accumulation of cellular cholesteryl ester in human monocyte/macrophages and mouse peritoneal macrophages incubated with chemically modified LDL (1–3). This uptake is nonsaturable, and is mediated by a pathway that appears to have a specificity related to the increased electronegativity of these modified particles: the "scavenger pathway." This pathway is unrelated to cellular uptake by the normal receptor mechanism for apoplipoproteins B and E (4).

Additional studies by Henriksen, Mahoney, and Steinberg (5) reported a modification of LDL incubated with arterial endothelial cells in vitro (EC-LDL), which results in enhanced uptake by both peritoneal macrophages and the J774 macrophage cell line. More recently Steinbrecher et al. (6) demonstrated that these alterations in EC-LDL result from LDL phospholipid peroxidation during the incubation process. In contrast, Tabas, Weiland, and Tall (7) have reported enhanced uptake and degradation of unmodified plasma LDL when incubated with J774 macrophages, as compared to resident mouse cells. These same workers also reported that LDL did not undergo modification during the incubation period to a form recognized by the scavenger receptor.

We have reported selective modification of LDL isolated from interstitial inflammatory fluid of the rabbit (IF-LDL), (8). Utilizing the polyvinyl sponge implant model, we have described IF-LDL as having an altered chemical composition, increased anodal electrophoretic mobility, and particle size heterogeneity when compared to LDL derived from homologous plasma (WP-LDL). Additional studies with leukopenic animals have implicated cells of the inflammatory response as responsible for these observed changes in IF-LDL (9). In these studies, pretreatment with cyclophosphamide prevented modification of

Abbreviations: LDL, low density lipoprotein; TLC, thin-layer chromatography; GLC, gas-liquid chromatography; EC, endothelial cells; WP-LDL, homologous plasma LDL; IF, interstitial inflammatory fluid; MDA, malondialdehyde; MPM, mouse peritoneal macrophages; Ac-LDL, acetyl-LDL; PBS, phosphate-buffered saline.

LDL isolated from inflammatory fluid. In additional support of these findings, we have also described modification of radioiodinated plasma LDL implanted directly into the extravascular space (10). Plasma LDL recovered from inflammatory fluid had an electrophoretic mobility and density profile similar to IF-LDL.

In the present studies, we examined the metabolism of IF-LDL by resident murine macrophages, and compared these results with those of homologous unmodified LDL (WP-LDL) as well as chemically modified LDL (Ac-LDL).

MATERIALS AND METHODS

Preparation of interstitial inflammatory fluid

Adult male New Zealand White rabbits were housed individually in a constant temperature and humidity environment on a 12-hr light/dark schedule. All animals were fed standard laboratory diet (Wayne) and water ad libitum. Interstitial inflammatory fluid (IF) was obtained from polyvinyl sponges (Ivalon, Unipoint) implanted subcutaneously under aseptic conditions for 48 hr as previously described (8). Prior to sponge explantation, blood was obtained by cardiac exsanguination, with the rabbits under mild ether anethesia, and transferred to tubes containing disodium ethylene diamine tetraacetate (Na_2EDTA, 1 mg/ml).

Lipoprotein preparation

Interstitial inflammatory fluid was squeezed from sponges into sterile, plastic tubes containing Na_2EDTA (1 mg/ml final concentration). Cell-free supernatants of IF and homologous plasma were utilized immediately for lipoprotein preparation. LDL was isolated by sequential density flotation in the density range 1.019–1.063 g/ml as described by Havel, Eder, and Bragdon (11). Each fraction was subjected to a wash-spin at maximum density, and exhaustively dialyzed against 0.15 M NaCl, 1 mM EDTA, pH 8.6, at 4°C prior to further use. Acetyl LDL (Ac-LDL) was derived from plasma LDL (WP-LDL) as originally described by Basu et al. (12). Lipoproteins were electrophoresed according to the method of Noble (13), and stained overnight in Oil Red O–Fat Red 7B 1:1. Iodination of lipoproteins with ^{125}I (New England Nuclear) was performed in the presence of Iodo-gen (Pierce Laboratories) (14). Greater than 95% of radioactivity was precipitable with 10% trichloroacetic acid (TCA), and less than 5% of TCA-precipitable radioactivity was extractable into chloroform–methanol 2:1 (v/v).

Isolation of peritoneal macrophages

Resident peritoneal macrophages were obtained from female Swiss Webster mice (Camm) by sterile lavage with phosphate buffered saline, pH 7.4. Cells ($2–4 \times 10^6$) were plated onto Linbro Multiwell plates (Flow) in Hams F-10 media (Gibco) with gentamicin (50 μg/ml, Gibco) and fungizone (250 μg/ml, Gibco) for 2 hr at 37°C in a humidified air (5% CO_2) incubator. Nonadherent cells were removed and the cells were incubated overnight in F-10 with 10% FCS and antibiotics prior to addition of test media.

Uptake and degradation of lipoproteins

Radioiodinated lipoproteins (100–300 cpm/ng) were incubated with cells in F-10 for up to 24 hr. For degradation studies, media was collected and degradation products were measured as the non-iodide, TCA-soluble radioactivity in the media as described by Henriksen, Mahoney, and Steinberg (15). Cells were washed twice in phosphate-buffered saline and dissolved in 0.25 M NaOH for determination of protein. Degradation in the absence of cells was taken into account utilizing duplicate cell-free dishes. For mass uptake studies, cells were extracted with hexane-isopropanol 3:2 (v/v) according to the method of Goldstein, Basu, and Brown (16), and subjected to gas–liquid chromatography (GLC) before and after saponification for measurement of nonesterified and total cholesterol, respectively. [4-^{14}C]Cholesterol (New England Nuclear) was used as a recovery standard.

Chemical assays

Cholesterol was measured by gas–liquid chromatography on 3% QF-1 in a Hewlett/Packard 5830 chromatograph fitted with a flame ionization detector, utilizing 5 α-cholestane as internal standard (17). Protein was measured by the method of Lowry et al. (18). Neutral lipids were extracted from lipoproteins and mouse cells according to the method of Folch, Lees, and Sloane Stanley (19) and separated on silica gel G TLC plates developed in hexane–diethyl ether–glacial acetic acid 146:50:4. Cholesteryl ester fatty acids were transmethylated as previously described and analyzed by GLC (20). Lipid peroxidation was measured as thiobarbituric acid-reacting substances (T BARS) as described by Morel et al. (21).

Electron microscopy

Macrophages were incubated for 24 hr in the presence of IF-LDL, WP-LDL, and Ac-LDL at 200 μg/ml. Cells were washed twice with PBS and removed with a rubber policeman. Cells were fixed in 2.5% gluteraldehyde, post-fixed in 1.33% osmium tetroxide, and ultrathin sections were stained with uranyl acetate and lead citrate. A Phillips EM-201 microscope was used.

RESULTS

Electrophoresis on agarose gel of IF-LDL and Ac-LDL demonstrated increased anodal migration when compared to LDL from homologous plasma; however, the increase in electronegativity with acetylation of plasma LDL substantially exceeds that of LDL from inflammatory fluid (**Fig. 1**).

Degradation of IF-LDL and WP-LDL was compared by 24 hr incubation of radioiodinated preparations with resident mouse peritoneal macrophages in culture. IF-LDL was degraded at approximately a threefold faster rate than WP-LDL (**Fig. 2**). In order to examine the pathway of IF-LDL uptake, we incubated radiolabeled IF-LDL with peritoneal macrophages in the presence of excess unlabeled lipoproteins. These data are shown in **Fig. 3**. Acetylated LDL derived from rabbit plasma inhibited IF-LDL degradation by MPM, but only minimal inhibition of IF-LDL degradation was observed with up to a tenfold excess of plasma LDL isolated from either rabbits or human subjects. These results indicate that IF-LDL binding and uptake is primarily mediated by the acetyl LDL receptor.

Incubation of Ac-LDL with peritoneal macrophages causes a massive accumulation of cellular cholesteryl ester; whereas no increase is seen with WP-LDL, even with levels as high as 200 μg/ml. IF-LDL was observed to cause a buildup in cholesteryl ester of macrophages in culture only at levels exceeding 100 μg/ml (**Fig. 4**).

Macrophages incubated with lipoproteins at 200 μg/ml were examined further by transmission electron microscopy. Cells incubated with Ac-LDL demonstrated massive lipid deposition, and the cells took on the appearance of foam cells (**Fig. 5a**). IF-LDL also caused an apparent lipid buildup in these cells. IF-LDL-incubated cells demonstrated multiple lipid droplets and evidence of extensive

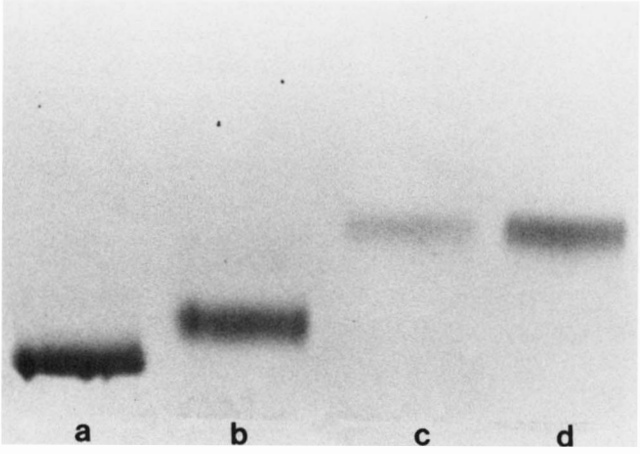

Fig. 1. Lipoprotein electrophoresis on 0.5% agarose gel of rabbit whole plasma LDL (a), inflammatory fluid LDL (b), rabbit acetylated LDL (c), and human acetylated LDL (d). Following electrophoresis, lipoproteins were fixed, dried, and stained overnight in Oil Red O, Fat Red 7B (1:1).

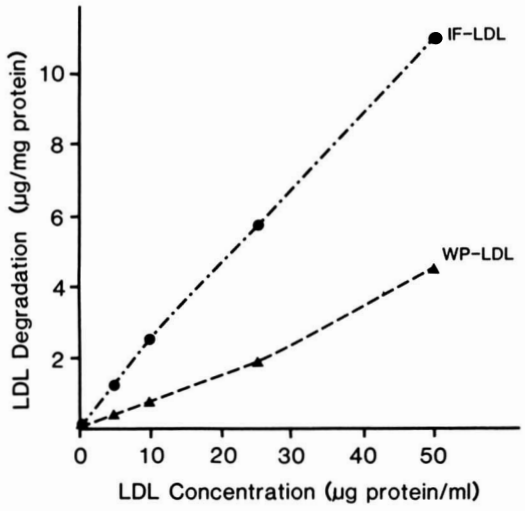

Fig. 2. Degradation of ^{125}I-labeled IF-LDL and WP-LDL by resident peritoneal macrophages. Cells were incubated with labeled lipoproteins (100–300 cpm/ng) at the indicated concentrations for 24 hr in serum-free F-10 media. Media was collected and degradation products were measured as outlined in Materials and Methods. Each value represents the average of three determinations. Duplicate dishes were utilized for assessment of degradation in the absence of cells.

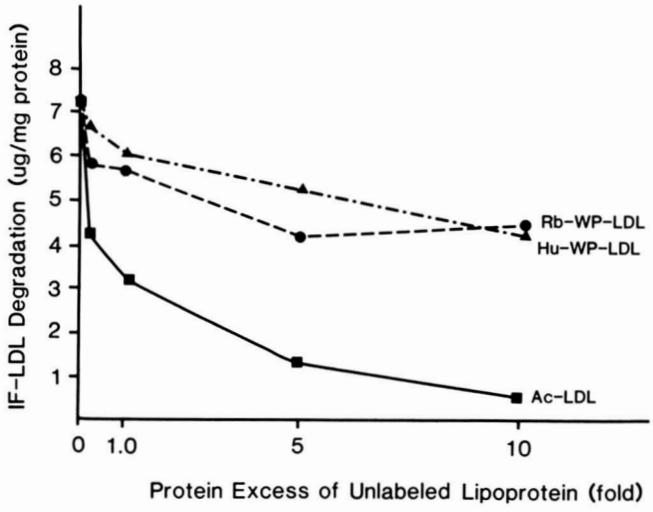

Fig. 3. The effect of increasing concentrations of unlabeled lipoproteins upon degradation of ^{125}I-labeled IF-LDL (10 μg/ml). Rabbit Ac-LDL or plasma LDL from rabbit (Rb-WP-LDL) or human subjects (Hu-WP-LDL) was added to media at the indicated concentrations. Cells were incubated for 24 hr, after which degradation products were measured. Each value represents the average of two determinations. Degradation in the absence of cells was taken into account (see legend, Fig. 2).

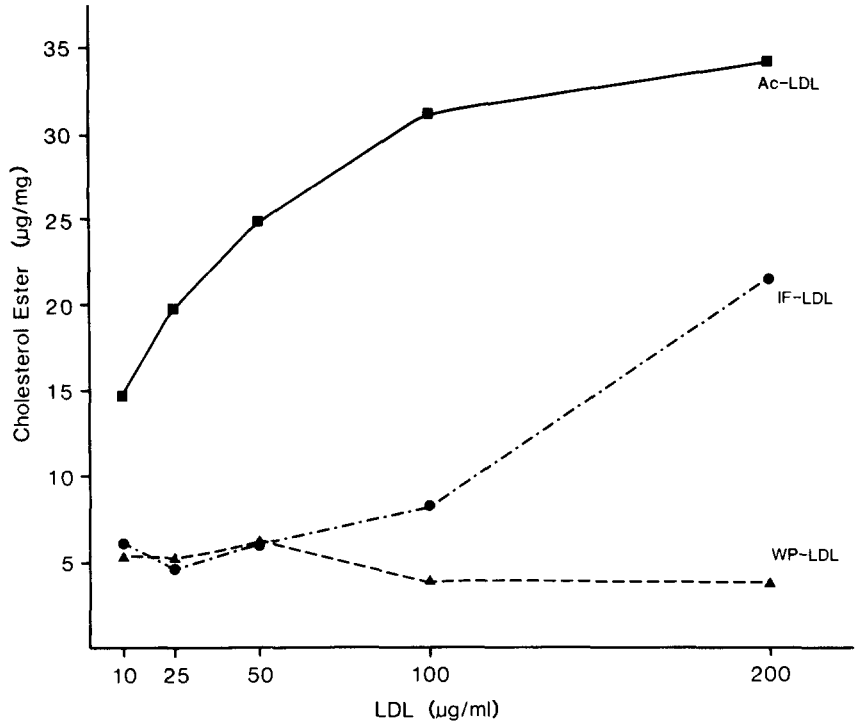

Fig. 4. Cholesteryl ester content of resident peritoneal macrophages incubated in the presence of inflammatory fluid LDL (IF-LDL), homologous plasma LDL (WP-LDL), or rabbit acetylated LDL (Ac-LDL). Cells were incubated with lipoprotein at the indicated concentrations for 24 hr, washed and measured for cholesteryl ester and protein content.

vacuole formation (Fig. 5b). Qualitative analysis of cholesteryl ester fatty acids in cells incubated with IF-LDL and Ac-LDL revealed a predominance of cholesteryl oleate (37.2 and 40.0%, respectively).

In order to examine the potential toxicity of these LDL fractions upon macrophages in culture, cells were incubated with each fraction for 24 hr at 200 µg/ml, washed with PBS, and tested for exclusion of 0.2% trypan blue. Although all of the LDL preparations provided somewhat less viability than 10% fetal bovine serum (99% viable), there were no appreciable differences between IF-LDL (89.2 ± 3.7%, $\bar{X}$ + SD), WP-LDL (86.5 ± 4.1%), and Ac-LDL (88.8 ± 4.5%). In addition, all three LDL preparations were found to be free of lipid peroxides, measured as malondialdehyde equivalents (data not shown).

DISCUSSION

In this study we have demonstrated that LDL can be modified in vivo to a form that causes accelerated uptake by resident peritoneal macrophages in culture with extensive lipid deposition. The results of this in vivo modification appear to be similar to results obtained with LDL modified in the laboratory by reaction with acetic anhydride or malondialdehyde (1, 2).

The common link between all reported forms of modified LDL taken up primarily by the scavenger receptor, appears to be a nonspecific increase in electronegativity of the particle, observed as increased migration on agarose gel. The amount of increase in electrophoretic mobility observed is quite variable between these modified lipoproteins. Interestingly, Smith and Ashall (22) have reported an increased net negative charge of LDL recovered from interstitial fluid of normal arterial intima.

The biological modification of LDL produced by incubation with arterial endothelial cells in vitro has been shown recently by Steinbrecher et al. (6) to be a result of autooxidation of LDL phospholipid fatty acids. In contrast to these findings, we were unable to demonstrate lipid peroxidation of IF-LDL, measured as MDA equivalents, thus ruling out this mechanism for the observed in vivo modification of IF-LDL.

The increase in cellular cholesteryl ester and the observed neutral lipid deposition in macrophages incubated

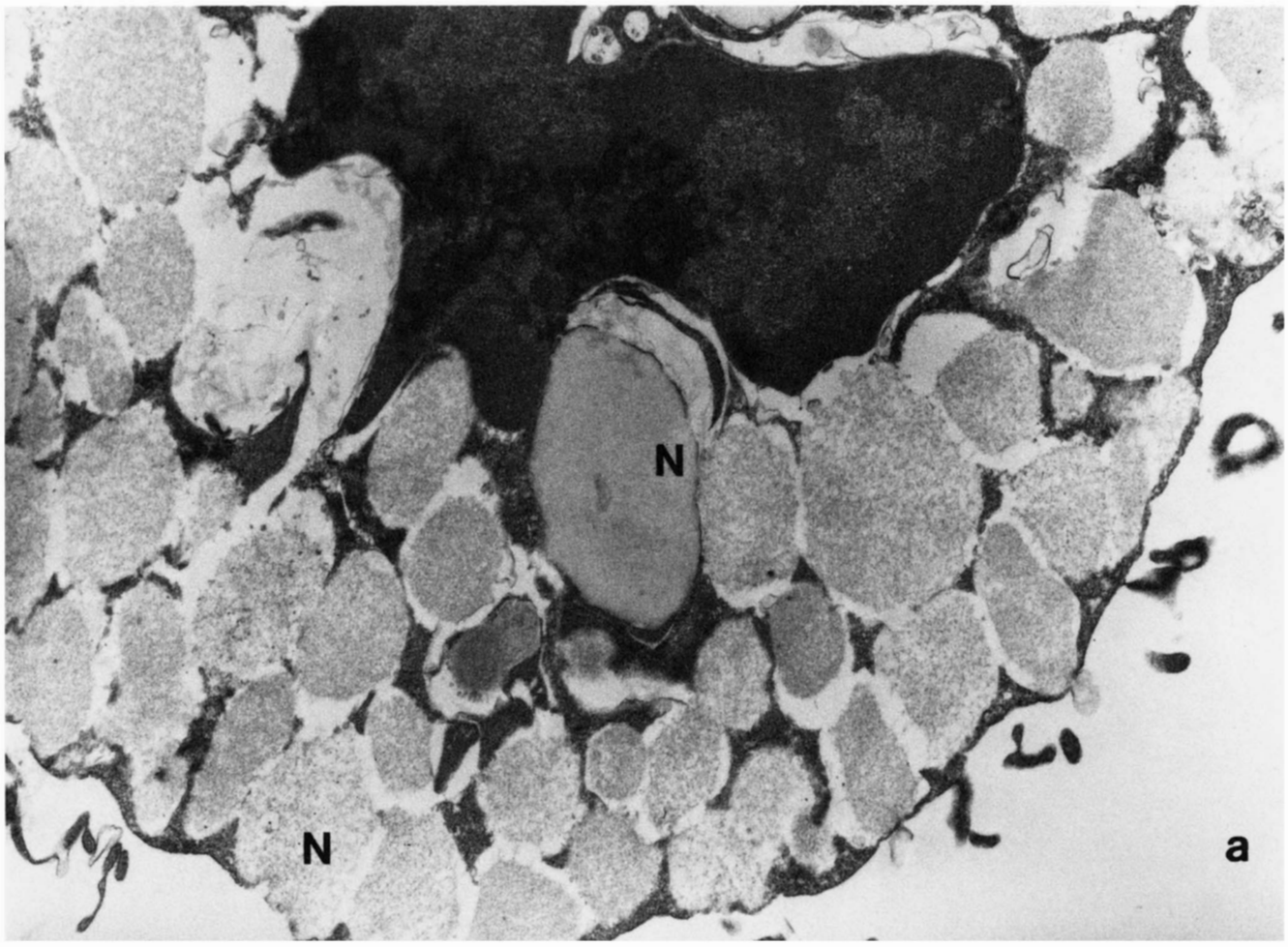

Fig. 5. Transmission electron microscopy of resident peritoneal macrophages incubated with Ac-LDL (a), and IF-LDL (b) for 24 hr at 200 μg/ml. N, neutral lipid droplets; F, flocculant material in vacuoles. Magnification: a, 22,000×; b, 10,230×.

with IF-LDL is less severe than the effect observed with equivalent amounts of rabbit Ac-LDL. Furthermore, this increase was not apparent below 100 μg/ml IF-LDL concentration in the medium, whereas these effects are easily observed in cells incubated with Ac-LDL at 10 μg/ml or less (1). This finding may be due, in part, to our previous observation of the reduced cholesterol content of IF-LDL (8).

In addition, these results suggest that only a portion of IF-LDL is suitably modified to cause enhanced uptake, degradation, and neutral lipid deposition in these cells. This conclusion is supported by our recent findings on the plasma decay of iodinated IF-LDL in recipient rabbits (23). In these studies, only 51.5% of IF-LDL was detected in plasma at 10 min following intravenous injection. In contrast, we observed over 92% of radioactivity remaining in plasma in rabbits receiving WP-LDL at this time point. Subsequent decay rates for WP-LDL and IF-LDL were quite similar, suggesting that approximately one-half of IF-LDL is actually modified and subjected to rapid removal by hepatic sinusoidal epithelial cells, as has been described for Ac-LDL following intravenous injection into rats and guinea pigs (24).

Considerable evidence has demonstrated the binding of LDL to connective tissue elements of the arterial wall, which may hold an important clue to the initiation of the atherosclerotic process (25). Falcone et al. (26) have reported increased macrophage uptake of LDL complexed with heparin, fibronectin, and denatured collagen. However, this uptake did not result in increased lipid deposition. It is attractive to postulate that LDL may complex in vivo with these macromolecules in the extravascular space, which may catalyze this uptake. Unfortunately, insoluble complexes are often formed by binding LDL to

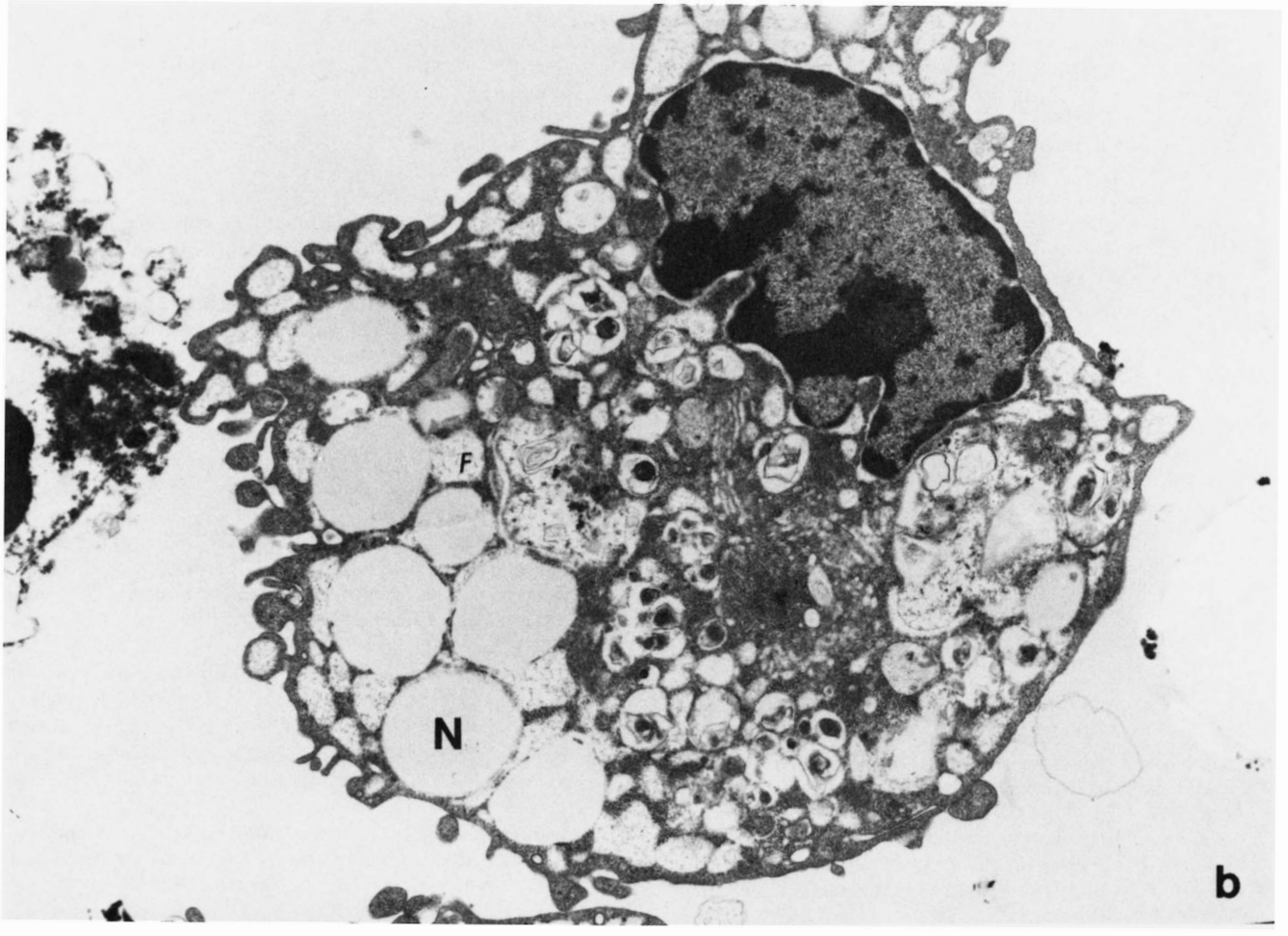

Fig. 5. (*Continued*)

these compounds in vitro (27). If connective tissue elements are bound to IF-LDL, it would be unlikely that these complexes would float in the ultracentrifuge at d 1.063 g/ml. One could postulate that, once into the extravascular space, LDL could interact reversibly with interstitial proteoglycans to cause modification and thus enhanced uptake by scavenger cell systems. Studies are currently under way to explore the possible in vivo association of IF-LDL with these compounds, and subsequent interaction with the peritoneal macrophage in culture.

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