Lipoproteins of the extravascular space: alterations in low density lipoproteins of interstitial inflammatory fluid[1]

Thomas L. Raymond and Scott A. Reynolds

Department of Internal Medicine, Quillen-Dishner College of Medicine, East Tennessee State University, Johnson City, TN 37614

Abstract Although extrahepatic degradation of low density lipoproteins (LDL) by peripheral cells is considered to be a significant component of daily cholesterol homeostasis, the nature of lipoproteins in the extravascular space has not been well described. Using a sponge implantation model in the rabbit, we examined lipoproteins prepared from interstitial inflammatory fluid. Inflammatory fluid cholesterol is correlated with plasma values, ($r = 0.735, P < 0.01$), but triglyceride values are not. Examination of inflammatory fluid lipoproteins by agarose gel electrophoresis, column chromatography, and density gradient centrifugation revealed a marked reduction in LDL concentration as compared to plasma LDL. Inflammatory fluid low density lipoproteins prepared by sequential density flotation had a larger mean diameter, they were erratic in shape, and contained more triglyceride and less cholesterol and cholesteryl ester than plasma LDL. Total cholesterol to protein ratio was significantly reduced in inflammatory fluid LDL (0.73 vs. 1.10, $P < 0.05$). Inflammatory fluid LDL migrated further than plasma LDL on agarose electrophoresis, despite similar apoprotein patterns. These data concur with findings of altered composition and electrophoretic mobility of plasma LDL modified in vitro by exposure to acetylating agents, malondialdehyde, or aortic cells in culture, and they may represent the actual form of LDL in the extravascular space.—**Raymond, T. L., and S. A. Reynolds.** Lipoproteins of the extravascular space: alterations in low density lipoproteins of interstitial inflammatory fluid. *J. Lipid Res.* 1983. **24:** 113–119.

Supplementary key words sponge implantation • rabbits.

The nature and appearance of lipoproteins in the extravascular space has not been well described. The findings of considerable degradation of low density lipoprotein (LDL) in hepatectomized animals (1), and the discovery of the LDL receptor pathway in skin fibroblasts and peripheral smooth muscle cells (2), point to the importance of ascertaining the nature of the lipoproteins available to these extravascular tissues. Recently, Fogelman et al. (3) and Henriksen, Mahoney, and Steinberg (4) have reported enhanced uptake of LDL by mononuclear macrophages following in vitro modification of LDL by treatment with malondialdehyde (MDA) or exposure to vascular endothelial cells, respectively.

There are few reports currently in the literature in which interstitial tissue fluid lipoproteins have been characterized both chemically and morphologically. The appearance of individual lipoprotein constituents has been reported in man for synovial fluid, peripheral lymph, ascitic fluid, and pleural effusions (5–8). Lipoproteins from peritoneal fluid of rabbits and pericardial lymph of dogs and swine have also been partially characterized (9, 10). Hoff and Gaubatz (11) as well as Hollander, Paddock, and Colombo (12) have recently reported isolation of lipoproteins retrieved from human atherosclerotic lesions. In this report we describe alterations in the spectrum of interstitial inflammatory fluid lipoproteins obtained by the polyvinyl sponge implant model in the rabbit.

MATERIALS AND METHODS

Seventy-five adult male New Zealand White rabbits housed individually and fed standard laboratory chow (Wayne, Chicago, IL) and water ad libitum were used in these experiments. The environment provided a 12-hour light/dark schedule as well as constant temperature and humidity.

Preparation of interstitial inflammatory fluid from subcutaneously implanted sponges has been previously published (13, 14). Briefly, polyvinyl sponge sheets (Unipoint, High Point, NC) of uniform thickness and size were exhaustively washed, dried, weighed, and frozen at −70°C prior to use. Prior to implantation,

Abbreviations: MDA, malondialdehyde; LDL, low density lipoproteins.

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sponges were sterilized in boiling water, transferred to sterile 0.9% saline, and squeezed to remove excess liquid. Twelve to 30 sponges were aseptically implanted into the subcutaneous areolar tissue across the dorsal surface of the animal's back through a 2-cm incision in the skin. Bleeding was prevented by use of electrocautery (Birtcher, Los Angeles, CA). Implantation was performed under intravenous pentobarbital sodium (Abbott) anesthesia, after which the animals were returned to their cages for 8–72 hr. At explantation the animals were exsanguinated by cardiac puncture under mild ether anesthesia. Sponges were removed under sterile conditions and transferred to 50-ml plastic tubes.

Interstitial inflammatory fluid was squeezed from the sponges in a second sterile plastic tube containing disodium ethylenediamine tetraacetate, (Na_2EDTA) to a final concentration of 1 mg/ml. Aerobic contamination was monitored by culturing each fluid on 5% sheep blood agar plates. Total cell and leukocyte counts were performed on a model ZBI Coulter counter (Coulter Electronics, Hialeah, FL). Differential counts were obtained from whole blood and inflammatory fluid smears stained with 0.4% Wright's solution. Fluids were centrifuged at 100 *g* for 10 min. The cell-free supernatant was then used for chemical analysis and lipoprotein preparation. Any fluids demonstrating aerobic bacterial contamination or bleeding into the extravascular space were discarded.

Distribution of lipoproteins according to particle size was measured by agarose column chromatography according to the method of Rudel et al. (15) as modified in our laboratory (16). Density profiles of lipoprotein distribution in plasma and inflammatory fluid were obtained by density gradient centrifugation of the d < 1.225 g/ml supernatants (15). Supernatants containing equal amounts of lipoprotein were overlayed with d 1.006 g/ml solution and centrifuged for 18 hr in a SW-41 rotor (Beckman, Fullerton, CA). The contents of the tube were eluted with a dense salt solution and monitored at 280 nm. The density of each fraction was obtained and the presence of apolipoprotein B was monitored by double immunodiffusion against anti-rabbit LDL. Antisera were prepared in our laboratory by inoculation of FP strain chickens with purified rabbit LDL derived from plasma. Low density lipoproteins (d 1.019–1.063 g/ml) were prepared by sequential density flotation according to the method of Havel, Eder, and Bragdon (17) in a L5-75B ultracentrifuge (Beckman) modified to allow operation without braking. Each fraction was subjected to an additional wash-spin and exhaustively dialyzed against 0.15 M NaCl, 1 mM Na_2EDTA, pH 8.6, at 4°C prior to analysis. Lipoprotein electrophoresis was performed according to the method of Noble (18).

All solvents were purchased as reagent grade and were redistilled in glass and checked by gas–liquid chromatography prior to use. Protein was measured by the method of Lowry et al. (19) as modified for lipoproteins by Kashyap, Hynd, and Robinson (20). Cholesterol was measured as the trimethyl silyl ether using 5-α cholestane as the internal standard in a Hewlett-Packard gas chromatograph (21). Triglyceride was measured by the method of Kessler and Lederer (22). Phosphorus was measured according to the method of Bartlett (23). Free to esterified cholesterol was determined following extraction of total lipids as described by Folch, Lees, and Sloane Stanley (24) and thin-layer chromatography of neutral lipids on Silica Gel G (Analtech, Newark, DE) in hexane–diethyl ether–acetic acid 146:50:4 (v/v/v). Lipoproteins were delipidated in ethanol–peroxide-free ether as described by Scanu and Edelstein (25) and electrophoresed in 7.5% polyacrylamide gels containing 8 M urea (26). Stained gels were scanned at 640 nm in a Gilford 250 spectrophotometer and apoproteins were quantitated by analytical weighing of chart paper areas. For electron microscopy, purified LDL fractions (d 1.019–1.063 g/ml) were overlayed on Formvar-coated grids and negatively stained with 1% phosphotungstic acid, pH 7.0 (27). A Phillips EM-201 microscope was used.

In order to examine possible modification of lipoproteins by the polyvinyl sponge, whole plasma was incubated at both 4° and 37°C under sterile conditions with polyvinyl sponges. Ten sponges, weighing 150–180 mg each were incubated with 15 ml of plasma to approximate the normal inflammatory fluid yield from sponge implants. LDL was prepared from sponge-incubated plasma, as well as from nonsponge-incubated (time control) and preincubated plasma. These LDL preparations were subjected to compositional, morphological, and electrophoretic analysis.

RESULTS

We have examined lipoproteins prepared from interstitial inflammatory fluid contained in sponges explanted at 8–72 hr after sponge implantation. Data presented herein are from lipoproteins prepared from 48-hr fluid. At the time of sponge implantation, cell-free supernatants of interstitial inflammatory fluid from four to six animals were pooled for lipoprotein analysis. Similar pools were prepared from autologous EDTA-plasma obtained at the time of exsanguination.

In these fluids, erythrocyte (1.81×10^6/ml) and leukocyte (2.74×10^6/ml) counts averaged 0.06 and 15%, respectively, of whole blood values. Differential analysis of inflammatory fluid low-speed cell pellets revealed an infiltration of 44–46% monocyte-macrophages with the

remainder of the cells consisting primarily of neutrophils and occasional lymphocytes (14).

Total cholesterol concentration in interstitial inflammatory fluid was 35–40% of plasma (16.78 ± 1.90 vs. 45.95 ± 3.31 mg/dl; mean ± SEM, $P < 0.01$), and a positive correlation was observed ($r = 0.735$, $P < 0.01$). Total protein of inflammatory fluid was in a similar ratio to total plasma protein (34.28 ± 1.61 vs. 63.07 ± 1.88 mg/dl; $P < 0.001$), but no relationship was observed. Inflammatory fluid triglyceride was only 5 to 15% of plasma and was not correlated with plasma values (17.86 ± 4.13 vs. 88.27 ± 8.38 mg/dl; $P < 0.001$). The ratio of free to esterified cholesterol was the same between inflammatory fluid and plasma, with 65–70% of cholesterol existing in the esterified form.

Initial examination of interstitial inflammatory fluid by agarose gel electrophoresis revealed an apparent absence of beta-migrating lipoproteins (**Fig. 1**). This finding was consistent for all fluids tested. Subsequently we examined lipoprotein particle size distribution by agarose column chromatography of ultracentrifugally floated lipoproteins. Representative chromatograms are shown in Fig. 1. Analysis of interstitial inflammatory fluid (**Fig. 2**) revealed an apparent increase in region I and II lipoproteins (VLDL and IDL) with a concomitant reduction in region III particles (LDL). The actual net increase in region I and II particles expressed as cholesterol is substantially less than apparent on the chromatogram due to light-scattering properties of these large macromolecules. Elution region I and II combined, region III, and region IV (HDL) contributed 23.2, 21.4, and 55.4%, respectively, of interstitial inflammatory fluid cholesterol as compared to 17.2, 45.0, and 37.8% for these same fractions isolated from autologous plasma.

Density gradient centrifugation of d < 1.225 g/ml lipoproteins from plasma and inflammatory fluid demonstrated alterations in the low density particle range (**Fig. 3**). Apolipoprotein B was detectable by immunodiffusion in fraction 19 of inflammatory fluid (d 1.070 g/ml) as compared to an upper limit of fraction 17 for plasma (d 1.064 g/ml).

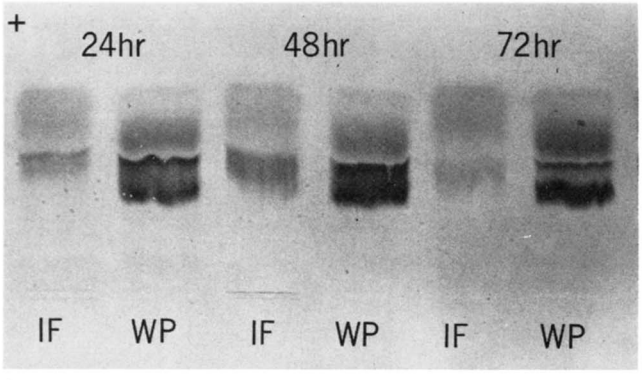

Fig. 1. Lipoprotein electrophoresis on 0.5% agarose gel of whole plasma (WP) and interstitial inflammatory fluid (IF) from three adult male rabbits from which polyvinyl sponges were explanted at 24, 48, and 72 hr, respectively. Following electrophoresis, lipoproteins were fixed, dried, and stained overnight in oil red-O, fat red 7B (1:1).

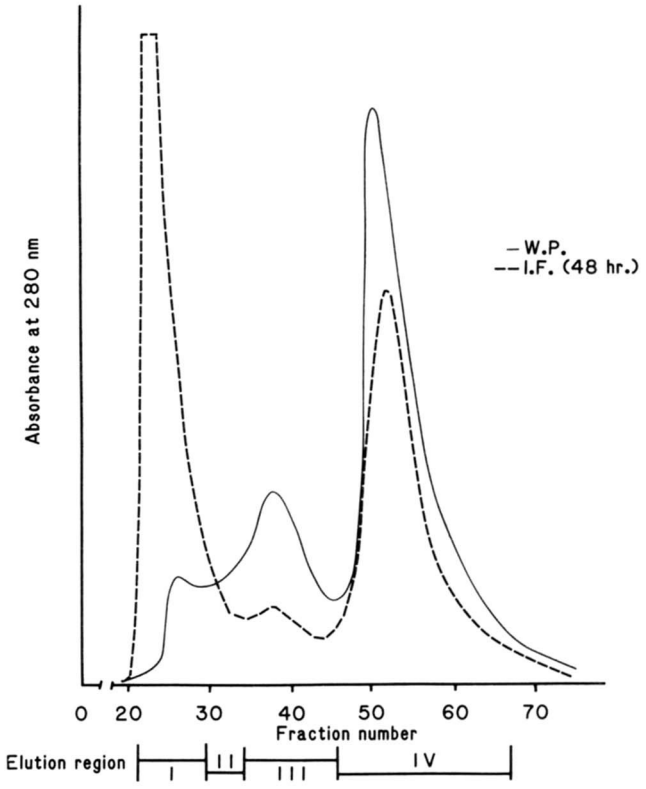

Fig. 2. Agarose column chromatography of ultracentrifugally floated lipoproteins from whole plasma (WP) and inflammatory fluid (IF) obtained at 48 hr after polyvinyl sponge implantation. Lipoproteins were initially floated at d 1.225 g/ml in the SW-41 rotor at 40,000 rpm for 40 hr at 15°C. The top 1.5 cm of the tube was sliced and lipoproteins were applied to a 2.6 × 60 cm column containing Bio-Gel A-5M in 0.1 M NaCl, 0.3 M K_2HPO_4, 0.01% Na_2EDTA, pH 8.0. Amount of lipoprotein applied to the column (5–15 mg), AUFS setting, and flow rate were identical for WP and IF.

We then embarked on a compositional and morphological examination of low density lipoproteins prepared from inflammatory fluid and plasma by sequential density ultracentrifugation. LDL was prepared in the density range 1.019–1.063 g/ml. Each fraction was subjected to an additional wash-spin, and exhaustively dialyzed prior to analysis. Compositional analysis of plasma and inflammatory fluid LDL is presented in **Fig. 4.** Inflammatory fluid LDL contained less total cholesterol (14.2 vs. 34.3%) and a concomitant increase in triglyceride (34.3 vs. 16.2%). Total protein remained relatively constant (22.5 vs. 21.8%), which resulted in a significant reduction in total cholesterol to protein ratio (0.73 ± 0.17 vs. 1.10 ± 0.4, $P < 0.05$). Both free and esterified cholesterol were reduced in inflammatory fluid LDL. However, the reduction in total cholesterol

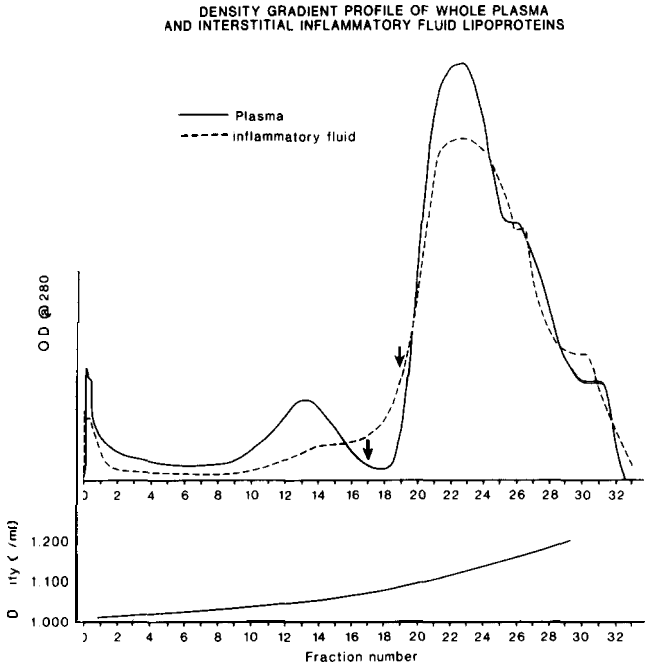

Fig. 3. Density gradient centrifugation of d 1.225 g/ml lipoproteins from plasma (——) and inflammatory fluid (– – –). Equal amounts of lipoprotein were overlayed with d 1.006 g/ml solution and centrifuged for 18 hr at 172,000 *g*. Arrows indicate upper density limit of apoprotein B detectable by double immunodiffusion.

was primarily due to the 4-fold reduction in cholesteryl ester (5.8 vs. 22.3%).

Polyacrylamide electrophoresis in 8 M urea of delipidated LDL revealed similar patterns (**Fig. 5**). Quantitation of individual apoproteins by weighing the area under the curve of scanned gels revealed 91 and 86% total protein in apoprotein B for plasma and inflammatory fluid LDL, respectively. Despite these similarities, inflammatory fluid LDL migrated further than plasma LDL on agarose electrophoresis (**Fig. 6**).

Morphological examination of concentrated LDL by negative staining and transmission electron microscopy revealed heterogeneity in inflammatory fluid LDL as compared to plasma (**Fig. 7**). Mean particle size was larger for inflammatory fluid LDL (34.2 ± 12.3 vs. 21.4 ± 3.2 nm) (**Fig. 8**). Almost all of plasma LDL was found between 20 and 30 nm while the majority of inflammatory fluid was found between 20 and 50 nm. Also noteworthy is the erratic shape of many inflammatory fluid particles, which may reflect compositional changes in free and esterified cholesterol with respect to protein and phospholipid.

In an attempt to create artifactual changes in lipoproteins by the sponge model, LDL was prepared from plasma incubated with polyvinyl sponges at both 4° and 37°C for 48 hr under aseptic conditions. No alterations were observed in chemical composition, electrophoretic mobility or morphology of LDL isolated from plasma incubated with polyvinyl sponges for up to 48 hr in vitro.

DISCUSSION

There is increasing evidence that lipoproteins may react under local stimulus with circulating aggregating agents to cause enhanced uptake of modified lipoproteins by mononuclear macrophages, which in turn may initiate the formation of foam cells in the arterial wall (28). Accelerated uptake of LDL modified in vitro by acetylation or reaction with malondialdehyde has been recently demonstrated (3, 29). Until this time there was no in vivo evidence for similar modification of LDL which might enhance removal by scavenger cells. We have now demonstrated in this study marked alteration in LDL of interstitial tissue fluid obtained in conjunction with an inflammatory response in the rabbit.

Our initial findings of marked reduction in inflammatory fluid LDL concentration as compared to plasma is in agreement with investigations of Reichl et al. (6) on apolipoprotein B in human leg lymph and the earlier studies of human synovial fluid by Small, Cohen, and Schmid (5). Reichl, Myant, and Pflug (30) have further demonstrated the appearance of apoplipoprotein B in d 1.063 g/ml infranatant from lymph. The authors postulated that LDL may indeed be modified in transit to the extravascular space (30). Concomitantly, Henriksen et al. (4) demonstrated an increase in the hydrated density of LDL incubated with rabbit aortic endothelial cells. Our finding of detectable apolipoprotein B at approximately d 1.070 g/ml in inflammatory fluid density gradient profiles is in agreement with these findings.

The observed electrophoretic migration of inflam-

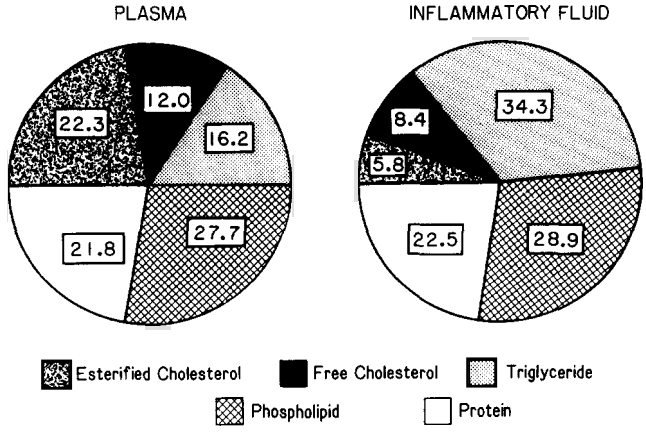

Fig. 4. Weight composition of low density lipoproteins prepared from plasma and interstitial inflammatory fluid by sequential density flotation. Numbers in boxes represent percentage by weight.

matory fluid LDL on agarose gel is not explained by total LDL protein or alterations in apolipoprotein patterns. It is interesting to note a similar change in electrophoretic mobility for malondialdehyde-LDL (3). In addition, the recent report of Henriksen et al. (4) for LDL harvested from media exposed to rabbit aortic endothelial cells showed a similar electrophoretic change. In addition, we were unable to demonstrate morphologic, compositional, or electrophoretic changes in plasma LDL by direct incubation with polyvinyl sponges for up to 48 hr at both 4° and 37°C, ruling out artifactual changes from sponge implants.

The altered morphology of inflammatory fluid LDL remains unexplained. Artifactual changes are unlikely due to similar treatment of LDL prepared from homologous plasma pools. The presence of naturally occurring larger particles, such as VLDL or VLDL remnants, is also unlikely due to the absence of C, D, and E peptides as observed by polyacrylamide electrophoresis (Fig. 5). Aggregation of small particles to create the observed large, erratically shaped particles in the LDL density range cannot be ruled out and may be a direct result of the observed changes in chemical composition (Fig. 4). For example, changes in total cholesterol to protein and cholesterol to phospholipid ratios in erythrocytes cause dramatic shape changes, such as the appearance of acanthocytes in familial abetalipoproteinemia (31).

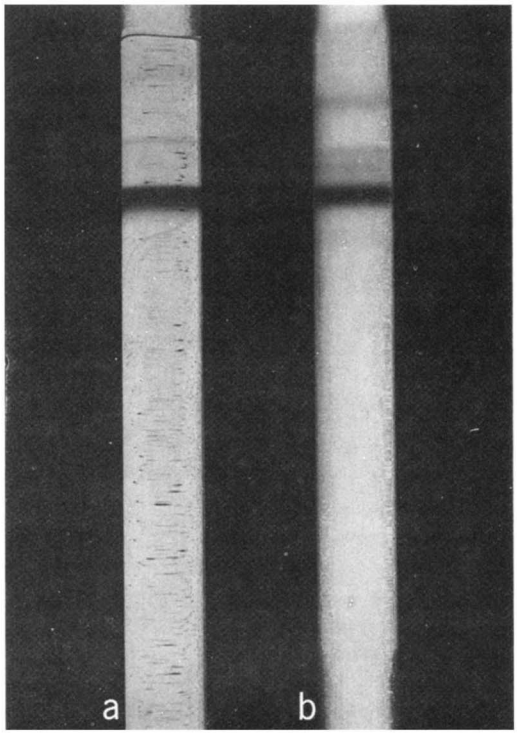

Fig. 5. Polyacrylamide electrophoresis in 8 M urea of delipidated low density lipoproteins prepared by sequential density flotation from whole plasma (a) and interstitial inflammatory fluid (b).

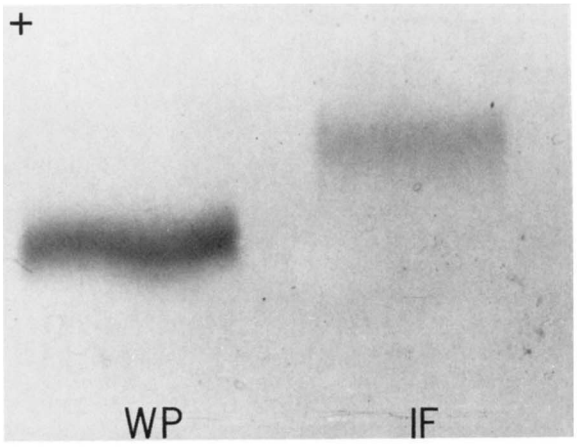

Fig. 6. Electrophoresis on 0.5% agarose gel of LDL prepared by sequential density flotation (d 1.019–1.063 g/ml) from whole plasma (WP) and interstitial inflammatory fluid (IF).

Compositional changes observed in low density lipoproteins from inflammatory fluid are similar to those described by Hollander et al. (12) for LDL retrieved from human atherosclerotic lesions. These workers reported particles in the 1.019–1.063 g/ml density range with only 45% of total sterol as cholesteryl ester as compared to 78% for plasma controls, a slight increase in triglyceride, and no change in phospholipid or protein content. They additionally described amino acid compositional changes, which have not been investigated in this study. Stein, Halperin, and Stein (32) recently reported substantial increases in percent free cholesterol (26 to 70% total sterol) in acetylated LDL incubated with mouse peritoneal macrophages.

The in vivo role of the scavenger pathway for uptake of LDL by monocyte-macrophages is yet to be examined. Fogelman et al. (3) have demonstrated the ability of these cells to bind LDL by both the apoprotein B receptor pathway and the scavenger route. Both native LDL and LDL modified with MDA are taken up, but only MDA-LDL results in accumulation of cholesteryl ester in the macrophage cytoplasm. The authors propose this finding to represent a pathway of foam cell formation stimulation by local injury, whereupon arachidonic acid is mobilized to thromboxane A_2 accompanied by an equimolar release of malondialdehyde. Our recent finding of an altered distribution of circulating phospholipids and a complete absence of arachidonic acid in inflammatory fluid phospholipids lends potential support to this hypothesis for the observed changes in inflammatory fluid LDL (33). One could postulate that enhanced production of thromboxanes and prostaglandins in response to sponge implantation

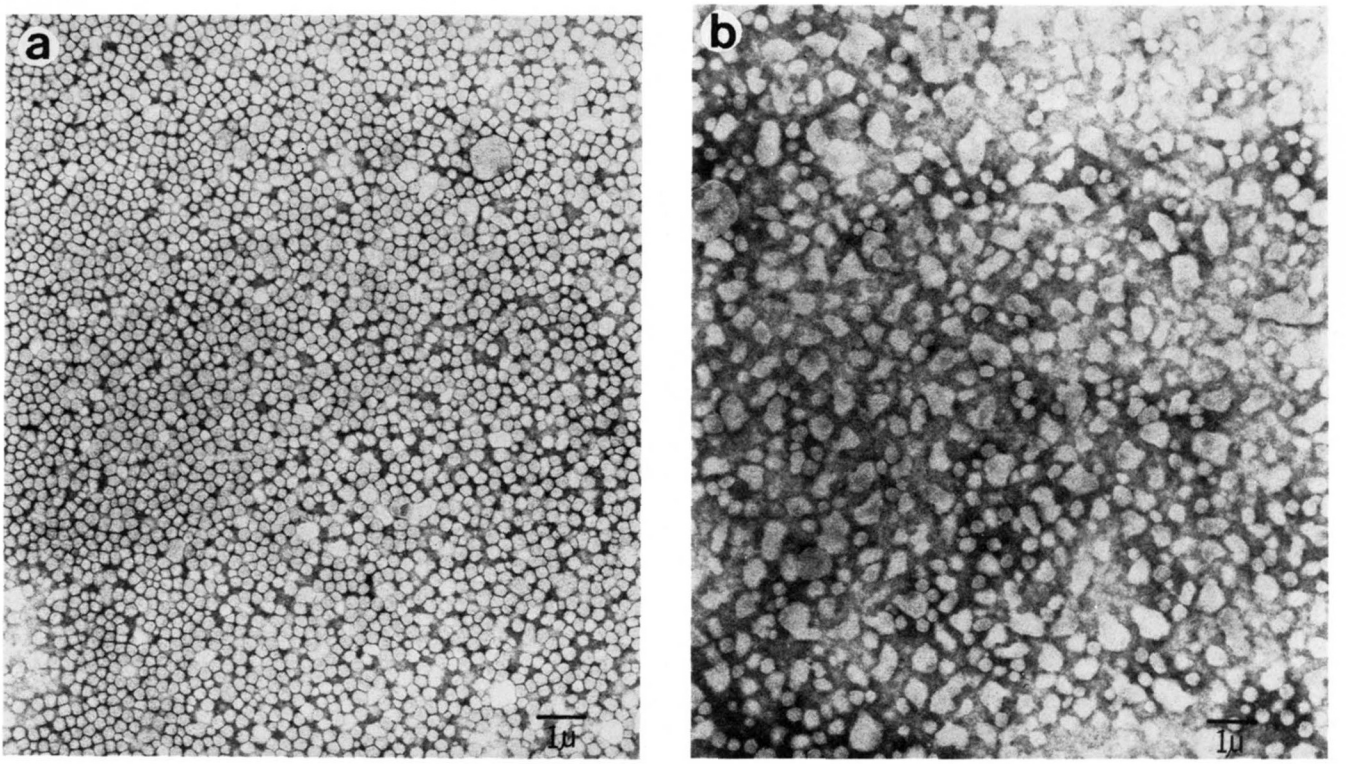

Fig. 7. Transmission electron microscopy of low density lipoproteins isolated by sequential density flotation from whole plasma (a) and interstitial inflammatory fluid (b). Lipoproteins were stained with 1% phosphotungstic acid, pH 7.0 Magnification 144,000×.

would increase the amount of malondialdehyde released into the interstitium, which upon reaction with low density lipoproteins would trigger their uptake by monocyte-macrophages infiltrating the extravascular space.

In this report we have described an altered spectrum of lipoproteins in interstitial inflammatory fluid, marked by significant modification of particles occurring in the LDL density range. These particles demonstrate alterations reported for low density lipoproteins following their incubation with arterial cells in vitro. Further investigation of these findings is required to elucidate the in vivo mechanism of these observed modifications and to examine the in vitro metabolism of inflammatory fluid lipoproteins by peripheral connective tissue and scavenger cells.

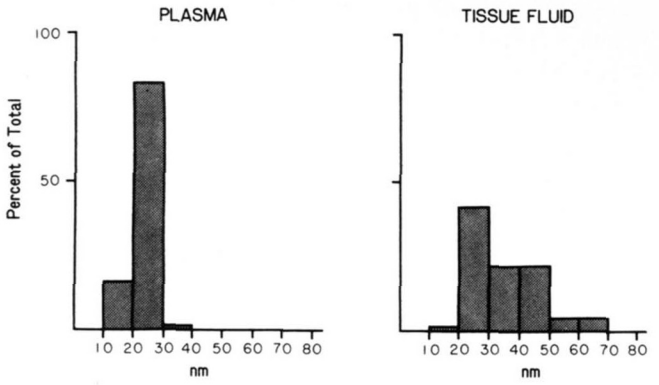

Fig. 8. Histogram plots of particle diameter measurements of low density lipoproteins from plasma and interstitial inflammatory fluid as observed in the electron microscope (Fig. 7). Each plot represents the mean diameter distribution of 100 particles measured at random.

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