Stimulation of Cholesteryl Ester Synthesis in Macrophages by Extracts of Atherosclerotic Human Aortas and Complexes of Albumin/Cholesteryl Esters

Joseph L. Goldstein, Henry F. Hoff, Y.K. Ho, Sandip K. Basu, and Michael S. Brown

Cholesteryl ester-rich particles extracted from human atherosclerotic plaques were shown to increase the rate of incorporation of $[^{14}C]$oleate into cholesteryl $[^{14}C]$oleate and to cause massive accumulation of cholesteryl esters in monolayers of mouse peritoneal macrophages. This stimulation showed saturation kinetics and susceptibility to competition by polyanions (polynucleic acid, fucoidin, dextran sulfate), suggesting that cell surface binding was required. Cellular uptake and lysosomal hydrolysis of the cholesteryl esters were also required, as indicated by the finding that stimulation of cholesteryl ester formation was prevented by the lysosomal inhibitor, chloroquine. The cholesteryl esterification-stimulating activity of the aortic extracts was excluded on a 2% agarose column and floated in the density range of 1.006 to 1.063 g/ml. Cholesterol-rich extracts from human adrenal glands and liver did not stimulate cholesteryl ester formation in macrophages. The aortic extracts did not stimulate cholesteryl ester synthesis in human fibroblasts. Complexes of $^{125}$I-labeled albumin and cholesteryl linoleate formed in vitro were taken up and degraded in macrophages, but not in fibroblasts, by a process resembling the uptake of the aortic extracts. The current data suggest that macrophages express mechanisms for internalizing certain types of cholesteryl ester-rich lipid/protein complexes, including those present in atherosclerotic plaques. (Arteriosclerosis 1981; 1:210-226)

Much of the cholesteryl ester that accumulates within atherosclerotic plaques is found within macrophage-derived foam cells.1-3 The cholesterol component of these esters is believed to be derived from the cellular uptake of cholesteryl ester-rich particles, primarily plasma lipoproteins, that have penetrated into the extracellular space.4 When studied in several in vitro systems, tissue macrophages such as those derived from the peritoneal cavity of mice do not take up large amounts of normal human or animal plasma lipoproteins. Yet, mouse peritoneal macrophages do ingest certain modified lipoproteins, such as acetylated low density lipoprotein (acetyl-LDL),1, 5-7 These modified lipoproteins are taken up by adsorptive endocytosis, and the cholesteryl esters are delivered to lysosomes wherein they are hydrolyzed. The liberated cholesterol is re-esterified and stored in the cyto-
plasm as cholesteryl ester droplets, causing cholesteryl ester accumulation.5-7

This sequence of uptake, hydrolysis, and re-esterification occurs when macrophages are incubated with LDL that has been rendered polyanionic by chemical derivitization, such as by maleylation,5, 7 or by reaction with malondialdehyde,7, 8 in addition to acetylation.5, 9 A similar uptake can be triggered by allowing LDL to form complexes with certain sulfated polysaccharides, such as dextran sulfate, which bind to the macrophage surface.10 The only unmodified plasma lipoproteins known to be recognized directly by mouse peritoneal macrophages are the β-migrating very low density lipoproteins (β-VLDL) that accumulate in the plasma of cholesterol-fed animals.11, 12 The β-VLDL bind to a discrete class of macrophage receptors that are distinct from the ones that bind polyanions. The intracellular metabolism of β-VLDL is the same as that of acetyl-LDL, i.e., lysosomal hydrolysis followed by cytoplasmic re-esterification.5, 7, 11, 12 Other cholesterol ester-rich particles such as native plasma LDL and high density lipoproteins (HDL) from humans or animals are not taken up with high affinity by mouse peritoneal macrophages.5, 11, 12

These studies raise the question as to whether any of the cholesteryl ester present in human atherosclerotic plaque is in a form recognized and taken up by macrophages. Hoff et al.13 and Hollander et al.14 have shown that cholesteryl-rich particles can be extracted from human atherosclerotic plaque after vigorous homogenization. Some of this extracted cholesterol is found in the form of large lipid/protein complexes that appear in the void volume of a Bio-Gel A-150 m column. Another portion of the cholesterol is found in particles in the molecular weight range of $3 \times 10^6$ as determined by gel filtration and electron microscopy (Gaubatz and Hoff, unpublished data).13 These particles, which are about the same size as plasma LDL, are reactive with an antibody directed against apoprotein B (apo-B), the major protein component of plasma LDL.15-17 By sodium dodecyl sulfate polyacrylamide gel electrophoresis, the protein component of these aortic LDL-like particles migrates similarly to apo-B.13 In contrast to plasma LDL, these aortic particles have an enhanced mobility on agarose gel electrophoresis, suggesting an increased negative charge (Gaubatz and Hoff, unpublished data).13, 14 Woodard et al.18 and Srinivasan et al.19 have isolated lipoprotein/glycosaminoglycan complexes from human atheroma. These complexes are excluded in the void volume of a Bio-Gel A-50 m column and float in the ultracentrifuge in the density range of 1.006-1.063 g/ml.19

Our current studies were undertaken to determine whether any of the cholesteryl ester/protein complexes extracted from human aortas are taken up efficiently by mouse peritoneal macrophages. As an index of uptake, we measured the ability of the aortic extracts to increase the rate at which the macrophages incorporate [14C]cholesterol into cholesteryl [14C]oleate. In the other macrophage lipoprotein uptake systems studied to date, the degree of stimulation of cholesteryl [14C]oleate synthesis has correlated with the amount of cholesteryl ester taken up by macrophages.5, 6, 11, 12 The current results show that the cholesteryl ester of aortic extracts is in a form that is susceptible to rapid uptake by macrophages. Rapid uptake is observed for the high molecular weight aortic cholesteryl ester complexes as well as for the lower molecular weight LDL-like particles purified by anti-apo-B affinity chromatography.

**Methods**

**Materials**

We obtained male and female Swiss Webster mice (25 to 30 g) from Simonsen Laboratories (Gilroy, California); [1-14C]oleic acid (56 mCi/mmol) and sodium [125I]iodide (carrier-free) from Amersham/Searle (Arlington Heights, Illinois); cholesteryl linolate from Applied Science (State College, Pennsylvania); bovine albumin (Fraction V from plasma) from Reheis Chemical Company (Phoenix, Arizona); and human albumin (crystallized, fatty acid free) from Miles Laboratories (Elkhart, Indiana). Newborn calf serum from Flow Laboratories was heat-inactivated (56° C, 30 min) prior to use. Dulbecco’s modified Eagle’s medium (DMEM, Cat. No. 320-1885), Eagle’s minimum essential medium (MEM, Cat. No. 410-1100), and Dulbecco’s phosphate-buffered saline (Cat. No. 310-4190) were purchased from Grand Island Biological Company (Grand Island, New York). Plastic petri dishes were obtained from Falcon. Pronase from Streptomyces griseus (B grade) was purchased from Calbiochem-Behring Corporation (LaJolla, California). Bio-Gel A-50 m (2% agarose) and Uni-pore polycarbonate membranes (1 μm pore diameter) were purchased from Bio-Rad (Richmond, California). Other supplies and reagents for assays were obtained from sources as previously reported.6, 11

**Lipoproteins**

Human LDL (density (d), 1.019 to 1.063 g/ml) and lipoprotein-deficient serum (d > 1.215 g/ml) were isolated from the plasma of individual healthy subjects by ultracentrifugation.5 LDL was acetylated with repeated additions of acetic anhydride.5 125I-Labeled acetyl-LDL was prepared as described.5
Preparation of Albumin/Cholesteryl Ester Complexes

Complexes of bovine albumin/cholesteryl linoleate (mass ratio 2:1) were prepared by sonication exactly as described by Werb and Cohn\textsuperscript{20} except that the bovine albumin was dissolved in DMEM rather than medium 199. \textsuperscript{125}I-labeled albumin/cholesteryl linoleate complexes were made by adding \textsuperscript{125}I-labeled albumin to the unlabeled cholesteryl linoleate. \textsuperscript{125}I-labeled albumin was prepared by the iodine monochloride method.\textsuperscript{21}

Tissue Extracts

Samples of atherosclerotic aortic intima, liver, and adrenal gland were obtained from the Harris County Medical Examiner (Houston, Texas) within 18 hours after death (table 1). The intimal lining of each aortic specimen was stripped from the underlying tunica media, and the intima was used for preparation of extracts as previously described.\textsuperscript{16,17} All operations were carried out at 4°C. Tissues were finely minced, and aliquots were weighed and placed in 1:1 or 1:2 (v/w) cold homogenization buffer (0.13 M Tris-HCl, 0.1% sodium EDTA, and 0.01% sodium azide at pH 7.4). The tissues were homogenized for 30 seconds with a Polytron homogenizer (Brinkmann Scientific, Westbury, New York) set at full power. Each homogenate was centrifuged at 3,000 \( \times g \) for 20 minutes. The pellet was discarded, and the supernatant fraction was designated "S-3 fraction." In some experiments, the S-3 fraction was further centrifuged for 30 minutes at 100,000 \( \times g \). The top cream layer and pellet were discarded, and the translucent supernatant fraction was saved and used for assays. This 100,000 \( \times g \) supernatant fraction is hereafter referred to as "S-100 fraction." All fractions were stored at 4°C for no more than 1 week. On the day before an experiment, the extracts were filtered through a Unipore polycarbonate membrane (1 \( \mu \)m pore diameter) and dialyzed overnight against a 20-fold excess of DMEM.

Affinity Chromatography

The immunoglobulin fraction of goat anti-human apo-B was purified on an LDL-Sepharose affinity column and then coupled to cyanogen bromide-activated Sepharose to form an anti-apo-B affinity column (Gaubatz and Hoff, unpublished data). Aliquots of the S-3 fraction of aortic extract (81 mg protein) were applied to the anti-apo-B affinity column in homogenization buffer. The column was washed with homogenization buffer until the optical absorption of the effluent at 280 nm reached baseline (nonadherent fraction). The adherent material was then eluted with 0.15 M NaCl adjusted to pH 11 with ammonium hydroxide. Immediately thereafter, the adherent and nonadherent fractions were dialyzed against buffer containing 0.15 M NaCl, 0.01% sodium azide, and 0.1% EDTA at pH 7.2. Pilot studies showed that the affinity column was capable of retaining at least 5 mg of LDL protein.

Mouse Macrophage Monolayers

Peritoneal cells were harvested from unstimulated mice in phosphate-buffered saline as previously described.\textsuperscript{5,6} The fluid from 20 to 40 mice (6 to 10 \( \times 10^6 \) cells/mouse) was pooled, and the

Table 1. Human Tissues Obtained at Autopsy

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age, Sex</th>
<th>Cause of death</th>
<th>Specimen*</th>
<th>Protein</th>
<th>Total cholesterol in soluble extract</th>
<th>Esterified cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>58 M</td>
<td>CHD</td>
<td>aortic plaque</td>
<td>0.75</td>
<td>0.68</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>62 M</td>
<td>CHD</td>
<td>aortic plaque</td>
<td>0.80</td>
<td>0.58</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>52 M</td>
<td>CHD</td>
<td>aortic plaque</td>
<td>0.38</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>30 M</td>
<td>Trauma</td>
<td>liver</td>
<td>0.002</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>84 M</td>
<td>CHD</td>
<td>aortic plaque</td>
<td>0.26</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>78 M</td>
<td>Neurological disease</td>
<td>aortic plaque</td>
<td>0.51</td>
<td>0.64</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>76 M</td>
<td>Trauma</td>
<td>aortic plaque</td>
<td>0.97</td>
<td>0.74</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>76 M</td>
<td>Trauma</td>
<td>adrenal gland</td>
<td>0.31</td>
<td>0.87</td>
<td></td>
</tr>
</tbody>
</table>

* Aortic plaques were obtained from the descending thoracic or abdominal aorta at regions where advanced atherosclerosis was apparent.
† For aortic specimens 4, 5, and 12 and adrenal specimen 18, the mass ratio was measured on the S-3 fraction (3,000 \( \times g \) supernate). For aortic specimens 16, 17, and 18 and liver specimen 14, the mass ratio was measured on the S-100 fraction (100,000 \( \times g \) supernate).
CHD = coronary heart disease, ND = not determined.
cells were collected by centrifugation (400 × g, 10 min, room temperature) and washed once with 30 ml of DMEM. The cells were resuspended in DMEM containing 20% (v/v) newborn calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) at a final concentration of approximately 2 × 10⁶ cells/ml. Aliquots (1 ml) of this cell suspension were dispensed into 35 × 10 mm plastic petri dishes and then incubated in a humidified CO₂ (5%) incubator at 37°C. After 1 to 2 hours, each dish was washed three times with 2 ml of DMEM without serum to remove nonadherent cells. The monolayers were incubated for 18 hours at 37°C in 1 ml of DMEM containing 20% newborn calf serum, after which the cells were washed with 2 ml of DMEM and then used for experiments.

**Human Fibroblast Monolayers**

Normal human fibroblasts (7 × 10⁴ cells dispensed into each 60 × 15 mm petri dish) were grown in monolayer with Eagle's minimum essential medium (MEM) and fetal calf serum, as previously described. The cells were used for experiments on Day 7 after a prior incubation for 48 hours in 10% human lipoprotein-deficient serum.

**Assays**

The incorporation of [¹⁴C]oleate into cellular cholesteryl [¹⁴C]oleate and [¹⁴C]triglycerides by cell monolayers was measured as previously described. The [¹⁴C]oleate (5000 to 11,000 cpn/mole) was added to the dishes as a complex with albumin (83 nmoles [¹⁴C]oleate per mg albumin); the final concentration of [¹⁴C]oleate was 0.2 mM. The [¹⁴C]-labeled lipids were extracted from hexane/isopropyl alcohol (3:2) directly from monolayers in situ in the plastic petri dish. The cellular content (surface-bound plus intracellular) of ¹²⁵I-acetyl LDL and ¹²⁵I-albumin/cholesteryl linoleate complexes was measured by previously described methods. Each monolayer was washed six times with an albumin-containing buffer, after which the cells were dissolved in 0.8 ml of 0.2 N NaOH and counted for their content of [¹²⁵I]-radioactivity. The proteolytic degradation of ¹²⁵I-acetyl LDL and ¹²⁵I-albumin/cholesteryl linoleate complexes was measured by assaying the amount of ¹²⁵I-labeled triglycerides and phospholipid content of samples was measured by gas-liquid chromatography. The triglyceride and phospholipid content of samples was measured as previously described.

Immunoreactive apo-B was quantified by electroimmunoassay with the use of plasma LDL as a standard.

**Reproducibility of Data**

The number of replicate assays for each data point is indicated in the legends. The average variation for replicate incubations in the different assays used in the cell incubation studies was less than 10%. Each of the experiments in figures 1, 2, 3, 4, 6, and 8 was repeated with aortic extracts from different individuals on three or more occasions with similar results. Each of the experiments in figures 7 and 9 and table 2 was repeated with aortic extracts from different individuals on two occasions with similar results. The experiments in figures 10 were performed with aortic extracts from different individuals on two occasions with similar results. The experiments in tables 3 and 5 and in figure 11 were each performed on one occasion.

**Results**

**Stimulation of Cholesteryl Ester Formation by Crude Extracts of Human Atherosclerotic Aortas**

Previous studies have shown that the delivery of cholesterol to macrophages increases the rate at which the cells synthesize cholesteryl ester. The degree of increase in the incorporation of [¹⁴C]oleate into cholesteryl [¹⁴C]oleate correlates with the amount of cholesterol that enters the cells. To determine whether human atherosclerotic plaques contain cholesteryl in a form that is taken up by macrophages, we prepared crude extracts of atherosclerotic intimas by Polytron homogenization followed by centrifugation at 3,000 × g. The 3000 × g supernate (S-3 fraction) contained high ratios of total cholesterol:protein, ranging from 0.38 to 0.80 (Specimens 4, 5, and 12 of table 1). About 60% of the cholesterol was in the esterified form. Aliquots of the S-3 fraction were incubated with monolayers of mouse peritoneal macrophages in the presence of [¹⁴C]oleate. After incubation for 4 hours, the cellular content of cholesteryl [¹⁴C]oleate was measured.

Figure 1 A shows that the S-3 fraction from the atherosclerotic intima of a human aorta (Specimen 5) stimulated cholesteryl [¹⁴C]oleate synthesis in macrophages. The stimulation was maximal when sufficient amounts of the aortic extract were added to produce a cholesterol concentration of about 25 μg/ml in the culture medium. On a milligram-for-milligram basis, the
cholesterol in the aortic extract was nearly as potent as the cholesterol of acetyl-LDL in stimulating cholesteryl \([^{14}C]\)oleate synthesis. In contrast, plasma LDL did not stimulate macrophage cholesteryl \([^{14}C]\)oleate formation. Similar negative results were obtained regardless of whether the LDL was prepared from the blood of living subjects or from postmortem blood obtained at the same time that the aortic specimens were obtained. An opposite result was obtained in cultured human fibroblasts (figure 1 B). In these cells, plasma LDL stimulated cholesteryl \([^{14}C]\)oleate synthesis, whereas the aortic extract was inactive.

In the experiment of figure 1 and in all subsequent experiments, we routinely measured the incorporation of \([^{14}C]\)oleate into cellular \([^{14}C]\)triacylglycerides. The values were generally in the range of 160 to 200 nmole/mg of protein per 4 hours of incubation and were not affected by the addition of tissue extracts or acetyl-LDL.

As expected from the \([^{14}C]\)oleate incorporation studies, the S-3 fraction of the aortic extract produced a marked increase in the cellular mass of esterified cholesterol after 48 hours of incubation (table 2). The increase was similar to that produced by acetyl-LDL. Both agents also raised the free cholesterol content of the cells, but the increase was not as great as the increase in cholesteryl esters.

To determine whether the cholesterol in the S-3 fraction was entering the macrophages by an uptake mechanism that was mediated by surface-binding sites similar to those for acetyl-LDL, we tested the ability of negatively-charged compounds to prevent the stimulation of cholesteryl \([^{14}C]\)oleate synthesis. These compounds block the binding of acetyl-LDL to the cell surface and thereby prevent its internalization by endocytosis and its stimulation of cholesteryl \([^{14}C]\)oleate synthesis. Two of the negatively-charged compounds, polyninosinic acid and fucoidin, inhibited the stimulation of cholesteryl \([^{14}C]\)oleate synthesis elicited by the aortic extract (figure 2 A). The maximum inhibition was in the range of 80% to 85%. Similar results were obtained with the S-3 fraction from all three aortic specimens that were tested (Specimens 4, 5, and 12). Polyninosinic acid and fucoidin inhibited the acetyl-LDL-mediated stimulation of cholesteryl ester formation by more than 95% (figure 2 B). Polycytidylic acid, a negatively-
Table 2. Cholesterol Content of Mouse Macrophages Incubated with Human Aortic Extract

<table>
<thead>
<tr>
<th>Addition to medium</th>
<th>Concentration added to medium (µg/ml)</th>
<th>Cellular content of cholesterol (µg sterol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cholesterol</td>
<td>Protein</td>
</tr>
<tr>
<td>None</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Aortic extract</td>
<td>112</td>
<td>75</td>
</tr>
<tr>
<td>(S-3 fraction)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetyl-LDL</td>
<td>37</td>
<td>25</td>
</tr>
</tbody>
</table>

Each monolayer received 1 ml of Dulbecco's modified Eagle's medium containing the indicated addition. After incubation for 24 hours at 37°C, fresh medium of the same composition was added. After a further 24 hours, the cells were washed and harvested for measurement of the cellular content of free and esterified cholesterol. Each value represents the average of triplicate incubations.

A charged compound that does not block acetyl-LDL binding, uptake, or degradation, did not inhibit the stimulation of cholesteryl ester formation elicited by acetyl-LDL (figure 2 B) or by the aortic extract (figure 2 A). As a control for nonspecific effects of fucoidin and polyinosinic acid, we showed that these compounds did not block the stimulation of cholesteryl [14C]oleate formation produced by incubation of the cells with 25-hydroxycholesterol plus cholesterol (figure 2 C).

To determine whether lysosomal digestion was a prerequisite for the aortic extracts to stimulate cholesteryl [14C]oleate synthesis in macrophages, we tested the ability of chloroquine to block this stimulation. Chloroquine, a weak base, concentrates in lysosomes and raises the lysosomal pH, thereby inhibiting lysosomal enzyme activity. By reducing the activity of the lysosomal acid lipase, chloroquine prevents the liberation of cholesterol from acetyl-LDL and thereby prevents the stimulation of cholesteryl ester formation. Figure 3 A shows that chloroquine prevented the aortic S-3 fraction from stimulating cholesteryl [14C]oleate synthesis at the same concentrations at which it prevented the effect of

![Figure 2. Cholesteryl ester formation in mouse macrophages incubated with human aortic extract: Inhibition by negatively-charged compounds. Each monolayer received 0.6 ml of Dulbecco's modified Eagle's medium (DMEM) containing 0.2 mM [14C]oleate-albumin; the indicated concentration of one of the following compounds (• = none; A = polyinosinic acid; O = fucoidin; or △ = polycytidylic acid); and one of the following stimulating agents: (A) 82 µg cholesterol/ml of aortic extract S-3 fraction; (B) 30 µg cholesterol/ml of acetyl-LDL; and (C) 5 µg/ml of 25-hydroxy-cholesterol plus 12 µg/ml of cholesterol added in 5 µl ethanol. After incubation for 4 hours at 37°C, the cellular content of cholesteryl [14C]oleate was determined. The “100% of control” values in the absence of any negatively-charged compound (•) were 54, 72, and 38 nmoles/mg protein for (A), (B), and (C) respectively. Each value represents a single incubation, except for the “100% of control” values which represent the average of triplicate incubations.](http://atvb.ahajournals.org/abstracts/215/0/0/0.png)
A. Cholesteryl [14C]oleate

B. [14C]Triglycerides

<table>
<thead>
<tr>
<th>Chloroquine (µM)</th>
<th>[14C]Oleate (%) of Control</th>
<th>[14C]Triglycerides (%) of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>75</td>
<td>75</td>
</tr>
<tr>
<td>5</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>10</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>25</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure 3. Effect of chloroquine on cholesteryl ester (A) and triglyceride (B) formation in mouse macrophages. Each monolayer received 0.6 ml of Dulbecco's modified Eagle's medium (DME) containing 0.2 mM [14C]oleate-albumin, the indicated concentration of chloroquine, and one of the following stimulating agents: ■ = none; ▲ = 55 µg cholesterol/ml of aortic extract 5 (S-3 fraction); ○ = 38 µg cholesterol/ml of acetyl LDL; or △ = 5 µg/ml of 25-hydroxycholesterol plus 12 µg/ml of cholesterol added in 5 µl ethanol. After incubation for 4 hours at 37°C, the cellular content of cholesteryl [14C]oleate (A) and [14C]triglycerides (B) was determined. In A, the “100% of control” values in the absence of chloroquine (■) were 43, 72, and 19 nmoles/mg protein for cells incubated with aortic extract, acetyl-LDL, and sterols respectively (no addition value, 0.45 nmole/mg). In B, the “100% of control” values in the absence of chloroquine (■) were 210, 175, and 200 nmoles/mg protein for cells incubated with aortic extract, acetyl-LDL, and sterols respectively (no addition value, 179 nmoles/mg). Each value represents the average of duplicate incubations.

Acetyl-LDL. Chloroquine had much less effect on cholesteryl esterification when it was stimulated by 25-hydroxycholesterol plus cholesterol, a combination that does not require hydrolysis to produce this stimulation. Chloroquine did not inhibit the incorporation of [14C]oleate into cellular [14C]triglycerides (figure 3 B).

**Stimulation of Cholesteryl Ester Formation by S-100 Fraction of Human Atherosclerotic Aortas**

The 3000 × g supernate from an additional set of aortic extracts was fractionated by ultracentrifugation at 100,000 × g. This centrifugation yielded a membranous pellet and a creamy layer at the top of the tube. Between these two fractions, there was a translucent white supernate. This supernate was designated the “S-100 fraction” and was used for further studies. The mass ratio of total cholesterol:protein in the S-100 fraction (aortic specimens 16, 17, and 18 of table 1) was in the same range as that of the S-3 fraction (aortic specimens 4, 5, and 12). About 70% of the cholesterol was esterified. Approximately 70% of the total esterification-stimulating activity present in the S-3 fraction was recovered in the S-100 fraction, which also contained about 70% of the total cholesterol. The S-100 fraction could be filtered through a 1 µm filter without loss of any esterification-stimulating activity.

Figure 4 shows that the addition of increasing amounts of the S-100 fraction of extracts from three different aortas stimulated cholesteryl ester synthesis in macrophages. Half-maximal activity was found at a cholesterol concentration of about 25 µg/ml. An extract (S-100 fraction) of liver obtained at autopsy did not stimulate cholesteryl ester synthesis in macrophages when added at cholesterol concentrations up to 100 µg/ml (figure 4 A). Similarly, an extract (S-3 fraction) of an adrenal gland obtained at autopsy also failed to stimulate cholesteryl ester synthesis in macrophages (figure 4 B). In the adrenal extract, more than 85% of the added cholesterol was present in the form of cholesteryl ester (table 1).
CHOLESTERYL ESTER FORMATION IN MACROPHAGES

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Figure 4. Cholesteryl ester formation in mouse macrophages incubated with human tissue extracts. Each monolayer received 0.6 ml of Dulbecco's modified Eagle's medium (DMEM) containing 0.2 mM [14C]oleate-albumin and the indicated concentration of one of the following: (A) ● = aortic extract 17 (S-100 fraction); ○ = aortic extract 16 (S-100 fraction); ■ = liver extract 14 (S-100 fraction). B: □ = aortic extract 18 (S-100 fraction); ● = adrenal gland extract 18 (S-3 fraction). After incubation for 4 hours at 37°C, the cellular content of cholesteryl [14C]oleate was determined. Each value represents a single incubation.

Fractionation of Aortic Extracts by Gel Filtration

The S-100 fraction of the aortic extract was further purified by gel filtration on agarose A-50 m (figure 5). The bulk of the esterification-stimulating activity appeared in a peak at the void volume of the column. On the other hand, most of the protein was in the included volume. The overall recovery of esterification-stimulating activity on the column was slightly greater than 100%. The active fractions in the void volume were pooled for further study, and are designated hereafter as "A-50 m fraction." This material had the following lipid and protein composition: free cholesterol (19%); esterified cholesterol (35%); phospholipid (11%); triglyceride (4%); and protein (31%).

The esterification-stimulating activity of the A-50 m fraction was similar in potency to the activity of the crude aortic extract, when compared on the basis of cholesterol content. A half-maximal increase in cholesteryl ester synthesis occurred by the Lowry method (○) and for their ability to stimulate the incorporation of [14C]oleate-albumin into cholesteryl [14C]oleate by monolayers of mouse macrophages (●). The cholesteryl esterification assay was carried out for 4 hours exactly as described in the legend to figure 1. Arrows indicate the elution peaks of molecular weight standards, which are 125I-labeled β-migrating very low density (β-VLDL) isolated from cholesterol-fed rabbits (see ref. 12), human 125I-LDL, and potassium dichromate (yellow color).
Figure 6. Cholesteryl ester formation in mouse macrophages incubated with A-50 m fraction of human aortic extract: Inhibition by negatively-charged compounds. A. Each monolayer received 0.6 ml of Dulbecco's modified Eagle's medium (DMEM) containing 0.2 mM [14C]oleate-albumin, 45 µg cholesterol/ml of A-50 m fraction, and the indicated concentration of one of the following negatively-charged compounds: ■ = none; ● = polyinosinic acid; ▲ = fucoidin; ○ = dextran sulfate; or △ = polycytidylic acid. B. Each monolayer received 0.6 ml of DMEM containing 0.2 mM [14C]oleate-albumin, the indicated concentration of A-50 m fraction, and one of the following concentrations of polyinosinic acid: ○ = none; △ = 1 µg/ml; ● = 3 µg/ml or ▲ = 20 µg/ml. After incubation for 4 hours at 37°C, the cellular content of cholesteryl [14C]oleate was determined. Each value represents a single incubation except for the no addition values (■) which represent the average of duplicate incubations.

at about 25 µg cholesterol/ml (figure 6 B). The maximal esterification-stimulating activity produced by the A-50 m fraction was about 60 nmoles/mg protein in 4 hours (figure 6 B), which is similar to that observed with the crude S-3 fraction (figure 1 A). The addition of increasing amounts of polyinosinic acid reduced the activity of the A-50 m fraction (figure 6 B). Dextran sulfate and fucoidin also reduced the activity, but polycytidylic acid had no effect (figure 6 A). These results are similar to those obtained with the S-3 aortic fraction (figure 2 A).

The inhibition of the esterification-stimulating activity of the A-50 m fraction by polyinosinic acid raises the possibility that the active component of this fraction may enter the macrophages by binding to the same site as that to which acetyl LDL binds. To explore this directly, we tested the ability of the A-50 m fraction to compete for the cellular uptake and degradation of 125I-labeled acetyl LDL (figure 7). Over the concentration range in which the A-50 m fraction stimulates cholesteryl esterification (i.e., up to 90 µg/ml in figure 6 B), the fraction reduced the uptake and degradation of 125I-acetyl LDL by only about 20% (figure 7). Thus, it appears that the two ligands enter the cell by binding to two different sites, both of which are blocked by polyinosinic acid.

In other experimental systems, it has been demonstrated that, in the presence of chloroquine, lipoproteins are internalized by the cells but not degraded, and, hence, cholesterol esterification is not stimulated. If cells are allowed to ingest lipoproteins in the presence of chloroquine and are then washed, the lipoprotein trapped within the lysosomes is degraded, cholesterol is liberated, and cholesterol esterification is stimulated. These experiments have provided strong evidence that cholesteryl esterification is stimulated by cholesterol released by lysosomal hydrolysis of cholesteryl ester. Figure 8 A shows that a similar phenomenon can be demonstrated with the A-50 m fraction. Macrophages were incubated for 14 hours with the A-50 m fraction in the presence of chloroquine and...
Figure 7. Uptake (A) and degradation (B) of $^{125}$I-acetyl-LDL by mouse macrophages: Competition by unlabeled acetyl-LDL (▲) and A-50 m fraction of aortic extract (●). Each monolayer received 0.6 ml of Dulbecco’s modified Eagle’s medium (DMEM) containing 10% human lipoprotein-deficient serum, 20 µg protein/ml of $^{125}$I-acetyl-LDL (58 cpm/ng), and the indicated concentration of either unlabeled acetyl-LDL (▲) or A-50 m fraction (●). After incubation for 5 hours at 37°C, the total cellular content (A) and degradation (B) of $^{125}$I-acetyl LDL was measured as described under Methods. The “100% of control” values in the absence of any addition (●) were 5.7 and 80 µg/mg protein for A and B respectively. Each value represents a single incubation except for the “100% of control” values, which represent the average of triplicate incubations.

Figure 8. Stimulation of cholesteryl ester formation by A-50 m fraction of aortic extract (A) and acetyl-LDL (B) released from cellular lysosomes after removal of chloroquine. Each monolayer of macrophages received 0.7 ml of Dulbecco’s modified Eagle’s medium (DMEM) containing 0.2 mM [14C]oleate-albumin, 16 µM chloroquine, and either 72 µg cholesterol/ml of the purified A-50 m fraction (A) or 15 µg cholesterol/ml of acetyl-LDL (B). After incubation for 14 hours at 37°C (zero time in A and B), the cells were washed three times with ice-cold phosphate-buffered saline (2 ml/wash). The cells were then subjected to a second incubation in 1 ml of DMEM containing 0.2 mM [14C]oleate-albumin in the absence (●) or presence (○) of 16 µM chloroquine. No aortic extract or acetyl-LDL was present. After incubation at 37°C for the indicated time, the cellular content of cholesteryl [14C]oleate was determined. Each value represents the average of duplicate incubations.
At the end of this time, only a small amount of \(^{14}\text{C}\)oleate had been incorporated into cholesteryl ester (zero time in Figure 8 A). At this point the cells were washed to remove the extracellular A-50 m fraction, and the dishes were divided into two groups, both of which received \(^{14}\text{C}\)oleate. In one group the chloroquine was maintained in the culture medium, and in the other group the chloroquine was removed. When the chloroquine was removed, the rate of cholesterol esterification rapidly increased. In the cells in which chloroquine was retained, cholesterol esterification continued to be blocked. Figure 8 B shows that chloroquine had a similar effect on the stimulation of cholesterol esterification with acetyl-LDL. That the chloroquine itself was not directly blocking the esterification reaction was indicated by the earlier experiment showing that chloroquine does not block the ability of 25-hydroxycholesterol plus cholesterol to stimulate cholesteryl ester synthesis (figure 3).

To determine whether the protein component of the A-50 m fraction was necessary for the uptake of the cholesteryl ester, we treated the fraction with increasing amounts of pronase. Figure 9 shows that pronase treatment lowered the ability of the fraction to stimulate cholesterol \(^{14}\text{C}\)oleate synthesis. Pronase treatment of acetyl-LDL did not significantly affect its ability to stimulate cholesteryl ester synthesis. Previous studies have shown that the portion of LDL protein necessary for binding to LDL receptors on fibroblasts and smooth muscle cells is not removed by protease treatment. The results in figure 9 indicate that a similar protection applies to acetyl-LDL, but not to the A-50 m fraction.

### Fractionation of Aortic Extracts by Ultracentrifugation

The S-3 fraction of aortic extract was subjected to sequential flotation at different densities in a manner similar to that used routinely for isolating lipoproteins (table 3). Each fraction was assayed for total protein and cholesterol as well as for its ability to stimulate cholesterol esterification in macrophages over a concentration range in which the rate of esterification was linearly proportional to the amount of extract added. The starting S-3 fraction contained 110 mg of cholesterol. About 8 mg of cholesterol was found in the fraction that floated at a density < 1.006 g/ml. The bulk of the recovered cholesterol (45 mg) and the bulk of the recovered esterification-stimulating activity were found in the fraction of density 1.006 to 1.063 g/ml, which is similar to the density of plasma intermediate and low density lipoproteins. In each lipoprotein fraction the specific esterification activity was in the range of 1.5 nmoles of cholesteryl \(^{14}\text{C}\)oleate formed/4 hrs/mg cell protein per \(\mu\)g of added cholesterol (table 3).

### Fractionation of Aortic Extract on an Anti-Apo-B Affinity Column

Recent studies have shown that a fraction of the cholesterol in crude aortic extracts is associated with immunoreactive apo-B and is retained on an anti-apo-B affinity column (Gaubatz and Hoff, unpublished data). To determine whether this apo-B-containing material accounted for the ability of the aortic extract to stimulate cholesteryl ester formation.

![Diagram](attachment:image.png)

**Figure 9.** Destruction by pronase of the ability of purified aortic extract to stimulate cholesteryl ester formation in mouse macrophages. Aliquots of the A-50 m fraction of aortic extract (60 \(\mu\)l containing 72 \(\mu\)g cholesterol) or acetyl LDL (60 \(\mu\)l containing 54 \(\mu\)g cholesterol) were incubated in a final volume of 150 \(\mu\)l containing 0.2 M 2-(N-morpholino)ethanesulfonic acid (MES) and 0.15 M NaCl at pH 6.8 for 24 hours at 37°C in the presence or absence of the indicated concentrations of pronase. Aliquots of the pronase-treated samples were then added to macrophage monolayers and assayed for their ability to stimulate cholesteryl ester formation. Each monolayer was incubated at 37°C for 4 hours with 1 ml of Dulbecco's modified Eagle's medium (DMEM) containing 40 \(\mu\)l of either pronase-treated aortic extract (final concentration, 19 \(\mu\)g cholesterol/ml) or pronase-treated acetyl-LDL (final concentration, 14 \(\mu\)g cholesterol/ml). The "100% of control" values were 42 and 72 nmoles of cholesteryl \(^{14}\text{C}\)oleate formed/4 hours per mg protein for the purified aortic extract (●) and acetyl LDL (○) respectively. Each value represents the average of duplicate incubations.
Table 3. Cholesterol Esterification-Stimulating Activity in Fractions of S-3 Extract Obtained by Ultracentrifugation at Varying Densities

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein (mg/fraction)</th>
<th>Cholesterol (mg/fraction)</th>
<th>Total (units X 10^-3/fraction)</th>
<th>Specific (units/µg cholesterol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-3 fraction</td>
<td>290</td>
<td>110</td>
<td>127</td>
<td>1.2</td>
</tr>
<tr>
<td>d &lt; 1.006 g/ml</td>
<td>4</td>
<td>8</td>
<td>15</td>
<td>1.9</td>
</tr>
<tr>
<td>d 1.006-1.063 g/ml</td>
<td>21</td>
<td>45</td>
<td>67</td>
<td>1.5</td>
</tr>
<tr>
<td>d 1.063-1.21 g/ml</td>
<td>9</td>
<td>6</td>
<td>6</td>
<td>1.0</td>
</tr>
<tr>
<td>d &gt; 1.21 g/ml</td>
<td>65</td>
<td>1</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

The S-3 fraction (3000 × g supernate) from aortic Specimen 12 was fractionated by sequential flotation in a Beckman preparative ultracentrifuge at 4°C according to standard techniques using solid KBr for density adjustment. Fractions that floated at each density were dialyzed against solutions containing 0.15 M NaCl, 0.01% sodium azide, and 0.01% EDTA (pH 7.4). The protein and cholesterol contents were determined as described under Methods. Esterification-stimulating activity was measured by determining the ability of each fraction to stimulate the incorporation of [14C]oleate-albumin into cholesteryl [14C]oleate by monolayers of mouse macrophages. The cholesteryl esterification assay was carried out for 4 hours exactly as described in the legend to figure 1. Each fraction was tested at five different cholesterol concentrations, activity increasing linearly in each case. A unit of esterification-stimulating activity is defined as the activity that stimulates the formation of 1 nmole of cholesteryl [14C]oleate per mg cell protein in a 4-hour incubation with macrophages in a volume of 0.6 ml per dish. The specific activity is defined as the units of esterification-stimulating activity per µg of cholesterol added to the culture medium.

Cholesteryl esterification, we passed the crude S-3 fraction over an anti-apo-B column. The material that failed to adhere to the column was collected, after which the adherent fraction was eluted from the column at alkaline pH and collected. Approximately 75% of the cholesterol of the extract was recovered in the nonadherent fraction, and 8% was recovered in the adherent fraction (table 4). All of the immunoreactive apo-B was recovered in the adherent fraction, where it constituted about one-third of the protein. The nonadherent fraction was as potent as the adherent fraction in stimulating cholesteryl [14C]oleate synthesis in macrophages when compared on the basis of cholesterol content (figure 10). Since about 10 times as much cholesterol was found in the nonadherent fraction as compared with the adherent fraction, these data indicate that most of the esterification-stimulating activity of the

Table 4. Fractionation of Human Aortic Extracts by Anti-Apo-B Affinity Column Chromatography

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein</th>
<th>Apo-B</th>
<th>Cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-3 fraction</td>
<td>81</td>
<td>1.2</td>
<td>81</td>
</tr>
<tr>
<td>Nonadherent</td>
<td>75</td>
<td>0</td>
<td>62</td>
</tr>
<tr>
<td>Adherent</td>
<td>3</td>
<td>1.1</td>
<td>6.6</td>
</tr>
</tbody>
</table>

The S-fraction (3000 × g supernate) of aortic extract 4 was subjected to affinity chromatography on an anti-apo-B column as described under Methods. The contents of total protein, apo-B, and cholesterol in the nonadherent and adherent fractions were measured as described under Methods. Each value represents the average of duplicate determinations.

Figure 10. Cholesteryl ester formation in mouse macrophages incubated with aortic extracts fractionated on an anti-apo-B affinity column. An S-3 fraction of aortic extract 4 was subjected to anti-apo-B affinity chromatography as described under Methods. Varying concentrations of the unfractuated S-3 (•) and the nonadherent (△) and adherent (■) fractions were tested for their ability to stimulate the incorporation of [14C]oleate into cholesteryl [14C]oleate by monolayers of mouse macrophages, as described in the legend to figure 1. The incubation was carried out at 37°C for 4 hours. Each value represents a single incubation. The protein and lipid content of the S-3 fraction and the nonadherent and adherent fractions is given in table 4.
Stimulation of Cholesteryl Ester Formation by Particulate Complexes of Albumin/Cholesteryl Ester

Previous studies by Werb and Cohn have shown that albumin/cholesteryl ester complexes are taken up by macrophages, leading to accumulation of cholesterol. To determine whether these complexes are metabolized in a fashion similar to that of the aortic extract and acetyl-LDL, we performed a series of experiments using \(^{125}\)I-albumin/cholesteryl ester complexes. Figure 11 A shows that the \(^{125}\)I-albumin/cholesteryl ester complex bound to the macrophage surface in a saturable fashion at 4°C. The binding was prevented by polyinosinic acid. Figure 11 B shows that the albumin/cholesteryl ester complex markedly stimulated cholesteryl esterification in macrophages at 37°C, again in a saturable fashion. This stimulation was prevented by polyinosinic acid, suggesting that surface binding was required. The albumin/cholesteryl ester complex did not stimulate the incorporation of \(^{14}\)C]oleate into cholesteryl \[^{14}\]C]oleate in cultured human fibroblasts, when tested at concentrations as high as 600 \(\mu\)g cholesterol/ml (data not shown).

When \(^{125}\)I-albumin was incubated with macrophages for 5 hours at 37°C, the cellular content of \(^{125}\)I-radioactivity was low, and only a trace amount of the \(^{125}\)I-albumin was degraded to trichloroacetic acid-soluble products (table 5). On the other hand, when the \(^{125}\)I-albumin was complexed with cholesteryl linolate, large amounts of the complex were taken up by the cells, and large amounts of \(^{125}\)I-albumin were degraded. The uptake and degradation of the labeled complex were blocked in the presence of an excess of unlabeled albumin/cholesteryl ester complex, suggesting that saturable binding was a prerequisite for both processes. Uptake and degradation were also blocked by polyinosinic acid and to a lesser extent by an excess of acetyl-LDL (table 5).

Figure 12 shows competition curves for the binding of \(^{125}\)I-albumin/cholesteryl ester complexes (upper panels) and \(^{125}\)I-acetyl LDL (lower panels).

Figure 11. Interaction of albumin/cholesteryl ester complexes with mouse macrophages. A. Surface binding of \(^{125}\)I-albumin/cholesteryl linolate complexes. Each monolayer was incubated with 0.6 ml of ice-cold Eagle's minimum essential medium (MEM) (without bicarbonate) containing 50 mM HEPES (pH 7.4), 10% human lipoprotein-deficient serum, and the indicated concentration of \(^{125}\)I-albumin/cholesteryl linolate (99 cpm/ng albumin) in the absence (O) or presence (●) of 10 \(\mu\)g/ml of polyinosinic acid. After incubation for 2 hours at 4°C, each monolayer was washed extensively with an albumin-containing buffer. The cells were then dissolved in 0.8 ml of 0.2 N NaOH, and the amount of cell-associated \(^{125}\)I radioactivity was determined. B. Stimulation of cholesteryl ester formation by albumin/cholesteryl linolate complexes. Each monolayer received 0.6 ml of Dulbecco's modified Eagle's medium (DMEM) containing 2 \(\mu\)g/ml of human serum albumin, 0.2 mM \[^{14}\]C]oleate-albumin, and the indicated concentration of albumin/cholesteryl linolate in the absence (●) or presence (△) of 30 \(\mu\)g/ml of polyinosinic acid. After incubation for 4 hours at 37°C, the cellular content of cholesteryl \[^{14}\]C]oleate was determined. Each value represents a single incubation.
Table 5. Uptake and Degradation of $^{125}$I-Albumin and $^{125}$I-Albumin/Cholesteryl Ester Complexes by Macrophages

<table>
<thead>
<tr>
<th>Addition to medium</th>
<th>$^{125}$I-Albumin (μg/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cellular content</td>
</tr>
<tr>
<td>$^{125}$I-Albumin (100 μg/ml)</td>
<td>0.07</td>
</tr>
<tr>
<td>$^{125}$I-Albumin/cholesterol linoleate (90 μg/ml)</td>
<td>9.3</td>
</tr>
<tr>
<td>+ albumin/cholesterol linoleate (3 mg/ml)</td>
<td>1.9</td>
</tr>
<tr>
<td>+ polyinosinic acid (30 μg/ml)</td>
<td>1.7</td>
</tr>
<tr>
<td>+ acetyl-LDL (300 μg/ml)</td>
<td>4.9</td>
</tr>
</tbody>
</table>

Each monolayer received 0.6 ml of DMEM containing 10% human lipoprotein-deficient serum and the indicated addition. The $^{125}$I-albumin and $^{125}$I-albumin/cholesterol ester complexes were added at specific activities of 139 and 75 cpm/ng albumin, respectively. After incubation for 5 hours at 37°C, the cellular content and degradation of $^{125}$I-albumin were measured as described under Methods. Each value is the average of triplicate incubations.

Panels) to the macrophage surface at 4°C. Polyinosinic acid inhibited the binding of both ligands by more than 95% (figures 12 A and C). At concentrations below 50 μg cholesterol/ml, acetyl-LDL inhibited the binding of $^{125}$I-albumin/cholesteryl ester. The maximum inhibition was about 80% (figure 12 B). Similarly, at low concentrations the albumin/cholesterol ester complex competed partially for the binding of $^{125}$I-acetyl-LDL, but only about 35% of the $^{125}$I-acetyl-LDL binding was susceptible to such competition (figure 12 D).

Figure 12. Inhibition of the surface binding of $^{125}$I-albumin/cholesterol linoleate complexes (A,B) and $^{125}$I-acetyl-LDL (C,D) to macrophages. Each monolayer was incubated with 0.6 ml of ice-cold Eagle's minimum essential medium (MEM) (without bicarbonate) containing 50 mM HEPES (pH 7.4), 10% human lipoprotein-deficient serum; either 50 μg protein/ml of $^{125}$I-albumin/cholesterol linoleate (82 cpm/ng albumin) (A,B) or 20 μg protein/ml of $^{125}$I-acetyl-LDL (237 cpm/ng protein) (C,D); and the indicated concentration of one of the following competing compounds: □ = none; ● = polyinosinic acid; △ = acetyl LDL; or ▽ = unlabeled albumin/cholesterol linoleate. After incubation for 2 hours at 4°C, each monolayer was washed extensively with an albumin-containing buffer. The cells were then dissolved in 0.8 ml of 0.2 N NaOH, and the amount of cell-associated $^{125}$I radioactivity was determined. Each value represents a single incubation, except for the no addition values (□) which represents the average of triplicate incubations.
Discussion

The current results demonstrate that human atherosclerotic plaques contain cholesterol in a form that is readily taken up by macrophages. Much of the cholesterol appears to be organized as large lipid/protein complexes in which about two-thirds of the cholesterol is in the ester form. These complexes enter macrophages by adsorptive endocytosis that is facilitated by their binding to sites on the plasma membrane. The complexes are digested in lysosomes and the liberated cholesterol stimulates the cholesterol esterification reaction, leading to the accumulation of esterified cholesterol within the cell. In all of these respects, the metabolism of the aortic extract by macrophages resembles the pathway followed by acetyl-LDL.5-7

The interaction of the aortic extracts with macrophages showed specificity at two levels. First, the aortic extract was taken up by macrophages, but not by cultured human fibroblasts, indicating specificity as to cell type. Second, the macrophages took up the aortic extract, but not plasma LDL, indicating specificity as to the form of cholesteryl ester/protein complexes that can be recognized.

Evidence that the active material in the aortic extracts is a lipid/protein complex comes from several experiments. First, the uptake could be prevented by prior treatment of the aortic extract with pronase. Second, the bulk of the active material floated in the density range of 1.006 to 1.063 g/ml, consistent with the density of many known plasma lipoproteins. Third, the active fraction in the aortic extract could be purified on an A-50 m column. The active fractions, which eluted in the void of the column, contained a mixture of free and esterified cholesterol, protein, triglycerides, and phospholipid in proportions that were similar to the composition of plasma lipoproteins, such as LDL, with the exception that the phospholipid content was somewhat lower. LDL contains about 25% phospholipid, whereas the A-50 m fraction of aortic extract contained about 11% phospholipid. It is likely that this material also contains sulfated polysaccharides,18 19 but the amounts and types are not yet known. Moreover, we do not know whether a sulfated polysaccharide plays a role in mediating the binding of the cholesteryl ester complexes in a manner analogous to that demonstrated for the complex between LDL and dextran sulfate.10

Recent studies have shown that human aortic extracts contain a lipoprotein that binds to an antibody directed against apo-B.15 and that shows an enhanced negative charge as compared with plasma LDL.13 14 While this apo-B-containing fraction was active in delivery of cholesterol to macrophages, it was not any more active than the material that did not contain immuno-reactive apo-B (figure 10). Thus, we could obtain no evidence in the current studies that apo-B was required for macrophage uptake of aortic cholesteryl ester complexes. We cannot exclude the possibility that these complexes contain apo-B, which is biologically active in binding to macrophages but which has been modified so that it no longer reacts with the anti-apo-B antibody.

Several important questions have not been resolved in this initial study of macrophage uptake of the cholesteryl ester of human aortic extracts. We do not know how much of the cholesteryl ester/protein complex is derived from extracellular deposits and how much is derived from intracellular cholesterol stores within the atheroma. The number and nature of the proteins present in the complexes also remain to be determined, and it remains to be determined whether any of these proteins are related to those of plasma lipoproteins. Further studies will also be necessary to determine whether the cholesteryl ester/protein complexes that have been isolated by our homogenization procedure were in fact present in the same molecular form in situ in the aortic wall.

Another unanswered question relates to the specificity of the cell surface binding site(s) that initiates the uptake of the aortic extract. This binding was susceptible to competition by certain polyanions, such as polyinosinic acid, dextran sulfate, and fucoidin, but not by polycytidylic acid. This pattern of competition is similar to that displayed by the binding site for polyanionic acetyl-LDL.1 In direct competition experiments, however, the aortic extract competed only to a small degree for the binding of acetyl LDL (figure 7). Thus, it would appear from the present data that the binding site for the aortic extract is similar, but not identical, to that for acetyl LDL.

The current studies also explored the ability of macrophages to ingest albumin/cholesteryl linoleate complexes that were formed in vitro. Whereas macrophages did not take up or degrade significant amounts of free 125I-albumin, they did take up and degrade large amounts of the protein when it was complexed with cholesteryl ester. The 125I-albumin and cholesteryl ester appeared to be taken up stoichiometrically in a 2:1 ratio, which is the same as the mass ratio of the two components of the complex in the culture medium. Thus, in the experiment of table 5, at 90 µg/ml a total of 44.4 µg of 125I-albumin had been taken up per mg of cell protein in 5 hours. This is calculated from the amount degraded (35.1 µg) plus the amount within the cell (9.3 µg). If the cholesteryl ester had been taken up together with the 125I-albumin, then 22.2 µg of cholesteryl ester should have entered the cell. Figure 11 B shows that, at a protein concentration of 90
μg/ml, about 23 nmoles of cholesteryl [14C]oleate were formed per mg of protein in 4 hours. This amount is equal to the hydrolysis of 14.9 μg of cholesteryl linoleate, with subsequent re-esterification of the cholesterol. Since some of the cholesterol is known to be excreted without re-esterification, the observed uptake of 14.9 μg of cholesteryl ester in 4 hours agrees quite well with the predicted uptake of 22.2 μg in 5 hours, based on the value for 125I-albumin uptake.

The uptake mechanism for albumin/cholesteryl ester complex showed specificity in that the binding was inhibited by polyinosinic acid (figure 11), but not by polycytidylic acid (data not shown). However, this uptake mechanism differed from the acetyl-LDL mechanism in that the albumin/cholesteryl ester complexes competed only partially for the uptake and degradation of 125I-acetyl LDL (figure 12). Moreover, unlabelled acetyl-LDL competed only partially for the degradation of 125I-albumin/cholesteryl ester complexes. Further studies will be necessary to determine whether the albumin/cholesteryl ester complexes are entering macrophages by binding to the same site as the material in the aortic extract. Inasmuch as the “albumin” preparations may well be contaminated with other proteins including apolipoproteins, it will also be important in future studies to be certain that it is the albumin in the albumin/cholesteryl ester complex that is the functional protein component. That the albumin itself is the active agent is suggested strongly by the stoichiometry of the uptake process, as discussed above.

Although polyinosinic acid blocked the uptake of all cholesteryl ester complexes used in the current study, this compound does not have a non-specific effect on the uptake of all cholesteryl ester/protein complexes by macrophages. Polyinosinic acid does not block the uptake of β-VLDL, which enters macrophages by binding to a site that is clearly distinct from all of the sites discussed above, nor does it block the uptake of LDL/dextran sulfate complexes. Moreover, polyinosinic acid does not prevent the stimulation of cholesteryl esterification that is achieved by incubation of macrophages with 25-hydroxycholesterol plus cholesterol (figure 2).

Our current studies, together with the earlier ones in this series, demonstrate that macrophages, as contrasted with fibroblasts, are armed with specific mechanisms for taking up specific types of cholesteryl ester/protein complexes. Yet, macrophages do not simply take up all lipid/protein complexes. For example, macrophages do not take up large amounts of LDL, HDL, or VLDL isolated from the plasma of normal humans and animals. Moreover, these cells do not apparently take up intracellular cholesteryl ester droplets isolated from the adrenal gland, as reflected by the failure of these complexes to stimulate cholesterol esterification (figure 4).

It seems likely that the various macrophage cholesteryl ester uptake systems constitute a defense mechanism for clearing the interstitial space of lipoprotein-derived cholesteryl esters in animal tissues, including the aortic wall. Further studies of this process in vitro should provide insight into its possible role in vivo in the pathogenesis of atherosclerosis.

Acknowledgments

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References


Index Terms: cell surface • polyanions • low density lipoproteins • endocytosis • lysosomes
Stimulation of cholesteryl ester synthesis in macrophages by extracts of atherosclerotic human aortas and complexes of albumin/cholesteryl esters.

J L Goldstein, H F Hoff, Y K Ho, S K Basu and M S Brown

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