Obligatory Role of Cholesterol and Apolipoprotein E in the Formation of Large Cholesterol-enriched and Receptor-active High Density Lipoproteins*

The formation of large cholesterol-enriched high density lipoproteins (HDL<sub>1</sub>/HDL<sub>4</sub>) from typical HDL<sub>3</sub> requires lecithin:cholesterol acyltransferase activity, additional cholesterol, and a source of apolipoprotein (apo-E). The present study explores the role of apo-E in promoting HDL<sub>1</sub>/HDL<sub>4</sub> formation and in imparting to these lipoprotein particles the ability to interact with the apo-B,E(low density lipoprotein (LDL)) receptor. Incubation of normal canine serum with cholesterol-loaded mouse peritoneal macrophages resulted in the formation of HDL<sub>1</sub>/HDL<sub>4</sub>, that competed with <sup>125</sup>I-LDL for binding to the apo-B,E(LDL) receptors on cultured human fibroblasts. Cholesterol efflux from macrophages was necessary because incubation of normal canine serum with nonloaded macrophages did not cause HDL<sub>1</sub>/HDL<sub>4</sub> formation. However, cholesterol delivery to the serum was not sufficient to result in HDL<sub>1</sub>/HDL<sub>4</sub> formation. Apolipoprotein E had to be available. Incubation of apo-E-depleted canine serum with cholesterol-loaded J774 cells, a macrophage cell line that does not synthesize apo-E, demonstrated that no HDL<sub>1</sub>/HDL<sub>4</sub> formation was detected even in the presence of significant cholesterol efflux. However, addition of exogenous apo-E to the serum during the incubation with cholesterol-loaded J774 cells promoted the formation of large receptor-active HDL<sub>1</sub>/HDL<sub>4</sub>. The receptor binding activity of these particles produced in vitro correlated with the amount of apo-E incorporated into the HDL<sub>1</sub>/HDL<sub>4</sub>. Apolipoproteins A-I and C-III were ineffective in promoting HDL<sub>1</sub>/HDL<sub>4</sub> formation; thus, apo-E was unique in allowing HDL<sub>1</sub>/HDL<sub>4</sub> formation. These results demonstrate that when lecithin:cholesterol acyltransferase activity, cholesterol, and apo-E are present in serum, typical HDL can be transformed in vitro into large cholesterol-rich HDL<sub>1</sub>/HDL<sub>4</sub>, that are capable of binding to lipoprotein receptors.

The inverse correlation between plasma high density lipoprotein (HDL) levels and the development of coronary artery disease (1-3) has sparked considerable interest in the role of HDL in cholesterol homeostasis. The postulated antiatherogenic effect of HDL may involve its ability to promote the efflux of cholesterol from peripheral tissues and transport the cholesterol to the liver for excretion (for review see Refs. 4 and 5). In 1968, Glomset (6) proposed the concept of reverse cholesterol transport, but there is a paucity of experimental evidence defining the mechanism whereby HDL could participate in this process. However, it has been shown that HDL can acquire cholesterol from cells (7-12), that the HDL become enlarged when enriched with cholesterol and cholesteryl esters (which promote an apparent expansion of the cholesteryl ester-rich core), and that during this process the HDL particles acquire apolipoprotein (apo-E) (12). The presence of apo-E could direct the metabolism of these cholesteryl ester-enriched HDL particles. Apolipoprotein E mediates lipoprotein uptake by extrahepatic and hepatic apo-B,E(LDL) receptors and hepatic apo-E receptors (for review see Refs. 5 and 13) and binds with very high affinity to both the apo-B,E(LDL) and the hepatic apo-E receptors; apo-E could therefore efficiently mediate the uptake of these lipoproteins.

The HDL can be fractionated into several subclasses using a variety of techniques. However, for discussion in this paper, it would be useful to consider two metabolically distinct subclasses of HDL, HDL-without apo-E (HDL<sub>2</sub> and HDL<sub>3</sub>) and HDL-with apo-E (HDL<sub>1</sub> and HDL<sub>4</sub>) (see Refs. 12 and 14 for a discussion of this nomenclature). The HDL-with apo-E can be isolated because they bind to heparin-Sepharose affinity columns (14), or in certain species, such as the dog, the HDL-with apo-E can be observed as distinct lipoprotein subclasses by Pevikon block electrophoresis (15). The HDL-with apo-E (HDL<sub>1</sub>/HDL<sub>4</sub>) are the largest of the HDL (∼120-300 Å in diameter). They are enriched in cholesteryl ester and possess apo-E (12, 16), usually in association with apo-A-I (sometimes along with apo-A-II and the C apolipoproteins). By virtue of the presence of apo-E, this subclass of HDL is capable of interacting with the apo-B,E(LDL) and apo-E receptors. The HDL-without apo-E are the smaller HDL (∼70-100 Å). They possess apo-A-I as their principal apolipoprotein and are thought to be the precursors of the larger, cholesteryl ester-rich HDL-with apo-E (12).

Several lines of evidence support the concept that the HDL-without apo-E (HDL<sub>2</sub>/HDL<sub>3</sub>) can be converted to the larger, cholesteryl ester-enriched HDL-with apo-E. When canine serum is incubated with an exogenous source of cholesterol (cholesterol-Celite particles or cholesterol-loaded mouse peritoneal macrophages), Gordon et al. (12) have shown that HDL<sub>2</sub>/HDL<sub>3</sub> are reduced in concentration as the HDL<sub>1</sub>/HDL<sub>4</sub> concentration increases. In addition, in experiments using canine 125I-HDL<sub>3</sub>, it was demonstrated that these particles were converted to HDL<sub>1</sub>/HDL<sub>4</sub> as they acquired cholesterol.
bating human serum for 24 h at 37 °C resulted in the formation of HDL-like lipoproteins and a marked reduction in the presence of HDLs. This conversion required lecithin:cholesterol acyltransferase, and the production of cholesteryl esters appeared to drive the reaction toward formation of cholesteryl ester-enriched HDL1. Gordon et al. (12) demonstrated that the formation of HDL1/HDL2 in vitro, using cholesterol-Celite as a source of cholesterol, also required the presence of HDL3. This conversion required lecithin:cholesterol acyltransferase. In contrast, the cholesteryl esters formed by the transacylation reaction of lecithin:cholesterol acyltransferase reaction.

The present study examined the conditions necessary for the formation of HDL1/HDL2 in vitro. Two major questions were addressed: are HDL1/HDL2 that are formed in vitro capable of binding to lipoprotein cell-surface receptors and is apo-E required for the production of HDL1/HDL2? The results demonstrated that apo-E is required for the formation of the large HDL1/HDL2 and that apo-E from various sources (exogenously added, redistributed from other lipoproteins, or newly synthesized by cultured macrophages) can participate in this process. Finally, it was found that HDL1/HDL2 particles, regardless of their source of apo-E, are able to bind to the apo-B/E(LDL) receptors.

**MATERIALS AND METHODS**

**Experimental Protocol**—A schematic representation of the general protocol followed in the present studies is shown in Fig. 1. Mouse peritoneal macrophages or cells of the murine macrophage-like cell line J774.2 were maintained in culture as described below. The cells were plated in tissue culture flasks 24 h prior to cholesterol loading. Macrophages or J774 cells were loaded with cholesterol by incubating the cells for 24 or 48 h at 37 °C with acetoxylated LDL added at a concentration of 100 μg of lipoprotein cholesterol/ml in Dulbecco’s modification of Eagle’s medium (DMEM) (GIBCO, Grand Island, NY). In some studies, acetoxylated LDL (18) was added in the serum for incorporation into newly synthesized proteins. Selected studies were performed with the addition of 10^−5 M monensin (Calbiochem-Behring) or 10 ng/ml endotoxin (Sigma) to the 10% canine serum.

The cell-free medium was collected at the end of the incubation period, and the d < 1.06 g/ml fraction was removed by ultracentrifugation in a 50 Ti rotor (Beckman Instruments, Fullerton, CA) for 18 h at 50,000 rpm (4 °C). The d > 1.06 g/ml fraction was then analyzed against three changes of 0.15 M NaCl, 0.01% EDTA, and concentrated using Amicon Centrifllo cones (Lexington, MA). In some experiments, the d = 1.006-1.21 g/ml fraction was used instead of the d > 1.06 fraction. This fraction was obtained by increasing the density of the d > 1.06 fraction to d = 1.21 g/ml by the addition of NaCl, followed by ultracentrifugation in a 50 Ti rotor for 18 h at 50,000 rpm (4 °C). The d > 1.06 or the d = 1.006-1.21 fractions were separated by Pevikon block electrophoresis and eluted from the block fractions with 0.15 M NaCl, 0.01% EDTA as previously described (15). The Pevikon block was divided into 20 1-cm-wide zones, with divisions beginning at the origin and extending through the zone known to contain the most rapidly migrating HDL. The major cholesterol peak marked the position of HDL on the Pevikon block. The fractions between LDL and the HDL corresponded to the HDL1/HDL2 fractions, which are labeled 1, 2, and 3 in the various figures. The position of the lipoproteins vary slightly in different Pevikon blocks. Control and experimental samples were separated on the same Pevikon block to ensure appropriate comparison of the different fractions in each experiment, and fractions of equivalent migration distances were compared to each other. The lipoproteins eluted from the Pevikon were characterized by distribution of radiolabeled proteins and cholesterol, chemical composition, polyacrylamide gradient gel electrophoresis, and receptor binding activity. The cholesterol content of the fractions and unfractionated serum was measured by the enzymatic spectrophotometric assay from Bio-Dynamics (Boehringer Mannheim), and the protein concentration was determined by the method of Lowry et al. (20). The various procedures are outlined below.

**Macrophage Cultures**—Mouse peritoneal macrophages were isolated from female Swiss Webster mice (Taconic, NY) 4 days after an intraperitoneal injection of 1 ml of thioglycolate medium (21). The peritoneal cells (10 ml) were suspended in DMEM (supplemented with penicillin G [100 units/ml] and streptomycin sulfate [100 μg/ml]) at a concentration of 4 × 10^7 macrophages/ml and allowed to adhere to 75-cm² culture flasks (Falcon, Oxnard, CA) for 1.5 h at 37 °C. The nonadherent cells were removed by three washes with DMEM, and the adherent cells were incubated overnight in DMEM supplemented with 10% fetal bovine serum before cholesterol loading. The murine cell line, J774.2, was a gift from Dr. Zenas Werb, University of California, San Francisco, CA. The cells were cultured in DMEM with 10% fetal calf serum. Cells were plated at a density of 5 × 10^5/ml 24 h before cholesterol loading.

**Preparation and Analysis of Lipoproteins**—Human LDL (d = 1.02-1.05 g/ml) were isolated from the plasma of normal, fasted donors by sequential ultracentrifugation in a 60 Ti (Beckman) rotor at 59,000 rpm for 18 h at 4 °C as previously described (17). The LDL were then washed by recentrifugation at 59,000 rpm for 16 h at 4 °C at d = 1.05 g/ml (The 3H-LDL were prepared by the iodine monochloride method of Bilheimer et al. (22). Plasma was obtained from a dog fed a high cholesterol diet (23). The plasma was first centrifuged at 59,000 rpm for 16 h at 4 °C in a 60 Ti rotor. The d > 1.06 fraction was raised to a density of 1.087 g/ml with KBr and then centrifuged at 59,000 rpm for an additional 20 h. The d = 1.006-1.087 g/ml fraction was then dialyzed against saline with 0.01% EDTA and subjected to Pevikon block electrophoresis.
was bound in the absence of any competitors.

values 01 determined by measuring the ability of the fractions to compete with serum, and incubation was continued for 48 h at 37 °C. Gels were then destained in 5% methanol and 7% acetic acid and scanned with a Beckman DU-8 spectrophotometer at 510 nm.

Preparation of Apo-E-depleted Serum - Normal canine serum was depleted of apo-E by incubation with the anti-canine apo-E monoclonal immunoglobulin 25D2 (27) complexed to Sepharose 4B. The apo-E monoclonal antibody was prepared by the procedure previously described (27). To remove the apo-E canine serum (20 ml) was incubated at room temperature for 2 h with 40 mg of anti-apo-E coupled to Sepharose. The unbound fraction (apo-E-depleted serum) was then used in the experiments. The apo-E level was determined by radioimmunoassay as previously reported (28).

The receptor binding activities of the lipoprotein fractions isolated by Pevikon block electrophoresis were determined by measuring the ability of the fractions to compete with 125I-LDL, for binding to apo-B/E(LDL) receptors on cultured fibroblasts. Binding assays were performed at 4 °C as previously described (29). Human fibroblasts (0.9 × 10⁷) were grown in 16-mm wells in DME with 10% fetal calf serum. After 5 days, the medium was replaced with DME containing 10% (v/v) lipoprotein-deficient serum, and incubation was continued for 48 h at 37 °C. The cells were then used in the binding assays. The medium used in the binding assays was composed of DME containing 20 mg Hepes and 10% lipoprotein-deficient serum. The fibroblasts were incubated at 4 °C in medium containing varying amounts of the lipoprotein fractions and 125I-LDL at 2 μg of protein/ml for 2 h. The cells were then washed three times with cold, phosphate-buffered saline containing 2% bovine serum albumin. This was followed by two 10-min washes with 4 °C using the same buffer. The cells were then washed three times with cold, phosphate-buffered saline and then dissolved with 20 μl of 0.1 M NaOH. The radioactivity contained in the 1 ml sample was measured in a γ counter. Non-specific binding was defined as the amount of 125I-LDL bound in the presence of 200 μg of unlabeled LDL protein/ml. The amount of radioactivity specifically bound in the presence of lipoprotein fractions was expressed as a percent of maximum specific binding, i.e., the amount of 125I-LDL specifically bound in the absence of any competitors.

Calculation of Particle Core Radius and Shell Width - The per cent core volume can be estimated from the composition of the core components (cholesterol ester and triglycerides) of the various lipoproteins by the relationship

\[
\frac{V_{core}}{V_{total}} = \frac{\text{TG} \cdot \alpha_1 + \text{CE} \cdot \alpha_2 + \% \text{FC} \cdot \alpha_3}{\text{TG} \cdot \alpha_1 + \text{CE} \cdot \alpha_2 + \text{FC} \cdot \alpha_3 + \% \text{PC} \cdot \alpha_4 + \% \text{PL} \cdot \alpha_5 + \% \text{AP} \cdot \alpha_6}
\]

where \(\alpha_1\), CE, FC, PL, and AP are the concentrations of triglycerides, cholesterol ester, free cholesterol, phospholipid, and apolipoproteins, and \(\alpha_2\), \(\alpha_3\), \(\alpha_4\), \(\alpha_5\), and \(\alpha_6\) are the partial specific volumes for TG, CE, FC, PL, and AP, respectively. The \(V_{core}\) is the core volume, and \(V_{total}\) is the volume for the entire particle. Two assumptions were made in arriving at the above relationship. First, the core is assumed to contain all of the triglycerides and cholesterol esters and a fraction of the free cholesterol because free cholesterol has limited solubility in a cholesterol ester-rich core. Second, the partition coefficient of free cholesterol between the surface and the core is assumed to be 0.1, which reflects the maximum free cholesterol solubility in the core. The values \(\alpha_1 = 1.10, \alpha_2 = 1.07, \alpha_3 = 0.95, \alpha_4 = 0.98, \alpha_5 = 0.74 (30, 31),\) and the per cent values reported in Table 1 for the composition of the various fractions may then be used to calculate \(V_{core}/V_{total}\).

RESULTS

Characteristics of HDL Fractions Separated by Pevikon Block Electrophoresis - As previously demonstrated by Gordon et al. (12), various subclasses of canine HDL can be isolated by Pevikon block electrophoresis. In the present studies, HDL subclasses obtained from the plasma of a cholesterol-fed dog were isolated by Pevikon block electrophoresis and characterized (Table 1). The HDL subclasses from the plasma of a cholesterol-fed dog resemble the fractions obtained after incubation of normal canine serum with a source of cholesterol (to be described below). The d = 1.006–1.087 fraction of the plasma from a cholesterol-fed dog was subjected to Pevikon block electrophoresis as described under "Materials and Methods." The LDL fraction was located 4–7 cm from the origin. One-centimeter fractions extending from the end of the LDL region were obtained, and the lipoproteins were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 2A), as well as by polyacrylamide gradient gel electrophoresis (Fig. 2B). The fractions were numbered 1–5, with Fraction 1 located immediately adjacent to the LDL fraction and Fraction 5 located farthest from the origin.

Fig. 2B (gradient gel patterns) demonstrates that the size of the HDL decreased as the distance of migration increased. The HDL migrating farthest from the origin (12–16 cm, Fraction 5) represented predominantly HDL₀, i.e., HDL equivalent to the small (~70–100 Å in diameter), dense particles that lack apo-E. Fractions migrating closer to the origin (7–11 cm) were larger particles (HDL₁/HDL₂) that floated at a lower density and contained more cholesterol (Table 1) and apo-E (Fig. 2A). The largest HDL particles, representing Fraction 1, are the HDL₀.

Fig. 2A illustrates the apolipoprotein content of the various lipoprotein preparations. The largest HDL (Fraction 1) contained the most apo-E. The apo-A-I content increased with decreasing particle size. The chemical composition of each fraction was determined, and the particle sizes were measured from electron micrographs (Table 1). The larger particles contained more free cholesterol, as well as cholesterol ester, whereas the percentage of protein was lower as compared to that of the smaller particles.

Formation of Receptor-active HDL₀ and HDL₁ by Incubation of Normal Canine Serum with Cholesterol-loaded Macrophages - Thioglycolate-elicted mouse peritoneal macrophages were loaded with cholesterol by incubation with human acetylacetlated LDL, as described under "Materials and Methods." After the cells were loaded with cholesterol, normal canine serum (10%) in tissue culture medium was added to promote cholesterol efflux and to serve as a cholesterol acceptor. After 20 h of incubation with the cholesterol-loaded macrophages (or nonloaded, control macrophages), the medium containing the 10% canine serum was removed from the cells. The d < 1.006 lipoproteins were removed from the medium by ultracentrifugation, and the lipoproteins in the d > 1.006 fractions were separated by Pevikon block electrophoresis.

The distribution of total cholesterol and [35S]methionine-labeled proteins obtained by Pevikon block electrophoresis of
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TABLE I
Per cent chemical composition and particle size of HDL fractions obtained by Pevikon block electrophoresis

| Fraction | Diameter* | Protein | Phospholipid | Free cholesterol | Cholesteryl ester | Triglycerides | Core radius | Shell width* |
|----------|-----------|---------|--------------|-----------------|------------------|--------------|-------------|-------------|
| 1        | 266 ± 43  | 18.0    | 39.7         | 14.1            | 25.6             | 2.6          | 92.7        | 40.3        |
| 2        | 225 ± 21  | 18.7    | 37.3         | 13.1            | 28.3             | 2.6          | 80.6        | 31.9        |
| 3        | 188 ± 41  | 27.5    | 38.1         | 7.4             | 22.1             | 4.9          | 64.5        | 29.5        |
| 4        | 107 ± 11  | 40.4    | 37.8         | 4.0             | 15.8             | 2.0          | 32.3        | 21.2        |
| 5        | 93 ± 15   | 43.7    | 36.9         | 3.3             | 16.1             | ND*c         | 27.2        | 19.3        |

*The size of the particles was determined from negative staining electron microscopy observations. In each fraction, the diameters of 200 free-standing particles that were observed in the micrographs were measured. The mean values and standard deviations are presented.

* Calculated as detailed under "Materials and Methods."

* ND, not detectable.

The d > 1.006 fraction after incubation with cholesterol-loaded or nonloaded macrophages is shown in Fig. 3. As previously demonstrated (12), incubating canine serum with cholesterol-loaded macrophages results in a shift of the lipoprotein cholesterol distribution to slower-migrating lipoproteins (HDL with apo-E, i.e. HDL1 and HDLc) (Fig. 3). Macrophages labeled with [35S]methionine secrete newly synthesized [35S]methionine-labeled proteins, one of which has been identified as apo-E (32, 33). As shown in Fig. 3, the cholesterol-loaded macrophages secreted more [35S]methionine-labeled proteins than did the nonloaded cells, and the majority of the newly synthesized [35S]methionine-labeled proteins migrated in the HDLc/HDL1 region of the Pevikon block. Fluorography of SDS-polyacrylamide gels of the d > 1.006 fractions revealed that apo-E accounted for a large portion of the observed augmentation in protein secretion induced by cholesterol loading (Fig. 3, inset). Densitometric scans of these fluorograms revealed that apo-E accounted for 35% of the total [35S]methionine-labeled proteins secreted by the cholesterol-loaded cells and only 14% of the proteins secreted by the nonloaded cells. Because the total secretion of [35S]methionine-labeled proteins by the cholesterol-loaded cells was approximately 2-fold greater than that of the nonloaded cells, and at least 2-fold more of the labeled protein was apo-E, the actual increase in apo-E secretion was at least 4-fold greater in the cholesterol-loaded macrophages. Thus, since a major portion of the secreted proteins was apo-E, the majority of the [35S]methionine-labeled proteins would indeed be located in the HDLc/HDL1 region (between LDL and HDLc) on the Pevikon block.

The lipoproteins in the HDLc/HDL1 region of the Pevikon block were isolated and tested for receptor binding to cultured human fibroblasts. As shown in Fig. 4 (representing a typical experiment), the HDLc subfraction (equivalent to Fraction 3, Fig. 3) isolated from serum after incubation with cholesterol-loaded cells was much more effective than an equivalent fraction obtained from serum incubated with nonloaded cells in competing with 125I-LDL for binding to fibroblast apo-
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**FIG. 3.** Pevikon block electrophoretic pattern of the distribution of total cholesterol (μg/ml) and [35S]methionine-labeled proteins (cpm) in the d > 1.006 fraction of canine serum (10%) after incubation with cholesterol-loaded (C, ■, L) or nonloaded macrophages (O, O, NL). The inset shows a fluorogram of a 5–20% SDS-polyacrylamide gradient gel separation of the d > 1.006 serum fraction. As determined by SDS-polyacrylamide gel electrophoresis, the majority of the [35S]methionine-labeled proteins co-migrating with HDL1/HDL2 were [35S]methionine-labeled apo-E. (Fractions 1–3 correspond to the HDL2/HDL3 fractions.) The [35S]methionine-labeled proteins migrating in the LDL region, however, were not apolipoproteins. When d = 1.006–1.21 fractions of the serum were analyzed, no radiolabeled protein migrated in this region (see Fig. 5). Furthermore, no [35S]methionine-labeled apo-B was observed in any serum fractions.

**FIG. 4.** Ability of HDL1/HDL2 fractions from serum incubated with cholesterol-loaded (■, L) or nonloaded (O, NL) peritoneal macrophages to compete with 125I-LDL for binding to fibroblasts. The binding activities of the fractions (Fraction 3) correlated with the content of [35S]methionine-labeled apo-E, as illustrated by the fluorogram of a 5–20% SDS-polyacrylamide gradient gel (see inset). The 100% binding represented 112 ng of [35I]-LDL bound/mg of cell protein.

B,E(LDL) receptors. A fluorogram of the SDS-polyacrylamide gel of the HDL1 isolated from serum incubated with cholesterol-loaded macrophages revealed a much greater amount of newly synthesized [35S]methionine-labeled apo-E than was present in the HDL1 obtained from serum incubated with nonloaded cells (Fig. 4, inset). Densitometric scans of the fluorogram revealed that there was approximately 40-fold more [35S]methionine-labeled apo-E in Fraction 3 from serum incubated with cholesterol-loaded macrophages than in the corresponding fraction from serum incubated with nonloaded macrophages. As shown in Fig. 4, Fraction 3 from the cholesterol-loaded macrophages also demonstrated about 40-fold more receptor binding activity.

In addition, it was possible to demonstrate that the HDL1/HDL2 subfractions obtained from the serum after incubation with cholesterol-loaded cells possessed variable binding activity that correlated qualitatively with the amount of [35S]methionine-labeled apo-E present. As shown in Fig. 5, Fractions 1 and 2 (see Fig. 3 for migration characteristics), which represented the largest HDL1/HDL2, were more potent in...
competing with *'I-LDL for binding to fibroblasts
receptors. Three HDL, fractions were isolated by Pevikon
block electrophoresis from serum after incubation with cholesterol-
loaded macrophages and were numbered 1 (△), 2 (●), and 3 (□). (See
Fig. 3 for migration characteristics of Fractions 1–3.) The 100%
binding represented 90 ng of *'I-LDL bound/mg of cell protein. The
inset shows a fluorogram of a 5–20% SDS-polyacrylamide electropho-
retic gel pattern of the three fractions, which demonstrates the
amount of [35S]methionine-labeled apo-E in each fraction.

Effect of Inhibition of Macrophage Apo-E Secretion on the
Generation of HDL, Fractions—The HDL, fractions that demonstrated receptor binding activity could have obtained apo-E by transfer from other plasma lipoproteins, as well as from the macrophages (as evidenced by the presence of [35S]methionine-labeled apo-E) (34). To determine the relative importance of newly synthesized macrophage apo-E versus transferred apo-E in the formation of the HDL, fractions, apo-E secretion by cholesterol-loaded macrophages was inhibited by the addition of monensin or endotoxin to the tissue culture medium. Monensin alters receptor-mediated endocy-
tosis and cellular protein secretion (35, 36). Endotoxin pro-
duces a variety of responses in macrophages, including the inhibition of apo-E secretion (37, 38). Addition of monensin (10^{-6} M) or endotoxin (10 μg/ml) to cholesterol-loaded macrophages did not significantly alter cellular cholesterol efflux. The total cholesterol concentration in the serum after incubation with the cholesterol-loaded macrophages in the absence or presence of monensin or endotoxin was significantly elevated compared to the cholesterol concentration in serum incubated with nonloaded macrophages (total cholesterol concentration was increased from 1.84 mg/ml after incubation with nonloaded macrophages to 2.3 mg/ml after incubation with cho-

FIG. 6. Pevikon block electrophoretic distribution of [35S]
methionine-labeled protein in the d = 1.006–1.21 fraction
after incubation of the serum with cholesterol-loaded mouse
macrophages in the absence (□) or presence (○) of 10^{-6} M
monensin or with nonloaded macrophages (●).
the secretion of apo-E by mouse peritoneal macrophages, significant quantities of apo-E were still secreted. To eliminate the possibility of macrophage apo-E production, a mouse macrophage-like cell line (J774) that does not secrete apo-E was used (40). The procedures described above for the analysis of HDL\textsubscript{a}/HDL\textsubscript{c} formation were used to demonstrate that the J774 cells released cholesterol and resulted in the generation of HDL\textsubscript{a}/HDL\textsubscript{c} particles that could be isolated by Pevikon block electrophoresis. The distribution of lipoprotein cholesterol in the $d > 1.006$ fractions was essentially identical to that shown in Fig. 2.

The isolated HDL\textsubscript{a}/HDL\textsubscript{c} fractions obtained by Pevikon block electrophoresis were tested for receptor binding activity. As shown in Fig. 8 (data from a representative experiment), the HDL\textsubscript{a}/HDL\textsubscript{c} fraction (Fraction 1) from the serum incubated with cholesterol-loaded J774 cells showed significant ability to compete with $^{125}$I-LDL for receptor binding activity. By comparison, an equivalent fraction from the nonloaded J774 cells showed less binding activity. These results, along with those obtained from the use of monensin and endotoxin, demonstrated that receptor-active HDL\textsubscript{a}/HDL\textsubscript{c} could be formed in the absence of apo-E secretion by cells, presumably because apo-E was redistributed from other serum lipoproteins, such as very low density lipoproteins, to the HDL\textsubscript{a}/HDL\textsubscript{c} but not in the absence of cholesterol released from intracellular stores.

Inhibition of HDL\textsubscript{a}/HDL\textsubscript{c} Formation by Depletion of Apo-E from the Serum—The apo-E in canine serum can be depleted by immunoeffectivity chromatography using an anti-cane apo-E-Sepharose column. As determined by radioimmunoassay, 60-80% of the serum apo-E could be removed by the immunoeffectivity column. Normal canine serum or canine serum depleted of apo-E was added to cholesterol-loaded cells at a protein concentration equivalent to 10% normal canine serum. The $d > 1.006$ lipoproteins obtained after the incubation were subjected to Pevikon block electrophoresis; the fractions were characterized by polyacrylamide gradient gels stained for lipid and scanned by densitometry. As previously described (12), polyacrylamide gradient gel electrophoresis has revealed the presence of large ($\sim 210$ Å) and small ($\sim 150$ Å) HDL\textsubscript{a} and HDL\textsubscript{c} ($\sim 250$ Å) in dog serum. As shown in Table II (data from a typical experiment), when apo-E-depleted canine serum was incubated with the cholesterol-loaded J774 cells, there was no significant production of HDL\textsubscript{a} or HDL\textsubscript{c}. However, when normal canine serum was incubated with cholesterol-loaded J774 cells, an increase in the amount of HDL\textsubscript{a} and HDL\textsubscript{c} was observed. These results indicated that a source of apo-E (either from macrophages or from serum) was essential for the formation of the large, cholesterol-enriched HDL\textsubscript{a}/HDL\textsubscript{c}.

The HDL\textsubscript{a}/HDL\textsubscript{c} subfractions obtained from normal serum after incubation with cholesterol-loaded J774 cells were very potent in competing with $^{125}$I-LDL for binding to apo-B,E(LDL) receptors of cultured fibroblasts (Fig. 9A, B). However, an equivalent fraction obtained after incubation of apo-E-depleted serum with the cholesterol-loaded J774 cells displayed little or no receptor binding activity (Fig. 9A, C). Parallel experiments using mouse peritoneal macrophages...
Fig. 9. The receptor binding activities of HDL$_{a}$/HDL$_{b}$ subfractions from normal and apo-E-depleted canine serum after incubation with cholesterol-loaded J774 cells (A) or cholesterol-loaded mouse peritoneal macrophages (B). The 100% value represented 95 ng of $^{125}$I-LDL protein bound/mg of cell protein. A, a HDL$_{d}$/HDL$_{c}$ fraction (Fraction 1) obtained after incubation of cholesterol-loaded J774 cells with normal (■) and apo-E-depleted (□) canine serum. B, a HDL$_{d}$/HDL$_{c}$ fraction (Fraction 1) obtained after incubation with cholesterol-loaded mouse peritoneal macrophages with normal (■) and apo-E-depleted (□) canine serum.

Instead of J774 cells) revealed that the HDL$_{a}$/HDL$_{b}$ subfractions obtained from normal canine serum after incubation with the cholesterol-loaded peritoneal macrophages displayed potent receptor binding activity (Fig. 9B, ■). Furthermore, when apo-E-depleted serum was incubated with the cholesterol-loaded peritoneal macrophages, the HDL$_{a}$/HDL$_{b}$ subfractions possessed almost equal receptor binding activity (Fig. 9B, □). Thus, it appears that the newly synthesized apo-E produced by the cholesterol-loaded peritoneal macrophages actively participated in the formation of HDL$_{a}$/HDL$_{b}$ and was responsible for the receptor binding activity.

Effect of Exogenously Added Apolipoproteins on the Formation of Receptor-active HDL—The results from the experiments described above suggested that apo-E from plasma or apo-E synthesized by macrophages could be used in the formation of cholesterol and apo-E-enriched HDL. To determine whether or not exogenously added apo-E could be incorporated into these particles, cholesterol-loaded J774 cells were incubated with apo-E-depleted canine serum with or without the addition of human apo-E3 (2.5 μg of protein/ml of serum). The d > 1.006 fractions obtained after the incubation were analyzed by 2–16% polyacrylamide gradient gel electrophoresis to obtain a better resolution of the HDL$_{a}$/HDL$_{b}$ region of the gel. The results from a representative experiment are shown in Fig. 10. In the absence of added human apo-E, there was a paucity of large HDL subclasses (HDL$_{c}$ and HDL$_{d}$) (Fig. 10, dashed line). When human apo-E was added to the serum, receptor binding activity was observed (Fig. 10, solid line).

The receptor binding activity of the Pevikon fractions was assayed (Fig. 11). The results confirmed that in the absence of added apo-E virtually no HDL$_{a}$/HDL$_{b}$ were formed; therefore, no binding activity was observed (Fig. 11B, □). When apo-E was added, large HDL$_{a}$ were formed, and they were highly active in competing with $^{125}$I-LDL for receptor binding (Fig. 11B, ■). The addition of human apo-E to normal serum incubated with cholesterol-loaded J774 cells resulted in increased binding activity of the HDL$_{a}$/HDL$_{b}$ fraction (Fig. 11A).

To determine the specificity of the effect of apo-E on the formation of large HDL during cholesterol addition, other apolipoproteins were also tested. Apolipoproteins A-I, C-III, and E2 were added separately to apo-E-depleted canine serum at concentrations of 2.5 μg/ml, and the serum was incubated with cholesterol-loaded J774 cells. The distribution of lipoproteins in the d > 1.006 fractions obtained after incubation was analyzed on 2–16% and 4–30% polyacrylamide gradient gels and quantitated by densitometric scanning of the lipid-stained gels. The results are summarized in Table III. The addition of apo-E3, as demonstrated above, resulted in a substantial increase in HDL$_{a}$ and HDL$_{b}$ formation during cholesterol delivery to the apo-E-depleted canine serum. Apolipoprotein E2(Arg$_{80}$ → Cys) was also effective in inducing the formation of large HDL. The addition of apo-A-I or apo-C-III did not result in a significant increase in HDL$_{a}$ or HDL$_{b}$. However, the addition of apo-A-I did result in the formation of HDL$_{d}$ and produced an increase in HDL$_{d}$. These experiments therefore support the conclusion that apo-E (either...
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### Table III
Summary of changes in lipoprotein distribution in apo-E-depleted canine serum containing exogenously added human apolipoproteins after incubation with cholesterol-loaded J774 cells

| Lipoprotein subclass* | Average increase ± S.E. | Lipid distribution | Apo-E-3 | Apo-E-2 | Apo-A-I | Apo-C-I11 |
|-----------------------|-------------------------|--------------------|---------|---------|---------|-----------|
| HDL,                  | 6.7 ± 5                 | ND                 | 10 ± 1  | ND      | ND      |           |
| HDL,                  | 9 ± 2                   | 2 ± 3              | 16 ± 0.8| 0.01 ± 0.01|         |           |
| HDL,                  | 72 ± 13                 | 23 ± 12            | 15 ± 20 | 0.1 ± 3 |         |           |
| HDL,                  | 55 ± 9                  | 55 ± 8             | 0.1 ± 0.1| 0.4 ± 0.4|         |           |
| LDL                   | 7 ± 3                   | 8 ± 8              | 0 ± 8   | 10 ± 30 |         |           |

*Defined according to Gordon et al. (12).

^Lipid distribution was determined by densitometric analysis of lipid-stained polyacrylamide gradient gels, and the results were calculated as average per cent increase in total area of each lipoprotein subclass over the area of each subclass in apo-E-depleted canine serum incubated with J774 cells without the addition of human apolipoproteins.

apo-E3 or apo-E2) is necessary for the formation of large HDL species during cholesterol delivery to canine serum.

### DISCUSSION
Data from tissue culture studies have shown that HDL can participate in the removal of cholesterol from cells (7–12). This might constitute an initial event in the process referred to as reverse cholesterol transport. According to this hypothesis, cholesterol leaves the cells as free cholesterol, which may be acquired by HDL either through the binding of the HDL particles to the cell surface (41, 42) or by diffusion of the cholesterol through the unstirred aqueous layer surrounding the cell and subsequent incorporation into the HDL particle (43). Eventually, the majority of the free cholesterol becomes esterified through the action of lecithin:cholesterol acyltransferase (6).

It has been shown that the HDL particles that acquire cholesterol from an exogenous source become cholesterol enriched, increase in size, and acquire apo-E (12). The studies by Gordon et al. (12) have demonstrated that the smallest HDL (HDL,) serve as precursors for the formation of the cholesterol-rich HDL, and HDL, which acquire apo-E as they become cholesterol enriched. The apo-E that associates with these particles could originate from other plasma lipoproteins (34) or may be newly synthesized and released from macrophages (32) or other peripheral cells (44).

The results of gradient gel electrophoresis, which was used to separate HDL particles on the basis of size, have demonstrated that three distinct subclasses of HDL-with apo-E could be formed when cholesterol was provided to serum either from cholesterol-Celite particles or cholesterol-loaded peritoneal macrophages (12). These included small HDL (140–160 Å in diameter), large HDL (180–200 Å), and HDLin (240–270 Å). These size increments appear to correlate with the formation of one, two, or three layers of cholesterol esters within the core of the small HDL,, large HDL,, and HDL,, respectively (12). It has also been demonstrated that lecithin:cholesterol acyltransferase activity was necessary for the enrichment of cholesterol esters in the formation of the HDL,,HDL, (12). The activation of lecithin:cholesterol acyltransferase by β-mercaptoethanol enhanced the formation of HDL,,HDL,. Conversely, when the lecithin:cholesterol acyltransferase was inhibited by N-ethylmaleimide, HDL,,HDL, formation was profoundly depressed. The cholesterol esters formed in the HDL,,HDL, were not due to cholesterol ester exchange activity since canine serum is noted for its lack of this enzymatic activity (45).

The presence of apo-E on these HDL particles could be expected to profoundly alter the metabolism of the lipoproteins by virtue of the ability of apo-E-containing lipoproteins to interact with both apo-B,E(LDL) and apo-E receptors (13). Theoretically, these lipoproteins could participate in the redistribution of cholesterol among the various cells that possess the apo-B,E(LDL) receptors, or they could be taken up by the liver by apo-B,E(LDL) or apo-E (remnant) receptors, subsequently eliminating cholesterol from the body. Alternatively, the cholesteryl esters may be transferred from the HDL to very low density lipoproteins or LDL, and the cholesterol could then be delivered to the liver by this indirect pathway. Either the direct or indirect delivery of the HDL cholesterol to the liver would be compatible with the reverse cholesterol transport hypothesis (for review see Refs. 4, 5, 13, and 46).

Large HDL enriched in cholesterol, cholesteryl esters, and apo-E are present in the plasma of animals and humans (5, 18). The HDL-with apo-E, which resemble the small HDL, formed in vitro, are present in significant concentrations in human cord blood (28); however, they represent a minor constituent of the HDL in adult humans (47). In patients with abetalipoproteinemia, a condition in which there is an absence of very low density lipoproteins and LDL and in which the HDL are the major cholesterol-carrying lipoproteins, the HDL-with apo-E (HDL,) represent a major subclass of the HDL (28, 48). In these patients, it appears that the HDL-with apo-E may be major lipoproteins responsible for transport and redistribution of cholesterol among peripheral cells possessing the apo-B,E(LDL) receptors or hepatic cells possessing both apo-B,E(LDL) and apo-E receptors. Diets high in fat and cholesterol induce an increase in the concentration of HDL-with apo-E in both humans (49) and various animals (4, 5, 18). In certain species, high fat and high cholesterol diets induce marked increases in the large, cholesteryl ester-rich HDL, in which apo-E becomes the major or exclusive apolipoprotein constituent (4, 5, 23).

The present study establishes that the apo-E-containing HDL, in the plasma of normal canine serum possess the ability to bind to the apo-B,E(LDL) receptors of fibroblasts. Furthermore, it has been shown that the tissue or species source of the apo-E does not make a significant difference. In some experiments, the apo-E acquired by the cholesterol-enriched HDL, was newly synthesized and secreted by mouse peritoneal macrophages. In other experiments using J774 macrophages, which do not synthesize apo-E, the apo-E acquired by the HDL, was either redistributed from other serum lipoproteins or was derived from apo-E added directly to serum previously depleted of apo-E. Likewise, monensin- or endotoxin-treated peritoneal macrophages produced much less (7- to 8-fold) apo-E than untreated macrophages, and in these studies, the HDL, in the macrophages acquired the apo-E from other lipoproteins. In all of these cases, the receptor binding activity of the HDL, correlated with the presence of apo-E, regardless of its source.

These studies also demonstrate an important role for apo-E in the formation of cholesteryl ester-enriched HDL, in the plasma of normal canine serum. The HDL,,HDL, could be formed only under conditions in which apo-E was synthesized by macrophages, redistributed by other serum lipoproteins, or added exogenously. In control studies in which apo-A-I or apo-C-I11 was substituted for apo-E, the addition of these apolipoproteins did not result in the formation of the HDL,,HDL, (Table IIII). Human apo-E2 was nearly as effective as human apo-E3 in eliciting the formation of the cholesterol-enriched HDL, in the plasma of normal canine serum. However, apo-E in...
When apo-E-depleted serum was incubated with cholesterol-loaded J774 cells that did not synthesize apo-E, the cells still released cholesterol; however, virtually no HDL\textsubscript{L}/HDL\textsubscript{H} were formed. Under these conditions, the cholesterol released (~75% of the level released in the presence of apo-E-containing serum) was principally associated with HDL\textsubscript{L}/HDL\textsubscript{H}. Hence the relative absence of apo-E in the serum did not substantially affect the release of cholesterol by cholesterol-loaded cells.

The formation of large HDL\textsubscript{L}/HDL\textsubscript{H} requires the acquisition of apo-E and cholesterol and the subsequent formation of cholesteryl esters. Previously, Gordon et al. (12) speculated that as the particles increase in size, there was an increase in the cholesteryl ester layers from 1 to 3 within the core of the HDL\textsubscript{L}/HDL\textsubscript{H}. An analysis of the particle size and chemical composition of various HDL subclasses within the plasma of cholesterol-fed dogs (Table I) suggests that, as the particle increases in size, not only does the core expand but also a concomitant increase in the shell or surface constituents occurs. The increase in surface constituents could be accommodated by the shell if the particles assume a nonspherical configuration. For example, a sphere has the smallest surface area to volume ratio while an ellipsoid of equal volume would have a greater surface to volume ratio and would therefore be able to accommodate larger amounts of surface constituents. However, if a spherical shape is retained, the surface constituent could be accommodated only if there is an increase in shell width (Table I). Of course a combination of shape change and increase in shell width could also allow the accumulation of the larger amounts of surface constituents. Regardless of the exact model, it appears that apo-E is necessary for the formation of an expanded cholesteryl ester core (12, 50) and an increase in the shell width or surface area.

In conclusion, these in vitro studies have demonstrated a mechanism whereby cholesteryl ester-enriched HDL can be formed in plasma and participate in reverse cholesterol transport. The acquisition of apo-E by the cholesterol-loaded HDL\textsubscript{L}/HDL\textsubscript{H} enables the lipoproteins to interact with apo-E receptors. By virtue of the presence of apo-E, these particles can participate in the redistribution of cholesterol to other cells, including those of the liver. Furthermore, it has been established that apo-E is essential for the formation of large HDL\textsubscript{L} and HDL\textsubscript{H}. Apolipoprotein E appears to be uniquely capable of eliciting the expansion of the shell and the core of these particles to accommodate free cholesterol and additional layers of cholesteryl ester. Precisely how apo-E functions in the formation of these large HDL particles remains to be determined.

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