papers on methodology

Identification of apolipoprotein B-100 low density lipoproteins, apolipoprotein B-48 remnants, and apolipoprotein E-rich high density lipoproteins in the mouse

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Abstract Plasma lipoprotein fractions from inbred C57BL/6J mice and outbred ICR mice were prepared by sequential density ultracentrifugation using density ranges that were optimized for separating mouse lipoproteins, or by Superose 6 HR10/30 fast performance liquid chromatography (FPLC). The lipoproteins were characterized by migration behavior in agarose, apolipoprotein (apo) composition, lipid composition, and particle size distribution. Both sequential density ultracentrifugation and Superose 6 FPLC were adapted for the separation of lipoproteins from a single mouse. In the plasma of ICR and C57BL/6J mice, in contrast to human plasma, α-migrating high density lipoproteins (HDL) and β-migrating low density lipoproteins (LDL) had overlapping density ranges. For example, β-migrating apoB-100 LDL, slow pre-β-migrating apoB-48 remnants, and α-migrating HDL_1 were found together in the d 1.02–1.04 g/ml fraction. The d 1.04–1.06 g/ml fraction contained β-migrating apoB-100 LDL and α-migrating HDL_1. Large HDL_1 that were found at d 1.02–1.06 g/ml were apoE-rich HDL_1, characteristic of cholesteryl ester transfer protein-deficient mammals. The d 1.10–1.21 g/ml fraction, in addition to α-migrating HDL, included unique slow β-migrating particles that contained apoE and apoA-I but was deficient in neutral lipids. These slow β-HDL eluted in the same FPLC fractions as dense α-migrating HDL. Compared to ICR mouse plasma, C57BL/6J mouse plasma contained more LDL and less HDL_1, which might contribute to the susceptibility of C57BL/6J and the resistance of ICR mice to the development of aortic fatty streak lesions when challenged with an atherogenic diet.—**de Silva, H. V., J. Más-Oliva, J. M. Taylor, and R. W. Mahley.** Identification of apolipoprotein B-100 low density lipoproteins, apolipoprotein B-48 remnants, and apolipoprotein E-rich high density lipoproteins in the mouse. *J. Lipid Res.* 1994. **35:** 1297–1310.

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The availability of many different genetically defined strains of mice is likely to provide valuable tools for studying the relationship of lipoprotein metabolism to cardiovascular disease. Atherosclerosis-susceptible and -resistant strains (1–3) have been identified with respect to the formation of aortic fatty-streak lesions in response to a cholesterol-supplemented diet. In addition, the ability to generate transgenic mice (4, 5) and the advancements made in gene ablation by homologous recombination in mice (6, 7) provide powerful tools to begin to address the in vivo role of specific apolipoproteins and other proteins involved in various aspects of lipid metabolism. However, detailed knowledge of mouse plasma lipoproteins and lipoprotein metabolism is lacking. Furthermore, the lack of cholesteryl ester transfer protein (CETP) activity in mouse plasma (8) results in a lipoprotein profile unlike that in humans. In the mouse, high density lipoprotein (HDL) is the primary cholesterol-carrying lipoprotein in plasma.

Preliminary surveys by LeBoeuf et al. (9) and by Lusis et al. (10) identified variations in lipoproteins between genetically defined inbred strains of mice. However, the characterization of mouse lipoproteins relied on the use of human plasma density ranges for lipoprotein separation, an approach that subsequently has proven inadequate for the isolation of mouse plasma lipoproteins. Camus et al. (11) undertook a more detailed characterization of mouse plasma lipoproteins using density gradient ultracentrifugation. Nine subfractions of mouse lipoproteins were examined, revealing an overlap in the density ranges of α-

Abbreviations: d, density; FPLC, fast performance liquid chromatography; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HDL, high density lipoproteins; LDL, low density lipoproteins; VLDL, very low density lipoproteins; IDL, intermediate density lipoproteins; CETP, cholesteryl ester transfer protein; LCAT, lecithin:cholesterol acyltransferase.

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migrating HDL and β-migrating low density lipoproteins (LDL). Camus et al. (11) also identified large HDL at low densities (HDL_1) similar to that of the rat (12) and dog (13). Subsequently, Jiao et al. (14) used Superose 6 fast protein liquid chromatography (FPLC) and nondenaturing gradient gel electrophoresis to identify LDL/HDL particle size variations in inbred strains of mice.

The goals of the current study were to characterize the specific lipoproteins found at different densities of plasma and to examine in detail the plasma lipoproteins from individual mice. The density ranges of mouse apoB-100 LDL, apoB-48 remnants, apoE-rich HDL_1, and dense HDL have been defined. Using both small-volume sequential density ultracentrifugation and gel filtration chromatography, we have established rapid methods for the isolation and characterization of lipoproteins from 200 μl to 1 ml of plasma. This characterization of the lipoproteins at each density range included their content of apolipoproteins, lipid compositions, and particle sizes as measured by negative staining electron microscopy. Two different strains of mice (C57BL/6J and ICR) were selected for study to determine the potential variation in lipoprotein profiles between strains. The C57BL/6J mice are susceptible to developing fatty-streak atherosclerotic lesions when maintained on a cholesterol-supplemented high-fat diet (3). In contrast, ICR mice are resistant to lesion formation when maintained on the same diet (H. de Silva, D. Sanan, J. M. Taylor, and R. W. Mahley, unpublished results). The ICR mouse is used widely for generating transgenic lines, and interest in this strain led to its inclusion in the present study.

EXPERIMENTAL METHODS

Lipoprotein isolation by sequential density ultracentrifugation

ICR mice were purchased from Charles River Laboratories (Wilmington, MA), and C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME). ICR and C57BL/6J mice 10–14 weeks of age, fed normal mouse chow (Purina 5001, 4% fat), were fasted for 4 h and bled by cardiac puncture using 1 mg/ml of EDTA.

Fasting was limited to 4 h to minimize stress to the animals prior to collection of blood. At this time, chylomicrons and their remnants are likely to have been cleared from circulation. Thus, the apoB-48 (as well as apoB-100) lipoproteins in plasma are derived almost exclusively from the liver by this time. In studies not described here, there was no significant difference in mouse plasma triglyceride levels between a 4 h and a 6 h fast, indicating that this time period had relatively stable lipid metabolism.

In the large volume analysis, lipoproteins from 60–200 ml of pooled mouse plasma were separated by sequentially adjusting the plasma to different densities by addition of potassium bromide followed by ultracentrifugation in a Beckman 60 Ti rotor, using tubes with a capacity of 39.5 ml. The d<1.006, d 1.006–1.02, and d 1.02–1.04 g/ml fractions were isolated after centrifugation at 50,000 rpm for 16 h at 4°C. The d 1.04–1.06 and d 1.06–1.08 g/ml fractions were centrifuged for 18 h; the d 1.08–1.10 and d 1.10–1.21 g/ml fractions were centrifuged for 24 h and 48 h, respectively, all at 59,000 rpm at 4°C. Lipoproteins that floated at each density were collected by tube-slicing and recentrifuged under the same conditions to remove contaminating plasma proteins. This analysis was carried out for four pooled volumes of ICR and C57BL/6J plasma.

In the small volume analysis, lipoproteins from single mice (n = 20) were separated by sequential density ultracentrifugation at the same density ranges in a TLA100.2 rotor (using a Beckman tabletop TL100 ultracentrifuge) with tubes having a capacity of 1 ml. Each sequential centrifugation was for 2.5 h at 4°C at 100,000 rpm, except the final d 1.10–1.21 g/ml centrifugation was for 4 h at 4°C at 100,000 rpm. Lipoproteins were not recentrifuged in this procedure. The recovered lipoprotein fractions were dialyzed against 150 mM NaCl, 10 mM Tris, pH 7.4, and 1 mM EDTA.

Lipoprotein isolation by Superose 6 fast performance liquid chromatography (FPLC)

Total plasma lipoproteins or lipoprotein fractions isolated by sequential density ultracentrifugation were separated by FPLC gel filtration using a single Superose 6 HR10/30 column (Pharmacia LKB, Piscataway, NJ) or two Superose 6 HR10/30 columns in series. Samples of 200 μl were applied to the column, and the eluate was collected in 0.5-ml fractions at a flow rate of 0.5 ml/min.

Agarose gel electrophoresis and immunoblot analysis

Equal volumes of isolated lipoprotein fractions were analyzed by electrophoresis in precast 1% agarose gels (Ciba Corning, Palo Alto, CA). The lipoproteins resolved by agarose gel electrophoresis were stained for neutral lipid by Fat Red 7B (Sigma, St. Louis, MO), according to the manufacturer's instructions, or they were transferred to nitrocellulose by blotting. For this latter procedure, wet nitrocellulose paper adjacent to three sheets of Whatman 3MM filter paper, all cut to the size of the gel, were placed on agarose gels for 30 min after electrophoresis. Following transfer, the nitrocellulose sheets were incubated with apolipoprotein-specific antibodies. The antibodies were detected by reaction with an ^{125}I-labeled secondary antibody followed by autoradiography, or with a horseradish peroxidase-conjugated secondary antibody followed by chemiluminescent detection (ECL Kit, Amersham, Arlington Heights, IL). Anti-human apoB and anti-human apoA-I were used to detect mouse apoB and

apoA-I, respectively; anti-rat apoE was used to detect mouse apoE.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroimmunoblot analysis

The apolipoproteins in equal volumes of each lipoprotein density fraction were analyzed by electrophoresis through denaturing 3–15% polyacrylamide gradient gels according to the method of Laemmli (15). The apolipoproteins that were separated by SDS-PAGE were stained with Coomassie Brilliant Blue or transferred to nitrocellulose sheets by electroblotting (16). Individual apolipoproteins were identified by reaction with specific antibodies as described above. The apolipoproteins in FPLC-separated fractions were identified by silver staining (17).

Negative staining electron microscopy

Lipoprotein fractions were diluted to 0.1 mg of protein/ml in 10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA and adjusted to 1% phosphotungstic acid, pH 7.0, and to 0.1% sucrose (18). The samples were dried onto Formvar carbon-coated grids and examined using a JEM 100 CXII transmission electron microscope (Jeol Ltd., Tokyo, Japan). The particle size distribution in each lipoprotein fraction was determined by measuring at least 200 particles using an Image-1/AT analysis system (Universal Imaging Corp., West Chester, PA).

Analytical methods

Protein concentrations were measured by the procedure of Lowry et al. (19). The triglyceride, total cholesterol, nonesterifed (free) cholesterol, and phospholipid contents of lipoprotein fractions were determined by colorimetric quantitation assays optimized for the necessary sensitivity range on an Abbott Spectrum Analyzer (Abbott Park, IL). The reagents for the determination of cholesterol (Product Number 290319) and triglycerides (Product Number 701912) were obtained from Boehringer Mannheim Diagnostics (Indianapolis, IN); the reagents for the determination of free cholesterol (Product Number 274-4109) and phospholipid (Product Number 996-54001) were purchased from Wako Chemicals USA, Inc. (Richmond, VA). The cholesteryl ester content of each lipoprotein fraction was determined by subtraction of the free cholesterol value from the total cholesterol value.

RESULTS

Distribution of mouse plasma lipoproteins

The lipoproteins in different density fractions that were separated by large volume ultracentrifugation followed by agarose gel electrophoresis and staining by Fat Red 7B are shown in **Fig. 1.** Separation of lipoproteins from small plasma volumes collected from individual mice yielded the same results as those from the large volumes (data not shown). A notable finding in both approaches was the appearance of both α-migrating and β-migrating lipoproteins in the d 1.02–1.08 g/ml range of fractions. This overlapping of α-migrating and β-migrating lipoproteins contrasts with the human plasma lipoprotein profile, where these lipoprotein classes have unique density ranges (20): human β-migrating LDL occur at d 1.019–1.063 g/ml, and human α-migrating HDL occur at d 1.063–1.21 g/ml (20). Consistent with the nomenclature used previously to describe rat (12) and canine (13) lipoproteins, mouse α-migrating lipoproteins that occur at $d < 1.06$ g/ml are referred to as HDL_1, and those at $d > 1.06$ g/ml as HDL.

In ICR and C57BL/6J plasma, the $d < 1.006$ g/ml fraction contained pre-β-migrating very low density lipoproteins (VLDL). The d 1.006–1.02 g/ml intermediate density lipoproteins (IDL) fraction consisted of two populations of particles migrating close together at a slow pre-β position. Three distinct particles were found at d 1.02–1.04 g/ml: β-migrating LDL (Fig. 1, band a), slow pre-β-migrating particles, shown subsequently to be apoB-48 remnants (Fig. 1, band b), and α-migrating HDL_1 (Fig. 1, band c). The d 1.04–1.06 g/ml fraction contained HDL_1 and LDL, while the d 1.06–1.08 g/ml fraction contained primarily HDL with minor amounts of LDL. The d 1.08–1.10 g/ml and d 1.10–1.21 g/ml fractions contained HDL.

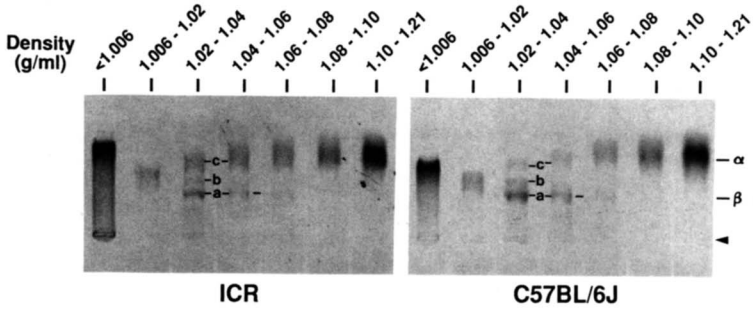

Fig. 1. Agarose gel electrophoresis of mouse plasma lipoproteins isolated by sequential density ultracentrifugation from ICR and C57BL/6J mice. Lipoproteins of the indicated densities were separated by electrophoresis on a 1% agarose gel and stained by Fat Red 7B. The labels are a, apoB-100 LDL; b, apoB-48 remnants; c, HDL_1. The arrowhead at the right indicates the migration origin of the agarose gels and the direction of migration is toward the α direction; the α and β symbols mark electrophoretic migration positions.

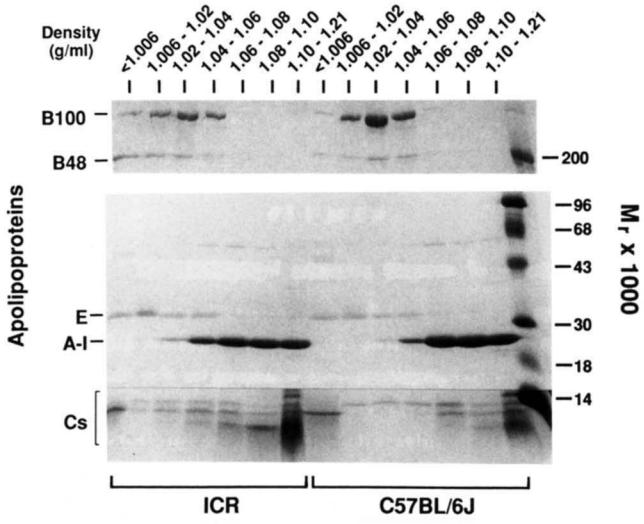

Fig. 2. SDS-polyacrylamide gel electrophoresis of isolated lipoprotein fractions of ICR and C57BL/6J mice. Lipoprotein fractions from ICR and C57BL/6J mice were isolated by sequential density ultracentrifugation and recentrifuged at the same density to remove plasma proteins. Aliquots of 15 μl for each density fraction were resolved by electrophoresis in denaturing 3–15% polyacrylamide gradient gels, then specific apolipoproteins were identified by electroimmunoblot analysis using apolipoprotein-specific antibodies.

While the lipoprotein profiles of ICR and C57BL/6J plasma were similar overall, there were subtle differences between the two strains. The lipoproteins in the d<1.006 g/ml fraction of C57BL/6J plasma migrated slower than those in the corresponding fraction of ICR plasma. In ICR plasma, HDL_1 (band c) was the predominant lipoprotein class in the d 1.02–1.06 g/ml fractions, whereas in C57BL/6J plasma, the LDL (band a) were the major lipoproteins in these density fractions.

Apolipoprotein composition of mouse plasma lipoproteins

Individual apolipoproteins in each fraction of ICR and C57BL/6J lipoproteins were separated by SDS-PAGE, then stained by Coomassie Brilliant Blue. **Figure 2** shows the apolipoprotein content of each density fraction isolated by large volume centrifugation. The d<1.006 g/ml fraction contained a mixture of apoB-48 and apoB-100. However, in the d 1.006–1.06 g/ml fractions, apoB-100 predominated. The d 1.02–1.06 g/ml fractions from C57BL/6J plasma contained increased levels of apoB-100 compared to those from ICR plasma. This finding was consistent with the increased levels of β-migrating LDL in these fractions of C57BL/6J plasma (Fig. 1). Apolipoprotein E was abundant in the d<1.06 g/ml fractions of both ICR and C57BL/6J plasma. Similar results were obtained after electrophoresis of density fractions isolated by small volume centrifugation of plasma from a single mouse. However, albumin was a major component of the d 1.10–1.21 g/ml fraction because these fractions were centrifuged only once (data not shown). Both apoA-I and the low molecular weight apoC components (apoA-II, apoC-I, apoC-II, and apoC-III) were abundant at higher densities. A relatively abundant peptide with an apparent M_r = 8,000 was detected in the d 1.06–1.21 g/ml fractions. As it was not observed in the lower density fractions, the M_r = 8,000 peptide could be apoA-II. It is noteworthy that this peptide is more abundant in ICR mice than in C57BL/6 mice, and may be associated with the minor differences observed in HDL size between the two strains.

Lipid composition of mouse plasma lipoproteins

Pooled plasma of fasting ICR mice (four separate pools, Tables 1 and 2) contained 130.0 ± 18.0 mg/dl of triglyceride, whereas C57BL/6J plasma contained 30.5 ± 5.7 mg/dl of triglyceride. Plasma cholesterol values were 91.3 ± 4.9 mg/dl and 60.5 ± 19.0 mg/dl for ICR and C57BL/6J mice, respectively. However, no major differences were observed in the percent distribution of individual lipid components among all lipoprotein fractions in each strain of mice (**Table 1**). Of the plasma triglyceride, 80% was in the d<1.006 g/ml fraction, whereas ~60% of the plasma cholesterol was in the d 1.10–1.21 g/ml fraction. Approximately 20% of the plasma cholesterol was recovered in the d 1.02–1.08 fractions that con-

TABLE 1. Percent distribution of lipid among plasma density fractions

Density	Lipoprotein Migration	Total Cholesterol		Phospholipid		Triglyceride	
		ICR	C57BL/6J	ICR	C57BL/6J	ICR	C57BL/6J
g/ml							
d < 1.006	preβ	8.3 ± 2.0	5.9 ± 1.0	11.8 ± 6.4	7.6 ± 2.0	82.0 ± 7.1	85.5 ± 1.2
1.006–1.02	slow pre β	3.2 ± 1.1	3.5 ± 1.1	2.7 ± 1.7	1.8 ± 0.8	8.5 ± 5.0	5.3 ± 2.4
1.02–1.08	α, slow pre β, β	22.5 ± 3.3	20.1 ± 3.3	17.6 ± 3.6	15.0 ± 3.6	5.7 ± 1.2	6.0 ± 1.2
1.08–1.10	α	10.7 ± 5.9	9.6 ± 4.5	11.4 ± 5.0	10.7 ± 5.3	2.0 ± 1.7	0.9 ± 0.7
1.10–1.21	α	55.4 ± 8.7	61.0 ± 8.4	56.4 ± 8.7	65.0 ± 10.7	1.2 ± 0.5	2.3 ± 1.0

The averages (± standard deviation) are values for density fractions from four pooled plasma separations (indicated as a, b, c, and d in Table 2) from ICR and C57BL/6J mice where the total lipid recovery was >80%. The relative amount of each major lipid class is indicated as a percent of the total amount of that lipid in all density fractions.

sisted of a mixture of β-, slow pre-β-, and α-migrating lipoproteins. The lipid composition of each density fraction is shown in **Table 2,** which shows the relative amount of the major lipid components in each fraction.

Size distribution of mouse lipoproteins

The lipoprotein size distributions in the various density fractions, as shown by negative staining electron microscopy, were similar in both ICR and C57BL/6J mice (**Fig. 3A**). Approximately 80% of the particles in the d < 1.006 g/ml fraction were 180–500 Å in diameter. Although three different classes of lipoproteins were found in the d 1.02–1.04 g/ml fraction, recognizable subpopulations of different sizes were not observed. In the d 1.10–1.21 g/ml fraction (Fig. 3G), the lipoproteins from ICR mice were distinctly larger than those from C57BL/6J mice.

Identification of mouse apoB-100 LDL, apoB-48 remnants, and apoE-rich HDL_1

The d 1.006–1.06 g/ml lipoprotein fractions contained α-migrating and β-migrating lipoproteins (Fig. 1) having apoB-100, apoB-48, apoE, and apoA-I components (Fig. 2). As these particles could not be separated by density centrifugation, they were resolved by agarose gel electrophoresis. Each of these different lipoprotein bands was excised from the gel, and the apolipoproteins were detected by SDS-PAGE and immunoblot analysis (**Fig. 4**).

The d 1.006–1.02 g/ml fraction had two major components (Fig. 4, left panel). The slower migrating particles (a) contained apoB-100 and apoE, and they were designated apoB-100 IDL. The faster migrating particles (b) contained primarily apoB-48 and apoE, and they were designated apoB-48 IDL. The d 1.02–1.04 g/ml fraction contained three distinct lipoproteins that were identified as apoB-100 LDL (band a), apoB-48 remnants (band b), and apoE-rich HDL_1 (band c) (Fig. 4, middle panel). In the d 1.04–1.06 g/ml fraction, the apoB-100 LDL contained almost no apoE, while the HDL_1 contained substantial amounts of both apoE and apoA-I (Fig. 4, right panel).

Apolipoprotein distribution among mouse plasma lipoproteins

The identification of apoB-100 IDL, apoB-100 LDL, apoB-48 IDL, apoB-48 remnants, and apoE-rich HDL_1 permitted a precise analysis of the apolipoprotein distribution among mouse lipoproteins. In this approach, the various ultracentrifugal fractions were resolved by agarose gel electrophoresis followed by the transfer of the lipoproteins from the agarose gel to nitrocellulose and reaction with specific antibodies (**Fig. 5**). The pre-β d < 1.006 g/ml lipoproteins contained both apoB-100 particles and apoB-48 particles (Fig. 2), and the d 1.006–1.02 g/ml fraction contained slow pre-β apoB-100 IDL and apoB-48 IDL. The apoB in the β-migrating lipoproteins (Fig. 5, band a) in the d 1.02–1.08 g/ml fractions is apoB-100 (Fig. 4), whereas the pre-β particles in the d 1.02–1.04 g/ml fraction (Fig. 5, band b) are apoB-48 remnants (Fig. 4).

Apolipoprotein E was found on all lipoproteins in the d < 1.006, d 1.006–1.02, and d 1.02–1.04 g/ml density fractions, but it was not present in apoB-100 LDL (band a) of the d 1.04–1.06 g/ml fraction. In the d 1.10–1.21 g/ml fraction that was isolated without recentrifugation, anti-apoE identified a slow β-migrating band in the lipoprotein fractions of both ICR and C57BL/6J plasma (Fig. 5, band d). This slow β particle is probably deficient in neutral lipids, as it did not stain with Fat Red 7B (Fig. 1). It was not present in the recentrifuged d 1.10–1.21 g/ml fraction (data not shown). Apolipoprotein A-I, in addition to its presence mainly in HDL, also was found in slow β-migrating lipoproteins of the d 1.10–1.21 g/ml fraction (Fig. 5, band d).

Characterization of lipoprotein density fractions by Superose 6 FPLC

The differences in size between the various plasma lipoprotein density fractions corresponded with their elution from Superose 6 columns by FPLC (**Fig. 6B**). To obtain maximum separation, two columns were used in series. Large triglyceride-rich VLDL and chylomicron remnants were eluted in the void volume, whereas lipoproteins of higher densities had successively greater retention times. Both ICR and C57BL/6J mouse plasma had essentially the same elution profiles; only the results of the ICR analysis are shown in Fig. 6. These results indicated that FPLC could be used as an alternative preparative method for isolating mouse plasma lipoproteins. However, similar to density centrifugation, gel filtration chromatography was unable to separate HDL_1, apoB-48 remnants, and apoB-100 LDL from each other in the d 1.02–1.04 g/ml density fraction. Additionally, gel filtration chromatography by FPLC was inadequate at separating apoB-100-containing particles from apoB-48-containing lipoproteins in all density fractions (Fig. 6C). These data, together with the results of negative staining electron microscopy (Fig. 3, panel C), suggested that these three classes of lipoproteins, at this density range, had similar sizes, making it necessary to use gel electrophoresis or other methods to separate these lipoproteins. Nevertheless, Superose 6 gel filtration is a rapid isolation method that avoids the potential artifacts of ultracentrifugation (21–25).

Analysis of whole plasma by FPLC confirmed that most triglyceride-rich lipoproteins eluted in the void volume, and cholesterol-rich lipoproteins had retention

TABLE 2. Lipid compositions of ICR and C57BL/6J mouse lipoprotein fractions

Density	Total Cholesterol	Phospholipid	Triglyceride	Free Cholesterol	Cholesteryl Ester
g/ml	*% of total lipid in each fraction*			*% of total cholesterol*	
ICR d < 1.006					
a	10	26	65	—	—
b	6	15	78	84	16
c	8	23	68	70	30
d	5	14	82	53	48
Mean ± SD	7.3 ± 2.2	19.5 ± 5.9	73.3 ± 8.1	69.0 ± 15.5	31.3 ± 16.0
ICR d 1.006–1.02					
a	19	29	52	—	—
b	21	28	51	52	48
c	23	36	41	50	50
d	30	5	65	59	41
Mean ± SD	23.3 ± 4.8	24.5 ± 13.5	52.3 ± 9.8	53.7 ± 4.7	46.3 ± 4.7
ICR d 1.02–1.04					
a	43	42	15	—	—
b	43	42	15	49	51
c	41	46	14	53	47
d	41	46	13	43	57
Mean ± SD	42.0 ± 1.2	44.0 ± 2.3	14.3 ± 1.0	48.3 ± 5.0	51.7 ± 5.0
ICR d 1.04–1.06					
a	45	48	7	—	—
b	48	44	8	41	59
c	39	56	5	39	61
d	32	43	25	46	54
Mean ± SD	41.0 ± 7.1	47.8 ± 6.0	11.3 ± 9.2	42.0 ± 3.6	58.0 ± 3.6
ICR d 1.06–1.08					
a	39	59	3	—	—
b	35	54	12	34	66
c	38	62	1	39	61
d	33	48	19	38	62
Mean ± SD	36.3 ± 2.8	55.8 ± 6.1	8.8 ± 8.3	37.0 ± 2.7	63.0 ± 2.7
ICR d 1.08–1.10					
a	31	56	12	—	—
b	34	54	12	28	72
c	39	61	0	29	71
d	33	54	14	31	69
Mean ± SD	34.3 ± 3.4	56.3 ± 3.3	9.5 ± 6.4	29.3 ± 1.5	70.7 ± 1.5
ICR d 1.10–1.21					
a	39	61	1	—	—
b	40	59	1	23	77
c	37	62	0	23	77
d	39	59	2	21	79
Mean ± SD	38.8 ± 1.3	60.3 ± 1.5	1.0 ± 0.8	22.3 ± 1.2	77.7 ± 1.2
C57BL/6J d < 1.006					
a	8	13	80	64	36
b	7	13	80	47	53
c	7	17	76	40	60
d	8	17	75	74	26
Mean ± SD	7.5 ± 6.0	15.0 ± 2.3	77.7 ± 2.6	56.3 ± 15.5	43.8 ± 15.5
C57BL/6J d 1.006–1.02					
a	41	27	32	59	41
b	41	27	32	35	65
c	28	30	42	49	51
d	33	29	39	45	55
Mean ± SD	35.7 ± 6.4	28.3 ± 1.5	36.3 ± 5.1	47.0 ± 10.0	53.0 ± 10.0
C57BL/6J d 1.02–1.04					
a	46	34	21	54	46
b	45	36	19	35	65
c	36	43	21	45	55
d	38	40	22	43	57
Mean ± SD	41.3 ± 5.0	38.3 ± 4.0	20.8 ± 1.3	44.3 ± 7.8	55.8 ± 7.8
C57BL/6J d 1.04–1.06					
a	42	37	21	47	53
b	38	44	18	36	64
c	43	45	11	43	57
d	43	44	13	45	55
Mean ± SD	41.5 ± 2.4	42.5 ± 3.7	15.8 ± 4.6	42.8 ± 4.8	57.3 ± 4.8
C57BL/6J d 1.06–1.08					
a	40	58	2	46	54
b	39	60	1	37	63
c	34	65	1	38	62
d	33	53	14	46	54
Mean ± SD	36.5 ± 3.5	59.0 ± 5.0	4.5 ± 6.4	41.8 ± 4.9	58.3 ± 4.9
C57BL/6J d 1.08–1.10					
a	37	60	3	41	59
b	37	60	3	31	69
c	34	66	0	32	68
d	33	62	5	40	60
Mean ± SD	35.3 ± 2.1	62.0 ± 2.8	2.8 ± 2.1	36.0 ± 5.2	64.0 ± 5.2
C57BL/6J d 1.10–1.21					
a	36	63	1	25	75
b	37	62	1	25	75
c	35	64	1	31	69
d	38	61	2	23	77
Mean ± SD	36.5 ± 1.3	62.5 ± 1.3	1.3 ± 0.5	26.0 ± 3.5	74.0 ± 3.5

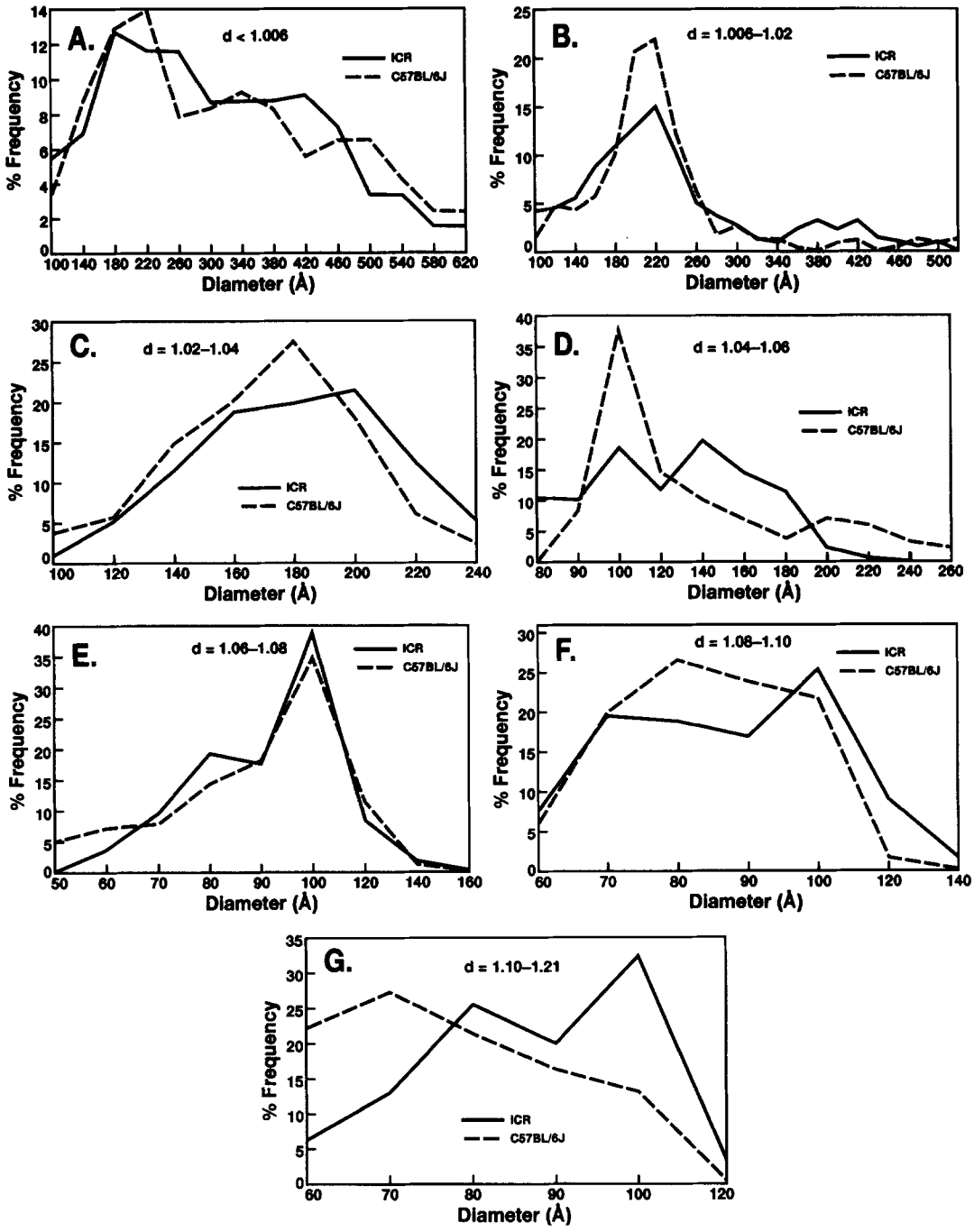

Fig. 3. Size distribution of lipoprotein particles. Plasma lipoprotein fractions from ICR (—) and C57BL/6J (---) mice were examined by negative staining electron microscopy as described in Methods.

times that corresponded to the elution of d 1.06–1.21 HDL (**Fig. 7A**). Both ICR and C57BL/6J plasma yielded the same results; only the ICR distribution profile is shown here. Immunoblot analysis of Superose 6 column fractions that had been separated by SDS-PAGE (Fig. 7B) showed that most of the apoE was in fractions 15–19, which corresponded to chylomicron remnants and VLDL, and in fractions 23–36, which corresponded to HDL_1. The remainder of the apoE was in smaller HDL (fractions 38–44). Apolipoprotein A-I was most abundant in fractions 30–48, corresponding to the elution positions of HDL other than HDL_1.

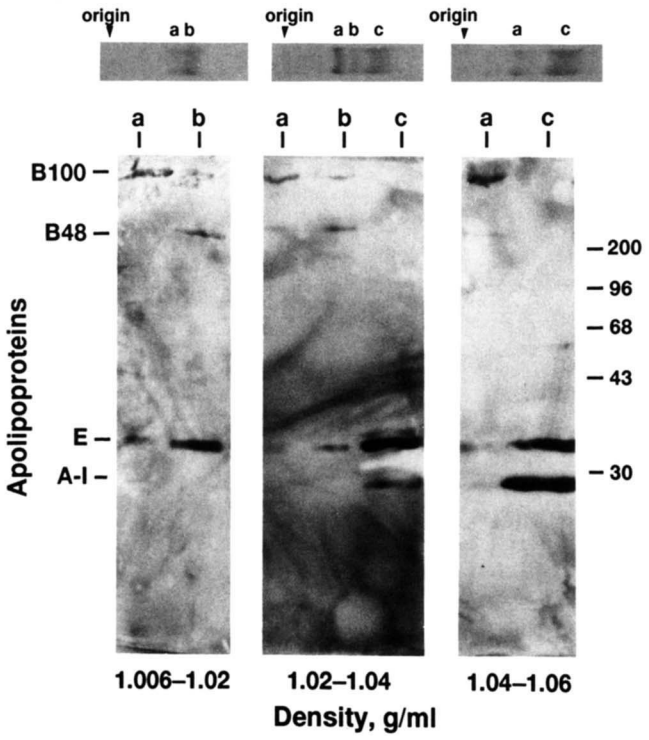

Fig. 4. Identification of apoB-100 LDL, apoB-48 remnants, and HDL_1. Top: ICR mouse d 1.006–1.02 (left panel), d 1.02–1.04 (middle panel), and d 1.04–1.06 (right panel) g/ml lipoprotein fractions (8 μl) were analyzed by agarose gel electrophoresis. The bands labeled a, b, or c were cut from agarose gels using an adjacent lane stained with Fat Red 7B for reference. Bottom: the lipoproteins in the agarose gel pieces were resolved by electrophoresis in denaturing 3–15% polyacrylamide gradient gels, transferred to nitrocellulose by blotting, and reacted with antibodies specific for apoB, apoE, and apoA-I.

Separation of mouse plasma lipoproteins by Superose 6 FPLC

As gel filtration is rapid and allows the separation of lipoproteins from as little as 200 μl of plasma, FPLC conditions using a single Superose 6 column were established. The elution of lipoproteins was monitored by the measurement of the cholesterol and triglyceride content of individual column fractions (**Fig. 8A**). Apolipoproteins B-48 and B-100 were in the VLDL eluate (fractions 17–20, Fig. 8B), whereas apoB-100 was more abundant than apoB-48 in the LDL/HDL_1-containing eluate (fractions 22–27, Fig. 8B). Apolipoprotein E was present in all fractions (in different amounts), reflecting its distribution among all classes of lipoproteins except LDL, whereas apoA-I was limited to smaller HDL classes. While this approach lacks the resolution of the two-column FPLC method, the single-column method permits rapid screening of mouse plasma.

The lipoproteins in each column fraction were separated by agarose gel electrophoresis, and the distribution of apoB, apoE, and apoA-I was examined by immunoblotting (Fig. 8C). The apoB was found on VLDL and IDL in fractions 16–20. In fractions 21–27, apoB-48 remnants (band b) could be distinguished from apoB-100 LDL (band a).

Apolipoprotein E was present on apoB-48 remnants (band b), HDL_1 (band c), smaller α-migrating HDL (fractions 28–33) and pre-β-HDL (band d) in fractions 29–31 (Fig. 8C). The pre-β-HDL (band d) correspond to the neutral, lipid-deficient slow β-HDL observed in the

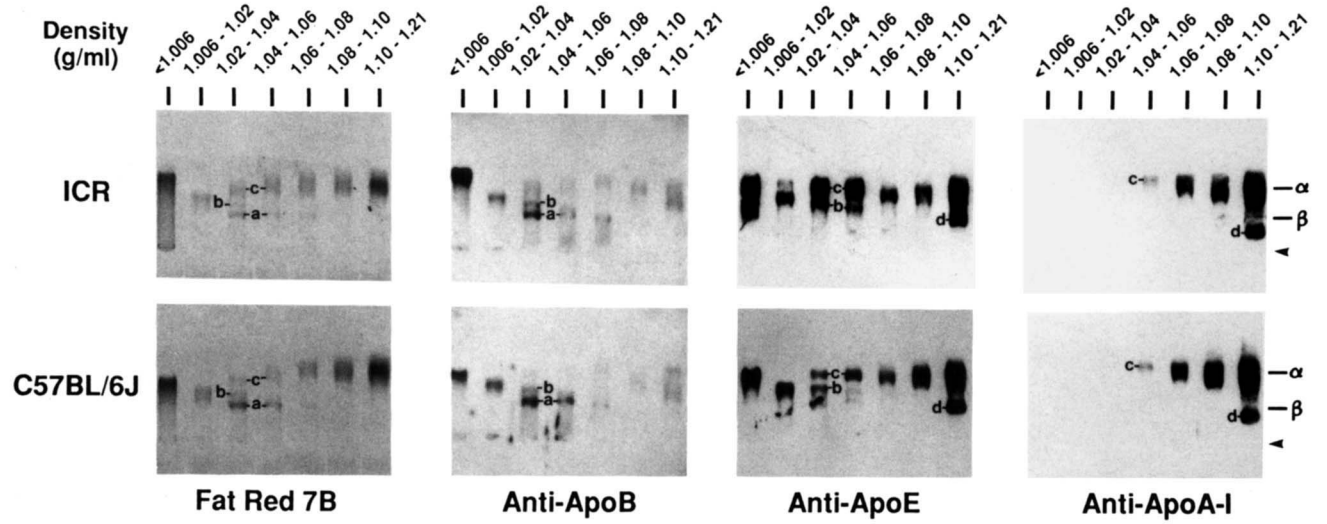

Fig. 5. Distribution of apolipoproteins among ICR and C57BL/6J mouse lipoproteins. Lipoprotein fractions (2 μl) from ICR (top) and C57BL/6J (bottom) mice were resolved by agarose gel electrophoresis, then detected by Fat Red 7B staining (left panel) or were transferred to nitrocellulose and reacted with antibodies specific to apoB, apoE, or apoA-I, as indicated. The labels indicate the following particles: a, apoB-100 LDL; b, apoB-48 remnants; c, HDL_1; d, neutral lipid-deficient HDL. The arrowhead at the right indicates the migration origin of the agarose gels; the α and β symbols mark electrophoretic migration positions.

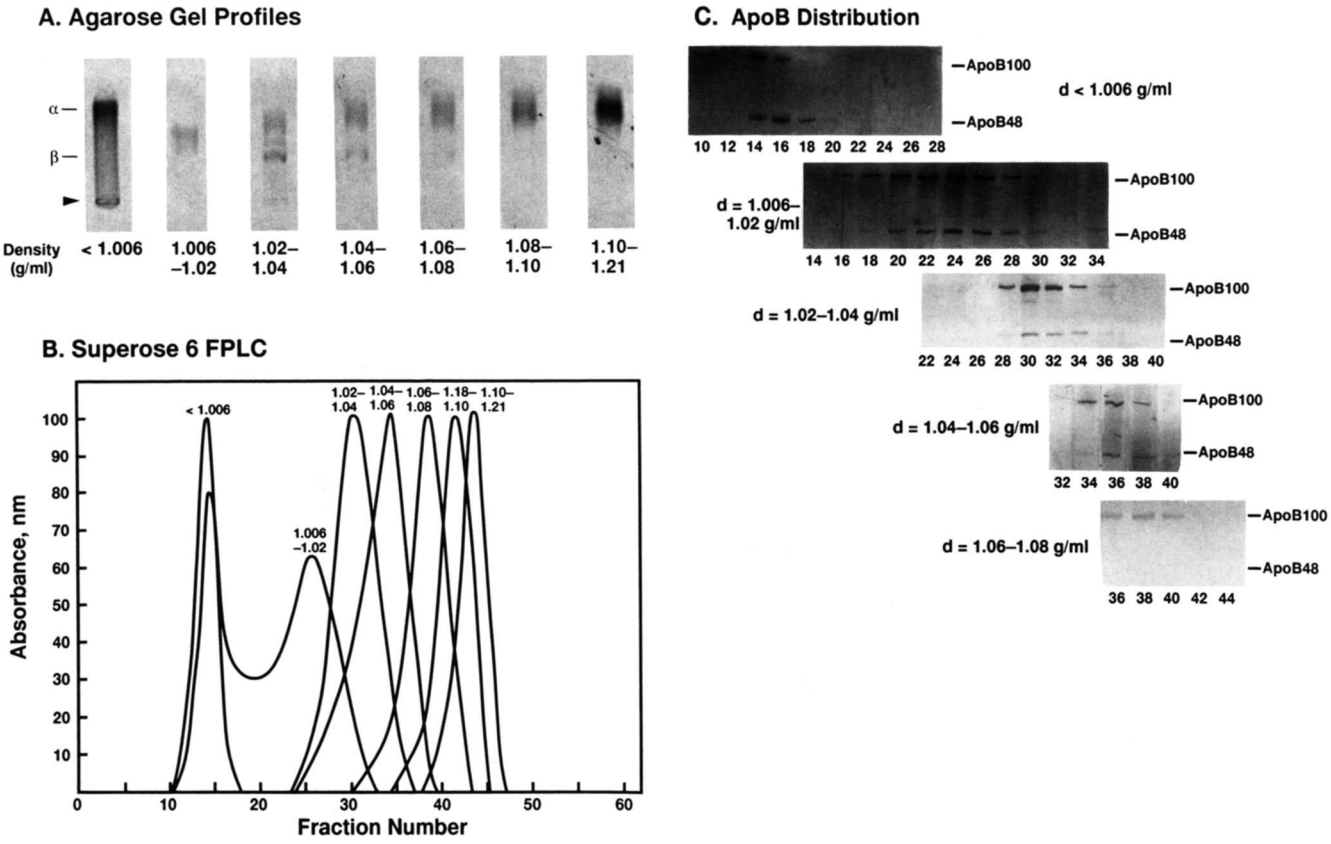

Fig. 6. ICR mouse plasma lipoprotein fractions separated by gel filtration after Superose 6 FPLC. Lipoprotein density fractions from the plasma of ICR mice (200 μl) were separated by agarose gel electrophoresis (panel A) and FPLC using two Superose 6 HR10/30 columns in series (panel B). The elution pattern of each lipoprotein fraction (labeled on top) after gel filtration is shown in an integrated profile. FPLC fractions from each profile were analyzed for apoB-100 and apoB-48 by 3–15% SDS-polyacrylamide gradient gel electrophoresis followed by silver staining (panel C).

d 1.10–1.21 g/ml density fraction that were identified previously (Fig. 5, band d). Apolipoprotein A-I also was found on these slow β-HDL, as well as on α-migrating HDL in fractions 28–33 as shown in Fig. 8C.

DISCUSSION

In this study we compared the normal lipoprotein profiles of two strains of mice: C57BL/6J, an inbred strain that is susceptible to atherosclerosis when placed on a diet high in fat and cholesterol (3), and ICR, an outbred strain that is resistant to developing fatty-streak lesions (H. de Silva, D. Sanan, J. M. Taylor, and R. W. Mahley, unpublished results) when placed on a defined atherogenic diet.

We have analyzed the development of fatty-streak lesions in female ICR mice fed a 30% fat, 1.25% cholesterol diet for 14 weeks (n = 20), 5 months (n = 16), and 13 months (n = 10). Cholesterol levels were increased 2- to 3-fold at each sampling interval as compared to age-matched ICR mice fed Purina 5001 mouse chow (4.5% fat). At 14 weeks, 5 months, and 13 months, the mice were exsanguinated and the heart and aortic arch were dissected and fixed in 4% paraformaldehyde. Serial sections of 10 mm were prepared starting at 100 mm below the aortic sinus and continued up to the curve of the aortic arch. No significant differences were observed in ICR mice maintained on the atherogenic diet and chow diet with regard to lipid deposition in the vessel wall as determined by Oil Red O staining (H. de Silva, D. Sanan, J. M. Taylor, and R. W. Mahley, unpublished results).

In addition, we have described the separation of mouse plasma lipoproteins using three approaches: conventional large-volume sequential density ultracentrifugation using density ranges optimized for mouse lipoproteins, small-volume sequential density ultracentrifugation, and Superose 6 FPLC. Small-volume sequential density ultracentrifugation offers significant advantages over conventional ultracentrifugation: small volumes of plasma from just one or two mice can be examined at one time, and the collection time for recovering density fractions is short.

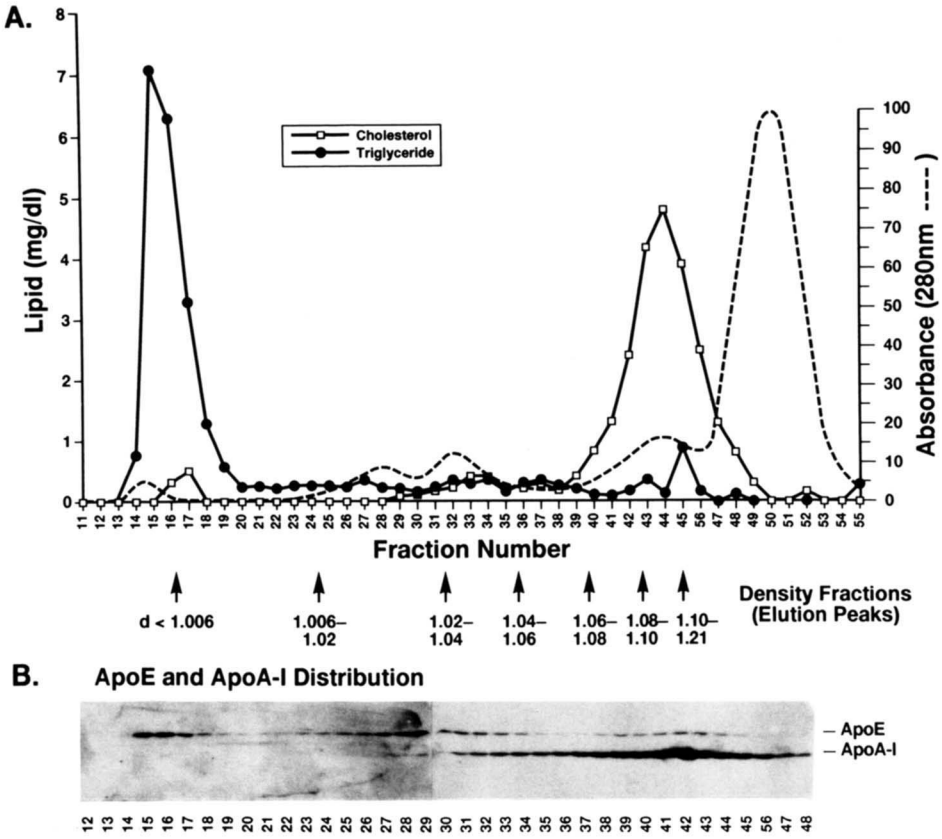

Fig. 7. The distribution of lipid, apoE, and apoA-I among lipoproteins separated by Superose 6 HR10/30 FPLC. ICR mouse plasma (200 μl) was resolved by FPLC using two tandem Superose 6 columns. Panel A: the cholesterol (□) and triglyceride (●) distribution in the plasma elution fraction was measured. The integrated absorbence profile at 280 nm for mouse plasma is indicated (---). The positions corresponding to the elution peaks for density fractions are included for reference. Panel B: the distribution of apoE and apoA-I across the FPLC profile (50 μl/fraction) was determined by SDS-PAGE and electroimmunoblot analysis using antibodies specific for apoE and apoA-I.

Furthermore, plasma lipoproteins isolated by density ultracentrifugation are relatively free of contaminating plasma proteins, and the lipoprotein samples are concentrated as a result of flotation. However, some apolipoprotein components might dissociate during flotation (21–25), and a lengthy processing time is required to obtain samples for analysis. As might be expected, gel filtration chromatography using Superose 6 FPLC is a rapid method for separating lipoproteins, and it is especially useful for studying lipoproteins in small volumes of plasma.

By themselves, each of these lipoprotein separation methods is inadequate to fully resolve mouse plasma lipoproteins. Each method must be used in combination with an additional separation technique to identify individual classes of lipoproteins in most fractions. For example, the d 1.02–1.04 g/ml density fraction contains three different lipoprotein classes (HDL_1, apoB-48 remnants, and apoB-100 LDL) that are not fully resolved by Superose 6 FPLC. Agarose gel electrophoresis can provide the next level of separation required for the analysis of the individual lipoproteins in each method. In addition, the combination of SDS-PAGE and electroimmunoblotting permits a rapid determination of the apolipoprotein composition of plasma lipoprotein fractions. Failure to include additional separation techniques after ultracentrifugation or FPLC can lead to a misinterpretation of experimental results.

Characterization of mouse lipoproteins prepared by the three isolation methods yielded identical results and showed that mouse apoB-containing particles (both apoB-100 LDL and apoB-48 remnants) and large apoE-rich HDL_1 overlapped substantially in both size and density. The apoB-100 LDL and apoB-48 remnants could be distinguished by agarose gel electrophoresis on the basis of their respective β- and pre-β-migrations. Similarly, apoB-100- and apoB-48-lipoproteins were resolved by agarose gel electrophoresis into faster-migrating apoB-48 IDL and slower-migrating apoB-100 IDL in the d 1.006–1.02 g/ml fraction. However, they were indistinguishable

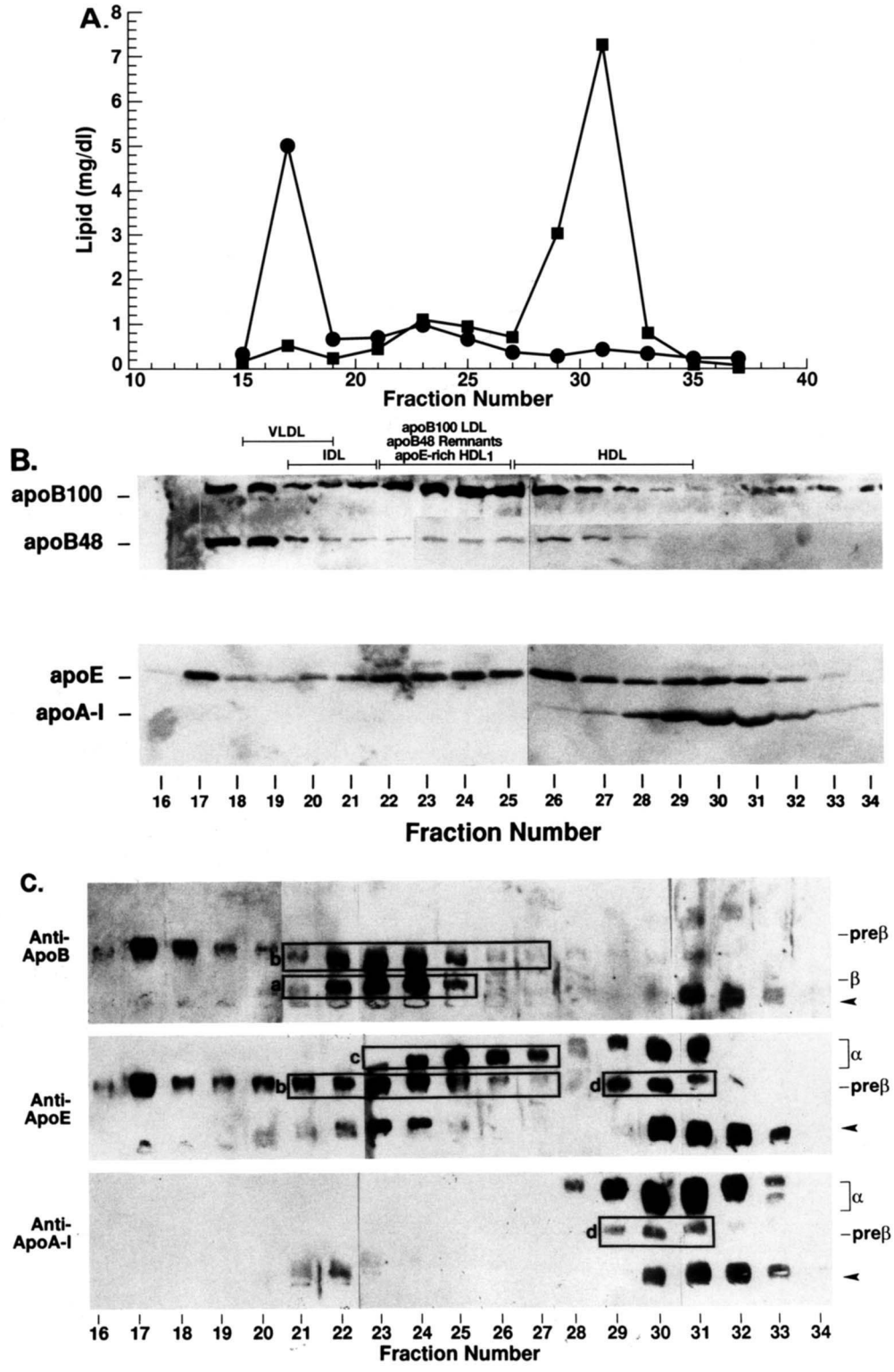

Fig. 8. Rapid separation and analysis of mouse plasma lipoproteins. Panel A: ICR mouse plasma (200 μl) was resolved by FPLC using a single Superose 6 column. The cholesterol (■) and triglyceride (●) distribution as determined by lipid analysis of the gel filtration fractions is shown. The fractions containing VLDL, IDL, apoB-100 LDL, apoB-48 remnants, apoE-rich HDL_1, and HDL, as determined by immunoblot analysis, are indicated below the plasma lipid profile. Panel B: electroimmunoblot analysis of lipoproteins separated by Superose 6 FPLC. Plasma gel filtration fractions (50 μl) were resolved by electrophoresis of 3–15% denaturing polyacrylamide gels, and the distributions of apoB-100, apoB-48, apoA-I, and apoE were determined by electroimmunoblotting using specific antibodies. Panel C: the FPLC fractions were resolved by agarose gel electrophoresis, transferred to nitrocellulose by passive diffusion, and immunoblotted using antibodies to apoB, apoE, or apoA-I. The labels are as follows: a, apoB-100 LDL; b, apoB-48 remnants; c, apoE-rich HDL_1; d, neutral lipid-deficient HDL. The α, pre β, and β migration positions are indicated at right, and the origin of the agarose gels is indicated by the arrowhead.

from one another in the d < 1.006 g/ml fraction. Unlike in human plasma, mouse apoB-48 particles extended to d 1.04 g/ml, and mouse apoB-100 particles were found up to d = 1.08 g/ml.

Mouse HDL were heterogeneous in size and composition and were found at d 1.02–1.21 g/ml. The large HDL_1 that occurred at d 1.02–1.06 g/ml were apoE-rich, while d > 1.06 g/ml HDL contained both apoE and apoA-I. Approximately 60% of the plasma cholesterol was found in the d 1.10–1.21 g/ml HDL. In addition to α-migrating HDL, slow β-migrating, neutral lipid-deficient HDL particles having both apoE and apoA-I were identified in the d 1.10–1.21 g/ml fraction. These particles also were found in the small HDL fractions eluted from the Superose 6 FPLC column. They are similar to the pre-β-migrating particles, described by Ishida, Frolich, and Fielding (26), isolated from mouse plasma after gradient density centrifugation at d > 1.08 g/ml. However, Ishida et al. (26) noted the association of only apoA-I with the pre-β-HDL particles. This apparent discrepancy in the data may be due to the presence of multiple forms of pre-β-HDL that contain apoE and/or apoA-I. The lipid-deficient HDL particles in the mouse may be analogous to human pre-β-HDL particles (27–29) postulated to be precursors to larger HDL. In hyperlipidemic patients, an increase in apoA-I pre-β-HDL was noted (27), suggesting a relationship between α-migrating HDL and small, lipid-deficient pre-β-HDL. The slow β-HDL in mice may be derived from the surface components of triglyceride-rich chylomicrons and VLDL as a consequence of catalysis by lipoprotein lipase and possibly hepatic lipase during circulation. However, the origin and function of the slow β-HDL require further study.

Several differences were observed in the lipoprotein profiles between ICR and C57BL/6J mice. The d < 1.006 g/ml fraction from C57BL/6J mice had a marked electrophoretic retardation on agarose gels, which is likely to be the consequence of a lipid or apolipoprotein composition difference. Additionally, the HDL of the d 1.08–1.10 and d 1.10–1.21 g/ml fractions had larger average diameters in ICR mice than in C57BL/6J mice. These size differences appeared to reflect the relative amount of the M_r = 8,000 putative apoA-II peptide found in these HDL (Fig. 2A). The HDL_1 levels were higher in ICR mice, whereas LDL levels were higher in C57BL/6J mice. ICR mice contained higher fasting plasma triglyceride levels than C57BL/6J mice. Overall, these differences may affect the susceptibility or resistance of each strain to the development of fatty-streak lesions when challenged with an atherogenic diet.

The distribution of plasma lipoproteins that we observed in both ICR and C57BL/6J mice can be interpreted according to the scheme illustrated in **Fig. 9.** The small HDL in the d 1.10–1.21 g/ml and d 1.08–1.10 g/ml fractions presumably acquire excess cholesterol from peripheral tissues and apoE from other lipoproteins or from HDL precursor particles. The cholesterol is esterified by lecithin:cholesterol acyltransferase (LCAT), an enzyme for which apoA-I is a cofactor (30, 31), and the dense HDL are converted to larger cholesteryl ester-rich HDL_1 particles. These large particles are apoE-rich but apoA-I-poor and are postulated to be cleared by a receptor-mediated mechanism that requires apoE. The d 1.02–1.04 g/ml fraction contains cholesteryl ester-rich, apoE-rich HDL_1 and apoB-48 remnants as well as apoB-100 LDL, accounting for ~20% of the total plasma cholesterol.

In the mouse, similar to other CETP-deficient mammals such as the rat (12) and dog (13), apoE-rich HDL_1 are postulated to function as a primary plasma source of

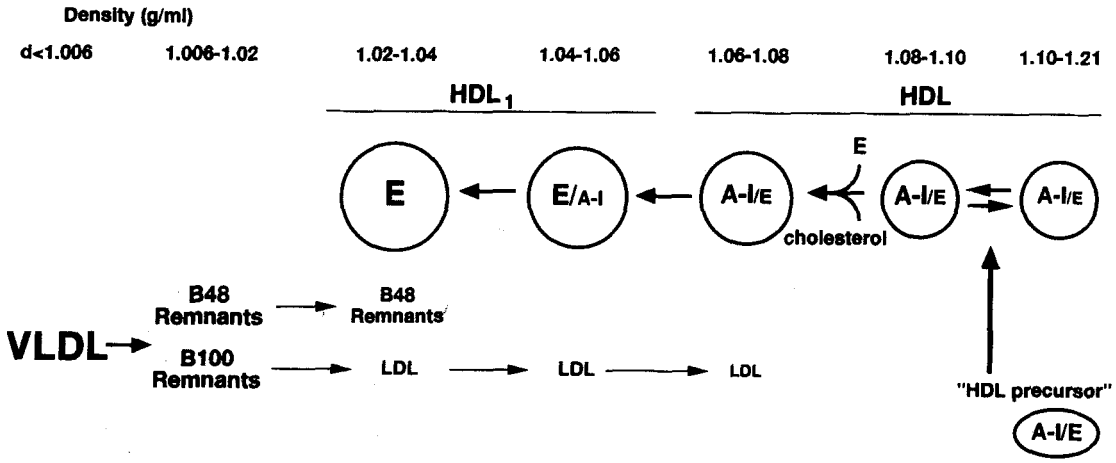

Fig. 9. Metabolic pathways of mouse plasma lipoproteins. These suggested pathways for mouse lipoprotein metabolism, as explained in the text, are consistent with the analysis of the composition and properties of plasma lipoproteins carried out in this investigation.

cholesterol for tissues. This postulate is supported by the results of Fig. 1, Fig. 5, and Table 1. In contrast to humans, HDL_1 in these animals is a major cholesterol-carrying lipoprotein, and apoE-containing large HDL_1 are prominent (32–35). In addition, humans with CETP deficiency (36, 37) have large HDL that are enriched in apoE and appear to be the human counterpart of mouse HDL_1 (36, 37). Cholesterol feeding of CETP-deficient animals results in a marked increase of large HDL_1 (also termed HDL_c), which can extend to low plasma densities ($d < 1.006$ g/ml) and may transport as much as 50% of the plasma cholesterol (38). The HDL_1 that are enriched in apoE bind to the LDL receptors with high affinity (39), and they can substitute for LDL as the major class of cholesterol-carrying plasma lipoproteins delivering lipids to peripheral tissues and the liver (38). The HDL_1 appear to be derived in plasma from small HDL that acquire cholesterol from extrahepatic tissues and are converted to the larger, lower density HDL_1 (13, 38).

Our understanding of the formation of large HDL from small HDL is influenced by the in vitro studies of Gordon, Innerarity, and Mahley (40). They used canine HDL and cholesterol-loaded macrophages or cholesterol-coated Celite particles to demonstrate that small HDL_3 were converted to large HDL_1 particles in the presence of excess cholesterol in an LCAT-dependent reaction. Koo, Innerarity, and Mahley (41) demonstrated that HDL_1 formation depended on the availability of apoE, which could be newly synthesized from cells, derived from other plasma lipoproteins, or exogenously added. In addition, apoE2, which binds the LDL receptor with reduced affinity (42, 43), was as effective as apoE3 in mediating HDL_1 formation. Apolipoprotein E was required for the expansion of the growing HDL core, but neither apoA-I nor apoC-III were sufficient to facilitate the formation of HDL_1 particles. The availability of transgenic mice and mutant mice null for the apolipoprotein genes and other genes involved in plasma lipid metabolism offers new experimental tools to delineate these mechanisms.

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