

# A species comparison of low density lipoprotein heterogeneity in nonhuman primates fed atherogenic diets

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**Abstract** Six male cynomolgus monkeys and five male African green monkeys were fed dietary cholesterol to induce hypercholesterolemia. The two groups studied had equivalent total plasma cholesterol concentrations. Low density lipoproteins (LDL) were isolated from whole plasma by ultracentrifugation and separated from other lipoprotein classes by agarose column chromatography. LDL were further subfractionated by density gradient ultracentrifugation in a VTi-50 vertical rotor. The material within five density regions was pooled from each sample and molecular weight, electrophoretic mobility, apoprotein heterogeneity, and percentage composition were determined for each subfraction. In general, cynomolgus monkey LDL were larger and more polydisperse than African green monkey LDL, and the LDL subfractions of cynomolgus monkeys were generally of lower densities although molecular weights at any density were in the same range for both species. ApoB-100 was the major apoprotein in each subfraction. ApoE was frequently present in the less dense subfractions while apoA-I was often seen in the more dense subfractions. Cynomolgus monkey LDL appeared to contain more apoE than African green monkey LDL. Over the entire spectrum of LDL, the percentage composition of the particles at any given density was indistinguishable between the species. In general, the average cynomolgus monkey LDL was larger, more polydisperse, less dense, and appeared to contain more apoE than the average African green monkey LDL. One or all of these differences might help explain the increased susceptibility to diet-induced atherosclerosis seen in cynomolgus monkeys. — Marzetta, C. A., and L. L. Rudel. A species comparison of low density lipoprotein heterogeneity in nonhuman primates fed atherogenic diets. *J. Lipid Res.* 1986. 27: 753-762.

**Supplementary key words** African green monkeys • cynomolgus monkeys • cholesterol • apoprotein B • ultracentrifugation • composition • molecular weight

The development of coronary artery atherosclerosis (CAA) has been positively correlated to cholesterol concentrations of low density lipoproteins (LDL) in humans (1) and to an increase in concentration as well as an increase in the average molecular weight of LDL in nonhuman primates (2-4). These observations point out the

importance of LDL in the pathogenesis of this disease and emphasize the need to understand more about factors that control LDL metabolism. Much has been learned about factors controlling LDL catabolism due to the pioneering work of Brown and Goldstein (reviewed in ref. 5) but factors regulating LDL formation are not well understood.

LDL are a heterogeneous class of lipoproteins that have been shown to vary in size (6, 7), density (7-12), composition (10-13), and metabolism (14, 15). This heterogeneity has been observed among normal and dyslipoproteinemic human subjects (6-10, 13, 16, 17), between males and females (13, 18), among various species of nonhuman primates (2, 11), and finally within individual human and nonhuman primates (6-15). Although LDL heterogeneity has been recognized for many years, very little is known about the mechanisms of formation or the relative atherogenicity of different LDL subpopulations. In the 1950s, Gofman et al. (16) and Lindgren et al. (17) reported higher concentrations of  $S_f$  10-20 lipoproteins in surviving myocardial infarct patients compared to patients without known cardiovascular disease. These data suggested that certain lipoprotein subfractions could be more atherogenic than others. More recently, Sniderman et al. (19-22) have reported the predominance of a "heavy" LDL subfraction in patients with elevated levels of LDL apoB-100, shown to be associated with CAA. Only one other study by Krauss, Lindgren, and Ray (23) has indirectly correlated an LDL subfraction ( $S_f$  0-7) to the development of CAA based on its inverse relationship to HDL F 2-9. The LDL

Abbreviations: apo, apoprotein; CAA, coronary artery atherosclerosis; DGUC, density gradient ultracentrifugation; HDL, high density lipoproteins; ILDL, intermediate-sized low density lipoproteins; LDL, low density lipoproteins; PAGGE, polyacrylamide gradient gel electrophoresis; SAA, serum amyloid protein; SDS, sodium dodecyl sulfate; VLDL, very low density lipoproteins; TLC, thin-layer chromatography.

subfractions described by Sniderman and Krauss are very different from the lipoprotein subfraction described by Gofman, suggesting that more than one atherogenic lipoprotein exists.

A variety of nonhuman primates have been used as animal models for studying the development of CAA (2, 3, 11, 12, 24–26). Most nonhuman primates develop hyperlipoproteinemia and atherosclerosis in response to dietary cholesterol; however, the magnitude and type of hyperlipoproteinemia, as well as the quantity and quality of CAA, vary among species and individual animals (2–4). *Cynomolgus* macaques are very responsive to dietary cholesterol and show marked elevations in total plasma and LDL cholesterol and decreased high density lipoprotein cholesterol concentrations. *Cynomolgus* monkeys develop concentric foam cell lesions that often involve extensive medial damage (24, 26). In contrast, African green monkeys are less responsive to dietary cholesterol and develop eccentric atherosclerotic lesions with well defined fibromuscular caps and lesion complications such as the necrosis, sterol clefts, mineralization, and vascularization seen in human atherosclerosis (24, 26). In spite of the differences between *cynomolgus* and African green monkeys in their response to dietary cholesterol and subsequent development of atherosclerosis, LDL molecular weight has been shown to be significantly correlated to the development of CAA in both species (2, 3). The present studies were done to compare LDL heterogeneity in *cynomolgus* and African green monkeys at equivalent plasma cholesterol concentrations and to determine whether there are structural characteristics or distinct LDL subfractions in *cynomolgus* monkeys that would appear to be more atherogenic than those seen in African green monkeys.

## MATERIALS AND METHODS

Six adult male *cynomolgus* monkeys (*Macaca fascicularis*) and five adult male African green monkeys (*Cercopithecus aethiops*) were used for these studies. The *cynomolgus* monkeys were fed diets containing 43% of calories as saturated fat (lard) with 0.3 mg of cholesterol/kcal. The African green monkeys were fed diets containing 42% of calories as saturated fat (lard) with 0.8 mg of cholesterol/kcal. All animals had consumed these diets for at least 1 year. These two groups of animals were selected to have equivalent total plasma cholesterol concentrations.

### Isolation and density subfractionation of LDL

Blood samples were drawn into tubes containing 1 mg/ml EDTA, 1 mg/ml  $\text{NaN}_3$ , and 0.4 mg/ml DTNB (final concentrations) and were collected from animals that had fasted overnight. Plasma was isolated from each

sample by centrifugation at 2000 *g* for 30 min at 4°C. Lipoproteins were isolated from plasma by ultracentrifugation at  $d$  1.225 g/ml (or 1.080 g/ml in some cases) and LDL were separated from other lipoproteins by agarose column chromatography (1.5 × 90 cm; Bio-Gel A-15m; 200–400 mesh) as described by Rudel et al. (27). Isolated LDL samples were adjusted to  $d$  1.019 or 1.030 g/ml by dialysis. All density solutions were made by adding solid KBr to 0.9% NaCl containing 0.01% EDTA and 0.01%  $\text{NaN}_3$ . Discontinuous salt gradients were set up in 39-ml quick-seal centrifuge tubes by first adding 10 ml of a  $d$  1.006 g/ml solution and then successively underlayering 19 ml of a  $d$  1.019 or 1.030 g/ml solution (including the lipoprotein sample) and 10 ml of a  $d$  1.050 or 1.060 g/ml solution. Volumes and density solutions were adjusted as needed for each LDL sample. A tube containing the same discontinuous gradient but without a lipoprotein sample was prepared at the same time. Ultracentrifugation was done in a VTi-50 vertical rotor at 50,000 rpm for 6 hr at 20°C. Six hours of ultracentrifugation were required for LDL to reach their equilibrium density. After centrifugation, each sample was drained from the top of the tube by pumping a dense solution (Fluorinert, 3M Company, St. Paul, MN; 1.85 g/ml) into the bottom of the tube using an ISCO (Lincoln, NE) tube-draining apparatus. The eluent was drained through a UV monitor and into a fraction collector at a rate of 0.8 ml/min. A gradient profile was obtained from recording absorbance at 280 nm. The refractive index (Abbe-3L Refractometer; Bausch & Lomb, Rochester, NY) was measured at 20°C on alternate fractions collected from the blank tubes and the density of each fraction was determined. Mean cholesterol recovery was  $98.3 \pm 2.1\%$  (mean  $\pm$  SEM;  $n = 9$ ). Fractions were pooled into five subfractions of LDL. The density ranges defining each subfraction varied among the animals and were based on the elution profile of each LDL sample (as shown in Fig. 1). All subfractions were dialyzed against distilled water containing 0.01% EDTA and 0.01%  $\text{NaN}_3$  for further analysis.

### Agarose electrophoresis, 4–30% SDS-PAGE, and 2–12% PAGE

The electrophoretic mobility of intact lipoproteins was characterized by agarose electrophoresis in 0.5% agarose as described by Noble (28). After fixation and drying, gel strips were stained for 2–3 hr at 37°C in 60% ethanol saturated with oil red O and fat red 7B.

Apoproteins were separated and characterized by electrophoresis in a horizontal slab gel system (LKB, Rockville, MD) with a 4% polyacrylamide stacking gel and a 4–30% gradient of polyacrylamide as the separating gel [polyacrylamide gradient gel electrophoresis (PAGE)]. Gels were poured and polymerized on Gel Bond (FMC, Rockland, ME) to prevent shrinkage and distortion during staining and destaining. Lipoprotein samples containing

50  $\mu\text{g}$  of protein were lyophilized and then resolubilized in 0.46% barbital, 5% mercaptoethanol, 3% SDS, and 0.001% bromophenol blue, pH 8.3, at 100°C for 5 min and 35  $\mu\text{g}$  of protein was applied to each well. Electrophoresis was done at 250 v for  $\sim 1$  hr (until the dye front entered the separating gel) and then at 400 v for 4 hr at 15°C using a running buffer containing 0.1% SDS, pH 8.3 (29). After electrophoresis, gels were fixed and stained in 50% methanol, 10% acetic acid, and 0.1% Coomassie blue (overnight) and destained in 500 ml of the same solution without the Coomassie blue stain ( $\sim 24$  hr). Apo-protein distributions were estimated by densitometric scanning using a Zeineh soft laser spectrophotometer (Bio-Med Instruments, Fullerton, CA).

Size heterogeneity of intact LDL subfractions was determined by electrophoresis in a horizontal slab gel system (LKB Instruments, Rockville, MD) with a 2% polyacrylamide stacking gel and a 2–12% gradient of polyacrylamide as the separating gel (30). Gels were poured and polymerized on Gel Bond. Lipoprotein samples were then dialyzed against distilled water containing 0.01% EDTA and 0.05%  $\text{NaN}_3$ , and samples containing 5–10  $\mu\text{g}$  of protein were applied to each well. Electrophoresis was done at 100 v for 5 hr and then at 125 v for 19 hr at 15°C using a running buffer consisting of 0.09 M Tris, 0.08 M boric acid, and 0.0025 M EDTA, pH 8.3. After electrophoresis, gels were fixed and stained with 5% trichloroacetic acid and 5% sulfosalicylic acid containing 0.05% Coomassie blue overnight and then destained in three changes of a 7% acetic acid, 5% methanol solution ( $\sim 8$  hr per solution). Each lane was scanned at 550 nm on an Acta CII spectrophotometer (Beckman Instruments, Palo Alto, CA) and the migration distance of each peak (from the origin to the center of the band) was used to determine its molecular weight based on the calibration curve of the standards run on the same gel.

Thyroglobulin (669,000 daltons), ferritin (440,000 daltons), and catalase (232,000 daltons) (Pharmacia, Piscataway, NJ) were used as standards, as was purified monkey Lp(a) (4.46  $\text{g}/\mu\text{mol}$ ). Purified monkey Lp(a) was obtained from a normolipemic rhesus monkey after isolation by gel filtration and density gradient ultracentrifugation as described above. The purity of the Lp(a) was documented by its behavior as a single, homogeneous band using agarose electrophoresis, density gradient ultracentrifugation, and PAGE, and its molecular weight was determined by agarose gel filtration (31). Purified Lp(a) was stable upon storage under nitrogen at 4°C for at least 1 month. On each gel, the standards and two LDL samples of known molecular weight were run, and a regression line between the distance of migration of these samples and the log of their molecular weight was calculated. The correlation coefficient between distance migrated and log molecular weight was consistently  $r > 0.9$ . The regression line was then used to calculate

the molecular weight of each sample. The interassay coefficients of variation for the molecular weights of individual standards averaged 5.1%.

### Chemical compositions

Lipoprotein cholesterol was measured according to the method of Rudel and Morris (32) and protein was determined by the method of Lowry et al. (33); the procedure used included hexane extraction after color development to remove lipid interference. Bovine serum albumin, fraction V, (Sigma Chemical Co.) was used as the protein standard. Lipid phosphorus was measured by the method of Fiske and Subbarow (34). Lipids were extracted from lipoprotein subfractions with chloroform–methanol 2:1 and were separated on thin-layer chromatography (TLC) silica gel 60F-254 plates (Brinkmann Instruments, Westbury, NY) using a hexane–diethyl ether–glacial acetic acid 146:50:4 solvent system. Isolated lipid classes were eluted from the gel (35) and triglycerides (36) and free and esterified cholesterol (32) were determined for each subfraction. Cholesterol recovery from the TLC plates was  $91.5 \pm 5.8\%$  (mean  $\pm$  SD,  $n = 44$ ).

## RESULTS

The lipoprotein cholesterol concentrations and distributions and average LDL molecular weights for the groups of each species are given in **Table 1**. No statistically significant differences were seen in total plasma cholesterol (TPC), VLDL + ILDL, LDL, or HDL cholesterol concentrations, although cynomolgus monkeys had higher average concentrations of LDL cholesterol and lower average concentrations of HDL cholesterol. In spite of similar LDL cholesterol concentrations, cynomolgus monkeys had significantly larger average LDL molecular weights (as determined by agarose column chromatography) than did the African green monkeys (4.10 vs. 3.46  $\text{g}/\mu\text{mol}$ , respectively;  $P < 0.02$ , Student's *t*-test).

Density gradient profiles for each LDL sample are illustrated in **Fig. 1**. In general, cynomolgus monkey LDL appeared more polydisperse than African green monkey LDL; however, a great deal of variability in the LDL elution profiles was seen in both species. The cumulative cholesterol distribution by density of LDL for each animal is illustrated in **Fig. 2**. Cynomolgus monkeys had more LDL cholesterol in less dense particles compared to African green monkeys, with only one African green monkey having a pattern that resembled those of the cynomolgus monkeys.

The electrophoretic mobility of whole plasma, LDL, and LDL subfractions was determined for each sample using agarose electrophoresis. Each LDL subfraction migrated in the  $\beta$  position, however, trace amounts of

TABLE 1. Comparison of lipoprotein cholesterol distribution between cynomolgus and African green monkeys

Monkeys	Plasma Cholesterol Concentrations (mg/dl)				LDL MW <sup>b</sup>
	TPC <sup>a</sup>	VLDL + ILDL	LDL	HDL	
Cynomolgus (n = 6)	430 ± 139 <sup>c</sup>	19.3 ± 8.0	366.2 ± 130.0	44.9 ± 22.0	4.10 ± 0.45 <sup>d</sup>
African green (n = 5)	424 ± 181	19.3 ± 17.0	344.6 ± 168.0	62.6 ± 24.0	3.46 ± 0.44

	% Cholesterol		
	VLDL + ILDL	LDL	HDL
Cynomolgus (n = 6)	4.9 ± 2.0	84.5 ± 5.0	10.7 ± 5.0
African green (n = 5)	3.9 ± 2.0	79.4 ± 11.0	17.1 ± 11.0

<sup>a</sup>TPC, total plasma cholesterol; VLDL, very low density lipoproteins; ILDL, intermediate-sized low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins.

<sup>b</sup>LDL molecular weights (g/μmol) were determined by agarose column chromatography.

<sup>c</sup>All values are means ± SD.

<sup>d</sup>P < 0.02, Student's *t*-test.

pre-β migrating and α-migrating material were sometimes seen in the most dense subfraction (data not shown).

LDL size heterogeneity was determined by 2–12% PAGGE. Fig. 3 illustrates densitometric scans of polyacrylamide gel separations and LDL subfractions from a selected cynomolgus monkey and African green monkey.

The starting LDL (isolated from the agarose column) is shown at the top of the profile (Fig. 3, LDL) and each density subfraction from least dense to most dense is shown sequentially (Fig. 3, I–V). The molecular weights of the major peaks are given for each sample. The calculated molecular weight of the starting LDL corresponded

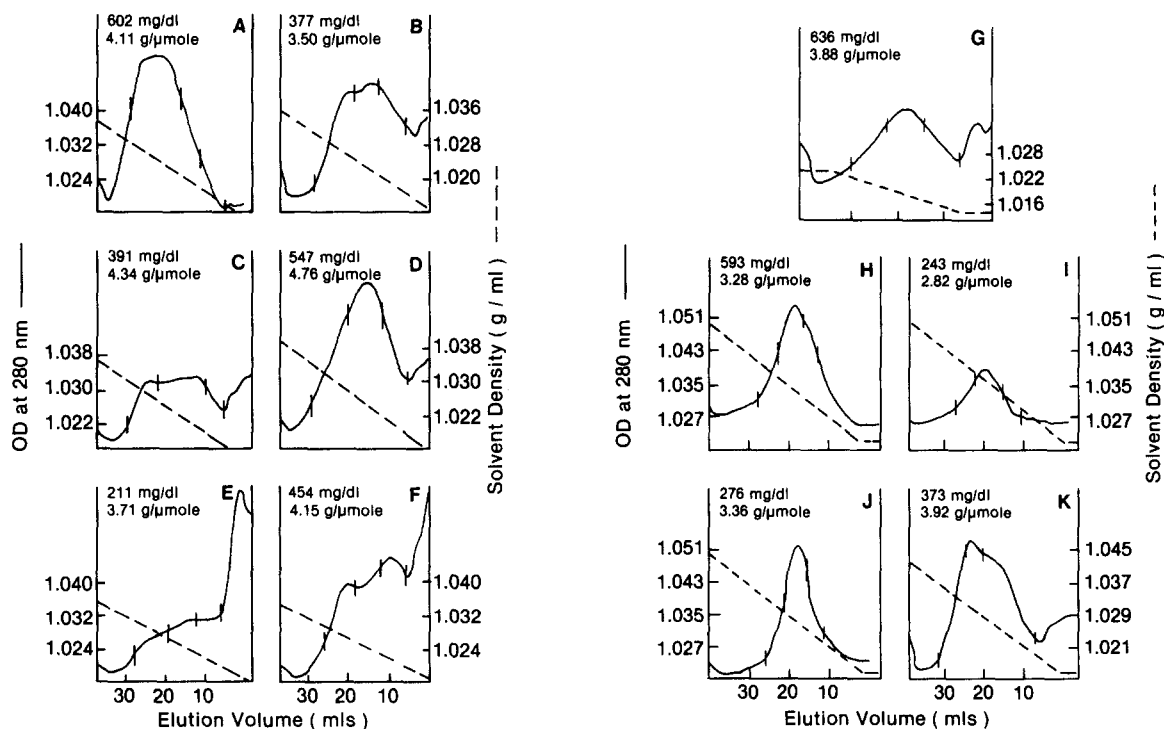


Fig. 1. Density gradient profiles of LDL samples isolated from six cynomolgus and five African green monkeys. Each sample was subjected to DGUC using the VTi-50 vertical rotor. Total plasma cholesterol (TPC) and average LDL molecular weight (LDL MW) are given for each sample. LDL molecular weights were determined by gel filtration. Five subfractions for each sample were identified based on the profile as indicated by the vertical lines. Fractions within each LDL subfraction were pooled for further analysis.

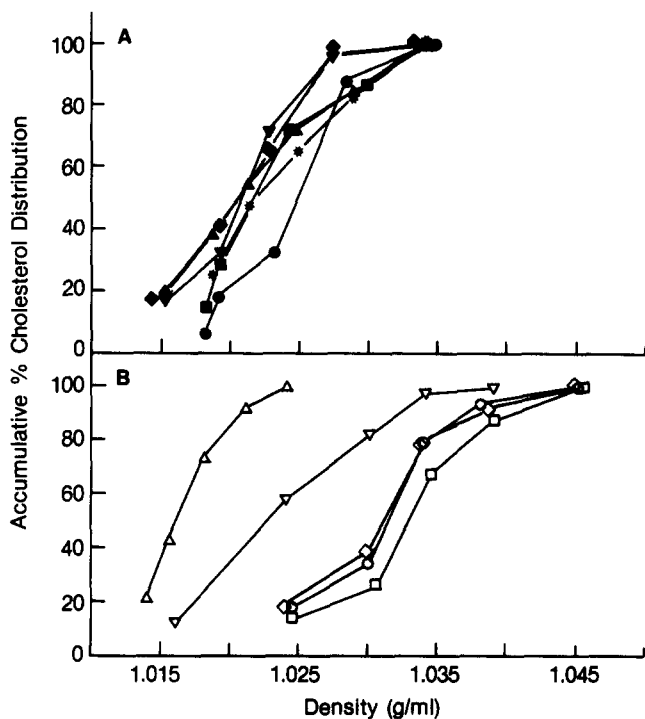


Fig. 2. Accumulative percent cholesterol distribution for each cynomolgus (A) and African green (B) LDL sample. The percent of total cholesterol in each density subfraction was summed from the least to most dense subfraction and plotted against its median density. Each symbol represents a different animal.

closely to the average LDL molecular weight determined by agarose column chromatography (3.76 vs. 3.71  $\text{g}/\mu\text{mol}$  and 3.30 and 3.28  $\text{g}/\mu\text{mol}$  for each sample, respectively). A correlation coefficient of 0.92 was found for several samples in which LDL molecular weights were determined by both methods (mean  $\pm$  SD,  $n = 9$ ,  $3.63 \pm 0.41$  vs.  $3.58 \pm 0.52$   $\text{g}/\mu\text{mol}$  for agarose column and PAGGE determinations, respectively). In general, the molecular weight of each successive subfraction decreased with increasing density; a large molecular weight peak often appeared in the most dense subfraction (Fig. 3, CM-V) and most likely represented Lp(a).

To determine whether the relationship between size and density was the same in both species, the molecular weight of the major subfraction as determined by PAGGE was plotted against its median density from DGUC (Fig. 4). The cynomolgus LDL subfractions distributed between  $d$  1.015 and 1.035  $\text{g}/\text{ml}$  whereas the African green LDL subfractions distributed between  $d$  1.015 and 1.046  $\text{g}/\text{ml}$ . At any one density or molecular weight, variability in the other measurement existed. For example, at  $d$  1.025  $\text{g}/\text{ml}$  the molecular weight of various subfractions ranged from 3.0 to 4.5  $\text{g}/\mu\text{mol}$ ; likewise, at 3.3  $\text{g}/\mu\text{mol}$ , the median density of the subfractions ranged from  $d$  1.0225 to 1.033  $\text{g}/\text{ml}$ . The variation in molecular weight at any density was seen within samples that were analyzed on the same gel and thus could not be explained by differences

between gels. The relationships between molecular weight and density were similar in both species.

Apoprotein composition among the density subfractions was analyzed using 4–30% SDS-PAGE. Fig. 5 shows the apoprotein profiles of LDL density subfractions from a selected cynomolgus monkey and African green monkey. ApoB-100 was the major apoprotein in all subfractions and smaller amounts of apoE and apoA-I were seen. Trace amounts of small apoproteins (including apoC, apoA-II and possibly apoSAA) were found in most of the subfractions. ApoA-I was frequently seen in the highest density subfraction. ApoE was visible in most of the cynomolgus monkey LDL subfractions, and these appeared to have more apoE than the African green monkey LDL subfractions. The gels were densitometrically scanned and the ratio for the area of the apoE band divided by the area of the apoB-100 band (apoE/apoB-100) was calcu-

## 2-12% PAGGE

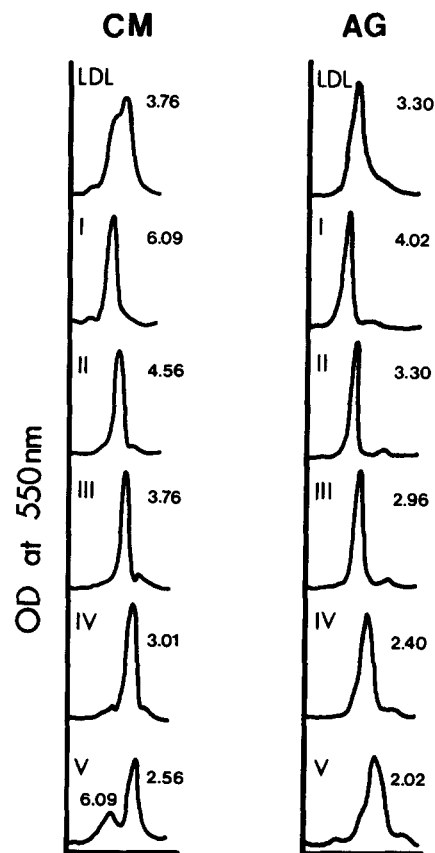
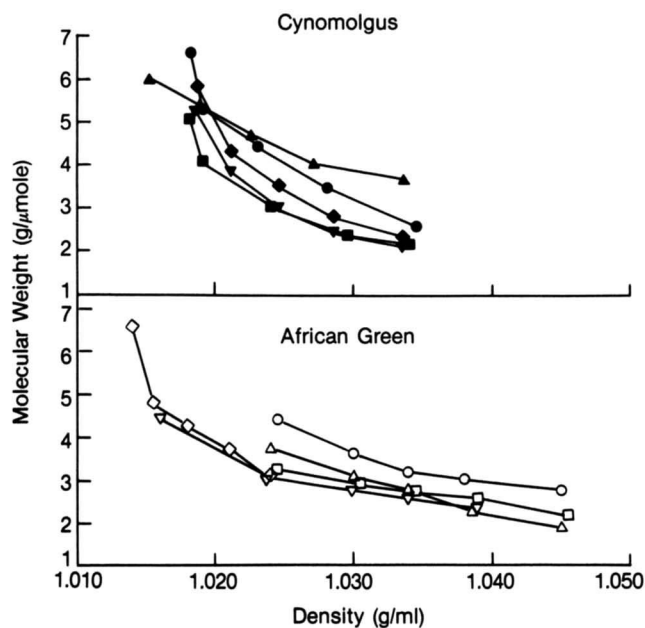


Fig. 3. Densitometric scans of LDL and LDL subfractions that were separated by 2–12% PAGGE. Samples from a representative cynomolgus monkey (left panel labeled CM) and African green monkey (right panel labeled AG) are shown. The LDL starting material is labeled LDL. LDL subfractions from density gradient centrifugation (Fig. 1) from least to most dense for each animal are labeled from I to V. The molecular weight ( $\text{g}/\mu\text{mol}$ ) of each major peak is given and was determined from the calibration curve of the standards for each gel.

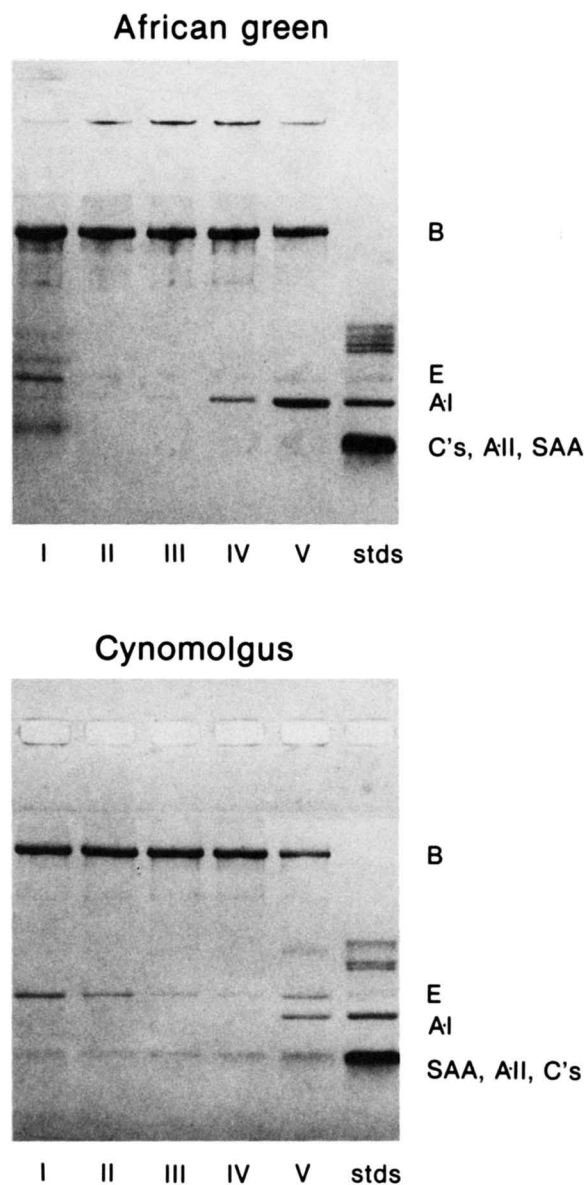


**Fig. 4.** The relationship between the median density and molecular weight for each LDL subfraction isolated from cynomolgus (closed symbols) and African green (open symbols) monkeys. Densities were determined from the density gradient profiles as the median density for each LDL subfraction (Fig. 1). The molecular weights were determined by 2-12% PAGE. Each symbol represents a different animal and each point is for a separate subfraction. All of the samples for a single animal were analyzed on the same gel. Each gel could accommodate 10 samples in addition to the standards. Three gels were run that contained all of the samples for two animals; ● and ■, ▼ and ◆, ○ and □ were paired on these gels. The samples for the remaining four animals were analyzed on separate gels.

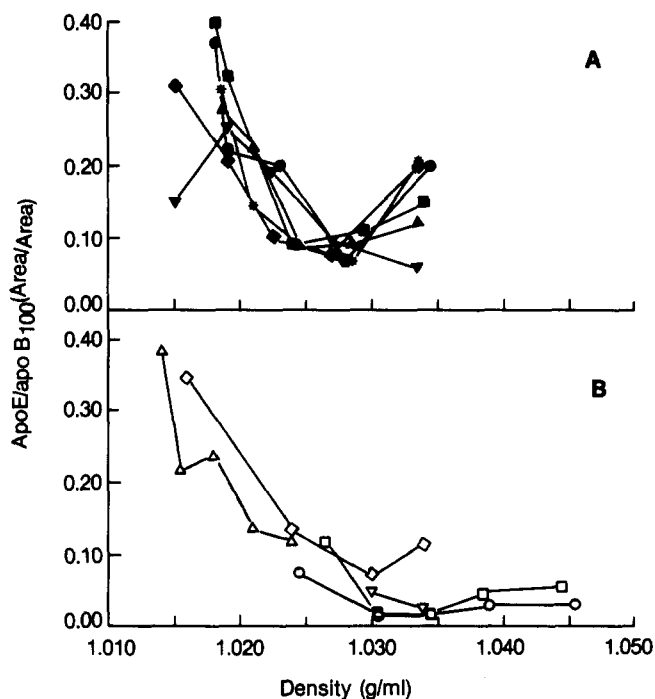
lated. **Fig. 6** shows the relationship between the apoE/apoB-100 ratio and the median density for each LDL subfraction. In both species, generally the apoE/apoB-100 ratio was inversely related to the density of the subfractions, although the ratio was higher in the most dense subfraction compared to the adjacent subfraction in cynomolgus monkey LDL subfractions (see Fig. 6-A). Two samples from African green monkeys had apoE/apoB-100 ratios similar to those of the cynomolgus samples, however, the other three African green monkey samples had much lower ratios of apoE/apoB-100. At d 1.030 g/ml, the mean apoE/apoB-100 ratio for the cynomolgus samples was 0.091 compared to 0.026 for the African green subfractions.

LDL subfractions that had one major band on 2-12% PAGE were characterized further by measuring the concentrations of phospholipid, free cholesterol, triglyceride, cholesteryl ester, and protein. In all but one sample, the most dense subfraction contained considerable amounts of a second band [Lp(a)] and was excluded from these analyses. **Fig. 7** illustrates the relationship between the percentage composition and the median density for LDL subfractions of African green monkeys (closed symbols) and cynomolgus monkeys (open symbols). The percentage

of TG was similar for all LDL subfractions and averaged  $0.9 \pm 0.9\%$ , mean  $\pm$  SD. The percentage of cholesteryl ester decreased from about 54 to 46% and the percentage of protein increased from about 14 to 24% with increasing density; the percentage of FC decreased slightly from 11 to 8% over the same density range. No compositional differences were apparent between the species.



**Fig. 5.** Patterns of the apoproteins of individual LDL subfractions from a representative African green monkey and cynomolgus monkey determined by 4-30% SDS-PAGE. The gel is labeled as follows: I-V, LDL subfractions from least to most dense; stds, chylomicron apoproteins; B, apoB-100; E, apoE; AI, apoA-I; C's, apoC; AII, apoA-II; SAA, apoSAA. The space at the top of each lane identified the well of the horizontal slab gel. Approximately 35  $\mu$ g of protein was added to each well. After electrophoresis, each gel was stained with Coomassie blue.



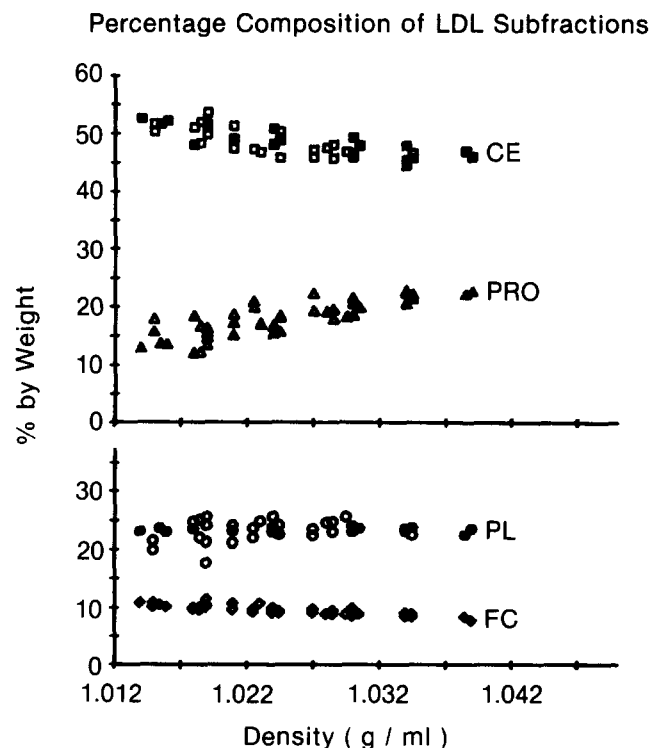
**Fig. 6.** The relationship between the median density and apoE/apoB-100 ratio for each LDL subfraction from cynomolgus monkeys (A) and African green monkeys (B). Each symbol represents a different animal and each point is for a separate subfraction. For one African green sample ( $\nabla$ ), only two subfractions are shown.

## DISCUSSION

The data of the present study characterized plasma LDL of two different species of nonhuman primates that are known to have different susceptibility to diet-induced hyperlipoproteinemia and atherosclerosis (2). The African green monkeys of the study were selected to be higher than average for this species in responsiveness to dietary cholesterol. In addition, the cynomolgus monkeys of the study were fed less cholesterol than the African green monkeys so that it was possible to study groups from both species that had equivalent degrees of hypercholesterolemia. By comparing the LDL when the extent of hypercholesterolemia was comparable, there was maximum opportunity to define differences in relative atherogenic potential of the LDL, per se, independent of the effects of concentration. This design eliminated one of the major differences in atherogenic potential between the species, namely, that given the same atherogenic diet challenge, cynomolgus monkeys become much more hypercholesterolemic than African green monkeys. For the study, the plasma concentrations of VLDL + ILDL, LDL, and HDL cholesterol were not statistically significantly different between the species although cynomolgus monkeys had higher average LDL and lower average HDL cholesterol concentrations. LDL from each animal was evaluated for

molecular weight, density, and composition. The percentage composition of the particles at any given density or molecular weight was indistinguishable between the species. However, the average cynomolgus monkey LDL was larger, more polydisperse, less dense, and appeared to contain more apoE than African green monkey LDL.

Variations in molecular weight at any given density and density at any given molecular weight were evident in both species. Some of these differences could be due to the limits of the methodology used to define the subfractions. The median density may not accurately reflect the peak density of each subfraction and, in fact, each isolated subfraction cannot be a single, homogeneous population since it is impossible to completely separate subfractions. Additionally, the limits of resolution of the gradient gel electrophoresis method could account for some of the variation. It seems unlikely, however, that the methodology could explain all of the molecular weight and density heterogeneity. Krauss and Burke (6) have reported similar findings for LDL subfractions in human beings. They have suggested that differences in density at any given size may be due to changes in the lipid content of the particle. Small variations in the composition of LDL subfractions were seen in the present study (Fig. 7) and may help explain some of the range of densities seen for any particular



**Fig. 7.** The relationship between the median density and percentage composition of cholesteryl ester (CE), protein (PRO), phospholipid (PL), and free cholesterol (FC) for each LDL subfraction. Open symbols represent LDL subfractions isolated from cynomolgus monkeys and the closed symbols represent LDL subfractions isolated from African green monkeys. Each point represents a separate subfraction.

size subfraction. The variation in molecular weight at any given density may also reflect some of the difference seen (Fig. 6) in the apoprotein content of the particles. The size and density of the LDL subfractions described in other studies in nonhuman primates (11–13, 37) fit the same curvilinear relationship shown for the cynomolgus LDL in the present study (see Fig. 4), in spite of the differences in methodology by which the data were obtained. In addition, the composition of the LDL density subfractions from cholesterol-fed rhesus monkeys (12) was superimposable with the compositional data presented for the cynomolgus and African green monkeys (Fig. 7).

The differences in the properties of LDL could conceivably reflect a difference in atherogenic potential between cynomolgus monkeys and African green monkeys. First, the average cynomolgus monkey LDL was larger than the average African green monkey LDL, and more cholesteryl ester per particle would be delivered to cells during binding and internalization of high molecular weight LDL by LDL receptors (38). Cholesteryl ester accumulation in cells of the artery wall could occur more quickly in cynomolgus monkeys in this case. In fact, large LDL were more effective in delivering cholesterol to cells in culture. Studies by St. Clair et al. (38, 39) have shown that large LDL from hypercholesterolemic rhesus monkeys were twice as effective in stimulating cholesterol esterification and cholesteryl ester accumulation in smooth muscle cells in culture than LDL from normocholesterolemic rhesus monkeys.

It is also possible that the properties of the LDL particles that convey increased "atherogenicity" are not properties that require LDL receptor recognition since most of the LDL receptors in the artery wall may be down-regulated (40). It may be that the larger, apoE-enriched LDL of cynomolgus monkeys are better substrates for binding to the macrophage  $\beta$ -VLDL receptor, a receptor known to bind  $\beta$ -VLDL and hypercholesterolemic rabbit IDL (41–43). Hypercholesterolemic rabbit IDL and the lower density LDL subfractions found in cynomolgus monkeys are similar in that they both contain apoB and apoE and both have relatively high TC/Pro ratios (4.3 vs. 3.4 for rabbit IDL and cynomolgus monkey LDL, respectively). The enlarged LDL of cynomolgus monkeys have also been noted to have a modified cholesteryl ester composition that is proportional to size and is associated with a shift in the physical property of the core lipid from a liquid to a liquid crystalline state (44). This change, and associated changes in the surface molecules, may contribute to increased atherogenicity. This could occur because the interaction of LDL with scavenger receptors in macrophages is enhanced, perhaps due to an increased complex formation between the enlarged LDL with the proteoglycans in the matrix of the artery wall (45). The atherosclerotic lesions seen in cynomolgus monkeys versus African green monkeys are much richer in

foam cells (24, 26) and it seems useful to consider that the "atherogenic" property of LDL of cynomolgus monkeys is one that stimulates LDL uptake by macrophages. In contrast, the LDL of cholesterol-fed African green monkeys remain more typical of normal plasma LDL (2) and a primary factor of atherosclerosis induction in this species could be the increased LDL concentration. However, even the more modest size changes that occur in African green monkeys must also be important, since the lipoprotein characteristic most highly correlated to coronary artery atherosclerosis in this species was LDL molecular weight (3).

The distribution of apoproteins among human or nonhuman primate plasma LDL subfractions has not been reported previously. The data presented here suggest apoprotein heterogeneity exists not only within an individual LDL sample but also among animals of the same species and between species. Of particular interest is the variability in the relative amounts of apoB and apoE (Figs. 5 and 6). The role of apoE in the catabolism of particles via specific receptors (LDL receptor and apoE receptor) has been well documented (reviewed in 46). It has been suggested that even a small amount of apoE on a particle may enhance its uptake by the B,E-receptor since the affinity of this receptor for apoE is 20-fold higher than its affinity for apoB (46). The differences in apoprotein composition among different density subfractions may help explain the metabolic heterogeneity reported in African green monkeys (14; and Marzetta, C. A., F. L. Johnson, L. A. Zech, and L. L. Rudel, unpublished observations) and influence the atherogenic potential of the particles.

The increased amounts of apoA-I seen in the most dense subfractions may have been due to the presence of trace amounts of HDL<sub>1</sub>-like particles similar to those described in baboons and man (47, 48). HDL<sub>1</sub> particles contain apoA-I as their major apoprotein (47, 48). Two observations suggest that much of the apoA-I in the most dense subfraction may be due to non-LDL particles. First, although most of the lipoproteins contained in the highest density subfraction exhibited beta migration, trace amounts of  $\alpha$ -migrating material were detected in some of the LDL samples (two out of eleven animals). Second, using 4–30% PAGE, detectable amounts of HDL<sub>1</sub>-like particles that banded between LDL and HDL were found in the most dense subfraction in three animals. It is also possible that the increase in the amount of apoE in the most dense LDL subfraction of some animals (Fig. 6) was also due to the presence of HDL<sub>1</sub> particles that contained apoE. However, in preliminary experiments in which HDL<sub>1</sub> from African green monkeys has been isolated and characterized, apoE was found to be a minor apoprotein while apoA-I was the major protein (Babiak, J. and L. L. Rudel, unpublished observations). The more likely possibility appears to be that the apoE at the bottom of the tube was dissociated from less dense



particles during centrifugation. In summary, it appears most likely that the apoA-I found in some LDL subfractions (Fig. 5) was not associated with apoB-100 LDL particles, but was part of HDL<sub>1</sub>-like particles. Some of the apoE in the most dense subfraction may also be on HDL<sub>1</sub> particles but some of it may represent apoE that dissociated from particles during centrifugation. In contrast, small amounts of low molecular weight apoproteins were seen in all LDL subfractions and presumably represent normal components of these particles.

In the cholesterol-fed animals of this study, the agarose column separated a larger-sized population of lipoprotein particles, termed intermediate-sized low density lipoproteins (ILDL), from the LDL peak. As a matter of convenience, we have not included a detailed analysis of this larger-sized subfraction in this report, but we have shown before that this region can contain intermediate density lipoproteins (IDL) as well as particles of the same size from other lipoprotein classes (18, 49). The material from the LDL peak of the agarose column includes material in the density range of 1.015–1.02 g/ml. This range overlaps the d 1.006–1.019 g/ml range usually defined for IDL. The difference between the “IDL” of the two column regions is size, but it is not clear that either of these “IDL” are the classic IDL, i.e., intermediates in the conversion of VLDL to LDL. Rather, these particles are cholesteryl ester-rich and triglyceride-poor (<2% triglyceride) (see Fig. 7). This fraction is usually enriched in apoE relative to the other LDL subfractions (Figs. 5, 6), however this alone does not characterize it as the metabolic equivalent of IDL in humans. Therefore, we have provided detailed information on subfractions isolated from the LDL peak; however, we can not be sure of, and have tried not to imply, the metabolic significance of the various subfractions. ■■

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