Effects of Saturated and Polyunsaturated Dietary Fat on the Concentrations of HDL Subpopulations in African Green Monkeys

John Babiak, Frank T. Lindgren, and Lawrence L. Rudel

The effect of the type of dietary fat on the concentrations and compositions of high density lipoprotein (HDL) subpopulations was studied in groups of African green monkeys consuming 40% of calories as fat supplied as saturated fat (P/S = 0.3) or polyunsaturated fat (P/S = 2.2) in the presence of either 0.8 mg or 0.03 mg cholesterol kcal. Plasma HDL cholesterol concentrations were lower in polyunsaturated fat-fed animals. The distribution of mass among HDL subfractions was assessed by analytic ultracentrifugation (AUUC), density gradient ultracentrifugation (DGUC), and polyacrylamide gradient gel electrophoresis (GGE). This made it possible to characterize and quantify the HDL subpopulations HDL_2a, HDL_2b, HDL_3a, HDL_3b, and HDL_3c (arranged in order of decreasing particle size and decreasing cholesterol content). Polyunsaturated fat-fed animals had lower concentrations of the large, cholesterol-rich HDL_2b subpopulation, as well as higher concentrations of intermediate size HDL (HDL_2a and HDL_3a) on the high cholesterol diet; HDL_3a and HDL_3b on the low cholesterol diet. Consistent with the observed fat-related redistribution of HDL mass, the saturated-fat-fed monkeys had higher apo A-I/apo A-II ratios. The larger HDL often contained detectable apo E; however, the concentration of apo E in HDL was low in both saturated- and polyunsaturated fat-fed animals. Thus, compared to saturated fat, dietary polyunsaturated fat induced the formation of smaller size HDL subpopulations and, therefore, an overall lower cholesterol content per particle for plasma HDL.

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assessed by three methods: analytic ultracentrifugation, density gradient ultracentrifugation, and gradient gel electrophoresis. Since these procedures subfractionate plasma HDL on the basis of different physical properties, we improved the possibility of detecting dietary effects on both the concentrations and the characteristics of individual HDL subpopulations. Data from all three methods demonstrated that polyunsaturated fat feeding resulted in lower concentrations of HDL\(_{2b}\), but higher concentrations of intermediate size HDL, independent of effects on total HDL mass or HDL cholesterol concentrations. In addition, the particular subpopulations elevated by polyunsaturated fat depended upon the level of dietary cholesterol. Previous work in this laboratory has demonstrated that African green monkey HDL subpopulations are quite similar to human HDL in chemical composition, hydrated density, flotation rate, and apoprotein content.\(^{12,13}\) In this paper we show by gradient gel electrophoresis that monkey HDL consist of five discrete subpopulations that correspond closely in particle size to human HDL\(_{2b}\), HDL\(_{2a}\), HDL\(_{3a}\), HDL\(_{3c}\), and HDL\(_{4c}\). These results further emphasize the propriety of this nonhuman primate species as a model in situations in which diet-induced hyperlipoproteinemia mimics that of human beings at increased risk for coronary artery disease.

Methods

Animals and Diets

Before the experiment, 60 male and 60 female feral African green monkeys (Cercopithecus aethiops) purchased from an animal importer were challenged with a diet containing 40% of calories as butter and 0.74 mg cholesterol/kcal that was identical to Diet X-75-5B diet containing 40% of calories as butter and 0.74 mg cholesterol/kcal. Before the experiment, 60 male and 60 female feral African green monkeys (Cercopithecus aethiops) purchased from an animal importer were challenged with a diet containing 40% of calories as butter and 0.74 mg cholesterol/kcal that was identical to Diet X-75-5B diet containing 40% of calories as butter and 0.74 mg cholesterol/kcal. All females were in a breeding colony and some were maintained on their diets for periods shorter than 5 years, but all measurements were made only after a monkey had been on its diet for at least 8 months. None of the monkeys was pregnant when studied. Twelve additional male monkeys were fed diets with 40% of calories as fat, but containing only 0.03 mg/kcal of cholesterol (Diets C and D in Table 1). All diets contained 40% of calories as fat, 40% as carbohydrate, and 20% as protein. Diets A and C contained 40% saturated, 47% monounsaturated, and 13% polyunsaturated fatty acids. Diets B and D contained 22% saturated, 30% monounsaturated, and 48% polyunsaturated fatty acids. Throughout the study the general good health of all monkeys was monitored and assured by routine clinical measurements including body weight, blood pressure, blood urea nitrogen, fasting blood glucose, hemoglobin, hematocrit, white cell blood count, and total serum protein.

Plasma Lipid and Apoprotein Measurements

Animals were fasted for 18 hours and were given ketamine intramuscularly (10 mg/kg) before blood collections. Blood was drawn from the femoral vein into tubes containing Na\(_2\)-ethylenediaminetetraacetate (EDTA) at a final concentration of 1 mg/ml, sodium azide (Na\(_3\)) at a final concentration of 1 mg/ml, and 5,5'-dithio(bis)-2-nitrobenzoic acid (DTNB) at a final concentration of 0.4 mg/ml. Within an hour of blood collection, plasma was isolated by centrifugation at 1,500 g and 4°C for 30 minutes.

Bimonthly measurements of cholesterol concentration in whole plasma and in the plasma supernatant after heparin-manganese chloride precipitation were made colorimetrically with an Autoanalyzer II (Technicon Instruments Corporation, Perytown, New York).\(^{16}\) Plasma concentrations of apo A-I and apo A-II were measured three times for each male monkey during the first 2 years of the study by radial immunodiffusion as previously described.\(^{19}\)

To estimate the amount of apo E within the HDL fraction, apo E concentrations in whole plasma, d<1.063 g/ml, and d>1.063 g/ml fractions were measured in a subset of animals by an enzyme-linked immunosorbent assay (ELISA) similar to our previously described method for apo A-I.\(^{20}\) Apo A-I and apo B concentrations were measured in the same samples as apo E to ensure that HDL were adequately separated from other lipoproteins by ultracentrifugation; 98.6 ± 0.3% (mean ± SEM) of the apo A-I was in the d>1.063 g/ml fraction, and 90.6 ± 1.5% of the apo B was in the d<1.063 g/ml fraction. Apoprotein patterns of HDL subfractions were also examined after polyacrylamide gel electrophoresis in 4% to 30% gradient gels containing 0.1% sodium dodecyl sulfate (SDS).\(^{21}\)

Analytic Ultracentrifugation

Concentrations of total HDL material and concentrations of the major HDL subpopulations HDL\(_{2b}\), HDL\(_{2a}\), and HDL\(_{3}\) were measured in all animals. For 5 years the two groups were fed diets (Diets A and B in Table 1) containing 0.8 mg/kg cholesterol and 40% of calories as fat derived primarily from lard (saturated fat diet, P/S = 0.3) or safflower oil (polyunsaturated fat diet, P/S = 2.2). All females were in a breeding colony and some were maintained on their diets for periods shorter than 5 years, but all measurements were made only after a monkey had been on its diet for at least 8 months. None of the monkeys was pregnant when studied. Twelve additional male monkeys were fed diets with 40% of calories as fat, but containing only 0.03 mg/kcal of cholesterol (Diets C and D in Table 1). All diets contained 40% of calories as fat, 40% as carbohydrate, and 20% as protein. Diets A and C contained 40% saturated, 47% monounsaturated, and 13% polyunsaturated fatty acids. Diets B and D contained 22% saturated, 30% monounsaturated, and 48% polyunsaturated fatty acids. Throughout the study the general good health of all monkeys was monitored and assured by routine clinical measurements including body weight, blood pressure, blood urea nitrogen, fasting blood glucose, hemoglobin, hematocrit, white cell blood count, and total serum protein.

Table 1. Diet Composition and Characteristics

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<th>Characteristic</th>
<th>Diet composition (g/100 g)</th>
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</thead>
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<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>Lard</td>
<td>11</td>
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<tr>
<td>Safflower oil</td>
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</tr>
<tr>
<td>Cholesterol*</td>
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<tr>
<td>Dried egg yolk</td>
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<td>Egg yolk replacement</td>
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</table>

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Diet composition (g/100 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>Casein</td>
<td>9</td>
</tr>
<tr>
<td>Lactalbumin</td>
<td>5</td>
</tr>
<tr>
<td>Wheat flour</td>
<td>35</td>
</tr>
<tr>
<td>Sucrose</td>
<td>12</td>
</tr>
<tr>
<td>Alphacel</td>
<td>7</td>
</tr>
<tr>
<td>Mineral mixture</td>
<td>3.5</td>
</tr>
<tr>
<td>Vitamin mixture</td>
<td>2.5</td>
</tr>
</tbody>
</table>

*Added to compensate for sterol content of the fat in the other diets.
†A low cholesterol (39 mg/100 g) substitute with a composition similar to egg yolks, consisting of (wt%) 50% lard, 22% casein, 16% soybean lecithin, 7.9% water, 2.5% sucrose, and 1.6% Hestled salt mixture.
were measured once during the third year of the study by analytic ultracentrifugation (AnUC). Plasma $d<1.20$ g/ml lipoprotein fractions obtained by ultracentrifugation at a density of 1.217 g/ml (Beckman Model Ti) were performed on these samples as described by Lindgren et al.\textsuperscript{22} in a Beckman Model E analytic ultracentrifuge with schlieren optics. All measurements were made on the 30-minute frame after attainment of 25,640 rpm. Concentrations of HDL subpopulations were calculated by a three-component analysis of digitized schlieren profiles, as described by Anderson et al.\textsuperscript{15} by using peak-F-rates of 1.56, 3.15, and 5.37 for HDL$_{3b}$, HDL$_{3a}$, and HDL$_{2b}$, respectively.

**Density Gradient Ultracentrifugation**

Density gradient ultracentrifugation (DGUC) was performed on the HDL of 10 male monkeys from each of diet Groups A and B and on six male monkeys from each of diet Groups C and D by the method of Rudel et al.\textsuperscript{12} HDL was determined by ultracentrifugation of whole plasma at a density of $d=1.225$ g/ml (Beckman 70.1 Ti rotor, 50,000 rpm, 24 hours, 15°C) followed by column chromatography on 4% agarose, as previously described.\textsuperscript{12} Discontinuous density gradients were poured by successively underlayering five NaCl-KBr solutions as follows: 9 ml of $d=1.06$ g/ml, 9 ml of $d=1.090$ g/ml, 3 ml of $d=1.115$ g/ml (containing the HDL sample), 9 ml of $d=1.120$ g/ml, and 9 ml of $d=1.150$ g/ml. After ultracentrifugation at 50,000 rpm for 18 hours at 20°C, the tubes were drained from the top by pumping Fluorinert (3M Company, St. Paul, Minnesota) into the bottom of the tube. The optical density of the eluted sample was continuously monitored at 280 nm, and individual fractions of 0.9 ml were collected. The density of each fraction was determined by refractometry.

The HDL was pooled into six fractions defined by the following density intervals: fraction 1, $d<1.086$ g/ml; fraction 2, $d=1.086$ to 1.098 g/ml; fraction 3, $d=1.098$ to 1.110 g/ml; fraction 4, $d=1.110$ to 1.122 g/ml; fraction 5, $d=1.122$ to 1.135 g/ml; fraction 6, $d>1.135$ g/ml. The amount of cholesterol, protein, and phospholipid was measured in each fraction and the plasma concentrations of these constituents were calculated by correcting for the recovery of total cholesterol (96 ± 2%; mean ± SEM).

**Gradient Gel Electrophoresis**

Gradient gel electrophoresis (GGE) according to the method of Nichols et al.\textsuperscript{24} was performed on plasma $d<1.225$ g/ml fractions from all samples characterized by DGUC as well as on selected HDL density subfractions. All samples and high molecular weight protein standards (Pharmacia Fine Chemicals, Piscataway, New Jersey) were electrophoresed in 4% to 30% polyacrylamide gradient gels (Pharmacia Fine Chemicals) for 24 hours at 125 V, 10°C. Gels were stained for protein with Coomassie G-250, and all lanes were densitometrically scanned with a Zeineh scanning densitometer (Biomed Instruments, Fullerton, California). The relative migration distance ($R_i$ value) for an individual band on each electrophoretic pattern was calculated by taking the ratio of the migration distance of the band relative to the migration distance of bovine serum albumin in the standard lane of the same gel. Plasma concentrations of the protein in each of the HDL subpopulations for each monkey were calculated from densitometric scans of the Coomassie-stained gradient gels. Each peak or shoulder was designated as consisting of a specific HDL subpopulation (HDL$_{2b}$, HDL$_{3a}$, HDL$_{3b}$, HDL$_{2a}$, or HDL$_{3a}$) and the area under each peak or shoulder was calculated by dropping vertical lines to the abscissa at the $R_i$ values that defined each subpopulation. The plasma protein concentration of each subpopulation was calculated by multiplying the area percent for each subpopulation by the total HDL protein concentration in plasma. The validity of quantitating HDL subpopulation concentrations from densitometric scans of stained gels was examined in separate experiments. Mixtures of DGUC-isolated HDL$_2$ and HDL$_3$ were electrophoresed, and gels were stained and scanned. There was a significant correlation ($p<0.001$, $r^2=0.992$) between the known mass ratio applied to the gel and the ratio of the densitometric scan areas of the two HDL populations.

**Chemical Analysis**

Cholesterol was measured in whole plasma samples, agarose column-isolated very low density, intermediate density, low density, and high density lipoprotein fractions, and HDL density subfractions by the method of Rudel et al.\textsuperscript{25} The protein content of column-isolated HDL fractions and HDL density subfractions was estimated by the method of Lowry et al.\textsuperscript{26} Phospholipid phosphorus was determined in HDL density subfractions by the method of Fiske and SubbaRow.\textsuperscript{27}

**Statistical Methods**

All data were analyzed by analysis of variance using the general linear model routine of the SAS statistical package. Because of differences in group sizes, the data derived from monkeys fed the high cholesterol diets (A and B in Table 1) was analyzed separately from data from monkeys fed the low cholesterol diets (C and D). The results from the high cholesterol and low cholesterol diets are presented separately in this article. The concentrations of total plasma cholesterol, HDL cholesterol, and HDL material (by AnUC) were tested for major effects of fat and sex. The fat-by-sex interaction term was not significant and was not included in the linear model. Apo A-I, apo A-II, HDL cholesterol (DGUC), and HDL protein (DGUC and GGE) concentrations were tested only for the effect of dietary fat. Since concentrations of HDL subfractions are not necessarily independent measurements, a multivariate analysis was performed on all HDL subfraction concentrations determined by each method (e.g., HDL$_{2b}$, HDL$_{3a}$, and HDL$_3$ concentrations by AnUC) to evaluate the overall effect of dietary fat. When a significant overall effect was observed, the effect of dietary fat on each HDL subfraction was then examined by univariate analysis of variance. Since many HDL subfraction concentrations were strongly correlated with total HDL concentrations, these data were tested for the effect of dietary fat with and without the appropriate total HDL concentration as a covariate. Covariate analysis
of variance indicated the effect of dietary fat on the distribution of HDL material independent of the concentration of total HDL. In most cases covariate and noncovariate methods yielded comparable results. In all covariate analyses performed, the fat-by-total HDL interaction term was not significant and was not included in the linear model. All of the above tests were repeated nonparametrically and yielded comparable results.

Results

Effect of Type of Dietary Fat on the Plasma Lipid and Apoprotein Concentrations of Monkeys

Table 2 lists the total plasma cholesterol and HDL cholesterol concentrations (mean ± SEM) for male and female African green monkeys fed either the saturated or polyunsaturated fat diet that contained 0.8 mg/kcal of cholesterol (Diets A and B). The monkeys consuming the saturated fat diet had significantly higher concentrations of both total and HDL cholesterol (p = 0.022 and p = 0.013, respectively). Plasma apo A-I and apo A-ll concentrations were measured for the male monkeys in the experiment and are also shown in Table 2. Apo A-I concentrations of monkeys consuming the saturated fat diet were 18% higher, but this difference was not statistically significant (p<0.07). The apo A-ll concentrations of the two diet groups were comparable. However, the ratio of apo A-l/apo A-ll was significantly higher (p<0.004) for the saturated fat-fed monkeys. No sex-related difference was observed for any parameter. Apo E concentrations were measured in the plasma of subsets of monkeys on each high cholesterol diet and were found to be 6.5 ± 1.4 mg/dl (mean ± SEM, n = 6) on the saturated fat diet and 2.9 ± 0.1 mg/dl (n = 7) on the polyunsaturated fat diet. The apo E contents of HDL were estimated by measuring apo E concentration in the ultracentrifugal d<1.063 g/ml fraction and were 2.2 ± 0.5 mg/dl and 1.8 ± 0.5 mg/dl, respectively, on the two diets. Therefore, no difference occurred in apo E concentration in HDL, in spite of a twofold higher plasma apo E concentration in the saturated fat-fed animals.

Table 3 shows the mean (± SEM) concentrations of total and HDL cholesterol, as well as apo A-I and apo A-ll for monkeys fed the two low cholesterol diets (Diets C and D). There were no statistically significant differences between the diet groups for these measurements, but the ratio of apo A-I/apo A-ll tended to be higher for the saturated fat (Diet C) monkeys (p = 0.08).

The importance of the type of dietary fat on HDL cholesterol concentration has been discussed before and was evaluated in our study by examining the relationship between HDL and total serum cholesterol concentrations. There was a positive relationship between HDL and total cholesterol among monkeys with total serum cholesterol concentrations below 235 mg/dl. This included all the monkeys fed the low cholesterol diets (Diets B and D) as well as some of the monkeys fed the high cholesterol diets (Diets A and C). In contrast, among monkeys having total serum cholesterol concentrations greater than 235 mg/dl, there was a negative relationship between HDL and total cholesterol. When the data were fit by polynomials, it was found that the greatest HDL cholesterol concentration was obtained at almost identical total serum cholesterol concentrations for the saturated (238 mg/dl) and polyunsaturated (235 mg/dl) fat-fed monkeys. The peak value of HDL cholesterol, however, was 128 mg/dl for the curve describing the saturated fat-fed monkeys and 100 mg/dl for the polyunsaturated fat-fed monkeys.

Definition of Plasma HDL Subpopulations by Gradient Gel Electrophoresis

The size distribution of the plasma HDL from a total of 26 monkeys on all four diets was analyzed by GGE followed by densitometric scanning of stained gels. As many as five bands were present within the HDL of each monkey. On profiles produced by densitometrically scanning sample lanes, each HDL band appeared as a peak or shoulder. Figure 1 shows densitometric scans of the HDL from a representative monkey from each diet group. Although it is apparent that the distribution of HDL mass differed among the profiles shown, the migration distances (Stokes' diameters) and number of bands within the HDL of each sample were similar, suggesting that similar HDL subpopulations were common to all diet groups. Further evidence for the presence of HDL subpopulations was obtained when the frequency of occurrence of bands in the profiles of all monkeys was plotted versus Rf value (migration distance relative to bovine albumin). Five distinct modes were apparent in the distribution (data not shown) and were designated HDL1a, HDL1b, HDL2a, HDL2b, and HDL3, by analogy to the human HDL subpopulations of equivalent size.

Table 2. Plasma Concentrations of Total and HDL Cholesterol, Apo A-I, and Apo A-ll of African Green Monkeys Fed High Fat, High Cholesterol Diets

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>TSC</th>
<th>HDL-C</th>
<th>Apo A-I</th>
<th>Apo A-II</th>
<th>Apo A-l/II</th>
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<tr>
<td>SAT-M</td>
<td>20</td>
<td>355±32</td>
<td>93±8</td>
<td>257±16</td>
<td>43±3</td>
<td>5.9±0.3</td>
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<tr>
<td>POLY-M</td>
<td>19</td>
<td>279±19</td>
<td>82±5</td>
<td>217±15</td>
<td>45±2</td>
<td>4.8±0.2</td>
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<tr>
<td>SAT-F</td>
<td>19</td>
<td>306±22</td>
<td>87±5</td>
<td>—</td>
<td>—</td>
<td>—</td>
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<tr>
<td>POLY-F</td>
<td>21</td>
<td>270±20</td>
<td>69±4</td>
<td>—</td>
<td>—</td>
<td>—</td>
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<tr>
<td>Fat, p value</td>
<td>0.022</td>
<td>0.013</td>
<td>0.07</td>
<td>NS</td>
<td>0.004</td>
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</table>

N = number of monkeys per group.
All values are given as mg/dl and are the means ± SEM.
SAT = saturated fat with cholesterol diet (diet A); POLY = polyunsaturated fat with cholesterol diet (diet B); M = male; F = female; NS = not significant.

Table 3. Plasma Concentrations of Total and HDL Cholesterol, Apo A-I, and Apo A-ll of African Green Monkeys Fed High Fat, Low Cholesterol Diets

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>TSC</th>
<th>HDL-C</th>
<th>Apo A-I</th>
<th>Apo A-II</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAT</td>
<td>6</td>
<td>164±11</td>
<td>79±3</td>
<td>214±13</td>
<td>37±4</td>
</tr>
<tr>
<td>POLY</td>
<td>6</td>
<td>143±7</td>
<td>74±5</td>
<td>201±13</td>
<td>44±4</td>
</tr>
<tr>
<td>Fat, p value</td>
<td>6.0±0.7</td>
<td>4.7±0.2</td>
<td></td>
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</table>

N = number of monkeys per group.
All values are given as mg/dl and are the means ± SEM.
Abbreviations: SAT = saturated fat diet (diet C); POLY = polyunsaturated fat diet (diet D).
Comparison of HDL Subpopulations Defined by Three Methods

The effect of dietary fat on the amounts and properties of individual HDL subpopulations was assessed by GGE, DGUC, and AnUC. These three methods subfractionate plasma HDL on the basis of different physical properties, so it was important to determine to what extent the HDL subpopulations defined by each method were comparable. Since DGUC was the only preparative method used to subfractionate HDL, density subfractions of HDL were analyzed by GGE and AnUC.

The six density fractions of the HDL from each of seven monkeys (four on the polyunsaturated fat diet, three on the saturated fat diet) were examined by GGE. Figure 2 shows the results from one monkey fed the low cholesterol and saturated fat diet (Diet C) and illustrates the degree of separation of HDL subpopulations achieved by density gradient ultracentrifugation. As this example shows, HDL as defined by GGE was the predominant HDL subpopulation.

Figure 1. Heterogeneity of monkey plasma HDL as observed by densitometric scans of samples stained with Coomassie G-250 after electrophoretic separation in 4% to 30% polyacrylamide gradient gels. Lipoproteins were isolated from plasma by ultracentrifugation at d = 1.225 g/ml, and an amount equivalent to 24 μL of plasma was electrophoresed at 125 V for 24 hours. Stained gels were scanned at 633 nm. Particle sizes measured as Stokes' diameter were determined by reference to the migration distances of high molecular weight protein size standards run in each gel. The profiles shown are of representative monkeys fed the saturated fat with high cholesterol diet (A), the polyunsaturated fat with high cholesterol diet (B), the saturated fat with low cholesterol diet (C), and the polyunsaturated fat with low cholesterol diet (D).

Figure 2. Separation of HDL subpopulations by density gradient ultracentrifugation as assessed by polyacrylamide gradient gel electrophoresis. All samples were electrophoresed in 4% to 30% polyacrylamide gradient gels, stained, and densitometrically scanned as described in Figure 1 and in Methods. HDL (top panel) was isolated from the plasma of one monkey fed diet C by a combination of ultracentrifugation and agarose column chromatography, as described in Methods. HDL density subfractions (panels 2 through 7) were obtained following density gradient ultracentrifugation of the whole HDL, as described in Methods.
tion in density fractions 1 and 2, while HDL$_{2b}$ was the major species in fraction 3. Fractions 4, 5, and 6 were heterogeneous, each containing more than one band within the HDL$_{2b}$, HDL$_{2a}$, and HDL$_{3}$ size ranges. In a separate experiment, aliquots of the six density gradient fractions pooled from the plasma of several monkeys fed Diets A and B were analyzed by AnUC to determine the relative amounts of HDL$_{2b}$, HDL$_{2a}$, and HDL$_{3}$ material within each HDL density fraction. As shown in Table 4, HDL density subfractions 1 and 2 contained predominantly HDL$_{2b}$ material; HDL$_{2a}$ was the major constituent in fraction 3 but was also present in fractions 2 and 4, while HDL$_{3}$ was the major species in fractions 4 through 6.

Quantitation by Analytic Ultracentrifugation of HDL Subpopulations of Monkeys Fed High Cholesterol Diets

The influence of dietary fat on the distribution of plasma HDL as a function of flotation rate was examined by AnUC. Figure 3 shows the mean schlieren profiles for all the monkeys in diet Groups A and B. Although the total amount of HDL material (measured as area within the solid lines) did not differ between the two diet groups, it was apparent that the distribution of HDL material was quite different. As can be seen in Figure 3, the saturated fat-fed group (Figure 3A) possessed more of the faster floating (higher flotation rate) HDL material than the polyunsaturated fat-fed group (Figure 3B).

Figure 4 shows the mean ± SEM levels of the three major HDL subpopulations, HDL$_{2b}$, HDL$_{2a}$, and HDL$_{3}$, as a function of dietary fat (Figure 4A) or of sex (Figure 4B) as determined by quantitation of the AnUC schlieren profiles of all the monkeys fed Diets A and B. There were no significant differences in total HDL material on the basis of either fat or sex. Among individual HDL subpopulations, saturated fat resulted in higher HDL$_{2a}$ levels and lower HDL$_{2b}$ concentrations. Males had higher HDL$_{3}$ levels than females. Covariate analysis also showed that HDL$_{2b}$ and HDL$_{2a}$ concentrations were significantly correlated with the total HDL concentrations ($p<0.0001$), but HDL$_{3}$ levels were not.

### Table 4. Relationships between Density Gradient Ultracentrifugation Fractions and Analytic Ultracentrifugation Subpopulations

<table>
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<th>Subfraction</th>
<th>% HDL$_{2b}$</th>
<th>% HDL$_{2a}$</th>
<th>% HDL$_{3}$</th>
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<tr>
<td>1</td>
<td>94</td>
<td>6</td>
<td>0</td>
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<tr>
<td>2</td>
<td>64</td>
<td>36</td>
<td>0</td>
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<tr>
<td>3</td>
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<tr>
<td>6</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
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</table>

The six HDL density subfractions isolated by density gradient ultracentrifugation from a pool of plasma HDL from several monkeys were analyzed by analytic ultracentrifugation. The percent distribution of HDL material among the three major HDL subpopulations was determined by deconvolution of the analytic ultracentrifugation profile of HDL present within each density subfraction as described in Methods.

Quantitation by Density Gradient Ultracentrifugation of HDL Subfractions of Monkeys Fed High Cholesterol Diets

To assess whether the effect of dietary fat on the distribution of HDL subpopulations was independent of the concentrations of HDL cholesterol, DGUC and GGE were performed on the plasma HDL from a set of male monkeys from diet Groups A and B that were selected to have comparable mean HDL cholesterol concentrations. Figure 5 shows typical absorbance profiles illustrating the distribution of HDL material by density. In general, the major peak of HDL material among saturated fat-fed monkeys (Figure 5A) was near the low density end of the gradient (mean ± SEM, $d = 1.086 ± 0.005$ g/ml, $n = 10$) while the peak of the HDL material among polyunsaturated fat-fed monkeys

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**Figure 3.** Mean analytic ultracentrifugal schlieren patterns of the HDL of all monkeys fed the high cholesterol diets containing either the saturated fat (A) or polyunsaturated fat (B). Each profile is deconvoluted into the three major HDL subpopulation components: HDL$_{2b}$ (---), HDL$_{2a}$ (- - -) and HDL$_{3}$ (---). The whole lipoprotein fraction was isolated from plasma by ultracentrifugation at $d = 1.217$ g/ml and was subjected to analytic ultracentrifugation at 52,640 rpm. The 30 minute frame of the schlieren profile from each monkey was digitized and stored on computer. Patterns shown are the unweighted arithmetic mean profiles of all monkeys in each diet group ($n = 39$ for diet A; $n = 40$ for diet B).
Figure 4. Mean ± SEM concentrations of the three major HDL subpopulations determined by analytic ultracentrifugation. A compares monkeys within the diet groups A (n = 38) and B (n = 39), while B compares male (n = 37) and female (n = 40) monkeys. Analytic ultracentrifugation was performed as described in Figure 3 and in Methods. Groups were compared both by analysis of variance (*) and by analysis of covariance (+), where total HDL concentration was the covariate. * and +, p<0.05; ** and ++, p<0.01; *** and ++++, p<0.001.

Figure 5. Density gradient absorbance profiles of the HDL from representative monkeys in (A) the saturated fat and high cholesterol diet A and (B) the polyunsaturated fat and high cholesterol diet B. HDL was isolated from plasma by a combination of ultracentrifugation and agarose column chromatography, and density gradient ultracentrifugation was performed on the HDL as described in Methods. The sample was eluted by pumping a dense solution into the bottom of the centrifuge tube, and absorbance at 280 nm was continuously monitored and recorded. The profiles shown are of the same monkey plasma HDL samples separated by GGE in Figure 1, A and B. (Figure 5B) was frequently near the middle of the density gradient (d = 1.102 ± 0.006 g/ml, n = 9). The density distributions of HDL cholesterol, protein, and phospholipid were quantitated after pooling the HDL recovered from each gradient into six predetermined density intervals.

Figure 6 shows the effect of dietary fat on the average cholesterol (Figure 6A) and protein (Figure 6B) concentrations of each of the six HDL density subfractions separated by DGUC. Among specific subfractions (Figure 6A), concentrations of cholesterol in fractions 1 and 2 were significantly higher among the saturated fat-fed monkeys, while fractions 3, 4, 5, and 6 were significantly higher among the polyunsaturated fat-fed monkeys. The cholesterol concentrations of all HDL density subfractions were positively correlated to the total HDL concentration, although the correlation for the concentration of fraction 5 was only of marginal significance (p = 0.059).

Figure 6B shows the effect of dietary fat on the mean ± SEM protein concentration of each of the six HDL density subfractions. The total HDL protein concentration of the polyunsaturated fat-fed group was 17% higher than that of the saturated fat-fed group, although this difference was not statistically significant. Among individual density fractions, fractions 1 and 2 were significantly higher among the saturated fat-fed monkeys, while fractions 3, 4, and 5 were higher among the polyunsaturated fat-fed monkeys. The protein concentrations of all HDL density subfractions were positively correlated with the total HDL protein concentration (all p<0.003).

To determine whether dietary fat affected the composition, as well as the concentrations, of individual HDL subfractions, we examined the ratios of the cholesterol, protein, and phospholipid content of HDL subfractions isolated by DGUC. Among individual subfractions, the cholesterol/protein ratio of fraction 2 was slightly higher in the saturated fat-fed group (p<0.008), the phospholipid/protein ratio of fractions 5 and 6 was greater in the polyunsaturated fat-fed group, and the cholesterol/phospholipid ratio of fractions 3, 4, 5, and 6 was higher for the saturated fat-fed monkeys, although these latter differences were of marginal significance.

The apoprotein patterns of density gradient subpopulations were analyzed by SDS-polyacrylamide gel electrophoresis (data not shown). Gross differences among subfractions were not apparent; apo A-I was the major band and an apo A-II, apo C band also was always present. Apo E, when present, was found only as a minor band, usually in the lower density subfractions.
Figure 6. Effect of type of dietary fat on the mean ± SEM of the protein concentrations of HDL subpopulations as determined by GGE. The average concentration of total HDL protein of the polyunsaturated fat-fed group was 19% higher than that of the saturated fat-fed group, although this difference was not statistically significant. Among specific subpopulations, the concentration of HDL$_{2b}$ protein was higher for the saturated fat-fed group, while HDL$_{2a}$ and HDL$_{3a}$ protein concentrations were higher for the polyunsaturated fat-fed monkeys. HDL$_{2b}$ and HDL$_{2a}$ protein concentrations were positively correlated with the total HDL protein concentration, although the correlation for the HDL$_{2b}$ subpopulation was of marginal significance ($p = 0.097$). Concentrations of none of the HDL$_3$ subpopulations (HDL$_{2b}$, HDL$_{2a}$, or HDL$_{3c}$) were significantly correlated with the whole HDL protein concentration.

### Quantitation of HDL Subpopulations of Monkeys Fed Low Cholesterol Diets

The effect of the saturation of dietary fat on HDL was also examined in a group of 12 monkeys consuming low cholesterol diets containing predominantly either saturated or polyunsaturated fat (Diets C and D in Table 1). Quantitation of the concentrations of each HDL subpopulation by AnUC (Figure 8) showed that the concentration of HDL$_3$ material was significantly higher ($p = 0.007$) among the polyunsaturated fat-fed monkeys although there was no difference in the concentration of total HDL material between the two groups.

When the HDL from all monkeys was subfractionated by DGUC, it was observed that the peak of the HDL material of the saturated fat-fed monkeys was near the center of the tube (mean ± SEM, $d = 1.110 ± 0.001$ g/ml, $n = 4$) while the peak of the HDL from the polyunsaturated fat-fed monkeys was near the bottom of the tube (mean ± SEM, $d = 1.117 ± 0.002$ g/ml, $n = 5$). Figure 9 shows the density distribution of cholesterol (Figure 9A) and protein (Figure 9B) for the two low cholesterol diets. There was no statistically significant difference in the amount of either cholesterol or protein in any density fraction. However, it was apparent that the distribution of each chemical constituent was shifted toward lower density among the saturated fat-fed monkeys, consistent with the trends observed in the AnUC profiles.

The fat-related difference in HDL$_3$ concentration observed by AnUC was also seen when the HDL distribution was quantitated by GGE (data not shown). These data showed that the polyunsaturated fat-related elevation in HDL$_3$ concentration observed by analytic ultracentrifugation was due to significantly higher concentrations of HDL$_{2b}$ and HDL$_{3c}$ ($p = 0.002$ and $p = 0.032$, respectively), but not of HDL$_{3a}$.

### Discussion

The data in this paper and previous studies$^{12, 16}$ demonstrate the similarity in composition and heterogeneity be-
 tween human and African green monkey HDL and emphasize the importance of these monkeys as models of human lipoprotein metabolism. In the present studies, most of the monkeys were fed diets designed to induce plasma cholesterol concentrations typical of human beings at increased risk for premature coronary heart disease. Therefore, the level of dietary cholesterol was higher than the average for our society; this is necessary to induce the modest hypercholesterolemia of these studies. In this case, our experiments specifically model humans at risk, not the population in general. In addition, we have fed two different dietary fat formulations, as 40% of calories, that differed in their content of saturated and polyunsaturated fatty acids. This was done to determine how effects of increased polyunsaturation in dietary fat would affect atherosclerosis and HDL composition and concentration.

We have previously shown that a group of African green monkeys fed polyunsaturated fat-rich diets had less coronary artery atherosclerosis than a saturated fat-fed group, in spite of lower HDL concentrations.

Specific relationships between diet-induced changes in HDL and the development of atherosclerosis are unknown, but several considerations seem important when reduced HDL concentrations do not correlate with atherosclerosis severity, as in polyunsaturated vs. saturated fat feeding. HDL are thought to protect against atherosclerosis by removing free cholesterol from the plasma membranes of cells within the arterial wall, thereby counteracting the influx of cholesterol, presumably from LDL. The saturated fat-fed monkeys had higher levels of LDL than the polyunsaturated fat-fed monkeys. Therefore, the HDL of the saturated fat-fed monkeys would need to transport more cholesterol away from the arterial wall simply to maintain the same degree of protection. The cholesterol enrichment of the HDL of the monkeys fed saturated fat may reflect an inability of these monkeys to adequately deliver HDL cholesteryl esters to the liver for excretion. In addition, the higher cholesterol/phospholipid ratios of the HDL3 in the saturated fat-fed monkeys would suggest that the HDL3 in these monkeys is less capable of removing cellular cholesterol than the HDL3 of the polyunsaturated fat-fed monkeys. In short, since the HDL of the saturated fat-fed monkeys were relatively cholesterol-enriched, they may have been less capable of transporting additional cholesterol away from the artery wall.

This paper provides the first demonstration that three common methods that fractionate high density lipoproteins on the basis of different physical characteristics each provide complementary information about HDL heterogeneity and identify similar diet-induced differences in HDL subfraction distribution. AnUC with computerized deconvolution of schlieren profiles was useful for quantitating whole lipoprotein mass, but did not resolve subpopulations of HDL3. GGE provided the best resolution of HDL subpopulations, especially HDL3, but this method was the least reliable for quantitation presumably because of uncertainties of uniform stain uptake by different HDL particles. DGUC was the only preparative method used. Analysis of density subfractions of HDL by GGE (Figure 2) and AnUC (Table 4) indicated that DGUC partially separated HDL subpopulations. This data also suggested that...
GGE fractionated HDL into comparable subpopulations consistent with the observations of Anderson et al. who studied human HDL.

The present report presents the first available data showing that the type of dietary fat results in a redistribution of material among HDL subpopulations distinct from any effects on the total HDL concentration. The data indicated that dietary saturated fat induced the accumulation in plasma of large, cholesterol-enriched HDL, while polyunsaturated fat induced elevations in the levels of intermediate size HDL. The particular subpopulations affected by polyunsaturated fat feeding depended upon the level of dietary cholesterol; HDL, and HDL, were elevated on high cholesterol diets, and HDL, and HDL, were elevated on low cholesterol diets. It should be noted that the observed fat-related redistribution of HDL mass was not merely the result of differences in HDL cholesterol levels, since the trends were apparent even when monkeys with comparable HDL cholesterol levels on the different diets were compared. Thus, it appears that dietary cholesterol and saturated fat each contribute to the enlargement and cholesterol enrichment of plasma HDL.

The mechanism for such diet-related modulations of plasma HDL distributions are unknown but could result from a direct effect of dietary fat on production of HDL precursors. HDL are presumed to arise from neutral lipid-rich precursors consisting of phospholipid, free cholesterol, and HDL apoproteins that are secreted by the liver and intestine, but additional components are derived from triglyceride-rich lipoproteins during lipolysis. Such lipoproteins are not typically observed in plasma because the plasma enzyme lecithin-cholesterol acyltransferase (LCAT) is thought to rapidly mediate the esterification of the free cholesterol in the surface of the precursor particles, with the formation of spherical particles typical of plasma HDL. Particles with the characteristics of HDL precursors, however, have been observed in medium following perfusion of rat and African green monkey livers when LCAT activity is negligible.

We have studied the effects of LCAT on the HDL precursor particles that are present in the perfusate of isolated African green monkey livers and have found that incubation of monkey liver perfusate with LCAT resulted in the formation of distinct subpopulations with the properties of plasma HDL, HDL, and HDL. The effect of the type of dietary fat on the characteristics and distribution of HDL subpopulations generated by LCAT from liver perfusate HDL precursors is not yet known. Data from this laboratory, however, has suggested that dietary polyunsaturated fat decreases apo A-I production by both the intestine and liver. Investigations to determine the effect of dietary fat on the production rates of apo A-II by the liver and intestine are in progress.

Apo E concentrations in both the plasma and HDL of the monkeys were low. None of the methods employed separated HDL on the basis of apo E content. It is likely that a small proportion of the particles designated as HDL contained apo E, while most did not. Apo E is primarily associated with the largest HDL subfractions, a phenomenon we have confirmed in these monkeys with SDS-PAGE. Extremely large, apo E-containing HDL, and apo E-HDL are commonly found in the plasma of cholesterol-fed animals that lack the cholesterol ester exchange protein (CEEP). The HDL of species that possess CEEP are smaller because the protein mediates an important process that results in the removal of HDL cholesteryl esters and a reduction in particle size. Studies in baboons have shown that diets rich in saturated fat and cholesterol can induce the appearance of HDL particles in the plasma of some individuals. The HDL itself was heterogeneous and only the largest particles (200 Å) contained as much as 10% of its protein as apo E. Particles with the characteristics of apo E-HDL were not observed. In the present studies with African green monkeys, only one of the more than 90 monkeys studied exhibited measurable levels of particles with the size and flotation properties of HDL.

Studies are currently in progress to determine situations that induce HDL in the African green monkey. In any event, it is apparent that apo E is not a major constituent of the five HDL subpopulations present in the plasma of African green monkeys or other primates, even when they are fed diets enriched in cholesterol.

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