Male–female variability in the dietary cholesterol-induced hyperlipoproteinemia of cynomolgus monkeys (*Macaca fascicularis*)[1]

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Abstract The characteristics of dietary cholesterol-induced hyperlipoproteinemia were studied in adult *Macaca fascicularis* to determine if significant male–female differences could be identified. The plasma lipoproteins from individual animals were separated by agarose column chromatography and the resulting size populations of lipoproteins were chemically characterized. The distribution of constituents among the lipoprotein classes was determined. The lipoproteins that increased most significantly in response to dietary cholesterol were the low density lipoproteins (LDL) which were those of region III of the column elution profile. The LDL mass concentrations (in mg/dl of plasma) were not different between males and females. However, these lipoproteins showed a characteristic difference between males and females in response to dietary cholesterol. The primary response in the males was an increased LDL molecular weight. In females, the primary response was an increased number of LDL particles (measured as μmolar concentration). Thus, at the same LDL mass concentration, males had significantly larger LDL particles and yet fewer of them. Analysis of the chemical composition data showed that size differences between LDL of males and females was due primarily to the incorporation of more cholesteryl ester into the LDL particles from male animals; the content per LDL particle of free cholesterol, phospholipid, and protein showed the same proportionality to size for both sexes. Another significant difference between lipoproteins of males and females was in the extent to which the plasma HDL (identified in these studies as the lipoproteins of region IV of the elution profile) decreased in response to dietary cholesterol. Chemical composition differences were also noted. The HDL particles of males and of cholesterol-fed animals contained more cholesteryl ester than did HDL of females and control animals, respectively.

Supplementary key words agarose gel chromatography · atherosclerosis · LDL molecular weight · cholesteryl ester · plasma cholesterol concentration

It has long been recognized that protection against coronary atherosclerosis exists among Caucasian females in the reproductively active years. The mechanism of this sparing is unknown. One possibility which has been suggested is that protection is related, at least in part, to the plasma lipoproteins, which probably function as mediators in the development of atherosclerosis. Accordingly, male–female differences in human plasma lipoproteins have been described (1–11). It is unknown if any or all of these male–female differences in lipoprotein concentration and composition are related to "female protection" against atherosclerosis. However, both older (12) and more recently reported (13, 14) findings of the relationship between HDL and coronary heart disease and the apparent male–female differences in HDL concentration are findings consistent with this possibility, as is the correlation between the sex difference in atherosclerosis and LDL concentration (15).

In view of potential relationships between plasma lipoproteins and male–female differences in coronary artery atherosclerosis, it seemed desirable that a primate model be identified so that knowledge of the physiopathological mechanisms could be developed. We have studied lipoprotein distribution and composition in a group of *Macaca fascicularis* (cynomolgus monkeys). The propriety of this species was suggested by at least two findings. These animals are known to be readily susceptible to experimental atherosclerosis (17–19) and the adult females of this species are known to experience a regular estrus cycle when housed indoors in a laboratory environment (16). In the present studies we have analyzed plasma lipo-

Abbreviations: HDL, high density lipoprotein(s); ILDL, intermediate-sized low density lipoprotein(s); LDL, low density lipoprotein(s); PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; VLDL, very low density lipoprotein(s); TLC, thin-layer chromatography; MW, molecular weight.

[1] This work was taken in part from the doctoral dissertation submitted by L.L.P. to Wake Forest University in 1976.

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proteins in detail so that individual animal differences and sex-specific characteristics would become apparent. Since detailed analyses were needed to characterize the entire plasma lipoprotein spectrum from numerous individual animals, the agarose column chromatographic method for lipoprotein isolation and characterization was chosen because of its time-saving feature (20) and its simultaneously preparative and analytical nature (21).

MATERIALS AND METHODS

Animals and diets

Forty feral *Macaca fascicularis* from Malaysia were purchased from a primate importer. All were adults at the start of the study. Of the 20 males and 20 females, 4 of each sex were randomly selected as control animals. Body weights were essentially constant throughout the study; the average for females was 3.4 kg and that for males was 5.0 kg. Vaginal swabs were done daily on all females to determine estrus cycle periodicity; all females cycled regularly ($\bar{x} = 28$ days) throughout the course of study. Blood samples for lipoprotein analysis were taken at mid-cycle. Animals were housed in group cages containing four animals each; males were housed directly across from females so that visual contact was maintained. A semipurified diet was fed, the composition of which is shown in **Table 1.** This diet has 40% of the calories as fat, 40% as carbohydrate, and 20% as protein. For the first 2 months of the study the test diet contained added cholesterol at the level of 0.4 mg/Cal (0.2% by wt) and, for the remaining 14 months of the study, 0.6 mg/Cal (0.3% by wt). The control diet contained only the cholesterol present in the lard (0.005 mg/Cal). Routinely, animals were fed each morning and evening. Before blood collection for serum cholesterol (22) or lipoprotein analyses (see below), animals were fasted for 18–24 hr, after which animals were caught and given Ketamine intramuscularly (10 mg/kg body weight) to facilitate handling and sampling, as described previously (23).

Lipoprotein isolation and characterization

Lipoproteins were initially separated from plasma by ultracentrifugation at a density (d) of 1.225 g/ml after which they were separated into classes by agarose column chromatography on Bio-Gel A-15m, 200–400 mesh, (BioRad Labs, Richmond, CA) by the method of Rudel, et al. (20). The determination of LDL molecular weight[4] was carried out during chromatographic separation according to the procedure previously described (21). Lipoprotein separation and characterization was carried out for each animal two or three times during the 14-month period of the 0.3% cholesterol diet. For each plasma sample, recovery of lipoproteins was quantitated by measuring the cholesterol recovered in each of the column fractions compared to that present in the original plasma sample; the percent recovery was 89.5 ± 0.88 (mean ± SEM, $n = 100$).

Column elution fractions within a size population of lipoproteins were combined and concentrated when necessary, as previously described (23). To check separation of peak III and peak IV lipoproteins, immunodiffusion was used with antisera prepared to rhesus monkey LDL and HDL. By this technique, peak III lipoproteins were judged free of HDL and peak IV lipoproteins free of LDL, as we have previously reported using human lipoprotein samples (20) and rhesus monkey samples (23). To characterize the size populations, the electrophoretic mobilities and density ranges were determined for lipoproteins included in each of the elution regions. Agarose electrophoresis, performed according to the method of Noble (24) in 0.5% agarose, was used to monitor the electrophoretic mobility of lipoproteins. Preparative ultracentrifugation was carried out as described previously (23). After lipoprotein separation, recovery was based on the distribution of cholesterol.

TABLE 1. Composition of diets[a]

	Control	Test
	% by weight	
Protein		
Lactalbumin	5.0	5.0
Casein USP	11.2	11.2
Gelatin	1.8	1.8
Carbohydrate		
Sucrose	23	23
Dextrin	23	23
Fat		
Crisco	8	8
Corn Oil	8	8
Lard	8	8
Hegsted's salt mix	4	4
Vitamin mix[b]	2.5	2.5
Alphacel	5.3	5.0
Cholesterol		0.3

[a] Dry ingredients were mixed with water (25% by wt), until a cake-like consistency was reached. Food was stored frozen as small 1-lb. cakes which were thawed just prior to use.
[b] Vitamin D_3 in corn oil was added separately.

[4] We recognize that a lipoprotein is not a molecule, per se, but is instead a complex of numerous lipid and protein molecules which is better termed a particle. Use of the term LDL molecular weight instead of LDL particle weight is at best an accommodation to assure clarity of concept.

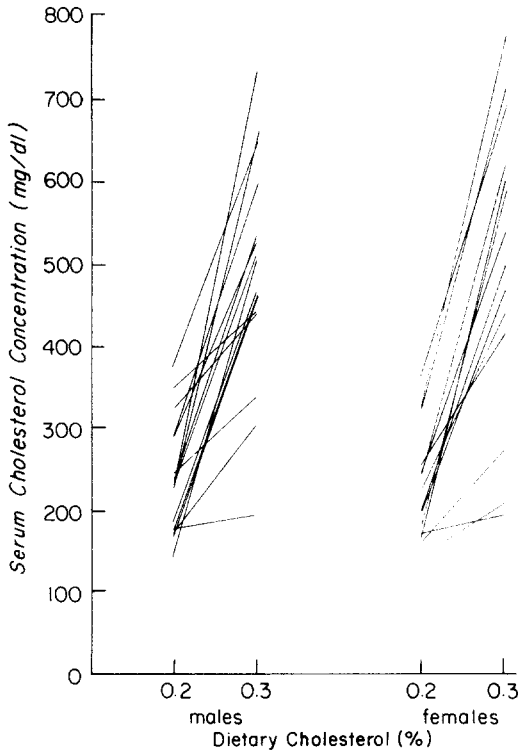

Fig. 1. Serum cholesterol concentration response to two levels of dietary cholesterol in male and female *M. fascicularis*. The serum cholesterol response to two diets different only in the level of cholesterol was observed at biweekly intervals for two months (0.2%) then at bimonthly intervals for 14 months (0.3%). For each animal, the average serum cholesterol concentration at each level of dietary cholesterol was plotted and these points were then connected with a line. The data are for 16 males and 14 females.

Chemical analyses

Lipoprotein fractions were chemically analyzed as previously described (21). Total cholesterol (25), phospholipid phosphorus (26), and protein (27) were determined directly on intact lipoproteins eluted from the column. After lipid extraction in chloroform–methanol 2:1 and TLC separation of lipid classes, free and ester cholesterol determinations were carried out by the method of Rudel and Morris (25). Triglycerides were quantitated after TLC separation by a modified method of Sardesai and Manning (28) in which the Florisil step was omitted. For apolipoprotein analysis, lipoprotein samples eluted from the column were dialyzed against distilled water containing 0.01% EDTA, after which they were lyophilized and extracted with ethanol–ether 3:1 according to the method of Scanu and Edelstein (29). The apoprotein samples were then solubilized in 0.025 M Tris–0.2 M glycine buffer (pH 8.3) containing 0.02 M sodium dodecyl sulfate, after which polyacrylamide gel electrophoresis (PAGE) was carried out in a 12.5% polyacrylamide gel horizontal slab, as previously described (21).

Statistical methods of analysis utilized in this study were taken from Snedecor and Cochoran (30). Unless otherwise indicated, statements of statistical significance refer to the $P < 0.01$ level.

RESULTS

The mean serum cholesterol concentrations, determined on multiple samples during the times of the two different levels of dietary cholesterol were plotted for individual animals as shown as **Fig. 1.** Among both males and females, considerable variability occurred in the serum cholesterol response to either diet. However, the rank order remained relatively constant from diet to diet, suggesting that the plasma cholesterol response was characteristic of the individual animal.

To develop a better understanding of this response, the plasma lipoprotein spectrum in each of the animals was characterized. Representative elution profiles of plasma lipoproteins from test and control animals are shown in **Fig. 2.** The shape of the elution profile showed that at least four size populations of lipoproteins were present. These were combined by pooling the fractions as indicated in Fig. 2. Overlap was clearly present for elution regions I, II, and III, especially in the test diet-fed animals.

The data in **Fig. 3** show the average lipoprotein mass concentrations for each of the four groups of

TABLE 2. Size, density, and electrophoretic characteristics of plasma lipoproteins of *Macaca fascicularis*

	Size Region	Density Range	Cholesterol[a] Distribution	Electrophoretic Mobility
		%		
Control diet	I	d < 1.006	>95	pre β (?)
	II	d < 1.006	10	pre $\beta_{1 \text{ and } 2}$
		1.006 < d < 1.050	80	
		1.050 < d < 1.10	10	
	III	1.019 < d < 1.063	>95	β
	IV	1.063 < d < 1.225	>95	α
Test diet	I	d < 1.006	>95	post β + β
	II	d < 1.006	50	β + pre β
		1.006 < d < 1.019	45	
		1.019 < d < 1.10	5	
	III	d < 1.006	5	β
		1.006 < d < 1.019	45	
		1.019 < d < 1.063	50	
	IV	1.063 < d < 1.225	>95	α

[a] Distribution among density ranges within the size region.

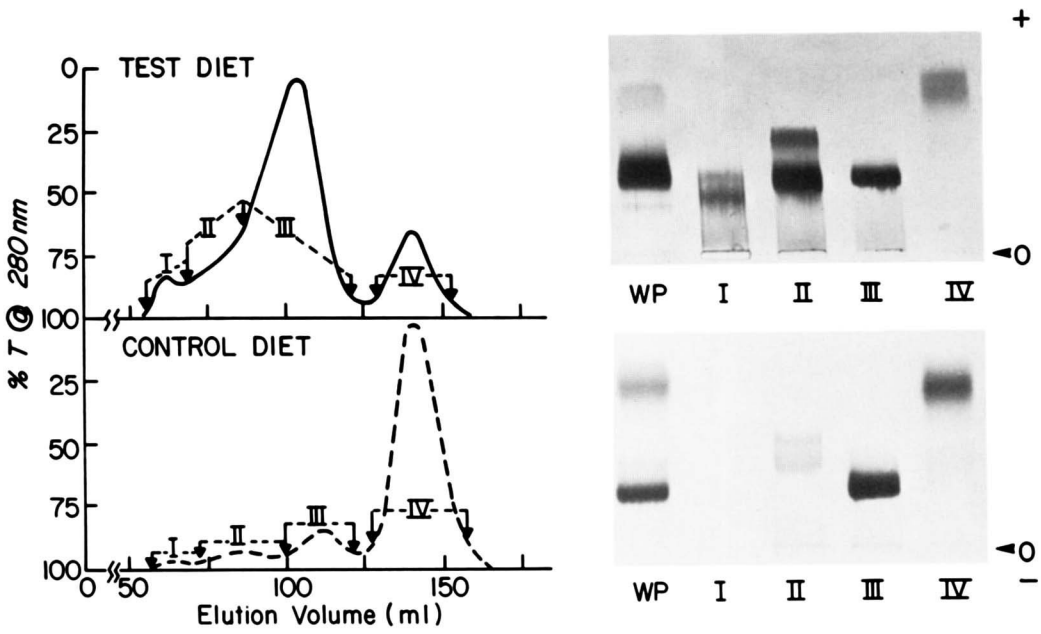

Fig. 2. Comparison of the agarose column chromatography elution profiles for plasma lipoproteins and the agarose electrophoresis patterns of elution regions. In the column profiles, the size distribution of lipoproteins isolated from 8 ml of plasma by ultracentrifugation is shown for representative control and test-diet animals. The material in the regions indicated by the roman numerals was then pooled and concentrated for electrophoretic analysis by ultracentrifugation at densities (in g/ml) of 1.006 (I), 1.063 (II), 1.063 (III), and 1.225 (IV). The electrophoresis strips were stained for 15 min at 37°C in a 60% ethanol solution saturated with oil red O and fat red 7B. The position of the sample wells is indicated by the arrows labeled *O*. Electrophoresis of whole plasma (WP) is shown for comparison.

this study. For region I and II lipoproteins, the concentrations were significantly higher in test diet animals compared to control diet animals; no significant differences between males and females were found. The largest dietary cholesterol-induced increase was in the concentration of region III lipoproteins which rose from an average of 140 mg/dl in control animals to nearly 1,100 mg/dl in test animals, a highly significant 8-fold increase. The average concentrations (mg/dl) of region III lipoproteins in males and females were similar in both diet groups. In contrast to the larger sized lipoproteins, the concentration of region IV lipoproteins was significantly reduced by dietary cholesterol. In males, the decrease was 425 mg/dl on the average, from 565 to 140 mg/dl. In females, the average decrease was less, from 534 to 204 mg/dl or 330 mg/dl.

The density ranges and electrophoretic mobility in agarose of the lipoproteins from each of the size regions indicated in Fig. 2 were evaluated. The results of these analyses are given in **Table 2.** Within each size region, the amount of cholesterol that floated at each density was used to approximate the lipoprotein distribution.

Over 95% of the region I lipoprotein cholesterol floated at $d < 1.006$, indicating these lipoproteins are VLDL. Lipoproteins with both post-β and β migration could be seen in this fraction from test animals by agarose electrophoresis (Fig. 2). Region I lipoproteins from control animals were not present in high concentration, and are not visualized in Fig. 2. Region II lipoproteins were lipoproteins with a broad density range. In control animals the distribution was quite variable. About 80% of the lipoprotein cholesterol in this region floated in the range $1.006 < d < 1.050$ g/ml, a fact that has led us to refer to this fraction as the intermediate-sized low density lipoproteins (ILDL). Two pre-β bands were found in region II lipoproteins of control animals. About 10% of the pre-β lipoprotein cholesterol of region II had a density range of $1.050 < d < 1.10$ g/ml, and about 10% of it was $d < 1.006$ g/ml. In test diet-fed animals, about half of the region II lipoprotein cholesterol floated at $d < 1.006$ g/ml and most of the remainder was in the range of $1.006 < d < 1.019$ g/ml. On the average, less than 5% was found in the $d > 1.019$ g/ml fraction. Both a wide β and a pre-β band were found when the region II lipoproteins of test animals were subjected to agarose electrophoresis (Fig. 2).

In control animals, region III lipoproteins were β-migrating lipoproteins with a density range of $1.019 < d < 1.063$ g/ml, i.e., low density lipoproteins. In test

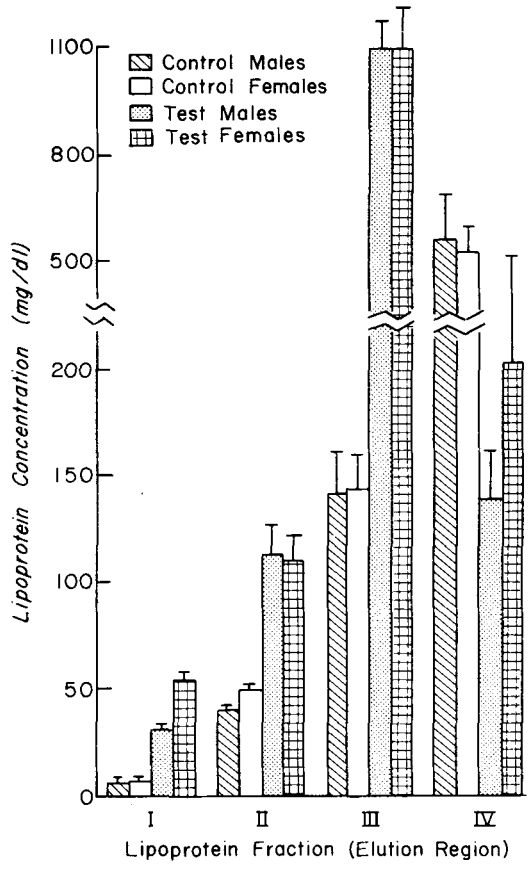

Fig. 3. Demonstration of the effect of dietary cholesterol (0.3%) on plasma lipoprotein mass concentrations in male and female *M. fascicularis*. Lipoproteins from each of the elution regions indicated (see Fig. 2) were analyzed for each constituent, including protein, phospholipid, free cholesterol, cholesteryl ester, and triglyceride. For each fraction, the lipoprotein concentration was determined as the sum of the constituents. The mean (±SEM) values are shown for each of the groups. At least two separate plasma samples per animal were analyzed. The number of animals in each group is given in Table 3; all 40 animals of the study are represented.

diet-fed animals, region III lipoproteins also were all β-migrating lipoproteins but the average density of the particles was less, as indicated by the fact that we found 45% of the cholesterol of these lipoproteins floated at d $1.006 < d < 1.019$ g/ml and 50% at $1.019 < d < 1.063$ g/ml. Less than 5% floated at d < 1.006 g/ml. Although we did not examine enough samples to establish a statistically significant relationship, the density distribution among region III lipoproteins appeared proportional to the total plasma cholesterol concentration, so that the higher the cholesterol concentration, the more the material that floated at a lower density. Region IV lipoproteins were α-migrating lipoproteins with a density range of $1.063 < d < 1.225$ g/ml, i.e., high density lipoproteins. For this fraction, no differences in density or electrophoretic mobility were found between control and test diet-fed animals.

The electrophoresis data of Fig. 2 suggested that the lipoproteins of regions I and II were heterogeneous and that further separation was needed to generate homogeneous fractions (based on density, size, and electrophoretic mobility). On the other hand, the lipoproteins of regions III and IV were reasonably free of electrophoretic heterogeneity. This fact together with the elution profile data and the density ranges indicated these lipoproteins were essentially pure LDL and HDL, respectively. These names for the lipoprotein fractions will be used subsequently for identification. Complete chemical analyses were carried out on these fractions.

The influence of dietary cholesterol on concentrations of each of the constituents of LDL and HDL is shown by the data in **Table 3.** Concentrations of each of the components of LDL were increased by dietary cholesterol and all of the increases except that of triglycerides were statistically significant; in contrast, no significant differences between males and females were found for these measurements as determined by analysis of variance. In both sexes, there were significant decreases in each of the components of HDL in response to dietary cholesterol. In addition, multivariate analysis of variance indicated that a significant difference between the HDL concentrations of males and females occurred in response to diet. HDL levels were generally higher in animals with lower total serum cholesterol concentrations. The correlation between serum cholesterol and the inverse of HDL concentration shown in **Fig. 4** was significant ($r = 0.61$). The females tended to have higher HDL concentrations (lower inverse values) than males at equivalent cholesterol concentrations (Fig. 4) although covariance analysis indicated that this difference was not statistically significant.

In addition to effects on concentration, we looked for differences in lipoprotein composition. For LDL, as shown in **Table 4,** the percentage composition appeared to have been modified by dietary cholesterol; in LDL of test animals cholesteryl ester was higher and protein was lower. The LDL of males were higher in cholesteryl ester and lower in protein than for those of females. By calculating ratios of constituents, analysis of variance for the compositional differences became feasible since ratio calculations are independent of each other. The six ratios shown illustrate the maximum variability among the groups. Multivariate analysis of variance indicated that differences induced by dietary cholesterol in LDL composition were statistically significant. Of the six ratios of con-

TABLE 3. Effect of dietary cholesterol on plasma concentrations of LDL and HDL from male and female *M. fascicularis*

	N	Plasma Concentrations					
		PL	FC	CE	TG	Pro	Total
				mg/dl			
Region III Lipoproteins (LDL)							
Test males	16	220.8[a] ± 16.5	93.7 ± 7.0	563.7 ± 44.5	23.5 ± 3.2	214.0 ± 14.9	1115.8 ± 83.4
Test females	16	219.7 ± 27.0	89.0 ± 10.4	531.0 ± 65.4	34.8 ± 18.0	219.3 ± 26.6	1093.8 ± 130.1
Control males	4	31.4 ± 4.6	12.1 ± 1.7	60.6 ± 9.0	5.7 ± 1.4	32.2 ± 5.1	141.9 ± 20.9
Control females	4	32.5 ± 3.5	11.1 ± 1.2	56.2 ± 5.8	8.8 ± 1.8	36.2 ± 3.9	144.9 ± 15.9
Region IV Lipoproteins (HDL)							
Test males	16	34.0 ± 6.3	4.1 ± 0.6	31.8 ± 5.1	1.9 ± 0.4	67.7 ± 12.1	139.5 ± 24.3
Test females	16	50.9 ± 14.2	6.1 ± 2.0	43.5 ± 9.8	3.9 ± 1.3	99.7 ± 21.6	204.1 ± 48.7
Control males	4	165.9 ± 36.9	18.8 ± 4.2	113.0 ± 20.2	11.6 ± 3.3	256.0 ± 44.8	565.4 ± 107.7
Control females	4	161.6 ± 24.9	14.9 ± 2.6	92.2 ± 12.8	15.1 ± 2.6	249.8 ± 26.3	533.6 ± 68.5

[a] Mean (±SEM) for at least two samples per monkey.

Abbreviations: *N*, number of individuals; PL, phospholipids; FC, free cholesterol; CE, cholesteryl ester; TG, triacylglycerol; Pro, protein.

stituents shown in Table 4, only the PL/Pro failed to increase significantly in LDL of animals receiving the test diet. The composition of LDL also was significantly different for males vs. females. The TC/Pro ratio and the molar FC/PL ratios were significantly higher in LDL of males vs. females; other ratios were not significantly different.

Further information about LDL was developed based on the molecular weights of the LDL particles. Molecular weights on all samples from individual animals were routinely measured chromatographically as described previously (21). For comparison, seven LDL samples were preselected to represent a wide range of values and molecular weight was determined by analytical ultracentrifugation. Results using the two procedures were well within the estimated 10% accuracy range for either method, i.e., column values, 5.45 ± 0.57 molecular weight units, mean ± SEM, range 3.8–7.8; analytical ultracentrifuge values, 5.68 ± 0.55 molecular weight units, range 4.2–7.8; correlation between the two methods, $r = 0.98$. As shown in **Table 5,** dietary cholesterol increased the average LDL molecular weight in both sexes; and males had higher average LDL molecular weights than females. Both the dietary-induced and sex-related differences were statistically significant. The significant LDL molecular weight differences occurred at equivalent plasma mass concentrations of

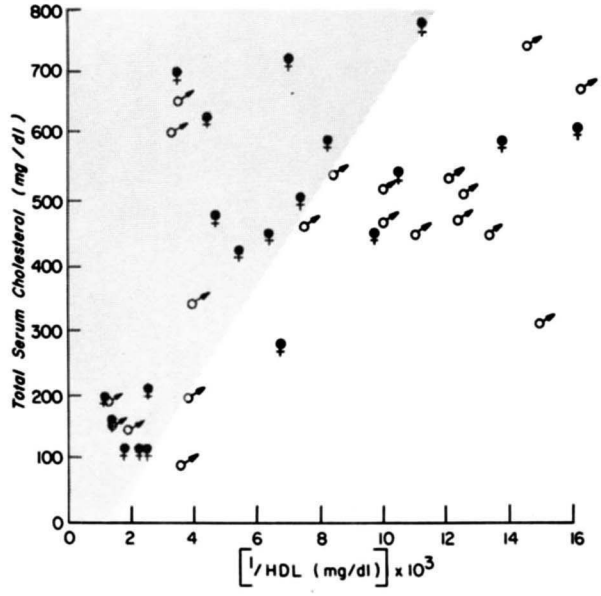

Fig. 4. Male–female comparison of the relationship between the inverse of plasma HDL concentration and total serum cholesterol concentration. For each animal, the HDL concentrations were determined on at least two separate plasma samples as described in Fig. 3. Average total serum cholesterol concentrations for each animal were determined on seven bimonthly samples taken during the last 14 months of the study. All 40 animals (test and control) of the study are included; each point represents the data for one animal. Because HDL concentrations decreased in test animals, the inverse of the HDL concentration was plotted as a more sensitive means of identifying differences. The graph was shaded in the region that had most of the data points for females.

TABLE 4. Effect of dietary cholesterol on the composition of LDL and HDL from male and female *M. fascicularis*

							Ratios of Constituents					
							Mass Ratios				Molar Ratios	
	N	PL	FC	CE	TG	Pro	PL/Pro	TC/PL	TC/Pro	EC/TC	FC/PL	EC/TG
				% by weight								
Region III Lipoproteins (LDL)												
Test males	16	19.8[a] ± 0.2	8.4 ± 0.1	50.2 ± 0.5	2.1 ± 0.2	19.4 ± 0.4	1.03 ± 0.02	1.92 ± 0.13	1.98 ± 0.06	0.777 ± 0.003	0.85 ± 0.02	38.6 ± 5.2
Test females	16	20.2 ± 0.4	8.2 ± 0.2	48.0 ± 1.0	3.3 ± 1.5	20.3 ± 0.4	1.00 ± 0.02	1.81 ± 0.18	1.80 ± 0.04	0.777 ± 0.004	0.82 ± 0.02	47.0 ± 10.6
Control males	4	22.2 ± 0.3	8.5 ± 0.1	42.7 ± 0.3	4.0 ± 0.8	22.6 ± 0.3	0.98 ± 0.02	1.52 ± 0.03	1.49 ± 0.02	0.747 ± 0.003	0.77 ± 0.02	16.2 ± 4.0
Control females	4	22.5 ± 0.2	7.7 ± 0.1	38.9 ± 0.6	5.9 ± 0.7	25.0 ± 0.3	0.90 ± 0.02	1.36 ± 0.05	1.22 ± 0.005	0.749 ± 0.004	0.69 ± 0.01	9.2 ± 1.2
Region IV Lipoproteins (HDL)												
Test males	16	23.7[a] ± 0.06	3.1 ± 0.1	23.8 ± 0.8	1.3 ± 0.1	48.1 ± 1.1	0.50 ± 0.02	0.73 ± 0.03	0.36 ± 0.02	0.820 ± 0.003	0.265 ± 0.015	26.8 ± 2.5
Test females	16	23.6 ± 0.6	2.8 ± 0.1	21.8 ± 0.4	1.7 ± 0.1	50.1 ± 0.7	0.48 ± 0.02	0.67 ± 0.02	0.31 ± 0.01	0.820 ± 0.007	0.237 ± 0.011	19.0 ± 1.7
Control males	4	28.6 ± 1.3	3.3 ± 0.1	20.3 ± 0.6	2.6 ± 0.6	45.9 ± 1.1	0.63 ± 0.04	0.53 ± 0.03	0.33 ± 0.01	0.785 ± 0.011	0.228 ± 0.001	16.9 ± 4.6
Control females	4	30.1 ± 0.9	2.7 ± 0.1	17.2 ± 0.3	2.8 ± 0.2	47.2 ± 1.0	0.64 ± 0.03	0.43 ± 0.01	0.27 ± 0.01	0.786 ± 0.006	0.183 ± 0.005	8.3 ± 0.4

[a] Mean (±SEM) for at least two samples per monkey.
Abbreviations as given in Table 3; EC, esterified cholesterol.

LDL (Table 5). As a result, the number of LDL particles per ml of plasma would be expected to be lower in males than females. This was found as shown in the column for μmolar concentration of Table 5.

The LDL molecular weight was significantly related to total serum cholesterol concentration in females ($r = 0.81$) and in males ($r = 0.75$), but, at equivalent cholesterol concentrations, the LDL molecular weight of males was larger than for females with few exceptions (**Fig. 5**). Covariance analysis showed that the serum cholesterol-LDL molecular weight relationship was significantly different for males vs. females. This finding, coupled with the information that average LDL mass concentrations were not different between males and females, suggested that the opposite relationship between μmolar concentration of LDL and total serum cholesterol should be present. This was found to be the case (**Fig. 6**). A significant relationship between total serum cholesterol and μmolar LDL concentration was found for females ($r = 0.96$) and for males ($r = 0.81$). At equivalent serum cholesterol concentrations, the males had significantly lower μmolar concentrations of LDL than females, as determined by analysis of covariance. Thus, at the same LDL mass concentration, males had significantly larger LDL particles and yet fewer of them.

TABLE 5. Effect of dietary cholesterol on LDL size and plasma concentration in male and female *M. fascicularis*

	N	mg/dl	MW (×10⁻⁶)	μmol/l
Test males	16	1116 ± 84[a]	5.32 ± 0.32	2.14 ± 0.018
Test females	16	1094 ± 130	4.12 ± 0.16	2.72 ± 0.028
Control males	4	142 ± 21	3.17 ± 0.08	0.45 ± 0.07
Control females	4	145 ± 16	2.65 ± 0.08	0.54 ± 0.05

[a] Mean (±SEM) determined on at least two samples per animal.

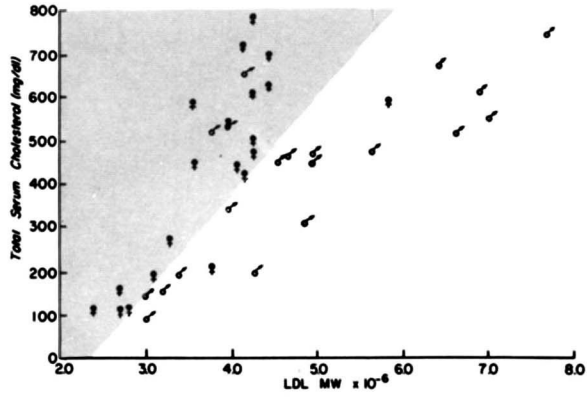

Fig. 5. Male–female comparison of the relationship between LDL molecular weight and total serum cholesterol concentration. The molecular weight for LDL was determined chromatographically based on a reference ^{125}I-labeled LDL standard (21) on at least two samples per animal. Other details are as given in Fig. 4.

We then asked if the composition of LDL would reflect the difference in LDL molecular weight. The chemical compositions were calculated on a per particle basis as g/μmol for both males and females, and the averages for each of the groups are shown in **Table 6.** The differences seen in these data were shown to be proportional to molecular weight when the data for each animal were plotted and the regression lines determined for each component of LDL. As shown in **Fig. 7,** the data for both males and females fit the same line in each instance.

The composition of HDL is also shown in Table 3. For HDL of test animals compared to those of controls, the average percentages of phospholipid and triglyceride were lower while those of cholesteryl ester and protein were higher. The average percentages of free cholesterol and cholesteryl ester were lower in HDL of females compared to those of males, while the average percentage of protein was higher. Multivariate analysis of variance indicated that both diet-induced and sex differences in HDL composition were statistically significant. For the dietary cholesterol effect, the TC/PL, EC/TC, molar FC/PL ($P < 0.05$) and molar EC/TG ratios were significantly higher while the PL/Pro ratio was significantly lower. The TC/Pro ratio was unchanged. Four ratios were significantly higher in males vs. females, i.e., TC/Pro, TC/PL, molar FC/PL ($P < 0.05$), and molar EC/TG; others were not significantly different.

We compared the apolipoprotein PAGE patterns for region III and IV lipoproteins of selected male and female animals of the study. The data shown in **Fig. 8** illustrate typical results. No male–female differences were apparent in the apolipoprotein patterns of either lipoprotein class. Detectable amounts of protein in the Arg-rich apoprotein, A-I apoprotein, and C apoprotein regions were found in all the LDL samples. There consistently appeared to be proportionally more protein in the A-I region and less in the Arg-rich region in LDL apoprotein samples from control animals compared to those of test animals.

TABLE 6. Effect of diet and sex on the within-particle composition of LDL

	N	LDL Composition				
		PL	FC	CE	TG	Pro
		g/μmol of LDL				
Test males	16	1.05[a] ± 0.06	0.45 ± 0.03	2.69 ± 0.19	0.11 ± 0.01	1.02 ± 0.05
Test females	16	0.83 ± 0.03	0.34 ± 0.02	1.99 ± 0.09	0.13 ± 0.06	0.84 ± 0.03
Control males	4	0.70 ± 0.03	0.27 ± 0.01	1.35 ± 0.05	0.13 ± 0.02	0.71 ± 0.03
Control females	4	0.60 ± 0.02	0.20 ± 0.01	1.03 ± 0.03	0.16 ± 0.02	0.66 ± 0.02

[a] Mean (±SEM) for at least two samples per animal. Abbreviations same as for Table 3.

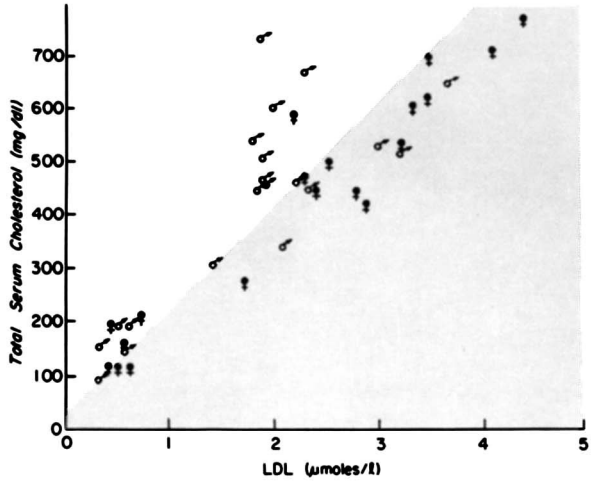

Fig. 6. Male–female comparison of the relationship between LDL μmolar concentration and total serum cholesterol concentration. The μmolar concentration was determined using the chromatographically determined molecular weights and the mass concentrations (Fig. 3). Other details are as given in Fig. 4.

DISCUSSION

We have characterized the hyperlipoproteinemic response to dietary cholesterol in adult *Macaca fascicularis* males vs. females. In general terms, the hypercholesterolemia induced by dietary cholesterol in this species was a hyperbetalipoproteinemia (Fig. 2). The extent of increase in concentration of β-lipoproteins was proportional to that of the total serum cholesterol concentration and occurred at the same time that a proportional decrease in the HDL concentration took place. On the average, the extent of hypercholesterolemia in males and females was the same, and the pattern of change in lipoproteins at first glance appeared quite similar for both sexes (Fig. 3). However, further evaluation revealed that this was not the case. It was recognized early in the study that significant individual animal variability in response to dietary cholesterol was present. By characterizing lipoproteins from each of the individual animals in the study, we capitalized on this variability to help us understand the distinctive features of the hyperlipoproteinemia. Specific aspects of lipoproteins were identified in which a patterned difference occurred among individuals within sexes and between sexes.

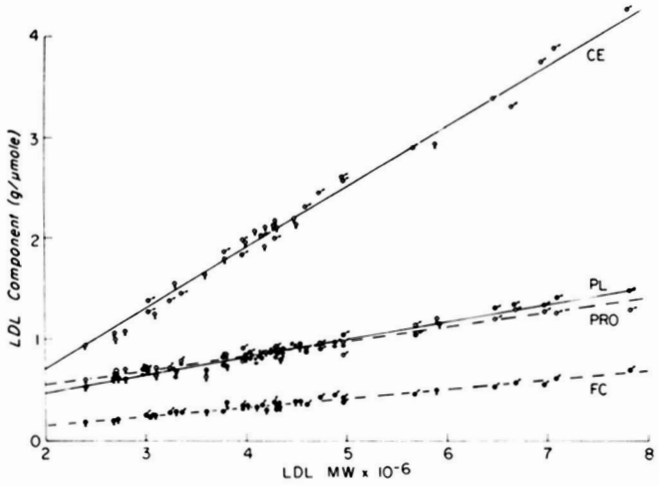

Fig. 7. Male–female comparison of the relationships between LDL molecular weight and the LDL within-particle composition. Percentages for each of the constituents of LDL were determined to obtain the g/μmol of the individual components (see Fig. 3). Molecular weights were determined chromatographically (see Fig. 5). Each point represents the data, determined in duplicate LDL samples, for one animal. Data from all 40 animals of the study are included. Abbreviations are defined in Table 3; CE, open symbols; PL, closed symbols; Pro, open symbols; FC, closed symbols. Lines shown are best-fit regression lines and the equations are: CE = 0.62 MW − 0.570; Pro = 0.14 MW + 0.26; PL = 0.18 MW + 0.12; FC = 0.09 MW − 0.02.

The β-lipoproteins most increased in concentration by dietary cholesterol were the region III lipoproteins, or LDL. While LDL mass concentration (in mg/dl of plasma) was not different between males and females, both LDL molecular weight and μmolar concentration (number of particles) were sex dependent. This observation is apparently without precedent, although some similarity may exist in the data suggesting a chemical composition difference for LDL from human males and females (6, 7) and a different flotation rate (6). Female monkeys had an increased LDL particle size in response to dietary cholesterol; one of 16 test females had an LDL molecular weight over 4.6×10^6 while 13 of 16 females had LDL MW between 3.8 and 4.6×10^6. By comparison, size increase was more marked in males, and 11 of 16 males had LDL molecular weights exceeding 4.6×10^6. In females, 11 out of 16 had μmolar concentrations exceeding 2.3 μmol/l, i.e., a significant part of the increase in LDL mass concentration was due to an increase in the number of LDL particles in plasma. On the other hand, the μmolar concentration of LDL in males exceeded 2.3 μmol/l in only 3 of 16 test animals, while 11 of 16 had concentrations between 1.8 and 2.3 μmol/l. These data suggest that, up to a point, both males and females increased their LDL concentration in response to dietary cholesterol by increasing both particle size and number. Males had a limited ability to increase particle number, but could increase LDL particle size in a more unlimited way. The situation in females was in contrast; LDL mass was elevated in part due to an increased particle size, but a limit in particle size increase was reached and females appeared to continue to increase LDL concentration by increasing the number of LDL particles.

These data indicated that two types of control mechanisms, i.e., for particle size vs. number, exist for increasing LDL concentrations which appeared to be modulated differently between the sexes. One potential control site would be that of apoprotein B

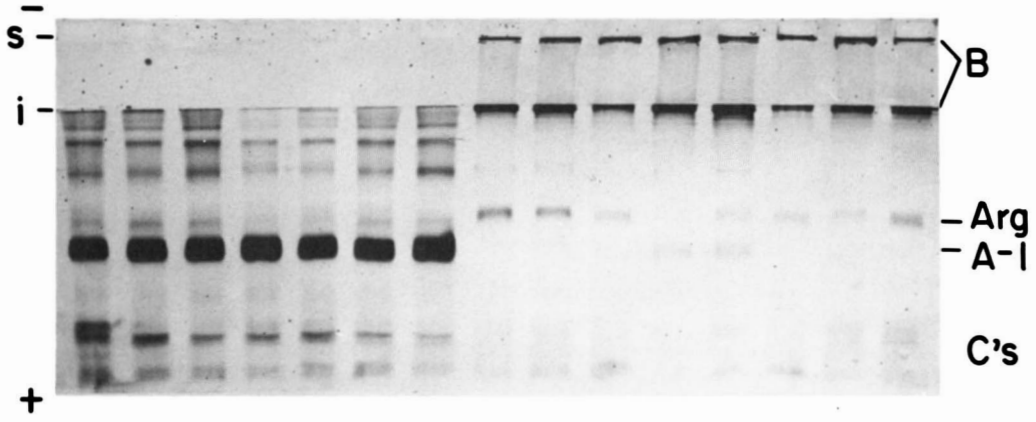

Fig. 8. Comparison of the apolipoprotein patterns for LDL and HDL of male and female *M. fascicularis*. Apoprotein samples from delipidated lipoproteins were placed in the sample wells (*s*) and electrophoresis in the horizontal slab was carried out. The interface (*i*) between the 5% polyacrylamide spacer gel and the 12.5% running gel is indicated. After electrophoresis, gels were stained with Coomassie Blue. The position of the specific apoprotein bands is indicated on the right side of the gel. Samples from a different animal were applied in each well and, numbered from left to right, were: *1*, apo-HDL, control male; *2–4*, apo HDL, test females; *5–6* apo-HDL, test males; *7*, apo-HDL, control male; *8–10*, apo-LDL, test females; *11*, apo-LDL, control female; *12*, apo-LDL, control male; *13–15*, apo-LDL, test males.

synthesis or secretion. Although our preliminary observations by PAGE analysis do not show major differences between apoproteins of LDL from males vs. females, much more information is needed before differences in apoproteins of LDL are ruled out. It is unfortunate that because of its extreme insolubility in the lipid-free state, apoprotein B has been among the apoproteins most difficult to characterize (31). Other potential control sites would appear to be among the enzymes involved in LDL synthesis. Currently, many data have accumulated which suggest that LDL arises as a result of intravascular catabolism of VLDL (32). If this is the case, then enzymes of LDL "synthesis" are also those of VLDL catabolism, namely lipoprotein lipase (33), lecithin:cholesterol acyl transferase (34), and possibly a liver parenchymal cell surface enzyme complex (35). How the action of any of these enzymes might select for size vs. particle number in the LDL is unclear.

One other possible control mechanism for LDL structure has been suggested by the studies of Atkinson et al. (36) and Deckelbaum, Shipley, and Small (37), namely that the physico-chemical requirements of cholesteryl ester transport may dictate lipoprotein conformation. For instance, the data of the present study clearly show that addition of cholesterol to the diet resulted in an increased LDL size which was due in large part to an increased cholesteryl ester content of the LDL particle (Fig. 7). As discussed earlier (21), the increase in cholesteryl ester content is consistent with an incremental enlargement process for LDL. Data recently published by Ross and Zilversmit (38) have shown that cholesteryl ester-rich, enlarged remnant lipoproteins that accumulate in plasma of cholesterol-fed rabbits are derived from the intestine. This occurred presumably as a result of increased absorption and esterification of dietary cholesterol, which was subsequently transported in chylomicra (or VLDL) prior to remnant formation. It is conceivable that the cholesteryl ester content of chylomicra in our cholesterol-fed monkeys might be increased, and the end product of catabolism of these particles might be LDL with an increased cholesteryl ester content. The additional phospholipid, free cholesterol, and protein of the increment, i.e., coat constituents (21), would presumably stem from the cholesteryl ester enrichment. In summary, this line of reasoning suggests that the requirement for increased cholesteryl ester transport results in an increased cholesteryl ester core, the size of which dictates overall LDL size. Control of LDL size would appear to be related to the extent of incorporation of cholesteryl esters into the intestinal chylomicra and/or liver VLDL.

The data of Fig. 7 are consistent with our prior findings (21). The size increase of LDL is probably due to incremental enlargement of the particle and occurs in similar fashion in both males and females. The components of the increment are in the same relative proportions as is indicated by the slopes of the lines in Fig. 7, and are essentially the same as described earlier. Cholesteryl ester of the core increases with size to a much greater extent than the protein, phospholipid, and free cholesterol of the coat, a finding consistent with the manner in which a sphere becomes enlarged. Using the partial specific volumes of the components and the formula for the volume of a sphere, we have calculated that the increase in LDL size can be adequately accounted for by an increase in the volume of cholesteryl ester (in the core); coat thickness remains constant at 21 Å (21). Based on the PAGE gel patterns (Fig. 8), the apoprotein component of the increment was principally apoB.

Another significant finding in this study was the marked decrease in HDL concentration induced by dietary cholesterol (Table 3 and Fig. 3) which was more marked in males than in females and which was associated with significant chemical composition differences (Table 4). A recent publication by Shen, Scanu, and Kézdy (39) has provided evidence that the appropriate structural model for HDL may be the same as proposed for other lipoproteins, namely that of a spherical core–coat configuration of constituents. The presence of a cholesteryl ester domain, presumably in the core as implicated by Tall and Small (40), is consistent with this possibility. Our findings suggested that, in response to diet, increased cholesteryl ester content occurred in the HDL fraction from males. Whatever the structural correlates of such chemical data, the composition of HDL in these animals is highly dependent on diet and the sex of the animal, a point which has not been often noted in the literature.

The HDL of this study were not subfractionated prior to chemical analyses, although across-the-column heterogeneity in chemical composition was detectable in region IV lipoproteins. We have looked for the discrete type of HDL_2–HDL_3 heterogeneity described for human HDL (9) but have not found evidence to suggest it is present in *M. fascicularis* males or females. Rather the region IV lipoproteins seem to represent a continuum of particles. Further data on this point are needed. We saw no clear indication of the presence of the HDL_c lipoprotein, described by Mahley and co-workers (41, 42) for dogs and swine, among the lipoprotein fractions of our animals. The HDL_c fraction contained arginine-rich and A-I apoproteins, no B apoprotein, and had pre-β migration on paper electrophoresis. The lipoproteins of

region IV contained essentially no detectable arginine-rich apoprotein (Fig. 8), and thus would not appear to include HDL_c. The region III lipoproteins from test animals contained detectably more Arg-rich apoprotein than those from control animals (Fig. 8), although this represented less than 5% of the total apoprotein. All of the region III material migrates β, however, which is atypical of HDL_c. It is possible that the region II lipoproteins might include an HDL_c lipoprotein fraction. We have not carefully subfractionated these lipoproteins in *M. fascicularis* to examine this point. However, we have not found an apparent analog to HDL_c in this region in *M. mulatta* (23, 43). We tentatively conclude that, if present, HDL_c is present only in low concentrations in *M. fascicularis*.

One of the reasons for studying male–female differences in plasma lipoproteins of *M. fascicularis* was to evaluate this species as a model for studies of "female protection" against coronary atherosclerosis. Although some similarities with human beings were noted, e.g., higher LDL concentrations concomitant with lower HDL concentrations in males vs. females, basically the response to dietary cholesterol in these monkeys was much exaggerated over that usually seen in human beings.

This exaggeration may be a time-compression feature which would not necessarily be undesirable in an animal model. However, to the extent that aspects are exaggerated, it becomes a larger step to draw an analogy with man. It remains to be seen if the LDL molecular weight differences and the alterations in HDL composition and concentration seen in *M. fascicularis* bear similarities to what occurs in human beings. Data for comparison in human beings are not yet available. The pertinence of the measurements of LDL molecular weight and total HDL concentration were underscored when it was found that these measurements were highly correlated with the severity of coronary atherosclerosis in the *M. fascicularis* of the present study (44).[5]

The authors wish to thank Mr. Ramesh Shah and Miss Pat Sisson for their excellent technical assistance and Ms. Pat DePaulis for her tenacious work in manuscript preparation. The assistance of Dr. Charles Nelson of the University of Arkansas Medical School in providing analyses by analytical ultracentrifugation is greatly appreciated. This work was supported by NIH grant number HL-14164 from the National Heart, Lung, and Blood Institute.

Manuscript received 1 November 1977; accepted 14 April 1978.

[5] Pitts, L. L., L. L. Rudel, T. E. Hamm, and B. C. Bullock. Manuscript in preparation.

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