Study of the atherogenic dyslipoproteinemia induced by dietary cholesterol in rhesus monkeys (*Macaca mulatta*)

Lawrence L. Rudel, Ramesh Shah, and Dianne G. Greene

Arteriosclerosis Research Center, Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, NC 27103

Abstract Hypercholesterolemia was induced in adult male rhesus monkeys with a high-fat diet containing an elevated cholesterol level (0.5%). Plasma lipoproteins were chromatographically separated into four size populations (regions) that were subdivided by density until fractions with single electrophoretic mobilities were obtained. The region III lipoproteins (LDL) contained 80% of plasma cholesterol and were present in the highest concentration of all fractions. Their molecular weight was increased over that of controls so that each particle averaged 1.8 times the number of cholesteryl ester molecules as did control LDL. Region II lipoproteins, a heterogeneous group, were present in next highest concentration. Most were cholesteryl ester-rich, β-migrating lipoproteins that overlapped the VLDL and LDL density ranges; apoB was the predominant apoprotein. One region II subfraction had preβ_2 migration and the density range $1.050 < d < 1.10$. Another subfraction, cholesteryl ester-rich VLDL including only about 1% of plasma cholesterol, had preβ_1 migration and apoB and apoC as the predominant apoproteins with no apoprotein E. Region I lipoproteins were larger sized, slow β-migrating cholesteryl ester-rich VLDL that included 5% of plasma cholesterol. ApoB and apoE were the predominant apoproteins. Region IV lipoproteins (HDL) contained 4% of the plasma cholesterol; their concentration was decreased to about ⅓ of the control level. Atherogenic features of the diet-induced dyslipoproteinemia included the increased plasma concentrations and cholesteryl ester contents of the region I, II, and III lipoproteins in addition to the decreased HDL concentration.

Supplementary key words agarose gel chromatography · agarose electrophoresis · apoproteins · cholesteryl esters · hypercholesterolemia

We have previously characterized the plasma lipoprotein spectrum present in rhesus monkeys (1). In that study, animals were fed a semipurified diet designed to approximate the typical North American diet in terms of calorie distribution, but which contained a low cholesterol level (0.05 mg/kcal). In the present study, the same basal diet was used except that the level of cholesterol was increased 20-fold. Hyperlipoproteinemia that results from the higher level of dietary cholesterol is marked in rhesus monkeys. Characterization of diet-induced dyslipoproteinemia has received much attention recently in several different species (2–8). In general, cholesteryl ester enrichment of lipoproteins has been noted to occur often in association with appearance of abnormal lipoprotein classes. The specific characteristics are of interest since dyslipoproteinemia usually occurs concomitantly with exacerbation of atherosclerosis. In the present study, we describe the distribution and composition of the spectrum of lipoprotein classes present in a group of dyslipoproteinemic male rhesus monkeys that were subsequently found by morphologic evaluation to have developed extensive atherosclerosis.

In control animals of the previous study (1), lipoprotein fractions were purified using the properties of size, density, and electrophoretic mobility as criteria with which to define individual lipoprotein fractions. The lipoprotein fraction present in highest concentration was the HDL (region IV lipoproteins), which had as the major apoprotein, apoA-I. The fraction present in next highest concentration was LDL (region III lipoproteins), which contained apoB as the primary apoprotein. It was necessary to use gel filtration chromatography in addition to density centrifugation to separate the LDL from larger preβ-migrating lipoproteins, a portion of which floated in the d 1.019–1.063 g/ml range. This lipoprotein fraction, operationally termed the II_{B100} lipoproteins, was similar in size and composition to human Lp(a) and significant concentrations were found in many control diet-fed rhesus monkeys. Lower concentrations of larger VLDL

Abbreviations: CE, cholesteryl esters; HDL, high density lipoproteins; LDL, low density lipoproteins; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; VLDL, very low density lipoproteins; TMU, tetramethyl urea.

and ILDL lipoproteins (region I and II lipoproteins) were identified and characterized including a cholesteryl ester-enriched, β-migrating VLDL. The β-migrating, intermediate sized d 1.006–1.050 g/ml lipoproteins appeared to represent cholesteryl ester-enriched remnants; this fraction contained less than 5% of the total plasma cholesterol. In the cholesterol-fed animals of the present study, almost all of the plasma lipoprotein fractions were found to have modified chemical compositions, size, and plasma concentrations.

METHODS AND MATERIALS

Animal colony

A group of six adult male rhesus monkeys were fed the test diet, which is the same semipurified diet (45% of calories as lard, 20% of calories as protein) as the control diet of reference 1, except that the cholesterol level was raised to 1 mg/kcal with crystalline cholesterol. All animals maintained or gained weight throughout the course of study and received this experimental diet for at least a year prior to initiation of the lipoprotein studies. The details of feeding, handling, and blood sampling were the same as previously reported (1).

Lipoprotein isolation procedures

Isolation of plasma lipoproteins, initially by ultracentrifugation at a density of 1.225 g/ml with subsequent separation by agarose column chromatography, has been described (9). Lipoprotein distribution was routinely monitored by the recovery of cholesterol among isolated fractions. For eight separate plasma samples, 99.0 ± 0.6% (mean ± SEM) of the plasma cholesterol was recovered in the initial centrifugation at d 1.225 g/ml, and 92.6 ± 1.1% was recovered among fractions separated chromatographically. LDL molecular weight determinations were carried out during preparative chromatography using ^{125}I-labeled LDL, according to the method of Rudel, Pitts, and Nelson (10).

Within populations of lipoproteins initially separated according to size, the density distribution was also determined. In these cases, samples were centrifuged sequentially at the appropriate densities, and the recovery of cholesterol was monitored. In most cases, ultracentrifugation served as the final purification and concentration step.

Lipoproteins were also isolated from plasma by sequential ultracentrifugation at successively increasing densities, namely 1.006, 1.019, 1.063, and 1.225 g/ml, using discontinuous gradients in the SW-40 rotor by the procedure described previously (1). The d < 1.006 g/ml fraction was heterogeneous as evidenced by the presence of three bands after agarose electrophoresis and was further subfractionated. In some studies, this fraction was first chromatographically separated into large (region I) and small (region II) components (see Fig. 2); then the fractions containing smaller components were pooled, concentrated, and subjected to a density gradient centrifugation. In other cases, the entire d < 1.006 g/ml fraction containing the slow β-migrating region I lipoproteins was used. A discontinuous gradient was prepared in which equal volumes of a solution of d 1.020 and 1.010 g/ml, and distilled water were successively layered over the sample (made to d 1.020 g/ml) in the SW-40 rotor tube. Samples were then centrifuged for 90 min at 200,000 g_{av}, 15°C, after which separate fractions were obtained by collecting through a needle from a hole punctured in the bottom of the tube. The β-migrating lipoproteins were found at the bottom of the tube, the preβ_1-migrating lipoproteins were found in the center of the tube, and slow β-migrating material was found at the top of the tube.

Lipoprotein compositional analyses

Determinations of the lipid composition of isolated lipoproteins were performed as described previously (1). Protein content of isolated lipoprotein fractions was determined directly by the method of Lowry, et al. (11), using bovine serum albumin, fraction V (Sigma Chemical Co., St. Louis, MO) as the standard. Turbidity in the larger lipoprotein fractions was removed by extraction with hexane after the color reaction was complete. Agarose electrophoresis of whole plasma and of isolated lipoproteins was carried out in 0.5% agarose (Bio-Rad Labs, Richmond, CA) essentially according to the method of Noble (12). Gels were stained at 37°C for 30 min in 60% ethanol saturated with oil red O and fat red 7B. Apoprotein analyses were carried out on isolated lipoprotein fractions that had been dialyzed against distilled H_2O containing 0.01% disodium ethylenediaminetetraacetate (EDTA) and lyophilized. Delipidation was carried out with chloroform–methanol 2:1, and after with ethyl ether. Resolubilization and polyacrylamide gel electrophoresis (PAGE) techniques have been described previously (1). The amount of apoB in each lipoprotein fraction was determined after precipitation of this apoprotein with tetramethylurea (TMU), as described by Kane (13). Monospecific antisera were used to identify apoA-1 and apoE (also called arginine-rich apoprotein)[1] in individual lipoprotein classes. In some cases,

[1] Antisera to patas monkey apoE and to human apoA-I were obtained through the generosity of Dr. Robert Mahley of the NIH.

intact lipoprotein preparations were iodinated with ^{125}I using the iodine monochloride technique of MacFarlane (14), as previously described (10). Individual apoproteins were then separated using PAGE to determine if apoproteins became iodinated in proportion to their relative amount on the lipoprotein particle.

RESULTS

Lipoprotein distribution

The dyslipoproteinemia induced by dietary cholesterol in rhesus monkeys was characterized initially by the distribution according to size. Typical lipoprotein elution profiles obtained by agarose column chromatography are shown in **Fig. 1.** In comparison with a profile from an animal fed the control level of cholesterol, much more material of the larger sizes (regions I, II, and III) was present in animals fed the higher level of cholesterol, and much less material was present in region IV. The amounts of cholesterol and protein in lipoproteins of each of the elution regions were then determined and the results are shown in **Table 1.** Data from animals fed the control level of cholesterol are shown for comparison. Lipoproteins in region III of test diet-fed animals were present in highest concentration and, for both protein and cholesterol, values were significantly higher than for those of control animals. The cholesterol concentration of the lipoproteins in regions I and II was also markedly elevated in test diet-fed animals. At the same time, the protein concentration of these lipoproteins was not increased over control levels to the same extent as was that of cholesterol, indicating that these lipoproteins were enriched in cholesterol. In contrast, the amount of both cholesterol and protein in region IV lipoproteins from test diet-fed animals was decreased to about ⅓ of the control level, and no change in the relative amount of cholesterol to protein was apparent.

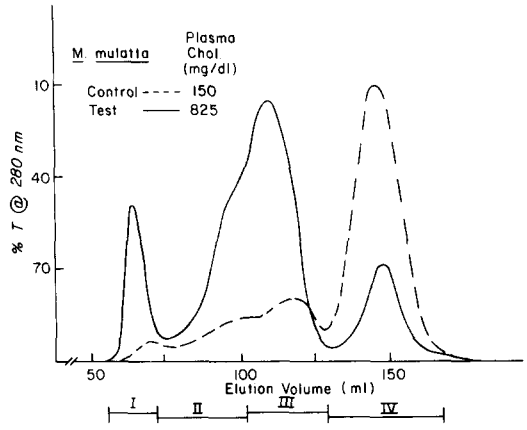

Fig. 1. Agarose column chromatography elution profiles of the plasma lipoprotein spectrum of a representative control and a test diet-fed rhesus monkey. Lipoproteins were isolated from 5 ml of plasma by centrifugation at d 1.225 g/ml, after which they were placed on a Bio-Gel A-15 m chromatography column, 1.5 × 90 cm, and eluted at the rate of 6 ml/hr with 0.9% NaCl, 0.01% EDTA, 0.02% NaN_3, pH 7.4, at 4°C. Two-milliliter fractions were collected in a fraction collector. In the test animal sample, fractions eluted within the regions indicated by roman numerals were pooled for further analyses. The cutoff for regions II and III was appropriately adjusted in samples from control animals (1). It is important to note that, for individual animal samples as in this example, the cutoff points between regions were set based on the information provided by the elution profile recording.

TABLE 1. Influence of dietary cholesterol on plasma lipoprotein distribution in rhesus monkeys

Diet Cholesterol Level	N	Plasma Lipoprotein Fraction I	II	III	IV	Total
		Cholesterol Concentration (mg/dl)				
Control[a]	7	0.9[b] ± 0.2	25.7 ± 5.2	53.7 ± 6.5	97.8 ± 6.7	191 ± 8.5
Test[c]	6	46.0[d] ± 4.2	92.3[d] ± 21.8	653.1[d] ± 35.2	34.1[d] ± 1.4	822[d] ± 25
		Protein Concentration (mg/dl)				
Control[a]	5	0.4 ± 0.7	23.7 ± 6.7	40.6 ± 3.4	310.7 ± 10.8	392.8 ± 16.2
Test	6	2.9[d] ± 0.5	25.4 ± 8.9	280.7[d] ± 23.7	112.2[d] ± 13.8	421.2 ± 28.6

[a] Data from reference 1.
[b] All values are mean ± SEM.
[c] Average plasma triacylglycerol concentration, 44 mg/dl.
[d] Significantly different ($P < 0.01$) from control.

Lipoprotein fractionation

In addition to size separation, we have used density differences as a means to characterize the lipoproteins of test diet-fed monkeys. The size–density relationships for the lipoproteins separated initially by density are shown in **Fig. 2.** Two size populations are apparent in the $d < 1.006$ g/ml lipoproteins, which represent the largest particles isolated. The size of the $1.006 < d < 1.019$ g/ml lipoproteins was intermediate between that of the largest $d < 1.006$ lipoproteins and the $1.019 < d < 1.063$ g/ml fraction. Considerable size overlap was observed among the $d < 1.006$, the $1.006 < d < 1.019$, and the $1.019 < d < 1.063$ g/ml fractions. The average size of the $1.019 < d < 1.063$ g/ml fraction was the same as for the region III lipoproteins, as indicated by coincidence at the center of the peaks. Very little overlap occurred between the $1.019 < d < 1.063$ and $1.063 < d < 1.225$ g/ml fractions.

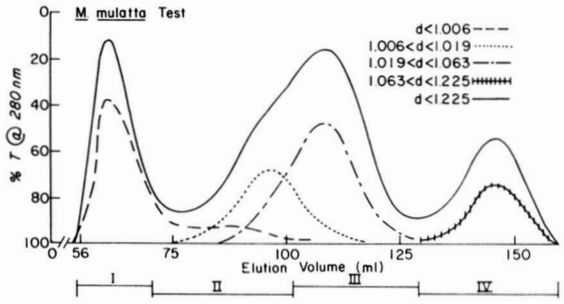

Fig. 2. Agarose column chromatography of rhesus monkey plasma lipoproteins which had been isolated by ultracentrifugation. The column separation was carried out as described in Fig. 1. The volumes of the individual density fractions were adjusted to approximate the same starting volume of plasma. All fractions were isolated from the same pooled plasma sample. The elution regions indicated by roman numerals are the same as for Fig. 1, and are shown for comparison.

Electrophoretic mobility was also used to characterize the lipoprotein fractions (see **Fig. 3**). Three electrophoresis bands were seen when the $d < 1.006$ g/ml (VLDL) fraction from plasma of fasted test diet-fed monkeys was subjected to agarose electrophoresis. The lipoproteins of region I, which were the largest of the isolated fractions, had a slow β mobility on agarose electrophoresis. Some material remained at the origin during electrophoresis. This may represent particles too large to penetrate the agarose gel matrix or possibly breakdown which occurred during concentration of the column fractions. In region II lipoproteins with $d < 1.006$ g/ml, fractions with preβ_1 and β migration were found. It was possible to separate the preβ_1 from β migrating VLDL by centrifugation in a density gradient as described in the methods section.

Region II showed further heterogeneity with respect to density (Fig. 2). Lipoproteins migrating β were found in both of the density ranges, $1.006 < d < 1.019$ and $1.019 < d < 1.050$ g/ml. In addition, a fraction representing the preβ_2 migrating material of whole plasma was isolated in the density range $1.050 < d < 1.10$ g/ml. In order to remove the β-migrating material that was also present in this density range, we subjected this fraction to a second chromatographic separation on Bio-Gel A-15m and pooled the larger fractions from this separation to obtain the purified preβ_2 lipoproteins shown in Fig. 3.

Region III lipoproteins were all β-migrating and represented particles in the $1.006 < d < 1.063$ g/ml (LDL) density range. Subfractionation at $d < 1.019$ g/ml was carried out, although it may represent an artificial separation of this population of lipoproteins. Region IV lipoproteins all had α migration and were isolated in the $1.063 < d < 1.225$ g/ml (HDL) density range.

Table 2 lists the features used to identify each of the separated lipoprotein fractions. A combination of size, density, and electrophoretic mobility has been used in defining separate fractions, and the designations shown in the last column, though cumbersome, appear to be the least redundant yet systematic way to define the isolated subfractions. The percentage of plasma lipoprotein cholesterol in each of the fractions was estimated. Variations may be expected from sample to sample, subject in part to losses incurred during purification procedures and to individual animal differences.

Lipoprotein chemical compositions

The chemical compositions of the isolated lipoprotein fractions were determined and the results are shown in **Table 3.** The region I lipoproteins were only 2.1% protein, and 70.4% of the lipid was cholesteryl ester. The TC/Pro ratio was 23.5, which is high compared to that of other lipoprotein fractions. This composition was different from any of the other isolated lipoprotein fractions. The percentage of protein in the $II_{6pre\beta_1}$, $II_{6\beta}$, and the II_{50} lipoproteins progressively increased. The $II_{6pre\beta_1}$ lipoproteins were the

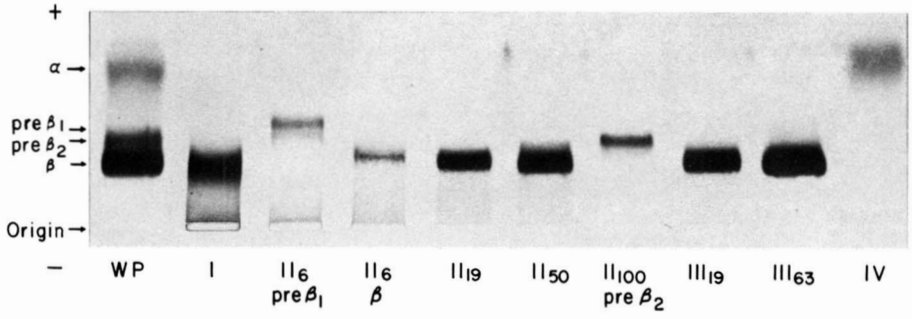

Fig. 3. Agarose electrophoresis of the plasma lipoproteins of test diet-fed rhesus monkeys. The designations for individual lipoprotein fractions are explained in Table 2. The pattern for whole plasma (WP) is shown for comparison. About 60 ml of pooled plasma from three animals was the starting sample. Individual fractions were concentrated to approximate a 5 to 10-fold increase over original plasma concentration.

TABLE 2. Characteristics of separated lipoprotein fractions of hyperlipoproteinemic rhesus monkeys

Lipoprotein Size Region	Agarose Electrophoretic Mobility	Density Range (g/ml)	Average Diameter[a] (Å)	% of Plasma Cholesterol Distribution[b] (%)	Designation[c]
I	slow β	$d < 1.006$	>650	5	I
II	preβ_1	$d < 1.006$	400	1	$II_{6pre\beta_1}$
II	β	$d < 1.006$	300	1	$II_{6\beta}$
II	β	$1.006 < d < 1.019$	300	3	II_{19}
II	β	$1.019 < d < 1.050$	300	6	II_{50}
II	preβ_2	$1.050 < d < 1.10$	300	1	$II_{100pre\beta_2}$
III	β	$d < 1.019$	245	13	III_{19}
III	β	$1.019 < d < 1.063$	245	66	III_{63}
IV	α	$1.063 < d < 1.225$	<125	4	IV

[a] Based on agarose column elution and chemical composition, as described by Sata, Havel, and Jones (26).
[b] Values were determined on a plasma pool that had a cholesterol concentration of 795 mg/dl. More cholesterol was generally found in $d < 1.006$ g/ml lipoproteins when plasma concentrations exceeded this value.
[c] This system is based on the procedural detail. Since size separation was carried out first, the size region is indicated first. Where density information is needed to distinguish further fractionation, this is added. Where electrophoretic mobility is needed to further distinguish between two fractions of the same size region and density, this information is also added. Where an abbreviated designation is given, e.g. region III lipoproteins, the reader can assume no further subfractionation was carried out.

fraction highest in percentage of triacylglycerol although this value was only 21%. Essentially no difference was found in the lipid composition of the $II_{6\beta}$, II_{50}, and III lipoproteins, although the lower TC/PL ratio of the region III lipoproteins was significantly different. The major component of these lipoproteins was cholesteryl ester. The $II_{100pre\beta_2}$ fraction had a higher percentage of protein than any of the other region II or III lipoproteins, but still contained cholesteryl ester as its major lipid component.

The region IV lipoproteins were divided at the peak into front and back subfractions. As can be seen in Table 3, there was a higher percentage of protein in the IV back compared to IV front lipoproteins, although the percentage composition of the lipid complement was similar. Interestingly, the percentage of free cholesterol was the only significant difference between the lipid percentages of IV front and back lipoproteins. This appeared to account for the significant EC/TC ratio difference.

TABLE 3. Chemical composition of plasma lipoprotein fractions isolated from hyperlipoproteinemic rhesus monkeys

Lipoprotein Fraction	% of Mass		% of Total Lipid				W/W		
	Lipid	Protein	FC	PL	CE	TG	TC/Pro	EC/TC	TC/PL
I[a]	97.9 ± 0.5	2.1 ± 0.5	6.9 ± 0.7	12.0 ± 0.5	70.4 ± 4.7	10.7 ± 4.9	23.46 ± 4.61	0.86 ± 0.02	4.02 ± 0.23
$II_{6pre\beta_1}$[a]	90.2 ± 0.4	9.8 ± 0.4	9.5 ± 0.2	18.5 ± 2.9	51.0 ± 8.6	21.0 ± 6.1	3.43 ± 0.35	0.76 ± 0.03	2.20 ± 0.58
$II_{6\beta}$[a]	88.4 ± 0.7	11.6 ± 0.7	12.4 ± 1.1	24.6 ± 1.5	62.1 ± 2.9	0.9 ± 0.7	3.59 ± 0.14	0.75 ± 0.02	2.00 ± 0.14
II_{50}[a]	86.1 ± 1.7	13.9 ± 1.7	11.8 ± 0.9	24.7 ± 2.0	61.2 ± 2.2	2.3 ± 1.3	3.00 ± 0.47	0.75 ± 0.02	1.94 ± 0.16
$II_{100pre\beta_2}$[b]	76.9	23.1	12.9	28.2	57.2	1.7	1.56	0.72	1.65
III[a]	82.9 ± 1.9	17.1 ± 1.9	11.6 ± 0.7	26.3 ± 1.2	60.9 ± 1.8	1.0 ± 0.5	2.40 ± 0.35	0.75 ± 0.01	1.82 ± 0.12
IV front[c]	54.4 ± 2.3	45.6 ± 2.3	6.1 ± 0.6	52.2 ± 2.2	35.5 ± 2.4	6.0 ± 1.5	0.33 ± 0.05	0.77 ± 0.05	0.52 ± 0.04
IV back[c]	47.1 ± 3.9	52.9 ± 3.9	3.7 ± 0.7	52.4 ± 5.8	36.3 ± 4.4	7.5 ± 2.9	0.23 ± 0.03	0.85 ± 0.03	0.48 ± 0.09

[a] Determined in duplicate on two samples, each pooled from three animals.
[b] Determined in duplicate on a pooled sample from four animals.
[c] Determined on individual samples from six animals.
All values are means (±SD). Abbreviations: FC, free cholesterol; PL, phospholipids; CE, cholesteryl esters; TG, triacylglycerols; TC, total cholesterol; EC, ester cholesterol; Pro, protein.

Lipoprotein apoprotein patterns

The apoprotein patterns from test and control animals are compared using SDS PAGE in **Fig. 4*A*.** The major apoprotein of control animal lipoproteins of regions I, II, and III remained in the sample well and near the interface between the stacking and separating gels, which is behavior characteristic of apoB. This was also characteristic of the region I, II, and III lipoproteins of the test diet-fed animals. Among

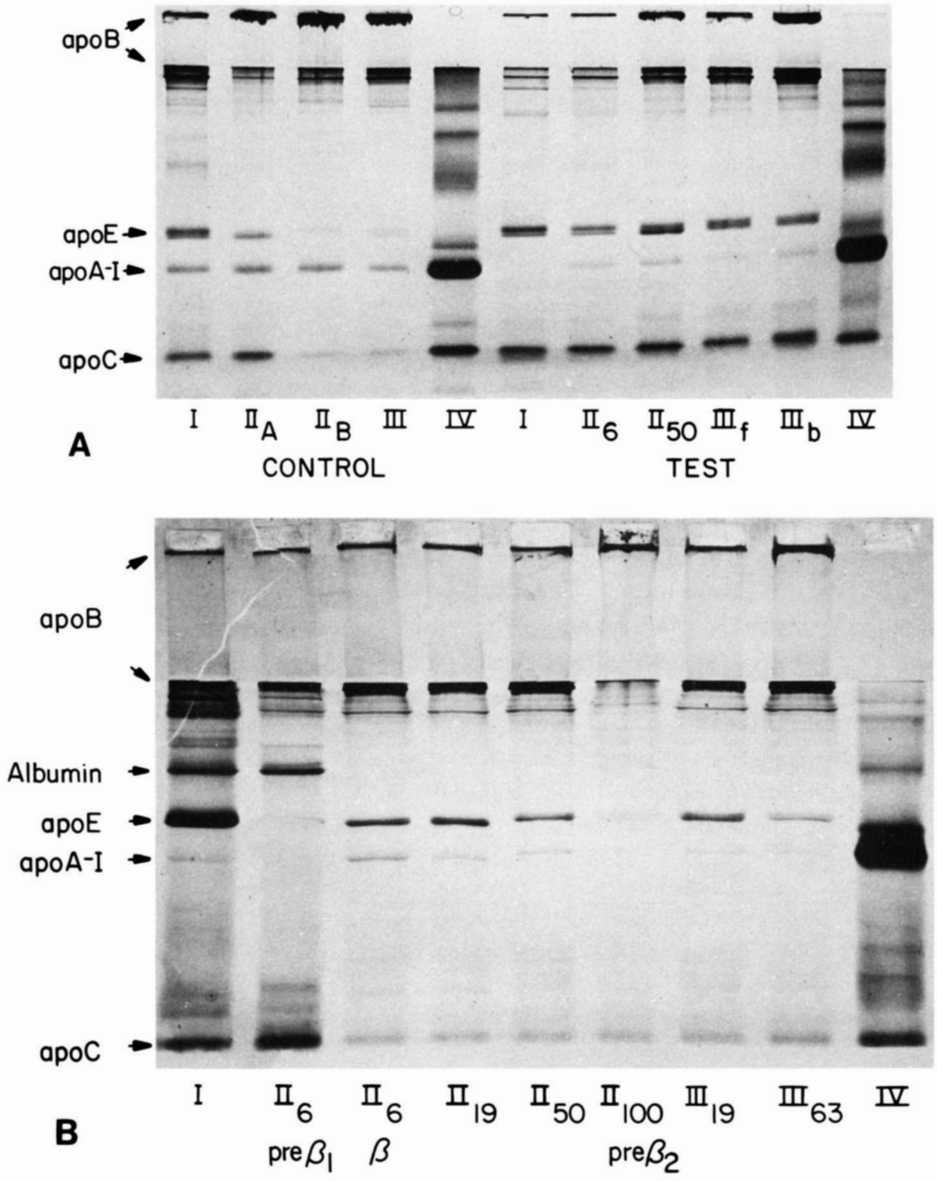

Fig. 4. *A*. A comparison of SDS PAGE apoprotein patterns of lipoprotein fractions isolated from pooled plasma samples of control and test diet-fed rhesus monkeys. Designations for individual fractions from test animals are explained in Table 2 and, for control animal samples, in reference 1. The region III lipoproteins were separated into a fraction from the front of the peak (III_f) and the back of the peak (III_b) to ascertain if heterogeneity within this fraction could be identified. The position of migration for individual apoproteins is identified at the left of the gel. Lipoprotein samples containing 100–150 μg of protein were delipidated, redissolved in 100 μl of 0.025 M Tris–0.2 M glycine buffer (pH 8.3) containing 0.02 M SDS, and 40 μl of this solution was placed in the sample well. Electrophoresis was carried out at 10°C for 5 hr at a constant current of 40 mA, after which the slab was stained with Coomasie blue, then destained in 7.5% glacial acetic acid containing 5% methanol. A 10% polyacrylamide separating gel containing 0.1% SDS, 0.4 M Tris, pH 8.8, with a 5% spacer gel was used in this experiment. *B*. SDS PAGE apoprotein patterns for individual lipoprotein fractions isolated from pooled plasma of test diet-fed rhesus monkeys. Designations for individual fractions are described in Table 2. Details are as given above for *A*, except that a 12.5% polyacrylamide separating gel with a 5% spacer gel was used in this experiment.

the apoproteins present in lesser amounts, consistently there appeared to be more protein migrating in the region of apoE and less in the region of apoA-I in the I, II, and III lipoproteins of test animals compared to those of control animals. The region II and III lipoproteins of test animals also contained more protein in the apoC region. Apoprotein patterns appeared similar for region IV lipoproteins of test and control animals.

In Fig. 4*B*, the apoprotein patterns for each of the separate fractions described in Fig. 3 and Table 2 are shown. There were differences among the d < 1.006 g/ml lipoproteins. A major difference was the amount of apoE present in the I vs. $II_{6pre-\beta_1}$ fraction. In the latter fraction, essentially no protein was present in the apoE region and relatively more protein migrating in the apoC region was found. Essentially no protein migrating as apoA-I was found in either fraction. Some protein migrating in the albumin region was consistently seen if these lipoproteins were isolated by centrifugation at d < 1.006 g/ml and then separated by density gradient centrifugation. Further washing caused a loss of albumin and other apoproteins.

The $II_{6\beta}$, II_{19}, and II_{50} lipoproteins all showed a similar apoprotein pattern on SDS PAGE and, upon visual inspection, appeared to contain slightly more protein in the apoA-I region and less in the apoC region than either of the larger lipoproteins. The $II_{100pre\beta_2}$ lipoproteins showed a pattern different from any of the other fractions. Minimal amounts of protein in the regions of apoA-I, apoE, or apoC were present, and the amount of protein that migrated out of the sample well, through the 5% spacer gel, and into the 12.5% separating gel was noticeably less than for other region I, II, and III lipoproteins. The apoprotein pattern for the region III lipoproteins was essentially the same as that of the other β-migrating lipoproteins. The region IV lipoproteins contained a major band in the region of apoA-I, and had significant amounts of protein in the apoC region as well as some unidentified proteins in the regions larger than apoA-I. It was consistently found with SDS PAGE that region IV lipoproteins had no protein in the apoE region. The protein that migrated just ahead of the apoE region was not apoE, as judged immunologically, although it migrated in the apoE region on 8 M urea PAGE (**Fig. 5**).

The SDS PAGE analyses indicated that apoB, or its equivalent, was a major apoprotein of each of the lipoproteins in regions I, II, and III. In order to quantitate the relative proportion of apoB among apoproteins of these lipoproteins, we made use of its property of insolubility in TMU. Intact lipoproteins were iodinated, and the distribution of ^{125}I among apolipoproteins was measured after delipidation. The insoluble TMU-precipitable protein, i.e., apoB, was 45, 65, 80, 91, 91, and <1% of total apoprotein mass of the region I, II_6, II_{50}, III_{front}, III_{back}, and IV lipoproteins, respectively. The percentage of ^{125}I-labeled protein that behaved as apoB was 41, 81, 89, 92, and 91% for region I, II_6, II_{50}, III_{front}, and III_{back} lipoproteins, respectively. These values are similar to the percentages of protein obtained for apoB by TMU precipitation. A comparison of radioactivity and Coomassie blue stain distribution was also made for the urea-soluble apoproteins. Densitometric scanning of the gels similar to those shown in Fig. 5 was carried out, and companion gels were sliced into regions and counted. The percentage distributions of radioactivity and stain were determined (**Table 4**) and were found to be similar, i.e., higher percentages of radioactivity

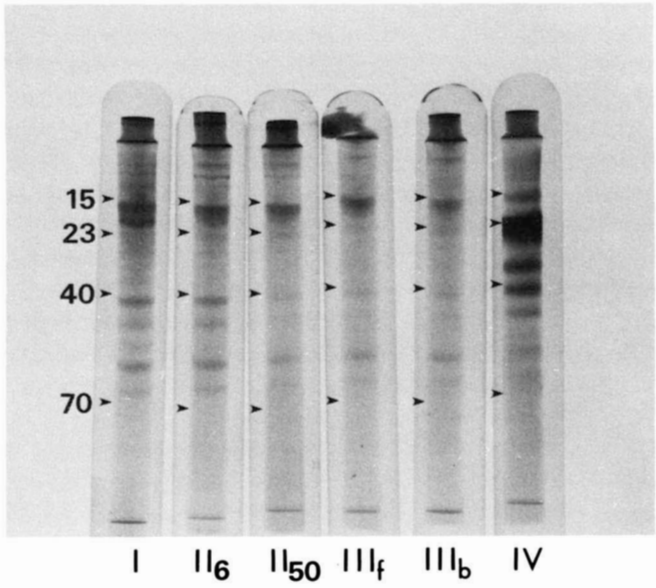

Fig. 5. Apoprotein patterns obtained by PAGE in 8 M urea for individual lipoprotein fractions of test diet-fed rhesus monkeys. Electrophoresis was carried out in 7.5% polyacrylamide separating gels containing 8 M urea, 0.375 M Tris, pH 8.9, with 3.5% spacer gels. Samples were redissolved in 0.01 M Tris buffer, pH 8.9, containing 8 M urea, and the equivalent of 50 μg of sample protein was applied to each gel. ApoB is sparingly soluble in this buffer. Designation of fractions is the same as in Fig. 4*A*. The numbers on the left indicate the relative migration index, r_m, (1) based on the bromphenol dye migration for the points at which gels were sliced for determination of ^{125}I radioactivity distribution (Table 4). Because the distance of bromphenol blue dye migration varied among gels, these points are indicated for each gel. The region IV gel was not sliced for radioactivity determination, but the points are indicated for reference. In this system, apoE migrates at r_m 17, apoA-I at r_m 25, apoA-II at r_m 35, and apoC's at r_m values between 40 and 70. Immunological identification by immunodiffusion of apoE in apolipoproteins of region I, II, and III lipoproteins was positive based on slices of gels with r_m values between 15 and 20. Immunological identification of apoA-I was positive for slices with r_m values between 20 and 30 for apolipoproteins of region, I, II, and IV lipoproteins.

TABLE 4. ^{125}I-Radioactivity and staining distribution among urea soluble apolipoproteins separated by polyacrylamide gel electrophoresis in 8M urea

Lipoprotein Fraction		Apolipoprotein Region (r_m) 0–15	15–23	23–40	40–70
		(Percentage)			
I	Stain	11	43	21	26
	^{125}I	10	64	12	14
II_6	Stain	12	37	22	29
	^{125}I	22	23	17	39
II_{50}	Stain	20	38	20	23
	^{125}I	15	33	21	31
III_{front}	Stain	10	44	22	24
	^{125}I	10	21	26	44
III_{back}	Stain	9	33	23	35
	^{125}I	13	11	33	43

All values are means for duplicate determinations.

and stain were present in the region of apoE (r_m 15–23) in region I lipoproteins than for region III lipoproteins and the percentages in the apoC region (r_m 40–70) were higher in region III than in region I lipoproteins.

Region III lipoproteins (LDL)

The region III lipoproteins (LDL) were the fraction present in highest concentration in test animals and were increased over control levels the most of any fraction. As shown in **Table 5,** this response occurred both as an increase in number of particles (μmolar concentration) and as an increase in particle size and molecular weight (compare the patterns of Fig. 1, region III). The size increase is reflected in the within-particle composition. It can be seen that each LDL particle from test diet-fed animals contained, on the average, 1.8 times as much cholesteryl ester, and more free cholesterol, phospholipid, and protein than did particles of control animals.

DISCUSSION

The data of the present study demonstrate that, in plasma of male rhesus monkeys made hyperlipoproteinemic with dietary cholesterol, many separate lipoprotein classes are present most of which bear chemical differences from their counterparts in control animals. In general, the lipoproteins that were present in elevated concentration in test animals (region I, II, and III lipoproteins) were larger in size and contained more cholesteryl ester as a percentage of total mass. Frequently, this was accompanied by an apparent decrease in average density for the fraction and less protein per unit mass. ApoB was the apoprotein present in highest amounts for these lipoprotein fractions, although some increase in the relatively small amounts of apoE was noted. Thus, the dietary cholesterol-induced dyslipoproteinemia of rhesus monkeys represents, predominantly, increased concentrations of large cholesteryl ester-rich lipoproteins that have apoB as the predominant apoprotein. These lipoproteins originate apparently as a result of an increased need to transport more cholesterol, presumably that derived from the diet.

The finding that cholesteryl ester-rich lipoproteins accumulate in plasma of cholesterol-fed animals is not restricted to rhesus monkeys. Such accumulations have previously been described in rabbits (2), pigs (4), rats (5), and patas monkeys (6), to name just a few. Previous reports of high molecular weight, cholesteryl ester-rich LDL of rhesus monkeys have appeared (8, 15). However, unique to the present study is the isolation, quantitation, and complete chemical characterization of the spectrum of plasma lipoproteins present in dyslipoproteinemic rhesus monkeys.

During this work, the inadequacy of the nomenclature system for lipoproteins was apparent, to wit, the identification of three VLDL ($d < 1.006$ g/ml) subfractions and three subfractions with densities overlapping the $1.019 < d < 1.063$ g/ml, or LDL, range. We found it necessary to develop a nomenclature system which was adequate to help during preparation to distinguish between fractions without redundancy (see Table 2). The chemical compositions of many of these fractions were different, i.e., I vs. $II_{6\beta}$ vs. $II_{6pre\beta_1}$, and II_{50} vs. $II_{100pre\beta_2}$. On the other

TABLE 5. Effects of dietary cholesterol on region III lipoproteins (LDL) of rhesus monkeys

Diet Group	N	Plasma Cholesterol (mg/dl)	Region III Lipoproteins (LDL) Concentration (μmol/l)	Molecular Weight ($\times 10^{-6}$)	Within Particle Composition[a] Pro	FC	PL	CE	TG
					(molecules/particle)				
Control	6	163 ± 16[b]	0.53 ± 0.02	3.2 ± 0.1	26	742	968	2038	177
Test	6	795 ± 37	3.61 ± 0.35	4.8 ± 0.2	33	1194	1350	3671	44

[a] Calculated from average compositions for Region III lipoproteins (LDL) of each diet group. Abbreviations as given for Table 3. Molecular weights assumed for these calculations were: Pro, 25,000; PL, 775; CE, 660; and TG, 900.

[b] Mean values ± SEM.

hand, separation of region II lipoproteins that migrated β on agarose electrophoresis ($II_{6\beta}$, II_{19}, and II_{50}) into density subclasses did not appear to generate fractions with different chemical compositions or different apoprotein patterns. Likewise, the lipoproteins of region III were separated into two density subfractions, III_{19} and III_{63}, although resulting chemical compositions and apoprotein patterns were minimally different. In comparing compositions of all of the β-migrating region II and III lipoproteins, the small differences present could have been primarily related to the size of the particles. This would account for the slight difference in core lipid (predominantly CE) to coat (Pro, FC and PL) ratio. Such a finding is consistent with the possibility that these lipoproteins represent remnant lipoproteins that were derived from catabolism of triglyceride-rich precursors with different cholesteryl ester contents. It remains for further structural and metabolic studies to help define whether or not these subfractions of β lipoproteins represent discrete metabolic entities rather than segments of a size continuum of lipoprotein particles.

One of the unexpected findings of these studies was that while the total plasma cholesterol increased over 4-fold in animals fed the higher levels of dietary cholesterol, the plasma level of lipoprotein protein remained almost the same (Table 1). This was due to the fact that apoprotein-rich region IV lipoproteins decreased in concentration at a time when increases occurred in the more cholesterol-rich, apoprotein-poor lipoproteins of regions I, II, and III. The fact that the level of total lipoprotein protein changed little may be coincidental. On the other hand, with the striking differences in apoprotein content of these two groups of lipoprotein fractions, the shift in relative amounts of the two major apoproteins, apoB and apoA-I, is quite remarkable. We have calculated the relative changes based on the data of Table 1 and the data for apoB and apoA-I distribution.[2] The concentration of apoB in lipoproteins was approximately 60 mg/dl in control animals compared to 280 mg/dl in test animals, values proportional to the increase in cholesterol concentration. On the other hand, apoA-I levels decreased from about 250 to 80 mg/dl.

Postulation of a metabolic relationship between this inverse proportionality between apoB and apoA-I seems reasonable. A relationship between HDL levels and responsiveness to dietary cholesterol in rhesus monkeys has been discussed previously (17). In the future, direct quantitation of individual apoprotein levels using available immunologic techniques (18) should provide valuable information on this type of metabolic relationship and help determine the relative significance to atherogenesis. Such studies are now in progress in our laboratory.

We carried out in vitro iodination of intact lipoproteins in this study to determine if selective changes in availability of apoproteins for iodination occurred among lipoproteins isolated from hyperlipoproteinemic animals. However, the distribution among apoproteins of radioactivity vs. stain was not significantly different, suggesting a surface location on the lipoprotein particle of all apoproteins and relatively similar conformations of apoproteins at the surface of the various particles.

Region I lipoproteins from test animals were predominantly slow β-migrating VLDL whereas, in control animals, only a subfraction of region I lipoproteins had this migration. This finding, together with the differences in percentage of cholesteryl ester (70 vs. 33%) and triacylglycerol (11 vs. 49%), and in apoE predominance, indicate that the region I lipoproteins of test animals may in fact represent a marked increase of the β-migrating region I subfraction over that found in the control animals. This subfraction may represent the rhesus monkey counterpart to the β-VLDL of cholesterol-fed patas monkeys, described by Mahley, Weisgraber, and Innerarity (6), although the percentages of protein and phospholipid are lower and the average particle size is larger in the fraction isolated from rhesus monkeys. The $II_{6pre\beta_1}$ may be the counterpart of the preβ VLDL of control animals. It was interesting to note that, when purified, the $II_{6pre\beta_1}$ fraction contained no detectable apoE (Fig. 4*B*) even though it was a cholesteryl ester-enriched VLDL (51% of lipid as CE vs. 21% as TG, Table 3).

The $II_{100pre\beta_2}$ lipoproteins we have isolated from test animals have similar size, density, and electrophoretic properties to the II_{B100} lipoproteins isolated from control animals (1), although they are chemically modified to contain more cholesteryl ester and less triacylglycerol and protein in test animals. The apoprotein patterns of II_{100} lipoproteins of test and control animals were similar in that essentially all of the apoprotein aggregated and poorly penetrated the SDS-containing polyacrylamide gel. Also similar was the relative absence of apoE, apoA-I, and apoC. The amount of the II_{100} fraction in test animals indicates that this fraction is not significantly increased in concentration in response to dietary cholesterol. The similarity between this lipoprotein and Lp(a) of human beings was discussed previously (1). Many suggestions have appeared that higher levels of Lp(a) in humans is a risk factor for clinically detected complications of coronary atherosclerosis (19). The relatively

[2] Based on the PAGE analyses and in agreement with the results of Edelstein, Lim, and Scanu (16), apoA-I was estimated to be 75% of the protein of HDL. It was no more than 1% of any of the other isolated fractions.

low levels of this lipoprotein in the hyperlipoproteinemic rhesus monkey suggest that this fraction may not be a major factor in the development of experimentally induced atherosclerosis in this species.

Based on the chemical and physical properties of each fraction, a similar conclusion was reached about region III and IV lipoproteins of test animals as for those of control animals (1), namely that these fractions represent, respectively, LDL and HDL. In this sense, the name LDL refers to the population of β-migrating lipoproteins with an average size centering in region III that has apoB as the predominant apolipoprotein and that has a chemical composition with the core–coat relationships as defined here. The metabolic origin is not implied.

Our data showed heterogeneity among LDL fractions from individual animals. Cholesteryl ester content of LDL particles increased with molecular weight out of proportion to free cholesterol and phospholipid which, in turn, increased proportionally more than protein. The fact that increases occurred in both the particle concentration (μmol/l) and the molecular weight is important in consideration of relationships between the dyslipoproteinemia and atherosclerosis. Not only are arteries exposed to more LDL particles per volume of plasma, but the chemical composition of each LDL particle that gets into the artery wall is modified (Table 4). The recent work of St. Clair and Leight (20), using smooth muscle cells in tissue culture, has suggested that the large molecular weight LDL of rhesus monkeys stimulated cholesteryl esterification and accumulation by the cells out of proportion to their particle number and mass of cholesterol. In other studies carried out in our laboratory in *Macaca fascicularis*, we have found that LDL molecular weight had a highly significant correlation with the extent of coronary atherosclerosis (21). Thus, the large molecular weight LDL resulting from dietary cholesterol induction appears to be an atherogenic lipoprotein both in terms of the increased concentration (μmol/l) and in the modified composition that is reflected in molecular weight enlargement.

Another shift induced by dietary cholesterol which is likely to be atherogenic is the decrease in plasma HDL concentration, especially since this response occurs at the same time that the LDL changes occur. Recent studies in humans have shown that HDL concentration is important in determining the relative risk to coronary heart disease (22). We have shown in *Macaca fascicularis* that a significant relationship existed between the plasma HDL concentration and experimentally induced atherosclerosis (21). Other aspects of the induced hyperlipoproteinemia that are atherogenic could be the appearance of apoE-rich lipoproteins, since Mahley and coworkers (23) have shown that apoE has a high affinity for the LDL receptor site described by Brown and Goldstein (24). The latter authors feel that this cell surface receptor is important in controlling atherogenesis (25).

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