Isolation and Characterization of Plasma Lipoproteins of Common Marmoset Monkey

Comparison of Effects of Control and Atherogenic Diets

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This study examines the potential of the common marmoset monkey (Callithrix jacchus) to serve as a model for human lipoprotein metabolism and atherosclerosis. The lipoproteins of animals fed a low-fat, low-cholesterol diet and a high-fat (12% wt/wt lard), high-cholesterol (0.34% wt/wt) diet were characterized by the combination of sequential ultracentrifugation and Pevikon block electrophoresis. Based on chemical and physical properties, equivalents of human very low density lipoproteins (VLDL), low density lipoproteins (LDL), and high density lipoproteins (HDL), including an HDL-apolipoprotein E subclass, were demonstrated. In control animals, whose plasma cholesterol concentration was 140.1 ± 20.2 mg/dl (means ± SD), approximately 40% of the plasma cholesterol was transported by LDL as compared with ~70% in humans. The cholesterol-fed marmosets segregated into two groups: hypo- and hyper-responders. The hyper-responders had plasma cholesterol levels of 450 to 970 mg/ml. The hypercholesterolemia was associated with elevated concentrations of VLDL, intermediate density lipoproteins, and LDL; in addition, these lipoproteins were enriched in cholesteryl esters relative to lipoproteins isolated from control animals. The HDL (d > 1.09 g/ml) levels did not change in response to cholesterol feeding, although the HDL-apolipoprotein E found in the d = 1.02 to 1.09 g/ml fraction increased approximately fivefold. Based on immunological characteristics and sialic acid content, the common marmoset appeared to lack a lipoprotein(a) equivalent. The results of a short-term feeding study (11 months) suggest that this monkey was susceptible to the development of diet-induced atherosclerosis. The hyper-responsive animals developed foam cell lesions and moderately proliferative intimal lesions, predominantly within the thoracic aorta. In summary, the results of our studies indicate that the common marmoset monkey potentially is a useful model for the study of both lipoprotein metabolism and diet-induced atherosclerosis. (Arteriosclerosis 10:633–647, July/August 1990)

Cholesterol metabolism—in particular, the transport of cholesterol by specific plasma lipoproteins—is known to influence the development of atherosclerosis in humans.1,2,3 The elucidation of the pathogenic mechanisms involved in atherosclerosis has been greatly aided by the availability of several animal models whose plasma lipoproteins resemble those of humans.4,5,6,7 Animal models can be maintained under conditions in which the effects of single variables—dietary cholesterol content, in particular—on cholesterol metabolism and associated atherosclerosis may be tested. It has been shown that several features of hypercholesterolemia are consistently induced by cholesterol feeding in dogs,7 swine,8 and rats: 1) the appearance of cholesteryl ester-rich β-very low density lipoproteins (β-VLDL) in the d < 1.006 g/ml fraction, 2) an increase in the plasma concentration of intermediate density lipoproteins (IDL) and low density lipoproteins (LDL), 3) an increase in the plasma concentration of high density lipoproteins (HDL) with E (HDL-E), and 4) a decrease in the levels of “typical” HDL (for a review, see references 1, 4, 5, and 6).

Nonhuman primates are good models for studies of lipoprotein and atherosclerosis research because of their close evolutionary relationship to humans.10,11 Both the experimentally induced atherosclerosis and the natural atherosclerosis found in nonhuman primates differ from that encountered in lower species primarily in the distribution of the lesions.12,13 In particular, nonhuman primates have less involvement of small arteries.14,15,16 In addition, the histological appearance of experimentally induced atherosclerosis in nonhuman primates tends to resemble that seen in humans, although generally the lesions are less likely to be complicated.14–17

The use of nonhuman primates has been impeded by factors such as expensive housing and maintenance requirements in laboratories and decreased availability of the monkeys due to bans on their export from some countries that had supplied them. However, the use of one particular species of nonhuman primate, the common marmoset monkey (Callithrix jacchus), has been less affected by such factors, and over the past 15 years...
the marmoset has become increasingly popular as a laboratory animal.\textsuperscript{18,19} The primary reasons for this are the small size of these New World monkeys (300 to 400 g for mature adults), their ease of handling, their high reproductive efficiency within a laboratory environment (normally twinning twice a year and often becoming sexually mature at 13 months), and the consequent relative ease of establishing breeding colonies.\textsuperscript{18,19,20}

Close similarities between the plasma lipoproteins and apolipoproteins of the common marmoset monkey (fed a diet low in cholesterol and fat) and those of humans have been described.\textsuperscript{21,22} Plasma lipoprotein classes corresponding to human VLDL, LDL, and HDL were identified by using a single density-gradient ultracentrifugal procedure. In the present study, we have extended the characterization by developing a fractionation procedure more suited to the distribution of the marmoset plasma lipoproteins and have compared changes in control plasma lipoproteins induced by feeding an atherogenic diet. Marmosets on the atherogenic diet were found to be susceptible to diet-induced atherosclerosis, and the induced lesions resemble early lesions seen in humans and other primates. In the companion article,\textsuperscript{23} we have shown that several marmoset apolipoproteins are highly homologous to their human counterparts. The close homologies between the lipoproteins of common marmoset monkeys and of humans indicate that this monkey is a promising model for the metabolism of human plasma lipoproteins and for diet-induced atherosclerosis.

Methods

Animals

Common marmoset monkeys (\textit{Callithrix jacchus}) were obtained from captivity-bred commercial stock (Charles River U.K., Margate, Kent, U.K.). Adult males between 2 and 3 years old were fed a standard low-fat, virtually cholesterol-free laboratory chow (Ralston Purina High Protein Monkey Chow #5405, St. Louis, MO) supplemented with a small amount of canned marmoset diet (Zu-preem, Hills Pet Products, Topeka, KS) or fruit (apples or oranges). The pelleted chow was fed in the morning. The supplements were varied daily and were given to the animals in the afternoon. Fresh water flavored with Tang (an orange drink) concentrate (LKS Products, Dublin, CA) and containing 0.02% (vol/vol) pediatric vitamin supplement (Tri-vi-sol, Bristol Labs, Evansville, IN) was supplied daily. For the cholesterol-fat feeding studies, eight monkeys that had been maintained on the control diet for 6 months were transferred from control chow to a high-cholesterol, high-fat diet (BioServ Basic Resource Atherogenic Diet, BioServ, Frenchtown, NJ) containing 0.73 mg of cholesterol/kcal (0.34% wt/wt) and 12% (wt/wt) lard, supplemented with small amounts of canned marmoset diet and fruit. The fat in the diet represented 42% of the total calories. A second group of four monkeys were fed a modified diet in which the BioServ pellets were coated with an emulsion of crystalline cholesterol in melted lard to give a final composition of 2.2 mg of cholesterol/kcal (1.0% wt/wt) and 16% (wt/wt) lard.

Lipoprotein isolation

Control plasma lipoprotein isolation was performed after the monkeys had been normalized on laboratory chow for a minimum of 3 months. In the case of the first group of cholesterol- and fat-fed animals (0.73 mg of cholesterol/kcal, 12% lard), lipoproteins were characterized between the third and the 11th month on the diet. In the case of the second group (2.2 mg of cholesterol/kcal, 16% lard), the lipoproteins were isolated after 3 weeks. After the animals had been fasted overnight (15 to 18 hours), blood was collected and transferred to chilled tubes (4°C) containing disodium ethylenediaminetetraacetic acid (final concentration 0.01% [wt/vol], pH 7.4), and plasma was prepared by low-speed centrifugation. A typical isolation for characterization started with 4.0 to 6.0 ml of plasma from 1 to 10 animals for the controls and 2.5 to 5.0 ml of plasma from 2 to 5 animals for the cholesterol-, fat-fed animals. Up to 3.5 ml of blood (routinely, 1 to 1.5 ml) could be obtained from the femoral vein of unseparated animals. In some instances, larger volumes of blood (8 to 15 ml) were obtained by exsanguination.

Plasma lipoprotein fractions were prepared by sequential preparative ultracentrifugation\textsuperscript{24} at 4°C in a 50 Ti rotor (Beckman Instruments, Palo Alto, CA) at 40 000 rpm. The d<1.006 and d=1.06 to 1.02 g/ml fractions were centrifuged for 18 hours and were recentrifuged under identical conditions to minimize plasma protein contamination. Lipoproteins of density 1.02 to 1.09 g/ml were isolated after a 24-hour centrifugation and were further subfractionated by Pevikon block electrophoresis as described previously\textsuperscript{25}; d=1.09 to 1.21 g/ml lipoproteins were centrifuged for 40 hours and recentrifuged for 24 hours. The recovery of cholesterol among lipoprotein density fractions ranged from 72% to 100%; the recovery of lipoprotein cholesterol from the Pevikon block procedure ranged from 60% to 70%. In some experiments, d=1.02 to 1.063 and d=1.063 to 1.21 g/ml lipoproteins were isolated after a 24- and 40-hour centrifugation, respectively, and the latter fraction was recentrifuged for 24 hours.

Lipoprotein characterization

Paper electrophoresis of the plasma and the various lipoprotein fractions was performed as previously described.\textsuperscript{26} Antiserum to human lipoprotein(a), Lp(a), was a gift of Christian Ehnholm of the National Public Health Institutes, Helsinki, Finland. Negative-staining electron microscopy was performed with freshly prepared carbon films and neutral 1% phosphotungstic acid as the contrast agent. The diameters of approximately 200 particles were measured from the photomicrographs and were printed at a final magnification factor of 100 000.

Chemical determinations

Protein concentrations were determined by the method of Lowry et al.\textsuperscript{27}; bovine serum albumin was used as a standard. The turbidity of triglyceride-rich lipoprotein fractions was removed by treatment with sodium deoxycholate. The total cholesterol and triglyceride concentrations were determined by using enzymatic procedures (BioDynamics, Boehringer Mannheim, Indianapolis, IN),
and the concentration of phospholipid was calculated from the phosphorus content. Cholesteryl esters were quantified by thin-layer chromatography and gas-liquid chromatography (Hewlett-Packard Model 5880, Palo Alto, CA). The sialic acid content was determined by the method of Warren, and the values were corrected for interference by unsaturated lipids when necessary.

Plasma lipoprotein cholesteryl ester content was determined by iatroscan analysis. Agerose gel chromatography on Biogel A15m (BioRad Laboratories, Richmond, CA) was used to fractionate d<1.006 g/ml lipoproteins from marmosets fed the 2.2 mg of cholesterol/kcal diet. The ability of these lipoproteins to stimulate cholesteryl 1,4-C-olate synthesis in mouse peritoneal macrophages was determined as previously described.

The apolipoprotein composition of marmoset plasma lipoproteins was investigated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After delipidation with chloroform/methanol (2:1, vol/vol), electrophoresis was performed in a three-tier gel system based on the method of Laemmli. The system comprised a 4.5% stacking gel (0.125 M Tris-HCl, pH 6.8) layered upon a 9.0% running gel (0.375 M Tris-HCl, pH 8.8), which in turn was layered upon an 11.0% running gel (0.375 M Tris-HCl, pH 8.8). In some instances, a running gel or one with a 5% to 20% gradient was used. Gels were stained with Coomassie blue R250 (BioRad), and proteins were identified by molecular weight standards or by comparison with purified human apolipoproteins.

Characterization of Diet-Induced Atherosclerosis

Necropsies were performed on two control marmoset monkeys and four monkeys fed the BioServ Atherogenic Diet for 11 months. The animals were anesthetized and were exsanguinated through the abdominal aorta. The upper body of the exsanguinated monkey was immediately flushed with ice-cold Minimum Essential Medium (GIBCO). The organs of interest (liver, lung, kidney, and spleen) were removed, trimmed of external fat and connective tissue, and weighed. For histological examination, the tissues were fixed in 4% formaldehyde in 0.1 M phosphate buffer, dehydrated, embedded, and sectioned in JB-4 (Polysciences, Warrington, PA). The entire aorta, from the aortic valve to the femoral arteries, was carefully excised, cleaned of adventitial fat, and cut longitudinally along its dorsal aspect. In one animal, the brachiocephalic trunk and the right and left common carotid arteries were opened as far as the external and internal bifurcation. The branch points of the aortic arch, the midthoracic aorta, the celiac trunk at its emergence, and the iliac bifurcation were fixed, embedded, and sectioned for histological examination. In addition, segments of the heart containing the coronary vessels, segments of the brain and upper spinal cord containing the major arteries of the head, and the tongue were embedded and sectioned for histological examination of their vessels.

Results

Plasma Lipids and Lipoproteins

The mean plasma cholesterol value for seven fasted marmoset monkeys, sampled after both 3 and 6 months on the control diet, was 140±20.2 mg/dl (means±SD). The corresponding plasma triglyceride values were 86.9±25.9 mg/dl. There was no significant difference between values obtained at the two time points. The percentage of esterified cholesterol in the plasma was 75% and 78% in the two samples in which this value was determined. Eight monkeys were started on the high-fat, high-cholesterol diet. Three of them developed a malabsorption syndrome and were withdrawn from the study. The remaining five monkeys remained in good health for the 11 months of the study. The plasma cholesterol values obtained for the five healthy animals over the first 42 weeks of the study are given in Figure 1. At the 20th week, four animals displayed a marked hypercholesterolemia (plasma cholesterol range, 450 to 970 mg/dl) in response to the diet; however, by the end of 42 weeks two of these animals had plasma cholesterol levels of 200 to 250 mg/dl. The first two monkeys were termed hyperresponders, and the second two, hypo-responders. The fifth monkey did not respond to the diet (plasma cholesterol range, 130 to 185 mg/dl). The percentage of esterified cholesterol in the plasma of the hypercholesterolemic monkeys was 76.0±7.6% (n=4). Plasma triglyceride levels were unaffected by the cholesterol-rich diet.

Plasma lipoprotein electrophoresis revealed two prominent bands in control marmoset plasma (Figure 2). These will be shown to correspond to LDL (β-migration) and HDL (α-migration). Lipoproteins with pre-β-mobility, characteristic of VLDL or LP(a), were not seen on routine electrophoresis of marmoset plasma. Dietary cholesterol induced several changes in the marmoset plasma lipoproteins (Figure 2). These included an increase in the staining intensity of both the LDL and HDL bands and,
notably in animals with more marked hypercholesterolemia, the emergence of a prominent $\alpha$-mobility band trailing the HDL. The electrophoretic patterns obtained for the single animal that failed to respond to the diet proved indistinguishable from those observed in control marmosets.

**Lipoprotein Distribution among Ultracentrifugal Fractions**

Marmoset plasma lipoproteins were separated into $d<1.006$, $d=1.006$ to $1.02$, $d=1.02$ to $1.09$, and $d=1.09$ to $1.21$ g/ml fractions. The use of these density intervals has been determined to be necessary to isolate HDL free of LDL in another nonhuman primate species, *Erythrocebus patas*. Analysis of the density fractions by paper electrophoresis (data not shown) indicated that VLDL ($d<1.006$ g/ml) had pre-$\beta$-mobility (corresponding to human VLDL), whereas the IDL ($d=1.006$ to $1.02$ g/ml), present in trace amounts and barely detectable by this technique, possessed $\beta$-mobility and will be shown to correspond to human IDL. The $d=1.02$ to $1.09$ g/ml fraction contained, as expected, two distinct lipoprotein classes: LDL ($\beta$-mobility) and HDL ($\alpha$-mobility). The HDL were also present in the $d=1.09$ to $1.21$ g/ml fraction; these denser HDL will be referred to as $d>1.09$ HDL to distinguish them from the HDL that were separated at $d<1.09$. In a second series of experiments, it was confirmed that marmoset LDL were present at densities greater than $1.063$ g/ml and that the use of this conventional density class was inappropriate for marmoset plasma lipoproteins. The LDL and $d<1.09$ HDL present in the broad $1.02$ to $1.09$ g/ml density range adopted for use in this study were separated by Pevikon block electrophoresis.

Paper electrophoretograms of the density class derived from cholesterol-fed marmoset plasma revealed several changes in the type and distribution of the lipoprotein classes (Figure 3). Cholesterol-fed marmoset $d<1.006$ g/ml lipoproteins contained a $\beta$-migrating fraction, in contrast to the pre-$\beta$-mobility band seen in control animals (not shown). In addition, a significant amount of the $d<1.006$ g/ml lipoproteins remained at the origin, a finding indicative of chylomicrons or large chylomicron remnants seen after cholesterol feeding of other species. The IDL ($d=1.006$ to $1.02$ g/ml) and LDL ($d=1.02$ to $1.09$ g/ml) were more prominent in the cholesterol-fed animals. Concomitant with the increase in the lipoproteins of lower density, there was a shift in the HDL density spectrum from the denser $d>1.09$ HDL to the $d<1.09$ HDL. The $d<1.09$ HDL of cholesterol-fed marmosets were characterized by $\alpha$-mobility on paper electrophoresis, in contrast to the $\alpha$-mobility of the $d>1.09$ HDL.

Values for the percentage distribution of plasma cholesterol and lipoprotein protein among the ultracentrifugal fractions are given in Table 1. In marmosets on a control diet, 60.8% of their plasma cholesterol was transported in lipoproteins of $d<1.09$ g/ml (study I) and 34.3% in lipoproteins of $d<1.063$ g/ml (study II). Lipoproteins of $d<1.03$ g/ml contributed less than 1% of the total lipoprotein cholesterol. As will be discussed, ultracentrifugation and Pevikon block electrophoresis enabled us to subfractionate plasma into three lipoprotein classes based on density and electrophoretic mobility: VLDL/LDL ($d<1.02$), LDL (all $\beta$-migrating lipoproteins), and HDL (all $\alpha$-migrating lipoproteins). Determination of the plasma cholesterol distribution in these three classes revealed that in the control marmoset 42.4% and 56.3% of the
Table 1. Percentage Distribution of Plasma Cholesterol and Lipoprotein Protein among the Ultracentrifugal Fractions of Control and Hypercholesterolemic Marmoset Plasma Lipoproteins

<table>
<thead>
<tr>
<th>Study and fraction</th>
<th>Control</th>
<th>Hypercholesterolemic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Percent cholesterol</td>
<td>Percent protein</td>
</tr>
<tr>
<td>I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>d&lt;1.006</td>
<td>0.2</td>
<td>0.4</td>
</tr>
<tr>
<td>d=1.006-1.02</td>
<td>0.4</td>
<td>0.3</td>
</tr>
<tr>
<td>d=1.02-1.09</td>
<td>60.0</td>
<td>29.4</td>
</tr>
<tr>
<td>d=1.09-1.21</td>
<td>39.3</td>
<td>70.0</td>
</tr>
<tr>
<td>II</td>
<td>34.3</td>
<td>13.6</td>
</tr>
<tr>
<td>d=1.063-1.21</td>
<td>65.7</td>
<td>86.4</td>
</tr>
<tr>
<td>III</td>
<td>1.1</td>
<td>—</td>
</tr>
<tr>
<td>VLDL/IDL d&lt;1.02</td>
<td>42.4</td>
<td>—</td>
</tr>
<tr>
<td>LDL (β-migrating lipoproteins, d=1.02-1.09)*</td>
<td>56.3</td>
<td>—</td>
</tr>
<tr>
<td>HDL (α-migrating lipoproteins, d=1.02-1.21)†</td>
<td>—</td>
<td>39.4</td>
</tr>
</tbody>
</table>

Pools of plasma from six and four animals were used in study I for the control and hypercholesterolemic groups, respectively; plasma cholesterol levels were 120 and 570 mg/dl, respectively. Plasma from a single animal and a pool from three animals were used in study II; plasma cholesterol levels were 151 and 640 mg/dl, respectively. Plasma from a single animal and a pool from four animals were used for study III; plasma cholesterol levels were 160 and 570 mg/dl, respectively.

*Total percentage of plasma cholesterol carried in low density lipoprotein (LDL) separated by a combination of ultracentrifugation and Pevikon block electrophoresis.
†Total percentage of plasma cholesterol carried in high density lipoprotein (HDL), including HDL-with apolipoprotein E, separated by a combination of ultracentrifugation and Pevikon block electrophoresis.

VLDL=very low density lipoprotein, IDL=intermediate density lipoprotein.

plasma cholesterol were transported in LDL and HDL, respectively (study III, Table 1).

Cholesterol and fat feeding resulted in marked shifts in the cholesterol distribution. In both studies I and II there was a shift of cholesterol from the higher to the lower density lipoproteins. As shown in study III, these shifts resulted in an increase in the percentage of cholesterol transported by VLDL/IDL and the LDL and a decrease in that carried by HDL. A comparison of the percentage distribution of total lipoprotein protein revealed a similar shift toward lipoproteins of lower density. After cholesterol feeding, there was a nearly twofold absolute increase in total lipoprotein protein over the values obtained with control marmosets (data not shown). This increase was associated solely with the d<1.09 g/ml fraction; absolute values of d>1.09 HDL protein remained constant (151.3 and 144.8 mg/dl for the controls and the hypercholesterolemic animals, respectively).

Isolation and Characterization of Plasma Lipoproteins

Control Marmosets

The chemical composition and particle sizes of control marmoset plasma lipoproteins are given in Table 2. Marmoset VLDL and IDL were both triglyceride-rich (64.9% and 52.2%, respectively). The IDL contained a higher percentage of protein and cholesterol than did the VLDL and were smaller.

The d=1.02 to 1.09 g/ml fraction, containing both LDL and d<1.09 HDL, was subjected to Pevikon block electrophoresis (Figure 4A). Zone 1 (migration from origin, 8 to 10 cm) contained LDL with β-mobility and represented 50% to 60% of the cholesterol; zone 2, a 2 to 3 cm region isolated directly ahead of zone 1, contained a minor LDL subfraction (approximately 5% of cholesterol) that had a slightly faster mobility on paper electrophoresis. Both LDL subfractions were rich in cholesterol and phospholipid (Table 2), although the minor, zone 2 lipoproteins contained less cholesterol than did those isolated from zone 1 (20.4% vs. 38.2%). Zone 2 LDL had a higher lipid-to-protein ratio than zone 1 LDL (2.9:1 vs. 2.3:1) and were larger (means±SD, 199±14 Å vs. 178±18 Å). The sialic acid content of zone 1 and zone 2 LDL was 10.7 and 13.0 g/mg, respectively; within the same assay, human LDL and Lp(a) gave values of 10.2 and 35.4 g/mg, respectively. No reaction was seen with either LDL class or unfractionated plasma when tested by immunodiffusion against a specific rabbit anti-human Lp(a) antiserum (protein concentration of purified lipoproteins, 0.1 to 0.4 mg/ml). Therefore, despite some physiochemical similarities to human Lp(a), the faster-moving zone 2 lipoproteins do not seem to represent Lp(a).

Zone 4 (migration distance, 15 to 19 cm) contained the d<1.09 HDL with α-mobility and represented 35% of the lipoprotein cholesterol. Zone 3, the region between zones 2 and 4, represented <5% of the recovered lipoprotein cholesterol and was not visualized by paper electrophoresis (Figure 4). Both d<1.09 HDL and d>1.09 HDL were rich in protein and phospholipid. The d<1.09 HDL were more than twofold enriched in triglyceride in
Table 2. Percentage Composition and Particle Size of Marmoset Plasma Lipoproteins

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Control marmosets</th>
<th>Hypercholesterolemic marmosets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Protein</td>
<td>Cholesterol</td>
</tr>
<tr>
<td><strong>d&lt;1.006</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(VLDL)</td>
<td>10.8</td>
<td>2.6</td>
</tr>
<tr>
<td><strong>1.006-1.02</strong></td>
<td>18.8</td>
<td>7.2</td>
</tr>
<tr>
<td>(IDL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>1.02-1.09</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zone 1 (LDL)</td>
<td>25.5</td>
<td>38.2</td>
</tr>
<tr>
<td>Zone 2</td>
<td>30.4</td>
<td>20.4</td>
</tr>
<tr>
<td>Zone 3 (HDL-with E)</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Zone 4 (d&lt;1.09 HDL)</td>
<td>35.8</td>
<td>17.6</td>
</tr>
<tr>
<td><strong>1.09-1.21</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(d&gt;1.09 HDL)</td>
<td>46.1</td>
<td>17.2</td>
</tr>
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<table>
<thead>
<tr>
<th>Fraction</th>
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</tr>
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<tbody>
<tr>
<td></td>
<td>Protein</td>
</tr>
<tr>
<td><strong>d&lt;1.006</strong></td>
<td>9.1</td>
</tr>
<tr>
<td>(VLDL)</td>
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</tr>
<tr>
<td><strong>1.006-1.02</strong></td>
<td>16.9</td>
</tr>
<tr>
<td>(IDL)</td>
<td></td>
</tr>
<tr>
<td><strong>1.02-1.09</strong></td>
<td></td>
</tr>
<tr>
<td>Zone 1 (LDL)</td>
<td>26.5</td>
</tr>
<tr>
<td>Zone 2 (LDL+HDL)</td>
<td>N.D.</td>
</tr>
<tr>
<td>Zone 3 (HDL-with E)</td>
<td>30.7</td>
</tr>
<tr>
<td>Zone 4 (d&lt;1.09 HDL)</td>
<td>35.5</td>
</tr>
<tr>
<td><strong>1.09-1.21</strong></td>
<td></td>
</tr>
<tr>
<td>(d&gt;1.09 HDL)</td>
<td>44.0</td>
</tr>
</tbody>
</table>

Values are given as means±SD.

*Diameters of approximately 200 particles were measured. Range disregards approximately 5% of the particles at either extreme.

VLDL=very low density lipoprotein, IDL=intermediate density lipoprotein, LDL=low density lipoprotein, HDL=high density lipoprotein, N.D.=not determined.

Comparison with their d>1.09 counterparts, had a higher lipid-to-protein ratio (1.8:1 vs. 1.2:1), and were larger (means±SD, 103±9 Å vs. 93±11 Å).

**Cholesterol- and Fat-fed Marmosets**

The chemical composition and particle sizes of plasma lipoproteins of cholesterol- and fat-fed marmosets (0.73 mg of cholesterol/kcal, 12% lard) are given in Table 2. Many of the marmoset plasma lipoproteins were drastically altered in response to a cholesterol-rich diet. Although similar in size to those found in control marmoset plasma, both the VLDL and IDL were enriched in cholesterol (30.3% and 47.0%, respectively) in comparison with the triglyceride-rich lipoproteins of control marmoset plasma. Cholesteryl esters accounted for 76.8% of the cholesterol-fed VLDL cholesterol and 74.2% of the IDL cholesterol.

The d=1.02 to 1.09 g/ml fraction from hypercholesterolomic animals contained a mixture of LDL and d<1.09 HDL, which were separated by Pevikon block electrophoresis. Paper electrophoresis of the Pevikon fractions (Figure 4B) revealed a spectrum of lipoproteins with electrophoretic mobilities ranging from β (zone 1) to α2 (zone 4). Zone 1 (7 to 9 cm migration) contained the LDL and consistently migrated more slowly than did control marmoset LDL (8 to 10 cm). The LDL of the cholesterol-fed animals (representing 60.6% of the cholesterol recovered from the Pevikon block) were similar in composition to those of control animals but were larger (means±SD, 228±20 Å vs. 178±18 Å).

Zone 2 lipoproteins, isolated directly ahead of zone 1 as a band 2 cm wide, represented a mixture of LDL and HDL that accounted for 3.5% of the recovered lipoprotein cholesterol. Further fractionation of this zone was not attempted. Zone 3 (6.7% of recovered cholesterol) was seen as a yellow band (migration, 11 to 15 cm) trailing the second major zone, the zone 4 band (migration, 15 to 18 cm). The zone 4 lipoproteins (28.5% of recovered cholesterol) of cholesterol-fed animals consistently displayed a slower migration than did their counterparts in control plasma (migration, 15 to 20 cm). Cholesterol-fed d<1.09 HDL were larger than control d<1.09 HDL (means±SD, 204±19 Å for zone 3 and 157±19 Å for zone 4 vs.
The apolipoprotein composition of marmoset monkey lipoproteins, as determined by SDS-PAGE (Figure 5), resembled that seen with human lipoproteins. Control marmoset VLDL and IDL (Figure 5A, lanes a and b) contained two high molecular weight apolipoproteins that comigrated with the apolipoproteins designated apo B-100 and apo B-48, according to the nomenclature of Kane et al. Proteins of $M_r=66$ 000, 48 000, 37 500, and 27 500 (corresponding to human albumin, apo A-IV, apo E, and apo A-I) were also present in VLDL and IDL, together with a number of low molecular weight apolipoproteins ($M_r>10$ 000) corresponding to human apo C (see companion article for further characterization). In many instances, apo A-IV was observed as a double band (Figure 5). The $d=1.02$ to 1.09 g/ml fraction contained apo B-100, along with apo A-I and traces of apo A-IV, apo E, and apo C (Figure 5A, lane c). The major LDL class (zone 1) contained exclusively apo B (lane d), whereas the minor class (zone 2) contained traces of apo A-IV, apo E, and an unidentified $M_r=68$ 000 protein in addition to apo B (lane e). The position of the $M_r=68$ 000 protein was not affected by $\beta$-mercaptoethanol incubation; thus, it does not appear to be albumin. Zone 3 and 4 lipoproteins (lanes f and g) contained predominantly apo A-I and low molecular weight apolipoproteins. Zone 3 lipoproteins consistently possessed apo E and apo A-IV and will be referred to as HDL-with apo E. The apolipoprotein composition of $d>1.09$ HDL (lane h) resembled that of $d<1.09$ HDL without apo E (zone 4; lane g), and contained apo A-I and low molecular weight apolipoproteins. The apolipoprotein compositions of the hypercholesterolemic lipoproteins were similar to those of control marmosets (Figure 5B). The identity of the apolipoproteins is based upon the detailed analyses reported in the accompanying article.

### Investigation of Heterogeneity of Hypercholesterolemic Marmoset $d<1.006$ g/ml Lipoproteins

Although paper electrophoresis of $d<1.006$ g/ml lipoproteins from hypercholesterolemic marmosets indicated the presence of $\beta$-migrating lipoproteins that trailed back to the origin (Figure 3), we were unable to isolate sufficient material for further fractionation. To induce a more severe hypercholesterolemia, a group of control marmosets were fed a modified atherogenic diet containing 2.2 mg of cholesterol/kcal (1.0% wt/wt) and 16% (wt/wt) lard. One monkey responded with a plasma cholesterol level of 2123 mg/dl (plasma triglycerides=164 mg/dl) within 2 weeks. Paper electrophoresis of plasma (data not shown) revealed the presence of lipoproteins resembling the $\beta$-VLDL of other species. The entire density class was subjected to agarose column chromatography. As demonstrated in Figure 6, two fractions were obtained. The fraction eluting with the void volume remained near the origin on paper electrophoresis, whereas the later-eluting peak exhibited a range of mobilities up to the $\beta$ position. These two populations will be referred to as fractions I and II, by analogy to the classes found in the $\beta$-VLDL of other species. SDS-
PAGE of fractions I and II (data not shown) revealed that fraction I contained apo B-48 almost exclusively, whereas fraction II contained apo B-100, as has been described for other species. In addition, the d < 1.006 g/ml fraction and fractions I and II stimulated cholesterol esterification in cultured mouse peritoneal macrophages (Table 3). These marmoset \( \beta \)-VLDL subclasses resemble those seen in other species.

**Necropsy Results**

Table 4 contains a summary of the necropsy findings in two control monkeys sacrificed at 2 years of age and in four monkeys which had been on a high-cholesterol, high-fat diet for 11 months. The four animals represent the two hypo-responders and the two hyper-responders. Cholesterol and fat feeding resulted in weight gain and a general increase in organ size. On gross examination, the livers showed fatty changes in parallel with increases in the circulating cholesterol levels of the animals. On histological examination, the livers of the two hyper-responsive animals were normal except for large amounts of parenchymal cell lipid. The lungs and kidneys were found to be normal. The spleens of both hyper-responsive animals, while grossly normal, contained numerous lipid-filled or foamy histiocytes. All other organs in all animals appeared to be normal upon gross examination.
Table 3. Stimulation of Cholesteryl Ester Formation In Mouse Peritoneal Macrophages by β-VLDL and β-VLDL Fractions

<table>
<thead>
<tr>
<th>Lipoprotein</th>
<th>Concentration of lipoprotein cholesterol in medium (mg/ml)</th>
<th>Cholesteryl ester synthesis (nmol/mg of cell protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control*</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>Rabbit β-VLDL†</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>Marmoset d&lt;1.006 g/ml</td>
<td>8.2</td>
<td></td>
</tr>
<tr>
<td>Marmoset fraction I</td>
<td>6.5</td>
<td></td>
</tr>
<tr>
<td>Marmoset fraction II</td>
<td>6.3</td>
<td></td>
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</table>

VLDL, very low density lipoprotein.
*Incubation in the absence of added lipoprotein. †d<1.006 fraction from cholesterol-fed rabbit.

Assessment of Atherosclerosis

Gross examination of the aortas from the cholesterol- and fat-fed marmoset revealed small, smooth, slightly raised plaques in the arch of the aorta in the two hyper-responsive animals, whose plasma cholesterol levels were 478 and 486 mg/dl. No plaques were visible in the two hypo-responsive animals, whose plasma cholesterol levels were 128 and 256 mg/dl, nor in the control animals. After the aorta was treated with the lipophilic dye, Sudan IV (Figure 7), the raised lesions of the hyper-responsive animals were stained. In addition, numerous small lipid-containing lesions were visible between the intercostal vessels of the thoracic aorta and surrounding the major abdominal branches. A diffuse staining of the ascending aorta, the aortic arch, and the thoracic aorta was evident in three of the four cholesterol- and fat-fed animals (animals 4, 5, and 6), particularly in the two hyper-responsive animals. This may represent diffuse lipid accumulation, as has been reported in other primates on cholesterol- and fat-rich diets.15 In one hyper-responsive animal, small fatty streaks were seen at the origin of the common carotids. No lesions or diffuse staining was evident in the iliac vessels or the carotid vessels.

The aortas were embedded and then examined histologically. In the two hyper-responsive marmosets, there were fatty streaks and what appeared to be early proliferative intimal atherosclerotic lesions in the arch of the aorta (Figure 8). Foam cells were found within the intima, often surrounded by matrix material (Figures 8B and 8C). As shown in Figure 8D, the beginnings of an organized fibrous cap was seen in one lesion. Numerous lipid-laden foam cells can be seen near the base of this lesion. Within sections from the lower thoracic and abdominal aorta, small collections of foam cells were frequently found within the intima. Only at the orifice of the large abdominal vessels was there significant intimal proliferation and the collection of large numbers of foam cells. Histological investigation of the major blood vessels of the brain, the lingual artery, and the coronary arteries yielded no evi-
dence of lesions, with the exception of a small foam cell-filled lesion at the origin of one of the coronary arteries. Longer term and more extensive studies to describe more completely the diet-induced atherosclerosis in the marmoset are under way.

**Discussion**

Common marmoset monkeys on a low-fat, low-cholesterol diet were found to have fasting plasma cholesterol concentrations (≈140 mg/dl) that agree well with those found by others studying this species. The values are also well within the range reported for both Old and New World monkeys. The fasted plasma triglyceride concentrations (≈87 mg/dl) were in good agreement with those recently reported for this species and within the range (62 to 108 mg/dl) found for six species of New World monkeys by Rudel and Lofland.

When marmosets were fed a high-fat, high-cholesterol diet, their responses varied considerably (Figure 1) but segregated into two general groups—hypo- and hyper-responders. The degree and range of response are similar to those observed in other primates and segre-gation of animals into hyper- and hypo-responders is a common finding in cholesterol feeding studies in primates as well as in other species. The underlying mechanism or mechanisms for this phenomenon are unclear at this time. As is the case with cholesterol- and fat-fed primates and other animal models, the plasma triglyceride levels remained virtually unchanged. The fact that three out of the eight animals started on the atherogenic diet had to be withdrawn raised the question of an intolerance of this species to the diet. With this in mind, a second group of 14 animals has been started on a modified diet in which the atherogenic diet is mixed with rice cereal (1:1, wt/wt), with the final percentage of cholesterol remaining at 0.5%. Seventeen animals have been on this modified diet for 8 months with no ill effects.

Paper electrophoresis of marmoset plasma revealed a lipoprotein pattern similar to that obtained with human plasma except for less intense staining of the β region and more intense staining of the α region. This difference was reflected in the distribution of lipoprotein cholesterol within the plasma. In marmosets, ~40% of the plasma cholesterol is transported by LDL, whereas in human plasma, ~70% of the cholesterol is found in this fraction. Classic ultracentrifugation techniques depend on the distribution of metabolically distinct lipoprotein classes within exclusive density intervals. This approach is unsuitable for the fractionation of nonhuman primate lipoproteins, as well as the lipoproteins of other species, e.g., rat, dog, or swine, that have lipoproteins with overlapping densities. Marmoset plasma lipoproteins have been previously fractionated by a single density-gradient ultracentrifugation, although this technique does not completely separate marmoset LDL and HDL. Cross-contamination of these lipoproteins was found within the density range 1.055 to 1.07 g/ml; this range contains the larger HDL with apo E as well as LDL. A combination of ultracentrifugation and Pevikon block electrophoresis in the present study enabled us to isolate these lipoproteins.

The plasma lipoproteins of the control marmoset monkey are similar to those of humans and include VLDL, IDL, LDL, and HDL subclasses. In the control as well as the cholesterol- and fat-fed animals, the d<1.09 to 1.21 HDL represented a prominent class of plasma lipoproteins whose concentration was unaffected by diet. Although marmoset VLDL contain more triglyceride and less cholesterol (64.9 and 2%, respectively), larger HDL with apo E as well as LDL. A combination of ultracentrifugation and Pevikon block electrophoresis in the present study enabled us to isolate these lipoproteins.

<table>
<thead>
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<th>Table 4. Necropsy Summary Findings in Six Marmosets</th>
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<tr>
<td>Parameters</td>
</tr>
<tr>
<td>Diet</td>
</tr>
<tr>
<td>Sex</td>
</tr>
<tr>
<td>Weight (g)</td>
</tr>
<tr>
<td>Age (years)</td>
</tr>
<tr>
<td>Cholesterol level (mg/dl)</td>
</tr>
</tbody>
</table>
| Lipoprotein pattern similar to that obtained with human plasma except for less intense staining of the β region and more intense staining of the α region. This difference was reflected in the distribution of lipoprotein cholesterol within the plasma. In marmosets, ~40% of the plasma cholesterol is transported by LDL, whereas in human plasma, ~70% of the cholesterol is found in this fraction. Classic ultracentrifugation techniques depend on the distribution of metabolically distinct lipoprotein classes within exclusive density intervals. This approach is unsuitable for the fractionation of nonhuman primate lipoproteins, as well as the lipoproteins of other species, e.g., rat, dog, or swine, that have lipoproteins with overlapping densities. Marmoset plasma lipoproteins have been previously fractionated by a single density-gradient ultracentrifugation, although this technique does not completely separate marmoset LDL and HDL. Cross-contamination of these lipoproteins was found within the density range 1.055 to 1.07 g/ml; this range contains the larger HDL with apo E as well as LDL. A combination of ultracentrifugation and Pevikon block electrophoresis in the present study enabled us to isolate these lipoproteins. The plasma lipoproteins of the control marmoset monkey are similar to those of humans and include VLDL, IDL, LDL, and HDL subclasses. In the control as well as the cholesterol- and fat-fed animals, the d<1.09 to 1.21 HDL represented a prominent class of plasma lipoproteins whose concentration was unaffected by diet. Although marmoset VLDL contain more triglyceride and less cholesterol (64.9 and 2%, respectively) than human VLDL (51 and 15%, respectively), they had similar apo-lipoprotein compositions, as evidenced by SDS-PAGE. Cholesterol and fat feeding dramatically increases the cholesterol content of the VLDL, mainly at the expense of triglyceride. Accumulation of IDL has been a consistent finding in studies on the effect of cholesterol feeding in animal models, including primates. As is the case with cholesterol feeding in several other primates, the increase in LDL concentration is also associated with a significant increase in particle size. In addition to apo B-100, SDS-PAGE gels of marmoset VLDL and IDL displayed a minor component co-migrating with human apo B-48. This minor apolipoprotein was consistently seen in preparations of marmoset VLDL and IDL despite fasting.
Figure 7. Sudan IV-stained aortas from six marmoset monkeys. These were collected from two monkeys fed a control, or normal, diet (animals 1 and 2) and four monkeys fed a cholesterol-fat-rich diet for 11 months (animals 3 to 6). The cholesterol concentrations in the animals at the time of necropsy were as follows: animal 1, 91 mg/dl; animal 2, 84 mg/dl; animal 3, 128 mg/dl; animal 4, 256 mg/dl; animal 5, 478 mg/dl; animal 6, 486 mg/dl. Aortas from animals 4, 5, and 6 show diffuse staining, but no staining in the major branches. In animals 5 and 6, intensely stained lesions are present in the aortic arch, along the intercostal vessels, and at the orifices of the large abdominal vessels.
Figure 8. Toluidine blue-stained plastic-embedded sections of marmoset monkey thoracic aorta. These sections were taken from regions of fatty streaks in the aortas of two monkeys showing the greatest response to a cholesterol- and fat-rich diet. A. At low magnifications, the adventitia and media appear normal, whereas there appears to be an increased thickening of the intima. × 203 B. At a higher magnification, typical foam cells (FC) are embedded in increased extracellular matrix of the intima. The endothelium (E) is still intact, as is the internal elastic lamina (EL). × 690 C. There are FCs and an increased number of smooth muscle cells (SMCs) within the intima. × 700 D. A fibrous cap has formed over the FCs of this lesion. × 413
of the animals for 18 hours before venipuncture. Preliminary data obtained with the perfused marmoset liver system indicate that hepatic VLDL contain only apo B-100, suggesting that the apo B-48 seen in fasted marmoset lipoproteins is derived from lipoproteins of intestinal origin. This is also the case in humans, but not in the rat. Similar results have been obtained with the perfused liver of the vervet monkey.

Preparative Pevikon block electrophoresis of marmoset d = 1.02 to 1.09 g/ml lipoproteins allowed the isolation of a minor LDL class (zone 2) that differed from the major LDL class in its faster electrophoretic mobility, larger particle size, lower lipid-to-protein ratio, and altered chemical composition. A similar LDL subclass, apparently corresponding to human LP(a), has been detected in the plasma of rhesus (Macaca mulatta) and patas monkeys. As with human LP(a), these subclasses isolated from Old World monkeys can be differentiated from typical LDL by their higher sialic acid content. In the case of the marmoset LDL, the subclasses did not differ in this respect. In addition, immunodiffusion analyses of marmoset plasma (both pooled from six to eight animals and from individual monkeys) and of isolated LDL subclasses failed to show immunochemical reactivity when tested against a rabbit anti-human LP(a)-specific antiserum. These results support those of Chapman et al., who failed to show LP(a) immunoreactivity in 10 marmoset sera measured individually. These authors also described a minor LDL band on agarose electrophoresis of serum that migrated slightly ahead of the major LDL band, and this band would appear to correspond to the LDL subclass that we have characterized in the present study. Other evidence that zone 2 lipoproteins represent an LDL subclass different from LP(a) is the fact that this subclass increases approximately fourfold with cholesterol feeding. The LP(a) equivalent in the patas monkey remained constant in response to cholesterol feeding.

Zone 3 lipoproteins of control plasma represented a minor class present in trace amounts. These lipoproteins correspond to the HDL-with apo E, which have been described in several species, including humans. Although this lipoprotein class showed a considerable increase with cholesterol feeding (~30-fold), the HDL-with apo E transport only 5% of the plasma cholesterol in the cholesterol- and fat-fed animals. This differs from several other species in which the HDL-with apo E become major carriers of cholesterol induced by cholesterol and fat feeding. However, the levels of HDL-with apo E in the cholesterol- and fat-fed marmoset are similar to those found in humans. The concentration of the HDL-with apo E appears to be inversely related to the activity of cholesteryl ester transfer protein, that is, species with low activity (dogs, rats, pigs) have high HDL-with apo E levels, while species with high activity (humans, rabbits, monkeys) have low levels. The low levels of HDL-with apo E observed in this study would be consistent with the marmoset having a moderately high plasma level of cholesteryl ester transfer activity. We have obtained evidence that marmoset plasma contains cholesteryl transfer activity. Marmoset plasma was capable of transferring 14C-cholesteryl ester from LDL to HDL at a level similar to that obtained with human plasma.

Our preliminary studies describing the arterial lesions that developed with fat and cholesterol feeding over 11 months suggest that common marmosets are susceptible to diet-induced atherosclerosis. The lesions seen in these animals resemble, in part, early lesions seen in other nonhuman primates. However, the short duration of the cholesterol and fat feeding (11 months) of this study and the relatively moderate elevation of plasma cholesterol levels attained in the animals are in keeping with the development of early, or immature, lesions. More prolonged feeding experiments are in progress. These should enable us to make a better evaluation of this animal model.

The results of this study differ from those of Dreizen et al., who investigated the cotton top marmoset, Saguinus oedipus, a related species. A diet containing 5% cholesterol and 23% lard (by weight) induced atherosclerotic disease in small arteries first, then in the aorta and larger vessels. There was little evidence of lesions in small arteries in our study. It has been suggested that the extremely high cholesterol levels eventually reached in the Dreizen et al. studies and their progressive increase over time are due to renal failure. The kidneys of our animals were found to be normal. Similarly, whereas Dreizen et al. reported that the cotton top marmoset can develop a lipodystrophy of the small intestine, we found no evidence of such lesions in the animals of our study.

In summary, the plasma lipoproteins of the common marmoset monkey, Callithrix jacchus, are similar in chemical and physical properties to human VLDL, IDL, LDL, and HDL subclasses. Several apolipoproteins have been characterized and are equivalent to human apo A-I, apo A-II, apo A-IV, apo C-II, and apo E. Feeding of a diet rich in cholesterol and saturated fat results in changes in the lipoprotein profile that are characteristic of other primate and animal models. Short-term cholesterol feeding resulted in the development of fatty streaks and what may be very early proliferative atherosclerotic lesions. Taken together, these results indicate that the common marmoset monkey is a suitable model for studies of both lipoprotein metabolism and atherosclerosis in humans.

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Density distribution
20. In: The common marmoset (Callithrix jacchus).


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Comparison of effects of control and atherogenic diets.
D Crook, K H Weisgraber, J K Boyles and R W Mahley

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