Structural and Functional Changes of Rhesus Serum Low Density Lipoproteins During Cycles of Diet-Induced Hypercholesterolemia

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Over the course of a 2-year study, two male rhesus monkeys underwent episodes of diet-induced hypercholesterolemia (from a diet supplemented with 25% coconut oil and 2% cholesterol) followed by regression phases in which the animals received a low fat Purina chow diet. During the induction of hypercholesterolemia, serum cholesterol, apo B, saturation of low density lipoprotein (LDL) cholesteryl ester fatty acyl chains, and the ability of the serum to stimulate cholesteryl esterification by smooth muscle cells rose immediately and in parallel, whereas there was a lag period before the serum became mitogenic to smooth muscle cells. Concurrently, there were important changes in the density, size, chemistry, and concentration of the LDL species in the rhesus serum; induced LDL shifted from the LDL-II to the LDL-I density region with increasing cholesterol concentration. Both structural and functional changes were reversed upon return to a normal Purina chow diet, although at different rates. Serum cholesterol, apo B, and the rate of cholesterol esterification in smooth muscle cells promoted by the serum declined in parallel while the mitogenicity of the serum to smooth muscle cells and the degree of saturation of LDL cholesteryl ester fatty acids took longer to return to normal values. In fact, there was an immediate and dramatic rise in saturation upon reversal before the LDL cholesteryl ester fatty acyl chains returned to their normal composition. The Lp(a) particles did not increase in either concentration or size in response to the test diet, although the change in their lipid composition was similar to those of the other LDL species. The studies indicate that dietary manipulations affect the physicochemical properties of the LDL particles, and that the resultant structural alterations are accompanied by changed in vitro cellular response, suggestive of a greater atherogenicity.

of monkeys and under chronologically unrelated experimental conditions. Because of the significant interanimal variability, we considered it important to investigate the relationship between structure and function of serum LDLs of individual animals to interanimal variability, we wished to determine the time course of the alterations reversed when the animals return to a regular Purina chow diet? The results of these studies are the subject of this account.

Methods

Animals and Diet

Two male rhesus monkeys were fed either a regular Purina primate chow diet (Ralston Purina Company, St. Louis, Missouri) or a diet of 70.5% modified, low fat Purina primate chow supplemented with 25% coconut oil, 2% cholesterol, 1% vitamin mix, and 1.5% gelatin. The monkeys were caged individually and were given 300 g of their diet each morning and allowed water ad libitum. At the start of the experiments, the animals were approximately 6 years old and weighed 8 kg. They gained body weight throughout the study and at the end of the 3-year experiment, they had almost doubled in weight. The monkeys were made to fast at least 16 hours before blood collection and were sedated with 5 to 7 mg Ketaset 1M (Ketamine HCl, Bristol Laboratories, Syracuse, New York) per kilogram of body weight. Blood (10-15 ml) was collected from the femoral vein and stored at 4°C for 1 to 2 hours to allow clotting. Serum was separated by centrifuging the blood at 4°C for 30 minutes at 1000 g.

Lipoprotein Preparation

LDL was isolated by a combination of rate-zonal and isopycnic equilibrium density gradient ultracentrifugation. Total lipoproteins were floated by adjusting serum to d = 1.21 g/ml with solid NaBr and centrifuging 20 hours in the Ti-60 Beckman rotor (Palo Alto, California) at 59,000 rpm. All solutions contained 0.01% Na₂ EDTA and 0.01% NaN₃, pH 7.0. The background density of the isolated total lipoproteins was raised to 1.4 g/ml by adding more NaBr, and the solution was layered under a linear 7.5% to 30% NaBr gradient. Sample volumes were usually 2 ml or less. LDL was separated from HDL at 20°C, either by spinning the SW-40 rotor at 20,000 rpm for 16 hours or at 35,000 rpm for 4 hours. The LDL species were then separated by centrifuging the total LDL fraction, obtained from the rate-zonal step, in a 0 to 10% NaBr gradient in the SW-40 rotor at 39,000 rpm at 20°C for 48 to 66 hours, at which time isopycnic equilibrium was reached.

Density Gradient Centrifugation of Whole Serum

The lipoprotein profile of the total low density lipoproteins was obtained by applying 1 ml of rhesus serum to the top of a 0 to 10% NaCl gradient and centrifuging in the SW-40 rotor for 66 hours at 20°C at 39,000 rpm. Serum and the gradient solutions contained 0.01% Na₂ EDTA and 0.01% NaN₃, pH 7.0. The gradient was pumped out at the rate of 1 ml/min through a ISCO UA-5 monitor (ISCO, Lincoln, Nebraska) set at 280 nm. Densities of fractions from a control tube were determined by analysis with a Zeiss/Abbe refractometer, model A (Carl Zeiss, Incorporated, Oak Brook, Illinois) which was thermostatted at 20°C.

Analytical Ultracentrifugation

Molecular weights were determined by high speed sedimentation and flotation equilibrium at 20°C in a Beckman model E ultracentrifuge (Palo Alto, California) equipped with a photoelectric scanner. For data collection, the output from the Model E at the dynograph chart input was fed into an 8 bit A/D converter (model APSET 1, Connecticut Microcomputers, Brookfield, Connecticut) and processed with a 48 KB Apple II plus microcomputer (Cupertino, California). The data were either stored on floppy discs or taken for immediate analysis using an Apple Silentype or a HIPILOT plotter (Houston Instruments, Austin, Texas) connected to the Apple II.

Before analysis, the LDL fractions were dialyzed exhaustively against three NaBr solutions of varying density and concentration (1.5% NaBr, 20% NaBr, and 30% NaBr) each containing 0.01% Na₂ EDTA, pH 7.0. Equilibrium centrifugation was carried out at the three different densities using six-channel charcoal-filled Epon centerpieces, and the six-place AN-G rotor.

Chemical Analysis

Protein content was determined by the method of Lowry et al. using bovine serum albumin as a standard. A correction factor of 0.91 was applied to the Lowry values obtained from the LDL samples to account for the higher chromogenicity of apo LDL relative to bovine serum albumin. Lipid phosphorus was measured essentially according to the method of Bartlett. Triglycerides were determined enzymatically using a Bio-Dynamics/BMC test kit (Indianapolis, Indiana) with some minor modifications. To increase the sample volume from 50 μl to 1.2 ml in specimens containing low amounts of triglyceride, we made up a buffer different from the one supplied with the reagent set. Our new buffer consisted of: 0.01 M glycycliglycerine, 0.4 mM MgCl₂, 6H₂O, 4 mg/ml fatty acid free albumin, and 0.2 mg/ml sodium dodecyl sulfate (SDS), pH 7.4. Total and free cholesterol...
were also determined enzymatically according to the slightly modified published procedures of Ailain, et al. and Gallo, et al. We added 200 µl of 1% Triton X-100 in saline to up to 2 ml of serum or lipoprotein solution in phosphate-buffered saline (pH 7.4). The reaction was initiated by adding 200 µl of reagent solution and incubating 1 hour at 37°C before measuring the absorbance at 500 nm. The reagent solution consisted of 20 units cholesteryl ester hydrodase, 12.5 units of cholesterol oxidase, 250 units of horseradish peroxidase, and 3000 units of lipase (Rhizopus arrhizus) in 100 ml of the following solution: 0.05% Triton X-100, 5 mM sodium cholate, 2 mM sodium azide, 15 mM phenol, 5 mM 4-aminopyridine, and 0.05 M potassium phosphate pH 7.4. For determination of free cholesterol, the cholesteryl ester hydrodase was left out. Cholesterol esterase, cholesterol oxidase, peroxidase, and Rhizopus arrhizus lipase were all from Boehringer Biochemicals (Indianapolis, Indiana).

Gas Liquid Chromatography

Different LDL fractions from the 0 to 10% NaCl gradients were pooled, dialyzed versus 0.15 M NaCl (containing 0.01% NaCl, and 0.01% Na₂EDTA, pH 7.0), and then lyophilized; then lipids were extracted with 2:1 chloroform-methanol. Neutral lipids were developed with hexane-diethyl ether-glacial acetic acid (90:20:2, v/v/v). The choleseryl ester fraction was scraped off the TLC plates (silica gel G, Analtech, Newark, New Jersey) and used for gas chromatography of the methylated fatty acyl chains on a Hewlett-Packard model 5840A gas chromatograph (Palo Alto, California) as previously described.

Radioimmunoassay for Rhesus Apo B

Apo B was measured by radioimmunoassay in serum or in samples obtained from density gradients as previously described. 

Electroimmunoassay

Rhesus Lp(a) (LDL-III) was quantitated by electroimmunoassay. Antisera against pure rhesus Lp(a) were raised in rabbits. To make the antiserum specific to the Lp(a) antigen, antibodies crossreacting with normal rhesus LDL were removed by affinity chromatography using rhesus LDL-I coupled to Sepharose-4B. Purified antiserum reacted only with rhesus Lp(a) but not with rhesus LDL-I, LDL-II, or HDL. By immunoelectrophoresis only one precipitin arc was observed with rhesus serum and was located in the region where Lp(a) bands. These antiserum also crossreacted with human Lp(a) serum but not with human Lp(a) serum (both kindly provided by Kare Berg, Oslo, Norway). To reduce the background of the stained immunoplates, the antiserum were precipitated with ammonium sulfate in order to obtain the immunoglobulin-enriched fraction that was used in the assay. The electrophoretic runs were performed at 18°C and 2.5v/cm for 16 to 18 hours using standard plastic immunoplates containing 1.5% agarose (Rel. endosmosis = -0.13, Aldrich, Milwaukie, Wisconsin) at a thickness of 1.5 mm. Electrophoresis was conducted with an LKB Multiphor unit (Rockville, Maryland) using 0.06 M Barbital buffer, pH 8.6, as both gel and electrophoresis buffer.

Tissue Culture

Proliferation

Primary explants of medial smooth muscle cells from the thoracic aorta of normolipemic rhesus monkeys were obtained according to the technique described by Fischer-Dzoga, et al. The cultured cells were allowed to grow to a stationary phase in 10% calf serum. At this time the experiments were initiated by replacing 5% calf serum with 5% rhesus serum test serum in groups of five to eight cultures for each experiment. Each experiment included three control groups: 10% calf serum, 5% pooled control rhesus serum, and 5% hypercholesterolemic rhesus serum. Increases in culture size in a given group were regularly accompanied by a relative increase in cells incorporating ³H-thymidine which is an indication of DNA synthesis. Both indicators of cellular proliferation were evaluated as previously described. Due to the considerable variability in primary culture systems which are mainly caused by differences in donor animals, the results of each experiment were expressed relative to the values of the normal monkey serum culture group.

Cholesterol Esterification

Secondary cultures of medial smooth muscle cells were used between the fourth to seventh cell generation for the measurement of cholesterol esterification. Experiments were carried out in duplicate 25 cm² flasks (Falcon) when the cells had reached near-confluency. The cells were thoroughly washed with sterile phosphate-buffered saline (PBS) and incubated for 24 hours with lipoprotein-deficient serum at 2.5 mg protein/ml. The cells were then refed with 5% calf serum plus 5% of the test serum for an additional 24 hours. 1-¹⁴C-sodium oleate (Amersham/Searle, Arlington Heights, Illinois) (0.2 µCi/ml, 0.15 mM) complexed to bovine serum albumin was prepared according to the procedure of St. Clair et al. and was added to the media for the final 4 hours. The cells were washed with PBS and harvested with 0.2 N NaOH. An aliquot of the cells in 0.2 N NaOH was extracted with mixtures of chloroform, methanol, and water using the method of Bligh and Dyer as a recovery standard. ¹²⁵I-H-cholesterol in chloroform was added to the extraction mixture. The lipids were separated using thin layer chromatography with silica gel 182 plates (Baker Chemical Company, Phillipsburg, New Jersey) and the results were expressed as the counts per minute incorporated into...
cholesterol ester as a percentage of the 10% calf serum-grown control cells. Another aliquot of the cells was used for a protein determination.

Results

Dietary Response of Serum Total Cholesterol and Apo B

Feeding a normal monkey chow diet supplemented with 25% coconut oil and 2% cholesterol to the two male rhesus monkeys resulted in a dramatic increase in their serum cholesterol level (figure 1). In both animals fully developed hypercholesterolemia was usually attained in 3 to 5 weeks and was maintained until they were returned to a normal diet. Changing the diet back to normal monkey chow caused a rapid decrease in the serum cholesterol levels to baseline values within 2.5 to 4 weeks. The time course of the hyperlipidemic response was reproducible in the two cycles of induction and regression in Monkey 6 and in the three cycles in Monkey 24. In the latter animal, we have no data on the second regression phase because the monkey was undergoing treatment for a severe shoulder injury. Although both monkeys responded to the test diet and to its withdrawal in a similar time frame, Monkey 6 responded to the test diet with cholesterol values of 600 to 1000 mg/dl; these values were consistently higher than those attained by Monkey 24 (values ranging from 400 to 600 mg/dl). The degree of hypercholesterolemia differed somewhat for each cycle. In the first cycle, Monkey 24 exhibited cholesterol values ranging from 550 to 700 mg/dl; in the second cycle, the values were lower (400 to 470 mg/dl) and in the last cycle, cholesterol level ranged from 520 to 600 mg/dl. In Monkey 6 the cholesterol varied between 600 and 700 mg/dl during the last 12 weeks of the first hypercholesterolemic period and the entire second period. However, during the initial induction period from the 6th to the 14th week, the serum cholesterol levels were extremely high (910 to 1050 mg/dl) before dropping to a level of 670 mg/dl after 16 weeks on the test diet. Throughout this study,

![Figure 1](http://atvb.ahajournals.org/)

**Figure 1.** Response of serum total cholesterol, triglyceride, and apo B over two cycles (Monkey 6) and three cycles (Monkey 24) of dietary induced hypercholesterolemia and subsequent regressions. **Arrow P** indicates the start of the progression period and **Arrow R**, the regression period.
serum triglyceride levels remained at normal levels, usually below 50 mg/dl.

Radioimmunoassay for serum apo B showed that in both monkeys the time course for the induction phase (3 to 5 weeks) and regression phase (2.5 to 4 weeks) of hypercholesterolemia was similar to the response of serum cholesterol (figure 1). In the fully developed hypercholesterolemia, serum apo B values for Monkey 6 were almost all above 200 mg/dl, whereas the other monkey had maximal values that ranged only between 120 to 140 mg/dl.

Density Gradient Profiles of LDL

Most of the increase in serum cholesterol was due to a rise in LDL, since HDL cholesterol in each monkey varied only between 65–85 mg/dl during fully developed hypercholesterolemia. This point is visually dramatized in figure 2 where LDL density gradient profiles are plotted as a function of time. Initially, when the serum cholesterol levels were less than 200 mg/dl, the concentration of LDL was so low that the various LDL species were barely detectable when 1 ml of serum was applied to a 0 to 10% NaCl gradient. However, with increasing serum cholesterol levels, the LDL peaks of both monkeys became progressively larger. During the first and second week, the induced LDL banded initially where LDL-II is found in the control animal, but moved steadily into the LDL-I region with increasing cholesterol levels. During the regression phase, the concentration of LDL decreased rapidly and the density of LDL shifted back to higher density in a reverse manner, the process being completed in 4 to 5 weeks. Although the sequence of events was similar for both Monkey 6 and Monkey 24, there was a marked difference in the degree of heterogeneity of their LDL. In Monkey 6,
the LDL induced by the cholesterol feeding was usually relatively homogeneous. In contrast, the LDL of Monkey 24 was consistently heterogeneous. In addition, the latter monkey often produced modest quantities of VLDL as a result of the test diet, whereas Monkey 6 had very low levels of VLDL. The absorbance (280 nm) profile of VLDL on the density gradient was greatly exaggerated since these particles scatter light due to their large size.

**Concentration of Lp(a)**

In each animal, the concentration of Lp(a) (i.e., LDL-III) was apparently unaffected by cholesterol feeding although there was a small change in hydrated density from 1.051 g/ml in the control state to 1.047 g/ml in the hyperlipidemic state for Monkey 6, and from 1.047 g/ml to 1.043 g/ml for Monkey 24. Since the density gradient profiles only gave a qualitative indication of the serum levels of the LDL and the Lp(a) particles (figure 2) we resorted to other methods to show that the levels of Lp(a) were insensitive to diet. By radioimmunoassay under baseline conditions, the ratio of apo B in the LDL to the apo B in Lp(a) (both pooled from the 0–10% NaCl gradients) was in the range of 1.0. This indicated that during the control diet the concentration of Lp(a) was approximately the same as that of LDL-I and LDL-II combined. During the cholesterol diet, the ratio of apo B (LDL)/apo B (Lp(a)) rose in a response pattern similar to that of the total serum cholesterol, indicating that the increase in concentration of LDL was greater relative to that of Lp(a). This evidence, taken together with the comparison of the sequential lipoprotein profiles obtained by density gradient centrifugation, supported the conclusion that the level of Lp(a) was not affected by the administration of the test diet. These results were corroborated by electroimmunoassay; analysis performed at weekly intervals for 4 weeks during the induction phase of the last cycle of cholesterol feeding indicated that the baseline level of Lp(a) (1.44 mg/ml for Monkey 6, 1.59 mg/ml of Monkey 24) remained essentially unchanged.

**Chemical and Physical Properties of LDL and Lp(a)**

Some of the chemical and physical properties of the LDL species obtained from the two monkeys before and after feeding with the coconut oil-cholesterol diet for 3 months are shown in table 1. The LDL distribution in the 0 to 10% NaBr density gradients is shown in figure 3, which indicates what fractions were taken for analysis. In the hyperlipidemic phase (cholesterol = 637 mg/dl for Monkey 6 and 520 mg/dl for Monkey 24) the LDL species from both monkeys were characterized by an increased content in free cholesterol and cholesteryl esters and by reduced amounts of triglycerides and phospholipid over control LDL species. Although the molecular weight and concentration of Lp(a) showed no significant changes when compared to baseline values, the lipid composition of these particles changed just like the other LDLs (see table 1). The main LDL fraction from Monkey 6 and its equivalent fraction (LDL-C) from Monkey 24 had a size and a hydrated density similar to normolipidemic LDL-I (although we have no evidence that these particles are LDL-I altered by the diet). In Monkey 24 the other two less dense LDL species with molecular weights of 4.8 × 10^6 and 6.0 × 10^6 had no equivalent in control serum

### Table 1. Effect of High Fat–High Cholesterol Diet on Some Physicochemical Parameters of Rhesus LDL and Lp(a)

<table>
<thead>
<tr>
<th>Monkey no:</th>
<th>Lipoprotein</th>
<th>Mean buoyant density (g/ml)</th>
<th>Molecular weight (g/mole LDL)</th>
<th>Protein (g/mole LDL)</th>
<th>Phospholipid (mole/mole LDL)</th>
<th>Free cholesterol (mole/mole LDL)</th>
<th>Cholesteryl ester (mole/mole LDL)</th>
<th>Triglyceride (mole/mole LDL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 6</td>
<td>LDL-I</td>
<td>1.028 3.26 × 10^6</td>
<td>612000</td>
<td>1081</td>
<td>691</td>
<td>1951</td>
<td>326</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LDL-II</td>
<td>1.036 2.70 × 10^6</td>
<td>586000</td>
<td>899</td>
<td>551</td>
<td>1554</td>
<td>232</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lp(a)</td>
<td>1.051 3.15 × 10^6</td>
<td>788000</td>
<td>988</td>
<td>586</td>
<td>1735</td>
<td>285</td>
<td></td>
</tr>
<tr>
<td>Control 24</td>
<td>LDL-I</td>
<td>1.031 3.14 × 10^6</td>
<td>562000</td>
<td>1098</td>
<td>690</td>
<td>1879</td>
<td>281</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LDL-II</td>
<td>1.037 2.85 × 10^6</td>
<td>550000</td>
<td>963</td>
<td>626</td>
<td>1675</td>
<td>262</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lp(a)</td>
<td>1.048 3.43 × 10^6</td>
<td>799000</td>
<td>1226</td>
<td>682</td>
<td>1805</td>
<td>274</td>
<td></td>
</tr>
<tr>
<td>Hypercholesterolemia 6</td>
<td>LDL</td>
<td>1.030 3.14 × 10^6</td>
<td>622000</td>
<td>871</td>
<td>755</td>
<td>2280</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lp(a)</td>
<td>1.047 3.27 × 10^6</td>
<td>811000</td>
<td>823</td>
<td>837</td>
<td>2203</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td>Hypercholesterolemia 24</td>
<td>LDL-A</td>
<td>1.017 6.0 × 10^6</td>
<td>720000</td>
<td>1690</td>
<td>1519</td>
<td>4892</td>
<td>233</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LDL-B</td>
<td>1.021 4.8 × 10^6</td>
<td>658000</td>
<td>1259</td>
<td>1214</td>
<td>3751</td>
<td>198</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LDL-C</td>
<td>1.025 3.5 × 10^6</td>
<td>546000</td>
<td>966</td>
<td>842</td>
<td>2720</td>
<td>131</td>
<td></td>
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<tr>
<td></td>
<td>Lp(a)</td>
<td>1.043 3.3 × 10^6</td>
<td>779000</td>
<td>881</td>
<td>776</td>
<td>2208</td>
<td>112</td>
<td></td>
</tr>
</tbody>
</table>

LDL-I = subfraction of normal rhesus LDL; LDL-II = subfraction of normal rhesus LDL; LDL-A, LDL-B, and LDL-C = subfractions of LDL from Monkey 24 in the hypercholesterolemic phase (see figure 3).
and carried a load of free and esterified cholesterol, which was 2 to 2.5 times higher than in control LDL-I. In this monkey, the concentrations of fractions A, B, and C relative to total LDL, not including Lp(a), were 16%, 34% and 42% respectively, while the main LDL fraction of Monkey 6 comprised about 64% of total LDL.

Saturation of LDL and Lp(a) Cholesteryl Esters

The increase in the saturated and monounsaturated fatty acyl chains of LDL cholesteryl esters seen in both animals during the test diet paralleled the increase in serum cholesterol and was due mainly to increased levels of 12:0 and 14:0 at the expense of 18:2 (figures 4 and 5). This response was reproducible in the two periods of hypercholesterolemia induced in Monkey 6 and the three which were induced in Monkey 24. In the second cycle of Monkey 6 and in the first two cycles of Monkey 24, the degree of saturation rose to maximal values ranging from 55% to 58%, and then dropped to levels of 50% and 52%. Returning the animals to the normal monkey chow diet did not lead to a quick drop in the degree of saturation, as was the case for serum cholesterol. Instead, within a day, there was a rapid and dramatic increase in saturation to between 58% and 60% which lasted from 1 to 3 weeks, and was caused by a further reduction in 18:2 and increased levels of 18:1 and 16:1 (figure 5). This increase in percentage of saturation occurred despite the fact that 12:0 and 14:0 dropped quickly to control levels when the test diet was removed (figure 5). However, unlike the response of serum cholesterol, the cholesteryl ester composition took much longer (7 to 10 weeks) to return to normal values (figure 4).

We also compared the fatty acid composition of Lp(a) cholesteryl esters with those of LDL at several
Figure 4. Percentage of saturation (percent 12:0, 14:0, 16:0, 16:1, 18:0, 18:1 vs 18:2, 18:3, and 20:4) of LDL cholesteryl esters in Monkeys 6 and 24. The data were obtained as a function of time on several cycles of induction and regression of hypercholesterolemia. The cholesteryl ester fatty acid composition determined by gas liquid chromatography was carried out on LDL fractions pooled from 0–10% NaCl gradients. Arrows P and R indicate the start of the progression and regression periods, respectively.

points during induction and regression of hypercholesterolemia and consistently found that they were similar.

**Tissue Culture: Proliferation**

Upon initiation of the atherogenic diet there was a definite time lag, lasting from 2 to 3 weeks, before the increasingly hypercholesterolemic sera became significantly mitogenic when compared to normal monkey control serum (figure 6). During this time, the increasing concentrations of LDL banded initially within the LDL-II density range. However, when the passage to the mitogenic phase was completed (3 to 5 weeks), LDL density had decreased to that of LDL-I, and its particle size and concentration had increased. These LDL species had markedly larger contents of free and esterified cholesterol. In the case of Monkey 24, the LDL species with molecular weights of 6.0, 4.8, and $3.5 \times 10^6$ had, respectively, 2.8, 2.2, and 1.5 times the total cholesterol content of control LDL-II. Serum from each animal remained mitogenic as long as the test diet was given. When the diet was changed to the control diet, the mitogenic effect of serum from both monkeys decreased, but at a slower rate than the decrease in the serum cholesterol, 3 to 5 weeks in the first two cycles of Monkey 24 and in the first cycle of Monkey 6. However, during the last cycle in both monkeys, it was 7 to 8 weeks before the proliferative effect of serum dropped into the normal range and stayed in a relatively high normal range for as long as 30 weeks. A LDL density profile resembling the normal distribution was usually attained after about 4 weeks, although the quality of the profile was not very good at low serum cholesterol values due to the low concentration of LDL. After the last regression period, we examined the LDL density profile repeatedly at five-to 10-fold greater sensitivity at monthly intervals for 6 months. During the second, third, and fourth months, we found LDL species floating in the density interval of 1.015 to 1.020 g/ml which constituted approximately 30% to 40% of the total LDL. Since these lipoproteins are less dense than normal LDL, they are probably larger in size with a greater total cholesterol content which might make them mitogenic. In this study we tested the whole serum and not the isolated LDL species and cannot be certain whether this unusual LDL fraction or some other serum factor was responsible for the extended period of the slightly above normal mitogenicity of these two rhesus sera.

**Tissue Culture: Cholesterol Esterification**

The ability of the serum from the two test monkeys to stimulate cholesterol esterification in secondary cultures of monkey smooth muscle cells was followed over the first cycle of dietary induction and reversal of hypercholesterolemia. As shown in figure 7, cholesterol esterification, which closely paralleled the serum total cholesterol and thus the apo B content of the serum during progression and regression, did not exhibit the lag phase seen in the proliferation studies.

**Discussion**

The design of our studies permitted us to follow in two monkeys changes in the distribution, structure, and function of serum LDL during five induction periods of diet-induced hypercholesterolemia and the subsequent regression phases. During the first 5 weeks after beginning the high fat-high cholesterol diet, peak serum values of total cholesterol and apo B were reached with a similar and parallel time course. At the same time, the LDL cholesteryl ester fatty acid chains became increasingly more saturated, and the LDL particles increased in size and decreased in buoyant density. These large and lighter LDLs, which we refer to as H-LDL, favored the proliferation of smooth muscle cells grown in culture and confirmed the previously observed correlation between size and mitogenic effect of cholesterol-rich LDL derived from both normo- and hyperlipidemic sera. In the latter, the large mitogenic LDLs (molecular weight > $3 \times 10^6$, $d < 1.030$ g/ml) are the
predominant species, whereas in control serum the large LDL-I is only a relatively small fraction of total LDL.

We are still unable to explain mechanistically the mitogenic effect of H-LDL or LDL-I contrary to the smaller species which we refer to as LDL-II. Interestingly, in the current studies there was a critical point both in the induction and regression periods when serum became or ceased to be mitogenic, which may correlate to the observed changes in structure and distribution of the LDL on account of the diet.

One could attribute this effect to the relatively large content of cholesterol in H-LDL over LDL-II (1.5 to 2.8 times more total cholesterol); however, we cannot discard the possibility that large LDLSs in circulation may serve as carriers of mitogenic factors, possibly of cell derivation. Regardless of their mode of action, these mitogenic LDLSs can be viewed as atherogenic if we assume that the in vitro effects also occur in vivo and accept the concept that arterial smooth muscle cell proliferation is an important event in the atherosclerotic process.²⁴ It has been
suggested that large cholesteryl ester-rich LDL stimulates cholesteryl ester accumulation in secondary cultures of smooth muscle cells and that the blood levels of these lipoproteins are correlated to the degree of coronary atherosclerosis in Macaca fascicularis.

From the data using secondary cultures, we found that the pattern of the stimulation of cholesterol esterification, which might be taken as an index of increased cholesterol delivery to the smooth muscle cells, closely paralleled the serum total cholesterol and the apo B content of the serum and did not show that lag phase seen in the proliferation studies. However, due to the biochemical and morphological differences between cells growing out of explants and subcultured cells, particularly in the number of lysosomes and their enzymatic enrichment in the latter cells, it is probably not possible to compare these two systems directly. Other factors besides the saturation of the LDL cholesteryl ester fatty acids and the relative quantities of large and small LDLs may have influenced the response of the secondary cultures to the serum. Thus, HDL has been shown to reduce the stimulation of cholesterol esterification promoted by hyperlipemic LDL and its presence might have masked differences between the effects of the various LDL species, particularly when the levels of LDL were reduced during regression. Therefore, the serum concentration of LDL and the ratio of HDL to LDL in the serum may have played the major role in the observed changes in cholesterol esterification.

Contrary to the important structural and functional changes in LDL which attended the administration of a high fat-high cholesterol diet, the Lp(a) particles remained unaffected in terms of their blood levels and size. We reported earlier that in spite of their mass approximating that of LDL-I, these particles have no mitogenic effect on cultured primary smooth muscle cells, probably due to their relatively high sialic acid content. Yet Lp(a) underwent changes in lipid composition, including the fatty acyl chains of cholesteryl esters, to the same extent as LDL. These fatty acid changes deserve a comment because of their implication in LDL structure and metabolism. The high fat diet led to an increase in the percentage of the saturated and monounsaturat-
ed fatty acyl chains of the LDL cholesteryl esters and thus to a net increase of the degree of saturation which raised the thermal transition of the neutral lipid core of these particles. This increase has been noted by Tall, et al. and ourselves and is particularly important since it is above body temperature and thus abnormal. Equally important, however, is the observation that returning the animals to a normal diet did not immediately correct this abnormal structural state. Instead there was a rapid and dramatic increase in fatty acid saturation which lasted from 1 to 3 weeks and appeared to be caused by a further reduction of 18:2 and increased levels of 18:1 and 16:1 fatty acids (figure 5). This occurred despite the fact that two of the prevalent fatty acid species in coconut oil (12:0 and 14:0), which were responsible for part of the increased saturation observed in the hypercholesterolemic state, quickly subsided.

In the early phase of induction, the hypercholesterolemic response occurred with the induced H-LDL banding in the LDL-II density range. However, with increasing time and serum cholesterol, the H-LDL gradually decreased in density until it banded in the LDL-I region, at which time it was the predominant LDL component. The mechanism for this redistribution of LDL species in hypercholesterolemia remains unclear.

Although LDL increased several fold, rhesus Lp(a) did not increase in concentration in response to dietary cholesterol. Similar findings have been reported by Albers in human subjects where Lp(a) levels remained unchanged after various dietary manipulations although apo B levels were substantially altered. Supportive evidence of a qualitative nature also comes from the studies of Rudel, et al. In agreement with these investigators, we found that the Lp(a) particles from the hypercholesterolemic animals contain more cholesteryl ester and less triglyceride; but in addition, we detected an increased amount of free cholesterol and a reduced content of phospholipid molecules. These chemical findings indicate that the Lp(a) particles underwent alterations of both surface and core lipid components, yet there were no changes in molecular weight. This suggests that the protein moiety of Lp(a) may be an important determinant of the
size of this particle, although this conclusion is limited by our modest knowledge of the Lp(a) protein. Our studies shed no new light on the metabolic role of Lp(a). However, it is clear that its fate is not divorced from that of the other lipoproteins as attested by the fact that diets can affect the chemical composition of Lp(a) in a manner analogous to that of the other LDL particles. Why then was there no increase in the number and size of these particles? This is a question which requires further studies.

In conclusion, the feeding of a high fat, high cholesterol diet induces both quantitative and qualitative changes in the LDL of rhesus monkeys, which may account for the documented atherogenicity of this diet. Returning the hyperlipidemic animals to a normal diet restores the baseline LDL profile, although after a prolonged period of time. It is interesting that this is also associated with the regression of the diet-induced atherosclerotic process.

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