Lipoprotein(a) and Apolipoprotein(a) in a New World Monkey, the Common Marmoset (Callithrix jacchus)  

Association of Variable Plasma Lipoprotein(a) Levels With a Single Apolipoprotein(a) Isoform

Heng-Chang Guo, Jean-Baptiste Michel, Yves Blouquit, and M. John Chapman

In an earlier report (Chapman et al, Biochemistry 1979;18:5096-5108), we suggested that the common marmoset may represent an important model for the study of human plasma lipoprotein metabolism. We now extend the interest of this monkey model to the study of lipoprotein(a) (Lp[a]) and apolipoprotein(a) (apo[a]). Density gradient ultracentrifugal fractionation of marmoset plasma revealed a bimodal distribution of Lp(a), with one peak of concentration occurring in association with very low density lipoproteins (VLDLs) and a second in the density range 1.040-1.080 g/ml. The dense Lp(a) subspecies displayed physicochemical properties (chemical composition, particle size, and electrophoretic mobility) that closely resembled those of its counterpart in humans and baboons but that were distinct from those of low density lipoprotein (LDL). Furthermore, the particle size of marmoset Lp(a) was invariant (31 nm) over the density interval 1.040-1.080 g/ml, whereas that of LDL decreased progressively with an increase in density (=26-25.2 nm). Use of polyclonal and monoclonal antibodies to human apo(a) and of a polyclonal antibody to marmoset Lp(a) allowed immunologic identification of a single apo(a) isoform in the marmoset whose size was similar to that of apo B-100; apo(a) and apo B-100 were associated in Lp(a) particles by a disulfide linkage. The total protein mass of apo-Lp(a) was estimated to be 800,000 or more by electrophoresis in sodium dodecyl sulfate-polyacrylamide-agarose gels. The amino acid compositions of marmoset and human apo(a) resembled each other but were distinct from those of the corresponding forms of apo B-100. Immunologic evidence is provided for a high degree of cross reactivity between apo(a) in marmosets, baboons, and humans, supporting the idea of the existence of a marked degree of structural homology between these proteins. In addition, electroimmunoblotting of marmoset apo(a) and marmoset plasminogen showed that these proteins shared certain epitopes in common, suggesting that marmoset apo(a) may possess kringle-like structural features. Finally, despite possession of a single apo(a) isoform, marmoset Lp(a) levels varied over a 100-fold range (0.5-49 mg/dl plasma). Considered together, our present findings suggest that the common marmoset monkey constitutes a unique model in which to study the regulation of apo(a) gene expression and the posttranslational processing of apo(a), as well as factors that modulate the synthesis, intravascular metabolism, and cellular catabolism of Lp(a). (Arteriosclerosis and Thrombosis 1991;11:1030-1041)
ate animal models. In the case of Lp(a), the availability of suitable animal species in which to undertake extensive studies of atherogenesis and of thrombotic phenomena is presently restricted to the nonhuman primates. Indeed, biochemical, genetic, and immunologic evidence has documented the occurrence of Lp(a) in species of both great and lesser apes such as the chimpanzee, orangutan, and gibbon and in numerous species of Old World monkey including the baboon, rhesus monkey, *Erthrocebus patas*, and pig-tailed and stump-tailed monkeys.3-11 Of these species, attention to date has been primarily focused on the baboon (genus *Papio*) and the rhesus monkey (*Macaca mulatta*).3,5,7-11

Despite the close phylogenetic relation of Old World monkeys to humans, use of the former for lipoprotein and atherosclerosis research has tended to be restricted to specialized primate centers, given the difficulties encountered in their handling, breeding in captivity, and the high cost of colony maintenance. Such limitations have favored the recent development of colonies of smaller species of nonhuman primates, in particular the common marmoset, *Callithrix jacchus*.12-14 This New World monkey, a member of the family Callithrichidae, breeds readily in captivity, is conveniently handled and bled in view of its small size (as much as ~400 g in adults), and is relatively easy and inexpensive to maintain.12-14 Indeed, it was such characteristics that originally prompted us to propose this species as an appropriate and promising model for lipoprotein research,15 in addition to our observation that both the qualitative and quantitative aspects of lipoprotein and apolipoprotein profile in *C. jacchus* closely resemble those found in normolipidemic humans.15,16 More recently, the susceptibility of the marmoset to diet-induced atherosclerosis, as originally described by Dreizen et al,17 has been confirmed18 and in addition has been extended to demonstrate the occurrence of both foam cell lesions and proliferative intimal plaques in monkeys hyperresponsive to diets supplemented with cholesterol and saturated fat.18

To date, both biochemical and immunologic studies have failed to reveal the presence of circulating Lp(a) in the plasmas of New World monkeys, including the common marmoset.3,15,18 As plasma Lp(a) levels may vary over a wide range both in humans and in nonhuman primates,2,4,7,9,11,19 it seems plausible to suggest that the apparent absence of Lp(a) in New World species may result at least in part from the chance selection of animals with low Lp(a) concentrations (≤5 mg/dl) and in part from a low degree of immunologic cross reactivity of the monkey Lp(a) particles with antisera to their human counterparts.3,4

In light of the foregoing considerations and with the aim of extending the marmoset model to studies of the atherogenic and thrombotic role of Lp(a), we undertook further investigation of the possible occurrence of this cholesterol-rich low density lipoprotein (LDL)-like particle in *C. jacchus*. We now describe the purification, physicochemical characterization, and plasma density profile of Lp(a) and of its specific protein component apolipoprotein (apo(a)) in the common marmoset monkey. Both the lipoprotein particle and its distinctive apo(a) showed marked homology with their human counterparts. The marmoset protein is, however, distinguished by its lack of polymorphism and presents as a unique size species whose circulating concentrations varied widely between monkeys. The results support and extend our earlier contention that the marmoset constitutes a highly valuable experimental animal model for comparative studies of the role of lipoproteins in atherosclerosis.

**Methods**

**Animals and Diets**

The marmosets used in this study form part of a breeding colony established at INSERM U.36, and the investigations described herein were made on blood samples taken from animals within this facility. Animals in our colony are genetically diverse. Thus, part of the original breeding population was derived from the CIBA-GEIGY colony in Basel, Switzerland, and the remainder from Shamrock Farms in the UK. Marmosets were caged in pairs, and adult animals weighed approximately 350 g; the temperature was maintained between 19°C and 23°C, and the relative humidity was kept between 60% and 70%. The animals were fed a diet that consisted of 20% protein, 7.5% fat, 52% carbohydrate, 3% cellulose, 5.5% mineral mixture, and 12% moisture; a mixture of vitamins was also included in this diet. The diet was made up as a pelleted preparation (UAR, Villemoisson-sur-Orge, France). Supplements of fruit (apples, oranges, or bananas) were offered daily.

**Blood Specimens**

Blood samples (as much as 1 ml) were routinely taken from restrained, unanesthetized trained animals that had been fasted overnight for approximately 15 hours. Blood was withdrawn into glass tubes containing Na2EDTA (final concentration, 1 mg/ml) by direct needle puncture of the femoral vein, and plasma was rapidly separated by low-speed centrifugation at 4°C. Lipoprotein fractionation was commenced within 3–4 hours of plasma separation, during which time the plasma samples were cooled on ice at 4°C, and NaN3 and sodium merthiolate (final concentrations, 0.01% and 0.001% wt/vol, respectively) were added. Lipoprotein studies were performed either on aliquots of individual plasma samples (as much as ~0.5 ml) or on pooled samples from six or more monkeys (~3 ml or more).

**Lipoprotein Isolation by Density Gradient Ultracentrifugation**

Marmoset lipoproteins were fractionated on the basis of their hydrated densities by the isopycnic ultracentrifugal density-gradient procedure described for human and marmoset serum lipoproteins.
by Chapman et al.\textsuperscript{15-20} and subsequently modified.\textsuperscript{21} Ultracentrifugation was performed in an SW41 rotor of a Beckman L8-55 ultracentrifuge at 40,000 rpm (56x10^3g/min) for 48 hours at 15°C; no brake was used at the end of the run. On completion of centrifugation, 20 0.5-ml fractions were collected successively from the meniscus of each tube by aspiration with a narrow-bore Pasteur pipette; the bottom fraction, essentially devoid of lipoproteins, was removed in a volume of approximately 2 ml. Our gradient fractionation did not take into account the position of visible lipoprotein bands, but their positions were noted relative to the number of the various fractions. The background density of each lipoprotein gradient subfraction was determined by reference to the density profile obtained from control gradients.\textsuperscript{20}

Lipoprotein fractions were exhaustively dialyzed in Spectrapor tubing (Spectrum Medical Industries, Los Angeles, Calif.; M, cutoff of 12,000) for 48 hours at 4°C against a solution containing either 0.1 M NaCl and 0.01% EDTA, and 0.01% NaN\textsubscript{3} (pH 7.4), at which time samples were destined for apolipoprotein analysis.

**Isolation of Lipoprotein(a)**

Lp(a) was isolated from pooled marmoset plasma samples (=50 ml) essentially according to the procedure of Ehnholm et al.\textsuperscript{22} After selection of the density interval over which the majority of Lp(a) was distributed in the marmoset (see "Results"), the plasma was brought to a solvent density of 1.050 g/ml by addition of solid KBr; centrifugation was then performed in the 50 Ti rotor of a Beckman L8-55 ultracentrifuge at 45,000 rpm for 24 hours at 10°C. After centrifugation the top fraction (=2 ml) was removed, and the lower fraction was brought to a density of 1.100 g/ml with solid KBr. After centrifugation under the same conditions as before but for 48 hours, the top fractions (=2 ml) containing lipoproteins of \(d=1.050-1.100\) g/ml were removed by aspiration with a narrow-bore Pasteur pipette and pooled. The pooled \(d=1.050-1.100\) g/ml subfractions were then concentrated to a volume of approximately 4 ml by use of a collodion thimble membrane under vacuum (Schleicher and Schuell, Darmstadt, FRG); the final protein concentration was in the range 2-5 mg/ml.

Lp(a) was purified from the \(d=1.050-1.100\) g/ml lipoproteins by gel-filtration chromatography\textsuperscript{24} on an agarose column (Bio-Gel A-5m; Bio-Rad, La Jolla, Calif.) with dimensions of 0.9x80 cm. This column was eluted under the conditions as described by Krempler et al.\textsuperscript{24} Fractions that eluted in distinct peaks were pooled (see Figure 3), and their purity was evaluated by chemical analysis and electrophoresis in agarose gel (see "Results").

**Purification of Apolipoprotein(a)**

Marmoset apo(a) was separated from Lp(a) by a modification of the methodology of Armstrong et al.\textsuperscript{28} and Laplaud et al.\textsuperscript{26} Dithiothreitol (final concentration, 20 mM) was added to 1 mg native Lp(a) in a final volume of 1 ml, and the solution was incubated at 37°C for 1 hour. The mixture was then adjusted to a solvent density of 1.100 g/ml with solid KBr and centrifuged at 40,000 rpm for 48 hours at 10°C in a Ti 50.3 Beckman rotor. After completion of centrifugation, apo(a) was found as a translucent pellet; this pellet was solubilized in a solution of 20 mM tris(hydroxymethyl)aminomethane (Tris), 1 mM EDTA, 10 mM dithiothreitol, and 2% sodium dodecyl sulfate (SDS) (pH 7.2). Possible contamination of apo(a) preparations by marmoset apo B-100 was excluded by further purification by immunoaffinity chromatography. Apo(a) preparations were loaded onto anti-LDL-Sepharose columns, and apo(a) was eluted in the unbound fraction; such preparations were dia lyzed against 0.5% HCOOH and subsequently lyophilized before chemical analysis.

**Plasminogen Purification**

The \(d=1.24\) g/ml bottom fraction of marmoset plasma was taken after density gradient fractionation and dialyzed against a buffer containing 0.1 M Na\textsubscript{2}HPO\textsubscript{4}, 0.01% NaN\textsubscript{3}, and 0.01% EDTA (pH 7.5). Plasminogen was then separated from this fraction by affinity chromatography on lysine-Sepharose (Pharcma Fine Chemicals, Upppsala, Sweden) by use of the protocol of Deutsch and Mertz.\textsuperscript{27}

**Monoclonal and Polyclonal Antibodies**

Monoclonal antibodies to human apo(a) (\(M,=570,000\)) were produced from hybridoma cells as described earlier.\textsuperscript{28} A monoclonal antibody to human apo B-100 (1.8. C4) was produced from hybridoma cells that were kindly provided by Thomas L. Innerarity. The characterization of this antibody has been reported earlier.\textsuperscript{29} A polyclonal antibody to marmoset apo(a) was produced by subcutaneous injection of 10 \(\mu\)g apo(a) emulsified in Freund's complete adjuvant into a mouse. The same amount in Freund's incomplete adjuvant was similarly injected at 2 and 4 weeks after the initial immunization. After immunologic testing of the antisera, the mouse was bled and the serum was separated. In addition to its specificity for marmoset apo(a), this polyclonal antibody reacted with both human and baboon apo(a) upon immunoblotting (see Figure 6 for baboon apo(a)).

**Chemical Analysis**

The protein content of lipoprotein and protein preparations was determined by the method of Lowry et al.\textsuperscript{30} with the use of bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, Mo.) as the working standard. Total and free cholesterol were estimated with the enzymatic kit of Biomérieux (Charbonnières-les-Bains, France). Ester cholesterol was calculated as the difference between total and free cholesterol; cholesteryl ester was then calculated as ester cholesterol times 1.67.\textsuperscript{20} Total triglyceride...
and phospholipid contents were also determined with enzymatic kits (Biomérieux).

Amino acid analysis of acid hydrolysates of purified marmoset apo(a) was performed on a Biotronik LC 6001 autoanalyzer. Hydrolysis of 15–30 μg lyophilized apo(a) was effected under the conditions outlined earlier.36

Electrophoresis of Native Lipoproteins

Agarose gel. Aliquots (2–5 μl) of whole plasma and of native lipoprotein fractions were electrophoresed for 40 minutes on agarose gel films (Universal electrophoresis film agarose, catalog No. 470100, Corning, Palo Alto, Calif.) with use of the Corning ACI system. On completion of electrophoresis, the sheets were stained for lipid with oil red O. This procedure is essentially the same as described by Noble.31

Polyacrylamide gradient gels. To assess lipoprotein heterogeneity by identification of specific particle size species and to determine lipoprotein particle diameters, continuous gradient gel electrophoresis of native lipoproteins was performed in a Pharmacia electrophoresis apparatus (GE-2/4 LS) loaded with gels containing a 2–16% gradient (PAA 2/16; Pharmacia). Approximately 15 μg lipoprotein protein was applied to each well, and electrophoresis was performed at 125 V for 12 hours at 4°C in a Tris–borate buffer (0.09 M Tris, 0.08 M boric acid, 0.003 M EDTA, pH 8.3).32 Gels were stained overnight in a solution containing 25% CH3OH, 0% acetic acid, and 0.1% Coomassie brilliant blue R-250; destaining was effected with a solution containing 25% CH3OH and 10% acetic acid. A set of standard proteins with known hydrated diameters (thryoglobulin, 170 Å; ferritin, 122 Å; catalase, 104 Å; lactic dehydrogenase, 81 Å; BSA, 71 Å; high molecular weight electrophoresis calibration kit, Pharmacia) together with latex beads (mean diameter, 38 nm; the kind gift of A.V. Nichols) were run in duplicate on each slab as reference markers. From the migration distances of the different lipoprotein subfractions and those of the proteins contained in the calibration kit, it was possible to calculate the Stokes’ diameters of lipoprotein mass. Continuous gradient gel electrophoresis of native lipoprotein fractions were electrophoresed for 4 hours at room temperature with 10 μg/ml of a monoclonal antibody to apo(a) or apo B-100 in TBS containing 3% BSA (vol/vol). After washing for 24 hours with TBS containing 0.1% BSA, the blots were incubated with an iodine-125–labeled conjugated sheep anti-mouse immunoglobulin G (Amersham) at 20°C for 4 hours. The nitrocellulose was washed for 24 hours with TBS containing 0.1% BSA. Radioactively labeled antibodies were visualized by exposure (−80°C) to x-ray film (Kodak Ortho G Film) for 24–48 hours and subsequent film development.

Dot Immunobinding Assays

For the initial detection of Lp(a) in lipoprotein subfractions isolated by density gradient ultracentrifugation, we employed a dot immunobinding assay as detailed elsewhere37 and adapted from that of Hawkes et al.38

Immunologic Quantification of Apolipoproteins

A quantitative estimate of apo B, A-I, and E concentrations in density gradient subfractions derived from marmoset plasma was performed by nephelometric assays based on the use of the BNA nephelometer analyzer (Behringwerke AG, Marburg, FRG). These assays were performed in accordance with the manufacturer’s protocol, employing a fixed-time nephelometric methodology with multipoint calibration. Monospecific rabbit antisera to human apo A-I and apo B (Behring) and a monoclonal antibody 39A1 (in biotin-labeled form) was employed as the second antibody instead of antibodies 14A12 and 53A9. For calibration purposes, marmoset Lp(a) purified as described above was used; the working concentration range of our assay was 0.02–1 μg Lp(a) purified as described above was used; the working concentration range of our assay was 0.02–1 μg Lp(a) lipoprotein mass.
Density gradient fraction number

Density (g/ml)

1.013 1.024 1.055 1.120 1.205

µg/0.5 ml

10 5 0

mg/0.5 ml

The quantitative distributions of Lp(a), apo B, total cholesterol, apo A-I, and apo E were determined as a function of density in three different plasma pools (Figure 1). A peak in cholesterol, apo E, and apo B concentrations occurred concomitantly with the presence of triglyceride-rich lipoproteins in fraction 1 (d<1.013 g/ml); a peak of Lp(a) concentration (=0.5 mg/0.5 ml) was also detected in this subfraction. To exclude the possibility that Lp(a) reactivity in the d<1.013 g/ml fraction might arise from bound plasminogen,40 we performed electrophoresis of the total d<1.013 g/ml protein content after reduction in agarose–acylamide gels (see “Methods”) followed by electroimmunoblotting with our polyclonal antibody to human apo(a); this antibody reacts strongly with plasminogen.28 A positive blot revealed a diffuse band with Mr in the range 90,000, and at the limit of our detection method in these subfractions; furthermore, recent sequence data have confirmed the close structural homology between human and marmoset apo(a).29

RESULTS

Density Distribution of Lipoprotein(a), Apolipoprotein B, Total Cholesterol, Apolipoprotein A-I, and Apolipoprotein E in Marmoset Plasma

Preliminary studies employing our polyclonal antibody to human Lp(a) or monoclonal antibodies to this same antigen28 revealed positive reactions with marmoset plasmas in dot immunobinding assays (data not shown); equally, immunoblots of marmoset plasma electrophoresed in an agarose-polyacrylamide gradient gel system and revealed with monoclonal antibody 39A128 permitted tentative identification of a high Mr counterpart of human apo(a) (see below). On the basis of these initial findings, we proceeded to fractionate marmoset plasma by density gradient ultracentrifugation (see “Methods”) and to apply our enzyme-linked immunosorbent assay for marmoset Lp(a) to the gradient subfractions.

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The major broad peak in cholesterol and apo B levels coincided with the distribution of LDLs over the density range 1.024–1.055 g/ml; over this same range, apo E levels varied from approximately 2–3 µg/0.5 ml fraction. Apo A-I levels were at the lower limit of our detection method in these subfractions; by contrast, the peak in apo A-I concentrations occurring over the density range 1.055–1.120 g/ml clearly defined the distribution of high density lipoprotein (HDL).

A broad Lp(a) peak of d=1.040–1.076 g/ml overlapped the density profiles of both LDL and HDL, the distributions of the latter being indicated by the profiles of apo B and apo A-I, respectively. Furthermore, low levels of Lp(a) (=0.1 mg/0.5 ml fraction) were found over the entire density range classically attributed to intermediate density lipoproteins and LDLs (d=1.013–1.065 g/ml). Immunologically reactive Lp(a) was, however, undetectable at gradient densities greater than 1.120 g/ml, thereby suggesting the absence of lipid-poor or free apo(a) (or apo[a]–B-100 complexes) in marmoset plasma.

The distinct density distributions of LDL and Lp(a) in gradient subfractions were further confirmed by immunologic identification of lipoprotein bands in nondenaturing gradient gel electrophoresis (Figure 2); such immunologic detection involved, on the one hand, electrophoretic transfer and immunoblotting and on the other hand, dot immunobinding, by employing monospecific antibodies to either apo(a) or apo B-100 in each case (see “Methods”). Thus, strong Lp(a) immunoreactivity was found in

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Purification and Characterization of
Marmoset Lipoprotein(a)

Purified Lp(a) was obtained by pooling the central fractions of the first peak eluted from the Bio-Gel A-5m column (Figure 3). This material presented a single band on gradient gel electrophoresis with a particle diameter of 31 nm, closely resembling that of purified human Lp(a) (Figure 3, inset). The second and third peaks from the agarose column were shown to contain primarily LDL and HDL, respectively, by electrophoresis in agarose gels and in nondenaturing polyacrylamide gradient gels (data not shown).

The chemical composition of marmoset Lp(a) markedly resembled that of its human counterpart (Table 1) but was slightly poorer in cholesteryl ester (32.4% compared with 37.8%). Marmoset Lp(a) and LDL isolated from the same plasma pool were distinguished by the elevated cholesteryl ester content and diminished proportions of both protein and phospholipid in the latter. As noted earlier, marmoset and human LDLs were of similar chemical composition. Purified marmoset Lp(a) and LDL were also distinct in their electrophoretic mobility in agarose gel, with Lp(a) migrating to a pre-β position and LDL to a β position (data not shown).

The apoprotein content of marmoset Lp(a) was examined by electrophoresis in SDS-agarose-acrylamide gels (Figure 4). Native delipidated Lp(a) presented as a single band of Mr, more than 800,000 in this system (Figure 4, lane 1); after reduction with 10 mM dithiothreitol (Figure 4, lane 2), this band disappeared and was replaced by a single smaller com-
component whose size (=550,000) was indistinguishable from that of marmoset apo B-100 (not shown). Moreover, the molecular weight of the band corresponding to the reduced form of apo-Lp(a) was approximately half that of the nonreduced apoprotein. These findings clearly suggested that marmoset apo Lp(a) might consist of two disulfide-linked subunits of similar size. This question was further evaluated by immunoblotting of SDS-agarose (0.5%)-polyacrylamide (2.75%) gels in which reduced plasmas from several individual monkeys had been electrophoresed (Figure 5). When apo(a) was specifically revealed by use of a mixture of five monoclonal antibodies to human apo(a) followed by autoradiography (see "Methods"), a single band was detected in each of 13 plasmas (Figure 5, pattern A, lanes 1–13); this component exhibited an $M_r$ of

\[ 1 \quad 2 \]

- $> 800 \text{ K}$

- $= 550 \text{ K}$

**TABLE 1. Chemical Composition of Lipoprotein(a) and Low Density Lipoprotein in the Common Marmoset and in Humans**

<table>
<thead>
<tr>
<th>Component</th>
<th>Marmoset†</th>
<th>Human (mean weight %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesteryl ester</td>
<td>32.4±1.2</td>
<td>37.8±1.1</td>
</tr>
<tr>
<td>Free cholesterol</td>
<td>11.5±1.1</td>
<td>8.9±1.3</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>4.8±0.5</td>
<td>3.9±0.0</td>
</tr>
<tr>
<td>Phospholipid</td>
<td>24.1±0.6</td>
<td>23.4±0.2</td>
</tr>
<tr>
<td>Protein</td>
<td>27.2±1.9</td>
<td>26.0±2.5</td>
</tr>
</tbody>
</table>

LDL and Lp(a) were isolated by agarose column chromatography (Figure 3).

*Values in humans are taken from Reference 41; LDL are of $d=1.02–1.05 \text{ g/ml}$ and Lp(a) of $d=1.05–1.08 \text{ g/ml}$.

†Data for marmoset lipoproteins are the mean±SD of triplicate analyses of three separate preparations.

**FIGURE 3.** Elution profile for purification of marmoset Lp(a) from d=1.050–1.100 g/ml lipoproteins by gel-filtration chromatography on Bio-Gel A-5m. Chromatography was performed essentially as described by Krempler et al, and the absorbance of the eluate was monitored at 280 nm (c). Lp(a) content of each fraction (µg/ml) (●) was estimated by ELISA (see "Methods"). Inset at left shows the nondenaturing gradient gel electrophoretic analysis of marmoset Lp(a) (lane a) purified by this procedure and of human Lp(a) (lane b) separated by a similar method; lane c shows protein standards used for size calibration purposes. Gel was stained with Coomassie brilliant blue R-250. Fractions containing Lp(a) were pooled as indicated by the shaded bar. S(t), Stokes' particle diameters; Lp(a), lipoprotein(a); ELISA, enzyme-linked immunosorbent assay; LDL, low density lipoprotein; HDL, high density lipoprotein.

**FIGURE 4.** Electrophoretic patterns in 2.75% SDS-agarose–polyacrylamide gels of purified marmoset apo-Lp(a) in native and reduced forms. Lane 1 shows native apo-Lp(a); this sample (40 µg protein) was solubilized in 5% SDS and running buffer (see "Methods") in the absence of a reducing agent. The same sample was electrophoresed in lane 2 but after solubilization under reducing conditions (10 mM dithiothreitol). A series of molecular weight markers was electrophoresed in parallel for calibration purposes; gels were stained with Coomassie brilliant blue R-250. The position to which marmoset apo B-100 migrates in this gel is indicated by the arrow at right. SDS, sodium dodecyl sulfate; Lp(a), lipoprotein(a); apo, apolipoprotein.
FIGURE 5. Electroimmunoblots of apo(a) and apo B-100 in plasmas from individual marmosets. Plasma (2 μl) was reduced with dithiothreitol, electrophoresed in sodium dodecyl sulfate–polyacrylamide (2.75%)-agarose (0.5%) gels, and electroblotted onto nitrocellulose. In pattern A, lanes 1–13, apo(a) was revealed by incubation with a mixture of five monoclonal antibodies to human apo(a) followed by an iodine-125-labeled anti-mouse immunoglobulin G. In pattern B, lanes 14–17, apo B-100 was revealed by incubation with a mouse monoclonal antibody (1.8.C4) to human apo B-100, followed by a 125I-labeled anti-mouse immunoglobulin G. Immunoblots in patterns A and B were visualized by autoradiography (see "Methods"). Note the difference in band intensities between individual marmoset plasmas in pattern A. apo(a), apolipoprotein(a).

approximately 550,000. Immunoblots from four of the same plasmas were reacted with a monoclonal antibody to apo human B-100 (Figure 5, pattern B, lanes 14–17) and equally revealed a single band (Mr≈550,000) whose migration closely resembled that identified as apo(a). These findings indicate that marmoset apo(a) and apo B-100 are of similar size and in addition that apo(a) is remarkably homogeneous within and between individual monkeys, presenting as a single molecular weight species.

The apparent presence of a single size isoform of apo(a) in C. jacchus might, however, result from the limited immunoreactivity of our monoclonal antibodies to human apo(a) with their counterparts in non-human primates. We therefore proceeded to produce a mouse polyclonal antibody to marmoset apo(a) (see "Methods") and to use this antibody in immunoblots of both marmoset and baboon plasmas (Figure 6). As many as five distinct apo(a) isoforms were identified in the baboon (Figure 6, pattern A, lanes 1–6), while the same murine polyclonal antibody to marmoset apo(a) detected only a single isoform in the homologous plasma (Figure 6, pattern B, lanes 7–13). Such data confirm the polymorphism of baboon apo(a).5,7-9 Control immunoblots were performed with an unrelated monoclonal antibody (IgG.C7) directed against the cellular LDL receptor.42 Such blots gave a uniform low background, attesting to the specificity of the positive blots at Mr≈550,000 obtained by use of either monoclonal antibodies to

FIGURE 6. Photograph showing determination of apo(a) isoforms by electroimmunoblotting of baboon and marmoset plasmas. Plasma samples (2 μl) were reduced with 10 mM dithiothreitol and electrophoresed in sodium dodecyl sulfate–polyacrylamide (2.75%)-agarose (0.5%) gels (see "Methods"). Protein bands were electrophoretically transferred onto nitrocellulose and immunoblotted initially with a mouse polyclonal antibody to marmoset apo(a) and subsequently with an iodine-125-labeled anti-mouse immunoglobulin G. Apo(a) bands were finally visualized by autoradiography (see "Methods"). Pattern A, lanes 1–7, individual baboon plasmas; pattern B, lanes 8–13, individual marmoset plasmas. apo(a), apolipoprotein(a).
TABLE 2. Comparison of Amino Acid Compositions of Apolipoprotein(a) and Apolipoprotein B-100 in the Marmoset and in Humans

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Marmoset</th>
<th>Human</th>
<th>Marmoset</th>
<th>Human</th>
</tr>
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<tbody>
<tr>
<td>Lys</td>
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<td>2.1</td>
<td>7.0</td>
<td>8.0</td>
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<tr>
<td>His</td>
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<td>Arg</td>
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<td>5.8</td>
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<td>3.4</td>
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<tr>
<td>Asp</td>
<td>9.7</td>
<td>8.9</td>
<td>10.3</td>
<td>10.7</td>
</tr>
<tr>
<td>Thr</td>
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<tr>
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<tr>
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<tr>
<td>Iso</td>
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<tr>
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<tr>
<td>Cys</td>
<td>ND</td>
<td>ND</td>
<td>0.6</td>
<td>0.4</td>
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</table>

Data are expressed as mol per 100 mol; data for marmoset apo(a) are representative of two analyses. Data in humans for apo(a) are taken from Reference 26 and for apo B-100 from Reference 43; values for marmoset apo B-100 are derived from our earlier report. Apo, apolipoprotein; ND, not determined.

The amino acid compositions of apo(a) and apo B-100 from the marmoset are compared with those of humans in Table 2. The amino acid profiles of marmoset and human apo(a) are remarkably alike and may only be distinguished by minor differences (=1 mol %) in histidine, aspartic acid, glycine, alanine, and isoleucine. An exception was the proportion of proline (10.3% and 7.0% in humans and marmosets, respectively).

The amino acid compositions of marmoset and human apo B-100 were characterized by elevated proportions of lysine, aspartic and glutamic acids, and leucine; their enrichment in lysine and leucine served to distinguish them from the homologous form of apo(a). By contrast, apo(a) in marmosets and humans featured elevated contents of threonine and proline, amino acids that were poorly represented in apo B-100 in these two species.

Immunologic Cross Reactivity of Lipoprotein(a) and Plasminogen

The marked homology of apo(a) and plasminogen in humans and the rhesus monkey has been documented immunochemically and by determination of cDNA-derived protein sequence. To examine the potential relation between apo(a) and plasminogen in the marmoset, we purified the latter protein from plasma by lysine-Sepharose chromatography. Subsequently, marmoset Lp(a) and plasminogen were electrophoresed in SDS-polyacrylamide gel, and the protein bands were transferred electrophoretically to nitrocellulose paper and immunoblotted (Figure 7). Our mouse monoclonal antibody to apo(a) (39A1) reacted positively with marmoset plasma to reveal a single high Mr band (apo[a]) and in addition a second component with Mr =90,000 (Figure 7, lane 1). When this same antibody was blotted to the purified homologous plasminogen (Figure 7, lane 2), a single band of Mr =90,000 was identified. Clearly, marmoset apo(a) and plasminogen are immunochemically related.

Distribution of Plasma Lipoprotein(a) Concentrations

By application of an enzyme-linked immunoassay for marmoset Lp(a) (see "Methods"), plasma levels were determined in 43 male and 64 female marmosets in which the mean concentrations (±SD) were 11.0±1.5 and 11.7±1.3 mg/dl, respectively. All individuals possessed immunologically detectable Lp(a) by this procedure. The overall range in Lp(a) levels was 0.5–49 mg/dl plasma, with a bimodal distribution.
of peaks occurring at 3 and at 25 mg/dl, respectively. Five animals were identified with levels in excess of 30 mg/dl. No sex differences were detectable.

Discussion

The present studies have documented for the first time the presence of a counterpart to human Lp(a) in a New World primate, the common marmoset. Several explanations may be proposed to account for the apparent absence of Lp(a) in New World monkeys in general, and in the common marmoset in particular; these potentially include the use of human polyclonal antisera with poor reactivity to the monkey lipoprotein, serendipitous selection of marmoset plasmas containing low Lp(a) concentrations, and use of relatively insensitive immunologic and electrophoretic detection methods.

Lp(a) in fasting marmoset plasma typically displayed a bimodal density profile (Figure 1), with approximately half of the total Lp(a) mass being associated with triglyceride-rich (d<1.013 g/ml) and intermediate density (d=1.013–1.016 g/ml) lipoproteins while the remainder presented as a more typical dense lipoprotein species distributed over the density interval from 1.040–1.080 g/ml. The interaction of Lp(a) with triglyceride-rich lipoproteins and the presence of apo(a) as a component of postprandial d<1.006 g/ml lipoproteins in humans are now well established. By contrast in the baboon, the only nonhuman primate in which the physicochemical properties of Lp(a) have been detailed until now, neither Lp(a) nor apo(a) has been found as a constituent of triglyceride-rich particles. An explanation for this species difference is lacking, especially because apo B-100 is a component of VLDL in the baboon, providing the potential for the interaction of its exposed lysine residues with lysine-binding sites in baboon apo(a).

As in the baboon, rhesus monkey, and human, a dense particle species is a significant component of the plasma Lp(a) profile in the marmoset monkey. The precise dimensions of the density interval over which the dense form of marmoset Lp(a) was primarily distributed (d=1.040–1.080 g/ml) closely resembled that in humans, baboons, and rhesus monkeys and in the European hedgehog. A major influence on the density profile of Lp(a) is exerted by apo(a) isofrom pattern. In this context it is relevant that we observed little variation in Lp(a) density profile among the individual marmosets studied, a finding consistent with the presence of a unique apo(a) isofrom.

In addition to hydrated density, a high degree of homology was evident after comparison of other physicochemical properties of the dense Lp(a) species in the marmoset with those of its counterpart in the baboon and in humans. Thus, the particle sizes of marmoset and baboon Lp(a)s as determined by nondenaturing polyacrylamide gradient gel electrophoresis were alike (30.8 nm and 31 nm, respectively) but somewhat larger than values reported for human Lp(a) (=23.6–25.5 nm²); such apparent differences may, however, reflect to some degree the nature of the analytical methodology applied, which in the case of the particles from humans, has primarily concerned ultracentrifugal procedures. Nonetheless, human, baboon, and marmoset Lp(a) particles are each of greater diameter than that of the corresponding LDL (Figure 2; References 2 and 5). The overall chemical compositions of human, baboon, and marmoset Lp(a) are alike and equally distinct from those of LDL (Table 1; References 5 and 41); in addition, Lp(a) particles in the latter species are all of pre-β electrophoretic mobility.

The remarkable finding in the current work was of a unique form of apo(a) in marmoset monkey Lp(a). This feature distinguishes the marmoset monkey from all other species (humans, rhesus monkeys, and baboons) in which apo(a) isofroms have been evaluated to date and in which multiple (as many as 11) forms have been detected. The molecular weight of marmoset apo(a) was indistinguishable from that of apo B-100 (each derived from marmoset Lp[a]) in the various electrophoretic systems used (Figures 3 and 4), and only immunoblotting proce-
dures facilitated its identification (Figure 4). The linkage between apo(a) and apo B-100 in marmoset Lp(a) appears to occur by means of disulfide bond(s), as disulfide reduction dissociated the high molecular weight protein complex (>800,000) into a single band of Mr~550,000 (Figure 3); similar behavior has been reported for Lp(a) in other species.5,25,26,50

Evidence for the identity and nature of marmoset apo(a) in addition to that provided by its immunoreactivity with polyclonal and monoclonal antisera to human apo(a) was derived by chemical analysis (Table 2); such analyses revealed marmoset apo(a) to be enriched (>10 mol%) in threonine and glutamic acid and deficient (=2 mol% or less) in lysine, methionine, and phenylalanine. Indeed, the contents of lysine, arginine, threonine, proline, leucine, tyrosine, and phenylalanine served to distinguish apo(a) from the homologous form of apo B-100 (Table 2); similar data have been described for apo(a) and apo B-100 in humans and the hedgehog.26,50,51 It is noteworthy that the proline content of marmoset apo(a) (7 mol%) resembles that detected in its human counterpart (8.1–11.4 mol%).50,51

Further insight into the structural organization of marmoset apo(a) was gained from examination of its relation with plasminogen. Earlier studies have clearly established the common possession of kringle IV and V, together with a carboxyl-terminal protease domain, in plasminogen and apo(a) in humans and in the rhesus monkey; hybridization of a rhesus monkey apo(a) cDNA probe with baboon apo(a) mRNA attests to a similar degree of homology between the baboon and rhesus proteins. On the basis of the cross-reactivity of our polyclonal and monoclonal antibodies to human apo(a) with marmoset apo(a) and marmoset plasminogen, a similar overall structural relation clearly exists between them, although protein sequencing data and identification of specific apo(a) epitopes recognized by our monoclonal antibodies are required before this relation may be delineated further.

Finally, it is remarkable that Lp(a) concentrations in our marmoset colony varied over a 100-fold range despite the apparent expression of a unique apo(a) isoform in C. jacchus. Indeed, an inverse relation between apo(a) isoform size and plasma Lp(a) concentration exists in humans.52 More recently, Gavish et al.53 have demonstrated that the variable number of kringle IV domains encoded by the apo(a) gene is the principal factor in determining both apo(a) protein size and plasma Lp(a) level in humans. By contrast, Rainwater and colleagues7 did not observe an inverse relation between apo(a) isoform size and Lp(a) level in baboons, and in addition they observed variability in the Lp(a) concentrations associated with a specific single isoform phenotype. Further interpretation of these fascinating—and apparently contradictory—findings may be premature, especially in light of the significant and apparently variable contribution of carbohydrate content to apo(a) isoform size.19

In conclusion, the detection of Lp(a) in the common marmoset, considered together with the presence of a single apo(a) isoform and variable plasma Lp(a) levels, makes this New World monkey an exciting and promising model for future research on the expression of the apo(a) gene, on the posttranslational processing of apo(a), and on the synthesis, intravascular metabolism, and cellular degradation of Lp(a).

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**Key Words** • apolipoprotein(a) isoform • monoclonal antibodies • immuno blotting • enzyme-linked immunosorbent assay • density gradient ultracentrifugation
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H C Guo, J B Michel, Y Blouquit and M J Chapman

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