Measurement of rhesus monkey (*Macaca mulatta*) apolipoprotein B in serum by radioimmunoassay: comparison of immunoreactivities of rhesus and human low density lipoproteins

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Abstract A sensitive and specific double antibody radioimmunoassay for the major apolipoprotein (apoB) of rhesus (*Macaca mulatta*) serum very low density lipoprotein (VLDL) and low density lipoprotein (LDL) is described. The antiserum was raised to LDL (d 1.030–1.040 g/ml) and the LDL_2 (d 1.020–1.050 g/ml) was labeled with ^{125}I by the chloramine-T or iodine monochloride method. The assay, which was sensitive to 0.02–0.5 μg of LDL_2, had an interassay coefficient of variation of 4.5%. This assay was successfully used to measure apoB in the whole serum and low density lipoproteins of control monkeys maintained on a standard Purina monkey chow (PMC) diet and of three groups of monkeys fed atherogenic diets: an "average American diet," a 25% peanut oil and 2% cholesterol-supplemented PMC diet, and a 25% coconut oil and 2% cholesterol-supplemented PMC diet. The control monkeys (n = 13) had a serum cholesterol of 146 ± 28 mg/dl and an apoB of 50 ± 18 mg/dl. In the monkeys maintained on the atherogenic diets the serum apoB was elevated: 103 ± 28 mg/dl (American), 102 ± 35 mg/dl (peanut oil), and 312 ± 88 mg/dl (coconut oil). The values for serum total cholesterol were 333 ± 65 mg/dl (American), 606 ± 212 mg/dl (peanut oil), and 864 ± 233 mg/dl (coconut oil) and were elevated relative to controls ($P < 0.001$). For each of the diets, total serum cholesterol correlated with serum apoB ($P < 0.001$). The slopes of the regression lines of serum apoB vs. cholesterol for the monkeys on the PMC, American, and coconut oil diets were similar (m = 0.531, 0.401, and 0.359, respectively), but differed from that of monkeys on the peanut oil diet (m = 0.121). The immunoreactivities of rhesus and human LDL were compared using specific antisera raised against these antigens. In homologous assay systems, monkey and human LDL exhibited unique immunological determinants. The same results were obtained with the delipidated preparations of the two LDLs using antisera raised against either monkey or human apoB. Crossover studies using a heterologous tracer with each antiserum resulted in the selection of a specific population of antibodies directed against antigenic sites shared by these two LDL species.

Supplementary key words low density lipoprotein radioimmunoassay · atherosclerosis · hypercholesterolemia.

The rhesus monkey (*Macaca mulatta*) is being increasingly employed as a laboratory model for studying the development of atherosclerotic lesions and lipoprotein metabolism. Experiments in which these monkeys have been fed various high cholesterol and high fat diets indicate that the development of atherosclerotic lesions is accompanied by abnormalities in circulating lipoprotein concentrations (1). Thus far, the data accumulated on changes in the serum lipids and lipoproteins suggest that the type of hyperlipoproteinemia as well as the nature of the aortic and coronary artery lesions varies with the composition of the diet (2). LDL prepared from hyperlipidemic monkey sera showed that these molecules stimulated proliferation of aortic medial cells in vitro (3).

In order to delineate the role of the serum lipoproteins in the atherosclerotic process, and also to gain some insight into their structural and functional roles in general, we have developed a radioimmunoassay for apolipoprotein B (apoB), the major component of the very low density (VLDL) and low density (LDL) lipoproteins. In this report we describe the development of this immunoassay, its validity and applicability to a large number of samples, as well as its use for comparing the immunoreactivities of rhesus and human apoB. A preliminary account of this work has appeared (4).

Abbreviations: VLDL, rhesus very low density lipoprotein of d < 1.006 g/ml; LDL_1, rhesus low density lipoprotein of d 1.006–1.020 g/ml; LDL_2, rhesus low density lipoprotein of d 1.020–1.050 g/ml; HDL, rhesus high density lipoprotein of d 1.063–1.21 g/ml; LDL, human low density lipoprotein of d 1.019–1.063 g/ml; apoB, the major apolipoprotein of low density lipoproteins; SDS, sodium dodecyl sulfate; PMC, Purina monkey chow diet; ^{125}I-LDL, ^{125}I-labeled LDL.

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MATERIALS AND METHODS

Isolation of lipoproteins

VLDL, LDL, and HDL were separated by preparative ultracentrifugation of plasma taken from fasting male monkeys (*Macaca mulatta*) and fasting normolipidemic human subjects according to previously published procedures (5–7). The lipoproteins were prepared from the pooled plasma of two donor monkeys maintained on a low fat Purina Monkey Chow (PMC) diet, from four rhesus monkeys fed a human diet representing an "average American" diet (8), and from five rhesus monkeys on a PMC diet supplemented with 25% coconut oil and 2% cholesterol (9). Rhesus VLDL was isolated at d < 1.006 g/ml; LDL_1 at d 1.006–1.020 g/ml, and LDL_2 at d 1.020–1.050 g/ml. Monkey HDL was usually prepared at d 1.063–1.21 g/ml. Electrophoresis of LDL_2 on 10% acrylamide containing 0.1% SDS (10) revealed that most of the apoprotein did not penetrate the gel, suggesting that it was mainly comprised of apoB. In addition, trace amounts of fast moving bands located in the positions of the arginine-rich and C-apoproteins could be detected in overloaded gels. LDL_1 exhibited a similar pattern but contained a slightly larger percentage of the arginine-rich and C-apoproteins. The identification of these polypeptides was based on electrophoretic criteria using standards available in this laboratory.

All of the C-apoproteins were isolated from a delipidated preparation of the VLDL from the two rhesus monkeys on the PMC diet by Sephadex G-200 column chromatography in urea (6).

Preparation of apoB

Monkey LDL (d 1.020–1.050 g/ml) and human LDL (d 1.030–1.040) in 0.001N sodium hydroxide was delipidated by extraction with mixtures of ether and ethanol.[2] The extraction was carried out at 4°C in graduated centrifuge tubes equipped with Teflon-lined screw caps. The LDL was extracted with an equal volume of diethyl ether–ethanol 1:1 (v/v) by vigorously injecting the organic solvent just below the surface of the LDL solution. The tubes were immediately placed on a rotator for 5–10 min and then centrifuged briefly to separate the phases. The lipid-containing upper phase was removed by aspiration and replaced with an equal volume of ether–ethanol 3:1 (v/v) by injecting as before. This procedure was repeated five to six times until the volume of the aqueous phase was reduced to its initial volume. After delipidation, the aqueous phase was dialyzed against 0.001 M sodium hydroxide, pH 11, to remove the organic solvent. The apoB preparation was maintained at pH 10, by addition of sufficient 1.0 M glycine, to give a final concentration of 0.02 M glycine. This solution was stored at 4°C. Electrophoresis of this preparation on SDS-polyacrylamide gel electrophoresis revealed that essentially all of the apoprotein did not penetrate the gel, suggesting that it was mainly comprised of apoB.

Preparation of antisera to LDL

To eliminate contamination by other apolipoproteins, narrow cuts of rhesus and human LDL (d 1.030–1.040 g/ml) were selected. Approximately 5.0 mg of this intact rhesus or human LDL, mixed with complete Freund's adjuvant, was injected intramuscularly into male albino New Zealand rabbits. After 4 weeks the rabbits were boosted with another 5.0 mg of LDL emulsified in incomplete Freund's adjuvant. The animals were bled 2 weeks later and one antiserum for each species of LDL was selected at the appropriate titre for use in these studies. The purity of the antiserum was tested by immunoelectrophoresis of whole serum. Only a single line of precipitation in the position of purified LDL was obtained.

Preparation of antisera to apoB

The protocol followed the same approach as that described for LDL except that 1 mg of unbuffered rhesus or human apoB at pH 11 was mixed with the Freund's adjuvant.

Assay buffer

A borate buffer (0.13 M) containing 0.5% bovine serum albumin (fraction V), 0.05% EDTA, and 0.5% sodium azide was used. It was adjusted to pH 8.0 with 12 N hydrochloric acid.

A 0.02 M glycine buffer containing 0.05% bovine serum albumin, pH 10, was used for preparing the apoB stock solutions for the standard curves. (To maintain the pH at 8.0 in the assay tubes, 0.1 ml of the apoB solution at pH 10 was added to 0.9 ml of borate–albumin assay buffer, pH 8.0.)

Iodination of LDL

Both monkey and human LDL (50 μg) were iodinated by a modification of the chloramine-T method as has been described for apoA-I (11). The labeled molecule, sp act 8.0 mCi/mg, was purified by gel filtration on 0.8 × 15 cm Superfine Sephadex G-75 columns equilibrated in borate–albumin buffer (fraction size 0.3 ml). The labeled LDL that eluted in the void volume was separated from the free iodide in the salt peak. The peak tubes of ^{125}I-LDL were combined and

[2] Fless, G. M. 1971. Preparation and physico-chemical characterization of apo low density lipoprotein of human plasma. Dissertation for Doctor of Philosophy, University of Illinois, Urbana, Ill.

rechromatographed on 1.2 × 50 cm Bio-Gel P-30 columns also equilibrated in the borate–albumin buffer (fraction size 1.0 ml). The ^{125}I-LDL eluted in the void volume and between 20 and 50% of the counts applied to the Bio-Gel P-30 column appeared as free iodide in the salt peak. The peak tubes of ^{125}I-LDL were pooled, stored at 4°C in the dark, and used in the assay for 4 weeks.

In the iodine monochloride method, 100 μg of monkey LDL was dissolved in 0.5 M glycine buffer, pH 10, in a total volume of 50 μl and 0.5 mCi Na^{125}I was added. The reaction was started by adding 0.4 nmol of free ICl (10 μl of 6.48 μg/ml in 0.5 M glycine buffer pH 10), and allowed to proceed for 3 min with gentle shaking. It was stopped by the addition of 5 μl of sodium metabisulfate (200 μg/ml in 0.5 M glycine buffer pH 10) and 100 μl of borate albumin buffer. The labeled lipoprotein, sp act 0.013 mCi/mg, was purified as described above for the chloramine-T method. As in the case of LDL labeled by chloramine-T, there was a significant amount of iodide present in the salt peak after the second purification on Bio-Gel P-30 columns.

The pooled monkey ^{125}I-LDL fractions from the Bio-Gel P-30 column were further analyzed for the presence of free iodide by precipitation with 5% TCA in the presence of carrier albumin. Twenty-five percent and 8% free iodide were found in the monkey ^{125}I-LDL labeled by the chloramine-T and iodine monochloride methods, respectively. The extraction method of Bligh and Dyer (12) was used to determine the extent of lipid labeling of the TCA precipitable LDL. Twenty-five and 10% of the radioactivity was lipid associated in the LDL labeled by the chloramine-T and iodine monochloride methods, respectively. The distribution of this lipid-bound radioactivity was determined by thin-layer chromatography.[3] In the lipids from both preparations the majority of ^{125}I remained at the origin with the phospholipids. Taking into account the free iodide present after TCA precipitation, 98–100% of the iodinated LDL from each preparation was bound by excess LDL antiserum.

Iodination of apoB

Monkey and human apoB (50 μg) were iodinated by the same iodine monochloride method described for LDL. The labeled apoprotein, sp act 3.92 mCi/mg, was purified on a 0.8 × 15 cm Superfine Sephadex G-75 column equilibrated in glycine albumin buffer. The ^{125}I-apoB eluted in the void volume and was completely separated from the free iodide. The peak tubes were pooled and stored at 4°C. More than 92% of the iodinated apoB was bound by excess anti-apoB antiserum.

Protein determinations

Protein was quantitated according to Lowry et al. (13) using bovine serum albumin, fraction V, as the standard. VLDL and LDL_1 were delipidated with diethylether after the development of color. As a control the bovine serum albumin standards and LDL_2 were also extracted with ether. Under these conditions the protein content of delipidated LDL was the same as that determined in the unextracted preparation. For all lipoproteins the protein content was determined directly from the Lowry et al. value.

The assay

The LDL (3–5 mg protein/ml) stock used for the standards was kept at 4°C under N_2 in the dark for up to one month. Except for the preparation of the stock solutions of the apoB standards (see Assay buffers), all dilutions were prepared in the borate albumin EDTA buffer. The LDL antiserum (1:50,000 final dilution) and the apoB antiserum (1:100,000 final dilution) were titrated with ^{125}I-LDL (10,000–30,000 cpm, equivalent to approximately 2–6 ng of LDL protein) or ^{125}I-apoB (15,000 cpm, equivalent to approximately 0.6 ng of apoB) to give 60–70% binding in the zero dose tubes. The duration of the first antibody reaction was 72 hr at 4°C. The second antibody reaction was carried out as previously reported for the apoA-I assay (11). One-tenth ml of normal rabbit serum (1:100 dilution) and 0.1 ml of goat antirabbit gamma globulin serum (1:30) were added to each tube and incubated for 48 or 72 hr at 4°C prior to centrifugation.

To control for the binding of the tracer to the glass as well as to the precipitate, tubes containing the tracer alone in the assay buffer were included. The nonspecific binding was approximately 5% and 6.5% with the ^{125}I-LDL and ^{125}I-apoB, respectively. To control for changes in nonspecific binding in the presence of varying concentrations of LDL or apoB, unlabeled LDL or apoB (0–50 μg) was mixed with tracer in the absence of antiserum. The nonspecific binding of ^{125}I-LDL did not increase in the presence of as much as 20 μg of LDL. However, the nonspecific binding of ^{125}I-apoB increased from 6.5 to 10% with 8.8 μg of apoB and continued to increase with higher concentrations of apoB. The apoB assays were therefore carried out using 10 μg of apoB as the highest standard.

Other procedures

Triglycerides and cholesterol were determined on an Auto Analyzer™11 (14) following procedures used

[3] Chen, R. M. 1973. Effects of hyperlipemic rabbit serum and its lipoproteins on proliferation and lipid metabolism of rabbit aortic medial cells in vitro. Dissertation for Doctor of Philosophy, University of Chicago, Chicago, Ill.

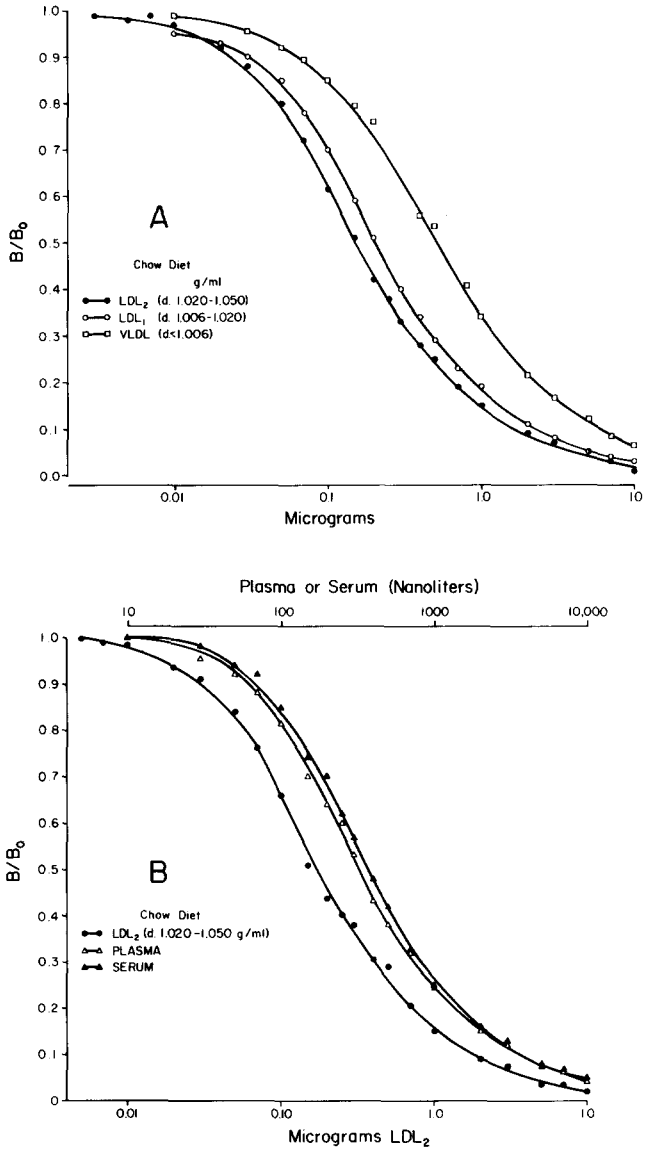

Fig. 1. Displacement of ^{125}I-labeled monkey LDL_2 from anti-monkey LDL antibodies (*A*) LDL_2 (d 1.020–1.050 g/ml) (● —— ●); LDL_1 (d 1.006–1.020 g/ml) (○ —— ○); VLDL (d < 1.006 g/ml) (□ —— □). Protein concentrations of all preparations were determined by the method of Lowry et al. (13). Stock solutions of each lipoprotein containing 0.01, 0.1, 1.0, 10, and 100 μg protein/ml were prepared in the borate–albumin EDTA buffer. Aliquots of 0.1–1.0 ml were assayed in duplicate. (*B*) LDL_2 (● —— ●); dilution of plasma (△ —— △) and serum (▲ —— ▲) from normolipidemic monkeys. Serial dilutions of 10^{-2}, 10^{-3}, and 10^{-4} of sera and plasma were prepared in borate–albumin EDTA buffer. Aliquots of 0.1–1.0 ml were assayed in duplicate.

by the Lipid Research Clinics Program (15). Serum phospholipids were extracted (16) and the phosphorous was measured according to Bartlett (17). Serum albumin was determined by the method of Watson (18). Statistical analyses were performed by standard methods (19). Unless otherwise indicated the results were expressed as mean ± SD.

Monkeys (*Macaca mulatta*)

Normolipidemic. There were 14 males weighing 6.05 ± 0.48 kg (range 4.5–8.4). These monkeys were maintained on the PMC diet.

Hyperlipidemic. These included four males, weighing 7.18 ± 0.61 kg (range 6.5–7.96), who had been eating an "average American" diet (8) for 2 years and 5 months. A PMC diet supplemented with 25% coconut oil and 2% cholesterol (9) was fed for 3 years to two males weighing an average of 8.36 kg and for 1 year and 3 months to three males weighing 4.7 ± 0.48 (range 3.9–5.6). Nineteen males weighing 4.86 ± 0.81 kg (range 3.80–7.24) were given a "peanut oil" diet, PMC diet supplemented with 25% peanut oil and 2% cholesterol (2), for 1 year.

RESULTS

Sensitivity and specificity of anti-LDL antibodies

The standard curve with purified LDL_2, prepared from monkeys fed a PMC diet, showed significant displacement at 0.02 μg (**Fig. 1*A***). The working range of the assay lay between 0.05 and 0.5 μg of LDL_2 and maximal displacement (greater than 98%) occurred with 10 μg.

Fig. 1*A* also shows the reactivity of the anti-LDL antibodies with LDL_1 and VLDL isolated from the same monkeys. Three preparations of LDL_1 were parallel to the standard curve over a 60-fold range and each contained 0.76 mg of immunoassayable apoB per mg protein. Reacting less well, and not completely parallel to the LDL_2 standard curve, was VLDL which contained an average of 0.32 mg of immunoassayable apoB per mg of protein (Fig. 1*A*). A mixture of the C-apoproteins, isolated from normolipidemic VLDL, did not react when added in a 5,900-fold excess.

The immunoreactivity of HDL isolated from the same monkeys was 0.06%. This degree of reactivity would represent less than one molecule of apoB per HDL molecule and therefore is consistent with contamination of HDL with very small amounts of LDL.

Immunoreactivity of fresh serum and plasma

Illustrated in Fig. 1*B* are dilution curves of fresh serum and plasma obtained from separate normolipidemic monkeys. Both serum and plasma were parallel to the LDL_2 standard (Fig. 1*B*). Comparison of the concentration of apoB in three sera vs. the corresponding plasma samples gave a ratio of 0.955 ± 0.021.

TABLE 1. Comparison of the logit-log slopes of LDL_2 standard curves, normolipidemic and hyperlipidemic sera[a]

	LDL_2		Normolipidemic Sera		Hyperlipidemic Sera[b]	
	B/Bo > 0.30	B/Bo < 0.20	B/Bo > 0.30	B/Bo < 0.20	B/Bo > 0.30	B/Bo < 0.20
	−1.0752	−0.9410	−1.0578	n.d.[c]	−1.0943	−0.7979
	−1.0934	−1.2258	−1.0587	n.d.	−1.0327	−0.7951
	−1.0195	−1.0073	−0.9959	n.d.	−1.0040	−0.8040
	−1.0746	−0.8945	−1.0103	n.d.	−1.1275	n.d.
	−1.0108	−0.7211			−0.8964	n.d.
					−1.0141	−0.8175
					−1.0643	n.d.
Mean ± SD	−1.0547 ± 0.0370[d,f]	−0.9579 ± 0.1834[e,f]	−1.0307 ± 0.0324[d]		−1.0333 ± 0.0747[d,g]	−0.8036 ± 0.0100[e,g]

[a] Normo- and hyperlipidemic sera were stored frozen (−20°C) and thawed several times.
[b] The first two sera are from monkeys fed an "average American" diet, the next three sera from monkeys fed the peanut oil diet and the last two sera from monkeys fed the coconut oil diet (see Methods).
[c] n.d., Not determined because the bend occurred towards the end of the standard curve and too few points were available for meaningful statistical analysis.
[d] NS upper region (B/Bo > 0.30): LDL_2 vs. normolipidemic sera and LDL_2 vs. hyperlipidemic sera.
[e] NS lower region (B/Bo < 0.20): LDL_2 vs. hyperlipidemic sera.
[f] NS upper region vs. lower region: LDL_2.
[g] $P < 0.001$ upper region vs. lower region: hyperlipidemic sera.

Logit transformation

The displacement curves of LDL_2, LDL_1, VLDL, and plasma and sera from normolipidemic monkeys were analyzed by logit-log transformation. This transformation should approximate a straight line when a single species of antibody reacts with a single antigen (20). The logit-log curves for LDL_2, LDL_1, VLDL, and plasma were resolved into two linear segments with the bend occurring between B/Bo 0.30 and 0.20. Because of the importance of ensuring that the slopes of the sera and LDL_2 standards were parallel, four normolipidemic and seven hyperlipidemic plasma samples that had been stored frozen (−20°C) for 5 months and thawed several times, were assayed in multiple dilutions. The dilution curves were compared with the LDL_2 standard curves by weighted regression analysis. The slopes of the standards and plasma did not differ in the upper [−1.0547 (LDL_2) vs. −1.0307 (normolipidemic) vs. −1.0333 (hyperlipidemic)] or the lower [−0.9579 (LDL_2) vs. −0.8036 (hyperlipidemic)] segments of the curves (**Table 1**).

Assay variability

The within-assay variation ranged from 13% in the region B/Bo 0.85–0.75 to 6% at B/Bo 0.50–0.35. The between-assay variation was 5.0%.

Validity of the apoB assay

Measurement of apoB in sera using ^{125}I-LDL_2 labeled by either the chloramine-T or iodine monochloride method. Parallel assays were performed using ^{125}I-LDL preparations labeled with chloramine-T and iodine monochloride, respectively. Normo- and hyperlipidemic sera were diluted over a 7-fold range and compared to the LDL_2 standard. The sera were parallel to the standards and their absolute values were similar in the two assays (ratio 0.94 ± 0.029).

Recovery of LDL_2 in plasma. Addition of LDL_2 (0.05–0.10 μg) to varying amounts of plasma (0.1–0.3 ml) resulted in recoveries of 104 ± 2% (n = 12).

Stability of apoB in serum and plasma. Normo- and hyperlipidemic sera and plasma samples were collected and assayed immediately (n = 8). Aliquots were then stored at −20°C for 3 weeks and thawed just prior to being diluted for the assay. A comparison of the immunoassayable apoB in the fresh and frozen and thawed sera and plasma samples gave an average ratio of 1.06 ± 0.09 (**Table 2**). In another experiment two normolipidemic plasma samples were assayed immediately (53.3 and 30.1 mg apoB/dl, respectively) and were then frozen (−20°C) and thawed seven times prior to assay over a 1½ year period (apoB range 50.4–57.3 and 29.6–33.9 mg/dl, respectively). The mean ± SD of the apoB values were 53.7 ± 0.02 and 31.8 ± 0.02 mg/dl and this amount of variation (4.1 and 5.0%, respectively) was within the between-assay variation. Similarly, for two hyperlipidemic sera that were frozen (−20°C), thawed, and assayed four times during a 1½ year period, the apoB value varied by 3.0%. Freezing and thawing sera or plasma did not alter the parallelism of immunoassayable apoB in the normolipidemic or hyperlipidemic samples relative to the LDL_2 standard (Table 1).

Plasma and sera were taken from normolipidemic and hyperlipidemic fasting monkeys (n = 13) on two occasions separated by at least 1 month. A comparison

TABLE 2. Comparison of apoB concentration in fresh and frozen sera and plasma

Sample No.	Sera[a]			Plasma[a]		
	Fresh	Frozen	Fresh / Frozen	Fresh	Frozen	Fresh / Frozen
	mg/dl			*mg/dl*		
6	96.6	95.8	1.01			
7	68.3	54.1	1.26			
1	41.3	42.2	0.98	43.1	43.0	1.00
2	35.3	31.7	1.11	35.7	35.3	1.01
3	45.2	41.7	1.08	49.3	47.1	1.05
Mean ± SD			1.09 ± 0.11[b]			1.02 ± 0.03[b]

[a] Sera and plasma samples were collected and aliquots were assayed immediately. Another aliquot was frozen (−20°C) for 3 weeks. Both sera and plasma were diluted 1:1000 in the borate–albumin buffer and five aliquots were assayed in duplicate over a three-fold range.
[b] The average ratio (fresh/frozen) for all the sera and plasma samples is 1.06 ± 0.09.

of the sera LDL values from the first and second bleedings gave a ratio of 0.97 ± 0.14.

ApoB and lipid concentrations in sera

Normolipidemic monkeys. The total serum cholesterol was 146 ± 8 mg/dl, triglyceride 44 ± 7 and phospholipid 223 ± 7 in the 13 healthy, male monkeys maintained on the PMC diet (**Table 3**). The apoB level was 50 ± 5 mg/dl (range 32–97) (Table 3).

Hyperlipidemic monkeys. Displacement curves of sera taken from monkeys on the three atherogenic diets were parallel to the LDL_2 standard (Table 1). In addition, LDL_2 isolated from the plasma of monkeys on the "average American" and coconut oil diets gave displacement curves that were parallel to the LDL_2 standard.

In monkeys eating the three atherogenic diets, serum apoB and total cholesterol levels were elevated relative to controls ($P < 0.001$, Table 3). On each of the diets, total serum cholesterol correlated with serum apoB ($P < 0.001$). The slopes of serum apoB versus total cholesterol in monkeys on the PMC diet ($m = 0.531$), average American ($m = 0.401$) and coconut oil ($m = 0.359$) diet were similar, but differed markedly from those on the peanut oil diet ($m = 0.121$) (**Fig. 2**).

Comparison of immunoreactivity of monkey and human apoB in intact LDL and isolated apoB

Monkey and human LDL. The immunoreactivities of monkey and human LDL were compared using antisera raised against these molecules. Monkey LDL cross-reacted poorly in the system using the human LDL antiserum and human ^{125}I-LDL (**Fig. 3*A***). With monkey LDL antiserum and monkey ^{125}I-LDL as the tracer, there was a greater degree of cross-reactivity between monkey and human LDL than in the homologous human system (**Fig. 4*A***), but the curves were not parallel. Substituting human ^{125}I-LDL for the monkey label improved the cross-reactivity of these two species of LDL (**Fig. 4*B***). A heterologous assay using human LDL antiserum with monkey ^{125}I-LDL resulted in identical displacement curves for monkey and human LDL (**Fig. 3*B***).

TABLE 3. ApoB and lipid levels in monkey sera and plasma

Group (n)	apoB	Total Cholesterol	Triglycerides	Phospholipids
		mg/dl (m ± SD)		
Normolipidemics (13)	50 ± 18[a]	146 ± 28[b]	44 ± 26[g]	223 ± 27
Hyperlipidemics				
1. Average American diet (4)	103 ± 28[c,d]	333 ± 65[e,f]	16 ± 2	
2. Peanut oil diet (19)	102 ± 35[c,d]	606 ± 212[e]	8 ± 9[g]	
3. Coconut oil diet (5)	312 ± 88[c]	864 ± 233[e,f]	43 ± 19[g]	360 ± 72

[a] $P < 0.001$ serum apoB: normolipidemics vs. hyperlipidemics.
[b] $P < 0.001$ serum total cholesterol: normolipidemics vs. hyperlipidemics.
[c] $P < 0.005$ serum apoB: coconut oil vs. American and coconut oil vs. peanut oil.
[d] NS serum apoB: American vs. peanut oil.
[e] NS serum total cholesterol: peanut oil vs. American and peanut oil vs. coconut oil.
[f] $P < 0.005$ serum total cholesterol: coconut oil vs. American.
[g] $P < 0.001$ serum triglycerides: peanut oil vs. normolipidemics and peanut oil vs. coconut oil.

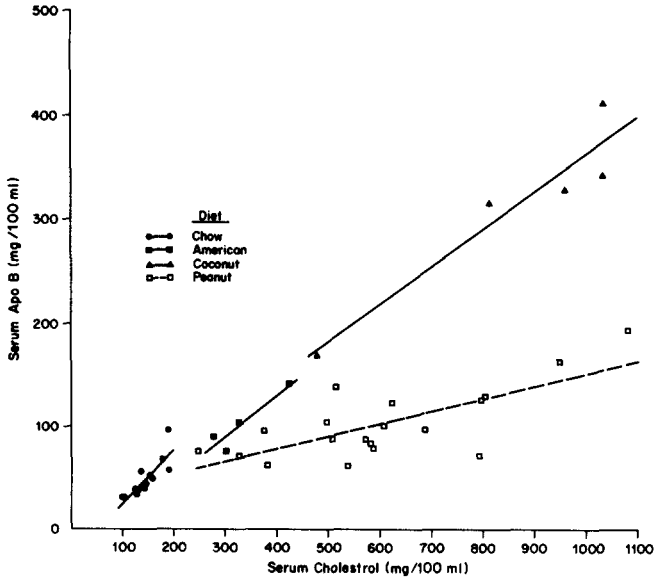

Fig. 2. The relationship between serum total apoB and total cholesterol concentration for monkeys maintained on the chow (● —— ●), average American (■ —— ■), coconut oil (▲ —— ▲), and peanut oil (□ —— □) diets.

Monkey and human apoB. Antisera were also raised against purified apoB isolated from the LDL of these two species. There was appreciable cross-reactivity between monkey and human apoB in systems using either monkey apoB antiserum with monkey ^{125}I-apoB or human apoB antiserum with human ^{125}I-apoB (**Fig. 5*A***). In both homologous assays, monkey and human apoB displacement curves were not parallel. Substituting monkey ^{125}I-apoB for the human label with the human apoB antiserum further improved the cross-reactivity of these two species of apoB (**Fig. 5*B***).

DISCUSSION

The radioimmunoassay

The assay described for monkey LDL is reproducible, specific, and sensitive for measuring apoB in whole serum and in isolated low density lipoproteins. The method is similar to those developed for measuring apoB in rat (21) and human sera[4] (22–24). We compared the apoB value in sera using the ^{125}I-LDL prepared by either the modified chloramine-T method of Frechet and Roth (25) or the iodine monochloride method of McFarlane (26). Though the amounts of free iodide and degree of lipid labeling were greater with the chloramine-T method, this did not alter the parallelism of dilutions of sera to the standard nor the absolute concentration of serum apoB measured in the radioimmunoassay. It thus appears that both iodination methods are adequate for labeling LDL_2 for use in this assay.

Analysis of the logit-log plots of the displacement curves of the LDL_2 standard and normo- and hyperlipidemic sera has important implications for the long-term goal of automating the assay procedure. Most computer programs for calculating radioimmunoassay results utilize this transformation. The logit-log lines for standards and serum dilutions derived from points over the range B/Bo 1.0–0.30 are parallel and give values for unknown samples that are identical to those obtained by hand calculation from direct plots

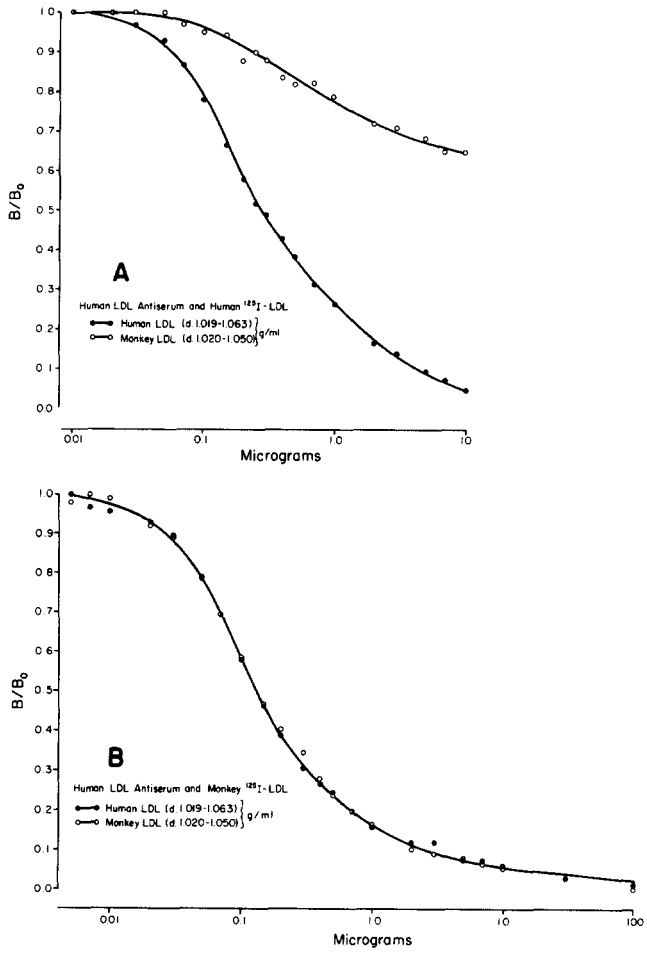

Fig. 3. (*A*) Displacement of ^{125}I-labeled human LDL from anti-human LDL antibodies (1:50,000, final dilution) by human LDL (● —— ●) and monkey LDL (○ —— ○). (*B*) Displacement of ^{125}I-labeled monkey LDL_2 from anti-human LDL antibodies (1:50,000, final dilution) by human LDL (● —— ●) and monkey LDL (○ —— ○). Protein concentrations were determined by the method of Lowry et al. (13). Stock solutions of each lipoprotein containing 0.01, 0.1, 1.0, 10, and 100 μg protein/ml were prepared in the borate–albumin EDTA buffer. Aliquots of 0.1 ml to 1.0 ml were assayed in duplicate.

[4] Karlin, J. B., D. J. Juhn, A. M. Scanu, and A. H. Rubenstein. Measurement of human low density lipoprotein apolipoprotein B in serum by radioimmunoassay. Unpublished data.

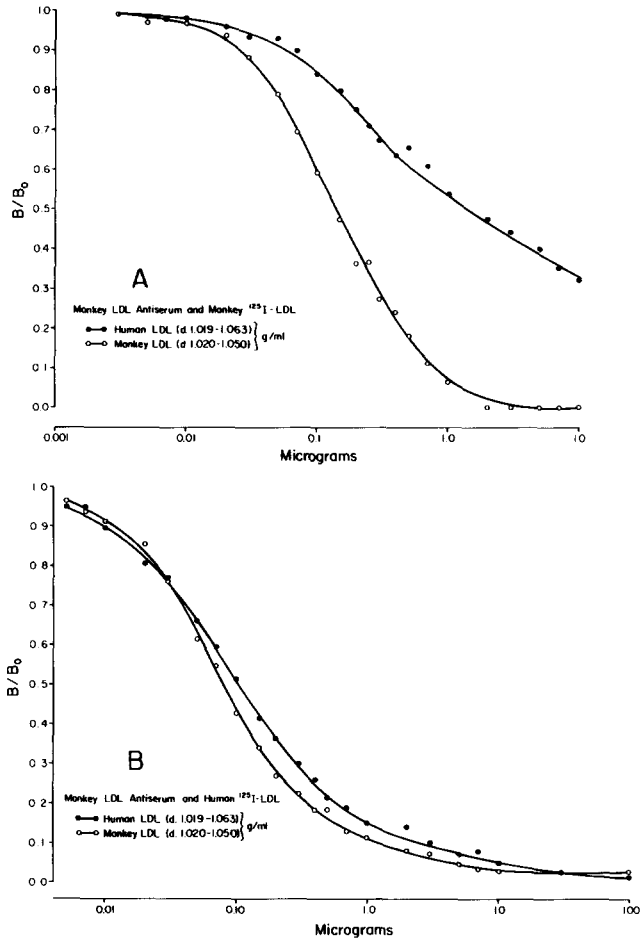

Fig. 4. (*A*) Displacement of ^{125}I-labeled monkey LDL from anti-monkey LDL antibodies (1:50,000, final dilution) by human LDL (● —— ●) and monkey LDL (○ —— ○). (*B*) Displacement of ^{125}I-labeled human LDL from anti-monkey LDL antibodies (1:50,000 final dilution) by human LDL (● —— ●) and monkey LDL (○ —— ○). Protein concentrations were determined by the method of Lowry et al. (13) and the stock solutions were prepared as described in Fig. 3.

of the data. On the other hand, there is considerable between-assay variation below B/Bo < 0.20 where the slope changes. Analysis of the logit-log plots of the displacement curves of isolated lipoproteins and sera is also useful for detecting small but potentially important differences in the immunogenicity of LDL. Thus far displacement curves of seven sera from monkeys on the three atherogenic diets have been examined. In the region B/Bo > 0.30, one serum from a monkey fed the peanut oil diet had a slope of $m = -0.8964$ which was lower than that of the other six hyperlipidemic sera (average $m = -1.0562$) and the LDL_2 standard (average $m = -1.0547$). Though these results are preliminary, they do illustrate the utility of the logit-log plots in the qualitative analysis of apoB and indicate that LDL in monkeys on different diets may not be immunochemically homogenous.

Because apoB in both normo and hyperlipidemic sera retains its full immunogenicity in frozen sera stored at −20°C, this assay may be conveniently adapted for routine use. Of interest is the stability of the apoB values in normolipidemic plasma and sera samples as well as hyperlipidemic sera samples which were frozen and thawed at least four times over a 1½ year period. In addition the apoB value in the control sera did not vary significantly when read against an LDL_2 standard (average protein concentration 5.0 mg/ml) which had been stored for at least 6 weeks under nitrogen in the dark at 4°C. In a preliminary experiment, an LDL_2 standard (protein concentration 7.9

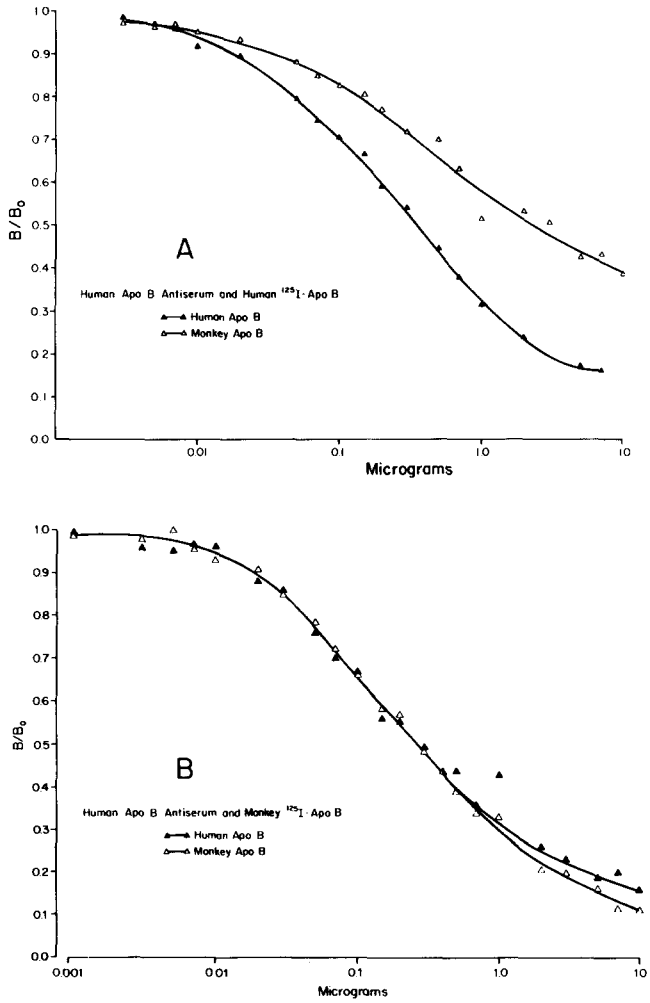

Fig. 5. (*A*) Displacement of ^{125}I-labeled human apoB from anti-human apoB antibodies (1:100,000 final dilution) by human apoB (▲ —— ▲) and monkey apoB (△ —— △). (*B*) Displacement of ^{125}I-labeled monkey apoB from anti-human apoB antibodies (1:100,000 final dilution) by human apoB (▲ —— ▲) and monkey apoB (△ —— △). Protein concentrations were determined by the method of Lowry et al. (13). Stock solutions containing 0.01–100 μg protein/ml were prepared in the glycine–albumin buffer, pH 10. Aliquots of 0.1 ml were added to 0.9 ml of borate–albumin buffer, pH 8.0 and were assayed in duplicate.

mg/ml) was stored at −20°C under nitrogen for 5 days and its displacement curve was indistinguishable for the same standard stored at 4°C under nitrogen. The ^{125}I-LDL_2, stored at 4°C, can be used in the assay for at least one month.

As in the case of human apoB[4] and apoA-I assays (11), appropriate dilutions of serum can first be made using automated pipetting equipment and stored for 24 hr at 4°C. The assay can then be set up using the same equipment. The ease of preparing the samples and the use of a computer program for calculating the results permit one person to process approximately 500 serum samples in triplicate each week.

ApoB value in serum

This is the first study in which the apoB concentration in sera of rhesus monkeys has been measured. In 13 fasting, healthy male monkeys maintained on a laboratory PMC diet, the serum apoB level was 50 ± 18 mg/dl (range 32–97). Assuming that 90–95% of apoB is in LDL_2, and a protein:lipid ratio of 24:76 (5, 28), the calculated mean LDL_2 concentration would be 197 mg/dl (range 125–380). This value is in reasonable agreement with the determination of LDL_2 (125 and 174 mg/dl) obtained by measuring the cholesterol distribution in serum and LDL_2 cholesterol content in two normolipidemic rhesus monkeys (27).

The total serum cholesterol (146 ± 28 mg/dl) and triglyceride (44 ± 26 mg/dl) concentrations in the normolipidemic rhesus monkeys are lower than that reported for normolipidemic human subjects (22, 24). The appreciably lower serum triglyceride level is reflected in the small amounts of VLDL that can be isolated from these sera. Based on the isolation of VLDL from plasmaphoresed monkeys, the VLDL protein content is estimated to be between 0.5 and 1.0 mg/dl. Because of the small number of normolipidemic monkeys in our colony and the very low concentrations of VLDL present in their sera, we were unable to isolate sufficient VLDL to be able to compare the apoB mass of VLDL, as determined by radioimmunoassay, with that quantitated by column chromatography (22) and/or tetramethyl urea precipitation (23). Consequently we have not yet been able to determine whether or not there is masking of the apoB determinants in triglyceride-rich particles. As more monkeys become available for study, we will attempt to devise experiments that will resolve this question. On the other hand preliminary experiments showed that the immunoassayable apoB and Lowry protein values of LDL_2 isolated from hyperlipidemic sera from monkeys fed the coconut oil diet were comparable. These results together with the observation that the VLDL content in both the normolipidemic and hyperlipidemic monkeys' sera is very low (Table 3) suggest that the direct measurement of apoB by radioimmunoassay is accurate.

The increase in serum apoB and cholesterol levels in monkeys eating the three atherogenic diets compared to the controls fed chow is consistent with previous reports (1). The slopes of the regression lines of serum apoB vs. cholesterol for the monkeys on the PMC, average American, and coconut oil diets were similar and differed from that for monkeys on the peanut oil diet (Fig. 2). This finding indicates a dissociation between the increase in serum cholesterol and apoB in monkeys fed various atherogenic diets and suggests that the composition of the lipoprotein particles containing apoB may vary significantly depending on the particular diet taken.

Comparison of immunoreactivity of monkey and human LDL

Studies in which monkey and human LDL were analyzed in homologous assay systems indicate that these two species of LDL have unique immunologic determinants. This result was confirmed in experiments comparing the immunoreactivities of isolated apoBs from these two species with antisera raised against monkey and human apoB. The lack of complete immunological identity between monkey and human LDL (apoB) is consistent with the results of Bautovich (24) and with the differences in the physicochemical properties noted in these molecules (5). However, the crossover studies using a heterologous tracer with each antiserum resulted in the selection of a specific population of antibodies directed against antigenic sites shared by the two species of LDL (apoB). One of the implications of these findings is the potential for purifying antibodies with defined immunological specificities from these antisera. These antibodies could then be used as probes for studying the physicochemical properties of isolated and reconstituted lipoproteins (28).

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