A NEW SERUM LIPOPROTEIN FOUND IN MANY RHESUS MONKEYS

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Summary: A new serum lipoprotein was found in about 10 out of 30 rhesus monkeys (Macaca mulatta) which contained 28% by weight of protein, 42% total cholesterol, 22% phospholipid, and 8% triglyceride. This is in contrast to LDL (which the ten monkeys also contained) which had 24% protein, 46% total cholesterol, 24% phospholipid and 7% triglyceride. An S_f, 1.063 in KBr of 3.0 to 3.7 and molecular weight of 3.5-3.7 million were observed compared to means of 8.1 and 3.0 million for normal rhesus LDL. The lower S_f was caused by its higher density. This new lipoprotein was most easily demonstrated and isolated by preparative ultracentrifugation of all serum lipoproteins at density 1.22 g/ml, followed by 6% agarose gel filtration at 6°. The new lipoprotein appeared as a distinct peak eluting before LDL.

Rhesus monkey (Macaca mulatta) LDL_2 has been the topic of recent detailed studies by several groups including ourselves (1,2,3).[2] Our studies are aimed at the effects of high cholesterol diets on the LDL chemical and physical alterations and their relation to the development of atherosclerosis. For the past four years, we have used a method of lipoprotein isolation which separates lipoproteins according to their molecular size (4). This procedure involves flotation of total serum lipoproteins at density 1.22 g/ml, followed by gel filtration on 6% agarose columns which separates the lipoproteins into the three major fractions, VLDL, LDL, and HDL. Application of this method for separation of normal (chow-fed) rhesus monkey serum lipoproteins revealed gross heterogeneity in the LDL for many monkeys. About one-third of our rhesus monkeys show some peak or shoulder lipoprotein eluting after the VLDL, but before the major LDL_2 peak. We have purified this new component and have found it of

1. To whom reprint requests should be submitted.
2. Abbreviations used are: VLDL, very low density lipoproteins; LDL and LDL_2, low density lipoproteins; HDL, high density lipoproteins; S_f, the flotation rate in the density given using KBr as the salt; PMSF, phenylmethylsulfonyl fluoride.

higher molecular weight, lower $S_{f,1.063}$, higher % protein, and a lower % cholesterol than LDL_2.

Materials and Methods: Serum preparation - The monkeys were cared for as described previously (1). The preparation of serum lipoproteins from fasted animals was also described in that paper. Lately, all samples have been treated with PMSF immediately to prevent proteolysis (5). However, we found no gross difference in lipoproteins with or without PMSF.

Lipoprotein isolation - Pure VLDL, the two forms of LDL, and HDL were isolated by the method of Rudel, et.al. (4), which involved flotation of the lipoproteins through a layer of pure solvent at density 1.22 in a swinging bucket rotor to remove albumin. The lipoproteins were then fractionated on a 2.4 x 85 cm column of 6% agarose (Bio-Gel 5m, Bio-Rad Labs) at 6°. The flow rate was 10 ml/hr, collecting 5 ml fractions.

The lipoproteins were pure as judged by agarose electrophoresis (6), immunoelectrophoresis (7), and analytical ultracentrifugation at density 1.063 or 1.2 g/ml.

Analytical procedures - Total cholesterol was measured according to the procedure of Rudel and Morris (8), triglycerides by the method of Sardesai and Manning (9), phospholipids by the method of Fiske and Subbarow (10), and protein by the method of Hartree (11) using bovine serum albumin as a standard, while standardizing the stock solution according to its absorbancy of a 1% solution of 6.67 at 280 nm.

Internal radioactive LDL standard - ^{125}I labeled LDL was obtained from Dr. L. L. Rudel of the Department of Comparative Medicine, Bowman Gray, Winston Salem, N.C. This was a narrow molecular weight, highly radioactive rhesus LDL which was included in many samples as an internal standard in the elution from the 6% agarose columns. The ratio of the elution volume of the sample LDL to that of the standard radioactive LDL was a sensitive indicator of the relative size of the sample LDL.

S_f - High salt flotation velocity experiments were carried out similar to methods used by Fisher et.al. (12) after the samples were concentrated by vacuum dialysis. Prior to analytical ultracentrifugation, the samples were dialyzed overnight vs. KBr[3] solutions containing 10^{-4} M EDTA and 0.01 g/100 ml azide at pH 7.4. The density of the solvent was determined either with a Westphal balance or by pycnometry. A double sector cell was filled with dialysate on one side and dialyzed lipoprotein on the other. An identical wedge cell contained another lipoprotein sample so that two solutions of lipoproteins could be run simultaneously. Flotation was carried out at 25° at speeds of 40,000 or 42,040 rpm in a Beckman/Spinco Model E analytical ultracentrifuge. Photographs were taken at known intervals and flotation rates determined by standard procedures. Partial specific volumes were determined from the variation of S_f with solvent density (12).

Molecular weight - Flotation equilibrium was carried out according to the methods previously described (13). A solvent of density 1.30 to 1.32 was used with and without fluorocarbon as a base fluid. No difference was found in the molecular weight obtained indicating the lipoprotein was not solubilized by the fluorocarbon as we suggested might be possible (13).

Results: Occurrance - Rhesus monkey serum lipoproteins isolated at density

3. KBr was used since its solvents have only small viscosity differences from water, whereas NaCl solvents have a much larger viscosity. In KBr, very little concentration dependence of the flotation rate (S_f) was observed in density 1.063 g/ml much as we and others (12) have found in density 1.2 g/ml. This is in contrast to a large concentration dependence of S_f in NaCl solvents (2).

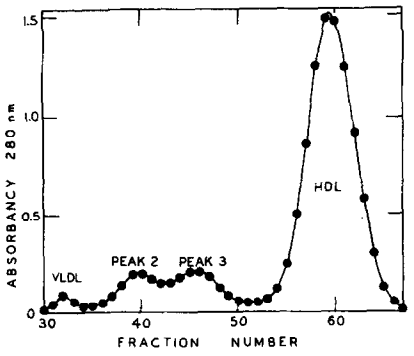

Fig. 1. The elution pattern from a 2.4 x 85 cm column of 6% agarose of all serum lipoproteins floating at density 1.22 g/ml. The serum (5 ml) was from monkey A22 whose serum total cholesterol was 151 mg/dl. The eluent was 0.15 M NaCl, 10^{-3} M EDTA at pH 7.4, eluting at a flow rate of 10 ml/hr at 6^{o}.

1.22 g/ml often elute from 6% agarose columns as four peaks rather than three (VLDL, LDL, and HDL). The additional peak (peak 2) was observed between the VLDL and the LDL peak as seen in Fig. 1. Rudel and Lofland also have noticed this phenomenon (3). About a third of the monkeys we have studied exhibited this additional peak 2 and the pattern was reproducible for each individual monkey studied at any time of the year, except for some variation in the amount of HDL. In all the monkeys with peak 2 lipoproteins, the amount of peak 3 was always in highest concentration, but the ratio of peak 2 to peak 3 varied with the individual monkey. We have studied two monkeys intensively, A22 and A31, in which the amount was nearly equivalent to peak 3. Their lipoprotein agarose column elution patterns were almost identical.

Chemical composition - The composition of peak 2 lipoproteins of monkey A22 or an additional five monkeys with peak 2 was different from their peak 3 lipoproteins and from the mean of LDL from 20 other monkeys (Table 1). The % protein of peak 2 lipoproteins was 28% compared to 24% for peak 3, while the total cholesterol was 42% in peak 2 compared to 46% in peak 3. Peak 3 lipoproteins had compositions more nearly like adult human LDL and identical with rhesus monkey LDL (Table 1).

Table 1

% Composition by Weight of Peak 2 and Peak 3 Lipoproteins

Lipoprotein	Pro	TC	PL	TG	% of serum cholesterol
Peak 2 (A22)	28.5	44.5	20	7	13
Peak 2 (N=5)	28	42	22	8	20
Peak 3 (A22)	23.5	47.5	23	6	20
Peak 3 (N=20)	24	46	24	7	33

Pro = protein; TC = total cholesterol; PL = phospholipid; TG = triglyceride

Table 2

Physical Properties of Peak 2 and Peak 3 Lipoproteins

Lipoprotein fraction	$S_{f,1.063}$		$S_{f,\ 1.2}$		MWx10^{-6}		$\bar{v}$ ml/g		LDL/^{125}I-LDL	
	A22	A31	A22	A31	A22	A31	A22	A31	A22	A31
Peak 2 Asc. Limb	3.6	4.5								
Peak 2 Top	3.0	3.7	35	36	3.5	3.7	0.946	0.952	0.871	0.881
Peak 2 Des. Limb	3.1	3.4								
Peak 3 Asc. Limb	7.6	8.9								
Peak 3 Top	6.6	8.5	34		2.7	3.1	0.962		1.013	1.004
Peak 3 Des. Limb	5.5	8.5								
LDL (mean from 19 animals not having Peak 2)	8.1 ± 0.6				3.0 ± 0.3		0.965		0.990±0.015	

Asc.,Top, and Des. refer to pooled fractions from 6% agarose gel filtration from the early eluting third of the peak, those having the highest absorbancy, and the late eluting third of the peak. The standard deviations are shown for multiple determinations. LDL/^{125}I-LDL is the ratio of the elution volume of Peak 2 or Peak 3 to the peak of ^{125}I-LDL.

Physical properties - Flotation velocity patterns of pure peak 2 lipoproteins and peak 3 lipoproteins are shown in Fig. 2 (left). On the right in Fig. 2, are impure lipoproteins which were obtained from adjacent agarose

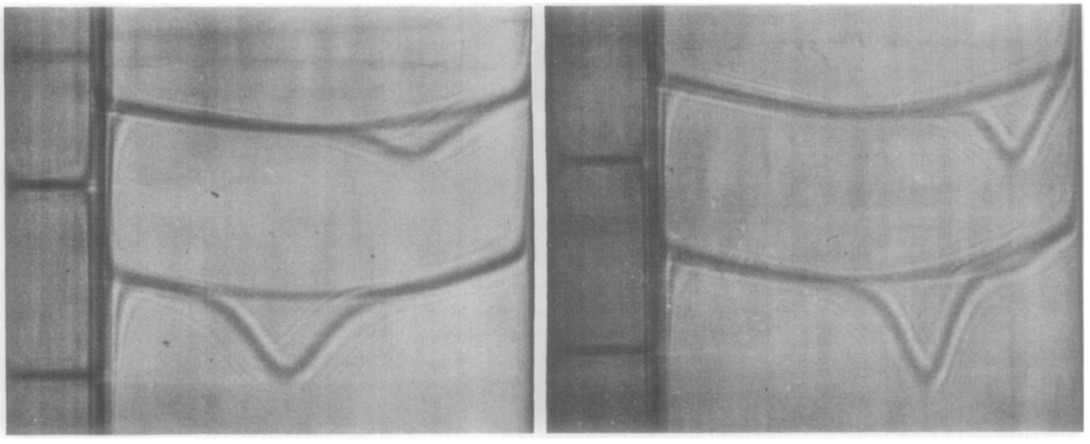

Fig. 2. The flotation velocity schlieren patterns of center agarose gel filtration fractions of Peak 2 (upper) and Peak 3 (lower) LDL on the left, and on the right, the descending gel filtration limb of Peak 2 (upper) and the ascending gel filtration limb of Peak 3 (lower) from monkey A31, whose lipoprotein agarose pattern was identical to that of monkey A22 shown in Fig. 1. The fractions at the trough between Peaks 2 and 3 were discarded. The density was 1.063 g/ml, and the pictures were taken 88 and 104 min, respectively at 25°. Flotation is from right to left, and the bar angle was 60°.

fractions of each peak. The $S_{f,1.063}$ of peak 2 lipoproteins was 3.0-3.7, which is roughly half of peak 3 lipoproteins (6.6-8.5) (Table 2). The lipoprotein density is greater for peak 2 lipoproteins due to their increased protein and decreased lipid content.

The reciprocal of the peak 2 lipoprotein density ($\bar{v}$) was also measured and is shown in Table 2. It was 0.95 ml/g while monkey LDL was 0.965. Since S_f is directly proportional to $(1-\bar{v}p)$ where p is the solvent density, this difference at p = 1.063 g/ml is -0.010 for peak 2 lipoproteins and -0.026 for rhesus LDL (peak 3). This predicts a 2.6 times slower rate for peak 2 lipoproteins if the molecular weight were the same. However, the molecular weight for peak 2 lipoproteins was about 700,000 higher than peak 2 lipoproteins from the same animal (Table 2). A size determination by ^{125}I-LDL revealed a larger size of peak 2 lipoproteins on each occasion as compared to normal LDL (Table 2).

Discussion: In this paper we describe the isolation and characterization of a new lipoprotein which, though similar, is easily distinguished from previously identified LDL. Evidence for its existence as a distinct species of LDL is provided by its persistence in the serum of certain monkeys over at least a 2 year period and a relatively constant quantitative relation to the normal LDL_2. For example, in monkey A22, this lipoprotein has been found in five separate serum samples and constituted about 65% as much cholesterol as was present in the LDL_2 on each occasion. The higher molecular weight and lower S_f rate also distinguished it from LDL_2. The chemical composition of this lipoprotein was also rather constant.

Rudel and Lofland have also observed this lipoprotein (3). However, the protein content of their lipoprotein was equal to or lower than normal LDL_2. Although these investigators did not study the S_f, because of the lower protein content, a slower S_f of the peak 2 compared to ours at density 1.063 g/ml would not have been observed. However, a higher molecular weight was found since the position of elution from agarose gel columns was similar to the peak 2 lipoproteins. The difference in chemical composition of our peak 2 lipoprotein and their lipoproteins might be due to the difference in dietary composition since Rudel and Lofland fed a control diet consisting of 45% of total calories as fat, while our diet was 10% of the total.

The relationship of this lipoprotein to the metabolism of VLDL and LDL in those rhesus monkeys, where it is found, is unclear. It could be a normal catabolic intermediate in all monkeys which accumulates only in certain animals because of a degradative limitation or it could be a genetically determined abnormal lipoprotein.

The new peak 2 lipoprotein is unlike LDL_1 of the human since the peak 2 lipoprotein and cholesterol content were higher than LDL_1, the triglyceride lower, and the flotation rate was only 3-4 compared to 12-20 for LDL_1. However, both peak 2 lipoprotein and LDL_1 have a higher molecular weight than

LDL_2. While it is possibly of genetic origin, we have not noted any striking evidence to this effect in siblings or offspring of those monkeys having this protein.

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