Properties of triglyceride-rich and cholesterol-rich lipoproteins in the remnant-like particle fraction of human blood plasma

Elisa Campos,[1,*] Leila Kotite,[*] Patricia Blanche,[§] Yasushi Mitsugi,[2,*] Philip H. Frost,[†] Umesh Masharani,[†] Ronald M. Krauss,[§] and Richard J. Havel[3,*]

Cardiovascular Research Institute,* Department of Medicine,† University of California San Francisco, 505 Parnassus Avenue, Rm L-1337, San Francisco, CA; and Lawrence Berkeley Laboratory,§ University of California Berkeley, Berkeley, CA

Abstract **An immunoassay procedure that quantifies remnant-like particle (RLP) cholesterol in human blood plasma has shown considerable promise as a clinically applicable risk marker for atherosclerotic disease. The lipoproteins included in this assay include not only certain TG-rich lipoproteins [all particles containing apolipoprotein B-48 (apoB-48) and a fraction of those containing apoB-100] but also a very small proportion of plasma cholesterol-rich lipoproteins. The TG-rich lipoprotein component of RLP has been partially characterized, but relatively little is known about the component cholesterol-rich lipoproteins. We have further characterized the properties of the TG-rich component that is included in RLP in which about 25% of the particles contain apoB-48 and the remainder apoB-100. We show that the cholesterol-rich component is comprised mainly of β-migrating LDLs that contain predominantly apoB-100. ApoE found in the LDL fraction of RLP resides on pre-β lipoproteins that lack apoA-I as well as apoB. The TG-rich component of RLP is responsible for increased RLP-cholesterol concentrations associated with hypertriglyceridemia. By contrast, the cholesterol-rich component is a major contributor to plasma RLP-cholesterol in individuals with low plasma TG. Our results suggest that particle heterogeneity in the RLP fraction is likely to affect the ability of RLP-cholesterol concentration to predict atherosclerotic risk. RLP-cholesterol concentrations in individuals with low plasma TG may not have the same clinical significance as they do in those with hypertriglyceridemia.**—Campos, E., L. Kotite, P. Blanche, Y. Mitsugi, P. H. Frost, U. Masharani, R. M. Krauss, and R. J. Havel. **Properties of triglyceride-rich and cholesterol-rich lipoproteins in the remnant-like particle fraction of human blood plasma.** ***J. Lipid Res.*** **2002.** 43: **365–374.**

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Following the original characterization of a fraction of human TG-rich lipoproteins (TRL) that fails to bind to a monoclonal antibody (Mab) to apolipoprotein B (apoB) (JI-H) as remnant-like lipoproteins (1), a facile procedure was developed to quantify cholesterol and TG in remnant-like particles as remnant-like particle cholesterol (RLP-C) and RLP-TG in blood plasma (2). Since that time, a considerable body of literature has supported the utility of RLP-C concentration as a risk marker for atherosclerotic disease (3). It has been recognized, however, that the test for RLP-C in particular includes not only the described fraction of TRL, but also certain lipoproteins of higher density (2, 4–6).

The epitope recognized by Mab JI-H is located just distal to the C-terminus of apoB-48 (4), so that all lipoproteins containing apolipoprotein B-48 (apoB-48) are included in RLP (1). In addition, a minor but significant fraction of TRL containing apoB-100 is also included. By contrast, more than 97% of the cholesterol in lipoproteins other than TRL in plasma from normolipidemic subjects is retained by the Mabs (JI-H and anti-apoA-I) that are utilized in the RLP test (4). In plasma from subjects with low TRL concentrations, however, these latter lipoproteins may comprise a major fraction of RLP (2, 4–6). Recent studies have shown that most non-TRL in the RLP fraction of plasma are of LDL size with relatively little contribution from HDL-sized particles (5, 6). As separated by gel filtration chromatography, the LDL-sized lipoproteins in the RLP fraction are enriched in apoE and apoC-III as well as apoB-100 (5), and are slightly larger on average than bulk LDL (6). These and other observations have led to the suggestions that RLP-LDL have special properties (5) and may represent LDL-sized remnant lipoproteins (6).

In the current study, we have evaluated the concentra-

Abbreviations: RLP, remnant-like particle(s); TRL, TG-rich-lipoprotein(s); GGE, gradient gel electrophoresis; LTG, low TG; HTG, high TG.

[1] Present address: Department of Biochemistry, Faculty of Medical Sciences, New University of Lisbon, 1169-056 Lisbon, Portugal.

[2] Present address: Department of Cardiology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan.

[3] To whom correspondence should be addressed.
e-mail: havelr@itsa.ucsf.edu

tion and lipid composition of RLP in plasma from normolipidemic and hyperlipidemic non-diabetic and diabetic patients. Our findings in these patients led us to undertake detailed characterization of the TG-rich and cholesterol-rich lipoproteins that do not bind Mab JI-H in individuals with varying plasma TG concentrations. For this purpose, we selected healthy subjects known to express lipoprotein phenotypes A and B, whose LDL exhibits distinctive properties (7). Our results confirm and extend previous data on the special properties of the TRL components of RLP and show that most LDL-sized RLP particles resemble bulk LDL.

MATERIALS AND METHODS

Subjects

Seventy-two patients with primary hyperlipoproteinemia and 75 patients with type II diabetes mellitus from the Lipid and Diabetes Clinics of the University of California San Francisco were selected for study. In addition to disorders causing secondary hyperlipoproteinemia, exclusion criteria included an E2/2 apolipoprotein phenotype, the use of lipid-modifying drugs other than thiazide diuretics and beta-blockers within 3 weeks, plasma TG >800 mg/dl, and LDL-C >300 mg/dl. Most diabetic patients were taking a sulfonylurea or insulin; patients taking metformin were excluded. Twenty healthy subjects known to express LDL subclass phenotypes A or B were recruited from the Cholesterol Research Center, Lawrence Berkeley National Laboratory, University of California Berkeley. Exclusion criteria were an E2/2 apolipoprotein phenotype, use of lipid lowering drugs, and plasma TG >300 mg/dl.

All subjects fasted overnight before blood was taken by venipuncture into 2 mM EDTA containing protease inhibitors (8) and immediately cooled in ice. Plasma was separated by centrifugation (2,000 *g*, 30 min, 4°C), and Trolox (Aldrich Chemical) was added to a final concentration of 0.25 μg/ml to inhibit lipoprotein oxidation. Plasma from five additional normolipidemic subjects (four with apoE phenotype 3/3, and one with phenotype 4/4) was used for detailed characterization of non-TRL lipoprotein species.

Protocols for blood drawing and use were approved by the Committee on Human Research of the University of California, San Francisco, and the Committee for Protection of Human Subjects of the University of California, Berkeley.

Reagents

JI-H Mab to apoB was a gift of Japan Immunochemical Laboratories. Polyclonal goat anti-human LDL and apoA-I were from International Immunology Corp. Polyclonal anti-human apoE was from a rabbit (9).

Immunoaffinity chromatography and ultracentrifugation

JI-H Mab bound to CnBr-activated Sepharose-4B (Amersham/Pharmacia) was used to prepare immunoaffinity columns, as described previously (1), except that multiple smaller columns (0.7 × 8 cm) with a bed volume of 3 ml were used. The capacity of the columns was approximately 4 mg of LDL apoB-100. These columns, maintained and run at 4°C, could be used at least 10 times without reduction in capacity. Plasma (2 ml) was applied to each column and the unbound fraction was eluted at a rate of 9 ml/h with 154 mM NaCl/1.3 mM EDTA, pH 7.4, containing NaN_3 (0.02 mg/ml), benzamidine (0.3 mg/ml), and Trolox (0.25 μg/ml) (buffer A). The bound fraction was eluted from the column at a rate of 30 ml/h with 3M NaSCN (pH 7.4) containing BSA (10 mg/ml), benzamidine (0.3 mg/ml), and Trolox (0.25 μg/ml), and immediately dialyzed against buffer A. The column was washed again with buffer A. Unbound and dialyzed bound fractions were concentrated overnight at 4°C in a dialysis/concentrator (Bio-Molecular Dynamics) containing buffer A, by using a dialysis membrane with a molecular weight cut-off of 50,000. Recoveries of cholesterol and TG applied to the column were approximately 85 ± 6% (mean and SD).

For lipoprotein fractionation, 5 ml of sample was first centrifuged at a density of 1.006 g/ml (40,000 rpm for 20 h at 12°C) in a 50.3 rotor of a Beckman ultracentrifuge to separate TRL. An additional 5 ml of sample was centrifuged under the same conditions at a density of 1.019 g/ml to separate IDL and TRL from LDL and HDL (10). To isolate IDL+LDL, the infranate of $P >$ 1.006 g/ml was collected and centrifuged under the same conditions at a density of 1.063 g/ml.

Nondenaturing polyacrylamide gradient gel electrophoresis and immunoblotting

Nondenaturing 2% to 16% polyacrylamide gradient gel electrophoresis (GGE) of whole plasma or isolated lipoproteins, with lipid staining, and calculation of lipoprotein particle size were performed as described (7, 11). Particle size measurement of lipoprotein controls run on each gel was within ±2.6 Å in the size range of IDL and LDL. GGE analysis was used to characterize LDL subclass phenotypes as A (large), B (small), or AB (intermediate) as described previously (7).

Plasma and JI-H unbound lipoproteins ($1.006 < \rho < 1.063$ g/ml) were separated by two-dimensional gel electrophoresis. For the first dimension, electrophoresis was carried out in 0.7% agarose gel in 50 mM barbital buffer (pH 8.6) by using vertical slab gel cassettes (BIO RAD mini gel apparatus). Sample (25–50 μl) containing 10% glycerol, and 0.01% bromophenol blue in barbital buffer (pH 8.6), was applied to each of 4 lanes, 1 cm in width. Electrophoresis (10 mA) was carried out at 4°C until the dye front had migrated 5 cm. A strip of agarose gel (0.5 cm wide) containing electrophoretically separated lipoproteins was placed at the top of 2.5–15% non-denaturing polyacrylamide gradient gel (8.3 × 6 cm, 0.15 cm thick) and sealed into position with a thin layer of 0.7% agarose. High molecular weight protein standards (7.1–17 nm, Amersham/Pharmacia), and LDL ($1.025 < \rho < 1.055$ g/ml) were mixed into 200 μl of melted agarose, allowed to cool on a glass slide, and one-half (about 1 × 1 cm) was placed at the top of the right edge of each gel. Electrophoresis was carried out at 4°C in buffer containing 25 mM Tris HCl, 190 mM glycine (pH 8.3) at 250 V for 20 h. The separated lipoproteins were electrotransferred (300 mA, 24 h, 4°C) into nitrocellulose membranes (Trans-Blot, Bio-Rad). The membranes were blocked in 5% non-fat cows' milk in TBS (20 mM Tris, 154 mM NaCl, pH 7.4) for 1 h at 37°C, rinsed three times with TTBS (0.1% Tween 20 in TBS). To detect apolipoproteins, each membrane was incubated for 1 h at room temperature with affinity-purified anti-apoE, affinity-purified anti-apoA-I or anti-LDL diluted 2,000-fold in TTBS containing 1% non-fat milk. The membranes were washed thrice with TTBS for 10 min and then incubated for 1 h at room temperature with peroxidase-labeled goat anti rabbit IgG (for apoE), or rabbit anti-goat IgG (for apoB and apoA-I), both diluted 10,000-fold in TTBS containing 1% milk. The membranes were finally washed thrice for 10 min with TTBS, and proteins were detected by enhanced chemiluminescence (Western blotting detection reagent, Amersham/Pharmacia). The sensitivity of this method was less than 100 ng for each protein, as estimated from electrophoresis of apolipoproteins in SDS.

Separation of RLP

RLP were separated by using the RLP-C assay kit (Japan Immuno Research Laboratories, Japan) (4). Five microliters of

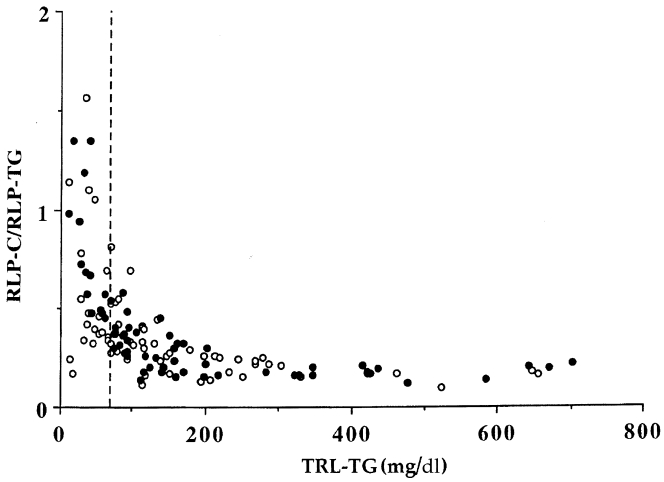

Fig. 1. Relationship between TG-rich-lipoprotein (TRL)-TG concentration and cholesterol-TG ratio in remnant-like particles (RLP) in patients with primary hyperlipoproteinemia (closed circle) and noninsulin-dependent diabetes mellitus (open circle). In the former group, a value for TRL-TG of 70 mg/dl discriminates those with ratios above and below 0.36.

plasma or lipoprotein fraction was added to 300 μl of gel suspension containing anti-human apoA-I and apoB-100 (JI-H) monoclonal antibodies bound to Sepharose. The suspension was gently mixed for 2 h at room temperature with a vertical magnetic bead oscillator. The Sepharose was allowed to settle for 30 min, and 200 μl of the supernate containing the RLP fraction was transferred for storage at −20°C until assayed for cholesterol and TG. When the separation procedure was applied to lipoprotein fractions, 5 μl of 7% human albumin were added to the gel suspension. The reproducibility of cholesterol measurement in plasma RLP is in the range of 3.5–6.1% (12).

Analyses of lipids and proteins

Total and free cholesterol and TG were estimated by enzymatic assays (13, 14) on an autoanalyser (Cobas Mira, Roche). HDL was separated by precipitation of non-HDL lipoproteins with dextran-sulfate, Mg^{2+} (15). The supernate was transferred for storage at −70°C until analyzed. IDL lipid concentrations were calculated as the difference between the measured values in the $\rho < 1.019$ g/ml and $\rho < 1.006$ g/ml fractions; LDL lipid concentrations were calculated as the difference between the measured values in whole plasma and the $\rho < 1.019$ g/ml + HDL fractions. Total phospholipids were determined as lipid $P \times 25$ (16) and protein concentration by the method of Lowry et al. (17) with BSA as stafrndard. Phospholipid species were separated by chromatography on silica gel layers (18) on glass plates (Whatman LK5D), visualized with 8-anilino-1-naphthalene sulfonic acid, and extracted with chloroform-methanol-water (10:10:9, v/v/v). A standard phospholipid mixture (Avanti) was included with each run. ApoA-I and apoB in plasma were quantified by immunonephelometry (19, 20). Individual apolipoproteins of isolated lipoproteins were quantified by an SDS gel electrophoresis procedure (21). ApoC-I and apoC-II in isolated lipoproteins were quantified by radial immunodiffusion (22). Enzyme-linked immunosorbent assay measurements for apoC-III and apoE were performed by using a modification of previously described methods (23, 24). Coefficients of variation for controls measured in each assay were within ±12%. ApoE phenotypes were determined by isoelectric focusing (25). The mean diameter of TRL particles was estimated from their composition (26).

Calculations

Known molecular masses of lipid and protein components (27) were used to calculate molar ratios. Differences between mean values for grouped data were evaluated by two-tailed Student's *t*-test. Linear regression analysis was used to evaluate relationships between variables.

TABLE 1. Characteristics of University of California San Francisco clinic patients

	Patients with Primary Hyperlipoproteinemia				Patients with Type II Diabetes Mellitus			
	Low TG TRL-TG ≤70 mg/dl (n = 19)		High TG TRL-TG >70 mg/dl (n = 53)		Low TG TRL-TG ≤70 mg/dl (n = 25)		High TG TRL-TG >70 mg/dl (n = 50)	
Age (years)	51.2 ± 11.1		50.4 ± 14.9		60.2 ± 11.8		57.5 ± 14.1	
% Males	58		64		44		46	
	Cholesterol	*Triglycerides*	*Cholesterol*	*Triglycerides*	*Cholesterol*	*Triglycerides*	*Cholesterol*	*Triglycerides*
Plasma lipids (mg/dl)								
Total	262.2 (55.7)	90.5 (16.8)	257.4 (53.5)	286.5 (179.8)	201.4 (53.3)	93.0 (22.3)	249.5 (52.7)	253.1 (138.0)
TRL	9.1 (3.1)	39.3 (16.5)	49.5 (40.6)	219.0 (169.4)	9.8 (3.8)	44.0 (19.4)	41.9 (28.1)	192.0 (138.2)
IDL	11.8 (6.1)	11.3 (5.5)	19.2 (11.5)	14.3 (16.1)	9.2 (4.0)	9.4 (3.4)	17.1 (9.1)	16.5 (12.4)
LDL	189.2 (55.1)	31.1 (10.6)	158.0 (54.4)	39.3 (20.4)	134.2 (47.7)	25.6 (10.8)	159.4 (45.2)	36.0 (23.1)
HDL	57.3 (15.8)	13.5 (4.8)	39.7 (14.2)	22.0 (15.3)	52.8 (16.6)	18.5 (6.4)	42.5 (10.1)	21.0 (8.5)
RLP	6.8 (2.7)	11.0 (3.8)[a]	18.1 (15.3)	88.5 (91.3)[b]	7.2 (5.2)	14.1 (5.8)[c]	16.4 (12.8)	83.8 (91.7)[d]
RLP-C/RLP-TG	0.75 (0.32)		0.26 (0.10)		0.57 (0.35)		0.28 (0.13)	
ApoE phenotype								
3/3	12		28		18		25	
4/3	6		18		4		12	
4/4							3	
3/2					1		4	
4/2							1	
ND	1		5		2		5	

[a] n = 15.
[b] n = 51.
[c] n = 23.
[d] n = 41.

RESULTS

RLP-cholesterol and TG concentrations in patients with primary or diabetic hyperlipoproteinemia

Among 130 patients, 66 with primary and 64 with diabetic hyperlipoproteinemia, plasma and TRL-TG concentrations varied widely. In both groups, the ratio of RLP-C to RLP-TG (hereinafter termed RLP ratio) was a hyperbolic function of TRL-TG levels, consistent with a variable contribution of TG-rich and cholesterol-rich lipoproteins to RLP-lipid concentrations (**Fig. 1**). To facilitate further analyses of data from these patients, we dichotomized the non-diabetic and diabetic groups based upon the following considerations: *1*) among the patients with primary hyperlipoproteinemia, the RLP ratio invariably exceeded 0.36 when TRL-TG concentrations were 70 mg/dl or less and, with 4 exceptions, were lower than 0.36 at higher concentrations of TRL-TG (Fig. 1); and *2*) as described below, a cut-point of 70 mg/dl (equivalent to plasma TG concentration of 120–130 mg/dl) effectively segregated, with one exception, our subjects with LDL pattern A from those with pattern B or A,B.

In **Table 1**, patients with primary or diabetic hyperlipoproteinemia are thus classified as low TG (LTG) or high TG (HTG), based upon the cut-point of 70 mg/dl for TRL-TG. Mean plasma RLP-C concentrations were similar in LTG primary and diabetic patients despite significantly lower mean concentrations of LDL-cholesterol in the latter group (189 vs. 134 mg/dl, $P < 0.01$). These RLP-C values (about 7 mg/dl) are within the reference range found in The Framingham Heart Study (28). Mean RLP-TG concentrations were about 20% lower in the primary group (11.0 vs. 14.1 mg/dl, $P = 0.08$). In each of the LTG groups, the RLP ratio was much higher than in the respective HTG groups ($P < 0.001$ for both). Among HTG primary and diabetic patients alike, RLP-C concentrations were 2–3-fold higher and RLP-TG concentrations were 6–8-fold higher than in their LTG counterparts.

The prevalence of one or two alleles for apoE-4 was higher in HTG than LTG primary and diabetic patients, but not significantly so, and lipoprotein-lipid concentrations in those expressing or lacking apoE-4 were similar (data not shown).

Relationships between the concentrations of RLP-C and TRL-TG and LDL-C, respectively, are shown in **Fig. 2**. Among both groups of LTG patients (Fig. 2A and C), RLP-C was a function of LDL-C, but not of TRL-TG. The regression of RLP-C on LDL-C was steeper in diabetic than primary patients, and only the former was clearly significant ($P < 0.01$ vs. $P = 0.06$). Of note, in four LTG diabetic patients but in none of the primary patients, RLP-C concentrations exceeded 13 mg/dl. By contrast, among both groups of HTG patients (Fig. 2B and D), RLP-C was highly correlated with TRL-TG ($P < 0.0001$), whereas there was

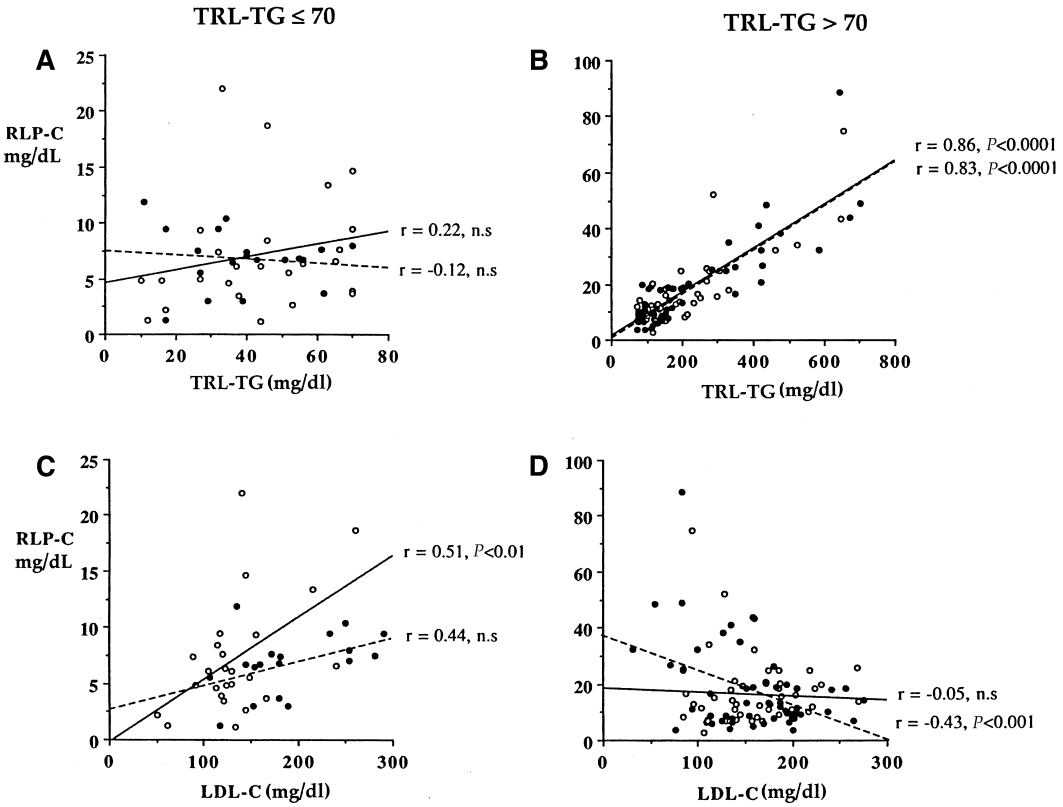

Fig. 2. Relationships between RLP-C and TRL-TG and LDL-C in patients with TRL-TG concentrations below (A, C) and above (B, D) 70 mg/dl. These relationships are shown separately for patients with primary hyperlipoproteinemia (solid circles and dashed lines) and noninsulin-dependent diabetes mellitus (open circles and solid lines). Regression coefficients (r) are indicated, where n.s. = not significant ($P > 0.05$).

TABLE 2. Characteristics of University of California Berkeley subjects according to LDL subclass phenotype

	Phenotype		
	A (n = 8)	A,B (n = 3)	B (n = 9)
Age (years)	46 (8)	39 (8)	45 (9)
Plasma lipids (mg/dl)			
Total C	219 (29)	185 (2)	211 (44)
Total TG	93 (26)	128 (44)	202 (62)[b]
TRL-C	10.4 (4.4)	21.2 (8.6)	33.1 (10.8)[a]
TRL-TG	49.0 (19.7)	77.5 (33.8)	137.6 (54.2)[a]
IDL-C	5.3 (2.4)	7.7 (3.2)	11.3 (4.2)[c]
LDL-C	152.3 (22.4)	111.8 (11.9)	135.3 (37.3)
HDL-C	49.0 (11.0)	44.0 (10.0)	32.0 (4.0)[a]
RLP-C	9.5 (3.8)	8.9 (4.4)	12.6 (4.0)
RLP-TG	17.5 (7.7)	28.9 (21.6)	61.1 (26.5)[a]
RLP-C/RLP-TG	0.70 (0.49)	0.37 (0.12)	0.22 (0.05)[c]
ApoE phenotypes			
3/3	4	1	5
3/4	2	1	2
4/4			
3/2	1		
ND	1	1	1
Apolipoproteins (mg/dl)			
ApoB	103 (12)	89 (10)	127 (28)[b,d]
ApoA-I	124 (19)	130 (16)	103 (11)[c,d]
ApoE	3.1 (0.79)	3.4 (0.91)	4.0 (0.61)[c]
LDL diameter (Å)	268 (3)	260 (4)	250 (4)[a]

Values are mean (SD). ND, Not determined.
Significant differences between A and B:
[a] $P < 0.001$.
[b] $P < 0.02$.
[c] $P < 0.05$.
[d] n = 7.

no relationship with LDL-C for diabetic patients, and a negative one for primary patients. The latter finding could reflect the inverse relationship between TRL-C and LDL-C observed in population studies when TRL-TG concentrations exceed 120 mg/dl (29), which was more common in primary than diabetic patients.

Taken together, these data suggest that, among hyperlipoproteinemic individuals with low plasma TG concentrations, RLP-C concentrations reflect primarily cholesterol-rich lipoproteins of the LDL class, whereas among those with higher plasma TG (exceeding 120–130 mg/dl) increased RLP-C concentrations may reflect exclusively higher concentrations of TRL. This conclusion is consistent with earlier observations (2, 12) and also suggest that, among patients with type II diabetes mellitus, high RLP-C concentrations may occur occasionally in the presence of low plasma TG concentrations (4, 30).

Distribution of RLP-C in plasma lipoproteins of healthy subjects with LDL patterns A and B

To evaluate further the properties of RLP in specific lipoprotein fractions of individuals with varying plasma TG, we selected 20 healthy subjects known to express LDL subclass phenotype A or B. When studied, three of these subjects expressed an intermediate pattern (AB), eight pattern A and nine pattern B, with the expected differences in mean LDL peak diameters (**Table 2**). Concentrations of plasma and LDL-C were similar in the A and B groups, but plasma and TRL-TG, TRL, and IDL-C as well as plasma apoB and apoE concentrations were higher, and HDL-C and apoA-I concentrations were lower in those with pattern B. These findings are consistent with published data (7). Plasma RLP-TG were higher and the RLP ratio was lower, as expected, in those with pattern B, but the concentration of RLP-C did not differ significantly in the two groups, a consequence of two subjects with pattern A who had unusually high RLP-C concentrations (15.9 and 14.0 mg/dl). Thus, as in LTG patients, RLP-C in healthy subjects with LDL pattern A evidently reflected a predominance of cholesterol-rich lipoproteins. To demonstrate this directly, RLP-C was measured in TRL and the HDL-containing lipoprotein fraction ($\rho > 1.063$ g/ml) from 15 of these subjects, and these values were subtracted from plasma RLP-C to calculate RLP-C in IDL+LDL (**Table 3**). RLP-C in HDL was a minor fraction of total RLP-C (~1 mg/dl), irrespective of LDL pattern. The concentration of RLP-C in IDL+LDL was also similar in the three groups (3–4 mg/dl) but accounted for about 40% of total RLP-C in subjects with pattern A, 30% in those with pattern AB, and 20% in those with pattern B. These data show directly that IDL+LDL account for most non-TRL RLP-C.

In the single outlier with pattern A (plasma RLP-C = 14 mg/dl) included in these 15 subjects, the high value was attributable to the IDL+LDL fraction (9.9 mg/dl). By contrast, in those with pattern B, increased concentrations of RLP-C were invariably attributable to TRL.

TABLE 3. Concentrations and distribution of RLP-cholesterol in University of California Berkeley subjects according to lipoprotein phenotype

	Phenotype					
	A (n = 6)		A,B (n = 3)		B (n = 6)	
	mg/dl	%	*mg/dl*	%	*mg/dl*	%
Lipoprotein fraction						
TRL	3.8 (1.5)	44.1 (14.6)	5.0 (1.6)	60.6 (15.9)	10.3 (2.8)[a,b]	71.0 (11.5)[a]
IDL+LDL	4.1 (3.1)	41.3 (19.5)	3.0 (2.5)	29.5 (12.8)	3.1 (1.5)	21.2 (9.2)[c]
HDL	1.3 (0.37)	14.6 (3.6)	0.94 (0.83)	9.9 (4.5)	0.98 (0.84)	7.8 (7.5)

Values are mean (SD).
[a] $P < 0.002$ B versus A.
[b] $P < 0.05$ B versus A,B.
[c] $P < 0.05$ B versus A.

TABLE 4. Composition of total TRL and TRL fractions

	Total	JI-H Unbound	JI-H Bound
% Mass (n = 10)			
CE	10.6 (2.6)	8.4 (2.0)	11.1 (2.9)[c]
TG	55.0 (4.4)	60.7 (3.3)	53.2 (4.3)[a]
FC	6.4 (0.48)	5.5 (0.94)	7.5 (0.71)[a]
PL	17.4 (2.0)	16.9 (1.0)	17.3 (1.3)
Protein	10.6 (1.4)	8.5 (1.6)	10.8 (1.7)[b]
ApoB100	3.9 (0.78)	2.2 (0.6)	4.6 (0.84)[a]
ApoB48	0.24 (0.06)	0.35 (0.1)	0
ApoE	0.99 (0.21)	0.86 (0.21)	1.2 (0.29)[c]
ApoC-III	3.7 (0.39)	2.9 (0.33)	3.1 (0.62)
Molar ratios (n = 10)			
CE/ApoB	1,928 (457)	2,307 (589)	1,865 (420)
ApoE/ApoB	3.4 (0.55)	4.5 (0.84)	3.9 (0.5)
ApoC-III/ApoB	50.6 (9.7)	60.0 (13.4)	40.4 (8.2)[a]
ApoE/ApoC-III	0.069 (0.17)	0.077 (0.022)	0.10 (0.024)[c]
Particle dimensions			
Diameter (nm) (n = 10)	38.0 (3.3)	43.0 (4.1)	36.1 (2.6)[a]
Surface area (nm^2) (n = 7)	4,533 (785)	5,853 (1,095)	4,091 (591)[b]
Surface density (n = 7) (molecules/$\mu m^2 \times 10^{-3}$)			
ApoE	0.80 (0.16)	0.78 (0.15)	1.0 (0.17)[c]
ApoC-I	3.5 (0.96)	3.8 (1.0)	3.4 (0.84)
ApoC-II	2.7 (0.49)	3.2 (0.48)	2.4 (0.46)[b]
ApoC-III	11.5 (2.1)	10.4 (1.1)	10.1 (2.2)
Phospholipid	628 (185)	660 (39)	607 (126)

Values are mean (SD). CE, cholesteryl esters (esterified cholesterol × 1.67); TG, triglycerides; FC, free cholesterol; PL, phospholipids.

Significant difference between JI-H unbound and JI-H bound:

[a] $P < 0.001$.

[b] $P < 0.01$.

[c] $P < 0.05$.

Properties of lipoproteins distinguished by affinity for Mab JI-H

The properties of TRL and IDL+LDL fractions, separated after immunoaffinity chromatography of plasma on JI-H columns, were similar in subjects with LDL patterns A, AB, and B. Hence, data from 10 subjects were pooled for analysis (**Table 4**). As described previously (1), unbound TRL was large and relatively enriched in cholesteryl esters and apoE as compared with bound TRL. In addition, the current data show that lipoproteins containing apoB-48 accounted for about one-fourth of the lipoprotein particles in JI-H unbound TRL.

The additional data on apoB components permitted a more precise assessment of the number of other components per average particle, expressed as molar ratio to total apoB (Table 4). These ratios indicate that the unbound (RLP) fraction of TRL was enriched in apoC-III as well as in apoE, whereas the ratio of apoE to apoC-III was lower in TRL-RLP than in the bound fraction (which contains no apoB-48). These data differ from those reported previously (1), in which the ratio of apoE to total apoCs was higher in TRL-RLP of three normotriglyceridemic subjects, and the same in five subjects with endogenous hypertriglyceridemia. The current data also permit an assessment of the average density of phospholipids and apolipoproteins on the surface of RLP-TRL as compared with TRL bound to Mab JI-H. No difference was found for total phospholipids, apoC-I and apoC-III, whereas the density of apoC-II was higher and that of apoE was lower in RLP-TRL (Table 4).

Lipoprotein lipase hydrolyzes glycerophospholipids in TRL, yielding particles that are relatively enriched in sphingomyelin (31). We analyzed phospholipid classes in total TRL and the Mab JI-H-unbound fraction of TRL from five hyperlipoproteinemic patients (one diabetic) with plasma TG concentrations ranging from 214–2920 mg/dl. The sphingomyelin-phosphatidyl choline ratios in total TRL and the unbound fraction of TRL did not differ significantly: mean values and SD were 0.119 ± 0.034 and 0.131 ± 0.031, respectively.

Consistent with the known properties of Mab JI-H (2, 4), the JI-H unbound fraction of IDL+LDL contained, on average, only 3.7% of apoB-100 in total IDL+LDL. The unbound fraction was relatively enriched in soluble apolipoproteins, which comprised only 7% of total IDL+LDL protein, but about one-half of that of the unbound fraction. Much of this was apoA-I (27% of apolipoprotein mass) with lesser amounts of apoE and apoCs (**Table 5**). Unbound IDL+LDL also contained a small amount of apoB-48. In molar terms, apoB-48 in the unbound fraction accounted for about 0.14% of apoB in total IDL+LDL (~0.1 mg/dl). The overall lipid composition of the unbound fraction of IDL+LDL resembled that of total IDL+LDL, except for an appreciably higher amount of TG (11% vs. 6% of total mass). The peak diameters of lipoproteins in unbound IDL+LDL, measured in seven subjects with pattern A and all nine subjects with pattern B,

TABLE 5. Composition of total and Mab JI-H unbound IDL+LDL

	% Mass	
	Total IDL+LDL	JI-H Unbound IDL+LDL
CE	40.0 (2.0)	35.2 (4.7)[b]
TG	6.1 (1.6)	11.0 (3.1)[a]
FC	8.4 (0.57)	7.2 (1.7)[c]
PL	24.3 (1.3)	23.6 (2.3)
Protein	21.2 (1.4)	23.0 (3.8)
ApoB100	19.7 (1.4)	11.2 (4.2)[a]
ApoB48	ND	0.6 (0.37)
ApoE	0.30 (0.076)	1.6 (0.68)[a]
ApoA-I	0.35 (0.29)	6.1 (4.1)[b]
ApoC's	0.81 (0.35)	3.4 (2.0)[c]
ApoC-III	0.57 (0.17)	1.2 (0.63)[d]

Values are mean (SD), n = 10. For abbreviations see Table 4. ND, not detectable.

Significant differences:

[a] $P < 0.001$.

[b] $P < 0.01$.

[c] $P < 0.02$.

[d] $P < 0.05$.

closely resembled those of LDL in whole plasma. In group A, mean peak diameters were significantly greater (271 Å) than in group B (251 Å) ($P < 0.01$), and the individual diameters for the 16 subjects were highly correlated ($R^2 = 0.91$). It should be noted that little of the apoA-I component of the unbound fraction (~2 mg/dl) would likely be present in total plasma RLP, as a Mab to apoA-I is included, together with Mab JI-H, in the RLP reagent (2). Marcoux et al. found only 0.3% of plasma apoA-I in plasma RLP from normolipidemic subjects (5).

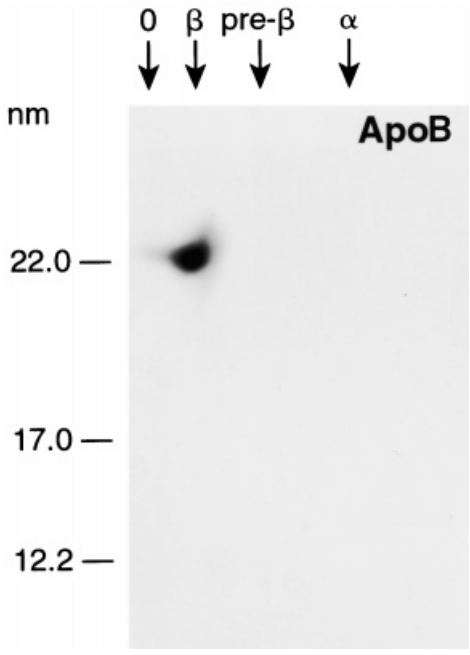

Fig. 3. Immunoblot of two-dimensional gel electrophoretogram of the JI-H unbound fraction of IDL+LDL with anti-apoB showing a single lipoprotein component of 22 nm (second dimension) and β mobility (first dimension).

To determine whether any of the apoA-I or apoE in the JI-H unbound fraction is associated with apoB-100, we separated the lipoproteins in this fraction by nondenaturing, two-dimensional gel electrophoresis followed by immunoblotting. As shown in **Fig. 3**, apoB in JI-H unbound IDL+LDL was associated exclusively with discrete particles of 22 nm and β mobility as expected for typical LDL (32). No detectable apoE was associated with these LDL particles, either in the total JI-H unbound fraction of plasma or in the IDL+LDL component (**Fig. 4**). ApoE in the JI-H unbound fraction of unfractionated plasma was associated with polydisperse particles, ranging from 10–20 nm and mainly with pre-β mobility. The apoE in the IDL+LDL component was in relatively discrete particles at the upper end of this size distribution. Particles containing apoA-I in the IDL+LDL fraction of unbound lipoproteins were smaller than those containing apoE (12 nm) and had α mobility. Thus, particles containing apoB-100, apoE, and apoA-I in the IDL-LDL component of JI-H unbound lipoproteins appear to represent three distinct species.

In order to assess the contribution of the various apolipoproteins in the unbound IDL+LDL fraction to the concentration of cholesterol and TG in this fraction, we performed correlation analyses. The concentrations of apoB-100 were well correlated ($P < 0.01$) with cholesterol

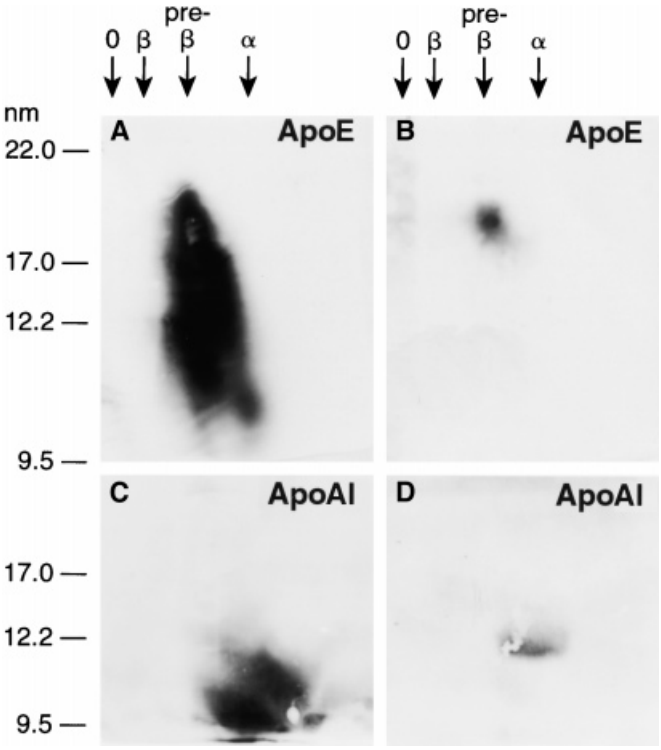

Fig. 4. Immunoblots of two-dimensional gel electrophoretogram of the total JI-H unbound fraction of whole plasma (A, C) and its IDL+LDL component (B, D) against anti-apoE (A, B) and anti-apoA-I (C, D). The blots for the whole plasma contain mainly polydisperse particles smaller than LDL (i.e., HDL), whereas the IDL+LDL component contains more homogeneous particles with pre-β mobility (containing apoE and apparently no apoA-I) and α mobility (containing apoA-I and apparently no apoE). TRL particles are not resolved in this system.

and TG (r = 0.89 and 0.63, respectively). By contrast, those of apoA-I and apoE with these lipids were much weaker and not significant. No correlation was found for apoB-48. Furthermore, apoB-100 as a percentage of protein mass in this fraction was a function of the cholesterol-TG ratio (r = 0.64, $P < 0.05$). These observations indicate that most cholesterol and TG in unbound IDL+LDL are associated with apoB-100 rather than with lipoproteins containing apoE, apoA-I, or apoB-48. Furthermore, the enrichment of TG in this fraction was mainly found in samples containing a low concentration of apoB-100, suggesting that "true" LDL particles in RLP are not TG enriched. By exclusion, the particles containing apoE, apoA-I, or apoB-48 in unbound−IDL+LDL may contain considerable TG. To assess this possibility, we carried out additional analyses. Inverse relationships between apoA-I and apoE as a percentage of total apolipoprotein mass and the cholesterol-TG ratio were observed (r = −0.56 and −0.43. These and other relationships were nonsignificant, however, and provide only weak support for TG enrichment of these particles.

DISCUSSION

Our observations provide new information about the contribution of TRL and other plasma lipoproteins to RLP-C, and about the properties of the TRL and LDL that invariably comprise most RLP-lipids. In people whose plasma TG concentrations are below approximately 120 mg/dl, LDLs provide a major fraction of RLP-C, whereas RLP-TGs reflect mainly a subpopulation of TRL. As plasma TG concentrations rise, the increment of RLP-C as well as RLP-TG is contributed essentially by TRL. Consistent with our earlier work (1), TRL that do not bind Mab JI-H are larger than the bound TRL. The extent to which this reflects the presence of apoB-48 containing TRL in the unbound fraction cannot be deduced from the current study. In other work, however, unbound apoB-100 containing TRL have also been found to be larger on average than bound TRL (Kovar and Havel, unpublished data). Also consistent with our earlier observations (1), unbound TRL are also enriched, on a per particle basis, in cholesteryl esters and apoE. In the current work, that observation has been solidified by quantifying apoB-48 as well as apoB-100, permitting an unambiguous estimation of TRL particle number. Such quantification is important because apoB-48 particles comprise about one-fourth of the lipoprotein particles in unbound TRL.

As compared with bound TRL, unbound TRL was found in our earlier study (1) to be depleted in total apoCs relative to apoE in normotriglyceridemic subjects and in those with familial dysbetalipoproteinemia. Only the latter difference was significant, however. The current data show that apoCs are not depleted, relative to apoE, in unbound TRL from normotriglyceridemic subjects. Rather, as compared with bound TRL, each of the major apoCs (C-I, C-II, and C-III) is enriched to a somewhat greater extent than apoE. In this respect, our current observations extend those reported for apoC-III by Marcoux et al. (5). In hypertriglyceridemic subjects, the ratio of total apoC/apoE (1) and of apoC-III/apoE (5) in unbound TRL has been found to resemble that of total TRL. In addition, our current data show no significant enrichment of sphingomyelin in unbound TRL phospholipids. Thus, taken as a whole, unbound TRL do not resemble classical remnants of chylomicrons and VLDL, which are on average smaller than their nascent precursors and are enriched not only in apoE relative to apoCs, but also in sphingomyelin relative to phosphatidylcholine. Our estimates of surface density (Table 4) suggest that the enrichment of soluble apolipoproteins in unbound TRL reflects primarily their greater average surface area rather than a higher surface concentration. Earlier work has indeed shown that larger TRL from normolipidemic and hyperlipidemic subjects alike are enriched in apoE and each of the apoCs, relative to apoB (33). Such particles would not be expected to turn over rapidly (34). Therefore, unbound TRL are likely to be preferred acceptors of cholesteryl esters transferred from higher density lipoproteins by CETP, accounting for their enrichment in this lipid. Unbound TRL, present in RLP, thus has distinctive properties that must be considered in relation to the utility of RLP-C as a marker of atherogenic risk. As discussed elsewhere (3), accumulating evidence suggests that RLP-C, as a measure of larger TRL in subjects with elevated TG, is a better risk marker than plasma TG.

In patients with familial dysbetalipoproteinemia, a high ratio of RLP-C/plasma TG is found despite the presence of hypertriglyceridemia (35). This observation undoubtedly reflects the cholesterol-enrichment of TRL of all sizes in this disorder (26). Under these circumstances, TRL-RLP are not only enriched in apoE, but also depleted of apoCs (1, 5), as are TRL as a whole. Furthermore, unbound TRL in familial dysbetalipoproteinemia, which may account for one-half or more of total TRL-C (1), are larger than those that bind Mab JI-H, consistent with an influence of particle size on the reactivity of this antibody with apoB-100. As virtually all particles in unbound TRL contain apoE (1) (E. Campos and R. Havel, unpublished data), TRL of whatever size that contain apoB-100 but lack apoE ("B particles") (8) evidently bind to Mab JI-H.

Are RLP particles of IDL/LDL density also distinctive? As with intermediate-size lipoproteins separated by gel chromatography (5), our observations of the overall composition of IDL/LDL separated by ultracentrifugation suggest that this may be the case. This fraction was enriched in TG and apoE, relative to total IDL/LDL from subjects with lipoprotein patterns A and B alike. It also contained detectable apoB-48, equivalent to about 0.1 mg/dl. Our further analysis revealed, however, that the apoE in this fraction is on particles distinct from those containing apoB-100. This observation resembles that of Marcoux et al. for the HDL fraction of RLP (5). They found that apoE particles in HDL-RLP lacked both apoB and apoA-I and we have confirmed this observation for the JI-H unbound fraction of whole plasma. Our analyses also strongly suggest that the particles containing apoB-

100 and lacking apoE, which account for most of the unbound cholesterol in this fraction, are not likely to be enriched in TG. Furthermore, the peak diameters of the major lipoprotein component of this fraction closely resembled those of LDL in whole plasma. We thus have obtained no evidence that the properties of unbound apoB containing IDL/LDL in normotriglyceridemic subjects differ from those of IDL/LDL as a whole.

Fielding and associates have reported elevated concentrations of apoE in a lipoprotein with an estimated density resembling LDL in plasma of patients with type II diabetes mellitus (36). This lipoprotein, which also contained apoB-100, was enriched in TG as compared with total LDL, thus resembling JI-H unbound IDL/LDL. This lipoprotein could account for the unusually high RLP-C concentrations found in some normotriglyceridemic type II diabetes patients.

Our observation that most JI-H unbound IDL/LDL lack apoE and resemble total LDL in healthy normotriglyceridemic individuals is consistent with earlier data showing that up to 3% of isolated LDL, in concentrations up to 210 mg/dl of LDL-C, remains unbound in the RLP test (4). At higher concentrations of LDL, larger percentages (for example, 5% at 300 mg/dl) remained unbound. We therefore excluded from our analysis of hyperlipidemic subjects those with very high LDL concentrations owing to familial hypercholesterolemia. We have found substantially elevated RLP-C concentrations in five patients with familial hypercholesterolemia whose mean LDL-C was 364 mg/dl, despite normal plasma TG. Available data, including that shown in Fig. 2 for subjects with low plasma TG concentrations, suggest that LDL binding to the JI-H antibody in the RLP test is incomplete and that binding efficiency falls progressively with increasing LDL concentrations in plasma. Taken together, these observations suggest that RLP-C concentrations in normotriglyceridemic individuals may not have the same significance as they do in those with hypertriglyceridemia. In a recently reported study of women participants in The Framingham Heart Study (37), the relationship of RLP-C to prevalent coronary heart disease was found to be nonlinear, even after logarithmic transformation. In an analysis using dichotomous cut-points, the concentration of this analyte predicted the prevalence of coronary heart disease only in women with RLP-C concentrations in the upper quartile of the total distribution after adjustment for known risk markers. The same conclusion was reached from logistic regression analysis for dichotomous measures. In comparable analyses, total serum TGs were not predictive. Of note, only in those women in the upper quartile of the RLP-C distribution was RLP-C/RLP-TG <0.36 (mean value = 0.22), indicating the most RLP-C was in TRL. By contrast, among women in the lower three quartiles, LDL evidently contributed the major portion of RLP-C. These data suggest that variation in RLP-C among individuals with low plasma TG, in whom RLP-C does not provide a valid measure of the concentration of larger TRL, may not predict atherosclerotic risk.

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