Effect of lipid transfer activity and triglyceride hydrolysis on apolipoprotein B immunoreactivity in modified low density lipoproteins

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Abstract Consequences of alterations in the size and the lipid composition of low density lipoproteins (LDL) on apolipoprotein (apo) B immunoreactivity were analyzed using two distinct anti-apoB monoclonal antibodies (Mabs), i.e., 4G3, which recognizes an epitope closed to the binding site to the LDL receptor, and 2D8, which is directed against a distal region. Inhibition analysis revealed that the lipid transfer-mediated triglyceride enrichment of LDL isolated from 12 native human plasmas is associated with significant reductions in the expression of 2D8 and 4G3 epitopes ($P < 0.05$ in both cases). In contrast, triglyceride hydrolysis of triglyceride-rich LDL significantly increased apoB immunoreactivity as compared with non-lipolyzed counterparts ($P < 0.05$ with 2D8 and 4G3 Mabs). Among all the modified LDL fractions studied (n = 36), immunoreactivity of 2D8 and 4G3 epitopes correlated negatively and significantly with the triglyceride content ($P < 0.01$ in both cases), but with neither the size nor the other lipid parameters of LDL particles. Furthermore, changes in the triglyceride content of LDL correlated significantly with changes in apoB immunoreactivity after in vitro treatment with either lipid transfer activity alone ($P < 0.01$ with 2D8 and 4G3 Mabs) or lipid transfer activity combined with triglyceride hydrolysis ($P < 0.01$ with 2D8 and 4G3 Mabs). Finally, both the triglyceride content of native LDL and the total triglyceride level in 12 normolipidemic human plasmas correlated negatively and significantly with the expression of 2D8 epitope ($P < 0.03$ in both cases) and 4G3 epitope ($P < 0.02$ in both cases). It is concluded that triglycerides constitute a major determinant of the immunoreactivity of 2D8 and 4G3 apoB epitopes in LDL.—**Viens, L., and L. Lagrost.** Effect of lipid transfer activity and triglyceride hydrolysis on apolipoprotein B immunoreactivity in modified low density lipoproteins. *J. Lipid Res.* 1997. **38:** 1129–1138.

Supplementary key words cholesteryl ester transfer protein • lipoprotein lipase • apoB immunoreactivity

Plasma low density lipoproteins (LDL) constitute several subpopulations with distinct size (1–3), density (4), and composition (2, 4). The biochemical processes that account for alterations in the size distribution of plasma LDL involve the replacement of cholesteryl esters by triglycerides in the LDL core, as mediated by the cholesteryl ester transfer protein (CETP), and the subsequent hydrolysis of triglycerides by endothelium lipases (5). Recent studies from our group (6) demonstrated that the latter mechanism can transform typical large-sized LDL pattern A into typical small-sized LDL pattern B (7) and can lead to alterations in the oxidation susceptibility of modified LDL (8). In addition to variations in their size and composition, plasma LDL particles have been shown to be heterogeneous in terms of apolipoprotein (apo) B immunoreactivity, as observed by using specific anti-apoB monoclonal antibodies. Interestingly, among the LDL fractions, alterations in apoB immunoreactivity were noted both between LDL preparations isolated from distinct subjects (9, 10) and between LDL subfractions from one single subject (11). An immunochemical heterogeneity of apoB has also been observed between the various plasma apoB-containing lipoprotein classes, i.e., very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL) and LDL (12–16), suggesting that the apoB conformation might be directly influenced by the structure and the composition of one given lipoprotein particle. In support of the latter view, the size as well as the lipid composition of LDL particles were alternatively shown to modulate the expression of apoB epitopes (9–11, 17–22). However, the major determinant of apoB immunoreactivity in plasma LDL has still not been clearly identified. In order to address further the effect of alterations in the structure and composition of LDL particles on apoB immunoreactivity, we chose in the present study to modify LDL in vitro by using a well-controlled experimental system involving lipid transfer and triglyceride hydrolysis

Abbreviations: LDL, low density lipoproteins; VLDL, very low density lipoproteins; CETP, cholesteryl ester transfer protein; LPL, lipoprotein lipase; PBS, phosphate-buffered saline; BSA, bovine serum albumin; TBS, Tris-buffered saline; Mabs, monoclonal antibodies.

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activities (6, 8). Thus, the size as well as the neutral lipid content of isolated LDL were gradually and significantly modified according to the biochemical sequence that is likely to occur in vivo. Subsequently, consequences of LDL modifications in terms of apoB immunoreactivity were analyzed by making used of two specific anti-apoB monoclonal antibodies that recognize two distinct regions of the apoB molecule. In addition, plasma lipid parameters and apoB immunoreactivity in normal plasma LDL were compared.

MATERIALS AND METHODS

Lipoprotein preparation

Lipoproteins were isolated from normolipidemic human plasma (total cholesterol lower than 2.5 g/L; total triglycerides lower than 1.4 g/L) by sequential ultracentrifugation. Densities were adjusted by adding potassium bromide and they were checked using a DMA 35 digital density meter (Paar, Graz, Austria). Very low density lipoproteins (VLDL) were isolated as the $d < 1.006$ g/mL plasma fraction with two successive runs, one 22-h run at 40,000 rpm (102,000 *g*) in a 65-Ti rotor on an L7 ultracentrifuge (Beckman, Palo Alto, CA), and one 2-h run at 70,000 rpm (339,000 *g*) in an NVT-90 rotor on an XL-90 ultracentrifuge (Beckman). Low density lipoproteins (LDL) were isolated as the $1.019 < d < 1.063$ g/mL plasma fraction on an NVT-90 rotor on an XL-90 ultracentrifuge (Beckman) with one 2-h/90,000 rpm (561,000 *g*) run at the lowest density and one 2.5-h/80,000 rpm (443,000 *g*) run at the highest density.

Modification of LDL by lipid transfer and triglyceride hydrolysis activities

Three types of LDL preparations, named 'Control', 'Transfer, −LPL' and 'Transfer, +LPL' were used in the present study. 'Control' LDL were directly isolated from total plasmas maintained at 4°C. 'Transfer, −LPL' LDL were obtained through the enrichment of the plasma LDL fraction with triglycerides. This was achieved by incubating total plasma with freshly isolated VLDL (triglyceride concentration added, 1g/L), according to the general procedure previously described (6). Briefly, VLDL-supplemented plasma was incubated for 24 h at 37°C to allow the substitution of VLDL-derived triglycerides for cholesteryl esters in the LDL core through the neutral lipid transfer reaction mediated by the cholesteryl ester transfer protein (CETP). Plasma samples contained 1.5 mmol/L iodoacetate in order to block lecithin:cholesterol acyltransferase (LCAT) activity. After reisolation of the LDL fraction by ultracentrifugation (see above), an aliquot of triglyceride-enriched, 'Transfer, −LPL' LDL was dialyzed overnight against Tris (hydroxymethyl)-aminomethane 0.2 mol/L, NaCl 0.08 mol/L, pH 8.4, buffer (TBS buffer). Subsequently, LDL were lipolyzed by incubation in the presence of purified bovine milk lipoprotein lipase (LPL) (specific activity 4 U/mg) (Sigma), according to the general procedure previously described (5). LDL (protein concentration, 0.5 g/L), LPL (final concentration, 10 μg/mL), heparin (2 U/mL), and fatty acid-poor bovine serum albumin (60 g/L) were incubated at 37°C for 2 h (6). At the end of the incubation period, LPL activity was inhibited by the addition of KBr, which raised the final density to d = 1.07 g/mL, and 'Transfer, +LPL' LDL particles were reisolated by one 2.5-h run at 100,000 rpm (356,100 *g*) in a 100.2 rotor on an TL100 ultracentrifuge (Beckman).

Anti-apoB monoclonal antibodies

Anti-apoB monoclonal antibodies (Mabs), 2D8 and 4G3, were a kind gift from Dr. Milne and Dr. Marcel (Heart Institute, Ottawa, Canada). They were obtained by immunizing mice with human LDL (23, 24). The specificity of these Mabs and the identification of their epitopes have been described (23–25). The 2D8 epitope is situated between amino acid residues 1438 and 1480 (26), and 2D8 Mabs react with both intestinal and hepatic apoB. The 4G3 epitope is located between residues 2980 and 3084 (26). In contrast to 2D8 Mabs, 4G3 Mabs react only with apoB-100, and they can block the binding of LDL to its cell-surface receptor (24, 25, 27).

ApoB enzyme-linked immunoabsorbent assay

The immunoreactivity of apoB in various LDL fractions was analyzed using an enzyme immunoassay as previously described (28). Control and modified LDL were added on the basis of their total protein content, as conducted in most of the previous studies (9–11, 17). In the present study, the LDL protein content was assayed by using bicinchoninic acid reagent (Pierce) according to the method of Smith et al. (29) adapted on a Cobas-Bio centrifugal analyzer (Roche). The intrassay and interassay coefficients of variation of the protein assay were 1.0 and 3.6%, respectively, with a working range of 0.03–2.00 g/l. As determined by electrophoresis in 15–250 g/l polyacrylamide gradient gels in the presence of sodium dodecyl sulfate, isolated LDL contained virtually only apoB-100. Finally, the protein content of various LDL preparations, treated or not with lipid transfer and triglyceride hydrolysis activities, did not vary in any systematic way, indicating that the estimates of protein content were independent of alterations in the structure and lipid content of LDL particles. All the steps of the immunoassay (pipetting, diluting, dispensing, washing, and photometry) were

carried out with a Biomek 1000 BioRobotics system (Beckman Instruments, Palo Alto, CA) coupled to a Zymark automated arm. Briefly, polystyrene microwell plates were coated overnight with isolated plasma LDL (protein concentration, 3 μg/mL) (28). In the meantime, equal volumes of LDL samples and monoclonal anti-apoB antibodies diluted to the desired concentrations in PBS buffer containing 10 g/L of bovine serum albumin were mixed in 96 deep-well titer plates (Beckman), and incubated overnight at 4°C. Aliquots (100 μL) of the mixture were pipetted into the coated immunoplate microwells, and incubated for 3 h at 37°C. The plates were then washed four times with a 150 mmol/L NaCl, 0.025% (v/v) Tween 20 washing solution. Subsequently, bound anti-apoB antibodies were detected by using peroxidase-conjugated anti-mouse antibodies (BioRad, Richmond, CA). A colorimetric reaction was developed by adding 100 μl of a freshly prepared 0.4 g/L *o*-phenylenediamine, 0.68 g/L hydrogen peroxide solution. Finally, the reaction was stopped by the addition of 50 μl of H_2SO_4 (2.5 mol/l), and absorbances were read at 490 nm with a photometry tool on the Biomek 1000 Biorobotics station. Data were saved on a PC-IBM computer and were treated by using the Beckman Immunofit EIA/RIA Data Analysis Software. The reactivity of Mabs was estimated by determining the LDL protein concentration required for 50% inhibition of maximal binding of Mabs to the LDL-coated plate (IC50) (28).

Electrophoretic analysis of LDL subfractions

The mean apparent diameter of plasma LDL particles was determined by nondenaturating electrophoresis in 20–160 g/L polyacrylamide gradient gels (PAA 2/16; Pharmacia, Uppsala, Sweden) (30). The electrophoresis conditions were as previously described (30). At the end of the electrophoresis, the gels were fixed, stained with Coomassie brilliant blue G, and destained (31). The distribution profile of LDL was finally obtained by densitometric scanning of the gels on a Bio-Rad GS-670 imaging densitometer. The apparent diameter of the predominant LDL subfraction was determined by comparison with ferritin (diameter, 12.20 nm; Pharmacia), thyroglobulin (diameter, 17.00 nm; Pharmacia), and carboxylated latex beads (diameter, 38.00 nm; Duke Scientific) that were subjected to electrophoresis with the LDL samples.

Lipid analyses

All chemical assays were performed on a COBAS-BIO centrifugal analyser (Roche, Basel, Switzerland). Total cholesterol, unesterified cholesterol, phopholipids, and triglycerides were measured by enzymatic methods using Boehringer Mannheim reagents. Plasma HDL-cholesterol concentrations were measured after selective precipitation of apoB-containing lipoproteins with Boehringer phosphotungstic acid/$MgCl_2$ reagent as recommended by the manufacturer. The plasma VLDL + LDL cholesterol concentrations were calculated as the difference between total cholesterol and HDL-cholesterol levels.

Statistical analysis

ANOVA was used to determine the significance of the difference between data means. Coefficients of correlation r and ρ were calculated by using linear regression and Spearman rank correlation analysis, respectively.

RESULTS

Effect of lipid transfers and triglyceride hydrolysis on the size and composition of LDL particles

LDL were either directly isolated from non-incubated human plasma ('Control' samples), modified in the presence of VLDL by plasma lipid transfer activity alone ('Transfer, −LPL' samples), or pretreated with both plasma lipid transfer activity and triglyceride hydrolysis ('Transfer, +LPL' samples) (see Materials and Methods). The size and the lipid composition of resulting LDL particles are presented in **Table 1.** In accordance with previous studies (6, 8), plasma lipid transfer activity induced significant decreases in the unesterified cholesterol, cholesteryl ester, and phospholipid contents of LDL. Conversely, the triglyceride content of 'Transfer, −LPL' LDL was markedly and significantly increased. As compared with non-lipolyzed LDL ('Transfer, −LPL' samples), incubation in the presence of LPL ('Transfer, +LPL' samples) induced a marked, significant reduction in the triglyceride content of LDL (Table 1). In addition, a slight but significant reduction in the phospholipid content of LDL was also observed (Table 1). In contrast, neither unesterified cholesterol nor cholesteryl ester contents of LDL were affected by the LPL treatment.

Changes in LDL composition were associated with changes in LDL size. Compared with 'Control' LDL, a slight but significant increase in the mean size of the major LDL subfraction was observed in 'Transfer, −LPL' samples (Table 1). Conversely, triglyceride hydrolysis ('Transfer, +LPL' samples) significantly reduced the mean apparent diameter of the major LDL subfraction as compared with both 'Control' and 'Transfer, −LPL' samples (Table 1). The combined effects of lipid transfer activity and triglyceride hydrolysis could result in the transformation of typical LDL pattern A in 'Control' samples (mean diameter greater than 25.5 nm) into typical LDL pattern B in 'Transfer,

TABLE 1. Effect of lipid transfers and triglyceride hydrolysis on the size and lipid composition of plasma LDL particles

	LDL Samples		
	Control	Transfer, −LPL	Transfer, +LPL
		nm	
Size of major LDL subfraction	25.8 ± 0.5	26.1 ± 0.4[a]	24.8 ± 0.3[a,b]
		g/g LDL protein	
Lipid content			
UC	0.66 ± 0.08	0.54 ± 0.09[a]	0.56 ± 0.09[a]
CE	1.91 ± 0.48	1.55 ± 0.38[a]	1.56 ± 0.41[a]
PL	1.15 ± 0.19	0.97 ± 0.17[a]	0.92 ± 0.20[a,b]
TG	0.24 ± 0.06	0.75 ± 0.15[a]	0.29 ± 0.09[b]

Plasma from 12 normolipidemic subjects was supplemented with a lecithin:cholesterol acyltransferase inhibitor (iodacetate, 1.5 mmol/L) and was either maintained at 4°C (Control) or incubated for 24 h at 37°C in the presence of VLDL (final concentration of added triglyceride, 1g/L) (Transfer, −LPL). Subsequently, LDL were isolated by ultracentrifugation, dialyzed against TBS, and incubated for 2 h at 37°C in the absence (Transfer, −LPL) or in the presence (Transfer, +LPL) of bovine milk lipoprotein lipase (LPL), and the size and lipid content were determined as described in Materials and Methods. The unesterified cholesterol (UC), cholesteryl ester (CE), phospholipid (PL), and triglyceride (TG) contents are expressed as gram per gram of LDL protein. Data means were compared using ANOVA.

[a] $P < 0.05$ versus homologous Control sample.

[b] $P < 0.05$ versus homologous Transfer, −LPL sample.

+LPL' samples (mean diameter lower than 25.5 nm) (7).

Effect of lipid transfer activity and triglyceride hydrolysis on apoB immunoreactivity

Alterations in apoB immunoreactivity induced by lipid transfers and triglyceride hydrolysis were assessed by inhibition analysis as described under Materials and Methods. **Figure 1** shows typical competitive curves obtained in native plasma LDL with either 2D8 or 4G3 monoclonal antibodies. The slopes of inhibition curves, which were determined by using the Beckman Immunofit EIA/RIA Data Software, did not differ significantly from one LDL preparation to another (slopes: 1.04 ± 0.16, 0.99 ± 0.24, and 1.04 ± 0.14 for twelve 4G3 curves obtained with 'Control', 'Transfer, −LPL', and 'Transfer, +LPL' LDL, respectively; 0.65 ± 0.14, 0.63 ± 0.10, and 0.65 ± 0.10 for twelve 2D8 curves obtained with 'Control', 'Transfer, −LPL', and 'Transfer, +LPL' LDL, respectively). As the affinity of Mabs for control and modified LDL did not differ significantly, the immunoreactivity of 2D8 and 4G3 epitopes was evaluated from the ELISA curves by the calculation of the IC50 value which corresponded to the LDL protein concentration required for 50% inhibition of maximal binding of Mabs to the LDL-coated plate. The IC50 values are inversely related to the apoB immunoreactivity.

As shown in **Table 2,** lipid transfer activity alone ('Transfer, −LPL' samples) induced a significant increase in the IC50 values obtained with either Mabs 2D8 or Mabs 4G3. In contrast, modification of LDL with both lipid transfer activity and triglyceride hydrolysis

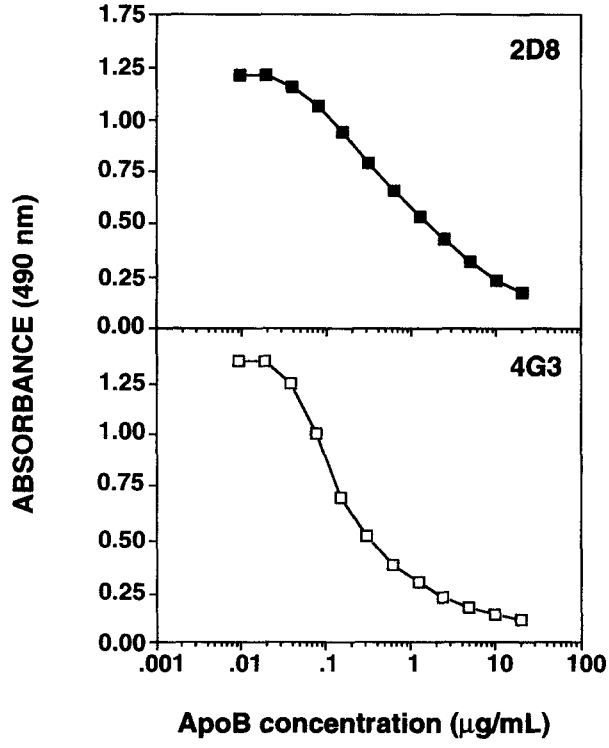

Fig. 1. Competitive inhibition curves obtained with anti-apoB monoclonal antibodies 2D8 and 4G3. Isolated plasma LDL were incubated overnight in 96-well plates at room temperature as described under Materials and Methods. Subsequently, serial dilutions of native plasma LDL were mixed with constant amounts of Mabs, and mixtures were added to coated microwells (see Materials and Methods). AntiapoB antibodies bound to the wells were detected by using peroxidase-conjugated, anti-mouse antibodies, and absorbances were read at 490 nm. Each point was determined in duplicate.

TABLE 2. Effect of lipid transfers and triglyceride hydrolysis on IC50 values

Monoclonal Antibodies	LDL Samples		
	Control	Transfer, −LPL	Transfer, +LPL
	μg/mL		
2D8	1.49 ± 0.62	2.15 ± 0.89[a]	1.59 ± 0.57[b]
4G3	0.18 ± 0.08	0.25 ± 0.12[a]	0.18 ± 0.07[b]

LDL samples are as described in Table 1. The immunoreactivity of 2D8 and 4G3 apoB epitopes was assessed using inhibition analysis, and IC50 values were calculated as described in Materials and Methods. IC50 values are expressed as μg/mL and are mean ± SD of 12 distinct LDL preparations. Data means were compared using ANOVA.

[a] $P < 0.05$ versus homologous Control sample.

[b] $P < 0.05$ versus homologous Transfer, −LPL sample.

('Transfer, +LPL' samples) significantly reduced the IC50 values as compared with 'Transfer, −LPL' LDL. No significant differences were found between 'Transfer, +LPL' LDL and 'Control' LDL.

As shown in **Table 3,** among all the modified LDL fractions studied (n = 36), significant relationships were observed between the triglyceride content of LDL and the IC50 values. Similar observations were made by using either 2D8 or 4G3 Mabs. In contrast, neither the size nor the other lipid parameters of in vitro modified LDL correlated significantly with IC50 values measured with 2D8 and 4G3 Mabs (Table 3). In support of a direct relationship between the triglyceride content of LDL and the immunoreactivity of apoB, significant correlations between changes in IC50 values and changes in the triglyceride content of LDL were observed after treatment with either lipid transfer activity alone or lipid transfer activity combined with triglyceride hydrolysis. Indeed, as shown in **Fig. 2,** the CETP-mediated increase in the triglyceride content of 'Transfer, −LPL' LDL as compared with 'Control' LDL correlated positively and significantly with the increase in IC50 values. Furthermore, the LPL-mediated changes in the triglyceride content of 'Transfer, +LPL' LDL as compared with 'Transfer, −LPL' LDL correlated positively and significantly with concomitant changes in the IC50 values. In all cases, consistent observations were made with 2D8 and 4G3 Mabs.

In summary, lipid transfer activity, which promotes the formation of large-sized, triglyceride-enriched LDL, results in a significant decrease in the immunoreactivity of 2D8 and 4G3 epitopes as compared with control, untreated LDL. In turn, triglyceride hydrolysis, which promotes the formation of small-sized, core-depleted LDL, leads to a significant increase in the immunoreactivity of 2D8 and 4G3 epitopes as compared with triglyceride-enriched, non-lipolyzed LDL. Overall, alterations in apoB immunoreactivity observed in the present study were shown to relate significantly to the triglyceride content of LDL but not to the other parameters measured.

Relationships between the size and lipid content of native plasma LDL and the immunoreactivity of apoB

In order to assess further the relevance of the observations made with in vitro modified LDL, native LDL were isolated from 12 distinct normolipidemic plasmas and relationships between the expression of 2D8 and 4G3 epitopes with the size and the lipid composition of LDL were investigated. In good agreement with data obtained with in vitro modified LDL, only the triglyceride content of plasma LDL, and none of the other parameters, correlated positively and significantly with IC50 values (**Table 4**). Again, consistent observations were made with 2D8 and 4G3 Mabs.

Relationships between the immunoreactivity of apoB in plasma LDL and the total plasma lipid parameters

Finally, the total plasma lipid compositions, including plasma triglyceride, total cholesterol, VLDL + LDL cholesterol, and HDL cholesterol levels, were compared to apoB immunoreactivity in corresponding isolated LDL fractions. As shown in **Table 5,** only the plasma triglycer-

TABLE 3. Correlation of IC50 values with the size and lipid content of in vitro modified LDL (n = 36)

Lipid Content of LDL	IC50			
	2D8		4G3	
	r	*P*	*r*	*P*
UC	−0.161	NS	−0.057	NS
CE	0.168	NS	0.242	NS
PL	0.130	NS	0.174	NS
TG	0.493	0.002	0.442	0.007
LDL size	−0.151	NS	−0.205	NS

LDL samples were as described in Table 1, and IC50 values, lipid content, and size of LDL were determined as described in Materials and Methods. UC, unesterified cholesterol; CE, cholesteryl ester; PL, phospholipid; TG, triglyceride. Coefficients of correlation *r* were determined using linear regression analysis; NS, non-significant.

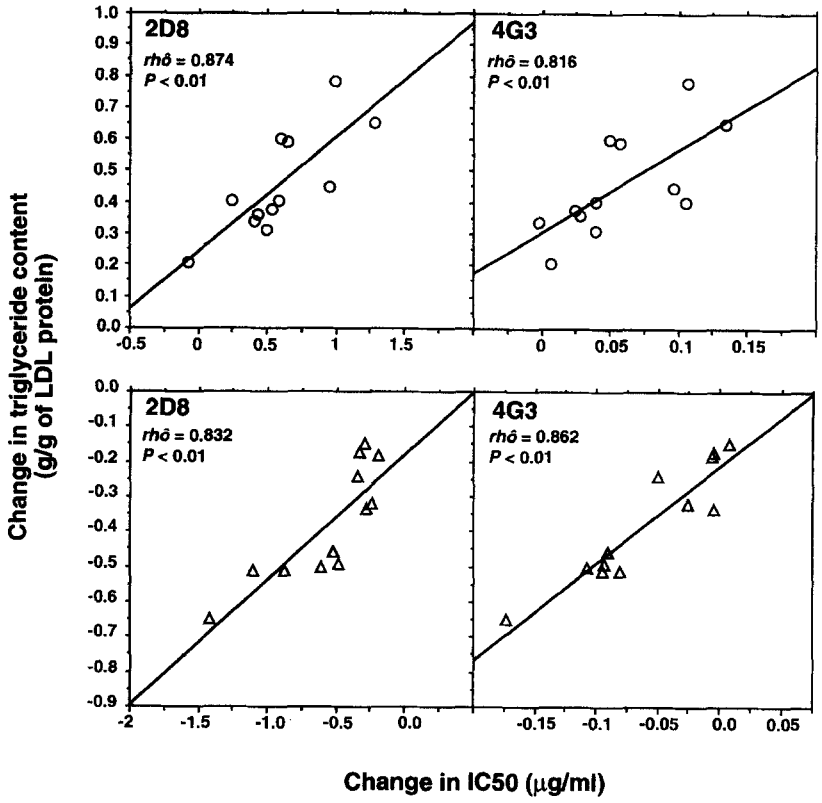

Fig. 2. Correlation between changes in the triglyceride content and changes in the immunoreactivity of 2D8 and 4G3 apoB epitopes in treated LDL. LDL were either directly isolated from non-incubated plasma ('Control' LDL), modified by lipid transfer activity alone ('Transfer, −LPL' LDL), or pretreated with both lipid transfer activity and triglyceride hydrolysis ('Transfer, +LPL' LDL). Upper panels present the relationships between changes in the triglyceride content of LDL and changes in apoB immunoreactivity (Δ IC50) in 'Transfer, −LPL' LDL as compared with 'Control' LDL. Lower panels present the relationship between changes in the triglyceride content of LDL and changes in apoB immunoreactivity (Δ IC50) in 'Transfer, +LPL' LDL as compared with homologous 'Transfer, −LPL' LDL. Correlations were analyzed by using Spearman rank correlation analysis.

ide levels correlated negatively and significantly with apoB immunoreactivity in the plasma LDL fraction, and this with both 2D8 and 4G3 Mabs.

DISCUSSION

Small, dense LDL, which predominate in the LDL pattern B described by Austin and Krauss (7), have been shown to be associated with a highly atherogenic plasma lipoprotein profile and with an increased risk for coronary artery disease (32–36). Although the reasons that account for the high atherogenic potential of small-sized, triglyceride-rich LDL still remain to be clearly established, studies during the last decade suggest that they might relate to the high interaction of small-sized LDL with intimal proteoglycans, to their lower binding affinity for the LDL receptor, and to their elevated oxidability (36). Interestingly, the mechanism that leads to the size reduction of plasma LDL particles, and, in particular, promotes the transformation of typical LDL

TABLE 4. Correlation of IC50 values with the size and lipid content of LDL directly isolated from normolipidemic plasma samples

	IC50			
	2D8		4G3	
Lipid Content of LDL	ρ	*P*	ρ	*P*
UC	−0.273	NS	−0.098	NS
CE	0.287	NS	0.448	NS
PL	0.267	NS	−0.168	NS
TG	0.746	<0.02	0.767	<0.02
LDL size	−0.524	NS	−0.517	NS

LDL were ultracentrifugally isolated from 12 normolipidemic plasma samples. IC50 values, as well as lipid content and the size of LDL, were determined as described in Materials and Methods. UC, unesterified cholesterol; CE, cholesteryl ester; PL, phospholipid; TG, triglyceride. Coefficients of correlation ρ were calculated using Spearman rank correlation analysis; NS, non-significant.

TABLE 5. Correlation of IC50 values with total plasma lipid parameters

	IC50			
	2D8		4G3	
Plasma Lipids	ρ	*P*	ρ	*P*
Triglycerides	0.662	<0.03	0.733	<0.02
Total cholesterol	0.196	NS	0.179	NS
HDL cholesterol	−0.425	NS	−0.256	NS
VLDL + LDL cholesterol	0.322	NS	0.294	NS

LDL were isolated from 12 normolipidemic plasmas. IC50 values were evaluated as described in Materials and Methods. Coefficients of correlation ρ were calculated using Spearman rank correlation analysis; NS, non-significant.

pattern A into typical LDL pattern B (7) has been now elucidated. It involves the CETP-mediated replacement of cholesteryl esters by triglycerides in the LDL core, followed by the lipase-mediated hydrolysis of newly acquired triglycerides (5, 6). In recent studies from our group, the combined effects of lipid transfers and triglyceride hydrolysis were shown to result in significant alterations in the size and lipid composition, as well as oxidability of LDL particles (6, 8). In the present study, we took advantage of the well-characterized and well-controlled system involving the combination of lipid transfer activity and triglyceride hydrolysis to investigate the effect of alterations in the size and lipid content of LDL particles on apoB immunoreactivity.

In agreement with previous observations (6, 8), when LDL were subjected to lipid transfer activity alone by incubation of total human plasma in the presence of VLDL supplementation, the replacement of cholesteryl esters by triglycerides in the LDL core was observed. The triglyceride enrichment of LDL core was accompanied by a slight but significant increase in LDL size. Conversely, the treatment of triglyceride-rich LDL by lipoprotein lipase was mainly characterized by significant decreases in both the size and the triglyceride content of LDL. It appears, therefore, that sources of lipid transfer activity and triglyceride hydrolysis, used in combination or not, provided us with convenient tools to induce controlled alterations in the size and triglyceride content of LDL. In the present report, the immunoreactivity of apoB was evaluated using two distinct monoclonal antibodies, 2D8 and 4G3, directed against epitopes located between amino acid residues 1438 and 1480 and between residues 2980 and 3084, respectively (26). We chose to investigate alterations in the reactivity of apoB in modified LDL by using these two distinct monoclonal antibodies for the following reasons: *i)* both 2D8 and 4G3 epitopes are conformational determinants that are dependent on the presence of lipids, and thus are susceptible to variation according to the structure and lipid composition of LDL (11, 37), and *ii)* unlike 2D8, the 4G3 epitope is located in the region of the binding site to the LDL receptor (24, 25, 27). By using inhibition analysis, we observed that IC50 values were significantly higher in lipid transfer-modified LDL than in control plasma counterparts, indicating that the replacement of cholesteryl esters by triglycerides can significantly decrease the expression of both 2D8 and 4G3 epitopes at the LDL surface. In a similar experimental system making use of isolated lipoprotein fractions and partially purified plasma lipid transfer proteins, Kinoshita, Krul, and Schonfeld (20) also reported a significant decrease in apoB epitope expression in triglyceride-enriched LDL. However, in the latter report, the CETP-mediated replacement of cholesteryl esters by triglycerides in the LDL core was surprisingly accompanied by a significant decrease in the mean diameter of LDL rather than an increase as reported in other studies (5, 6, 8). In another study (21), raising the triglyceride content of LDL in vitro by transferring triglycerides from an excess of VLDL significantly decreased the immunoreactivity of the apoB region closed to residue 1022, but not of the ligand region to the LDL receptor. In previous studies (20, 21), in vitro production of small-sized LDL particles by lipolysis of triglyceride-rich LDL (5, 6, 8), as it actually occurs in vivo in hypertriglyceridemia, was not achieved. In the present report, we chose to study alterations in apoB immunoreactivity not only after triglyceride enrichment of LDL, but also after lipolysis of resulting triglyceride-rich LDL. Thus, in an attempt to reproduce what may occur in vivo, the triglyceride-rich LDL resulting from the action of CETP were incubated in the presence of lipoprotein lipase as a source of triglyceride hydrolysis activity. The present report demonstrated that triglyceride hydrolysis decreased IC50 values calculated with both 2D8 and 4G3 antibodies, indicating a significant increase in the immunoreactivity of corresponding epitopes on the apoB molecule. In support of the effect of triglyceride hydrolysis in inducing changes in the local conformation of apoB, Aviram and coworkers (18) demonstrated that the arrangement of apoB lysines is perturbed by the hepatic lipase-mediated hydrolysis of LDL triglycerides,

and the immunoreactivity of a monoclonal antibody directed against the LDL-receptor recognizing domain of apoB was higher in lipolyzed LDL than in non-lipolyzed counterparts. Although alterations in apoB immunoreactivity were also observed in lipoprotein lipase-treated VLDL, conclusions in that particular case were rather confusing with both enhanced and reduced apoB immunoreactivity being reported (15, 38).

From data obtained with LDL modified in the presence of lipid transfer activity associated or not with triglyceride hydrolysis, both the size and the triglyceride content of LDL arose as two putative candidates in determining apoB immunoreactivity. Indeed, the lipid transfer-mediated increase in LDL size was accompanied by a significant decrease in apoB immunoreactivity while, in turn, the lipoprotein lipase-mediated decrease in the size of triglyceride-rich LDL was associated with a significant increase in apoB immunoreactivity, suggesting that LDL size is inversely related to apoB epitope expression. In parallel, decreased apoB immunoreactivity in LDL was associated with increased triglyceride content, whereas enhanced apoB immunoreactivity was observed when the triglyceride content of triglyceride-rich LDL was substantially reduced, suggesting that LDL triglycerides might reduce the apoB epitope expression. In addition to the compositional analysis conducted in the present study, one recent study from our group (8) showed some significant differences in the non-esterified fatty acid (NEFA) content of modified LDL, with significantly greater NEFA contents of both 'Transfer, −LPL' and 'Transfer, +LPL' LDL than 'Control' LDL. As lipid transfers and triglyceride hydrolysis were shown to exert opposite effects on apoB immunoreactivity in the present study, NEFAs are unlikely to influence the immunoreactivity of 2D8 and 4G3 epitopes in modified LDL. Interestingly, the size and the triglyceride content of LDL were alternatively described as determinants of apoB immunoreactivity in previous studies when LDL were modified in vitro by using various experimental systems, i.e., the CETP-mediated triglyceride enrichment of LDL by incubation with VLDL (18, 20, 21) or with triglyceride emulsion (22), the CETP-mediated reduction of the triglyceride content of triglyceride-rich LDL by incubation with normal HDL (19), and the lipase-mediated hydrolysis of LDL triglycerides (18). In fact, previous data are controversial and either the size (22) or the triglyceride content (18–21) of LDL were reported to constitute the most important determinant of apoB immunoreactivity. This might relate, at least in part, to the fact that the size and the triglyceride content of LDL constitute two linked parameters. In contrast to the marked changes in size and triglyceride content of LDL that were induced in previous studies (22), we chose in the present study to promote controlled alterations that were compatible with what may occur in plasma. Under those conditions, only changes in the triglyceride content of LDL, but not changes in their size, correlated significantly with changes in the reactivity of 2D8 and 4G3 epitopes. Therefore, in the present study, the triglyceride content of LDL constituted the single major determinant of the immunoreactivity of 2D8 and 4G3 epitopes, suggesting that LDL size is only second to triglycerides in determining immunoreactivity of both epitopes. Although the latter statement is compatible with previous observations indicating that the immunoreactivity of 2D8 and 4G3 epitopes is dependent on the lipid composition of LDL (37), the mechanism by which triglycerides can alter apoB immunoreactivity remains to be elucidated, and it might relate to the ability of core triglycerides to influence either the conformation of apoB directly or its implication in LDL structure. Finally, it must be noted that the absence of significant relationships between differences in apoB immunoreactivity and differences in LDL size observed in the present study might relate to the fact that changes in the triglyceride content of modified LDL were of greater magnitude than concomitant changes in LDL size.

In support of a specific role of LDL triglycerides in determining the immunoreactivity of apoB, the IC50 values calculated with isolated plasma LDL fractions correlated significantly with their triglyceride content, but not with their size. Consistent relationships between the triglyceride content of LDL core and apoB immunoreactivity have been previously reported with some but not all anti-apoB Mabs (9, 10). Finally, by using 2D8 and 4G3 Mabs, we observed a positive and significant correlation between the total plasma triglyceride levels and the IC50 values calculated for LDL isolated from normolipidemic plasmas. In agreement with a direct relationship between total plasma triglyceride levels and LDL apoB immunoreactivity, the triglyceride content of LDL has been shown to be a predictable function of total plasma triglycerides (21), and the hypotriglyceridemic treatment of hypertriglyceridemic patients, which is associated with a significant reduction in the triglyceride content of LDL, has been shown to increase the binding affinity of specific antibodies to apoB (17).

In conclusion, controlled alterations in the structure and lipid composition of LDL, by using lipid transfer activity and triglyceride hydrolysis, revealed that the triglyceride content of LDL can constitute the major determinant of immunoreactivity of 2D8 and 4G3 apoB epitopes. Whereas the physiological impact of reduced immunoreactivity of 2D8 and 4G3 epitopes in triglyceride-rich LDL remains to be clearly established, previous studies demonstrated that the weaker exposition of apoB epitopes, and in particular of the ligand domain to the LDL receptor, can secondarily affect the ability of LDL to interact with its cell surface receptor (17, 18,

20–22). Whether the lower immunoreactivity of some apoB epitopes might relate to the longer residence time of small-sized, triglyceride-rich LDL in vivo deserves further investigation.

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