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Immunological properties of apoB-containing lipoprotein particles in human atherosclerotic arteries

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Abstract In this study, we have documented immunochemical properties of apolipoprotein (apo) B-containing particles (LpB) extracted from human atherosclerotic lesions obtained during vascular reconstructive surgery of patients. These properties were compared to those of particles purified from corresponding atherosclerotic plasma and healthy control plasma. LpB immunoreactivities were tested in solid phase competitive binding radioimmunoassays using five anti-apoB monoclonal antibodies (MAb) for which epitopes have been previously located on the protein. The regions encompassed amino acids 405 to 539 (MAb B1), 1854 to 1879 (MAb B4), 3506 (MAb BA11), and 4355 (MAb BL3). The fifth antibody (MAb BL5) recognizes a conformationally expressed epitope. LpB from lesions presented a significantly decreased immunoreactivity as compared to LpB from respective plasma except for the epitope recognized by MAb BA11 located precisely in the low density lipoprotein (LDL) receptor binding site. The accessibility of the four sequential epitopes was similar on LpB from atherosclerotic and healthy plasma while it was decreased for the conformational one in LpB from atherosclerotic samples. These altered immunoreactivities were not related to changes in chemical composition of LpB as this was quite comparable in all preparations. With regard to electronegativity, apoB fragmentation, immunological accessibility, and size distribution of the particles, changes seem to increase in the following order from healthy plasma, atherosclerotic plasma, and the corresponding lesions. The results confirm some structural characteristics of oxidatively modified particles from human atherosclerotic lesions and to a lesser degree from respective plasma, but more specifically demonstrate a global conformational change in LpB from lesions, this change being perhaps initiated in the plasma.—**Tailleux, A., G. Torpier, B. Caron, J-C. Fruchart, and C. Fievet.** Immunological properties of apoB-containing lipoprotein particles in human atherosclerotic arteries. *J. Lipid Res.* 1993. **34:** 719–728.

Supplementary key words monoclonal antibody • immunological accessibility • in vivo oxidative process • atherogenesis

Early atherosclerotic lesions are characterized by a massive accumulation of foam cells. In the progression of atherosclerosis, these lesions can develop into fatty streaks and plaques. Careful morphological and immunohistologic chemical studies have shown that foam cells in early lesions are derived predominantly from macrophages (1). The accumulation of lipid droplets that characterizes the foam cells is today explained by the uptake of modified low density lipoproteins (LDL) via the scavenger-receptor (2). Indeed, chemical modifications of LDL (acetylation, acetoacetylation, modification by malonaldehyde or copper ions) have been shown to lead, in vitro, to the accumulation of cholesteryl esters in macrophages. However, these modifications do not occur physiologically, therefore another form of LDL modification may be implicated. A sequence of events that could occur under physiological conditions has been described, and so the modification of LDL by oxidative processes has been postulated (3). This was reinforced by the fact that oxidatively modified LDL has been actually extracted from atherosclerotic lesions in rabbits and in humans (4–8). These LDL-like particles have some of structural and functional properties of in vitro oxidized LDL (9) and, further, these react with antibodies specific for oxidatively modified LDL (10). Very little data about immunochemical properties of such in vivo modified LDL, expressed as changes in apoB epitope accessibility, are, however, available. Such an approach has been already published by three different groups using in vitro oxidized LDL, but their results differ quite considerably (11–14). The oxidative procedures performed in this way were different, generating, then, heterogeneous

Abbreviations: VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; LpB, apoB-containing particles; MAb, monoclonal antibody; LpB-A, LpB-P, and LpB-C, LpB particles from patients' arteries, patients' plasma, and control plasma, respectively.

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lipoproteins that were modified to variable degrees and their epitopes were probably exposed differently. Having at our disposal anti-apoB monoclonal antibodies (MAbs) for which determinants have been precisely located on the apoB molecule (15, 16), we have compared the immunological properties of apoB-containing lipoprotein particles extracted from human arteriosclerotic lesions and from the respective plasma.

MATERIALS AND METHODS

Subjects

The patients (n = 10, mean age 58 ± 10 yr) were men admitted to hospital for vascular reconstructive surgery. They were not included if they *1)* had concomitant renal or hepatic insufficiency of thyroid dysfunction; *2)* had been affected by a myocardial infarction in the 3 previous months; or *3)* took lipid-lowering agents.

The control subjects (n = 5, mean age 63 ± 6 yr) were men who had come voluntarily to the Lille Pasteur Institute (Preventive Medicine Center) for a health-control examination. They were not included if they *1)* had a history of atypical chest pain, angina pectoris, electrocardiographic signs indicative of coronary heart disease, concomitant renal or hepatic insufficiency, or thyroid dysfunction; or *2)* took lipid-lowering agents or nitrates. At this age range, it is highly likely, statistically, that some or all of these controls had subclinical atherosclerosis. However, the choice of such persons tends to diminish the apparent differences we would obtain between patients and controls, lending credence, therefore, to our results that there are no spurious associations. On a technical ground all the subjects were selected on the absence of Lp[a] (see below).

Sample acquisition and tissue processing

Blood samples were obtained from patients just before surgery and from controls after a 12 h overnight fast, in Vacutainer tubes containing preservatives as follows: D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK) (1 μM), ethylenediamine-tetraacetic acid (EDTA) (3.2 mM), sodium azide (NaN_3) (2% wt/vol) gentamicin (0.08%), aprotinin (10,000 U/l), and sodium chloride (NaCl) (0.15 M). Blood cells were sedimented by low speed centrifugation, after which preservatives were added to the plasma : phenylmethanesulfonyl fluoride (PMSF) (1 mM) and benzamidine (1 μM) (17). Plasma was stored at 4°C in the dark under nitrogen gas until further processed within 48 h.

Samples of arteries were obtained at the time of surgery. They were derived from carotid (n = 3), iliac (n = 1), and femoral arteries (n = 3), and abdominal aorta (n = 3). No biopsies of severe plaque areas or complicated lesions were examined.

To prevent proteolytic enzyme degradation, bacterial growth, and hydroperoxidation of the lipoproteins, the tissues, after excision, were placed immediately in a cold buffer-saline solution containing HEPES (N-2-hydroxyethyl piperazine-N'2-ethanesulfonic acid) (10 mM) and NaCl (15 mM) supplemented with butylhydroxytoluene (BHT) (10 μM), EDTA (5 mM), benzamidine (2 mM), ε-aminocaproic acid (4 mM), PMSF (1 μM), gentamycin sulfate (0.01% wt/vol), and NaN_3 (0.02% wt/vol). Samples were then rapidly transferred to the laboratory in a refrigerated package.

Tissues were rinsed briefly in several changes of the buffer-saline solution to remove any loosely adherent blood components. The intima was dissected from the media and rinsed once with saline. Cleared pieces were then minced finely with a scalpel. The resulting gruel remained in the buffer for about 48 h, at 4°C, in the dark and under nitrogen gas, to permit diffusion of lipoproteins into the buffer. Such a gentle extraction procedure has been advised (6). The extract was sedimented by centrifugation (2000 rpm, 20 min, 4°C) and the supernatant, containing the lipoproteins, was pipetted off and kept for further separation.

Plasma determinations

Total plasma cholesterol, triglycerides, and phospholipids were automatically measured by enzymatic test kits from Boehringer-Mannheim (Mannheim, Germany), adapted to a Hitachi 705 analyzer. High density lipoprotein (HDL) cholesterol content was determined after precipitation of apoB-containing lipoproteins by phosphotungstic acid/Mg (Boehringer Mannheim).

Total apoB and apoA-I were quantified by an immunonephelometric assay adapted to a Behring Nephelometer Analyzer (BNA) (Behring Werke, Marburg, Germany) using commercial reagents. ApoC-III, apoE, and Lp[a] were measured by established immunoenzymometric assays (18-20).

ApoB-containing particles isolation

The lipoprotein-containing buffer aliquots derived from the arteries, from patients' plasma, and from control subjects' plasma were subjected to affinity chromatography on an anti-apoB-100-Sepharose column as previously described (21). The eluted LpB particles were concentrated by low-speed centrifugation (Centricon filters, Amicon Division, Beverly, MA), sterile-filtered (filter 0.22 μm, Minisart, Sartorius, Germany), and stored at 4°C, in the dark, under nitrogen gas. Particles obtained from patients' arteries, respective (patients') plasma, and control plasma were designated as LpB-A, LpB-P, and LpB-C, respectively.

From a pool of fresh normolipemic plasma, which also contained preservatives as above, an LpB standard batch was likewise purified, concentrated, and sterilized. It was stored at 4°C in sterile aliquots under nitrogen gas. We

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observed no proteolytic degradation or oxidative processes occurring throughout the time of study.

Iodination of LDL

LDL was isolated from a pool of fresh normolipemic plasma using sequential preparative ultracentrifugation (1.019 < d < 1.063 g/ml) (22). These lipoproteins were radioiodinated with $Na^{125}I$ according to a modification of McFarlane's procedure as described (23). ^{125}I-labeled LDL used in radioimmunoassays were kept in tightly capped vials, in the dark, under nitrogen gas at 4°C for no longer than 2 weeks. We needed to use three different preparations to perform the study. The mean specific activity of such material was 0.45 μCi/μg of protein.

Physical and chemical analysis of LpB preparations

Electrophoretic mobility. Cellulose acetate strips (Cellogel) were used. Electrophoresis, fixing, and protein staining were performed according to the instructions of the manufacturer (Sebia, Issy les Moulineaux, France). R_f was measured relative to LpB-C.

Lipid analysis. Total cholesterol, triglycerides, and phospholipids were automatically measured using the same procedure as for plasma determinations. Free cholesterol was determined manually with a modified Boehringer-Mannheim kit in which cholesteryl ester hydrolase was omitted. Cholesteryl esters were calculated as the difference between total and free cholesterol multiplied by a factor of 1.68.

Lipids were extracted with chloroform–methanol 2:1 (v/v) and phospholipids were further separated by high performance thin-layer chromatography (Silica Gel H, Merck, Germany) in a migrating solvent as previously described (24). Phospholipid subfractions were visualized by zinzade reagent and then quantified by densitometry (CD 60 densitometer, Desaga, Flotec, France).

Apolipoprotein quantification. Because of a different immunological behavior between LpB particles and the commonly used standard for apoC-III and apoE immunoassays, we had to separate out and quantitate both these apolipoproteins linked with apoB in the LpB particles by a non-immunological procedure. In contrast to apoE and apoC-III, the apoB content determination was possible using an immunological method that utilized a secondary plasma standard. Nevertheless, in order to validate the expression of results as molar ratios of apoC-III and apoE linked to apoB, it was necessary to measure apoB concentration in the same non-immunological system as other apolipoproteins. For that, a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 3–19% gels in Tris-HCl (0.1 M), glycine buffer (0.8 M) (pH 8.3), containing SDS (0.1% wt/vol) (150 V, 25 mA, 45 min, SE 250 Hoefer Shlighty II electrophoresis apparatus). Before the electrophoresis, the samples were heated for 3 min in a boiling water bath. The sample buffer contained Tris-HCl (0.05 M), SDS (1.5% wt/vol), glycerol (10% wt/vol), and bromophenol blue (0.001% wt/vol) (pH 6.8). No reducing agent was included in the sample buffer, in order not to reduce apoA-II; otherwise the R_f values of its monomer would be confused with that of apoCs. Protein bands were visualized by staining in 0.03% Coomassie brilliant blue. We first studied the proportionality that could exist between the intensity of the coloration and the quantity of each apolipoprotein present in the samples. For that, the distribution was analyzed by densitometry and peak areas were integrated automatically (Digital Imaging Station, Biocom 500, Touzart and Matignon, France). We then established a standard curve by running several different amounts of lipoprotein fraction prepared by ultracentrifugation at a density below 1.21 g/ml in which apoB had been previously measured by an immunonephelometric assay (see above), as well as apoCs and apoE (18, 19, 25). We plotted peak areas versus concentrations. Using this linear curve as reference, the concentration of each apolipoprotein in the samples could be determined.

It was, therefore, important to select only subjects without Lp[a] detectable levels to avoid any overestimation of apoB due to the presence of an apo[a] isoform B in plasma.

Fragmentation of apoB. After SDS-PAGE performed as above, separated proteins were transferred to nitrocellulose paper (0.45 μm pore size, Hoefer Sciences Instruments) for 1 h, at 4°C in Tris (25 mM), glycine (192 mM), methanol (20%, vol/vol), pH 8.3, using a "Temblot" apparatus (Hoefer Sciences Instruments). The remaining protein binding sites were then saturated with Tris (20 mM), NaCl (150 mM), Tween (0.1% wt/vol), powdered nonfat milk (1% wt/vol) (TSM buffer), pH 8.00, for 90 min at room temperature. To detect the apoB immunoreactive bands, the replicas were then incubated overnight at 4°C with polyclonal anti-apoB antibodies diluted in the saturating buffer without milk (TS buffer). After three washings in the TS buffer, rabbit anti-immunoglobulins (Diagnostic Pasteur) diluted in the TS buffer were added. After 1 h incubation at room temperature, nitrocellulose papers were washed two times in TS buffer and one time in phosphate-buffered saline (PBS) (0.1 M), pH 7.4. Bands were revealed by staining the nitrocellulose paper in the substrate solution containing 1 ml chloro-1-naphtol (1.8% wt/vol) in methanol, 50 μl H_2O_2 (110 vol), and 100 ml PBS (0.1 M), pH 6.5.

Size determinations. Lipoproteins were negatively stained and analyzed by electron microscopy as previously described (26). The fractions were first dialyzed against a volatile buffer containing ammonium acetate (0.125 M), ammonium carbonate (2.6 M), and EDTA (0.26 mM). Dilutions of lipoproteins that contained approximately 200–400 μg/ml protein were mixed with equal proportions of phosphotungstic acid (2% wt/vol, pH 7.5). Ali-

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TABLE 1. Lipid and lipoprotein values in atherosclerotic patients and controls

Parameters	Patients (n = 10)	Controls (n = 5)	Reference Normal Range[a]
		mg/dl ± SD	
Total cholesterol	179 ± 42	224 ± 35	(180-270)
Triglycerides	99 ± 29	95 ± 33	(65-150)
HDL-cholesterol	23 ± 9[b]	54 ± 23	(34-62)
ApoB	96 ± 22	98 ± 24	(55-130)
ApoA-I	98 ± 17[c]	171 ± 16	(120-215)
ApoC-III	1.4 ± 0.3[c]	2.2 ± 0.6	(1.8-6.1)
ApoE	4.2 ± 1.6	5.6 ± 0.7	(1.3-6.1)

[a]Reference normal range values for 55-70-year-old males. The values were determined in a presumably healthy population of about 1000 subjects.
[b]$P < 0.05$.
[c]$P < 0.001$.

quots of this mixture were placed on coated carbon grids. The excess fluid was removed with blotting paper, and the grids were allowed to air-dry for 5 min. Electron micrographs were obtained with a Philips 420 electron microscope at an accelerating voltage of 80 kV and a magnification of 51,000.

Mean diameters of particles were obtained by measuring the diameters of 500 particles per sample.

The particles were statistically compared by a median test followed by a Chi-Square test.

Immunological analysis

The immunoreactivity of apoB epitopes was determined in each LpB preparation by a radioimmunoassay using five previously described anti-apoB MAbs (B1, B4, BA11, BL3, BL5) (15, 16).

The localization of the specific epitopes along the apoB molecule has been mapped (16) between residues 405 and 539 (B1), 1854 and 1879 (B4), at residues 3506 (BA11), and 4355 (BL3). BL5 failed to react with any fragments, suggesting it only recognizes conformationally expressed epitopes.

Microtiter wells were coated with each MAb. After washing and saturation steps, serial triplicate increasing amounts of competitors (LpB-A, LpB-P, LpB-C, and LpB standard) (expressed as protein apoB content) were then added with a constant amount of ^{125}I-labeled LDL. After incubation the wells were washed and radioactivity was counted.

B/Bo ratios (where B and Bo are specific cpm bound in the presence and absence of competitors, respectively) versus lipoprotein concentrations were plotted. The displacement curves were linearized by logit-log transformation of the data (27) and the slopes were calculated. These correspond to the apparent affinity of the LpB preparations for each antibody. These slopes were compared by a test for heterogeneity based on the general linear model procedure (28). The apparent apoB content (number of epitopes) was calculated from competitive displacement curves. The results were expressed as a percentage of the standard to which an arbitrary expression of 100% was assigned to each epitope. Thus, a percentage value below 100 indicates that less competitor is needed to achieve the same degree of displacement of the labeled LDL ligand and reflects a better accessibility of the measured epitope. In contrast, a percentage value above 100 indicates a lower immunological accessibility of an epitope for its competitor. The reproducibility of the procedure was assessed from LpB standard data; the coefficients of variation between assays were around 10%. A statistical comparison of apoB contents between the different LpB preparations was performed using the Mann-Whitney test (U-test).

Correlation between chemical composition and immunoreactivity of LpB preparations was assessed by the Spearman rank correlation coefficients.

RESULTS

Characteristics of subjects

The plasma lipid and lipoprotein parameters from the patients and controls were measured (**Table 1**). The comparison of both groups showed significant decreases in HDL-cholesterol ($P < 0.05$), apoA-I ($P < 0.01$), and apoC-III ($P < 0.01$) in patients, whereas the other parameters were within the normal range values.

Extraction of lipoproteins from vascular tissue

To ensure the extraction procedure we performed on vascular tissue did not produce artifactual modifications of the lipoproteins, we compared LpB purified from three different plasma samples after their incubation for 48 h in extraction buffer with LpB directly extracted from the same three native plasma. We found no evidence for

TABLE 2. Chemical composition of LpB preparations from arteries, respective plasma, and control plasma

	LpB-A (n = 10)	LpB-P (n = 10)	LpB-C (n = 5)
Total cholesterol/apoB	2230 ± 230	2328 ± 445	2428 ± 460
Free cholesterol/apoB	706 ± 99	696 ± 136	736 ± 158
Cholesteryl esters/apoB	2729 ± 318	2743 ± 554	2843 ± 518
Triglycerides/apoB	587 ± 136	568 ± 290	558 ± 188
Phospholipids/apoB	710 ± 63[a]	810 ± 154	935 ± 224
ApoE/apoB	0.40 ± 0.35	0.64 ± 0.60	0.50 ± 0.32
ApoCs/apoB	0.44 ± 0.39	0.47 ± 0.58	0.41 ± 0.45

Results are expressed as molar ratios using 388, 877, 775, 38,000, and 7,977 as the mean molecular weights for cholesterol, triglycerides, phospholipids, apolipoprotein E, and apolipoproteins C, respectively. LpB-A: LpB from arteries; LpB-P: LpB from respective plasma; LpB-C: LpB from control plasma. Values are means ± standard deviation.
[a]$P < 0.05$, compared with LpB-P and LpB-C (U-test).

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TABLE 3. Phospholipid subclasses of LpB preparations from arteries, respective plasma, and control plasma

	LpB-A (n = 10)	LpB-P (n = 10)	LpB-C (n = 5)
	% of total phospholipids		
Lysolecithin	0.7 ± 0.8[a]	1.5 ± 0.8[a]	4.5 ± 0.9
Sphingomyelin	36.2 ± 4.7	34.2 ± 4.9	25.4 ± 7.2
Lecithin	59.5 ± 5.6	61.0 ± 5.5	66.5 ± 8.5
Others	3.6 ± 2.7	3.3 ± 2.2	3.6 ± 1.4

Values are means ± standard deviation. LpB-A: LpB from arteries; LpB-P: LpB from respective plasma; LpB-C: LpB from control plasma.
[a] $P < 0.05$, compared with LpB-C (U-test).

modification as regards chemical composition, electrophoretic mobility on cellulose acetate strips, and apoB immunoreactivity (data not shown). Moreover, when a LpB preparation was added to a pool batch of atherosclerotic intimal tissues, there was likewise no evidence for any oxidative modification.

The amount of LpB protein isolated from the tissues varied from 500 to 1000 μg of apoB per piece. This recovery allowed us to perform all subsequent experiments for each individual sample.

Extraction of lipoproteins from vascular tissue

To ensure the extraction procedure we performed on vascular tissue did not produce artifactual modifications of the lipoproteins, we compared LpB purified from three different plasma samples after their incubation for 48 h in extraction buffer with LpB directly extracted from the same three native plasma. We found no evidence for modification as regards μg of apoB per piece. This recovery allowed us to perform all subsequent experiments for each individual sample.

Physical and chemical properties of LpB from particles

In the ten cases studied, the electrophoretic mobility on cellulose acetate strips of LpB-A was more elevated than that of LpB-P (1.23 ± 0.15 vs. 1.10 ± 0.06, respectively, $P < 0.05$). Also, LpB-P showed a significantly greater electrophoretic mobility than LpB-C ($P < 0.05$). These results assumed that LpB-A and, to a lesser extent, LpB-P are more electronegative than LpB-C.

Table 2 shows the chemical composition of the particle preparations. Assuming there is only one apoB molecule (mol wt 549,000) per lipoprotein particle (29), the results are expressed in terms of molar ratios. Except for phospholipid content, which was slightly lower ($P < 0.05$ U-test) in LpB-A as compared with LpB-P, no significant difference was found with regard to the lipid or apolipoprotein content of the LpB particles, whatever their origins. Elevated values of standard deviation, particularly for apoE and apoCs, indicated a large heterogeneity in the particle compositions.

Table 3 shows the phospholipid subclass proportions in the different particle preparations. Lecithin seemed to decrease progressively from LpB-C to LpB-P to LpB-A and the relative amount of sphingomyelin increased in LpB-A and LpB-P as compared with LpB-C, but not significantly. The percentage composition of lysolecithin revealed a significant lower proportion in the LpB-A and LpB-P than in the LP-C.

The electrophoretic patterns of LpB-A, LpB-P, and LpB-C confirmed that the major protein was apoB-100, but also revealed other proteins of molecular masses 68, 46, 33, 28, 17, and 8 kDa identified by Western blot as serum albumin, apoA-IV, apoE, apoA-I, apoA-II, and apoCs, respectively (data not shown). No traces of Lp[a] were confirmed. A slight breakdown of apoB was observed in LpB-A, and the blot analysis confirmed the lower molecular weight fragmentation products to be related to apoB. This fragmentation was apparent to a lesser extent in LpB-P, but not in LPB-C (**Fig. 1**).

Particle sizes

Each particle sample was visualized by negative staining and electron microscopy. All of the preparations showed monomeric particles, without clusters defined by aggregated material thus permitting the measurement of particle diameters.

The statistical comparison of median sizes (U-test) indicates a shift towards larger sizes in LpB from arteries. This displacement is significant for LpB-A versus LpB-P ($P < 0.01$) and for LpB-P versus LpB-C ($P < 0.05$) (**Table 4**). Elevated values of standard deviation indicate once more the heterogeneity of LpB preparations. Thus on an

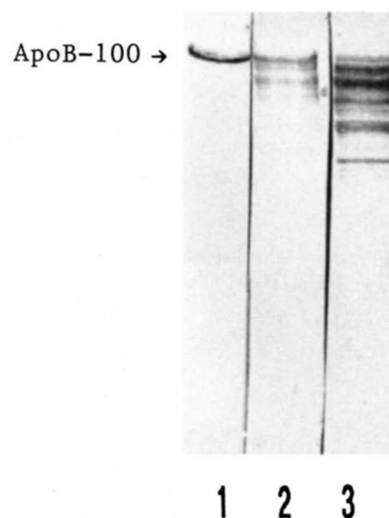

Fig. 1. SDS-PAGE and Western blot analysis of LpB isolated from the artery (LpB-A) and respective plasma (LpB-P) of one atherosclerotic subject and from one control plasma (LpB-C). Apolipoproteins were separated in 3–19% SDS-PAGE under nonreducing conditions and transblotted to a nitrocellulose membrane. Western blots were done using polyclonal apoB antisera (dilution 1 μg/ml). Lane 1: LpB-C; lane 2: LpB-P; lane 3: LpB-A. An arrow indicates the position of apoB-100. Each lane contains 15 μg protein.

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TABLE 4. Median sizes of LpB particles from arteries, respective plasma and control plasma

	LpB-A (n = 10)	LpB-P (n = 10)	LpB-C (n = 5)	LpB-A vs LpB-P	LpB-P vs LpB-C
Median size (Å)[a]	285 (± 21)	270 (± 20)	257 (± 3)	P < 0.01[b]	$P < 0.05$[b]

LpB-A: LpB from arteries; LpB-P: LpB from respective plasma; LpB-C; LpB from control plasma.
[a]Results are expressed as a mean median size ± standard deviation of 500 randomly selected LpB particles from each preparation.
[b]*P* values represent significance levels (U-test).

individual basis, three samples of LpB-A did not exhibit significant differences regarding size when compared to LpB-P. A larger heterogeneity occurred in LpB-P samples when compared to LpB-C, five samples being effectively larger, but five others having comparable sizes.

Immunoreactivity of LpB preparations

The abilities of the different preparations to compete with ^{125}I-labeled LDL for binding to five anti-apoB MAbs were compared. After linearization of the curves by logit-log transformation of the data, the calculation of the slopes provided the apparent affinity of the different LpBs for each MAb. We observed that one of the curves produced by LpB-A with MAb B1, two of those produced with MAb BA11, three of those produced with MAb BL3, as well as two of those produced with MAb BL5 had slopes that were statistically decreased from those of their corresponding LpB-P. In other LpB-A samples as well as LpB-P and LpB-C samples, no significant differences were found between the slopes of the samples and standard, demonstrating a similar apparent affinity in all these preparations. Thus, we could determine the apparent apoG content of each epitope in LpB fractions, reflecting their accessibility. That apparent apoB content corresponded to the relative capacities of the particles to displace ^{125}I-labeled LDL from binding to MAbs. It was expressed as a percentage of LpB standard to which an arbitrary expression of 100% was assigned to each epitope. Results are shown in **Table 5** and correspond to mean values.

LpB-P and LpB-C did not differ from each other in their immunoreactivities for the epitopes recognized by B1, B4, BA11, and BL3 antibodies and the number of epitopes expressed was around 100%. However, with BL5, this percentage was higher in LpB-P, thus reflecting a lower accessibility of the epitope ($P < 0.001$). The apoB content was significantly lower in LpB-A as compared with LpB-P for all epitopes except for those recognized by BA11. The high standard deviation value indicated, moreover, that LpB-A and, to a lesser extent, LpB-P greatly varied in their immunoreactivities among patients.

Relation of LpB composition to immunoreactivity

No significant correlation (Spearman rank test) was found between chemical composition and immunoreactivity for any LpB samples, except for apoB content in LpB-A determined by antibody BL5, which correlated significantly ($P < 0.05$) and positively with triglyceride molar ratios.

TABLE 5. Immunological accessibility of LpB preparations from arteries, respective plasma, and control plasma

Monoclonal Antibody	LpB-A	LpB-P (n = 10)	LpB-C (n = 5)	LpB-A versus LpB-P	LpB-P versus LpB-C
B1	136 ± 27 (n = 9)[a]	100 ± 30	89 ± 26	$P < 0.01$[b]	NS
B4	173 ± 66 (n = 10)	122 ± 19	110 ± 8	$P < 0.05$	NS
BA11	116 ± 43 (n = 8)	104 ± 25	101 ± 15	NS	NS
BL3	193 ± 120 (n = 7)	120 ± 34	111 ± 11	$P < 0.05$	NS
BL5	163 ± 52 (n = 8)	110 ± 44	74 ± 8	$P < 0.01$	$P < 0.001$

Results are expressed as a percentage of LpB standard to which an arbitrary expression of each epitope was assigned as 100 percent. Data are the mean apparent apoB content ± standard deviation calculated from displacement curves as described in Materials and Methods. These correspond to LpB preparations from arteries (LpB-A), respective plasma (LpB-P), and plasma from controls (LpB-C). Results are the compiled results of triplicate experiments.
[a]n = Number of preparations. Cases that exhibited displacement curves not parallel with the LpB standard were not included.
[b]*P* values represent significance levels; NS, not significant.

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DISCUSSION

In a clinical situation such as atherosclerosis, lipoproteins and particularly apoB-containing particles may be modified in a fashion that confers atherogenic properties (4–8). The modifications may concern size, chemical composition and/or charge, inducing alterations of apoB configuration, and receptor interaction (30–32). These changes in apoB conformation can be investigated through immunological studies using MAbs. The present study examined the immunological properties of apoB-containing particles isolated from human vascular tissue of atherosclerotic patients compared with particles that are present in plasma from the same patients or healthy controls.

As others (4–8), we have observed an increase in electrophoretic mobility for lipoproteins from arteries (LpB-A) and a fragmentation of apoB as compared with LpB from control plasma (LpB-C). Moreover, we documented both of these characteristics in LpB from atherosclerotic patients plasma (LpB-P) compared with LpB-C.

Despite a decrease in phospholipid content in LpB-A, the mean relative lipid composition of these particles was very close to that of particles from patients or from control plasma. This similarity as a whole may appear inconsistent with a previously published study on LDL from atherosclerotic lesions of humans (6) but consistent with data reported by Clevidence et al. (4) on lipoproteins extracted by immunoaffinity from lesions of human aortas. However, contrary to both these earlier studies, we did not show increases in the cholesterol to protein ratio in LpB from lesions.

In the present study, LpB-A and LpB-P had slight changes in lecithin and sphingomyelin proportions as compared with LpB-C composition. The decrease in relative amounts of lecithin and the increase in the sphingomyelin content observed in LpB-A seem consistent, although to a different extent, with earlier reports on lipoproteins extracted from rabbit lesions (5, 6). Nevertheless, we were surprised by the decrease of lysolecithin in LpB-A and LpB-P because its precursor, lecithin, also decreased. The reason for this unexpected result may arise from the relatively small amount of lipoprotein extractable from human arterial tissue on living subjects combined to a relatively small amount of lysolecithin. Thus, the plausibility of the results may be discussed, but any comparison with other data becomes difficult because phospholipid composition has been performed in very few studies. In two previously published findings, an increase in lysolecithin proportion was described in lesion LDL from WHHL rabbits (5, 6) as compared with LDL from plasma samples. Nevertheless, the relative amounts of lecithin and sphingomyelin appear quite different between both studies. From our knowledge, one unique report has concerned LDL-like particles from human grossly lesion-free aortic intima (33) that showed no change in lysolecithin content but a tendency for decreased sphingomyelin and increased lecithin as compared with plasma LDL. These tendencies, as a whole, appear to conflict with the other studies (5, 6) and all of these discrepancies may, perhaps, reflect a technical problem in measuring phospholipid class distribution with a good reproducibility under standard conditions. In contrast to previous observations (8, 34), no tendency to aggregate was seen on electron photomicrographs of lesioned particles, as confirmed by the absence of high molecular weight protein in SDS gels (Fig. 1). Two major reasons may explain this conflicting result: first, the very gentle extraction procedure of lipoproteins that we performed allowed only the liberation of readily removed material soluble in the aqueous buffer, possibly leaving behind a more tightly bound fraction in an aggregated state. Second, the concentration of particles in our preparations never exceeded 1 mg/ml, above which in vitro aggregation may occur (8). Also, another argument may be formulated as SDS-PAGE patterns of apoB-containing particles showed concomitantly associated apolipoproteins such as apoA-I and apoE which have been proposed as apolipoproteins with amphipathic properties capable of preventing aggregation of lipoproteins (35).

We observed a significantly larger median particle size in the order of LpB-C, LpB-P, and LpB-A (Table 4). This shift towards larger sizes cannot be related in our study to different lipoprotein status between the patients and the controls because all were normotriglyceridemic. The electrophoretic separation of lipoproteins on polyacrylamide disc gels (36) has confirmed the absence of hyper-very low density lipoproteinemia in any particular sample (data not shown). The presence of particles larger than LDL in lesion-free human aortic tissue or extracts from human atherosclerotic aortas has already been described (8, 33, 37). These lipoproteins have been associated with VLDL or VLDL remnants. Also, Avogaro, Bittolo Bon, and Cazzolato (38) have characterized a modified form of LDL in the plasma of normolipemic subjects whose mean diameter was larger than the normal unmodified fraction.

In addition to confirming a number of previously reported physico-chemical characteristics shared by human lesion lipoproteins, we documented some immunological properties of these modified lipoproteins. The approach used MAbs for which epitopes have been located on the apoB molecule. Therefore it enabled us to advance the understanding of the functional disorder that accompanies atherosclerosis. It is important to refer to the studies of Avogaro et al. (38) and Ylä-Herttuala et al. (6, 33). These three studies indicate a more marked alteration of the antigenic sites of apoB close to the LDL-receptor recognition domain: the first in modified LDL isolated from normolipemic plasma, the second in human lesion LDL, and the third in lipoproteins extracted from

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lesion-free human aortic intima. In the present study, we have chosen to study immunoreactivity changes on apoB of lipoproteins extracted from vascular tissue and corresponding plasma by means of a quantitative radioimmunoassay. This allowed us to calculate slopes and apparent apoB content which reflect the affinity and accessibility of epitopes specific for five different MAbs. A lower slope value has been obtained for a few LpB-A preparations with some antibodies as compared with corresponding LpB-P. Probably this may be explained by a greater heterogeneity of particles extracted from arteries than that from plasma. Nevertheless, the affinity analysis indicates that in our study LpB-P and LpB-C were less heterogeneous than LpB-A with respect to the epitopes studied. This indicates the absence of apoB polymorphic variation in the samples studied. We have obtained a significant decrease in accessibility of all apoB epitopes on vascular lipoproteins when compared to those isolated from the respective plasma, except for the epitope specific for BA11 previously located in the receptor binding domain of apoB at residue 3506 (15). Because previous studies have shown that human lesion LDL is recognized and degraded poorly by the LDL-receptor of fibroblasts and, inversely, degraded more rapidly by macrophages (4, 6, 8, 39), it may be surprising that the expression of one epitope situated in the LDL-receptor recognition domain was not affected. We can easily explain this apparent conflicting result because one epitope involves a smaller region than the LDL-receptor recognition domain.

A lack of significant difference between the apoB epitopes accessibility to LpB from atherosclerotic patients and controls was reported with MAbs B1, B4, BA11, and BL3 (Table 5), which recognize sequential determinants located on the apoB-100 protein (15, 16). However, a significant decrease ($P < 0.001$) of BL5 epitope (conformationally expressed epitope) immunoreactivity seems to indicate that some modifications of apoB conformation may be initiated in the plasma of atherosclerotic patients. A cascade of events would follow, leading to the presence in arteries of completely reorganized particles.

Modulation of apoB epitope expression depends on the conformation of lipoproteins, which, in turn, is subjected to alterations induced by either the lipid compositions and/or sizes of the particles (31, 32). Other apolipoproteins present on the particles may also interact with apoB and modify epitope expression, as suggested by lipolysis experiments (40) which also induce exchange of proteins (41). No statistical difference was noted between lipid and protein composition of LpB from arteries and respective plasma or control plasma (Table 2), suggesting that the decrease of immunoreactivity we observed for LpB from arteries may not be related to a different chemical environment. Only phospholipid content was slightly decreased in LpB from arteries, but the lack of correlation in our study between the parameters and any apoB content measured with the MAbs does not permit us to rely on some perturbation at the surface of the particles and conformational changes.

Because the expression of apoB epitopes is modulated by the chemical composition and/or size and by the density of the lipoprotein in which the apoB is found (31, 32), individual epitopes may be expressed differently in chylomicrons, VLDL, IDL, and LDL. We have previously shown that MAbs B4 and BA11 bind VLDL, IDL, and LDL with the same affinity, while B1 recognizes VLDL to a lesser extent (15). Others have reported a high affinity of BL3 for VLDL and LDL, and a poor recognition of VLDL by BL5 (42). Therefore, the decreased immunoreactivity of LpB from arteries may not be entirely related to the heterogeneity of lipoprotein size. Nevertheless, a decrease in BL5 epitope expression in LpB from atherosclerotic plasma may be linked to the presence of larger particles than the LDL-sized ones. Further research is needed to define the role of these larger particles in the atherosclerotic arteries.

With regard to electronegativity, apoB fragmentation, immunological accessibility, and size, modifications of apoB are shown to increase in the following order: LpB from control plasma, LpB from atherosclerotic plasma, and LpB from the respective arteries. A previously reported study has even indicated that LDL extracted from human atherosclerotic plaques were derived and modified from plasma LDL. These modifications of LpB seem to be a prerequisite to their atherogenicity in vivo (43).

The physical and immunochemical properties of LpB from atherosclerotic plasma that we have reported in this study seem to indicate that an initial event of lipoprotein modification may start in the plasma.

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REFERENCES

1. Schaffner, T., K. Taylor, E. J. Bartucci, K. Fisher-Dzoga, J. H. Beenson, S. Glagov, and R. Wissler. 1980. Arterial foam cells with distinctive immunomorphologic and histochemical features of macrophages. *Am. J. Pathol.* **100:** 57-80.
2. Brown, M. S., and J. L. Goldstein. 1983. Lipoprotein metabolism in the macrophage: implications for cholesterol deposition in atherosclerosis. *Annu. Rev. Biochem.* **52:** 223-261.

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3. Witztum, J. L., and D. Steinberg. 1991. Role of oxidized low density lipoprotein in atherogenesis. *J. Clin. Invest.* **88:** 1785–1792.
4. Clevidence, B. A., R. E. Morton, G. West, D. M. Dusek, and H. F. Hoff. 1984. Cholesterol esterification in macrophages. Stimulation by lipoproteins containing apoB isolated from human aortas. *Arteriosclerosis.* **4:** 196–207.
5. Daugherty, A., B. S. Zweifel, B. E. Sobel, and G. Schonfeld. 1988. Isolation of low density lipoprotein from atherosclerotic vascular tissue of Watanabe heritable hyperlipidemic rabbits. *Arteriosclerosis.* **8:** 768–777.
6. Ylä-Herttuala, S., W. Palinski, M. E. Rosenfeld, S. Parthasarathy, T. E. Carew, S. Butler, J. L. Witztum, and D. Steinberg. 1989. Evidence for the presence of oxidatively modified low density lipoprotein in atherosclerotic lesions of rabbit and man. *J. Clin. Invest.* **84:** 1086–1095.
7. Rosenfeld, E. R., W. Palinski, S. Ylä-Herttuala, S. Butler, and J. L. Witztum. 1990. Distribution of oxidation specific lipid-protein adducts and apolipoprotein B in atherosclerotic lesions of varying severity from WHHL rabbits. *Arteriosclerosis.* **10:** 336–349.
8. Hoff, H. F., and J. O'Neil. 1991. Lesion derived low density lipoprotein and oxidized low density lipoprotein share a ability for aggregation, leading to enhanced macrophage degradation. *Arterioscler. Thromb.* **11:** 1209–1222.
9. Esterbauer, H., M. Dieber-Rotheneder, G. Waeg, G. Striegl, and G. Jürgens. 1990. Biochemical, structural, and functional properties of oxidized low-density lipoprotein. *Chem. Res. Toxicol.* **3:** 77–92.
10. Palinski, W., S. Ylä-Herttuala, M. E. Rosenfeld, S. W. Butler, S. A. Socher, S. Parthasarathy, L. K. Curtiss, and J. L. Witztum. 1990. Antisera and monoclonal antibodies specific for epitopes generated during oxidative modification of low density lipoprotein. *Arteriosclerosis.* **10:** 325–335.
11. Zawadzki, Z., R. W. Milne, and Y. L. Marcel. 1989. An immunochemical marker of low density lipoprotein oxidation. *J. Lipid Res.* **30:** 885–891.
12. Zawadzki, Z., R. W. Milne, and Y. L. Marcel. 1991. Cu^{2+}-mediated oxidation of dialyzed plasma: effects on low and high density lipoproteins and cholesteryl ester transfer protein. *J. Lipid Res.* **32:** 243–350.
13. Kleinman, Y., E. S. Krul, M. Burnes, W. Aronson, B. Pfleger, and G. Schonfeld. 1988. Lipolysis of LDL with phospholipase A_2 alters the expression of selected apoB-100 epitopes and the interaction of LDL with cells. *J. Lipid Res.* **29:** 729–743.
14. Gandjini, H., P. Gambert, A. Athias, and C. Lallemant. 1991. Resistance to LDL oxidative modifications of an N-terminal apolipoprotein B epitope. *Atherosclerosis.* **89:** 83–93.
15. Fievet, C., C. Durieux, R. Milne, T. Delaunay, G. Agnani, H. Bazin, Y. Marcel, and J. C. Fruchart. 1989. Rat monoclonal antibodies to human apolipoprotein B: advantages and applications. *J. Lipid Res.* **30:** 1015–1024.
16. Pease, R. J., R. W. Milne, W. K. Jessup, A. Law, P. Provost, J. C. Fruchart, R. T. Dean, Y. L. Marcel, and J. Scott. 1990. Use of bacterial expression cloning to localize the epitopes for a series of monoclonal antibodies against apolipoprotein B. *J. Biol. Chem.* **265:** 553–568.
17. Edelstein, C., and A. M. Scanu. 1986. Precautionary measures for collecting blood destined for lipoprotein isolation. *Methods Enzymol.* **128:** 151–155.
18. Parsy, D., V. Clavey, C. Fievet, I. Kora, P. Duriez, and J. C. Fruchart. 1985. Quantification of apolipoprotein C-III in serum by a noncompetitive immunoenzymometric assay. *Clin. Chem.* **31:** 1632–1636.
19. Koffigan, M., I. Kora, V. Clavey, J. M. Bard, J. Chapman, and J. C. Fruchart. 1987. Quantification of human apolipoprotein E in plasma and lipoprotein subfractions by a noncompetitive enzyme immunoassay. *Clin. Chim. Acta.* **163:** 245–256.
20. Vu Dac, N., H. Mezdour, H. J. Parra, G. Luc, I. Luyeye, and J. C. Fruchart. 1989. A selective bi-site immunoenzymatic procedure for human Lp[a] lipoprotein quantification using monoclonal antibodies against apo[a] and apoB. *J. Lipid Res.* **30:** 1437–1443.
21. Bard, J. M., L. Candelier, G. Agnani, V. Clavey, G. Torpier, A. Steinmetz, and J. C. Fruchart. 1991. Isolation and characterization of human LpB lipoprotein containing apolipoprotein B as the sole apolipoprotein. *Biochim. Biophys. Acta.* **1082:** 170–176.
22. Havel, R. J., W. A. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34:** 1345–1353.
23. McFarlane, A. S. 1958. Efficient trace labelling of proteins with iodine. *Nature.* **182:** 53–57.
24. Nouvelot, A., G. Sezille, P. Dewailly, and J. C. Fruchart. 1977. Chromatographie monodimensionnelle des lipides polaires sur couche mince de gel de silice en gradient discontinu d'humidité. *Rev. Fr. Corps Gras.* **7:** 365–366.
25. Delpierre, C., C. Fievet, V. Equagoo, H. Mezdour, C. Cachera, J. C. Gesquiere, A. Tartar, and J. C. Fruchart. 1988. Antibodies of predetermined specificity to apolipoprotein C-II elicited with a synthetic peptide. Characterization and use for immunoassays. *J. Immunol. Methods.* **109:** 61–68.
26. Forte, A. M., and R. W. Nordhausen. 1986. Electron microscopy of negatively stained lipoproteins. *Methods Enzymol.* **128:** 442–457.
27. Zettner, A. 1973. Principles of competitive binding assays (saturation analysis). 1. Equilibrium techniques. *Clin. Chem.* **19:** 699–705.
28. Snedecor, G. W., and W. G. Cochran. 1967. Statistical Methods. 6th ed. Iowa State University Press, Ames, IA. 342–343.
29. Watt, T. S., and R. M. Watt. 1983. Detection of unique antigenic determinants on human plasma low density lipoprotein and on delipidated apolipoprotein B. *Proc. Natl. Acad. Sci. USA.* **80:** 124–128.
30. Galeano, N. F., R. W. Milne, Y. L. Marcel, E. Levy, R. A. McIntosh, E. L. Curtin, Y. A. Carpentier, M. Aviram, L. D. Wittle, and R. J. Deckelbaum. 1989. Does LDL size or lipid composition determine apoprotein B configuration and binding to the LDL receptor. *Circulation.* **80** (Suppl. II): 334.
31. Schonfeld, G., W. Patsch, B. Pfleger, J. L. Witztum, and S. W. Weidman. 1979. Lipolysis produces changes in the immunoreactivity and cell reactivity of very low-density lipoproteins. *J. Clin. Invest.* **64:** 1288–1297.
32. Krul, E. S., Y. Kleinman, M. Kinoshita, B. Pfleger, K. Oida, A. Law, J. Scott, R. Pease, and G. Schonfeld. 1988. Regional specificities of monoclonal anti-human apolipoprotein B antibodies. *J. Lipid Res.* **29:** 937–947.
33. Ylä-Herttuala, S., O. Jaakkola, C. Ehnholm, M. J. Tikkanen, T. Solakivi, T. Särkioja, and T. Nikkari. 1988. Characterization of two lipoproteins containing apolipoproteins B and E from lesion-free human aortic intima. *J. Lipid Res.* **29:** 563–572.
34. Frank, J. S., and A. M. Fogelman. 1989. Ultrastructure of the intima in WHHL and cholesterol-fed rabbit aortas pre-

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pared by ultra-rapid freezing and freeze-etching. *J. Lipid Res.* **30:** 967-978.

35. Khoo, J. C., E. Miller, P. McLoughlin, and D. Steinberg. 1990. Prevention of low density lipoprotein aggregation by high density lipoprotein or apolipoprotein A-I. *J. Lipid Res.* **31:** 645-652.
36. Sezille, G., J. C. Fruchart, J. Jaillard, P. Dewailly, and C. Desreumaux. 1975. Improved serum lipoprotein electrophoresis procedure in polyacrylamide gradient gel. *Biomedecine.* **23:** 315-317.
37. Rapp, J. H., H. W. Harris, R. L. Hamilton, W. C. Krupski, L. M. Reilly, W. K. Ehrenfeld, R. J. Stoney, J. Goldstone, and J. P. Kane. 1989. Particle size distribution of lipoproteins from human atherosclerotic plaque: a preliminary report. *J. Vasc. Surg.* **9:** 81-88.
38. Avogaro, P., G. Bittolo Bon, and G. Cazzolato. 1988. Presence of a modified low density lipoprotein in humans. *Arteriosclerosis.* **8:** 79-87.
39. Steinbrecher, U. P., J. L. Witztum, S. Parthasarathy, and D. Steinberg. 1987. Decrease in reactive amino groups during oxidation or endothelial cell modification of LDL. Correlation with changes in receptor-mediated catabolism. *Arteriosclerosis.* **7:** 135-143.
40. Tikkanen, M. J., T. G. Cole, K. S. Hahm, E. S. Krul, and G. Schonfeld. 1984. Expression of apolipoprotein B epitopes in very low density lipoprotein subfractions. Studies with monoclonal antibodies. *Arteriosclerosis.* **4:** 138-146.
41. Eisenberg, S. 1984. High density lipoprotein metabolism. *J. Lipid Res.* **25:** 1017-1058.
42. Salmon, S., S. Goldstein, D. Pastier, I. Theron, M. Berthelier, M. Ayrault-Jarrier, M. Dubarry, R. Rebourcet, and B. Pau. 1984. Monoclonal antibodies to low density lipoprotein used for the study of low- and very-low-density lipoproteins, in "ELISA" and immunoprecipitation technics. *Biochem. Biophys. Res. Commun.* **125:** 704-711.
43. Shaikh, I., S. Martini, J. R. Quiney, P. Baskerville, A. E. La Ville, N. L. Browse, R. Duffield, P. R. Turner, and B. Lewis. 1988. Modified plasma-derived lipoproteins in human atherosclerotic plaques. *Atherosclerosis.* **69:** 160-172.

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