paper on methodology

Isolation of pure LpB from human serum

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Abstract Low density lipoproteins (LDL), even after isolation from a narrow density cut and after several washes by preparative ultracentrifugation, are contaminated by 3–5% non-apoB proteins. Incubation of these LDL with artificial triglyceride-rich lipid emulsions (TGRP) removed all contaminating apoC and also, under certain conditions, apoA proteins. TGRP treatment did not, however, change the lipid composition and the flotation behavior of LDL. Residual apoE and albumin, amounting up to 0.5% of the apoB mass, were resistant to removal by TGRP treatment as well as by heparin-Sepharose column chromatography. ApoE and albumin could only be removed by immunoabsorption. – **Zechner, R., R. Moser, and G. M. Kostner.** Isolation of pure LpB from human serum. *J. Lipid Res.* 1986. **27:** 681–686.

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Low density lipoproteins (LDL) represent the major lipoprotein fraction in normal plasma from fasting humans. LDL are considered as the catabolic end product of endogenous triglyceride-rich lipoproteins (VLDL) (1). Although it has been postulated in the past that LDL contains a single apolipoprotein, apoB, later studies have clearly revealed a considerable microheterogeneity with respect to the protein content and the lipid composition (2–4).

For metabolic studies in vitro and in vivo, narrow density cuts of LDL prepared in the ultracentrifuge are commonly used. Data are then interpreted in view of LpB metabolism, neglecting the presence of non-apoB proteins on the order of 5% of the protein mass. These additional proteins may become a major problem if displacement of ligands from specific surface receptors is studied with excess LDL, or when evaluating the role of LDL as a substrate for enzymes. Finally, contaminating non-apoB proteins are a hindrance in the production of monospecific antibodies. Here we report on methods for preparing LDL which is virtually free of non-apoB proteins, designated in this study as LpB.

MATERIALS AND METHODS

Blood was obtained from normolipidemic and, in a few cases, from hyperlipidemic volunteers in the morning after an overnight fasting period. After allowing the blood to clot at room temperature for 30 min, serum was separated by low speed centrifugation. In some cases, citrate-plasma was used instead of serum. The following preservatives were added and their action was studied: 1 mg/ml of Na_2-EDTA; 1 mg/ml NaN_3; 0.2 mM diisopropyl fluorophosphate; 0.5 mg/ml glutathione; 0.5 mg/ml butylated hydroxytoluene; and 0.5 mg/ml gallyl propionate (all from Sigma).

The separation of LDL was started immediately by preparative ultracentrifugation: *1*) 22 hr at d 1.025 g/ml (solution density) and 120,000 *g*; *2*) 22 hr at d 1.070 g/ml and 140,000 *g*; and 3) 24 hr in a density gradient at 200,000 *g*. Steps *1* and *2* were carried out in fixed-angle rotors (Beckmann 50.1 or 70 Ti). The third step was carried out in the Beckman SW-41 rotor. Five ml of the d 1.025–1.070 g/ml density fraction containing 50–100 mg of LDL was placed in a tube and overlayered with 3.5 ml each of an NaCl solution of d 1.040 and d 1.020 g/ml, respectively. In a few cases, a linear gradient ranging from d 1.00–1.70 g/ml was used. After the last centrifugation step, a narrow cut of LDL was aspirated with a syringe from the very visible yellow lipoprotein band on top of the d 1.040 g/ml layer. All densities were adjusted by adding solid NaCl and centrifugation was carried out at 15°C in a Sorvall OTD-2B. In control experiments, LDL + VLDL were precipitated with Na-phosphotungstate-

Abbreviations: TGRP, triglyceride-rich particles; LDL, low density lipoproteins; LpB, LDL which is virtually free of non-apoB proteins; HDL, high density lipoproteins; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TMU, tetramethylurea; TG, triglycerides.

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Mg^{2+} (5), solubilized in 1% Na-citrate, pH 8.5, and LDL was isolated by ultracentrifugation identically as above. Occasionally LDL was washed several times in the SW-41 or in the fixed-angle rotor.

All LDL preparations revealed the presence of various amounts of non-apoB proteins. The most apparent ones, as revealed by polyacrylamide gel electrophoresis (PAGE), were apoC-I, -C-II, and -C-III. Since artificial TG-rich lipid emulsions are known to avidly adsorb apoproteins, this system was used to prepare apoC-free LDL. Commercial lipid emulsions: Intralipid (Vitrum), Lipofundin (Brown, Melsungen), "Lipase Substrate" (Sigma), as well as four other local materials were studied. In order to get rid of the phospholipid-containing mesophase, the emulsions containing 10–50% TG were floated by high speed centrifugation (20,000 *g* for 40 min), resuspended in bidistilled water, and centrifuged again. These washings were repeated three times and the TG-particles were resuspended in distilled water to yield a 10% TG-emulsion. When salt solutions or buffers were used, we observed disintegration of some of the emulsions during the washing procedures.

Incubation of LDL with TG-rich particles (TGRP)

The LDL fractions isolated by density gradient ultracentrifugation containing 30–50 mg of LDL/ml were mixed with equal volumes of washed TGRP and incubated for 1 hr at 37°C. The mixture was then centrifuged at 20,000 *g* for 30 min to remove the TGRP. The floating TG-layer was removed with a spatula and the whole incubation procedure was repeated.

Removal of non-apoB proteins by immunoabsorption

LDL, pretreated or not with TGRP, were mixed with the IgG fraction of monospecific antisera against apoA-I, apoE, and albumin. IgG was prepared by ammonium sulfate precipitation, dialysis, and lyophilization, and was usually added to the LDL solutions in dry form. In previous experiments the titer of the antibody was calculated and antibodies were added in ca. twofold excess. The antisera were mostly from horse or sheep. Alternatively, the LDL fractions were passed over an immune specific adsorber loaded with various non-apoB antibodies. After immunoabsorption, the LDL were again spun in the SW-41 rotor under the same conditions as above.

Analytical methods

Polyacrylamide gel electrophoresis in SDS or urea-containing gels was performed as described earlier (6). Protein was determined by the method of Lowry et al. (7) using HSA as a standard. Non-apoB proteins in LDL were determined according to Egusa et al. (8). Individual lipids were quantitated by commercial enzymatic tests. Immunoquantitation of apolipoproteins was performed by Laurell electrophoresis using monospecific antibodies as described (9). The electrophoresis was carried out on 7 × 7 cm glass plates, in 6 ml of 1% agarose. For apoE quantitation we normally used 100 μl of antiserum from rabbit. The sensitivity of our Laurell electrophoresis was in the range of 1–10 ng of protein, depending on the antibody used, with linearity down to ca. 5 ng. Five μl of LDL or LpB with a protein concentration of ca. 10 mg/ml corresponding to 50 μg of protein was applied to the plates. Assuming a sensitivity of 5 ng, we thus were able to detect impurities amounting to 0.01% of the apoB mass, and to quantitate impurities up to 0.05%.

Experiments with the analytical ultracentrifuge were carried out as outlined earlier (6). Binding studies of LDL and of LpB to the B/E receptor were performed as described earlier (10).

RESULTS

By ultracentrifugation alone, it is impossible to obtain 100% pure LpB. This was found in more than 50 experiments even after multiple washings in swinging-bucket or fixed-angle rotors. Non-apoB protein amounted in all cases to ca. 3–5%. This was determined by treatment of LDL with isopropanol and measuring the protein in the supernatant solution. The isopropanol-soluble protein consisted mainly of apoC, apoA, apoE, and albumin as determined by immunodiffusion and PAGE. **Fig. 1A** shows the protein pattern of TMU-soluble proteins of a LDL preparation after ultracentrifugation. ApoE and albumin are not visible under these conditions, probably due to partial aggregation; aggregated proteins with higher molecular weights cannot enter 10% urea-containing gels. ApoE and albumin, however, can be demonstrated immunochemically and by SDS-PAGE (Fig. 1B).

The content of individual non-apoB proteins in LDL, except for apoC, was quantitated by Laurell electrophoresis. ApoC was estimated by subtracting the amount of apoA, apoE, and albumin from the total isopropanol-soluble protein, and thus might include small amounts of undefined proteins. **Table 1** lists quantitative data of four different LDL preparations, indicating that apoC was the major contaminating protein followed by apoA-I, apoE, albumin, and apoA-II.

Treatment of LDL with TGRP, as described above, quantitatively removed all TMU-soluble proteins as judged by PAGE (Fig. 1B). We tested several brands of commercial TG-emulsions and found that Lipofundin was the most efficient, followed by Intralipid. Thus, in all further experiments we used Lipofundin.

The apoC contaminants of LDL could be removed most easily and were almost undetectable after one incubation with TGRP (Table 1). For removal of all apoA-I, we had to incubate twice. If, on the other hand, LDL was

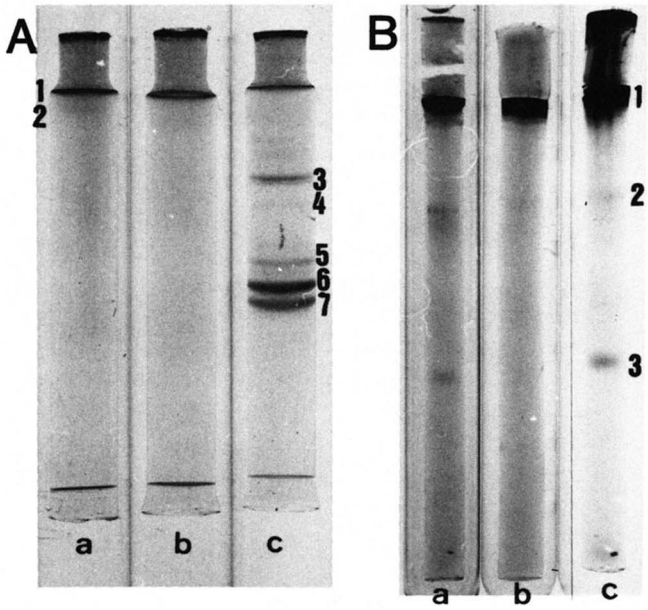

Fig. 1. Polyacrylamide gel electrophoresis (PAGE) of LDL isolated by ultracentrifugation solely (LDL) and of LDL treated with Lipofundin (LDL-Li). A: PAGE in 10% gels containing 8 M urea. TMU-soluble proteins from 200 μg of apoLDL were applied to the gels a and b, and 100 μg of apoLDL to gel c. a) LDL treated with Lipofundin; b) the same as a) but absorbed with antibodies against LpE and anti-albumin; c) parent LDL. The bands are: 1, residual apoB; 2, albumin; 3, apoA-I; 4, apoA-II; 5, apoC-II; 6, apoC-III-1; 7, apoC-III-2. B: PAGE in 12.5% SDS gels. The samples are the same as in A except that 200 μg of total apoLDL was applied to all gels; 1, apoB; 2, albumin; 3, apoE.

contaminated with HDL (alpha-migrating apoA-I-containing lipoproteins), the incubation with TGRP (even for three or four times) was ineffective. Similarly, if LDL was prepared by phosphotungstate precipitation in the first step, there always remained detectable amounts of apoA-I which could not be removed by TGRP. LDL prepared from hypertriglyceridemic individuals also were more resistant to apoC and apoA removal. In these cases, LDL had to be passed over immunoabsorbers containing anti-apoA-I, or occasionally with anti-apo-C (only if hypertriglyceridemic LDL was purified).

The LDL prepared by incubation with TGRP were further tested by SDS-PAGE (Fig. 1) and immunodiffusion (**Fig. 2**) for the presence of apoE, albumin, and other possible proteins. Untreated LDL contained, on average, 0.4% apoE (fraction of total LDL-protein) as determined by Laurell electrophoresis. An apoE-free preparation was never obtained by ultracentrifugation, even after repeated washings. Treatment with TGRP for up to four times had little influence on the apoE content. Only 20–30% of the apoE impurities could be removed at maximum by TGRP.

In further experiments, crude LDL or TGRP-treated LDL were chromatographed over heparin–Sepharose as proposed by Shelburne and Quarfordt (11). None of the fractions eluting with a NaCl salt gradient were free of apoE, but there were minor quantitative differences. In order to remove apoE, it was absolutely necessary to apply immunoabsorption either over columns or by addition of solid IgG. As the amount of apoE in LDL is relatively small, the antibody consumption is not tremendously high, and we preferred to use the latter procedure. LDL was therefore incubated with the IgG fraction of a sheep anti-apoE antiserum. In order to remove the IgG from LDL after absorption, the mixture was ultracentrifuged again in a density gradient using the SW-41 rotor. The resulting LDL was apparently free of apoE (Table 1), and did not contain any detectable amount of IgG.

Albumin behaved identically to apoE. There were always low but detectable amounts present and these could only be removed by immunoabsorption. This was achieved by passing either the starting LDL or the apoA-, apoC-, and apoE-free LDL over an immunoabsorber column containing anti-albumin. Since albumin may not interfere in many experiments, this last step was only carried out occasionally in our laboratory.

TABLE 1. Apolipoprotein content of LDL isolated by ultracentrifugation solely, and after treatment with Lipofundin and with specific antibodies

Fraction[a]	Non-ApoB Protein[b]	Apolipoprotein Content					Recovery of ApoB[c]
		ApoA-I	ApoA-II	ApoC	ApoE	Albumin	
	% of total protein	*% of non-apoB protein*					*%*
L-1	4.2 (1.8)	8.7 (2.4)	3.9 (1.1)	73.0 (6.7)	8.3 (2.3)	4.8 (2.1)	100
L-2	1.3 (0.5)	2.5 (1.0)	1.3 (0.5)	5.4 (1.6)	54.5 (6.8)	36.8 (7.7)	84.7 (3.4)
L-3	0.7 (0.2)	n.d.	n.d.	n.d.	56.8 (5.7)	43.6 (8.3)	75.8 (5.0)
L-4	0.5 (0.1)	n.d.	n.d.	n.d.	n.d.	100[d]	68.4 (3.2)
L-5	0.2 (0.05)	n.d.	n.d.	n.d.	n.d.	n.d.	63.7 (3.1)

The values are means ± SD obtained from four different LDL preparations; n.d., not detectable, i.e., less than 0.01% of the apoB mass.

[a] L-1: LDL isolated from pooled serum by density gradient ultracentrifugation. L-2: L-1 treated once with Lipofundin. L-3: L-1 treated twice with Lipofundin. L-4: L-3 treated with anti-apoE and recentrifuged. L-5: L-4 passed over an immunoabsorber specific for albumin.

[b] Lowry protein in the supernatant after isopropanol treatment (8).

[c] Determined by radial immunodiffusion.

[d] We assumed that all residual non-apoB protein consisted of albumin since no other proteins could be detected.

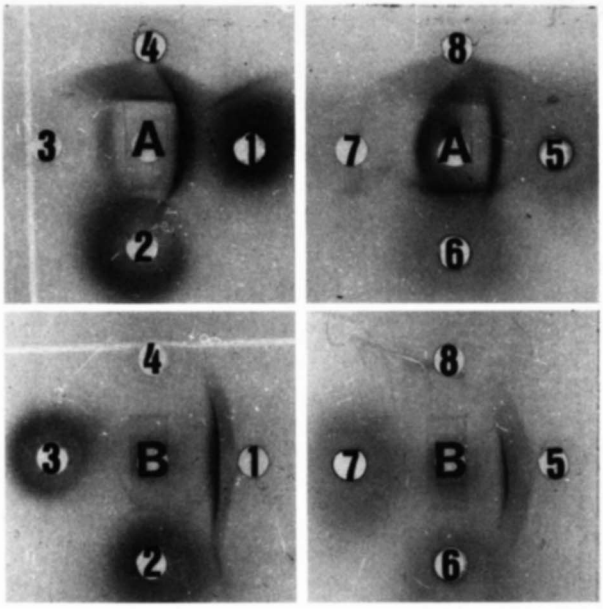

Fig. 2. Immunodiffusion of LDL (A) and LDL treated with Lipofundin and absorbed with antibodies against LpE and albumin (B). The following antibodies were used: 1 and 5, anti-apoB; 2, anti-apoA-II; 3, anti-apoC-II; 4, anti-apoA-I; 6, anti-apoC-III; 7, anti-apoE; and 8, anti-albumin. The lipoproteins were applied in intact form.

Table 1 not only lists the apoprotein content after each purification step, but also the total yield of apoB as determined by radial immunodiffusion (5, 6). In our experiments, applying all four purification steps, we had an apoB yield on the order of 60%. It must be emphasized here, however, that we paid more attention in these experiments to an optimal purification rather than to a maximal yield.

Albumin and apoE both contain cysteine and since there was the possibility that these proteins were linked to apoB by -S-S- bridges (12), the whole isolation and incubation with TGRP was repeated in the presence of 5–50 mM dithiotreithol. This disulfide cleaving agent, however, had no influence on the apoE and albumin contamination of LDL.

Thus we obtained an LpB(LDL) preparation that was virtually free from contaminating apoproteins, albumin, or other serum proteins. There was a small residual, isopropanol-soluble fraction, corresponding to 0.2% of the apoB mass. Since we failed to demonstrate in this fraction any apolipoprotein or serum protein by PAGE or immunochemically, we considered this as the blank value in the isopropanol method.

The LDL prepared by TGRP-incubation and immunoabsorption were analyzed chemically and by analytical ultracentrifugation (**Table 2**). The only significant difference between the parent LDL and the TGRP-incubated LDL resided in the content of total and non-apoB protein. The TG and the phospholipid content as well as the flotation in the analytical ultracentrifuge remained virtually unchanged.

We also investigated the influence of the various preservatives listed in the Methods for possibly deteriorating the purification of LDL. None of the agents detectably influenced the contamination of LDL with non-apoB proteins. In final experiments we compared the LDL and LpB preparations from serum with that of plasma. There were virtually no differences with respect to contaminations with non-apoB proteins.

Since this work was aimed at preparing the most homogeneous LDL possible for studying the interaction with cultured cells, it was of importance to investigate LpB for possible changes in binding to the B/E receptor. This was studied in cultured human fibroblasts. **Fig. 3** shows the binding, internalization, and degradation of LDL as compared to LpB. LpB exhibited a somewhat greater binding affinity and a higher internalization rate as compared to LDL. The degradation was identical for both lipoproteins (data not shown).

DISCUSSION

For receptor binding studies as well as in in vivo experiments aimed at the investigation of metabolic parameters, LDL purified solely by ultracentrifugation is used in most laboratories. Occasionally, heparin-Sepharose chromatography is additionally used to remove apoE (11). In our experiments investigating specific B/E- and E-receptors in liver cells (F. Krempler and G. Kostner, unpublished

TABLE 2. Chemical composition of LDL isolated by ultracentrifugation solely, and of LDL treated with Lipofundin and anti-apoE and anti-albumin (LpB)

Sample	S_f, 1.063[a]	Protein[b]	CE	FC	PL	TG
LDL	5.2 ± 0.5	22.3 ± 1.6	42.7 ± 1.3	9.1 ± 1.7	21.0 ± 0.6	4.8 ± 1.6
LpB[c]	5.4 ± 0.5	20.5 ± 1.4	43.8 ± 1.5	8.9 ± 1.0	22.4 ± 0.9	5.4 ± 1.7

The results are means ± SD of four different LDL/LpB preparations. Values are in % (w/w).
[a] S_f, 1.063: flotation constant at d 1.063 g/ml in negative Svedberg units.
[b] Protein, Lowry protein; CE, cholesteryl ester; FC, free cholesterol; PL, phospholipids; TG, triglycerides.
[c] LpB is identical to L-5 in Table 1.

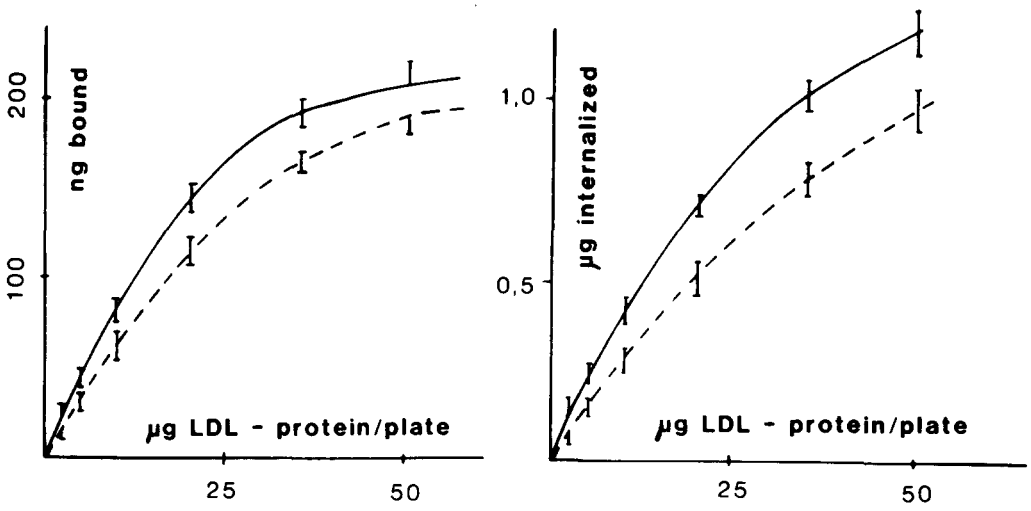

Fig. 3. Binding and internalization of LDL (- - -) and of LpB (——). This experiment was carried out as described in reference 10. Monolayers of human skin fibroblasts were incubated for 6 hr at 37°C in the presence of DMEM medium containing 5% LPDS and increasing amounts of ^{125}I-labeled lipoproteins. After incubation, the medium from each dish was removed and the content of trichloroacetic acid-soluble material was measured (degradation). Heparin-releasable radioactivity was determined after incubation of the cells for 30 min at 4°C with 1% sodium-heparin solution, and refers to binding. Radioactivity remaining associated with the cells was defined as being internalized.

data), where a 500–1000-fold excess of LDL had to be used to prove the specificity of the E-receptor, it became apparent that LDL are contaminated by small amounts of apoE which could not be removed by size exclusion (13) or heparin-Sepharose column chromatography. There were also detectable amounts of apoC and apoA-I present. These latter impurities have been shown to influence markedly the LDL metabolism in vitro and in vivo (10, 14).

Searching for fast and convenient methods to obtain pure LpB, we made use of the observation that TGRP avidly take up non-apoB apolipoproteins (15). With these emulsions we succeeded in removing most of the non-apoB proteins including all apoC, and, in most of the cases, all apoA proteins. The obtained LDL consisted of ca. 99% apoB. ApoE and albumin, however, could not be removed by this treatment. For the removal of these proteins, immunoabsorption had to be used. The LDL treated by anti-apoE and anti-albumin in addition to TGRP consisted exclusively of apoB in the protein moiety, as tested immunochemically and by SDS-PAGE.

The LpB purified according to this procedure exhibited a higher affinity to the B/E-receptor and also an increased internalization when studied in cultured human fibroblasts. We did not determine the significance of these differences since the data were derived from only one triplicate experiment. The result, however, was in accord with our previous study where we demonstrated that LCAT-treated LDL, which contains a higher amount of non-apoB material as compared to reference LDL, showed a significantly lower binding to the B/E-receptor despite the higher apoE content (10).

Finally it might be worth noting that TGRP are ineffective in removing apoC from HDL or from VLDL.

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