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References

Allen, L., Vanecek, J., & Wolfe, R. G. (1971) *Arch. Biochem. Biophys. 143*, 166-174.

Anderson, C. M., McDonald, R. C., & Steitz, T. A. (1978a) *J. Mol. Biol. 103*, 1-13.

Anderson, C. M., Stenkamp, R. E., & Steitz, T. A. (1978b) *J. Mol. Biol. 123*, 15-33.

Banaszak, L. J., & Bradshaw, R. A. (1975) *Enzymes (3rd Ed.) 11A*, 369-396.

Barry, C. D., Bosshard, H. E., Ellis, R. A., & Marshall, G. R. (1974) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 33*, 2368-2372.

Birktoft, J. J., & Banaszak, L. J. (1983) *J. Biol. Chem. 258*, 472-482.

Birktoft, J. J., Fernley, R. T., Bradshaw, R. A., & Banaszak, L. J. (1982a) in *Molecular Structure and Biological Activity* (Griffin, J. F., & Duax, W. L., Eds.) pp 37-55, Elsevier/North-Holland, New York.

Birktoft, J. J., Fernley, R. T., Bradshaw, R. A., & Banaszak, L. J. (1982b) *Proc. Natl. Acad. Sci. U.S.A. 79*, 6166-6170.

Hendrickson, W. A., & Konnert, J. H. (1981) in *Biomolecular Structure, Function, Conformation and Evolution* (Srinivasan, R., Ed.) Vol. 1, pp 43-57, Pergamon, Oxford.

Hermans, J., & McQueen, J. E. (1974) *Acta Crystallogr., Sect. A: Cryst. Phys., Diffr., Theor. Gen. Crystallogr. A30*, 730-739.

Herriott, J. R., Watenpaugh, K. D., Sieker, L. C., & Jensen, L. H. (1973) *J. Mol. Biol. 80*, 423-432.

Hill, E., Tsernoglou, D., Webb, L., & Banaszak, L. J. (1972) *J. Mol. Biol. 72*, 577-591.

Hopper, P., Harrison, S. C., & Sauer, R. T. (1984) *J. Mol. Biol. 177*, 701-713.

Lederer, F., Glatigny, A., Bethge, P. H., Bellamy, H. B., & Mathews, F. S. (1981) *J. Mol. Biol. 148*, 427-448.

Lipscomb, W. N., Hartsuck, J. A., Quiocho, F. A., & Reeke, G. N. (1969) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2619-2623.

Matthews, B. W., Colman, P. M., Jansonius, J. N., Titani, K., Walsh, K. A., & Neurath, H. (1972) *Nature (London), New Biol. 238*, 41-43.

McLachlan, A. D. (1972) *J. Mol. Biol. 64*, 417-437.

Miller, J. R., Abdel-Meguid, S. S., Rossmann, M. G., & Anderson, D. C. (1981) *J. Appl. Crystallogr. 14*, 94-100.

Rao, S. T., & Rossmann, M. G. (1973) *J. Mol. Biol. 76*, 241-256.

Taylor, S. S., Oxley, S. S., Allison, W. S., & Kaplan, N. O. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 1790-1794.

TenEyck, L. F. (1973) *Acta Crystallogr., Sect. A: Cryst. Phys., Diffr., Theor. Gen. Crystallogr. A29*, 183-191.

Tsernoglou, D., Hill, E., & Banaszak, L. J. (1972) *J. Mol. Biol. 69*, 75-87.

Watenpaugh, K. D., Sieker, L. C., Herriott, J. R., & Jensen, L. H. (1973) *Acta Crystallogr., Sect. B: Struct. Crystallogr. Cryst. Chem. B29*, 943-956.

Watson, H. C. (1969) in *Progress in Stereochemistry*, Vol. 4, pp 229-333, Butterworth, London.

Webb, L. E., Hill, E. J., & Banaszak, L. J. (1973) *Biochemistry 12*, 5101-5108.

Wright, C. S., Gavilanes, F., & Peterson, D. L. (1984) *Biochemistry 23*, 280-287.

Selective Isolation of Human Plasma Low-Density Lipoprotein Particles Containing Apolipoproteins B and E by Use of a Monoclonal Antibody to Apolipoprotein B†

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ABSTRACT: A monoclonal antibody to human plasma apolipoprotein B was used in a single-step immunoaffinity chromatography procedure to isolate a subpopulation of low-density lipoprotein particles from normolipidemic human plasma. The isolated particles were homogeneous in terms of size (20 nm), flotation coefficient (S_f = 9.5), and electrophoretic mobility (β band). Their protein moiety consisted of apolipoproteins B and E in a molar ratio close to 2. The lipid moiety consisted of 47.3% cholesterol, 4.7% triglycerides, and 48.0% phospholipids. To indicate its characteristic apolipoprotein composition and hydrated density properties, this family of particles was named LP-B:E_{L2}. In most normolipidemic subjects, LP-B:E_{L2} particles accounted for less than 10% of the total plasma apolipoprotein B content. The LP-B:E_{L2} particles bound to the membranes of the human hepatoma HepG2 cells in a specific and saturable manner indicative of receptor-mediated binding. Their binding was significantly higher than that of low-density lipoprotein particles containing only apolipoprotein B.

Human plasma low-density lipoprotein (LDL) are considered to be potentially the most atherogenic of all plasma lipoproteins (Fredrickson et al., 1978). In fact, the recently released results of the Lipid Research Clinics trial have shown that reduction of LDL-cholesterol is associated with a significant decrease in the morbidity and mortality of coronary heart disease (Lipid Research Clinics Program, 1984). Physicochemically, LDL represent a system of particles heterogeneous with respect to hydrated density, size, and lipid

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and protein composition (Lee & Alaupovic, 1970; Hammond et al., 1977; Krauss & Burke, 1982). Immunological characterization of LDL subfractions has also revealed a marked antigenic heterogeneity of this density class identifying apolipoprotein B (ApoB) as the major and apolipoproteins A-I, A-II, C-I, C-II, C-III, and E as the minor protein constituents (Lee & Alaupovic, 1974a; Rubenstein, 1978; Alaupovic, 1980). To reconcile this physical and chemical heterogeneity, we have proposed that LDL consist of a mixture of discrete lipoprotein particles with similar hydrated densities but different apolipoprotein composition (Alaupovic et al., 1972; Lee & Alaupovic, 1974b). Studies on the fractionation of LDL by immunoprecipitation procedures have shown the occurrence of two types of ApoB-containing lipoproteins. The major subspecies of lipoprotein particles contains ApoB as the sole protein constituent (LP-B), while the minor types of lipoprotein particles contain ApoB in association with ApoC and/or ApoE (LP-B:C, LP-B:E, etc.) (Lee & Alaupovic, 1974b; Alaupovic et al., 1986).

We have shown previously that monoclonal antibodies offer significant advantages over polyclonal antibodies for the isolation of lipoproteins by immunoaffinity chromatography (Koren et al., 1986a). In this study, we describe the use of a monoclonal antibody to ApoB for a selective single-step isolation from whole plasma of a family of particles referred to as LP-B:E_{L2} characterized by apolipoproteins B and E as their protein moiety.

Materials and Methods

Plasma and Lipoprotein Density Classes. The isolation of LP-B:E_{L2} particles was carried out with human blood samples obtained by venipuncture from asymptomatic, normolipidemic male and female donors who had fasted overnight. The blood was collected into 10-mL vacutainer tubes containing 0.5 mL of 5 mmol/L ethylenediaminetetraacetic acid (EDTA) and centrifuged at 2500 rpm for 15 min at 4 °C to separate cells from plasma. Plasma samples were kept at 4 °C and always used within 48 h.

Plasma used for the isolation of major lipoprotein density classes was collected by plasmapheresis from normolipidemic subjects or patients with type V hyperlipoproteinemia characterized according to the procedure recommended by the Lipid Research Clinics (1974). Chylomicrons ($d < 0.94$ g/mL), very low density lipoproteins (VLDL; $d = 0.94$–1.006 g/mL), low-density lipoproteins 1 (LDL_1; $d = 1.006$–1.019 g/mL), low-density lipoproteins 2 (LDL_2; $d = 1.019$–1.063 g/mL), high-density lipoproteins 2 (HDL_2; $d = 1.063$–1.125 g/mL), and high-density lipoproteins 3 (HDL_3; $d = 1.125$–1.21 g/mL) were isolated as previously described (Alaupovic et al., 1972).

Production and Characterization of Monoclonal Antibody. Production and characterization of monoclonal antibodies to human plasma ApoB, in this laboratory, have been previously described in detail (Koren et al., 1986b). Briefly, after immunization with LDL_2, mouse spleen cells were fused with mouse myeloma cells (Sp 2/0) by using the poly(ethylene glycol) method. Resulting hybridomas were cloned by the limiting dilution method. Clones were screened for reactivity with chylomicrons, VLDL, LDL_1, LDL_2, HDL_2, HDL_3, and apolipoproteins A-I, A-II, E, C-III, C-II, and C-I by using the enzyme-linked immunosorbent assay on microtiter plates (micro-ELISA). The clone (C_1) selected for this study produced antibody with high affinity for LDL_2. Binding to VLDL, LDL_1, HDL_2, and ApoB was lower, while no significant binding was observed in the case of chylomicrons, HDL_3, and apolipoproteins A-I, A-II, E, C-III, C-II, and C-I. Specificity of C_1–antibody was also analyzed by immunoblotting against large molecular weight ApoB (ApoB-100) which resulted in a positive reaction. Monoclonality of C_1–antibody was confirmed by double immunodiffusion against subclass-specific antisera and by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of the purified antibody under reducing conditions (Koren et al., 1986b). Three subsequent subclones of the C_1 clone produced antibodies of identical properties (IgG_1 κ with a single heavy and light chains on SDS–PAGE). It was, therefore, concluded that C_1–antibody was monoclonal, specific for ApoB, and showing high affinity for LDL_2.

Immunoaffinity Chromatography. C_1–monoclonal antibody was produced in ascites, purified, coupled to agarose and used in an immunoaffinity chromatography procedure as described previously (Koren et al., 1986a). The capacity of the immunoaffinity column containing 10 mL of antibody-coupled gel was determined by saturating the column with increasing volumes of plasma until no further increase in the amount of ApoB retained by the column was observed (Koren et al., 1986a). This was achieved with 10 mL of plasma which corresponded to a total of 0.6 mg of ApoB retained on the column.

Characterization of the Lipoprotein Retained by C_1–Antibody. The lipoprotein isolated from whole plasma by the above immunoaffinity chromatography procedure was characterized in terms of apolipoprotein and lipid composition, flotation coefficient, size, and electrophoretic mobility. Concentrations of apolipoproteins A-I and A-II (Curry et al., 1976a), B (Curry et al., 1978), C-I and C-II (Curry et al., 1981), C-III (Curry et al., 1980), D (Curry et al., 1977), and E (Curry et al., 1976b) were measured by previously described electroimmunoassays. Double immunodiffusion against monospecific antisera and immunoelectrophoresis were performed as described previously (Alaupovic et al., 1966). Neutral lipids were analyzed by gas–liquid chromatography according to the procedure of Kuksis et al. (1975). Phospholipids were determined according to Gerlach and Deuticke (1963). Lipoprotein electrophoresis on cellulose acetate and staining with Oil Red O were performed according to the method described by Halpern and Miguel (1973).

The analytical ultracentrifugation of the lipoprotein sample isolated by C_1–monoclonal antibody was performed in a Spinco Model E ultracentrifuge equipped with a schlieren optical system, an electronic speed control unit, and an RTIC temperature-control unit. Plate measurements were made with a Nikon microcomparator (Nikon W., Tokyo, Japan). The lipoprotein samples were dialyzed against a NaCl solution of $d = 1.063$ g/mL containing 0.02% EDTA for 48 h at 4 °C under N_2 with five changes. Flotation velocity experiments were performed as described previously (Lee & Alaupovic, 1974b) at 52000 rpm and at 20 °C. The observed flotation coefficient, S_f, was calculated as outlined by DeLalla and Gofman (1954).

Gradient SDS–Polyacrylamide Gel Electrophoresis. Linear gradient (2.5–10%) polyacrylamide slab gels (140 × 130 × 1.5 mm) were prepared by using a gradient gel former (LKB 2001-500, LKB, Bromma, Sweden). Lipoprotein samples (150 μL per well) were electrophoresed without reducing agent as previously described (Lee et al., 1984). Silver staining of gel slabs was done by using Bio-Rad's staining kit according to the producer's instructions. Immunoblotting was carried out as previously described (Koren et al., 1986b). The molecular weight of ApoE was estimated from a linear plot of logarithms

of molecular weights of protein standards vs. the R_f values of protein bands.

Electron Microscopy. Lipoprotein particles were analyzed in terms of shape and size distribution by the use of a negative staining procedure. The Formvar-coated grids were floated on 1% poly(L-lysine) (Sigma Chemical Co., St. Louis, MO) solution for 1 h. After the grids had been dried, fresh, lipoprotein-containing samples were applied immediately after immunoaffinity chromatography. Particles were allowed to settle on the grid for 2 min, and the excess of sample was removed by filter paper. This was followed by the exposure of grids to 2% phosphotungstic acid (pH 6.6) for 2 min. The samples were analyzed by using a JEOL-100 cx (JEOL, Tokyo, Japan) electron microscope.

Binding of LP-B:E_{L2} to HepG2 Cell Membranes. Cells of the human hepatoma cell line HepG2, proven to express functional LDL receptor (Dashti et al., 1984; Havekes et al., 1983), were grown in culture and preincubated in medium containing 5% lipoprotein-deficient plasma as described previously (Dashti et al., 1984). Membranes were isolated from cultured cells according to the procedure described for human fibroblasts and hepatic biopsy specimens by Hoeg et al. (1986). LP-B:E_{L2} was radiolabeled with $Na^{125}I$ using Iodo-Beads (Pierce Chemical Co., Rockford, IL) as described by Markwell (1982). Specific binding of radiolabeled LP-B:E_{L2} to HepG2 cell membranes and the affinity of binding (K_d) were analyzed by using the method of Hoeg et al. (1986). An identical study was also performed with radiolabeled LP-B, a lipoprotein containing only ApoB, which was isolated from LDL_2 and characterized as previously described (McConathy et al., 1985).

Quantification of LP-B:E_{L2}. The quantification of LP-B:E_{L2} in plasma was carried out by immunoaffinity chromatography on a column containing the C_1–antibody. The capacity of the column was found to be sufficient to remove virtually all LP-B:E_{L2} from 10 mL of normolipidemic plasma in a single run. To quantify LP-B:E_{L2}, 2 mL of plasma was run over the column at a flow rate of 1 mL/h. Both unretained and retained fractions were collected and concentrated to the original volume as described previously (McConathy et al., 1985). Concentrations of ApoB and ApoE were determined in both fractions by using electroimmunoassay. When the values of unretained and retained fractions were added, the average recoveries of both ApoB (96% ± 15%, n = 20) and ApoE (94% ± 8%, n = 20) were close to 100%. Once removed, the LP-B:E_{L2} could not be detected in the unretained plasma fraction.

The quantity of ApoB retained by the C_1 column under the above conditions was used to calculate the percentage of total plasma ApoB present in the form of LP-B:E_{L2}.

Immunoprecipitation of LP-B:E_{L2} from LDL_2. Immunoprecipitation of LP-B:E from LDL_2 was carried out by a modification of a previously described procedure (Lee & Alaupovic, 1974b; Alaupovic et al., 1986). The LDL_2 preparations isolated from plasma of normolipidemic subjects were tested by immunodiffusion with antisera to apolipoproteins B, C-I, C-II, C-III, and E; only LDL_2 preparations showing partial identity between apolipoproteins B and E, but not between ApoE and ApoC peptides, were used for the immunoprecipitation. Such selected LDL_2 preparations were analyzed for the contents of ApoB and ApoE by electroimmunoassay. One milliliter of LDL_2 was mixed with an equivalent amount of a polyclonal antiserum to ApoE (IgG fraction). The reaction mixture was incubated for 2 h at 4 °C, and the precipitated antigen–antibody complex was collected by low-speed centrifugation (2500 rpm) for 10 min. The supernatant

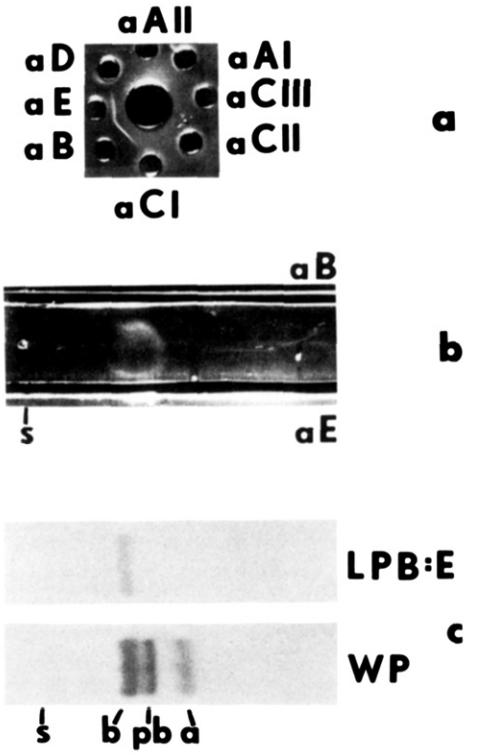

FIGURE 1: Immunochemical and electrophoretic characterization of LP-B:E_{L2}. Pattern a shows double immunodiffusion of LP-B:E_{L2} (central well) against monospecific antisera to apolipoproteins A-II (aAII), A-I (aAI), C-III (aCIII), C-II (aCII), C-I (aCI), B (aB), E (aE), d D (aD). Pattern b represents the immunoelectrophoresis of LP-B:E_{L2} against antisera to ApoB (aB) and ApoE (aE); starting point is indicated by s. Pattern c shows electrophoresis of LP-B:E_{L2} and whole plasma (WP) on cellulose acetate. Only one lipoprotein band can be seen in the case of LP-B:E_{L2}. This band has an electrophoretic mobility identical with that of the β-lipoprotein band (b) seen in whole plasma (WP). In the case of whole plasma, pb and a indicate the positions of pre-β- and α-lipoprotein bands.

fraction was tested for the presence of apolipoproteins B, C-I, C-II, C-III, and E by double diffusion analysis and electroimmunoassays. In the supernatant fraction, no ApoE and a reduced concentration of ApoB were found. Concentrations of apolipoproteins C-I, C-II, and C-III did not change. The difference between the concentrations of apolipoproteins B and E in the original LDL_2 preparation and supernatant fraction after precipitation with antibodies to ApoE was taken as the amount of apolipoproteins B and E precipitated in the form of LP-B:E_{L2} complex.

RESULTS

Characterization of Lipoprotein Particles Isolated by C_1–Monoclonal Antibody. Immunoaffinity chromatography of normolipidemic whole plasma over a column with C_1–monoclonal antibody resulted in the isolation of a lipoprotein containing apolipoproteins B and E and all major plasma lipids. Double-diffusion analysis (Figure 1a) and electroimmunoassays with antisera to apolipoproteins A-I, A-II, B, C-I, C-II, C-III, and D only showed the presence of ApoB and ApoE. Double-diffusion analysis also showed a reaction of complete identity between immunoprecipitation lines of ApoB and ApoE. The immunoelectrophoresis of LP-B:E_{L2} (Figure 1b) against antisera to ApoB and ApoE showed that both immunoprecipitation lines occurred at the same distance from the antigen, well displaying again the reaction of complete identity. The gradient SDS–polyacrylamide gel electrophoresis and immunoblotting of LP-B:E_{L2} (Figure 2, pattern C) showed the presence of two bands. The faster migrating band detected in the lower part of the gel had an apparent molecular weight

Table I: Chemical Composition of LP-B:E_{L2} Isolated from Whole Plasma and LDL_2 of Normolipidemic Subjects by Use of the Monoclonal Antibody Immunoaffinity Column and from LDL_2 by Immunoprecipitation in the Fluid Phase[a]

	[ApoB] (mg/dL)	[ApoE] (mg/dL)	% total lipids: unesterified cholesterol	cholesterol esters	triglycerides	phospholipids
LP-B:E_{L2} isolated by monoclonal antibody from						
whole plasma	6.40 ± 0.45[b]	0.68 ± 0.08	9.35 ± 0.13	37.88 ± 0.56	4.75 ± 0.52	48.00 ± 0.91
LDL_2	6.15 ± 0.32	0.64 ± 0.05	8.98 ± 0.11	39.00 ± 0.32	4.21 ± 0.36	47.81 ± 0.57
LP-B:E_{L2} isolated from LDL_2 by immunoprecipitation	5.0 ± 0.30	0.53 ± 0.04	ND[c]	ND	ND	ND

[a] Identical plasma samples were used in all three isolation experiments. No measurable quantities of apolipoproteins A-I, A-II, C-I, C-II, C-III, and D were found in LP-B:E_{L2} isolated by either method. [b] Mean from five subjects ± SD. [c] ND = not determined.

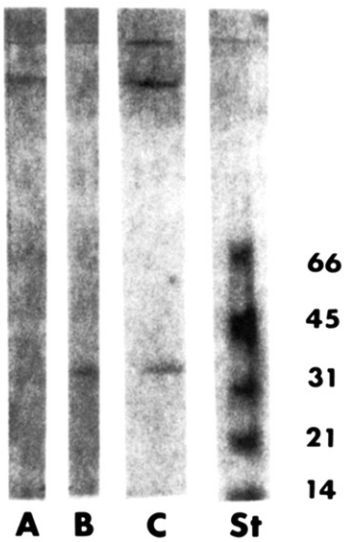

FIGURE 2: Gradient SDS–polyacrylamide gel electrophoresis and immunoblotting of LP-B:E_{L2}. The isolated LP-B:E_{L2} was electrophoresed on a 2.5–10% gradient gel. One strip of the gel was silver stained (pattern C). The other gel strips were subjected to Western transfer and immunoblotting with polyclonal antisera to ApoB (pattern A) and ApoE (pattern B). The pattern St represents protein bands used as the molecular weight standards: bovine serum albumin with a molecular weight of 66 200 (66); ovalbumin with a molecular weight of 45 000 (45); carbonic anhydrase with a molecular weight of 31 000 (31); soybean trypsin inhibitor with a molecular weight of 21 500 (21); and lysozyme with a molecular weight of 14 400 (14).

of 34 000 and gave a positive reaction with an antiserum to ApoE (Figure 2, pattern B) but not with an antiserum to ApoB (Figure 2, pattern A); these properties were consistent with its identification as ApoE. The slow migrating band detected close to the top of the gel (Figure 2, pattern C) had a mobility characteristic of ApoB-100 (Dashti et al., 1986) and only reacted with an antiserum to ApoB (Figure 2, pattern A). Since no reducing agents were used in the polyacrylamide gel electrophoresis, apolipoproteins B and E detected in LP-B:E_{L2} did not appear to be covalently bound. The lipid composition of lipoprotein particles was characterized by equal weight percentages of total cholesterol and phospholipids and a relatively small percentage of triglyceride (Table I). The lipoprotein nature of ApoB- and ApoE-containing particles was confirmed by electrophoresis on cellulose acetate, showing a single band in the β position stained with Oil Red O (Figure 1c). Electron microscopy of LP-B:E_{L2} revealed a uniform population of spherical particles (Figure 3). A detailed morphometric analysis showed that 9% of all particles were in the size range of 15–20 mm, 88% in the range of 20–25 nm, and only 3% in the range of 25–30 nm. This size distribution indicated a higher degree of uniformity of LP-B:E_{L2} particles in comparison with the size distribution (49%, 45%, and 6%, respectively) of the conventionally isolated LDL_2 particles (Koren et al., 1986a). Analytical ultracentrifugation also demonstrated the uniformity of LP-B:E_{L2} particles. The

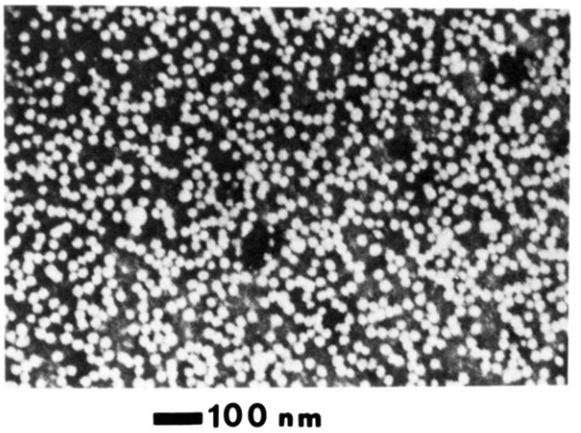

FIGURE 3: Negatively stained electron micrograph of LP-B:E_{L2}. Spherical particles have an average diameter of 20 nm.

schlieren pattern exhibited a single, symmetrical peak with an S_f(obsd) of 9.5. These results indicate that LP-B:E_{L2} particles constitute the S_f = 7–12 subfraction of LDL_2 described by Shen et al. (1981). Both ApoB and ApoE were found to reside on the same lipoprotein particles as demonstrated by the reaction of completed identity on double immunodiffusion and immunoelectrophoresis (Figure 1a,b). The apparent molar ratio between ApoE and ApoB was close to 2 (1.7) using 520 000 as the molecular weight for ApoB and 33 000 for ApoE. This indicated that the protein moiety of these lipoprotein particles may contain two molecules of ApoE and one molecule of ApoB.

Gradient SDS–polyacrylamide gel electrophoresis revealed ApoB-100 as the only molecular form of this apolipoprotein. It was concluded that C_1–antibody retained a homogeneous population of lipoprotein particles characterized by the presence of apolipoproteins B and E as the unique chemical feature. This lipoprotein was named on the basis of its apolipoprotein composition LP-B:E_{L2}. LP stands for lipoprotein, the letters B and E represent apolipoproteins B and E, and L2 indicates the LDL_2 nature of particles which should be distinguished from lipoproteins containing also ApoB and ApoE but having density properties of VLDL or LDL_1. To ascertain that LP-B:E_{L2} particles were not generated as artifacts, this lipoprotein was isolated by immunoaffinity chromatography from whole plasma and LDL_2 and by fluid-phase immunoprecipitation of LDL_2 with a polyclonal antiserum to ApoE. All three methods resulted in the isolation of lipoprotein particles that were similar in their composition (Table I). Rechromatography of isolated LP-B:E_{L2} over the same column had no effect on its physical and chemical properties. These results excluded artifactual generation of LP-B:E_{L2} and confirmed its stability.

Binding of LP-B:E_{L2} to HepG2 Cell Membranes. A binding study of radiolabeled LP-B:E_{L2} and LP-B to the HepG2 cell membrane in the presence and absence of excess unlabeled LDL_2 demonstrated that both lipoprotein particles bind to the membrane in a specific and saturable manner indicative of

Table II: Average Concentrations of Apolipoproteins B and E, Cholesterol, Triglycerides, and LP-B:E_{L2} in Plasma of Normolipidemic Female and Male Subjects

	[ApoB] (mg/dL)	[ApoE] (mg/dL)	[cholesterol] (mg/dL)	[triglycerides] (mg/dL)	[LP-B:E_{L2}] (% total plasma ApoB)
women (n = 12)	71.9 ± 13.3[a]	9.06 ± 2.2	168 ± 31	59 ± 22	4.95 ± 2.03
men (n = 8)	85.15 ± 25.3[b]	11.31 ± 2.5[c]	193 ± 44[b]	85 ± 37[c]	6.49 ± 2.77[b]

[a] Mean ± SD. [b] Difference is not significant. [c] Difference is significant, $p < 0.05$.

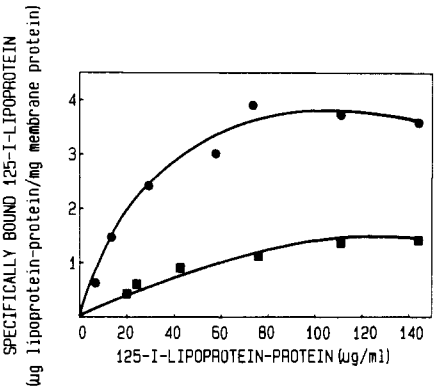

FIGURE 4: Specific binding of radiolabeled LP-B:E_{L2} (●) and LP-B (■) to the HepG2 cell membrane.

receptor-mediated binding (Figure 4). Apparent K_d values indicated a higher binding affinity of LP-B:E_{L2} (K_d = 21 nM for the LP-B:E_{L2} apolipoprotein) compared to that of LP-B (K_d = 69 nM for the LP-B apolipoprotein). The maximum specific binding of radiolabeled LP-B:E_{L2} to the cell membrane (3.8 μg of LP-B:E_{L2} apolipoprotein/mg of cell membrane protein) was also higher than that of LP-B (1.5 μg of LP-B apolipoprotein/mg of cell membrane protein). The addition of excess unlabeled LDL_2 resulted in a 72% displacement of labeled LP-B:E_{L2} and 64% displacement of LP-B. Unlabeled HDL did not compete significantly with either LP-B:E_{L2} or LP-B for binding to the cell membrane.

Concentration of LP-B:E_{L2} in Normolipidemic Plasma. The concentration of LP-B:E_{L2} was expressed as a percentage of the total plasma ApoB found in the form of LP-B:E_{L2}. In normolipidemic women, the LP-B:E_{L2} particles accounted, on an average, for 4.95% of the total plasma ApoB and in normolipidemic men for 6.49% (Table II). These differences, however, were not statistically significant. It should be pointed out that in one male and two female normolipidemic subjects, relatively high percentages (20%, 18%, and 17%, respectively) of total plasma ApoB were found in the form of LP-B:E_{L2}. These subjects were not taken into account when the average value was calculated for each group. The concentration of LP-B:E_{L2} showed significant positive correlation with plasma triglyceride concentration (r = 0.46, $p < 0.05$). No significant correlations were found with plasma cholesterol, ApoB, and ApoE.

Discussion

Results of this study have demonstrated that normolipidemic human plasma LDL_2 contain a family of lipoprotein particles characterized by apolipoproteins B and E as the constituents of its protein moiety. To emphasize its characteristic apolipoprotein composition and hydrated density properties, this family of particles was named LP-B:E_{L2}.

The use of a monoclonal antibody (C_1) to ApoB allowed the isolation of LP-B:E_{L2} by single-step immunoaffinity chromatography. This procedure also permitted a semiquantitative determination of ApoB present in these lipoprotein particles. These cholesterol-rich lipoprotein particles exhibited β-electrophoretic mobility and a narrow range of density [S_f(obsd) = 9.5] and size (average particle diameter of 20 nm) distributions. ApoB-100 was found to be the only molecular form of this apolipoprotein. In most normolipidemic subjects, LP-B:E_{L2} particles accounted for less than 10% of the total plasma ApoB content.

Although results of several studies have suggested the possible presence of LP-B:E in human plasma (Alaupovic et al., 1986; Gibson-Carey et al., 1984; Castro & Fielding, 1984), the direct evidence for the actual existence of LP-B:E particles and the description of some of their physical and chemical properties are now presented for the first time.

To exclude the possible artifactual generation of LP-B:E_{L2} particles during the isolation procedure, precautions were made to shorten the exposure time of LP-B:E_{L2} particles to the dissociating agent used for their elution. A number of studies have amply demonstrated the well-preserved structural and metabolic integrity of lipoprotein particles eluted from immunosorbers by 3 M NaSCN (Koren et al., 1986a; McConathy et al., 1985; Gibson-Carey et al., 1985). Results of this study confirm such findings by demonstrating that immunoaffinity chromatography yields LP-B:E_{L2} particles of stable and reproducible composition from the whole plasma as well as LDL_2. Moreover, LP-B:E_{L2} particles of similar apolipoprotein composition were isolated from LDL_2 by immunoprecipitation with a polyclonal antiserum to ApoE, a procedure which included neither the chromatography nor the dissocating agents. Since it is quite unlikely that all three isolation experiments would generate identical artifacts, LP-B:E_{L2} appear to represent relatively stable lipoprotein particles. What is the origin of these particles? LP-B:E_{L2} particles were identified in rat liver perfusates (Dolphin et al., 1978), rat hepatocytes (Davis et al., 1979), and the culture medium of human hepatoma HepG2 cells (Dashti et al., 1985). In hypercholesterolemic rats, cholesterol ester rich lipoproteins containing ApoB and ApoE were identified as the major lipoprotein species in both VLDL and LDL (Noel et al., 1979); deficient in ApoC peptides, these particles were considered to be secretory liver products rather than catabolic remnants of VLDL. However, under normal conditions, secreted LP-B:E particles most probably interact in the extracellular compartment with ApoC peptides to generate triglyceride-rich LP-B:C:E particles (Dolphin et al., 1978). It is quite possible that LP-B:E_{L2}, described in this study, represents one of the degradation products formed during the lipolysis of triglyceride-rich lipoproteins secreted by liver. A recent study, for example, showed that in vitro lipolysis of triglyceride-rich VLDL particles containing apolipoproteins B, C-III, C-II, C-I, and E, i.e., LP-B:E:C-I:C-II:C-III, resulted in the formation of LDL_2 particles, some of which were identified as LP-B:E:C-I (Alaupovic et al., 1986).

The use of monoclonal antibodies for the isolation of ApoB-containing lipoproteins has been reported (Koren et al., 1983, 1986a; Milne et al., 1984). Milne et al. (1984) used monoclonal antibody to separate VLDL particles containing ApoB-100 from VLDL particles containing ApoB-48. The basis for this separation was the specificity of monoclonal antibody which reacted with ApoB-100 but not with ApoB-48. There are two possible explanations for the separation of

LP-B:E_{L2} by a monoclonal antibody to ApoB described in this study. One of these is based on the microheterogeneity of ApoB. In this case, C_1–antibody would be specific for a hypothetical isoform of ApoB-100 present exclusively in LP-B:E_{L2}. However, a more likely explanation is that C_1–antibody is specific for a particular conformation of ApoB expressed in LP-B:E_{L2} particles. It has generally been accepted that monoclonal antibodies can distinguish global conformational states of a protein, such as might be mediated by allosteric effectors (Berzofsky et al., 1984). More specifically, monoclonal antibodies to ApoB have been shown to be sensitive to conformational differences of ApoB in various lipoprotein particles or induced by various lipids (Patten et al., 1982; Marcel et al., 1984). According to the conformation hypothesis, epitope recognized by C_1–antibody is fully and most favorably expressed in LP-B:E_{L2}, allowing the highest binding affinity between C_1 and the LP-B:E_{L2} particle. Other types of ApoB-containing particles, with the corresponding epitope altered or hidden, are bound with an insufficient affinity to be retained or they pass through the column completely ignored by the C_1–antibody. The net result is retention of LP-B:E_{L2} and its separation from other ApoB-containing particles. The mechanism responsible for the induction in LP-B:E_{L2} of a conformation of ApoB that matches requirements of the C_1–antibody remains to be established. Specific microcomposition and organization of lipid molecules adjacent to the epitope and/or the presence of ApoE may induce the most favorable conformation of ApoB. This study offers new possibilities for the isolation of lipoprotein subspecies. It would be very difficult, if not impossible, to isolate LP-B:E_{L2} from the bulk of ApoB-containing particles by the conventional methods. While these particles were also isolated from LDL_2 by immunoprecipitation with polyclonal antibodies to ApoE, this method was not applicable to the whole plasma because of the simultaneous precipitation of other ApoE-containing lipoproteins. The obvious advantage of C_1–monoclonal antibody is to provide a means whereby the LP-B:E_{L2} particles can be isolated from whole plasma in a single-step procedure.

The higher binding of LP-B:E_{L2} to the HepG2 cell membranes than that of LP-B indicates that these lipoprotein particles may be metabolically different from other LDL_2 particles. Further studies on the occurrence and concentrations of LP-B:E_{L2} in various dyslipoproteinemic states and their interaction with LDL receptors are currently in progress in this laboratory.

Registry No. Cholesterol, 57-88-5.

REFERENCES

Alaupovic, P. (1980) *Ann. Biol. Clin. (Paris) 38*, 83–93.

Alaupovic, P., Sanbar, S. S., Furman, R. H., Sullivan, M. L., & Walraven, S. L. (1966) *Biochemistry 5*, 4044–4053.

Alaupovic, P., Lee, D. M., & McConathy, W. J. (1972) *Biochim. Biophys. Acta 260*, 689–707.

Alaupovic, P., Wang, C.-S., McConathy, W. J., Weiser, D., & Downs, D. (1986) *Arch. Biochem. Biophys. 244*, 226–237.

Berzofsky, J. A. (1984) in *Monoclonal and Anti-Idiotypic Antibodies. Probes for Receptor Structure and Function*, pp 1–19, Alan R. Liss, Inc., New York.

Castro, G. R., & Fielding, C. J. (1984) *J. Lipid Res. 25*, 58–67.

Curry, M. D., Alaupovic, P., & Suenram, C. A. (1976a) *Clin. Chem. (Winston-Salem, N.C.) 22*, 315–322.

Curry, M. D., McConathy, W. J., Alaupovic, P., Ledford, J. D., & Popovic, M. (1976b) *Biochim. Biophys. Acta 439*, 413–425.

Curry, M. D., McConathy, W. J., & Alaupovic, P. (1977) *Biochim. Biophys. Acta 491*, 232–241.

Curry, M. C., Gustafson, A., Alaupovic, P., & McConathy, W. J. (1978) *Clin. Chem. (Winston-Salem, N.C.) 24*, 280–286.

Curry, M. D., McConathy, W. J., Fesmire, J. D., & Alaupovic, P. (1980) *Biochim. Biophys. Acta 617*, 505–513.

Curry, M. D., McConathy, W. J., Fesmire, J. D., & Alaupovic, P. (1981) *Clin. Chem. (Winston-Salem, N.C.) 27*, 543–548.

Dashti, N., Wolfbauer, G., Koren, E., Knowles, B., & Alaupovic, P. (1984) *Biochim. Biophys. Acta 794*, 373–384.

Dashti, N., Koren, E., Knight-Gibson, C., & Alaupovic, P. (1985) *Arteriosclerosis (Dallas) 5*, 495a.

Dashti, N., Lee, D. M., & Mok, T. (1986) *Biochem. Biophys. Res. Commun. 137*, 493–499.

Davis, R. A., Engelhorn, S. C., Pangburn, S. H., Weinstein, D. B., & Steinberg, D. (1979) *J. Biol. Chem. 254*, 2010–2016.

DeLalla, O., & Gofman, J. (1954) in *Methods of Biochemical Analysis* (Glick, D., Ed.) Vol. I, pp 459–478, Wiley-Interscience, New York.

Dolphin, P. J., Wong, L., & Rubinstein, D. (1978) *Can. J. Biochem. 56*, 673–683.

Fredrickson, D. S., Goldstein, J. L., & Brown, M. S. (1978) in *The Metabolic Basis of Inherited Disease* (Stanbury, J. B., Wyngaarden, J. B., & Fredrickson, D. S., Eds.) pp 604–655, McGraw-Hill, New York.

Gerlach, E., & Deuticke, B. (1963) *Biochem. Z. 337*, 477–479.

Gibson-Carey, J., Rubinstein, A., & Brown, W. V. (1984) *Clin. Chem. (Winston-Salem, N.C.) 30*, 1784–1788.

Gibson-Carey, J., Rubinstein, A., Ngai, N., Ginsberg, H. N., Le, N. A., Gordon, R. E., Goldberg, I. J., & Brown, V. W. (1985) *Biochim. Biophys. Acta 835*, 113–123.

Halpern, M. J., & Miguel, M. J. (1973) *Nouv. Presse Med. 2*, 3124–3129.

Hammond, M. G., Mengel, M. C., Warmke, G. L., & Fisher, W. R. (1977) *Metab., Clin. Exp. 26*, 1231–1242.

Havekes, L., Van Hinsbergh, V., Kempen, H. J., & Emeis, J. (1983) *Biochem. J. 214*, 951–958.

Hoeg, J. M., Demosky, S., Jr., Lackner, K. J., Osborne, J. C., Jr., Oliver C., & Brewer, B. H., Jr. (1986) *Biochim. Biophys. Acta 876*, 13–21.

Koren, E., Solter, D., Knight-Gibson, C., & Alaupovic, P. (1983) *Arteriosclerosis (Dallas) 3*, 510a.

Koren, E., Knight-Gibson, C., Wen, G., DeBault, L. E., & Alaupovic, P. (1986a) *Biochim. Biophys. Acta 876*, 101–107.

Koren, E., Solter, D., Lee, D. M., Reiner, Z., McConathy, W. J., Dashti, N., & Alaupovic, P. (1986b) *Biochim. Biophys. Acta 876*, 91–100.

Krauss, R. M., & Burke, D. J. (1982) *J. Lipid Res. 23*, 97–104.

Kuksis, A., Myher, J. J., Marai, L., & Geher, K. (1975) *J. Chromatogr. Sci. 13*, 423–430.

Lee, D. M., & Alaupovic, P. (1970) *Biochemistry 9*, 2244–2252.

Lee, D. M., & Alaupovic, P. (1974a) *Atherosclerosis (Shannon, Irel.) 19*, 501–520.

Lee, D. M., & Alaupovic, P. (1974b) *Biochem. J. 137*, 155–167.

Lee, D. M., Koren, E., Singh, S., & Mok, T. (1984) *Biochem. Biophys. Res. Commun. 123*, 1149–1156.

Lipid Research Clinics Laboratory Manual (1974) 1, *DHEW Publ. (NIH) 75-628*, National Heart and Lung Institute, Bethesda, MD, pp 74–81.

Lipid Research Clinics Program (1984) *JAMA, J. Am. Med. Assoc. 251*, 351–364.
Marcel, Y. L., Hogue, M., Weech, P. K., & Milne, R. W. (1984) *J. Biol. Chem. 259*, 6952–6957.
Markwell, M. A. K. (1982) *Anal. Biochem. 125*, 427–432.
McConathy, W. J., Koren, E., Wieland, H., Campos, E. M., Lee, D. M., Kloer, H. U., & Alaupovic, P. (1985) *J. Chromatogr. 342*, 47–66.
Milne, R. W., Weech, P. K., Blanchette, L., Davignon, J., Alaupovic, P., & Marcel, Y. L. (1984) *J. Clin. Invest. 73*, 816–823.
Noel, S.-P., Wong, L., Dolphin, P. J., Dory, L., & Rubinstein, D. (1979) *J. Clin. Invest. 64*, 674–683.
Patton, J. G., Alley, M. C., & Mao, S. T. J. (1982) *J. Immunol. Methods 55*, 193–203.
Rubenstein, B. (1978) *Can. J. Biochem. 56*, 977–980.
Shen, M. S., Kraus, R. M., Lindgren, F. T., & Forte, T. M. (1981) *J. Lipid Res. 22*, 236–244.

Purification and Characterization of *Rhodobacter sphaeroides* Acyl Carrier Protein†

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ABSTRACT: Acyl carrier protein (ACP) has been purified from the facultative phototrophic bacterium *Rhodobacter sphaeroides*. The ACP preparation was >95% homogeneous as determined by native and disodium dodecyl sulfate (Na_2DodSO_4)–polyacrylamide gel electrophoreses and N-terminal amino acid analysis. Amino acid compositional analysis revealed that the protein contains approximately 75 amino acids, has a calculated minimum molecular weight of 8700, and lacks the amino acids tyrosine and tryptophan. The presence of the characteristic 4′-phosphopantetheine prosthetic group was indicated by the occurrence of equimolar quantities of β-alanine and taurine in amino acid hydrolysates and was confirmed by independent chemical analysis. The protein displayed a p*I* of 3.8 and had a calculated partial specific volume of 0.732 mL/g. The primary structure of the protein has been determined for the first 46 amino acid residues from the N terminus of the molecule, and the region of the molecule encompassing the amino acids from residues 31 to 44 was found to have 100% homology with the identical residues in *Escherichia coli* ACP. In contrast to *E. coli* ACP, *R. sphaeroides* ACP migrated according to its molecular weight during Na_2DodSO_4 gel electrophoresis, was resistant to pH-induced denaturation, and comigrated with the *cis*-vaccenoyl-ACP derivative during native gel electrophoresis. It is proposed that the basis for these properties is the enhanced hydrophobic character of the protein.

Organisms possessing a type II (nonaggregated) fatty acid synthetase complex (bacteria and plants) utilize a freely dissociable acyl carrier protein (ACP)[1] to bind the intermediates and products of fatty acid synthesis (Vagelos, 1971; Prescott & Vagelos, 1972; Bloch & Vance, 1977). In addition to their role in bacterial fatty acid synthesis, acyl-ACP derivatives are known to directly serve as acyl donors for de novo phospholipid biosynthesis in bacteria (Ailhaud & Vagelos, 1966; van den Bosch & Vagelos, 1970; Goldfine et al., 1967; Goldfine & Ailhaud, 1971; Lueking & Goldfine, 1975a; Cronan, 1978; Rock & Cronan, 1982) and, most recently, have been shown to participate as acyl donor substrates for the production of lipid A (Anderson et al., 1985) and for the reacylation of lysophosphatidylethanolamine in cells of *Escherichia coli* (Rock, 1984). The central role played by ACP in bacterial lipid metabolism was recently highlighted by the results of Rock and Jackowski (1982), who showed, via a series of in vivo studies, that the size and composition of the intracellular acyl-ACP pool responded to the metabolic status of cellular phospholipid biosynthesis.

The non-sulfur purple facultative phototrophic bacterium *Rhodobacter sphaeroides* provides an attractive system for investigations of the regulation of bacterial fatty acid and phospholipid biosynthesis (Kaplan, 1978; Lueking et al., 1978; Niederman et al., 1978; Takemoto, 1974; Donohue & Kaplan, 1985). In contrast to *E. coli*, *R. sphaeroides* displays an obligate requirement for acyl-ACP substrates as acyl donors for de novo phospholipid synthesis (Lueking & Goldfine, 1975b; Cooper & Lueking, 1984), and both temporal and light-mediated controls of phospholipid synthesis have been demonstrated in this organism (Lueking et al., 1978; Fraley et al., 1978; Campbell & Lueking, 1983). In view of the central importance of ACP and acyl-ACP in the lipid metabolism of *R. sphaeroides* (Boyce & Lueking, 1984; Cooper & Lueking, 1984), we have purified and examined the physical

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[1] Abbreviations: ACP, acyl carrier protein (reduced form); Na_2DodSO_4, disodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DEAE, diethylaminoethyl; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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