

Human low density lipoprotein subfractions separated by gradient gel electrophoresis: composition, distribution, and alterations induced by cholesteryl ester transfer protein

Philippe Gambert,^{1,*} Catherine Bouzerand-Gambert,* Anne Athias,* Michel Farnier,[†] and Christian Lallemand*

Laboratoire de Biochimie Médicale,* Faculté de Médecine, Dijon, and Unité des Maladies Métaboliques,[†] Hôpital du Bocage, Dijon, France

Abstract Low density lipoprotein (LDL) subfractions were studied in sera from 208 normolipidemic, 22 hypercholesterolemic, and 33 hypertriglyceridemic subjects. Whole serum without preliminary ultracentrifugation was submitted to electrophoresis in a nondenaturing polyacrylamide gel. Three main LDL patterns were observed in normolipidemic sera: type 1, characterized by the presence of only one major band; type 2, characterized by the presence of two close major bands; and type 3, where LDL were more dispersed and presented at least three distinct bands. Type 1 was more frequent in men (43%) than in women (19%). The tendency for a higher potential coronary disease risk profile sera, namely higher triglyceride level, higher very low density lipoprotein + LDL fraction and lower high density lipoprotein (HDL) fraction, was type 3 < type 2 < type 1. The LDL patterns found in hypercholesterolemic sera were of type 1. Hypertriglyceridemic sera were characterized by the presence of a major band of small size. Separated LDL subfractions were collected by electroelution and analyzed for composition. In all subspecies, the mass ratio of core to surface components was constant as well as the molar ratio of the two lipid surface components, phospholipids and free cholesterol. Surface lipid to apolipoprotein B ratio, cholesteryl ester to triglyceride ratio, and cholesteryl ester to apoB ratio increased with particle size increment. Incubation of LDL with HDL and purified cholesteryl ester transfer protein induced a transfer of lipids, mainly cholesteryl esters and phospholipids, to LDL and an increase of the sizes of LDL subfractions. This suggests that lipid transfers from HDL to LDL might be a process of intravascular LDL remodeling and a factor of LDL polymorphism. —Gambert, P., C. Bouzerand-Gambert, A. Athias, M. Farnier, and C. Lallemand. Human low density lipoprotein subfractions separated by gradient gel electrophoresis: composition, distribution, and alterations induced by cholesteryl ester transfer protein. *J. Lipid Res.* 1990. 31: 1199–1210.

Supplementary key words gel filtration • electroelution • hypertriglyceridemia • hypercholesterolemia

The heterogeneity of the main plasma lipoprotein fractions is a revealing sign of their complex synthesis process and intravascular remodeling. A detailed knowledge of

lipoprotein subclass composition and distribution should be highly informative for the understanding of lipoprotein metabolism and atherogenic disorders. That is especially true for LDL, the main transport particles for cholesterol in human plasma. LDL have been defined as lipoproteins of density comprised between 1.019 and 1.063 g/ml. They are particles constituted of a core of apolar lipid components, cholesteryl esters and triglycerides, surrounded by a polar coat of phospholipids, unesterified cholesterol, and proteins. On average, the LDL mass comprises approximately 40% cholesteryl esters, 25% proteins, essentially apoB-100, 20% phospholipids, 10% unesterified cholesterol, and 5% triglycerides (1). LDL heterogeneity has been demonstrated by ultracentrifugation (1–6) and by nondenaturing gradient gel electrophoresis (3, 7–9). Analysis of the subfractions separated by ultracentrifugation revealed that a single copy of apoB-100 is present per particle (5) and that mean particle diameter, molecular weight, lipid to protein ratio, cholesteryl ester, unesterified cholesterol, and phospholipid contents decrease progressively with increasing density of the particles (2, 5). In contrast, the variations of the relative proportions of the core lipids show an inflection in the middle of the density range, namely a higher ratio of cholesteryl ester to triglyceride in the intermediate fractions compared with those of highest and lowest densities (2, 5).

The aspects of LDL heterogeneity, as observed after ultracentrifugation, are matters of controversy. Some

Abbreviations: apo, apolipoprotein; VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; CETP, cholesteryl ester transfer protein; EDTA, ethylenediamine tetraacetic acid.

[†]To whom reprint requests should be addressed at: Laboratoire de Biochimie Médicale, Hôpital du Bocage, 21034 Dijon, France.

studies have shown multiple isopycnic bands (2, 3) whereas others indicated a unimodal distribution of LDL subspecies (5). Lipoprotein separation according to size by gradient gel electrophoresis clearly revealed multiple LDL bands whose distribution varies with plasma lipid status (3, 8). Different LDL patterns have already been individualized (7-9). Their study is promising but would be strengthened by chemical and structural analysis of the observed subfractions. To this aim we have studied LDL polymorphism in sera of normo- and dyslipidemic subjects. Whole serum was submitted to electrophoresis in a nondenaturing polyacrylamide gradient gel without preliminary ultracentrifugation. Separated fractions were collected by electroelution and analyzed for composition. A classification of the observed LDL profiles was attempted and we explored the effects of CETP-induced transfers of cholesteryl esters to LDL to provide information on the mechanism of LDL subspecies formation.

MATERIALS AND METHODS

Subjects

Normolipidemic subjects (107 women, 101 men) were 20 to 35 years old. Their serum cholesterol and triglyceride concentrations were below 230 mg/dl and 140 mg/dl, respectively. They did not take drugs other than oral contraceptives.

The group of hypertriglyceridemic subjects included 33 women and 48 men, 14 to 82 years old. Their serum triglyceride concentrations ranged from 180 to 1700 mg/dl (mean \pm SD: 349 \pm 157 mg/dl) and their serum cholesterol concentrations were below 266 mg/dl (mean \pm SD: 203 \pm 14 mg/dl).

The group of hypercholesterolemic subjects included 22 women and 19 men, 16 to 62 years old. Their serum cholesterol concentrations ranged from 258 to 579 mg/dl (mean \pm SD: 351 \pm 81 mg/dl) and their serum triglyceride concentrations were below 145 mg/dl (mean \pm SD: 79 \pm 35 mg/dl).

The dyslipidemic subjects were not taking lipid-lowering drugs for at least 2 months.

Blood samples

Blood was collected from all subjects after a 12-h overnight fast. Blood samples were drawn into plain glass tubes and placed at 4°C. They were kept at this temperature during all subsequent operations. Sera were collected by a 5-min centrifugation at 3,000 *g* and analyzed within a few hours.

Electrophoretic separation of LDL subfractions

Serum lipoproteins were separated by electrophoresis on nondenaturing polyacrylamide gradient gels according

to the procedure previously described (10). The electrophoresis was carried out in a 7.4 cm \times 7.4 cm \times 2.7 mm vertical slab gel casted in a GSC-8 apparatus (Pharmacia, Uppsala, Sweden). The polyacrylamide gradient gel ranged from 23 to 180 g/l. The migration buffer was a Tris-glycine 14:110 mmol/l (pH 8.3) solution. Lipoproteins were prestained by mixing equal volumes of serum and 5 g/l solution of Sudan black B in ethylene glycol. After a 30 min 50 V preelectrophoresis, an 8- μ l portion of each mixture was applied to the gel in a 3-mm large slot. The electrophoresis was performed in a Pharmacia GE-2/4 LS cell at 4°C for 21 h, 1 h at 30 V, 2 h at 60 V, and 18 h at 140 V. The gels were scanned at 633 nm with a 2202 Ultrosan laser densitometer (LKB, Bromma, Sweden) coupled with a CR4A Chromatopac reporting integrator (Shimadzu, Kyoto, Japan).

Lipoprotein size calibration

The apparent diameters of the separated LDL subfractions were determined as indicated by Nichols, Krauss, and Musliner (11) by comparison with a standard protein mixture and calibrated latex particles. The calibration preparations, high molecular weight protein calibration kit from Pharmacia and carboxylated latex beads from Duke Scientific (Palo Alto, CA) were placed in lateral slots of the polyacrylamide gel and electrophoresed together with the stained sera. After completion of the electrophoresis, each lipoprotein fraction was located by cutting guide lines on the surface of the gel. The lateral portions of the gel containing the calibration preparations were cut off and stained with Coomassie G 250 (Sigma, St. Louis, MO). The apparent diameters corresponding to the guide lines were calculated from a calibration curve constructed with catalase (10.4 nm), apoferritin (12.2 nm), thyroglobulin (17.0 nm), and latex beads (38.0 nm).

Preparation of LDL subfractions

An aliquot of 200 μ l of serum mixed with 100 μ l of Sudan black B solution was layered on one slab gel in a 7-cm large slot. After electrophoretic separation, conducted as indicated above, the portions of the gel containing individualized LDL subfractions were cut off and placed into the cells of an Isco (Lincoln, NE) concentrator where lipoproteins were extracted from the gels by electroelution. The elution was performed for 4 h at 2 mA per cell with a 16 mmol/l Tris, 4 mmol/l glycine, 1.4 g/l sucrose concentration buffer and a 160 mmol/l Tris, 40 mmol/l glycine elution buffer. Each band was collected in about 200 μ l of buffer.

Lipoprotein component assays

Total cholesterol, unesterified cholesterol, triglyceride, and choline-containing phospholipid concentrations were measured on a Cobas Bio analyzer (Roche, Basel, Swit-

zeland) by enzymatic methods using Boehringer (Mannheim, FRG) reagents. Serum HDL cholesterol was measured after selective precipitation of apoB-containing lipoproteins by concanavalin A. The technique was derived from that of Chicaud et al. (12): 25 μ l of serum was added to 250 μ l of a 1 g/l concanavalin A (Sigma), 500 g/l polyethylene glycol (Sigma) solution; after 15 min at room temperature the mixture was centrifuged for 5 min at 10,000 *g* and cholesterol was assayed in the supernatant by an enzymatic method. Serum VLDL + LDL cholesterol concentrations were obtained by difference. LDL apoB content was measured by electroimmunoassay in a 0.6 g/l agarose, 3.75 g/l polyacrylamide gel (13). The polyclonal anti-apoB antibodies were from Atlantic Antibodies (Scarborough, ME). The primary standard was an LDL fraction obtained by sequential ultracentrifugation between densities 1.040 and 1.050 g/ml. Its protein content, assumed to be 95% apoB, was assayed by the Lowry et al. method (14) using bovine serum albumin as a standard. ApoA-I was assayed by enzyme immunoassay with Behring (Marburg, FRG) anti-apoA-I antibodies.

Quantitation of HDL subfractions

The two main HDL subfractions separated from pre-stained sera by electrophoresis in polyacrylamide gradient gels, small size fraction HDL_S (mean apparent diameter 9.3 nm) and large size fraction HDL_L (mean apparent diameter 10.6 nm), were quantified by scanning the gels at 633 nm with a 2202 Ultrascan laser densitometer (LKB) coupled with a HP 3390 reporting integrator (Hewlett-Packard, Palo Alto, CA) according to the method previously described (15). HDL_S cholesterol and HDL_L cholesterol were calculated from HDL cholesterol and from the proportions of HDL_S and HDL_L obtained by densitometry. Experiments conducted with HDL subfractions separated by ultracentrifugation showed a close relationship of HDL_L and HDL_S with HDL₂ (d 1.063–1.125 g/ml) and HDL₃ (d 1.125–1.210 g/ml), respectively (10, 15).

Preparation of CETP

CETP was purified from human serum by a procedure derived from those of Pattnaik et al. (16) and Bastiras and Calvert (17). A 200-ml volume of serum was treated with ammonium sulfate between 25 and 55% saturation. The precipitate was redissolved by extensive dialysis against water and, after adjustment of the density to 1.21 g/ml with solid NaBr, lipoproteins were removed by ultracentrifugation at 250,000 *g* for 24 h in a Beckman (Palo Alto, CA) 70 Ti rotor. All the further steps of purification were done by chromatography on a Pharmacia Fast Protein Liquid Chromatography system at room temperature. First, hydrophobic chromatography was carried out on a

1.6 \times 40 cm column of phenyl-Sepharose CL-4B (Pharmacia) equilibrated with a 150 mmol/l NaCl, 1 mmol/l EDTA, 200 mg/l sodium azide solution. Once the absorbance of the effluent decreased to 0.3, the eluent was changed to water. Proteins eluted with water, in a 20-ml fraction, were submitted to a cation exchange chromatography on a 1 \times 40 cm carboxymethyl Sepharose column (Pharmacia) equilibrated with a 50 mmol/l sodium acetate, 1 mmol/l EDTA, 200 mg/l sodium azide, pH 4.5, solution. Proteins were eluted with a 0–1 mol/l NaCl gradient. The 150–200 mmol/l NaCl fraction (10 ml) was immediately concentrated to 0.5 ml and washed with a 20 mmol/l sodium phosphate, 150 mmol/l NaCl, 1 mmol/l EDTA, 200 mg/l sodium azide, pH 7.4, buffer in a micro-concentrator Centricon-30 (Amicon, Danvers, MA). After dialysis against a 10 mmol/l Tris-HCl pH 7.4 buffer, this fraction was loaded onto a MonoQ HR 5/5 anion exchange column (Pharmacia) equilibrated with the same buffer and eluted with a 0–1 mol/l NaCl gradient. CETP was recovered in the 0.15 mol/l NaCl fraction (2 ml). The CETP activity, assayed as described by Pattnaik et al. (16) with the modification of Abbey, Calvert, and Barter (18), was 60–80 units/ml (1 unit = 100 times the fraction of LDL cholesteryl ester replaced per 3 h). There was no lecithin:cholesterol acyltransferase activity.

Preparation of lipoprotein fractions by ultracentrifugation

Lipoprotein fractions were isolated by sequential ultracentrifugation at desired densities. Centrifugations were performed for 4 h at 100,000 rpm (275,000 *g*) in a TL 100 centrifuge (Beckman) equipped with a 100.2 rotor.

Gel permeation chromatography of lipoproteins

Gel permeation chromatography was performed as previously described (19) in a Pharmacia Fast Protein Liquid Chromatography system on a 30 cm Superose 6 HR column (Pharmacia) calibrated with ultracentrifugally prepared VLDL, LDL, and HDL.

Anti-CETP monoclonal antibodies

Specific TP1 monoclonal antibody was prepared at the Clinical Research Institute of Montreal by immunizing mice with purified CETP. TP1 (or 2H4) has been found to neutralize the cholesteryl ester and triglyceride transfers catalyzed by CETP (20).

Statistical evaluations

Statistical comparisons of two means were made with the unpaired *t* test or Mann-Whitney test. Multiple comparisons were computed using one-way analysis of variance.

RESULTS

LDL patterns

The LDL patterns were studied after electrophoresis in a nondenaturing polyacrylamide gradient gel of sera prestained for lipid components with Sudan black B. From the number of main bands observed in the LDL range, the patterns obtained after analysis of 208 sera from normolipidemic subjects were divided into three main classes

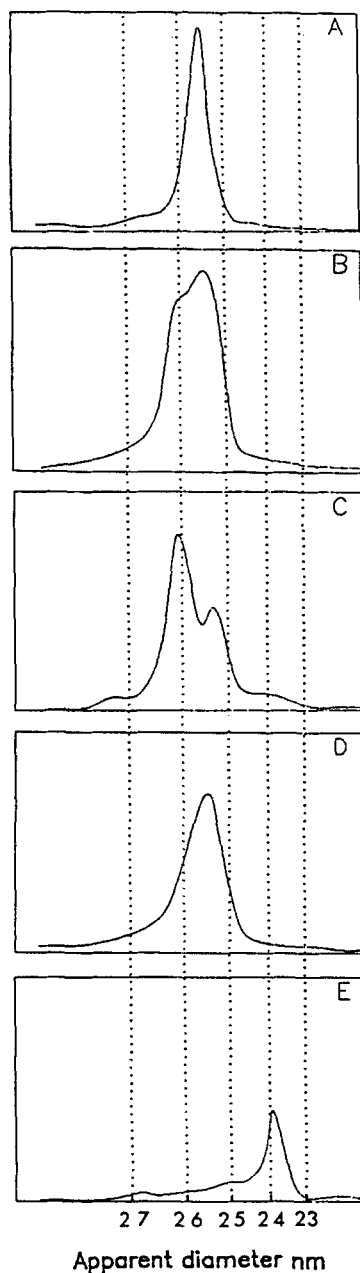


Fig. 1. LDL densitometric scannings of normolipidemic and dyslipidemic sera. Whole sera were submitted to gel electrophoresis after prestaining with Sudan black B. A: normolipidemic serum, type 1 pattern; B: normolipidemic serum, type 2 pattern; C: normolipidemic serum, type 3 pattern; D: hypercholesterolemic serum; E: hypertriglyceridemic serum.

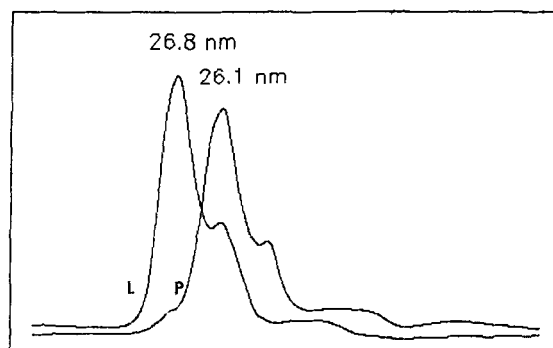


Fig. 2. LDL densitometric scannings of a normolipidemic serum (type 3 LDL pattern) prestained for lipids with Sudan black B (L) or post-stained for proteins with Coomassie blue (P). Indicated values are apparent diameters of the major peaks.

(Fig. 1). Type 1 was characterized by the presence of only one major LDL band. This band had an apparent diameter varying from 24.5 to 26.0 nm and after scanning gave an almost symmetrical peak. The type 1 pattern was present in 30% of the sera but was more frequent in men (43%) than in women (19%). Type 2 was characterized by the presence of two close major bands in the 24.5–26.5 nm range. It was present in about 50% of the sera (59% in women, 43% in men). The remaining profiles were classified as type 3 patterns. LDL were more dispersed and presented at least three distinct bands in the 24–27 nm range. The larger size band was usually the more abundant. There was frequently an additional minor fraction of small size at about 22.5 nm.

The LDL patterns found in the 41 hypercholesterolemic sera were of type 1 (Fig. 1). The mean (\pm SD) apparent diameter of the LDL band was 25.5 ± 0.4 nm. The 81 hypertriglyceridemic sera that were studied were characterized by the presence of a major band of small size (Fig. 1). The mean (\pm SD) apparent diameter of this small size band was 23.3 ± 0.5 nm.

The effect of prestaining was studied by comparing LDL profiles from prestained and nonprestained sera. The LDL patterns of prestained sera did not differ notably from the LDL patterns observed after poststaining with Coomassie blue, except for a slight shift of about 0.7 nm towards the high sizes (Fig. 2).

LDL subfraction composition

For composition analysis, LDL subfractions separated by electrophoresis of whole prestained sera were collected by electroelution. The validity of the method was assessed by several preliminary experiments. The purity of LDL obtained by gradient gel electrophoresis was estimated by analyzing the protein composition of delipidated fractions. After SDS gel electrophoresis, apoB appeared to represent more than 90% of the total protein content. By reapplying isolated LDL subfractions on a polyacrylamide

gradient gel it was observed that the extraction did not change their original size and profile characteristics (Fig. 3). To evaluate a potential effect of prestaining, analyses of LDL collected from prestained and unstained samples of the same serum were compared. As indicated in Table 1 the sample pretreatment did not notably alter LDL composition. Finally, it was checked that recoveries in the electroelution process were similar for all the LDL components. In a typical experiment the recovery was $79 \pm 6\%$ for total cholesterol, $84 \pm 6\%$ for unesterified cholesterol, $76 \pm 7\%$ for phospholipids, $80 \pm 7\%$ for triglycerides, and $82 \pm 8\%$ for apoB (mean \pm SD, $n = 4$).

In 15 normolipidemic sera with type 2 or type 3 patterns, two to four individual LDL bands were collected and classified into four categories according to their sizes. Likewise, the single bands from the 9 normolipidemic sera with type 1 pattern, the single bands from 21 hypercholesterolemic sera, and the small size major bands from 9 hypertriglyceridemic sera were collected and analyzed. The lipid composition of LDL subfractions varied according to their size when the results were expressed as ratio of lipid component to apoB mass concentrations (Table 2). LDL subfractions of similar size had similar composition, whether they were from normo- or dyslipidemic sera. Cholesteryl ester, unesterified cholesterol, and phospholipid contents decreased from the large particles to the small ones. Inversely, there was a slight increase of the triglyceride content as the size decreased. On the assumption that only one molecule of apoB-100 ($M_r = 550,000$) was present in every LDL particle, mass (Fig. 4) and molar (Table 3) particle compositions were calculated from the analytical data. Some interesting features appeared. In each LDL subclass the total mass of surface components, apoB, phospholipids, and unesterified cholesterol was nearly equal to the total mass of core components, cholesteryl esters, and triglycerides. Molar amounts of the two lipid surface components, phospholipids and unesterified cholesterol, were similar; in subspecies from normolipidemic sera their ratio varied from 1.00 to 1.19. Relative to the protein surface component, apoB, their amounts decreased with the size of the particles. From the largest to the smallest particles the molar ratio phospholipids + unesterified cholesterol to

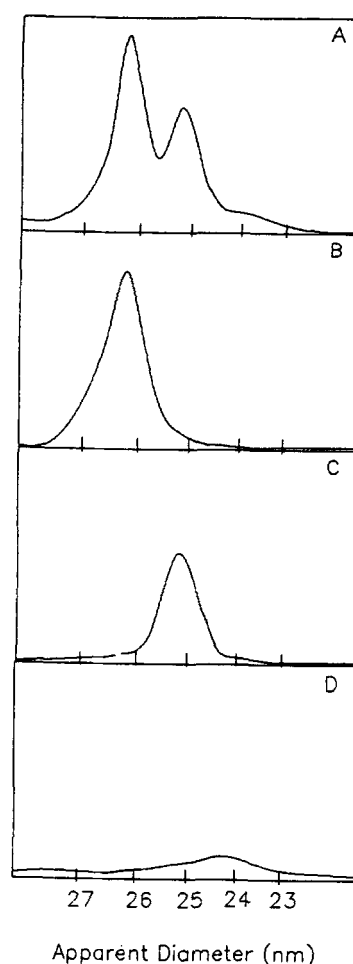


Fig. 3. Gel electrophoresis of total LDL and LDL subfractions. A: LDL densitometric scanning of a prestained whole serum. B, C, D: densitometric scanning of LDL subfractions collected by electroelution from the same serum.

apoB varied from 1300 to 500 and the mass ratio varied from 1.39 to 0.54. The sum of the amounts of the core components varied as a function of the particle size but the molar ratio cholesteryl esters to triglycerides increased from 3.9 to 10.7 from the smallest to the largest LDL subspecies. The LDL fraction of type 1 normolipidemic sera was similar in size and lipid composition to the 25.5

TABLE 1. Influence of serum prestaining on LDL mass composition

| | CE | TG | UC | PL | ApoB |
|----------------|----------------|---------------|----------------|----------------|----------------|
| | % | | | | |
| Prestaining | 39.1 ± 0.5 | 2.5 ± 0.1 | 16.0 ± 0.4 | 18.0 ± 0.4 | 24.4 ± 0.5 |
| No prestaining | 39.1 ± 0.7 | 3.1 ± 0.3 | 15.0 ± 0.9 | 19.6 ± 0.5 | 23.2 ± 0.7 |

LDL were extracted by gel electrophoresis and electroelution from 100- μ l samples of the same serum, either prestained with Sudan black or not. Values are mean of duplicates \pm extreme deviation (difference between the mean and each of the duplicate values); CE, cholesteryl esters; TG, triglycerides; UC, unesterified cholesterol; PL, phospholipids.

TABLE 2. Mass composition (mean \pm SD) of LDL subfractions from normo- and dyslipidemic sera

| LDL Subfraction | n | Apparent Diameter | CE/ApoB | TG/ApoB | UC/ApoB | PL/ApoB | TL/ApoB |
|---------------------------------------|----|-------------------|------------------|-----------------|------------------|------------------|------------------|
| <i>nm</i> | | | | | | | |
| Type 2 and type 3 normolipidemic sera | | | | | | | |
| a | 11 | 26.5 \pm 0.2 | 2.28 \pm 0.27 | 0.29 \pm 0.08 | 0.45 \pm 0.06 | 0.94 \pm 0.16 | 4.00 \pm 0.41 |
| b | 15 | 25.5 \pm 0.3 | 1.61 \pm 0.36 | 0.24 \pm 0.08 | 0.35 \pm 0.05 | 0.70 \pm 0.10 | 2.92 \pm 0.48 |
| c | 15 | 24.3 \pm 0.5 | 1.16 \pm 0.29 | 0.35 \pm 0.17 | 0.23 \pm 0.03 | 0.49 \pm 0.08 | 2.26 \pm 0.36 |
| d | 7 | 22.4 \pm 0.4 | 1.13 \pm 0.37 | 0.40 \pm 0.19 | 0.16 \pm 0.02 | 0.38 \pm 0.03 | 2.06 \pm 0.27 |
| a vs b | | | <i>P</i> < 0.01 | n.s. | <i>P</i> < 0.01 | <i>P</i> < 0.01 | <i>P</i> < 0.01 |
| b vs c | | | <i>P</i> < 0.05 | <i>P</i> < 0.05 | <i>P</i> < 0.01 | <i>P</i> < 0.01 | <i>P</i> < 0.01 |
| c vs d | | | n.s. | n.s. | <i>P</i> < 0.01 | <i>P</i> < 0.01 | n.s. |
| Variance analysis | | | <i>P</i> < 0.001 | <i>P</i> < 0.05 | <i>P</i> < 0.001 | <i>P</i> < 0.001 | <i>P</i> < 0.001 |
| Type 1 normolipidemic sera | | | | | | | |
| e | 9 | 25.3 \pm 0.4 | 1.76 \pm 0.32 | 0.33 \pm 0.15 | 0.35 \pm 0.06 | 0.85 \pm 0.13 | 3.22 \pm 0.45 |
| e vs b | | | n.s. | n.s. | n.s. | <i>P</i> < 0.01 | n.s. |
| Hypercholesterolemic sera | | | | | | | |
| f | 21 | 25.4 \pm 0.4 | 1.58 \pm 0.55 | 0.21 \pm 0.15 | 0.37 \pm 0.14 | 0.73 \pm 0.27 | 2.90 \pm 1.00 |
| f vs b | | | n.s. | n.s. | n.s. | n.s. | n.s. |
| Hypertriglyceridemic sera | | | | | | | |
| g | 9 | 23.4 \pm 0.5 | 0.97 \pm 0.22 | 0.29 \pm 0.20 | 0.15 \pm 0.04 | 0.40 \pm 0.09 | 1.81 \pm 0.35 |
| g vs d | | | n.s. | <i>P</i> < 0.05 | n.s. | n.s. | n.s. |

Apparent diameters are those of prestained lipoprotein fractions. CE, TG, UC, PL, as in Table 1; TL, total lipids; n.s., not significant at *P* < 0.05.

nm class from type 2 and type 3 sera, except for a moderate enrichment in phospholipids. LDL from hypercholesterolemic sera was also similar to this 25.5 nm LDL subfraction (Table 2). In hypertriglyceridemic sera the small size major band was comparable to the smaller size fraction of normolipidemic sera except for a smaller amount of triglycerides (Table 2).

LDL patterns and lipoprotein distribution

Lipid components and lipoprotein fractions were assayed in 208 normolipidemic sera and studied according to the pattern and the sex of the subjects (Table 4). From type 1 to types 2 and 3 patterns there was, in both sexes, a significant decrease of the mean serum triglyceride concentration. The decrease of total cholesterol was not significant but the cholesterol distribution varied significant-

ly. There were inverse variations of VLDL + LDL cholesterol and HDL cholesterol levels. From type 1 to type 3 patterns a decrease of VLDL + LDL cholesterol was associated to an increase of HDL cholesterol. The increase of HDL cholesterol reflected that of HDL_L, the large size HDL subfraction, whereas the small size HDL fraction, HDL_S, was constant (in males) or decreased slightly (in females). Although the mean triglyceride level was higher and the HDL cholesterol and HDL_L cholesterol levels were lower in the whole male population than in the whole female population, there was almost no difference between men and women within a given LDL pattern. The differences between the whole male and female populations were consequently due to a difference in the LDL pattern distribution, principally a much higher occurrence of the type 1 pattern in the male subjects.

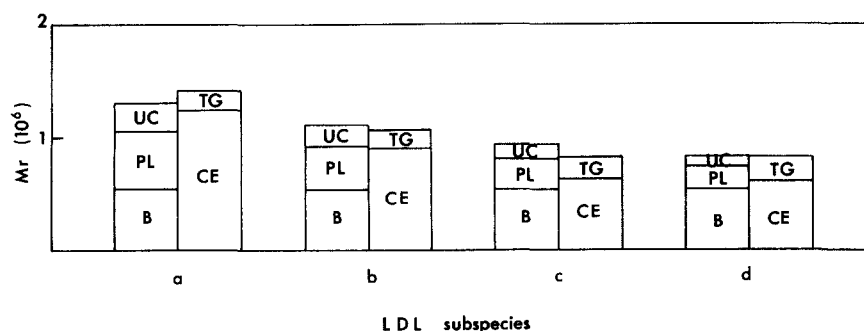


Fig. 4. Particle mass composition of LDL subspecies. It was assumed that there was one mole of apoB-100 ($M_r = 550,000$) per LDL particle. Values for lipid components were calculated from mean values of Table 1. Left bars: surface components. Right bars: core components. B, apoB-100; CE, TG, UC, PL, LDL subspecies as in Table 2.

TABLE 3. Molar composition of LDL subfractions from normo- and dyslipidemic sera

| LDL Subfractions | $M_r \times 10^6$ | CE | TG | CE/TG | UC | PL | PL/UC |
|------------------|-------------------|---------|-----|---------|-----|-----|-------|
| | | mol/mol | | mol/mol | | | |
| a | 2.75 | 1924 | 180 | 10.7 | 640 | 668 | 1.04 |
| b | 2.16 | 1364 | 149 | 9.2 | 497 | 497 | 1.00 |
| c | 1.80 | 983 | 218 | 4.5 | 327 | 348 | 1.06 |
| d | 1.68 | 958 | 249 | 3.8 | 227 | 270 | 1.19 |
| e | 2.32 | 1492 | 205 | 7.3 | 497 | 604 | 1.22 |
| f | 2.15 | 1339 | 131 | 10.2 | 526 | 519 | 0.99 |
| g | 1.54 | 822 | 180 | 4.6 | 213 | 284 | 1.33 |

Values were calculated from mean values of Table 2. They are expressed per mole of LDL. It was assumed that there is one mole of apoB-100 per mole of LDL. Molecular weights of 550,000, 649, 885, and 774 for apoB-100, cholesteryl esters, triglycerides, and phospholipids, respectively, were used for calculations. LDL subfractions, CE, TG, UC, PL as in Table 1.

Effect of lipid transfers on LDL patterns

In view of the differences in cholesteryl ester content of LDL subspecies, the effect of a transfer of cholesteryl esters to LDL was evaluated. To this end, LDL + HDL fractions (d 1.006–1.210 g/ml) prepared by ultracentrifugation were incubated with purified CETP (4–6 units/ml in the incubation mixture) for 24 h at 37°C. After completion of the incubation the lipoprotein fractions were separated by gel permeation chromatography and the changes in their lipid contents were measured. Concurrently, the alterations of the LDL pattern were studied by gradient gel electrophoresis. The gel filtration lipoprotein profiles demonstrated a transfer of cholesteryl ester from the HDL fraction to the LDL fraction (Fig. 5). This transfer occurred whatever the LDL pattern was (Fig. 6). It was associated with transfers, in the same direction, of

phospholipids, unesterified cholesterol and, in minor amounts, of triglycerides (Table 5). In contrast, when VLDL instead of LDL were incubated with HDL and CETP, the transfer of cholesteryl esters from HDL to VLDL was associated with a minor transfer of phospholipids and with an inverse transfer, from VLDL to HDL, of triglycerides (Table 5). In a set of five experiments with various proportions of LDL and HDL (LDL to HDL cholesteryl ester ratios ranging from 0.60 to 1.40), cholesteryl esters accounted, on a molar basis, for $54.5 \pm 5.3\%$ (mean \pm SD) of the total lipid transfer to LDL, phospholipids for $28.1 \pm 3.5\%$, unesterified cholesterol for $12.2 \pm 2.4\%$, and triglycerides for $5.2 \pm 1.2\%$. According to LDL patterns and LDL to HDL ratios, the increase of the LDL cholesteryl content ranged from 10 to 40%. There was no transfer of apoA-I detectable by enzyme immunoassay. In addition to the lipid transfers, the gel filtration profiles showed an increase of the average LDL size and a redistribution of the HDL fraction towards larger and smaller particles (Fig. 5C). The LDL size increase was more clearly demonstrated by gradient gel electrophoresis (Fig. 7). Whatever the primitive LDL pattern was, the incubation brought about a LDL profile shift of at least 1 nm towards the high size range whereas the general features of the LDL profiles were not markedly altered. The LDL size increase was especially marked with small size LDL from hypertriglyceridemic sera. The increase of the LDL apparent diameter compared well with the LDL mass increase induced by the lipid transfers. For example, in the experiment reported in Table 5, both the particle mass increase, calculated from the observed lipid transfers, and the particle volume increase, calculated from a 1.3 nm LDL apparent diameter shift, were about 15%. These changes were comparable to the size, mass, and composition differences between the LDL subfrac-

TABLE 4. Lipid concentrations and distribution (mean \pm SD) according to LDL patterns in normolipidemic sera

| LDL Pattern | n | Age | Total Cholesterol | Triglycerides | VLDL + LDL Cholesterol | HDL Cholesterol | HDL _S Cholesterol | HDL _L Cholesterol |
|-------------------|-----|----------------|----------------------------|---------------------------|----------------------------|----------------------------|------------------------------|------------------------------|
| | | yr | mg/dl | | | | | |
| Females | | | | | | | | |
| All | 107 | 26.0 \pm 4.9 | 186 \pm 29 | 58 \pm 24 | 125 \pm 29 | 61 \pm 14 | 33 \pm 7 | 28 \pm 1 |
| Type 1 | 20 | 24.4 \pm 4.4 | 195 \pm 31 | 81 \pm 29 | 144 \pm 32 | 51 \pm 8 | 38 \pm 6 | 13 \pm 7 |
| Type 2 | 63 | 25.9 \pm 4.6 | 185 \pm 25 | 58 \pm 21 | 125 \pm 26 | 60 \pm 11 | 33 \pm 7 | 27 \pm 10 |
| Type 3 | 24 | 27.7 \pm 5.6 | 180 \pm 35 | 42 \pm 13 | 107 \pm 26 | 73 \pm 17 | 30 \pm 8 | 43 \pm 18 |
| 1 vs 2 | | n.s. | n.s. | $P < 0.01$ | $P < 0.01$ | $P < 0.01$ | $P < 0.01$ | $P < 0.001$ |
| 2 vs 3 | | n.s. | n.s. | $P < 0.001$ | $P < 0.01$ | $P < 0.001$ | n.s. | $P < 0.001$ |
| Variance analysis | | n.s. | n.s. | $P < 0.001$ | $P < 0.001$ | $P < 0.001$ | $P < 0.001$ | $P < 0.001$ |
| Males | | | | | | | | |
| All | 101 | 27.6 \pm 4.4 | 180 \pm 30 ^{ns} | 69 \pm 29 ^{**} | 127 \pm 30 ^{ns} | 53 \pm 17 ^{***} | 32 \pm 9 ^{ns} | 20 \pm 16 ^{***} |
| Type 1 | 43 | 27.4 \pm 4.9 | 183 \pm 28 ^{ns} | 85 \pm 27 ^{ns} | 141 \pm 27 ^{ns} | 42 \pm 10 ^{**} | 33 \pm 8 [*] | 9 \pm 6 ^{ns} |
| Type 2 | 43 | 28.0 \pm 4.0 | 178 \pm 31 ^{ns} | 58 \pm 24 ^{ns} | 123 \pm 26 ^{ns} | 55 \pm 11 [*] | 32 \pm 7 ^{ns} | 23 \pm 10 ^{ns} |
| Type 3 | 15 | 27.1 \pm 4.1 | 175 \pm 36 ^{ns} | 56 \pm 23 [*] | 100 \pm 26 ^{ns} | 75 \pm 22 ^{ns} | 32 \pm 12 ^{ns} | 43 \pm 18 ^{ns} |
| 1 vs 2 | | n.s. | n.s. | $P < 0.001$ | $P < 0.01$ | $P < 0.001$ | n.s. | $P < 0.001$ |
| 2 vs 3 | | n.s. | n.s. | n.s. | $P < 0.01$ | $P < 0.01$ | n.s. | $P < 0.001$ |
| Variance analysis | | n.s. | n.s. | $P < 0.001$ | $P < 0.001$ | $P < 0.001$ | n.s. | $P < 0.001$ |

Significance of difference between males and females: *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; n.s., not significant at $P < 0.05$.

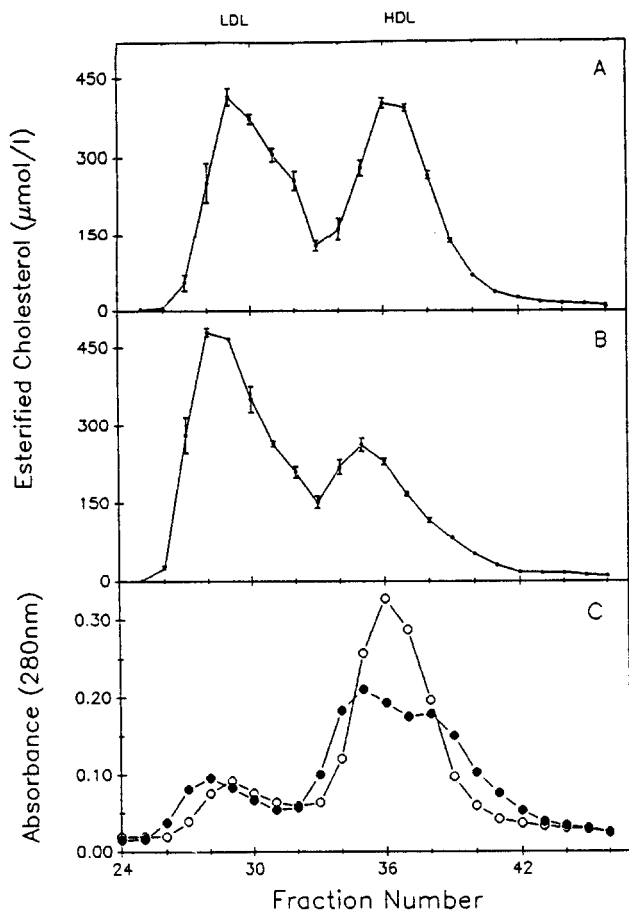


Fig. 5. Effect of CETP on LDL and HDL gel filtration profiles. LDL + HDL fractions were incubated with CETP (5.3 U/ml) in pH 7.4 phosphate buffer (20 mmol/l sodium phosphate, 150 mmol/l NaCl, 1 mmol/l EDTA, 0.2 g/l sodium azide) for 24 h at 4°C (A and ○) or 37°C (B and ●). Incubation mixtures were injected (200 µl) onto a Superose 6 HR column and eluted with a 150 mmol/l NaCl, 1 mmol/l EDTA, 0.2 g/l sodium azide, pH 7.4 solution (flow rate: 0.2 ml/min; elution fractions: 0.4 ml). Esterified cholesterol values are mean \pm SD of four experiments.

tions separated from native sera. The actual implication of CETP in lipid transfers and LDL size alterations was assessed by control experiments. Incubation of LDL and HDL in the absence of CETP did not induce any LDL alteration (Fig. 8C). A 1-h heating at 56°C did not inactivate the CETP preparation (Fig. 8B). On the other hand, preincubation with TP1 monoclonal antibody almost completely blocked lipid transfers and LDL size modifications (Fig. 9).

DISCUSSION

The results reported herein show that electrophoresis, in a nondenaturing polyacrylamide gradient gel, of whole

serum prestained by Sudan black B is a convenient method for the study of LDL subspecies composition and distribution. The exploration of normo- and dyslipidemic sera revealed four LDL patterns: a type 1 pattern present in hypercholesterolemic sera and in some normolipidemic sera, types 2 and 3 patterns, both observed in normolipidemic sera, and a pattern specific for hypertriglyceridemia, characterized by LDL of small size. The composition analysis of the separated LDL subspecies pointed out a size-related variation of the particle cholesteryl ester content which suggested a role of CETP in the LDL size-modification process. This hypothesis was sustained by the results of *in vitro* incubations of LDL and HDL with a purified CETP preparation.

The experiments presented in this report were primarily designed for composition analysis of LDL subfractions

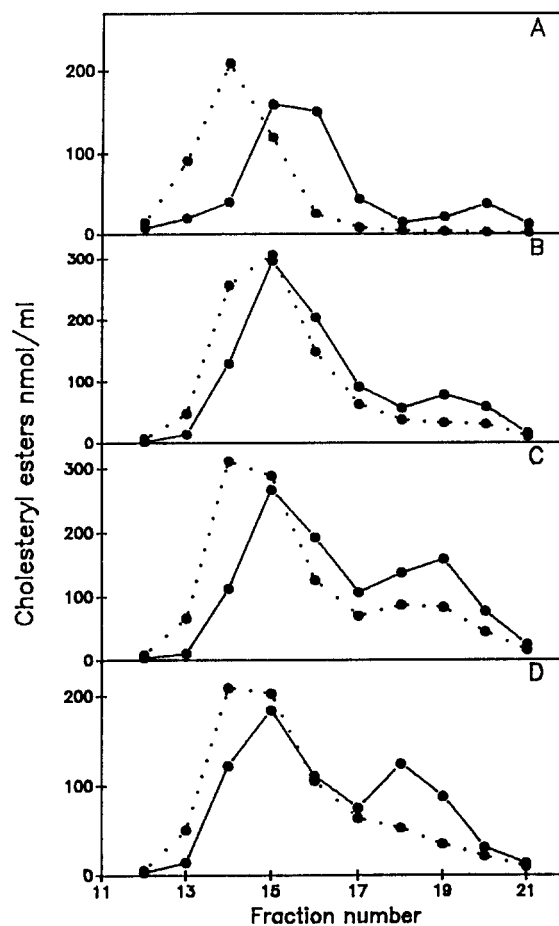


Fig. 6. Effect of CETP on LDL and HDL cholesteryl ester distribution. LDL + HDL fractions (total cholesterol: 1.65–1.80 mg/ml) were incubated with CETP (6.0 unit/ml) for 24 h at 37°C (dotted line) or at 4°C (continuous line). Conditions of incubation and gel filtration as in Fig. 5 (except for elution fractions: 0.8 ml). A was from a hypertriglyceridemic serum; B from a hypercholesterolemic serum; C from a normolipidemic serum, type 2 LDL pattern; D from a normolipidemic serum, type 3 LDL pattern. LDL: fractions 12–17; HDL: fractions 18–21.

TABLE 5. Effect of CETP on HDL, LDL, and VLDL lipid composition

| Fraction | Time | CE | TG | UC | PL |
|---------------|----------|-------|-------------------|-------|-------|
| | <i>h</i> | | $\mu\text{mol/l}$ | | |
| LDL | 0 | 2059 | 359 | 1004 | 942 |
| HDL | 0 | 1595 | 224 | 379 | 1300 |
| LDL | 24 | 2450 | 389 | 1148 | 1111 |
| HDL | 24 | 1204 | 194 | 235 | 1131 |
| Δ LDL | | + 391 | + 30 | + 144 | + 169 |
| VLDL | 0 | 978 | 1734 | 545 | 741 |
| HDL | 0 | 1600 | 355 | 407 | 1312 |
| VLDL | 24 | 1742 | 1456 | 641 | 746 |
| HDL | 24 | 836 | 633 | 311 | 1307 |
| Δ VLDL | | + 764 | - 278 | + 96 | + 5 |

HDL were incubated for 24 h at 37°C with either LDL or VLDL in the presence of CETP (5.0 unit/ml). After completion of the incubation, the lipoproteins were separated by gel filtration. Conditions of incubation and gel filtration as in Fig. 5. Values, from one experiment, are expressed as concentrations in the incubation mixtures; they were corrected for gel filtration sampling variations. CE, TG, UC, PL as in Table 1. LDL mean apparent diameter increased from 24.0 to 25.3 nm during incubation.

separated by gradient gel electrophoresis. To avoid denaturation of lipoprotein fractions, preliminary separations were eliminated and whole serum was submitted to electrophoresis. To allow detection of LDL bands, lipoproteins were prestained for lipid components by Sudan black B. The present methodology, suggested by the work of Krauss and Burke (3), gave results similar to those obtained by gel electrophoresis of ultracentrifugally separated LDL and protein poststaining (3, 8). The LDL domain appeared heterogeneous from one serum to another, with generally one to four distinct bands. The size ranges of the separated LDL subspecies were in agreement with those previously published, provided that the size shift brought about by lipid prestaining was taken into account. We have already shown (10) that serum prestaining did not modify HDL size distribution, as estimated by gradient gel electrophoresis, apart from a slight shift of about 0.5 nm towards the high sizes. The data now presented show that this conclusion can be extended to LDL distribution. The LDL patterns observed after lipid prestaining or after poststaining by Coomassie differed only by a 0.7-nm shift. The LDL size of the four main LDL fractions defined in this study were approximately, after correction for prestaining, those generally admitted for LDL subspecies LDL-I + LDL-II, LDL-III, LDL-IV A and LDL-IV B (11). Thus, gradient gel electrophoresis of whole serum prestained for lipids by Sudan black B appeared to be a reliable and rapid procedure for the study of LDL subspecies distribution.

LDL heterogeneity in band number and size gives rise to a large spectrum of patterns. Austin and Krauss (7) have already defined two LDL patterns, pattern A and pattern B. Pattern A was characterized by a major peak

of LDL subclasses with large diameters, greater than 25.5 nm. Pattern B was characterized by the presence of a major peak of smaller, denser LDL particles and was associated with increased levels of triglycerides, VLDL, and IDL (7, 9). Accordingly, in our study, this pattern B was characteristic of hypertriglyceridemic sera which all presented a major band in the small size range. Other sera, either normolipidemic or hypercholesterolemic, could have been classified as pattern A sera but were different enough to be divided into subsections. As noted earlier (8), some LDL appeared almost homogeneous with only one distinct band and were classified as type 1 whereas others were heterogeneous. In spite of the various aspects of the latter category it was possible to divide it into type 2 and type 3 according to the number of bands and to the LDL dispersion. Hypercholesterolemic sera were all of the type 1 pattern. Type 1, 2, and 3 patterns were observed within the normolipidemic population. The distinction between these three patterns was confirmed a posteriori by the study of the variations of serum lipids

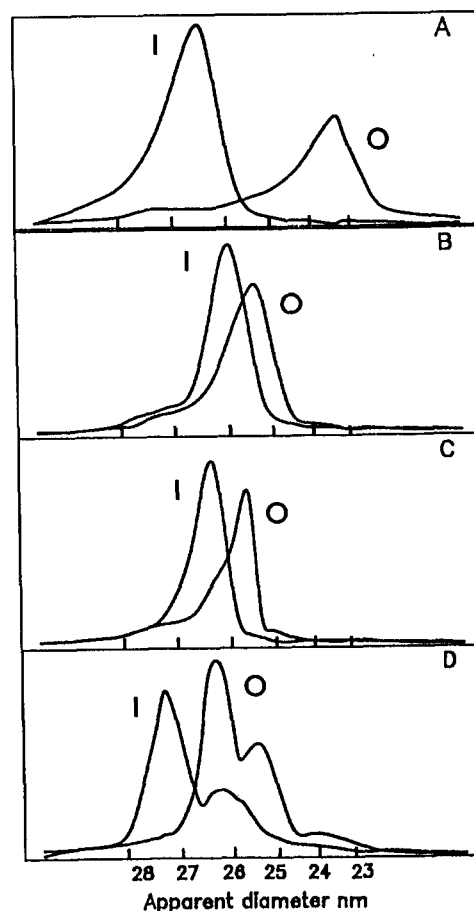


Fig. 7. Effect of CETP on gel electrophoretic LDL profiles. Incubation mixtures were those of Fig. 6. They were submitted to electrophoresis without prestaining. Gels were scanned after poststaining with Coomassie blue. A, B, C, D as in Fig. 6. I: mixtures incubated at 37°C; O: mixtures kept at 4°C.

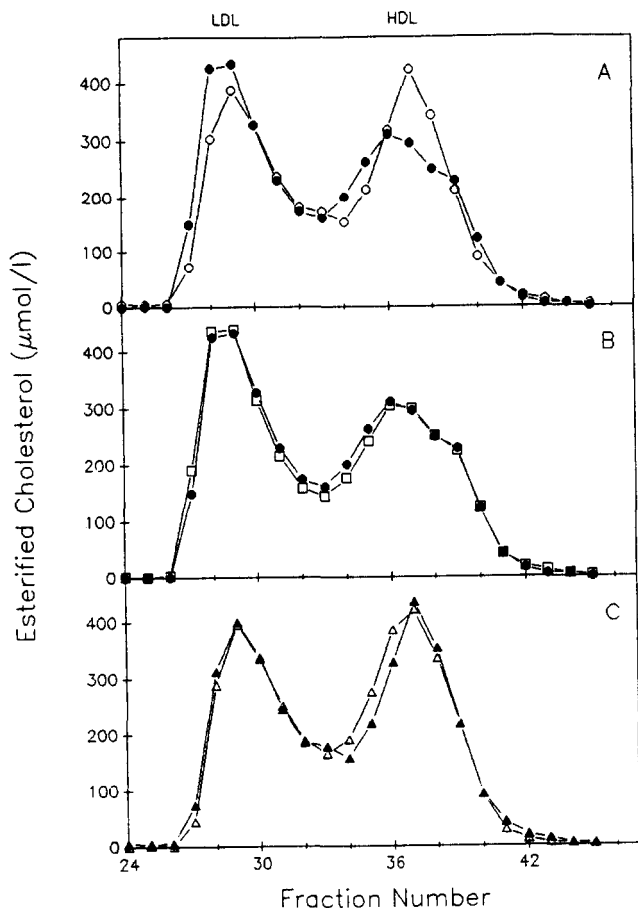


Fig. 8. CESTP-induced LDL and HDL alterations (control experiments). LDL and HDL were incubated for 24 h and fractionated by gel filtration as in Fig. 5. A: Incubation at 37°C (●) or 4°C (○) in the presence of CESTP (5.3 U/ml). B: incubation at 37°C in the presence of CESTP (5.3 U/ml) preheated (□) or not (●) for 1 h at 56°C. C: Incubation at 37°C (▲) or 4°C (△) in the absence of CESTP.

and lipoproteins according to LDL patterns. These variations were very suggestive of a link between LDL subfraction patterns and the general metabolism of plasma lipoproteins. The link is evident in dyslipidemic sera, especially hypertriglyceridemic sera, but is perhaps more interesting in the normolipidemic group. Indeed, from type 3 to types 2 and 1 patterns there was an increase of the VLDL + LDL: HDL ratio, likely a consequence of variations in interlipoprotein exchanges and transfers. Moreover, from type 3 to type 1 patterns, sera tended to have a higher potential coronary disease risk lipid and lipoprotein profile, namely higher triglyceride level, higher VLDL + LDL fraction, lower HDL fraction, and lower large size, light HDL subfractions. In addition, LDL pattern and composition of type 1 normolipidemic sera were very similar to those of highly atherogenic hypercholesterolemic sera. We have previously shown (21) that, in a group of normolipidemic subjects undergoing coronary catheterization, 90% of the patients with at least

50% obstruction of two or three major vessels were of the LDL type 1, whereas no type 1 profile was observed in subjects with normal coronaries. The predominance of the type 1 LDL pattern in the male subjects of our study, compared with the female subjects, could be put together with the fact that men are more prone to cardiovascular disease than women. All these observations postulate a pejorative connotation for the type 1 LDL pattern. More generally, the study of LDL pattern could be a precious tool for the detection of borderline disorders of lipoprotein metabolism.

Composition analysis of the lipoprotein subspecies separated by gel electrophoresis is a new contribution of this work. Electroelution of the LDL bands cut out from the gradient gel allowed subspecies recovery without loss of resolution and made possible comparison studies of LDL according to their sizes. There were two conclusions from the results. On the one hand, some features appeared as common characteristics of the entire LDL fraction. Mass equivalence of core and surface components and equimolarity of the two lipid surface components, phospholipids and free cholesterol, belong to this category. On the other hand, there were progressive

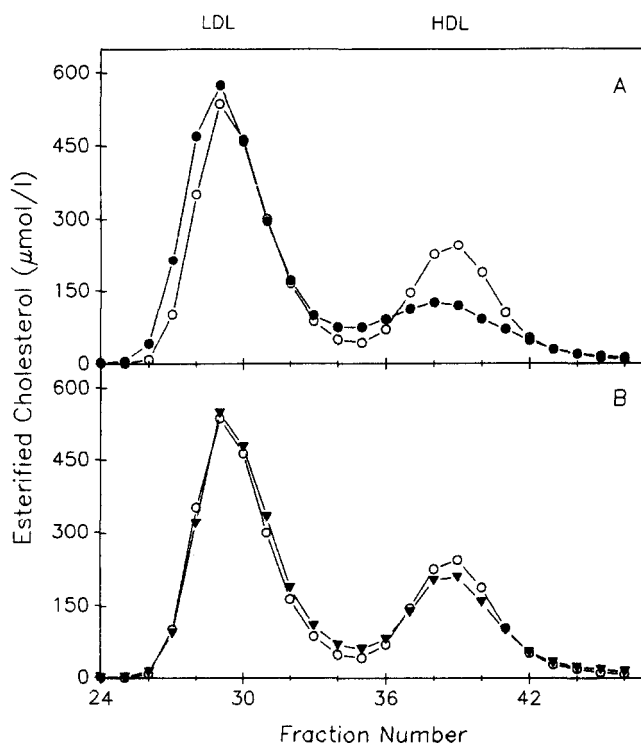


Fig. 9. Effect of anti-CESTP monoclonal antibodies on CESTP-induced LDL and HDL alterations. LDL + HDL fractions were incubated for 24 h at 37°C (● and ▼) or 4°C (○) with CESTP (6.0 U/ml). CESTP was preincubated (B, ▼) or not (A, ●) for 3 h at 37°C with TP1 anti-CESTP monoclonal antibody (25 μl of 60 U/ml CESTP + 50 μl of 0.50 g/l TP1 solution). Conditions of incubation and gel filtration as in Fig. 5.

changes with the apparent size of the particles. The increase of LDL total lipid content with size increment confirmed the well-established inverse density-size relationship (3). The variation of the respective proportions of lipid surface components and apoB underlines the structural alteration of the LDL surface compartment occurring as the size of the particles varies. By the change in apoB conformation and the lipid environment that it is likely to bring about, this variation can be classified with the reported changes in apoB immunoreactivity according to LDL size, especially the reduced immunoreactivity in small, dense LDL of the antigenic determinants close to the LDL receptor recognition site (22). With regard to the core components, there was an increase with increasing particle size of the cholesteryl ester to apoB ratio and, in contrast with variations observed in terms of particle density (3, 5), of the cholesteryl ester to triglyceride ratio. These features might be directly related to the mechanism of LDL size remodeling.

The metabolic process leading to LDL polymorphism is not well understood. It is generally admitted that conjugate effects of lipoprotein lipase activity and lipid transfers between triglyceride-rich lipoproteins and LDL produce a conversion of large LDL subspecies to smaller ones (23). *In vitro* (24) and *in vivo* (25) studies indicate that lipoprotein lipase contributes to the formation of large LDL by removing triglycerides from VLDL and IDL. In the presence of CETP, LDL cholesteryl esters can be transferred to triglyceride-rich particles in exchange for triglycerides. The subsequent removal of triglycerides by lipoprotein lipase would lead to denser, smaller LDL species. Conversely, interactions of HDL with LDL during lipolysis (26) may give rise to LDL size increases by facilitating transfer of both lipid and HDL apolipoprotein components to LDL. The size-related variations of the cholesteryl ester content of LDL subspecies, observed in this study, led us to consider CETP-induced transfers of cholesteryl esters from HDL to LDL (27) as another potential process of LDL remodeling. Indeed, the experiments presented in this report showed that an LDL size increase occurred when LDL and HDL were incubated in the presence of physiological amounts of CETP. The LDL size increase was associated with transfer of lipids, mainly cholesteryl esters and phospholipids, from HDL to LDL. These lipid transfers were not the consequence of a fusion process of HDL with LDL since there was no apoA-I transfer. Nor was it due to a contaminating lipid transfer protein-2 activity since transfer was not suppressed by heating of the lipid transfer protein preparation (28). Inhibition by specific anti-CETP monoclonal antibodies confirmed that the transfers were actually induced by CETP. The changes these transfers brought to LDL size and composition were similar to the differences observed between LDL subfractions separated from native sera. That the HDL to LDL lipid transfer might

be of importance *in vivo* is suggested, in particular, by the normalization of the size of LDL from hypertriglyceridemic sera after incubation with CETP. Knowing that VLDL are preferential acceptors of CETP-induced transfers of HDL cholesteryl esters (27), one can hypothesize that in hypertriglyceridemia the presence of small-sized LDL is, in part, the consequence of competitive inhibition, by VLDL in excess, of HDL cholesteryl ester transfers to LDL. The physiologic role of CETP in LDL subspecies formation is also supported by the recent observation of small and cholesteryl ester-poor LDL particles in patients deficient in CETP activity (29). In our experiments, LDL, after incubation with CETP, retained some features of their primitive pattern, in spite of a general shift towards large sizes. This seems to indicate that multiple factors, such as formation of LDL from different precursor lipoproteins (23), conformational changes in apoB (26), apoB polymorphism, or the presence of minor apolipoproteins, account for LDL subspecies and pattern polymorphism. However, lipid transfers from HDL to LDL might be an important factor in the complex process of intravascular LDL remodeling. ■

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