Characterization of apolipoprotein B-containing lipoproteins separated by preparative free flow isotachophoresis

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Abstract Preparative free flow isotachophoresis (ITP) was used for the fractionation of apoB-containing lipoproteins ($d < 1.063$ g/ml) from fasting and postprandial sera derived from normolipidemic individuals. According to their net electric mobility, four major particle groups (I–IV) have been recognized. The fast-migrating particles in group I, which correspond predominantly to very low density lipoproteins (VLDL), are rich in triglycerides, free cholesterol, phosphatidylcholine, and apoE and C apolipoproteins. This group expresses nonspecific binding to fibroblasts but binds to HepG2 cells with high affinity (K_D = 3.6 μg/ml, B_{max} = 37 ng) to a single class of binding sites. The particles migrating in group II, which are related to intermediate density lipoproteins (IDL), are richer in cholesteryl esters and apoB than those in group I. They interact specifically with a single site on fibroblasts (K_D = 7.8 μg/ml, B_{max} = 54 ng) while on HepG2 cells two binding sites, one with a higher (K_D = 3.5 μg/ml, B_{max} = 22 ng) and one with a lower affinity component (K_D = 16.9 μg/ml, B_{max} = 53 ng), are involved. The particles migrating in groups III and IV correspond to low density lipoproteins (LDL). The protein moiety of both fractions consists almost exclusively of apoB. Group III represents cholesteryl ester-rich LDL particles, while the particles in group IV contain smaller amounts of cholesteryl esters. The lipoproteins of both groups are ligands for apoB,E-receptors. However, the particles in group IV interact with fibroblasts with the highest affinity (K_D = 2.3 μg/ml, B_{max} = 58 ng) and with the biphasic HepG2 cell binding sites with the lowest affinity of all analyzed groups (K_{D1} = 11.2 μg/ml, B_{max1} = 58 ng, K_{D2} = 68 μg/ml, B_{max2} = 170 ng). When apoB-containing lipoproteins were isolated from postprandial sera of the same individuals, significant changes in the lipid composition were observed only in particle groups I and II, where the triglyceride and phospholipid content was enhanced. Group I particles from postprandial serum bind to HepG2 cells with a higher affinity (K_D = 2.5 μg/ml) than group I particles from fasting serum. Postprandial group II particles bind with the same affinity to the biphasic HepG2 cell receptor as fasting group II particles, while the affinities of postprandial group III (K_{D1} = 4.1 μg/ml, K_{D1} = 47 μg/ml) and group IV particles (K_{D1} = 3.9 μg/ml, K_{D2} = 38 μg/ml) to the high affinity binding site of the biphasic receptor are enhanced. However, in contrast to HepG2 cells, the binding properties of each particle group to fibroblasts did not change significantly. The preparatively isolated subfractions of apoB-containing lipoproteins correspond to eight peaks in the analytical capillary ITP pattern obtained from whole serum or lymph. Groups I and II relate to the first four peaks in the analytical capillary ITP pattern obtained from whole serum or lymph. Groups I and II relate to the first four peaks; the next four belong to groups III and IV. The pattern of 80 sera from normolipidemic individuals with different apoE phenotypes indicates that the apoE2 allele is associated with higher concentrations of VLDL and IDL particles migrating in group I and II, while the LDL particles in group IV are diminished. The apoE4 allele determines the opposite pattern with lower concentrations of groups I and II, and enhanced concentrations of group IV particles when compared with the apoE3 allele. The data indicate that analytical ITP of whole serum lipoproteins may be a helpful tool for the analysis of subpopulations of apoB-containing lipoproteins with different metabolic properties directly in patient sera, but needs further confirmation by patient analysis. —**Nowicka, G., T. Brüning, B. Grothaus, G. Kahl, and G. Schmitz.** Characterization of apolipoprotein B-containing lipoproteins separated by preparative free flow isotachophoresis. *J. Lipid Res.* 1990. **31:** 1173–1186.

Supplementary key words capillary isotachophoresis • preparative isotachophoresis • serum lipoproteins • lymph lipoproteins • VLDL-, IDL-, LDL-subfractions • fibroblasts • HepG2 cells

ApoB-containing lipoproteins as separated by density ultracentrifugation have been further subfractionated into multiple discrete subspecies by gradient gel electrophoresis, immunochemical methods, and other techniques (1–3). It was shown that each of the major apoB-containing fractions is structurally and functionally heterogeneous. Large triglyceride-rich VLDL (>35 nm in diameter) are cleared rapidly from the circulation and only small por-

Abbreviations: VLDL, very low density lipoproteins; IDL, intermediate density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; apoB, apolipoprotein B; apoC, apolipoprotein C; apoE apolipoprotein E; Lp[a], lipoprotein[a]; TC, total cholesterol; TG, triglycerides; UC, free cholesterol; EC, esterified cholesterol; PC, phosphatidylcholine; SPM, sphingomyelin; HDL-C, HDL-cholesterol; ITP, isotachophoresis; HPMC, hydroxypropylmethylcellulose; ammediol, 2-amino-2-methyl-1,3-propanediol; SDS, sodium dodecyl sulfate; DMEM, Dulbecco's modified Eagle medium; PBS, phosphate-buffered saline; FCS, fetal calf serum; LPDS, lipoprotein-deficient serum; BSA, bovine serum albumin.

tions are converted into low density lipoproteins, while fractions containing smaller cholesteryl ester-enriched VLDL (diameter of 32–35 nm) have a longer plasma circulation time and form greater amounts of LDL (4, 5). Among intermediate density lipoproteins two subclasses of overlapping buoyant density and with a particle diameters of 29.1 nm and 28.3 nm have been isolated: IDL_1, which appear to form a continuous spectrum with small VLDL, and the smaller more dense IDL_2 (5, 6). IDL_1 and IDL_2 may be the precursors for two functionally different LDL subclasses (6). LDL are constituted of particle species that differ from each other in terms of size, buoyant density, lipid composition, apolipoprotein content, and metabolic properties. It was shown that the turnover of lighter LDL ($d < 1.038$ g/ml) subspecies is faster than that of heavier LDL ($d > 1.038$ g/ml) subfractions (7). The heterogeneity of LDL has been characterized in normal and hyperlipidemic individuals (8–10). Large, cholesterol-rich LDL particles accumulate in many patients with familial hypercholesterolemia, while in hypertriglyceridemia or hyperapobetalipoproteinemia the accumulation of smaller, more cholesteryl ester-poor LDL were observed when compared to normal subjects (10–12).

The catabolism of apoB-containing lipoproteins at least in part, is mediated by specific cell receptors (13). Extrahepatic cells possess specific binding sites (apoB,E-receptors) that recognize apoB-100 and apoE. These apoB,E-receptors are responsible for uptake and catabolism of LDL, but they also recognize IDL and VLDL. However, VLDL binding is significantly correlated with the particle composition and apoB and apoE conformation (13–15). In normal subjects only the smallest VLDL (VLDL-3) possess the ability to interact specifically with extrahepatic apoB,E-receptors (15).

Liver, which plays a central role in lipoprotein metabolism, possesses classical apoB,E-receptors and receptors that are suggested to recognize apoE and thereby are critically involved in the clearance of remnants originating from the metabolism of triglyceride-rich lipoproteins (16). VLDL seem to be recognized also by these receptors in addition to their specific binding to hepatic apoB,E-receptors (17).

Recently we have reported that free flow ITP can be used for lipoprotein separation (18, 19). We fractionated HDL by this method and described the chemical composition of isolated subfractions as well as their binding properties to HDL receptors and their ability to release cholesterol from cells (19). In the present study we used preparative ITP for the fractionation of apoB-containing lipoproteins. We have characterized the chemical composition of the isolated subfractions and studied their interaction with fibroblasts and HepG2 cells.

Free flow ITP is an electrophoretic technique with a high resolution by which ionic sample components are separated in a discontinuous electrolyte system based on differences in their net electric mobility and without molecular sieve effects (20, 21). Self-focusing of the separated zones and concentrating effects on sample ions are major characteristics of ITP. The leading electrolyte must contain an ion species with a mobility higher than that of any one of the sample ions and the terminating electrolyte contains an ion species with a mobility lower than that of any one of the sample ions of interest. The common counter ion must have a good buffering capacity in the pH range within which the separation takes place. When the system has reached equilibrium, all ions move with the same speed, individually separated into a number of zones. The mobility of the sample ions is related to the defined mobility of spacers added to the sample solution, and the resolved peak pattern represents reproducible net mobility profiles. For the lipoprotein separations presented in the paper, amino acids and dipeptides are used as spacers and they strictly define the position of the separated subfractions in the isotachophoretic lipoprotein patterns.

MATERIALS AND METHODS

Plasma and serum collection

Blood was drawn from healthy volunteers and patients attending the University Hospital. Plasma or sera were prepared by low speed centrifugation at 4°C. Na_2EDTA was added to serum (final concentration 0.01%) to avoid peroxidation during storage. The samples used for analytical ITP were either stored at 4°C and analyzed within 24 h or immediately frozen and thawed only once before use. Fractionation of lipoproteins by ultracentrifugation and preparative ITP was performed from freshly prepared samples.

Postprandial plasma was drawn 4 h after a 1600 kcal breakfast consisting of bread with butter and cheese and 50 ml of milkshake containing orange juice, sunflower oil, fat cream, and milk powder. The total breakfast contained 57% fat, 20% protein, and 23% carbohydrates (by energy) (22).

Analytical capillary isotachophoresis of lipoproteins

Isotachophoretic lipoprotein patterns were obtained from sera/plasma that were preincubated for 30 min at 4°C with the nonpolar dye Sudan black B (1% solution in ethylene glycol) and mixed with spacers (2:1, v/v; final concentration of each spacer 0.2 mg/ml). The following compounds were used as spacers: glycylglycine, alanylglycine, valylglycine, glycylhistidine, serine, glutamine, methionine, histidine, glycine, 3-methyl-histidine, and pseudouridine (Serva, Heidelberg). For separation, 2 μl of the mixture was injected into the capillary system between the leading and terminating electrolyte. The leading elec-

trolyte consisted of 5 mM H_3PO_4, 0.25% HPMC, and 20 mM ammediol, pH 9.2. The terminating electrolyte contained 100 mM valine and 20 mM ammediol, pH 9.4. The separation was started with a constant current of 150 μA. During the run the current was reduced to 100 μA (at 6 kV) and then to 50 μA (at 6 kV) before detection. The separated zones were monitored at 570 nm. Analyses were performed in a LKB 2127-Tachophor System. However, similar results have been obtained using a fully automated, free flow capillary electrophoresis instrument, which has recently been developed in our laboratory. In both instruments the capillary is cooled with air or a nonconductive organic solvent, and separation can be performed at a temperature from 4° to 10°C. The ITP method presented here for lipoprotein analysis can be directly adapted also to the Capillary Isotachophoresis Analyzer from Shimadzu, Japan.

Preparative isotachophoresis of lipoproteins

Preparative subfractionation of lipoproteins was carried out in an Elphor VAP 22 (Bender & Hobein, Munich) (23). For analysis, leading and terminating electrolytes of the same composition as for analytical ITP were used. The sample in the volume of 50 ml of terminating buffer containing spacers (final concentration of each 1 mg/ml) was separated at a voltage of 1000 V and a current of 60 mA at 10°C using a sample flow of 10 ml/h. The collected fractions were further analyzed.

Lipoprotein preparation

Lipoprotein fractions were isolated from plasma by sequential ultracentrifugation (24) using a Beckman 50.3 Ti rotor at 4°C and 48,000 rpm. The following lipoprotein fractions were isolated: VLDL at density <1.006 g/ml, IDL at a density of 1.006–1.019 g/ml, and LDL at a density of 1.019–1.063 g/ml. The fractions were dialazed against 0.9% NaCl/0.01% Na_2EDTA (pH 7.4) at 4°C.

Isolation of LDL subfractions

Low density lipoproteins isolated by sequential preparative ultracentrifugation were dialyzed overnight against NaBr solution of d 1.006 g/ml containing 0.01% Na_2EDTA. A continuous sodium bromide gradient d 1.006–1.09 g/ml was prepared using the LKB 11300 ULTROGRAD gradient mixer. One-ml aliquots of the dialyzed LDL were overlayered on top of the gradient solution and the gradient was centrifuged in an SW 40 Beckman rotor at 38,000 rpm for 24 h at 4°C. Ten fractions of 1 ml were collected according to Groot et al. (25) and scanned at 280 nm. A background salt tube was included with each centrifugation, and the density of the fractions was determined from the sodium concentration using the empirical formula: $d\,(g/ml) = 2.307 \times [Na^+]\,mmol/l = 1.00155$ (26). Four of the collected fractions belonged to the LDL density class. The average densities of the LDL subfractions were 1.025 g/ml, 1.036 g/ml, 1.046 g/ml, and 1.057 g/ml.

Analytical capillary ITP of lipoprotein subfractions

Capillary ITP of VLDL, IDL, and LDL subfractions was carried out under standard conditions used for serum analysis. In order to have serum matrix conditions during ITP analysis, the subfractions were mixed with appropriate aliquots of the lipoprotein-free $d > 1.21$ g/ml serum fraction.

Analysis of the protein and lipid compositions

Total cholesterol, free cholesterol, triglycerides, phosphatidylcholine, and sphingomyelin were determined by enzymatic methods (27, 28) using reagents from Boehringer Mannheim and a Cobas-Bio centrifugal analyzer (Hoffman-La Roche, Zürich). Esterified cholesterol was calculated as the difference between total and free cholesterol.

ApoB was determined by a turbidimetric method using antiserum and calibration serum from Behring, Marburg. Turbidity was measured at 340 nm by an ELISA reader (Dynatech Laboratories, Inc., Alexandria, VA) after 90 min incubation of standards (calibration serum) and samples (diluted in 0.15 M NaCl) with antiserum. ApoE and C apolipoproteins were quantified by an enzyme-linked immunosorbent assay (ELISA) developed as a sandwich type assay. Individual wells of microtiter plates were coated with affinity-purified anti-apoE, -apoC-II, or -apoC-III antibodies by a standard procedure. Coating was performed after 1:1000 dilution in 0.1 M $NaCO_3$, pH 9.5. A reference standard (from Immuno AG, Vienna) was used for quantitation of samples diluted in PBS containing 0.5% BSA, pH 7.7. The reaction of the antibody-peroxidase conjugate, which was prepared following the procedure described by Nakane and Kawai (29) using *o*-phenylendiamine–H_2O_2, was measured at 490 nm with an ELISA reader.

ApoE polymorphism was determined as previously described from our laboratory (30). Protein concentration was measured by the method of Lowry et al. (31).

Binding experiments

After isolation by preparative ITP, the subfractions of apoB-containing lipoproteins were iodinated by the IODO-Bead method (32). Aliquots of these subfractions (1 mg protein/1 ml) were mixed with 0.5 mCi of Na ^{125}I, six IODO-Beads were added, and the mixture was incubated for 10 min at room temperature. Unbound iodine was removed by chromatography on a PD-10 column (Pharmacia) and the lipoprotein subfractions were dialyzed against PBS. The specific activity of the iodinated subfractions was between 200 and 400 cpm/ng protein.

Normal human skin fibroblasts were grown in monolayers in DMEM culture medium supplemented with 10% fetal calf serum and used after 8–10 passages. Cells from the established HepG2 cell line were cultured in DMEM containing 10% FCS. For the binding experiments both cell types were preincubated with culture medium containing 10% LPDS for 48 h prior to analysis.

Binding experiments were performed according to the method described by Goldstein and Brown (33). The cells were chilled to 4°C for 30 min and incubated for 2 h at 4°C with the indicated concentrations of ^{125}I-labeled lipoprotein subfractions in the presence or absence of a 20-fold excess of nonlabeled subfractions. After incubation, the cells were rapidly and extensively washed with ice-cold PBS containing 2% BSA and PBS without BSA, transferred to a tube, and dissolved in 1N NaOH. Aliquots were used for the determination of cellular protein content, and cell-associated ^{125}I radioactivity was measured in a Compugamma 1282 automatic counting system (LKB).

RESULTS

Analytical capillary ITP of lipoproteins under fasting conditions

We have reported (19) that whole serum or plasma lipoproteins can be separated into fourteen subfractions by analytical capillary ITP (**Fig. 1A**). Six of them (peaks 1–6) correspond to HDL. In peak 7, chylomicron-derived particles are recognized, and VLDL and IDL create three peaks (peaks 8–10) in the serum/plasma pattern, while LDL migrate in the last four peaks (peaks 11–14). The biochemical characterization of the HDL subfractions (peaks 1–6) separated by ITP from fasting and postprandial samples of normolipidemic donors has been described (19). Here, we have focused on the subfractionation of apoB-containing lipoproteins from normolipidemic subjects.

In addition to capillary ITP of serum lipoproteins (Fig. 1A), we have analyzed the lymph lipoproteins (truncus co-

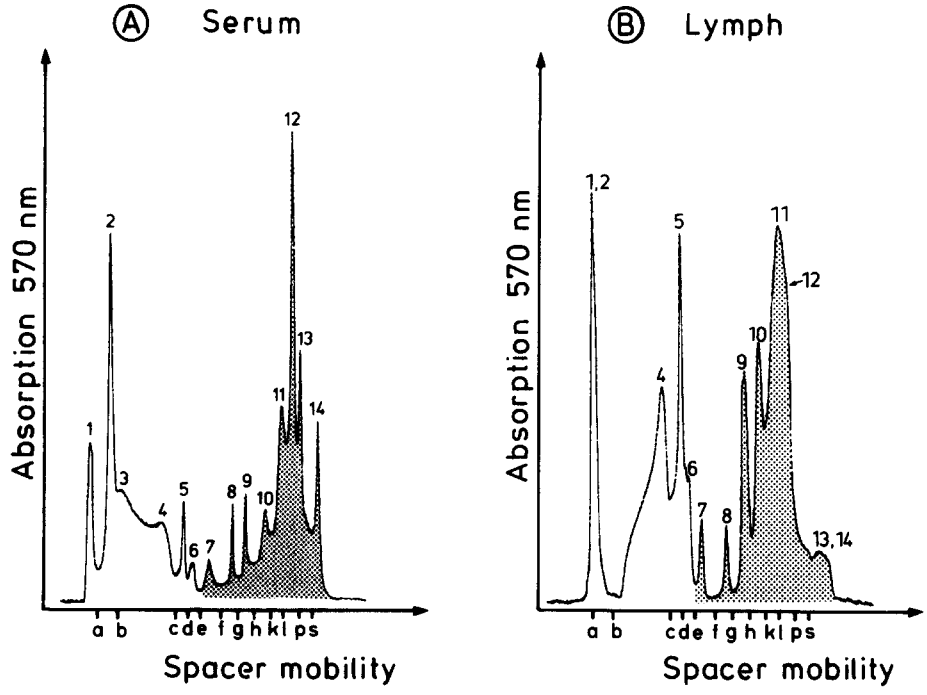

Fig. 1. A. ITP pattern of normal serum lipoproteins. Fasting serum was incubated with Sudan black B for 30 min at 4°C and mixed (2:1, v/v) with the following spacers: glycylglycine (a), alanylglycine (b), valylglycine (c), glycylhistidine (d), histidylleucine (e), serine (f), glutamine (g), methionine (h), histidine (k), glycine (l), 3-methyl-histidine (p), pseudouridine (s), final concentration of each: 2 mg/ml. The spacers are presented according to their mobility which is marked in Fig. 1. Two μl of this mixture was used for the analysis. The leading electrolyte contained 5 mM H_3PO_4, 0.25% HPMC adjusted to pH 9.2 with ammediol. The terminating electrolyte contained 100 mM valine adjusted to pH 9.4 with ammediol. The detection was monitored at 570 nm. Peaks 1–6 correspond to HDL, peak 7 to chylomicron-derived particles, peaks 8–10 to VLDL and IDL, 11–14 to LDL. B. ITP pattern of lymph lipoproteins. Fasting lymph was collected from the truncus coeliacus upon abdominal surgery from the same donor from which the serum ITP pattern was analyzed in panel A. Sample preparation was identical as described in panel A and peak numbers correspond to numbers used in the serum profile.

eliacus) from the same donor and the data are presented in Fig. 1B. In the abdominal lymph, the major constituent of apoB-containing lipoproteins under fasting conditions is formed by particles that correspond in the serum lipoprotein pattern to peaks 11 and 12. The other LDL subfractions, related to peaks 13 and 14 in serum, are minor constituents in the lymph lipoproteins.

Analytical capillary ITP of serum lipoproteins in individuals with different apoE polymorphism

The serum lipoprotein profile was analyzed by capillary ITP in 80 normolipidemic sera from healthy subjects (students) with serum cholesterol levels of 178 ± 21 mg/dl, serum triglyceride levels of 87 ± 31 mg/dl, and with different apoE phenotypes: apoE3/3 (n = 50), apoE3/4 (n = 6), apoE3/2 (n = 8), apoE4/4 (n = 5), apoE4/2 (n = 5), and apoE2/2 (n = 6). The results for the relative distribution of apoB-containing lipoproteins are shown in **Fig. 2**. There are differences in the ITP profile of apoB-containing lipoproteins between normolipidemic subjects with different apoE phenotypes and the data indicate that apoE2/2 subjects have higher concentrations of VLDL and IDL particles migrating in peaks 8, 9, and 10 as compared to apo 3/3 homozygotes. In the LDL profile of apoE2/2 subjects, peaks 11 and 12 dominate, while peaks 13 and 14 are less abundant than in apoE3/3 homozygotes. A tendency for the appearance of enhanced VLDL/IDL related peaks is observed also in apoE3/2 and apoE4/2 subjects. On the other hand, in individuals with apoE4/4 and apoE3/4 phenotypes, the opposite phenomenon appears and VLDL/IDL peaks are lower in concentration while LDL peaks 13 and 14 are enhanced as compared to apoE3/3 subjects.

Analytical capillary ITP of postprandial serum lipoproteins

The pattern of apoB-containing lipoproteins was monitored by capillary ITP in sera from 11 normolipidemic apoE 3 homozygote subjects under fasting conditions and 2, 3, 4, 5, and 6 h after ingestion of a fat meal. However, proteins. ITP offers a new discrimination principle for apoB-containing lipoproteins based on net charge. In addition to the analysis of the flotation properties of lipoprotein particles, it offers new insights into the compositional and functional relationship of lipoprotein subclasses with changes in the lipoprotein concentration and found a linear relationship within the individual lipoprotein classes when three calibration curves were used (VLDL/IDL: r = 0.91; LDL: r = 0.96, HDL: r = 0.94). The relative changes in the serum concentration of apoB-containing lipoproteins (peaks 7–14) in postprandial sera are shown in **Fig. 3**. A significant increase in the concentration of chylomicron-derived particles migrating in peak 7 was observed reaching a maximum (470%) 2 h after the fat meal, while peaks 8 and 9 reached their maximum (230% and 170% increase) after 4 h. The particles migrating in peak 10 show a 20% increase and the particles migrating in peak 11 show only a 10% increase after 5 h and no changes in the concentrations for the LDL-related peaks 12–14 are visible. In order to determine whether indivi-

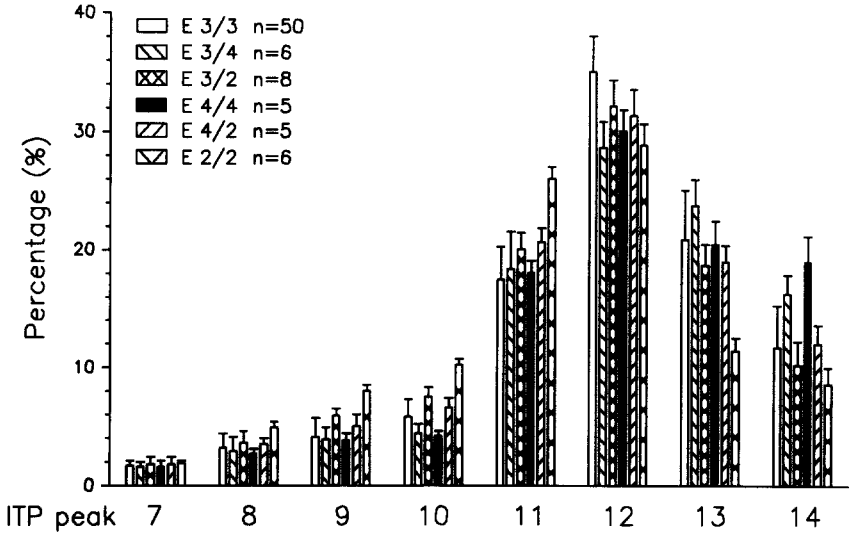

Fig. 2. Relative distribution of apoB-containing lipoproteins among individual ITP subpopulations in normal subjects with different apoE phenotypes. The ITP analyses were made predominantly from fresh fasting sera. Only a few separations from the apoE 3/3 group were performed from one-time frozen–rethawed samples. The integration was done using a Spectra Physics SP4270 Integrator. The total peak area corresponding to apoB-containing lipoproteins (peaks 7–14) was taken as 100% and the percentage distribution of individual ITP peaks was calculated. The data are presented as mean ± SD. The significance of differences among apoE phenotypes was assessed by variance analysis: $P < 0.02$ for peak 8 and $P < 0.001$ for peaks 9–14.

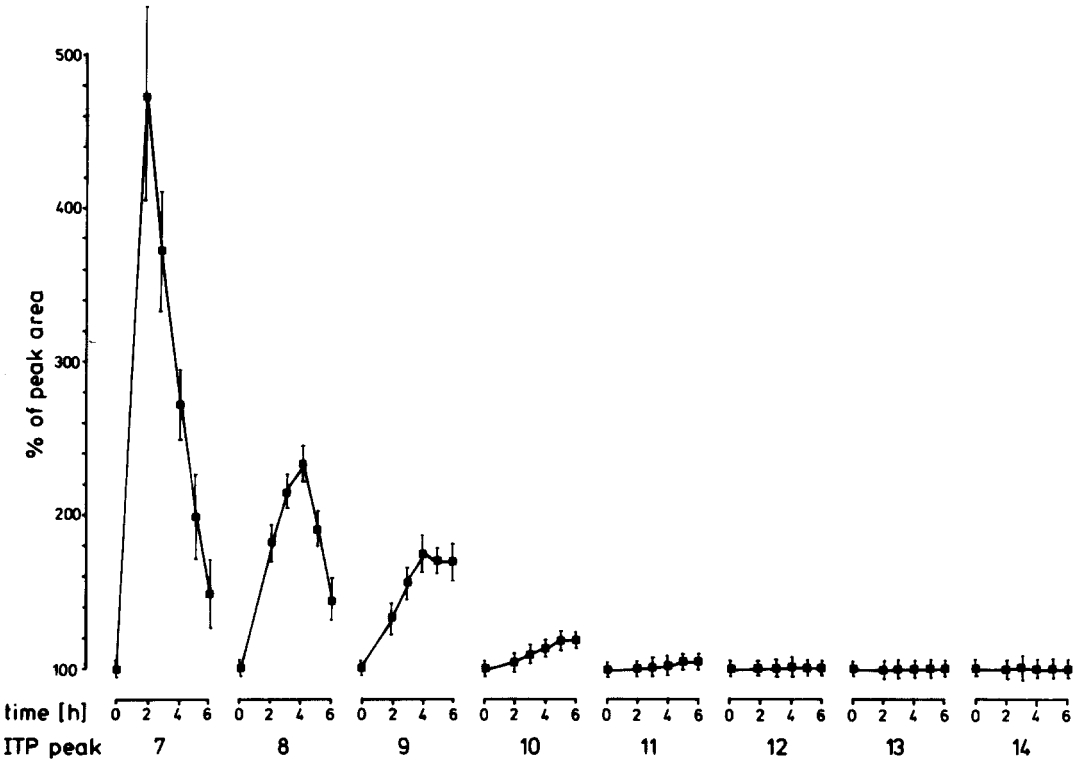

Fig. 3. Changes in area of ITP peaks corresponding to apoB-containing lipoproteins in ITP patterns from postprandial serum lipoproteins. The data are presented as percentage changes in individual peak area using the corresponding peak area in the fasting state (0 h) as 100%. Analysis was made using fasting (0 h) and 2, 3, 4, 5, and 6 h postprandial serum of the same normolipidemic donors with apoE3/3 phenotype (n = 11). The ITP analysis conditions are mentioned in the legend to Fig. 1. The peak numbers correspond to numbers presented in the serum pattern (Fig. 1 A). The integration was done using a Spectra Physics SP4270 Integrator.

dual subjects have a characteristic and reproducible pattern of postprandial lipoproteins, arbitrarily selected subjects (n = 4) from the same group were fed the same meal on a second occasion and they responded similarly. However, in postprandial sera from patients with various forms of hypertriglyceridemia, significant differences in the metabolic behavior of the individual subclasses, especially in peaks 7–11, can be observed (36). The data indicate that capillary ITP allows the analysis of postprandial changes in the apoB-containing lipoprotein subclasses directly in whole serum.

ITP patterns of VLDL, IDL, and LDL subfractions derived from normal subjects

The relationship between the hydrated densities of apoB-containing lipoproteins and their corresponding isotachophoretic mobilities was studies and the results are shown in **Fig. 4**. We compared the ITP lipoprotein profiles of ultracentrifugally isolated lipoprotein subfractions from 40 normolipidemic apoE3 homozygote subjects and recognized two major patterns, indicated as pattern I (upper panel) and pattern II (lower panel) in Fig. 4, which are representative for the analyzed individuals. In the VLDL density range (Fig. 4A) two major groups and one minor group of particles appear. The major groups correspond to peaks 8 and 9 in the whole serum lipoprotein pattern. The minor group has the mobility of peak 7. In all analyzed ultracentrifugation fractions in the ITP profile of VLDL, peak 8 dominates followed by peaks 9 and 7. In 30% of the analyzed subjects, the additional peak 10 is also visible (Fig. 4A-1). In the IDL density range (Fig. 4B) particles dominate with the mobility of peak 10. However, in all subjects, faster migrating particles related to peaks 8 and 9 were detected. In 25% of the normal subjects, particles with a slower mobility than peak 10 which resembles peaks 11–13 are also visible (Fig. 4B-I). In the LDL density range (Fig. 4C–F) particles are found which create predominantly peaks 11–14, but also particles with mobilities characteristic for VLDL/IDL appear in varying concentrations. The lightest LDL subfraction with a density of 1.025 g/ml (Fig. 4C) contains particles corresponding to peaks 13 and 14. In 60% of the normal subjects this fraction also contains particles with the mobility of peak 10 (Fig. 4C-II), and in 40% of the subjects particles are present which correspond to peaks 9 and 10 (Fig. 4C-I) while peaks 11 and 12 are absent. In the LDL subfraction of d 1.036 g/ml (Fig. 4D) four groups of particles occur related to peaks 11–14. In 51% of LDL of d

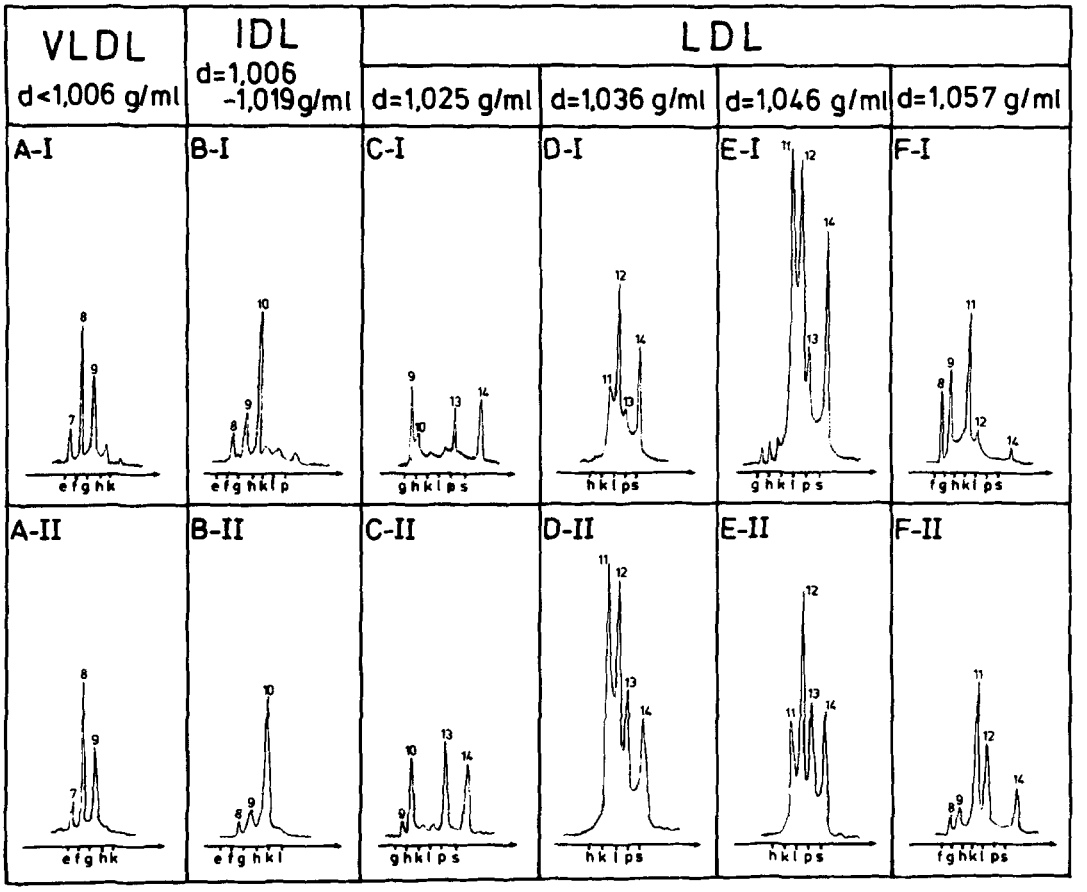

Fig. 4. ITP patterns of VLDL (A), IDL (B), and LDL density subfractions (C–F) representative for normal subjects. Based on the analysis of ITP patterns of individual lipoprotein fractions from 40 normolipidemic E3 homozygote subjects, two major patterns indicated as pattern I (upper panel) and pattern II (lower panel) were recognized. The ultracentrifugally isolated lipoprotein subfractions (obtained as described in Methods) were mixed (1:1, v/v) with the lipoprotein-deficient d > 1.21 g/ml serum fraction in order to have serum matrix conditions during ITP analysis. ITP analysis was performed under standard conditions described in the legend to Fig. 1. The peak numbers correspond to numbers used in the serum pattern and the spacer mobilities are shown on the axis.

1.036 g/ml from normal subjects, 12 and 14 dominate, followed by peaks 11 and 13 (Fig. 4D-I). In 49% of the samples peaks 11 and 12 prevail, followed by peaks 13 and 14 (Fig. 4D-II). In the subfraction of d 1.046 g/ml (Fig. 4E) a similar profile is seen as compared to the latter fraction. In 45% of the normal subjects peaks 14 and 13 dominate (Fig. 4E-I), while in 55% peak 12 prevails followed by peaks 11, 13, and 14 (Fig. 4E-II). In the most dense LDL subfraction (d 1.057 g/ml) in 60% of the normal subject, peak 11 prevails followed by peaks 12 and 14 (Fig. 4F-II), while peak 13 is absent. In this subfraction also, particles with a faster mobility similar to peak 8 and 9 are visible. In 40% of the samples in the densest LDL subfraction, peak 11 dominates followed by peaks 9 and 8 (Fig. 4 F-I); however, slower migrating particles related to peaks 12 and 14 are also detectable in varying concentrations.

The isotachophoretic analysis of isolated density subfractions shows that in the VLDL and IDL density range particles appear with a mobility typical for LDL; on the other hand, in the LDL density range particles occur with a mobility characteristic for VLDL and IDL. In order to recognize possible artefacts that could be formed during ultracentrifugation, each subfraction was mixed with serum, and changes in the serum ITP profile were monitored (data not shown). In all these experiments no additional peaks were observed. The mobility of the particle groups detected in the isolated subfractions strictly corresponds to the mobility of the individual peak groups detectable in whole serum. The data indicate that for the apoB-containing lipoproteins, and especially for LDL, the net electric mobility of the individual particles does not always correspond to the flotation properties of these lipoproteins, and it is also visible that the individual peak groups between different subjects and different density classes resemble a variability in concentration of defined peaks rather than changes in peak mobilities. Therefore compositional and functional analyses were performed for the subclasses of apoB-containing lipoproteins separated by preparative free flow ITP.

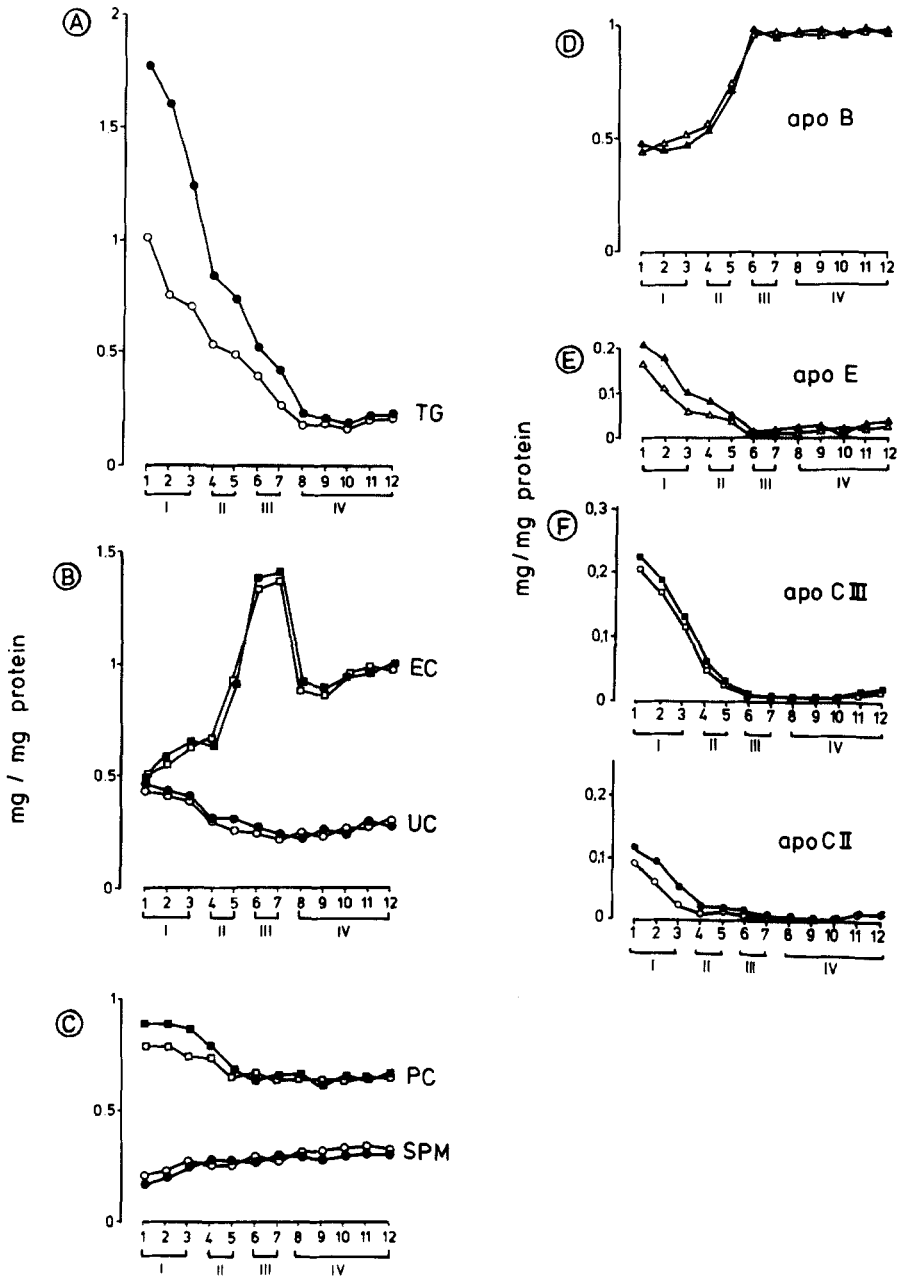

Fig. 5. Lipid and apolipoprotein composition of apoB-containing subfractions separated by preparative ITP. ApoB-containing lipoproteins (d < 1.063 g/ml) were isolated from fasting and 4 h postprandial plasma of normolipidemic volunteers (n = 5, TG = 91 ± 22 mg/dl; TC = 187 ± 18 mg/dl; HDL-C = 51 ± 5 mg/dl) by ultracentrifugation and fractionated by preparative ITP as described in Methods. Open symbols represent the data from fasting, closed symbols from postprandial subfractions. The data represent the mean value from five experiments that differed less than 5% and are presented in mg/mg total protein. Protein concentration was determined by the Lowry method. A. Triglyceride distribution. TG concentration was determined enzymatically. B. Distribution of esterified (EC) and free (UC) cholesterol. EC concentration was calculated as the difference between total and free cholesterol determined by enzymatic methods. C. Distribution of phosphatidylcholine (PC) and sphingomyelin (SPM). The concentrations were determined by an enzymatic assay. D. Apolipoprotein B content. E. Apolipoprotein E content. F. Apolipoprotein C-II and apoC-III distribution. ApoB, C-II, C-III, and E concentrations were measured by immunological methods as described in detail under Methods.

Composition of apoB-containing lipoprotein subpopulations separated by preparative free flow ITP

ApoB-containing lipoproteins (d < 1.063 g/ml) were separated by preparative ultracentrifugation from fasting and 4 h postprandial sera of the same normolipidemic volunteers (apoE3/3) and then further subfractionated by preparative free flow ITP. Twelve fractions were collected, and the lipid and apolipoprotein composition of the isolated fractions is presented in **Fig. 5A–F**. According to

their net electric mobility, four major groups of particles (I–IV) can be differentiated. In additional experiments similar to those described in the previous paragraph it was found that group I corresponds predominantly to peaks 7 and 8 and in part to peak 9 in analytical capillary ITP. Group II contains particles creating peaks 9 and 10. Particles from group III migrate in peaks 11 and 12, while particles from group IV are related to peaks 13 and 14 in analytical ITP. The fast migrating subfractions (group I) are rich in triglycerides and their content decreases with decreasing mobility of the fractions related to groups II and III (Fig. 5A). Higher triglyceride concentrations occur in the corresponding fractions when preparative ITP is performed from postprandial $d < 1.063$ g/ml lipoproteins. However, the largest difference in the triglyceride content between fasting and postprandial state ($90 \pm 19\%$) is observed for particles with the mobility of group I (Fig. 5A). This fast migrating group I is also relatively rich in free cholesterol and phosphatidylcholine (Fig. 5B and C) and contains low amounts of esterified cholesterol (Fig. 5B) and sphingomyelin (Fig. 5C) as compared to other groups. The phosphatidylcholine concentration is even higher ($12.5 \pm 1\%$) when group I is separated from postprandial samples (Fig. 5C). In the protein profile of these subfractions apoB represents the major apolipoprotein (Fig. 5D). However, these fractions are also rich in apoE (Fig. 5E) and C apolipoproteins (Fig. 5F) as compared to other groups. In the second (II) group of particles an increase in the apoB and cholesteryl ester content and a decrease in apoE, C apolipoproteins and PC are observed when compared to group I. Particles in group III have the highest cholesteryl ester content of all apoB-containing lipoproteins and apoB is their dominating apolipoprotein component. The particles migrating in group IV contain smaller amounts of cholesteryl esters and are poorer in triglycerides than in group III. ApoE and C apolipoproteins are also detectable in group IV. However, the particles migrating in groups III and IV obviously have apoB as their major apolipoprotein component.

Cellular interaction of apoB-containing lipoprotein subfractions isolated by ITP

The 4°C binding properties of the apoB-containing lipoprotein subpopulations fractionated by preparative free flow ITP were measured in normal human fibroblasts and the hepatoma cell line HepG2, which has some similarities with liver cells. The binding data presented here are related to groups I–IV. However, before these groups were formed, binding was analyzed for all isolated fractions eluting from preparative ITP. Based on the similarities found in these studies, the fractions were pooled into the four groups.

The 4°C binding properties of the apoB-containing lipoprotein subfractions isolated from fasting samples to fibroblast receptors are represented in **Fig. 6**. In addition, **Table 1** contains the corresponding equilibrium dissociation constants (K_D) and maximal binding values (B_{max}) for fibroblasts and HepG2 cells calculated by Scatchard analysis from analogous binding experiments with postprandial samples.

The lipoproteins related to group I, which in analytical capillary ITP migrate in peaks 7 and 8, reveal nonspecific binding to fibroblasts when fasting as well as postprandial serum is used for the isolation of the subfractions. The particles migrating in group II, that create peaks 9 and 10 in analytical ITP, bind with high affinity to the cells (K_D = 7.8 μg/ml, B_{max} = 54 ng). When postprandial samples are used, the affinity of group II particles to fibroblasts slightly decreases (K_D = 9.5 μg/ml, B_{max} = 45 ng) (Table 1). The particles migrating in group III (peaks 11 and 12 in analytical ITP) interact with fibroblasts with a higher (K_D = 4.4 μg/ml, B_{max} = 75 ng) than particles related to group II, and the affinity increases again (K_D = 2.3 μg/ml, B_{max} = 58 ng), when particles corresponding to group IV (peaks 13 and 14 in analytical ITP) are analyzed. No significant differences are observed in the affinity and B_{max}, when group III and group IV particles are isolated from postprandial samples (Table 1).

The binding properties of these lipoprotein subfractions isolated from fasting sera on HepG2 cells are shown in **Fig. 7**, the binding characteristics of postprandial subfractions are summarized in Table 1.

The particles related to group I reveal high affinity binding to HepG2 cells (K_D = 3.6 μg/ml, B_{max} = 37 ng). The affinity increases, when these particles are isolated from postprandial sera (K_D = 2.5 μg/ml, B_{max} = 59 ng). For particles belonging to groups II, III, and IV, the Scatchard plots show that a high-affinity and a low-affinity binding component exist for HepG2 cells. For the high- and low-affinity component the highest affinity to the cells is found for particles related to group II (*1*: K_D = 3.5 μg/ml, B_{max} = 22 ng; *2*: K_D = 16.9 μg/ml, B_{max} = 53 ng) followed by lipoproteins corresponding to group III (*1*: K_D = 7.7 μg/ml, B_{max} = 50 ng; *2*: K_D = 50 μg/ml, B_{max} = 160 ng) and IV (*1*: K_D = 11.2 μg/ml, B_{max} = 58 ng; *2*: K_D = 68 μg/ml, B_{max} = 170 ng). No changes in the affinity and B_{max} occur, when particles belonging to group II are isolated from postprandial sera. Differences compared to fasting samples can be observed with particles of group III and IV: the K_D values of the high affinity component of postprandial group III and IV particles (III: K_D = 4.1 μg/ml; IV: K_D = 3.9 μg/ml) are significantly lower than the high affinity components of fasting group III and IV particles (III: K_D = 7.7 μg/ml; IV: K_D = 11.2 μg/ml), while the corresponding B_{max} values do not differ from each other.

The presented data indicate that the particle groups isolated from apoB-containing lipoproteins by isotachophoresis possess different binding properties to peripheral and hepatic cells.

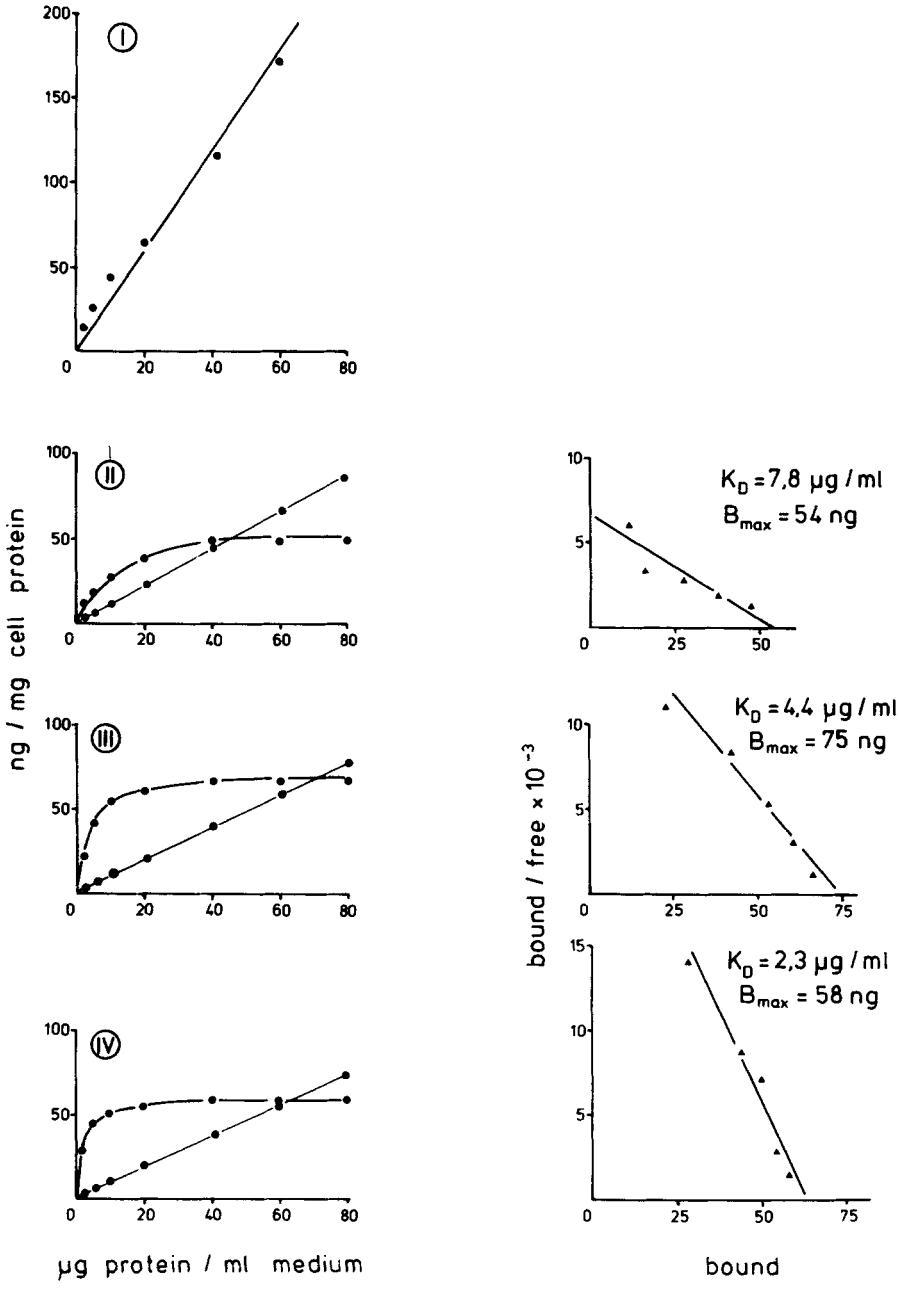

Fig. 6. Binding of apoB-containing lipoprotein subfractions to fibroblasts. ApoB-containing lipoproteins were fractionated from pooled fasting samples of three normolipidemic donors by preparative ITP as described in Methods, and four (I–IV) groups of particles were recognized. Cells were preincubated for 48 h with culture medium containing 10% LPDS, washed with medium, cooled, and incubated with the indicated concentrations of ^{125}I-labeled subfractions (I–IV) for 2 h at 4°C, in the presence or absence of a 20-fold excess of nonlabeled subfractions. Specific and nonspecific binding curves are shown for all groups except for group I where total binding is presented. Specific binding was obtained by subtracting nonspecific binding from total binding and the K_D values were calculated by Scatchard analysis. K_D and B_{max} values represent mean values from five experiments which differed less than 6% for each individual value.

DISCUSSION

In our previous studies (18, 19) we established the conditions for lipoprotein separation by analytical capillary and preparative free flow ITP and characterized the various HDL subfractions (19). In the present report we have fractionated the apoB-containing lipoproteins ($d < 1.063$ g/ml) and characterized the isolated subfractions.

In the analytical capillary ITP from whole serum, apoB-containing lipoproteins create eight peaks. Three of

TABLE 1. Equilibrium dissociation constants (K_D) and maximal binding values (B_{max}) from binding experiments of apoB-containing lipoproteins from postprandial sera to fibroblasts and HepG2 cells

	Fibroblasts		HepG2		
Group	K_D [μg/ml]	B_{max} [ng]		K_D [μg/ml]	B_{max} [ng]
I	nonspecific			2.5	59
II	9.5	45	1	3.5	42
			2	14	78
III	4.8	79	1	4.1	35
			2	47	150
IV	2.2	53	1	3.9	38
			2	38	150

ApoB-containing lipoproteins (d < 1.063 g/ml) from pooled postprandial samples of three normolipidemic donors were fractionated by preparative ITP as described in Methods, and the fractions were pooled into four (I-IV) major groups. Binding to preincubated (10% LPDS, 48 h) cells (2 h, 4°C) was performed for each (I-IV) postprandial group. K_D and B_{max} were calculated by Scatchard analysis; *1* represents the high-affinity binding component, *2* represents the low-affinity binding component. K_D and B_{max} values represent mean values from five experiments that differed less than 6% for each individual value.

them are related to VLDL and IDL particles, and the others correspond mainly to LDL. However, the isotachophoretic analysis of isolated density fractions has clearly indicated that apoB-containing particles with identical mobilities in ITP may be found in smaller or larger quantities across the whole density range of VLDL, IDL, and LDL subclasses. One could argue that this observation may be a result of sample preparation or due to the isotachophoretic separation system. However, if during ultracentrifugation or upon isotachophoresis artefacts are formed, it is unlikely that they would have the same reproducible mobility as apoB-containing particles occurring in whole serum. On the other hand our previous results (19) have shown that no significant changes can be recognized in the lipoprotein pattern that may be caused by degradation processes during ITP analysis. In addition, other laboratories have clearly demonstrated that plasma proteins underwent no detectable modification in this technique (37). In the case of the most dense LDL subfraction, in which very fast particles occur, Lp[a] and HDL_1 or prebeta-migrating HDL (38) are likely to be present in some subjects. The analysis of sera from patients with high Lp[a] concentrations has shown that Lp[a] is heterogeneous and has VLDL and LDL mobility. Further work with purified Lp[a] and Lp[a]-positive sera is currently underway in our laboratory and in those patients who have elevated Lp[a] concentrations we observe an increase in the fast migrating particle groups to which the Lp[a]-positive subfractions correspond (Nowicka, G., et al., unpublished results). Lp[a] has been determined in all sera used for ITP analysis and all subjects presented here were Lp[a]-negative (Lp[a] <5 mg/dl). Additional experiments have also been performed to estimate the occurrence of prebeta-migrating HDL.

In the preparative ITP separation profile of apoB-containing lipoproteins, four (I–IV) major groups of particles are recognized. Group I is rich in triglycerides, free cholesterol, and phosphatidylcholine. It contains apoB, apoE, and C apolipoproteins. When this group is isolated from postprandial samples it is significantly enriched in TG, PC, and apoE. As is obvious from lipid and apolipoprotein analysis and capillary ITP, group I contains the bulk of VLDL-1, VLDL-2, and chylomicron-derived particles. It expresses nonspecific binding to fibroblasts, which is in agreement with reports from other laboratories that, in normal subjects, only VLDL-3 but not VLDL-1 and VLDL-2 possess the ability to interact with apoB,E receptors on fibroblasts (14, 15). On the other hand, this group binds with high affinity to receptors on HepG2 cells, which possess both apoB,E and remnant receptors that may recognize apoE. It was found that normal VLDL reveal high affinity binding to hepatic receptors and that this process is mediated by apoE (17). In group I, isolated from postprandial samples, a higher apoE content is recognized and concomitantly these particles express a higher binding capacity to hepatic receptors.

Group II is richer in cholesteryl esters and apoB than group I; however, apoE is still a significant component of its protein moiety. Particles from group II correspond in their composition to small VLDL-3 and IDL. This fraction binds specifically to apoB,E-receptors on fibroblasts. It interacts with hepatic receptors with a high-affinity and a low-affinity component. However, due to the mixture of different kinds of particles in this group, the resulting binding curves for HepG2 cells may be a product of remnant receptor binding and binding to the hepatocyte LDL receptor which seems to have two binding components. Previous studies have shown that the hepatocyte LDL receptor differs from the fibroblast LDL receptor (39, 40), and, moreover, that two components of LDL binding to HepG2 cells (41) and rat hepatocytes (42, 43) may exist, which is in agreement with our results.

According to their lipid and apolipoprotein composition, groups III and IV correspond to low density lipoproteins. Group III represents cholesteryl ester-enriched LDL, while group IV contains predominantly cholesteryl ester-depleted LDL particles. These groups reveal high affinity binding to fibroblast apoB,E-receptors. However, the K_D for cholesteryl ester-poor LDL is about twofold lower than the K_D for cholesteryl ester-enriched LDL. Groups III and IV interact with the biphasic HepG2 cell receptor with affinities lower than those migrating in group II; there is almost no contamination of particles that interact with the remnant receptor. The K_D values are in the same range as they are described by Salter, Saxton, and Brindley (43). When these particle groups are isolated from postprandial serum, the affinities to fibroblasts do not change, while the affinities to HepG2 cells increase by about two- to threefold.

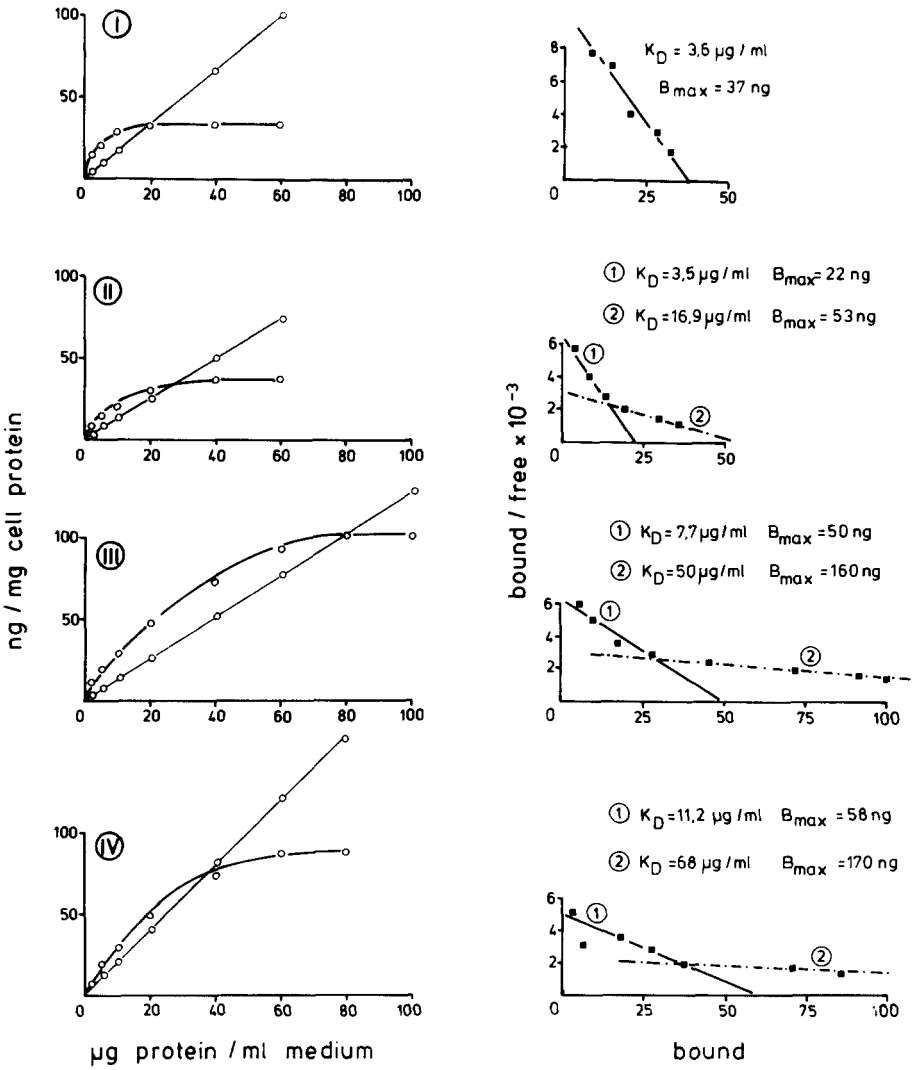

Fig. 7. Binding of apoB-containing lipoprotein subfractions to HepG2 cells. ApoB-containing lipoproteins were fractionated from pooled fasting samples of three normolipidemic donors by preparative ITP as described in Methods, and four (I-IV) groups of particles were recognized. Cells were preincubated for 48 h in culture medium containing 10% LPDS and, after washing, incubated with the indicated concentrations of ^{125}I-labeled subfractions (I-IV) for 2 h at 4°C in the presence or absence of a 20-fold excess of nonlabeled subfractions. Specific and nonspecific binding curves are shown for all groups. Specific binding was obtained by subtracting nonspecific binding from total binding and the K_D values were calculated by Scatchard analysis. K_D and B_{max} values represent mean values from five experiments which differed less than 6% for each individual value.

The binding results show that the remnant receptor of HepG2 cells interacts with particles that correspond to peaks 7 and 8 and, in part, to peak 9 (chylomicron-derived particles, VLDL-1, VLDL-2) in analytical ITP, while particles corresponding to peaks 11–14 (cholesteryl ester-rich and -poor LDL) bind to the B,E-receptor of both fibroblasts and HepG2 cells. Particles represented by peaks 9 and 10 (VLDL-3, IDL) in analytical ITP seem to be a mixture of lipoproteins that interact with the remnant as well as with the B,E-receptor.

The ITP patterns of sera from normolipidemic individuals with different apoE phenotypes indicate that the apoE2 allele is associated with higher concentrations of VLDL and IDL particles migrating in groups I and II, while the LDL particles in group IV are diminished. The apoE4 allele determines the opposite pattern with low groups I and II, and an enhanced concentration of group IV particles when compared with the apoE3 allele. This is in agreement with recent reports (44, 45) that indicated that apoB-containing lipoprotein metabolism is influenced by the apoE phenotype.

We have presented here the characterization of apoB-containing lipoproteins fractionated by preparative free flow ITP and we have shown that this method is a

helpful tool for the analysis of the most atherogenic lipoproteins. ITP offers a new discrimination principle for apoB-containing lipoproteins based on net charge. In addition to the analysis of the flotation properties of lipoprotein particles, it offers new insights into the compositional and functional relationship of lipoprotein subclasses with respect to their biological role. Based on the demonstrated biochemical and functional characterization of apoB-containing lipoprotein subfractions separated by preparative ITP, we think that analytical capillary ITP is a potent method for lipoprotein subclass analysis in patient samples with a high discrimination for functionally important lipoprotein subclasses.

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