Macrophage interaction of HDL subclasses separated by free flow isotachophoresis

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Abstract Preparative isotachophoresis (ITP) was used for the fractionation of fasting and postprandial high density lipoproteins (HDL) according to their net charge in the absence of molecular sieve effects. Three major HDL subpopulations with fast, intermediate, and slow mobility have been recognized. Particle size analysis by gradient gel electrophoresis has shown that in the fast-migrating subpopulation particles dominate with a size of HDL_{3a} and HDL_{2b}. The subpopulation with intermediate mobility contains particles with a size between HDL_{2a} and HDL_{3b}, while in the slow migrating subpopulation particles dominate with a size of HDL_{2b}, HDL_{3a}, and HDL_{3c}. The fast-migrating subpopulation is rich in apoA-I and phosphatidylcholine. The particles of this fraction bind at 4 °C to HDL receptors on macrophages with high affinity (K_D = 7.71 μg/ml; B_{max} = 245.6 ng). The subpopulation with intermediate mobility is rich in apoA-II, apoE, C apolipoproteins, cholesteryl esters, and sphingomyelin. Its affinity to HDL receptors (K_D = 17.7 μg/ml; B_{max} = 198.4 ng) is lower than that of the HDL particles in the fast-migrating subfraction. The slow-migrating subpopulation consists of particles rich in apoA-IV and is associated with a high LCAT activity. This fraction expresses the highest nonspecific binding to mouse peritoneal macrophages compared to the other HDL fractions and contains only a small amount of particles that interact with HDL receptors by high affinity binding (K_D = 7.3 μg/ml; B_{max} = 95.9 ng). In 37 °C binding experiments the fast-migrating subfraction reveals the highest total cell-associated activity, 72% of which is trypsin-resistant. The other subfractions express a lower total cell-associated activity and 45% of the activity of the intermediate- and 43% of the activity of the slow-migrating fraction is trypsin-sensitive. When the HDL fractions are isolated from postprandial sera of the same donor, the fast-migrating particles bind at 4 °C with a higher affinity (K_D = 4.6 μg/ml) while no significant changes are observed in the intermediate- and slow-migrating subpopulations. The slow- and the fast-migrating HDL subpopulations isolated from fasting serum have a high capacity to promote cholesterol removal from macrophages. We hypothesize that the HDL subpopulations rich in apoA-I promote cholesterol removal predominantly via the interaction with HDL receptors, while apoA-IV-rich HDL particles receive their driving force for cholesterol efflux from the concomitant action of LCAT via a predominantly nonspecific interaction of the particles with the cell surface. The preparatively isolated HDL fractions correspond to six HDL peaks in analytical capillary ITP obtained from whole serum and lymph. The technique may be a helpful tool for the analysis of HDL subfractions but needs further confirmation by patient analysis.—**Nowicka, G., T. Brüning, A. Böttcher, G. Kahl, and G. Schmitz.** Macrophage interaction of HDL subclasses separated by free flow isotachophoresis. *J. Lipid Res.* 1990. **31:** 1947-1963.

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High density lipoproteins (HDL) represent a series of dynamically changing HDL populations differing in density, size, and composition; HDL_2 (d 1.063-1.125 g/ml) and HDL_3 (d 1.125-1.210 g/ml) are the major HDL subpopulations (1). In addition, several other HDL subspecies have been identified by gradient ultracentrifugation, gradient gel electrophoresis, isoelectric focusing, and chromatographic methods (2-9). HDL contain several apolipoproteins including apoA-I, A-II, A-IV, C-I, C-II, C-III, D, E, and other proteins such as cholesteryl ester transfer protein (CETP), serum amyloid A protein, and beta-1-glycoprotein (1, 8, 10, 11). Two principal HDL particle groups have been isolated according to their content of apoA-I with and without apoA-II as the major apolipoproteins (7, 8, 10). They occur within the HDL_2 and HDL_3 density classes and consist of particles that are heterogeneous with respect to their size and apolipoprotein content (7, 8, 11, 12).

It has been proposed that HDL play an important role in the transport of cholesterol from peripheral cells to the liver for bile acid formation and excretion (1). Hepatic and extrahepatic cells such as fibroblasts, endothelial cells, smooth

Abbreviations: VLDL, very low density lipoproteins; IDL, intermediate density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; apo, apolipoprotein; TC, total cholesterol; TG, triglyceride; UC, free cholesterol; EC, esterified cholesterol; PC, phosphatidylcholine; SPM, sphingomyelin; HDL-C, HDL-cholesterol; CETP, cholesteryl ester transfer protein; LCAT, lecithin:cholesterol acyltransferase; ACAT, acyl-CoA:cholesterol acyltransferase; ITP, isotachophoresis; ammediol, 2-amino-2-methyl-1, 3-propanediol; HMPC, hydroxypropylmethylcellulose; HPTLC, high performance thin-layer chromatography; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; FCS, fetal calf serum; BSA, bovine serum albumin.

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muscle cells, adipocytes, and macrophages possess specific high affinity receptors for HDL (13-20). HDL binding increases when cells are loaded with cholesterol or incubated with ACAT inhibitors (21), suggesting that these receptors are regulated in response to changes in cellular cholesterol metabolism. Apolipoprotein A-I-containing HDL particles bind to high affinity receptor sites on cells and it has been suggested that they have a high capacity to promote cholesterol efflux (13-20). Synthetic apoA-IV-containing particles have been found to be very effective in removing cellular cholesterol, while synthetic particles rich in apoA-II and C apolipoproteins reveal a very low capacity or are even ineffective in this process (22).

Changes in the HDL particle composition observed under postprandial conditions (23, 24) may have an effect on interactions between HDL and cells. Postprandial plasma was found to be more effective than fasting plasma in stimulation of net cholesterol transport from cells (25).

As our laboratory reported before (26, 27) plasma lipoproteins can be separated by isotachophoresis into a number of subpopulations according to the net charge of the particles in the absence of molecular sieve effects. Isotachophoretic separation of apoB-containing lipoproteins revealed four major groups that differed in their biological properties (27). Such charge-dependent separation of HDL particles may also more adequately reflect their biological activity than does classical separation according to density using ultracentrifugation.

ITP is an electrophoretic technique by which ionic sample components are separated in a discontinuous electrolyte system based on differences in their net electric mobility (28, 29). 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 must contain 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 in immediate contact with each other. Therefore, to achieve an optimal separation, intermediate-mobility compounds, so-called spacers, are added to the sample solution. The sample mobility is related to the defined spacer mixture and the sample peak pattern represents reproducible net mobilities. The optimal selection of spacer ions has to be determined experimentally. Under the conditions presented in this paper for lipoprotein separation, amino acids and dipeptides were used as spacers and they strictly defined the position of isolated subfractions (ITP-peaks) in lipoprotein patterns.

In the studies presented here, we used preparative and analytical isotachophoresis for the subfractionation of HDL and have characterized the chemical composition and biological function of isolated subfractions.

MATERIALS AND METHODS

Plasma and serum collection

Blood was drawn from fasting healthy volunteers and patients at the University Hospital. Plasma and sera were separated by low speed centrifugation at 4 °C. The samples were either stored at 4 °C and analyzed within 24 h or immediately frozen and thawed only once before use. To protect serum lipoproteins against lipid peroxidation during storage, Na_2EDTA (pH 7.1) was added to the sera at a final concentration of 0.01% immediately after separation.

Postprandial plasma was drawn 4 h after a 1600 kcal breakfast. Within 10 min each subject consumed a meal that consisted of bread with butter and cheese and 500 ml of milkshake containing orange juice, sunflower oil, low fat cream cheese, and milk powder. The total breakfast contained 57% fat, 20% protein, and 23% carbohydrate (by energy) (30).

Lipoprotein preparation

Lipoproteins were isolated from plasma by sequential ultracentrifugation (31) using a Beckman 50.3 Ti rotor at 4 °C and 48,000 rpm. HDL and HDL subfractions were separated according to densities as follows: HDL, d 1.063-1.21 g/ml; HDL_2, d 1.063-1.125 g/ml; HDL_3, d 1.125-1.21 g/ml. The fractions were dialyzed against 0.9% NaCl-0.01% Na_2EDTA (pH 7.4) at 4 °C.

Analytical isotachophoresis of lipoproteins

Isotachophoretic patterns of lipoproteins were obtained from whole sera/plasma or isolated subfractions that had been preincubated for 30 min at 4 °C with the nonpolar dye Sudan black B (1% solution in ethylene glycol) mixed with serum-plasma (3:1, v/v). Before analysis, prestained samples were mixed with spacers (2:1, v/v, final concentration of each spacer 0.2 mg/ml) to achieve a better separation of individual zones. The following compounds (reported according to their mobility) were used as spacers: glycylglycine, alanylglycine, valylglycine, glycylhistidine, histidylleucine, serine, glutamine, methionine, histidine, glycine, 3-methylhistidine, and pseudouridine (Serva, Heidelberg). For separation, 2 μl of the mixture was injected into the 20-cm long capillary system between the leading and terminating electrolyte. As the leading electrolyte, 5 mM H_3PO_4 was used. The electrolyte contained 0.25% HPMC to increase the viscosity and to suppress unwanted transverse electroendosmotic movement of the electrolyte to the capillary wall. Ammediol was added as a common counter ion to achieve pH 9.2. The terminating electrolyte contained 100 mM valine and was adjusted with ammediol to pH 9.4. The separation was started with a constant current of 150 μA. During the 10-min run the current was reduced to 100 μA (at 7 kV) and then to 50 μA (at 6 kV) before detection. The separated zones were monitored at 570 nm. Analyses

were performed either with an LKB 2127-Tachophor System or by a fully automated free flow capillary electrophoresis instrument that has been recently developed in our laboratory. In both instruments the capillary was cooled with air or with a nonconductive organic solvent and analyses were performed at a temperature from 4° to 10°C. The ITP method for lipoprotein analysis presented here can be directly adapted to the HPE-capillary electrophoresis system from Beckman (Palo Alto, CA). The capillaries are available from Dr. Knauer, Säulentechnik, Berlin.

Preparative isotachophoresis of lipoproteins

Preparative subfractionation of lipoproteins was carried out in an Elphor VAP 22 apparatus (Bender & Hobein, Munich) (32). The surface of the separation chamber was covered by thin Teflon membranes (DuPont) instead of silica laminates to reduce electroendosmosis and to prevent remixture of separated zones by counter flow. For analysis, leading and terminating electrolytes of the same composition as for analytical ITP were used. The sample (50 mg of protein) in 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 30 ml/h. Under these conditions the separation time of the sample volume present in the 10-cm broad chamber was 10 min. The collected fractions were passed through a Sephadex G-25, PD-10 column (Pharmacia), in order to modify the electrolyte with normal saline, and further analyzed.

Analysis of the fractionated lipoproteins

Total cholesterol, free cholesterol, triglycerides, phosphatidylcholine, and sphingomyelin were determined by enzymatic methods (33, 34) using Boehringer Mannheim reagents and a Cobas-Bio centrifugal analyzer (Hoffmann-La Roche, Zurich). Esterified cholesterol was calculated as the difference between total and free cholesterol.

ApoA-I and apoA-II were determined by a turbidimetric method using reagents (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, apoC-II and apoC-III 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 standard procedures. Coating was performed after 1:1000 dilution in 0.1 M $NaHCO_3$, pH 9.5. A reference standard (from Immuno AG, Vienna) was used for quantification of samples diluted in PBS containing 0.5% BSA, pH 7.7. The reaction of antibody-peroxidase conjugate, which was prepared according to the procedure described by Nakane and Kawai (35) using *o*-phenylenediamine H_2O_2, was measured at 490 nm with an ELISA reader. ApoA-IV concentration and LCAT activity were determined according to methods described before (36, 37).

Gradient gel electrophoresis was performed according to the method described by Blanche et al. (5) using 4–30% gradient gels. Electrophoresis was carried out in 9 mM Tris, 80 mM boric acid, 3 mM EDTA buffer, pH 8.35, at constant voltage of 125V, at 10°C for 24 h. Gels were stained with Coomassie Blue G-250, destained in 7% acetic acid, and scanned by a laser densitometer (Pharmacia, LKB). The reference protein mixture (Calibration Kit, Pharmacia LKB, containing thyroglobulin, apoferritin, catalase, lactate dehydrogenase, and BSA) was included in each gel run. The molecular diameter was calculated from a calibration curve and the relative distribution of particles among HDL_{2b} (12.9–9.7 nm), HDL_{2a} (9.7–8.8 nm), HDL_{3a} (8.8–8.2 nm), HDL_{3b} (8.2–7.8 nm), and HDL_{3c} (7.8–7.2 nm) particle size intervals (38) were calculated by a computer-assisted procedure.

Binding, uptake and degradation experiments

Lipoprotein subfractions freshly isolated by preparative ITP were iodinated by the IODO-Bead method (39) as follows. Aliquots of these subfractions (1 mg/1 ml) were mixed with 0.5 mCi of $Na^{125}I$ (DuPont de Nemours, Germany); five IODO-Beads (Pierce) 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 activities of the iodinated HDL subfractions ranged between 200 and 400 cpm/ng protein. No significant differences between labeled HDL subfractions in the percentage distribution of total radioactivity found in the apolipoprotein (95–98%) and lipid moiety (2–5%) were observed based on TCA precipitation and SDS-PAGE of labeled HDL subpopulations.

The binding experiments were performed according to the method described by Goldstein and Brown (40) on mouse peritoneal macrophages. The cells were cultured in DMEM containing 10% FCS. Before the experiments the cells were preincubated for 18 h with acetyl-LDL (50 μg/ml medium). The cells were washed with DMEM, chilled on ice in a 4°C cold room for 30 min, and incubated with the indicated concentrations of ^{125}I-labeled lipoprotein subfractions for 1 h at 4°C in the presence or absence of a 20-fold excess of nonlabeled subfractions. After incubation, the cells were rapidly and extensively washed with PBS containing 2% BSA and PBS without BSA, transferred to glass tubes, centrifuged at 200 *g* for 10 min, and dissolved in 1 N NaOH. The aliquots were used for determination of cellular protein content and cell-associated ^{125}I-radioactivity, which was measured in a Compugamma 1282 automatic counting system (LKB). Scatchard analysis of the binding data was performed using the Ligand PC computer program (41).

For uptake and degradation measurements mouse peritoneal macrophages were incubated at 37 °C with the indicated amounts of radiolabeled HDL subfractions for the indicated times (42). After incubation, the medium was removed and collected for determination of lipoprotein degradation (see below). The cells were washed and cell-associated activity was determined. To give an insight into the process of internalization of isolated HDL subpopulations by mouse peritoneal macrophages, the trypsin treatment assay was performed. To measure trypsin-sensitive (cell surface) and trypsin-resistant (internalized) pools of cell-associated ligand we followed the procedure described by Oram, Johnson, and Brown (42). The cells were divided into two groups. The first group of cells was incubated at 4 °C to perform the binding assay, washed, and 1 ml of trypsin buffer (PBS containing 1 mM EDTA, 4 mg/ml trypsin) was added. The second group of cells was incubated at 4 °C to perform the binding assay, washed, incubated at 37 °C, cooled, washed, and incubated with 1 ml of trypsin buffer. After 1 h incubation at 4 °C the medium was removed for activity measurement (trypsin-releasable activity) and the cells were washed with medium containing 2 mg/ml soybean trypsin inhibitor and dissolved in 1N NaOH. The aliquots were used for counting (trypsin-resistant activity) and protein determination.

Lipoprotein degradation was assayed by the amount of trichloroacetic acid-soluble ^{125}I-labeled material in the culture medium (43). In all degradation experiments the rate of release of free iodine from ^{125}I-labeled HDL in the absence of cells was measured.

Cholesterol efflux from macrophages

Mouse peritoneal macrophages were obtained from unstimulated mice as described previously (30). Cells were incubated in DMEM containing 10% FCS, penicillin (100 U/ml), and streptomycin (100 μg/ml) in a humidified incubator (5% CO_2) at 37 °C. On the second day each dish was extensively washed with DMEM and the cells were preincubated for 18 h with DMEM containing 50 μg/ml of acetyl-LDL or [^{3}H]cholesteryl oleate-labeled acetyl-LDL. The labeled lipoproteins were prepared according to Sparks et al. (44). The mixture of 200 μl of acetyl-LDL (4 mg protein/ml) and 50 μCi of the radiolabel was dried under nitrogen, then 1 ml of acetyl-LDL was added, and the sample was stored for 6 h at room temperature. The lipoproteins were reisolated by filtration through a 0.22 μm pore-size filter. The loaded cells were washed and incubated for 1 h with culture medium. After this hour of preincubation an aliquot of medium was taken to measure medium ^{3}H activity (in an automatic beta counting system from LKB), which was in the range of blank samples. Then the cells were incubated with the HDL subfractions isolated by preparative ITP. At the end of the incubation period medium was removed and the cells were washed with DMEM and PBS. The cells were harvested with a rubber policeman in 500 μl of PBS, transferred to glass tubes and sonicated with a Branson sonifier three times for 20 sec in ice-water. One part of the homogenate was used for protein determination and the second part was delipidated by a modified Folch procedure (45). The organic solvents were evaporated and the samples were dissolved in 50 μl of chloroform. Aliquots of this extract were used to determine the total cell-associated activity. The lipids were chromatographically separated on HPTLC silica plates as previously described (45, 46). Spots were detected using a manganese chloride–sulfuric acid reagent and quantification was performed by densitometry with a Camag TLC-scanner. The distribution of radioactivity between free and esterified cholesterol was also determined. The removal medium was centrifuged to pellet residual cells. Aliquots of the medium were taken to measure medium ^{3}H radioactivity and for thin-layer chromatography to confirm that only free cholesterol appeared in the medium.

Other methods

Proteins were measured by the method of Lowry et al. (47). LDL were acetylated by repeated additions of acetic anhydride as described by Basu et al. (48) and dialyzed against PBS (pH 7.4) at 4 °C. All acetyl-LDL preparations were examined for their increased electrophoretic mobility in 1% agarose gels.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed by the Laemmli method (49) using a 14% gel. For SDS-PAGE analysis of cell-associated proteins, the washed cells were solubilized in SDS buffer containing β-mercaptoethanol and boiled for 3 min prior to electrophoresis. Protein bands were identified by staining of gels with Coomassie Blue. Radiolabeled protein bands were identified by autoradiography and, in addition, gels were cut into 2-mm pieces and ^{125}I activity associated with each piece was measured and related to the molecular weight standards.

RESULTS

Analytical capillary ITP of whole serum lipoproteins

As reported from our laboratory (26), whole serum lipoproteins can be directly separated into distinct zones by analytical free flow capillary ITP. Three major lipoprotein fractions were recognized in whole serum, VLDL, LDL, and HDL, and they could be separated into additional subpopulations. Previously (26), we focused on the influence of pH changes of the leading and terminating buffer on lipoprotein separation, evaluated the specific detection of lipoproteins in native sera, and established the optimal staining time and stability of the lipoprotein–dye complexes. In the meantime, we have improved the separation conditions of the electrolyte system and additional spacer ions have been added to further subfractionate each of the earlier recognized lipoprotein fractions (27).

Lipoprotein analysis by analytical capillary ITP can now be performed from whole serum, plasma, or lymph. The ITP pattern of lipoproteins from normal serum/plasma consists of 14 subfractions (**Fig. 1A**), which have been identified by the addition of ultracentrifugally isolated lipoprotein fractions (VLDL, d < 1.006 g/ml; IDL, d 1.006–1.019 g/ml; LDL, d 1.019–1.063 g/ml; HDL_2, d 1.063–1.125 g/ml; HDL_3, d 1.125–1.21 g/ml) to serum or plasma as previously shown (26). In the HDL range, which has the highest mobility, six subpopulations (peaks 1–6 in Fig. 1A) are detectable. An increase in individual peaks in the HDL mobility range was observed after addition of either isolated HDL_2 or HDL_3. VLDL and IDL particles correspond to peaks 8–10 and LDL to the last four peaks, 11–14 (27).

In addition to capillary ITP of serum lipoproteins shown in Fig. 1A, we analyzed the lymph lipoproteins (truncus coeliacus) from the same donor and the data are presented in Fig. 1B. The lymph lipoprotein pattern differs considerably from that of serum lipoproteins. To relate the individual lipoprotein subpopulations separated from lymph to the corresponding serum lipoproteins, we compared the net mobilities of isolated subfractions. We also collected the separated subfractions on a cellulose membrane using a Tachophrac microfraction collector and determined the apolipoprotein distribution by two-dimensional immunoelectrophoresis (data not shown here). In lymph the major HDL fractions are related to peaks 1 + 2 and peaks 4 + 5, while peak 3 is absent and peak 6 is almost invisible. In the mobility range of apoB-containing lipoproteins, peaks 9 and 10 are significantly enhanced compared to the serum profile. In the lymph, peak 11 is a major constituent of apoB-containing lipoproteins, while peak 12, the predominant LDL peak in the serum lipoprotein pattern, appears in lymph only as a shoulder of peak 11. The other serum LDL fractions related to peaks 13 and 14 are much reduced in lymph. The metabolic basis of the differences in the HDL subclasses between serum and lymph will be elaborated later in this report, while the analysis of apoB-containing lipoproteins is described in another manuscript (27).

We compared the ITP patterns of serum and EDTA-plasma lipoproteins from the same individual obtained from freshly prepared samples stored for 24 h at 4°C and in frozen-thawed samples and found a satisfactory reproducibility (c.v. < 5%) for individual peaks (**Table 1**). We also used heparin and citrate plasma (**Fig. 2**) to recognize changes in the pattern caused by degradation processes occurring upon sample preparation and during analysis. The results (Fig. 2) show that no significant changes can be observed in the ITP profiles between serum and plasma samples and between fresh, 4°C-stored, and freshly frozen and once thawed samples.

Analytical capillary ITP of HDL_2 and HDL_3 fractions

Since differences in the metabolic behavior of HDL_2 and HDL_3 as the major HDL subclasses have been established (1), we determined the electrophoretic mobilities of these subfractions obtained from normal subjects by analytical capillary ITP. In **Fig. 3** the representative ITP patterns of ultracentrifugally isolated HDL subfractions from normal healthy volunteers are presented. In the ITP profile of both HDL_2 (d 1.063–1.125 g/ml) and HDL_3 (d 1.125–1.21 g/ml) six major groups of particles with comparable mobilities appear. This indicates that HDL subfractions characterized by differences in their hydrated density can have identical electrophoretic mobilities. However, significant differences in the relative distribution of detected fractions are observed. Peaks 1 and 2 and peaks 4 and 5, which are major fractions in lymph, are significantly higher in HDL_3

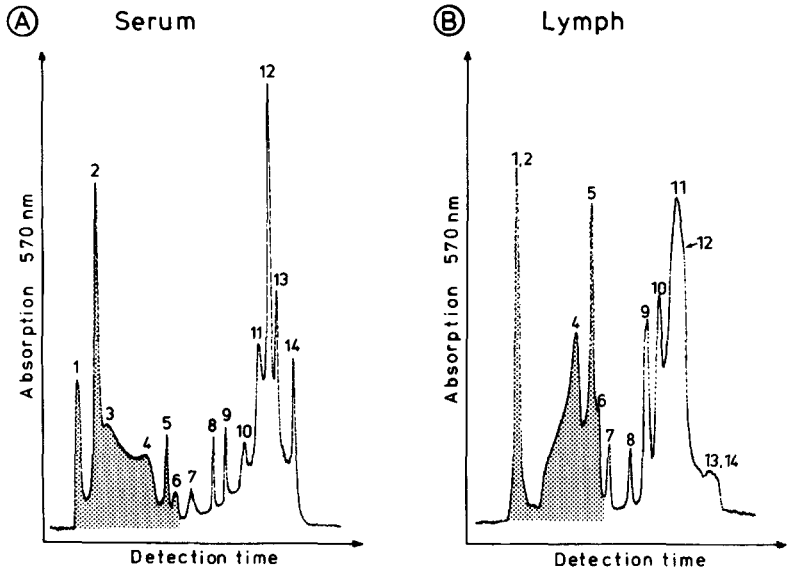

Fig. 1. Panel A. ITP pattern of normal whole serum lipoproteins. Serum was incubated with Sudan black B for 30 min at 4°C and mixed (2:1 v/v) with the following spacers presented according to their mobility: glycylglycine, alanylglycine, valylglycine, glycylhistidine, histidylleucine, serine, glutamine, methionine, histidine, glycine, 3-methyl-histidine, and pseudouridine (final concentration of each 2 mg/ml). 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. Monitoring was at 570 nm. Peaks 1–6 correspond to HDL, peak 7 to chylomicron-derived particles, peaks 8–10 to VLDL and IDL, and peaks 11–14 to LDL. Panel 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 has been analyzed in panel A. Sample preparation was identical as described in panel A and peak numbers correspond to numbers used in serum profile.

TABLE 1. Total peak area and its percentage distribution among individual peaks of serum/plasma lipoprotein isotachophoresis pattern

Sample	Total Area in Integrated Scanner Units	Peak Area as % of Total Area													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
Freshly prepared															
Serum	3.26×10^6	5.53	15.39	24.33	6.41	2.12	0.71	0.80	2.24	2.11	2.64	7.98	15.99	8.63	5.12
	±0.14	±0.25	±0.69	±1.09	±0.29	±0.10	±0.03	±0.04	±0.10	±0.10	±0.12	±0.35	±0.72	±0.39	±0.23
Plasma	3.26	5.50	15.21	24.60	6.37	2.07	0.73	0.79	2.22	2.15	2.70	8.00	15.79	8.85	5.02
	±0.15	±0.25	±0.70	±1.13	±0.30	±0.10	±0.03	±0.04	±0.10	±0.10	±0.12	±0.37	±0.73	±0.41	±0.23
Stored 24 h at 4°C															
Serum	3.25	5.60	15.00	25.01	6.38	2.10	0.70	0.82	2.20	2.10	2.70	7.60	16.01	8.55	5.23
	±0.14	±0.24	±0.65	±1.08	±0.27	±0.09	±0.03	±0.03	±0.09	±0.09	±0.11	±0.33	±0.69	±0.37	±0.22
Plasma	3.25	5.55	15.40	24.40	6.50	2.20	0.70	0.80	2.30	2.10	2.62	7.85	16.00	8.28	5.30
	±0.15	±0.25	±0.70	±1.10	±0.29	±0.10	±0.03	±0.04	±0.10	±0.09	±0.12	±0.35	±0.72	±0.38	±0.24
Frozen-thawed															
Serum	3.27	5.50	15.52	24.40	6.35	2.20	0.74	0.80	2.33	2.20	2.60	8.10	15.27	8.79	5.20
	±0.15	±0.25	±0.72	±1.12	±0.30	±0.10	±0.03	±0.04	±0.11	±0.10	±0.12	±0.38	±0.70	±0.40	±0.24
Plasma	3.27	5.44	15.30	25.00	6.49	2.05	0.71	0.77	2.21	2.10	2.70	7.95	15.53	8.60	5.15
	±0.14	±0.23	±0.66	±1.08	±0.28	±0.09	±0.03	±0.03	±0.10	±0.09	±0.12	±0.34	±0.67	±0.37	±0.22

The total peak area is presented in integrated scanner units. Data were calculated for ITP patterns obtained under standard conditions as described in Methods using freshly prepared, stored (24 h at 4°C), and frozen-thawed (only one time before use) serum and plasma samples from the same donor.

than in HDL_2. Peak 3, which is absent in lymph, is found in both HDL_3 and HDL_2. In addition, peak 6 is relatively higher in HDL_2 than in HDL_3. In both HDL subfractions, very low and varying concentrations of particles comigrating with peak 7 are detectable in different individuals.

Lipid and apolipoprotein composition of HDL subpopulations separated by preparative free flow ITP

Preparative free flow ITP was used to characterize the HDL subfractions obtained in analytical capillary ITP.

HDL (d 1.063-1.21 g/ml) were prepared from fasting and 4-h postprandial sera of the same normolipidemic volunteers by ultracentrifugation and then subfractionated by preparative free flow ITP. Ten fractions containing HDL particles were collected from preparative free flow ITP and further analyzed.

As presented in **Fig. 4** (A–K) the separated fractions can be divided into three major groups according to their composition: fast, intermediate, and slow migrating fractions. The fast-migrating HDL subfractions (I) contain particles rich in apoA-I, phosphatidylcholine, and triglycerides, and poor in cholesteryl esters, sphingomyelin, apoA-II, apoA-IV, apoE, and C apolipoproteins. The intermediate fractions (II), which dominate in the HDL pattern, are rich

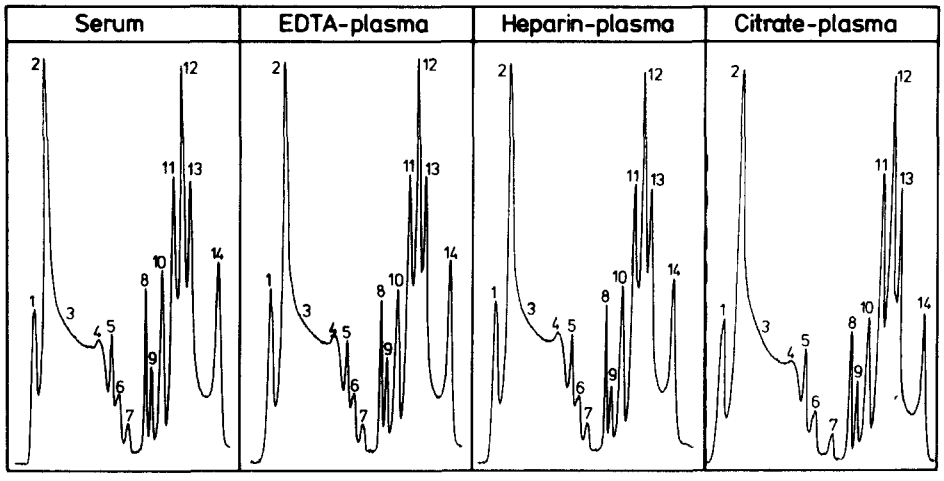

Fig. 2. Comparison of ITP lipoprotein patterns obtained from serum and plasma samples. Analysis was made using serum, and EDTA; heparin- and citrate-plasma of the same donor. The samples were prepared as described in Fig. 1. Analysis conditions and peak numbers are mentioned in legend in Fig. 1.

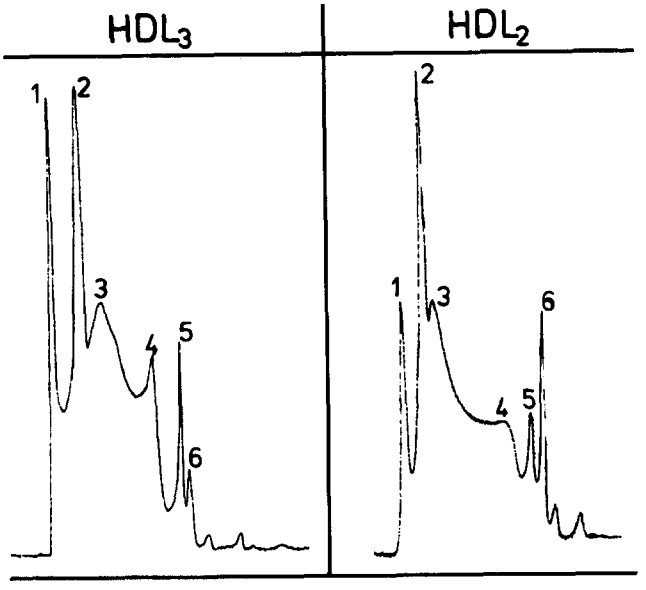

Fig. 3. ITP patterns of HDL_2 and HDL_3. The profiles are representative for normal, healthy subjects. ITP of isolated fractions was performed under standard conditions as described in the legend to Fig. 1 and peak numbers correspond to numbers used in the whole serum lipoprotein profile.

in cholesteryl esters, sphingomyelin, apoA-II, apoE, and relatively poor in apoA-I, apoA-IV, and phosphatidylcholine. The C apolipoproteins are represented by apoC-III and apoC-II, which are almost absent in the fast- and slow-migrating subfractions. The slow-migrating HDL subpopulation (III) consists of particles poor in cholesteryl esters, triglycerides, and sphingomyelin, but contains the bulk of HDL apoA-IV (68.8% of total HDL apoA-IV) and HDL-associated LCAT activity (75.4% of total HDL-associated LCAT activity) (**Fig. 5**). ApoA-I is lower in concentration than in the fast-migrating fraction. In addition, the slow-migrating HDL subpopulation (III) contains higher amounts of apoE than the fast-migrating HDL subfraction (I).

Based on the determination of the total HDL protein concentration we have found a protein distribution of 18 ± 2.7% in the fast-migrating fraction (I), 75 ± 3.2% in the intermediate fraction (II), and 7 ± 2.1% in the slow-migrating fraction (III). We calculated the percentage composition of isolated HDL subfractions and found that particles in subfraction I contain 53% of protein, in II 48%, and in III 51%. The fast-migrating subfractions collected as group I from preparative ITP correspond to peaks 1 and 2 in analytical ITP. Particles from group II migrate predominantly in peak 3 and particles collected as group III migrate in analytical ITP in peaks 4, 5, and 6.

In fast- and slow-migrating subpopulations separated from 4 h-postprandial serum, the protein concentrations were 5 ± 1.9% higher than the corresponding HDL fractions separated from fasting samples.

Under postprandial conditions, the fast-migrating HDL fractions (I) are richer in phosphatidylcholine and apoA-I as compared to fasting HDL. In the slow-migrating subpopulations (III) a higher content of phosphatidylcholine, apoA-IV, and apoE is recognized. The triglyceride concentration and LCAT activity is enhanced in all subfractions prepared from postprandial HDL.

When separated subpopulations were submitted to isotachophoresis, the protein and lipid profiles were similar to the original profiles. This indicates that stable particle populations had been isolated.

Gradient gel size analysis of HDL particles separated by preparative free flow ITP

The size of the fast-, intermediate-, and slow-migrating HDL subpopulations separated by preparative ITP was calculated based on densitometric scans after gradient polyacrylamide gel electrophoresis of individual subpopulations. As shown in **Table 2** in the fast-migrating HDL subpopulation, particles with a size of HDL_{3a} and HDL_{2b} dominate. The subpopulation with intermediate mobility consists of particles with a size between HDL_{2b} to HDL_{3b}. In the slow-migrating HDL subpopulation, particles prevail with a size of HDL_{2b} and HDL_{3a} and also higher amounts of HDL_{3c} particles are observed as compared to the fast-migrating subpopulation. Gradient gel electrophoretic analysis of HDL subpopulations isolated under postprandial conditions showed that in the fast-migrating subpopulation higher amounts of large particles with the size of HDL_{2b} and HDL_{2a} occur, while in the slow-migrating subpopulation slightly higher amount of particles appear with HDL_{3b} and HDL_{3c} size as compared to the fasting state (data not shown). These particles may represent large triglyceride-rich HDL particles (see panel E in Fig. 4) and newly secreted, small apoA-IV-rich HDL particles, respectively. (1, 50)

Interaction of HDL subpopulations separated by preparative free flow ITP with mouse peritoneal macrophages

We studied the interaction of the HDL subfractions separated by preparative ITP with mouse peritoneal macrophages. HDL binding, uptake, degradation, and HDL-mediated cholesterol efflux were initially monitored for all subfractions to elaborate optimal concentrations and time intervals. From these experiments it was evident that three major groups (I, II, III) could be formed and data for these subpopulations are summarized in Figs. 6–10.

Analysis of HDL binding at 4°C. The fast-migrating HDL subpopulation (I), as isolated from fasting HDL, revealed high affinity for the HDL binding sites (K_D = 7.71 μg/ml, B_{max} = 245.6 ng) on mouse peritoneal macrophages (**Fig. 6**). The affinity of this subfraction to HDL receptors increases under postprandial conditions (K_D = 4.6 μg/ml, B_{max} = 212 ng). The subpopulations with intermediate mobility (II) interact with HDL binding sites with a lower affinity (fasting K_D = 17.7 μg/ml, B_{max} = 198.4 ng, postprandial K_D = 15.9 μg/ml, B_{max} =195.6 ng) as com-

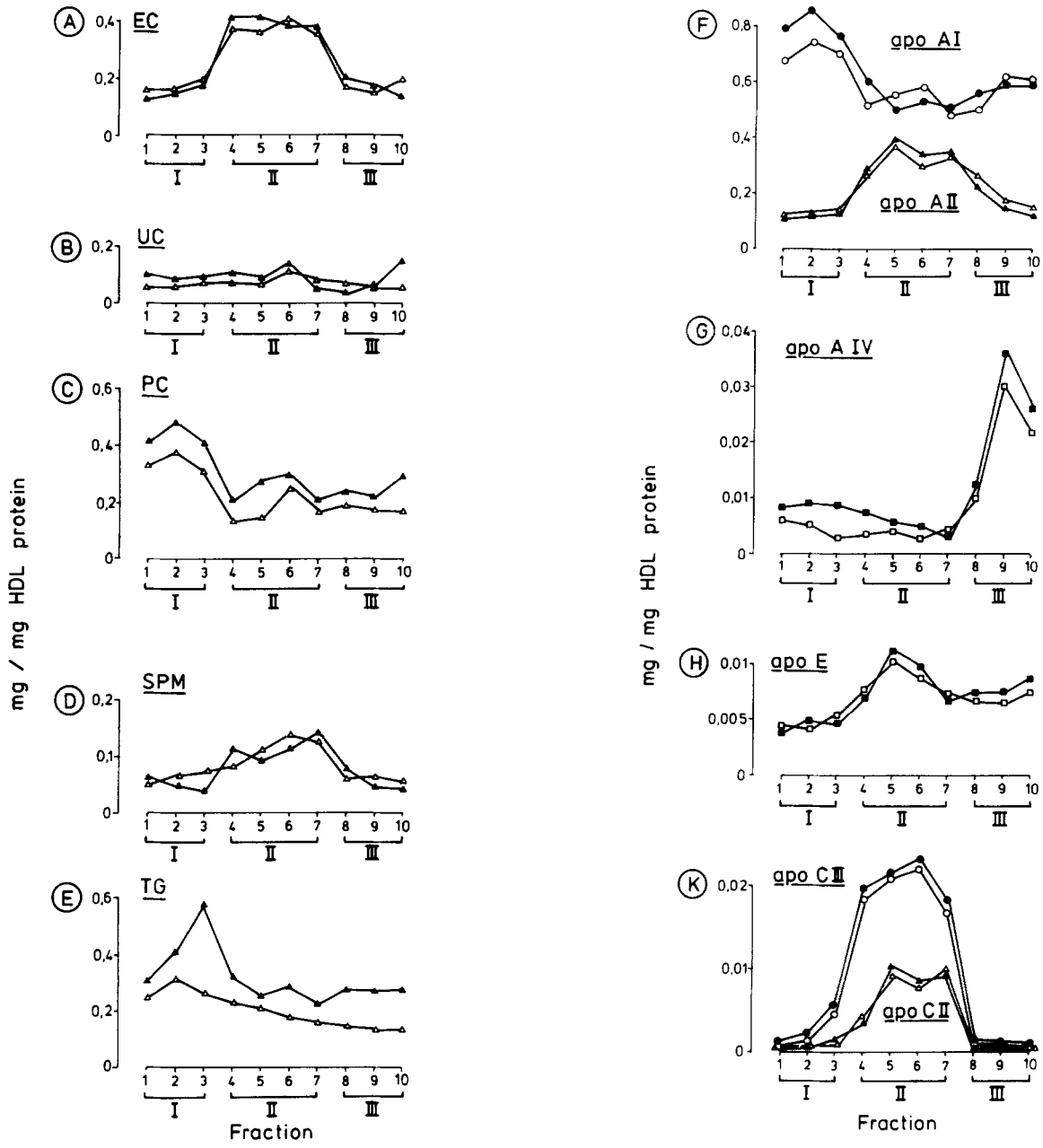

Fig. 4. Lipid and apolipoprotein composition of HDL subfractions separated by preparative ITP. HDL were isolated from fasting and 4-h postprandial plasma of normolipidemic volunteers (n = 5, TG = 91 ± 22 mg/dl, TC = 187 ± 18 mg/dl; HDL-C = 51 ± 5 mg/dl) by sequential ultracentrifugation and fractionated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

pared to the fast-migrating HDL subpopulation (I). The slow-migrating HDL subfraction (III) expresses the highest nonspecific binding component compared to fractions I and II, but also contains particles that interact with HDL binding sites with a high affinity (fasting K_D = 7.3 μg/ml, postprandial K_D = 7.0 μg/ml) but a very low B_{max} (fasting B_{max} = 95.9 ng, postprandial B_{max} = 87.9 ng).

Analysis of HDL uptake and degradation in mouse peritoneal macrophages at 37°C. **Fig. 7** shows specific 37°C cell-associated activity (○) and degradation (●) as related to either the

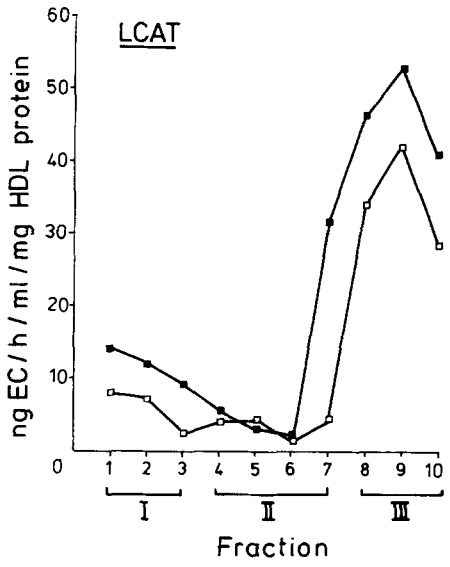

Fig. 5. LCAT activity distribution in HDL subfractions separated by preparative ITP. HDL subfractionation and LCAT activity determination were performed as described in Methods. The data represent the mean values from five experiments which differed less than 5%. Open symbols represent the data from fasting HDL, closed symbols from postprandial HDL.

concentration (panel A) or the incubation time (panel B) of the added HDL-subfraction. In these experiments the apoA-I-rich fraction I revealed the highest total cell-associated activity as compared to the other subfractions which were about 43% (fraction II) and 64% (fraction III) lower.

Degradation of HDL subfractions was very low in relation to total cell-associated activity and no significant differences in the degradation rate were observed between the subfractions that were examined. However, when the HDL subclasses were treated with neuraminidase, an enhanced uptake and degradation was observed only with the intermediate migrating apoC-III-containing subfraction II, while uptake and degradation of the fast- and slow-migrating subfractions did not change (data not shown).

In Figs. 8 and 9 additional data on trypsin-resistant and trypsin-releasable cell-associated radioactivity are presented. The trypsin treatment assay gives insights into the internalization process of HDL particles when trypsin-resistant activities after 4 °C binding and 4 °C binding plus 37 °C uptake are compared. The trypsin-resistant cell-associated activity is assumed to represent internalized ligand, while the trypsin-sensitive cell-associated activity should represent cell surface-associated ligand. When the 4 °C binding assay on mouse peritoneal macrophages was followed by trypsin incubation, about 20% of cell-associated activity was trypsin-resistant and no differences were observed between analyzed HDL subpopulations. This indicates that trypsin treatment does not fully release cell surface-associated ligands. However, when the cells were incubated for another 1 h at 37 °C after the 4 °C binding assay and then treated with trypsin, the apoA-I-rich fraction (I) revealed the highest total cell-associated activity compared to the subfractions II and III. Seventy-two % of the cell-associated fast-migrating fraction (I) was found to be trypsin-resistant, while 55% of the activity of the intermediate fraction (II) and 57% of the activity of the apoA-IV-rich fraction (III) were trypsin-resistant (**Fig. 8**). The relative distribution of radioactivity in gels after SDS-PAGE of solubilized cells after 4 °C and 37 °C binding experiments shows (**Fig. 9**) that the highest cell-associated activity was found in apoA-I bands. When the 4 °C binding was followed by trypsin treatment assay, there was still some activity in bands corresponding to apolipoproteins. The comparison of relative distributions of radioactivity in gels after SDS-PAGE of cells incubated at 37 °C with SDS-PAGE of cells incubated at 37 °C and treated with trypsin (Fig. 9) indicates that trypsin-resistant activity associated with apoA-I band in experiments with fraction I was significantly higher as compared to experiments with other fractions (II and III). This indicates that the apoA-I-rich

TABLE 2. Size distribution of particles from fast-(I), intermediate-(II), and slow-(III) migrating HDL

Gradient Gel Electrophoresis		Particle Distribution		
HDL Subpopulation	Particle Diameter	I Fast HDL	II Intermediate HDL	III Slow HDL
	nm		%	
HDL_{2b}	12.9-9.7	30 ± 7	21 ± 9	32 ± 8
HDL_{2a}	9.7-8.8	10 ± 4	29 ± 8	9 ± 3
HDL_{3a}	8.8-8.2	41 ± 8	25 ± 9	39 ± 7
HDL_{3b}	8.2-7.8	12 ± 3	22 ± 9	8 ± 2
HDL_{3c}	7.8-7.2	7 ± 3	3 ± 2	12 ± 4

HDL subpopulations (I-III) isolated by preparative isotachophoresis were analyzed by polyacrylamide gradient gel electrophoresis as described in Methods. The relative distribution of particles with different size as defined by gradient gel electrophoresis was calculated based on the densitometric scans of gels using a computer-assisted procedure. For details, see Materials and Methods.

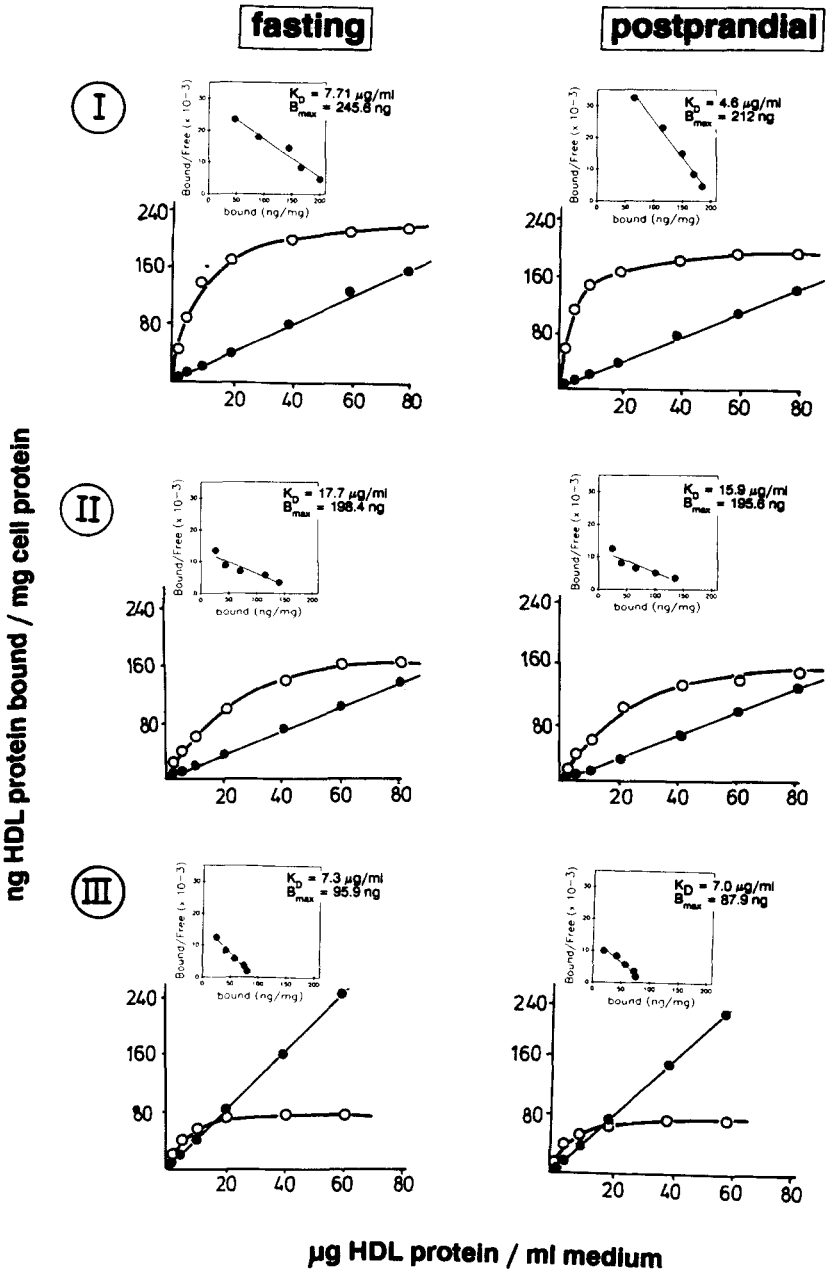

Fig. 6. Specific and nonspecific binding of HDL subfractions to cells from mouse peritoneal macrophages. Cells were preincubated with acetyl-LDL (50µg/ml, 18 h), washed with DMEM, cooled, and incubated with indicated concentrations of ^{125}I-labeled HDL subfractions for 1 h at 4°C in the presence or absence of 20-fold excess of nonlabeled subfractions. Assays were performed with fast-(I), intermediate-(II), and slow-(III) migrating HDL subpopulations separated by preparative ITP from fasting (left panel) and postprandial (right panel) samples as described in Methods. Specific binding was obtained by subtracting nonspecific binding from total binding. Nonspecific binding was also analyzed by linear regression analysis with the Ligand PC computer program. The K_D and B_{max} values were calculated by Scatchard analysis using the same computer program. Three individual experiments were performed and each point of analysis represents triplicate determinations. The results presented here represent mean values from three experiments which differed less than 8% for each individual value. Statistical comparisons of the K_D and the B_{max} values of the specific binding curves and the slopes of nonspecific binding between all three HDL subclasses analyzed were highly significant ($P < 0.001$).

subfraction (I) is taken up by the cells at a higher rate than are the other subfractions.

Analysis of HDL-mediated cholesterol efflux from mouse peritoneal macrophages. HDL-mediated cholesterol efflux was measured by mass analysis of cholesterol and release of [^{3}H]cholesterol from prelabeled cells. To measure the removal of cholesterol from cells, we performed long-time incubation experiments (**Fig. 10**). The fast-(I) and the slow-(III) migrating subpopulations were more effective in removing cholesteryl esters and free cholesterol from preloaded

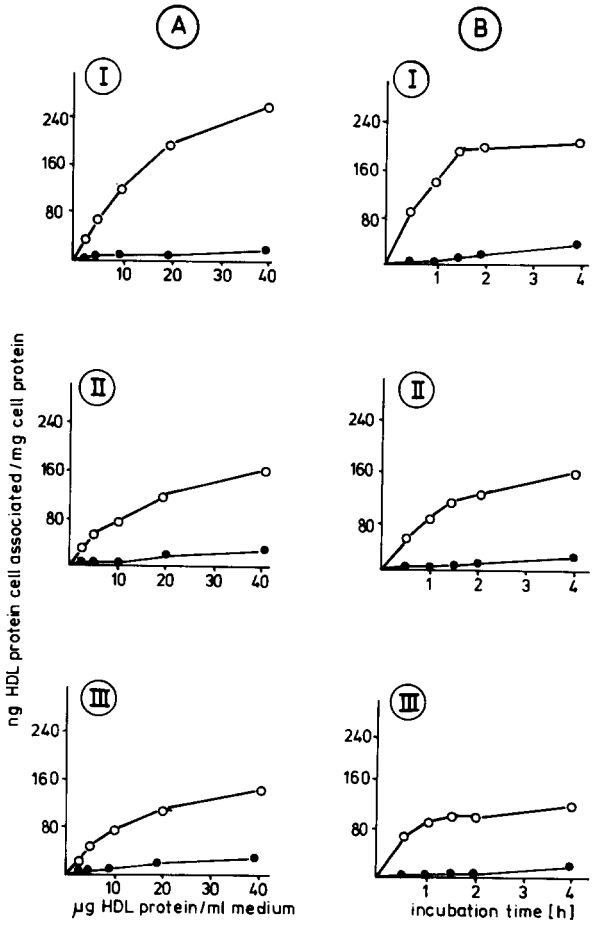

Fig. 7. Cell-associated activity at 37°C of ^{125}I-labeled HDL subfractions. Mouse peritoneal macrophages plated on 35-mm dishes were loaded with cholesterol by 18 h incubation with 50 μg/ml acetyl-LDL. Binding of ^{125}I-labeled HDL subfractions was measured at 37°C as described under Methods with the indicated concentrations of ^{125}I-HDL subfractions plus or minus a 20-fold excess of nonlabeled HDL for 1 h (panel A) or 20 μg/ml of iodine-labeled HDL subfractions plus or minus a 20-fold excess of nonlabeled HDL for the indicated times (panel B). After binding, the medium was collected for determination of lipoprotein degradation. After washing of the cells, specific 37°C binding was obtained by subtracting nonspecific binding from total binding. The cell-associated radioactivity is represented by open circles (○); lipoprotein degradation is represented by closed circles (●).

mouse peritoneal macrophages than subpopulations of intermediate mobility (II). At a concentration of 40 μg HDL protein/ml medium, they reduced cellular cholesteryl ester content to 60% and 58% and free cholesterol to 68% and 64%, while at a concentration of 80 μg HDL protein/ml medium they caused a reduction of cholesteryl esters to 51% and 47% and free cholesterol to 55% and 42%, respectively. The HDL subpopulation with intermediate mobility under the same conditions reduced cellular cholesteryl esters to 75% and 69% and free cholesterol to 81% and 79%, respectively. The fast-(I) and slow-(III) migrating subfractions separated from postprandial sera had a higher ability to promote cholesterol efflux than subfractions obtained from fasting samples and reduced cellular cholesteryl ester content to 56% and 50%, and free cholesterol to 50% and 41%, respectively, when added at a concentration of 40 μg HDL protein/ml medium. When added at a concentration of 80 μg HDL protein/ml medium they caused a cholesteryl ester reduction to 47% and 36%, and a loss of free cholesterol to 42% and 32%, respectively. The increase in the ability to promote cholesterol efflux under postprandial conditions is higher for the slow-(III) than for the fast-(I) migrating HDL subpopulation, while the affinity of the fast-migrating fraction to the HDL binding sites is significantly enhanced as compared to the fasting state.

The appearance of cholesterol in the culture medium measured as [^{3}H]cholesterol occurring in the medium during a short time incubation with HDL subfractions is shown in **Fig. 11**. It is obvious that the fast-(I) and slow-(III) migrating HDL subfractions are more effective in the promotion of cholesterol efflux than the subfraction of intermediate mobility (II). After 1 and 2 h of HDL exposure to the cells, the slow-migrating fraction (III) was more effective in cholesterol efflux promotion than the fast-migrating HDL (I). However, after 4 h of incubation no difference was observed between the fast-(I) and the slow-(III) migrating fractions, while the efflux induced by the intermediate fraction (II) was 50% lower.

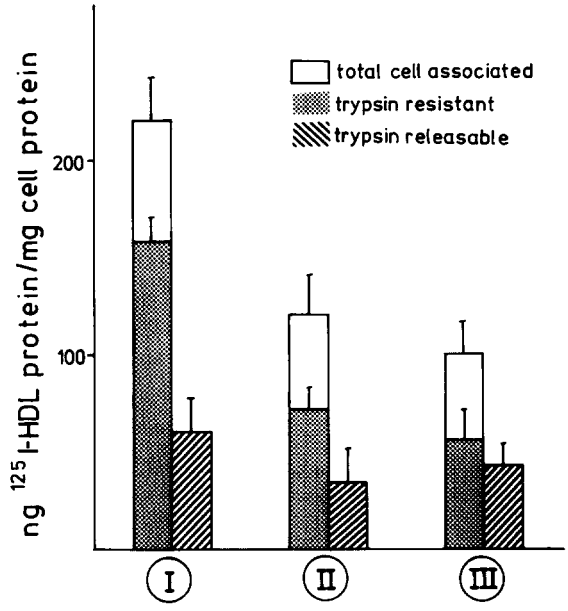

Fig. 8. Release of HDL from mouse peritoneal macrophages after 37°C incubation. Mouse peritoneal macrophages were preincubated for 18 h at 37°C with 50 μg/ml acetyl-LDL, washed, and incubated at 4°C for 1 h with 20 μg/ml of ^{125}I-labeled HDL subfractions, washed, and incubated at 37°C for 1 h in DMEM. After incubation, the standard wash procedure was performed and cell-associated activity was determined. To measure trypsin-sensitive and trypsin-resistant HDL binding, the cells were cooled to 4°C, washed, and incubated for 1 h at 4°C with PBS containing 1 mM EDTA and 4 mg/ml trypsin. Cell-associated and released (medium) activity were determined in an automated counting system (LKB).

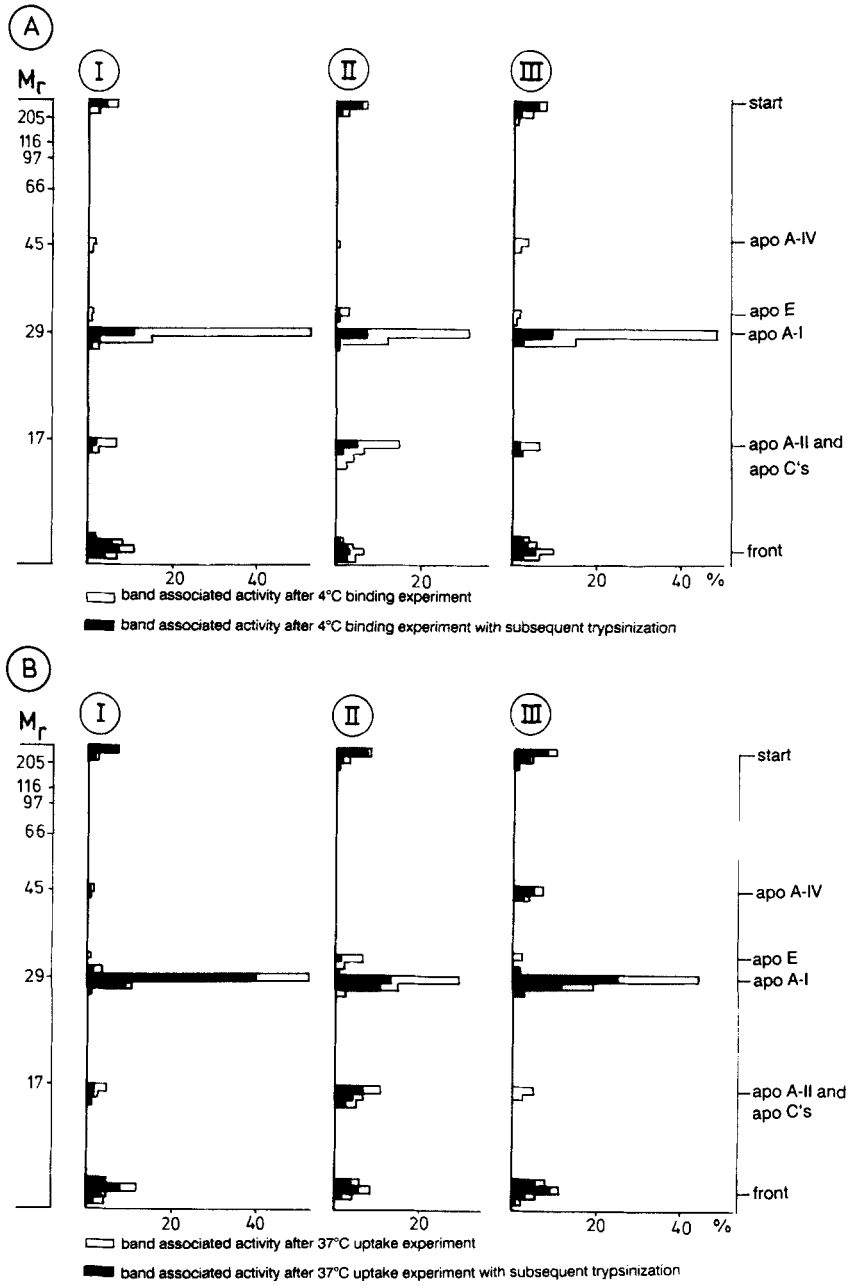

Fig. 9. Binding and internalization of ^{125}I-labeled HDL subpopulations separated by preparative ITP with mouse peritoneal macrophages. Cholesteryl ester-loaded mouse peritoneal macrophages were washed with DMEM, cooled, and incubated with 20 μg of the indicated ^{125}I-labeled HDL subfractions for 1 h at 4 °C. Cells were then washed and treated with trypsin at 4 °C (panel A) or incubated 1 h in DMEM at 37 °C and treated with trypsin (panel B) as described under Methods. Control cells were treated identically as the trypsin-treated cells except that the enzyme was omitted. Treated cells were solubilized in SDS buffer and subjected to SDS-PAGE. For separation, equal amounts of sample radioactivity were used. Apolipoprotein bands were identified by the addition of delipidated apoHDL and staining the gels with Coomassie Blue. Non-labeled apoHDL and molecular weight markers were applied to the gel as a standard. Then gels were cut into 2-mm pieces and radioactivity associated with each piece was measured. The background radioactivity was subtracted, total gel activity was taken as 100%, and relative distribution of radioactivity was calculated.

DISCUSSION

It is obvious from previous studies (15, 51) that both HDL_2 and HDL_3 contain particles rich in apoA-I with a high affinity for HDL receptor sites. Therefore, we decided to subfractionate HDL by isotachophoresis, since we expected that a charge-dependent separation of HDL particles might better correspond to their biological function than classical density separation by ultracentrifugation. HDL subclass separation by preparative ITP has been performed previously (52).

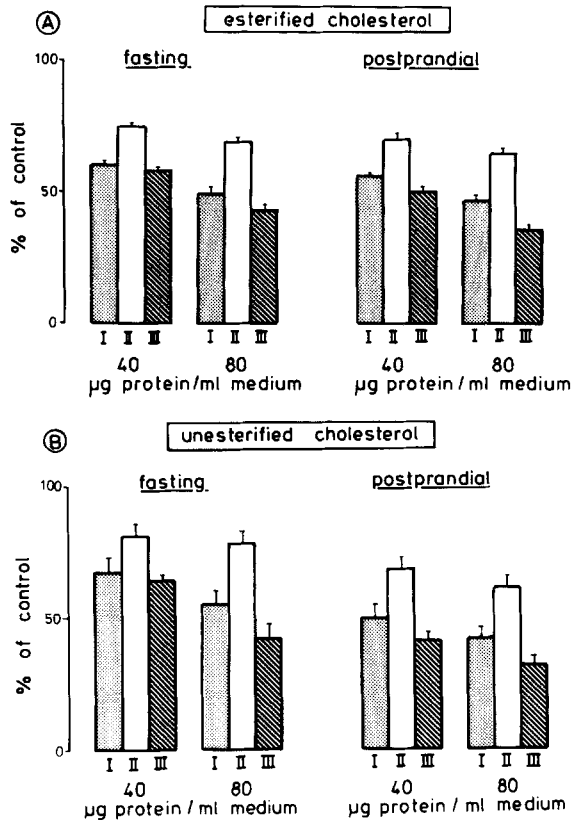

Fig. 10. Reduction of esterified (A) and unesterified cholesterol (B) content in mouse peritoneal macrophages by HDL subpopulations. The fast-(I), intermediate-(II), and slow-(III) migrating HDL subpopulations were separated by preparative ITP from fasting and postprandial HDL as described under Methods. Mouse peritoneal macrophages were preloaded with cholesterol by preincubation with acetyl-LDL (50 µg/ml, for 18 h) and incubated for 18 h with 40 and 80 µg protein/ml medium of the indicated HDL subfractions. At the end of the incubation period the cells were harvested in PBS and homogenized. The protein concentration was determined by Lowry method, the free cholesterol and cholesteryl ester contents were determined by HPTLC as described in Methods. The results are presented as % of cholesteryl ester or free cholesterol content of control cell samples containing cells preincubated with acetyl-LDL and medium without addition of HDL subfractions.

However, those data were obtained using a gel matrix that exerted molecular sieve effects and ampholytes that bound to the HDL particles and caused charge changes. In addition, no functional studies were done. The technique used here is carrier-free, contains no ampholytes, and can be performed using currently available instruments (32).

In the preparative ITP separation profile of HDL we recognized three major groups of particles that differed in their chemical composition and biological properties. The fast-migrating subpopulation (fraction I) is rich in apoA-I and phosphatidylcholine. The subpopulation with intermediate mobility contains the bulk of apoA-II, apoCs, apoE, and cholesteryl esters, while the slow-migrating subpopulation consists of particles enriched with apoA-I with which apoA-IV and LCAT activity are associated. The particle size analysis of our HDL subpopulations showed that, in the fast- and slow-migrating subpopulations, particles dominate with a size of HDL_{3a} and HDL_{2b} and, in addition, in the slow-migrating subpopulation there were higher amounts of particles with the size of HDL_{3a} as compared to the fast-migrating subpopulation. In the subpopulation with intermediate mobility, particles dominate with the size of HDL_{2a} to HDL_{3a}. Our particle size distributions found after preparative ITP are consistent with results obtained by other authors for HDL subpopulations separated by immunoaffinity chromatography (8, 10, 53-55). The size of particles found in the slow- and fast-migrating subpopulations corresponds to the size of particles that contain apoA-I without apoA-II. In our experiments a high LCAT activity was found in the slow-migrating fractions. This is in agreement with results obtained by Cheung et al. (55) who found that the majority of LCAT activity and also the majority of HDL-associated CETP (personal communication) was located in particles with diameter of 11.6 ± 0.4 nm (HDL_{2b}). Our slow-migrating subpopulation also contains the bulk of apoA-IV associated with HDL. This is consistent with results reported by James et al. (10) who found apoA-IV in an HDL subpopulation with apoA-I but without apoA-II and with results obtained by Bisgaier et al. (50) who found that apoA-IV is localized in small HDL particles (7.8-8.0 nm).

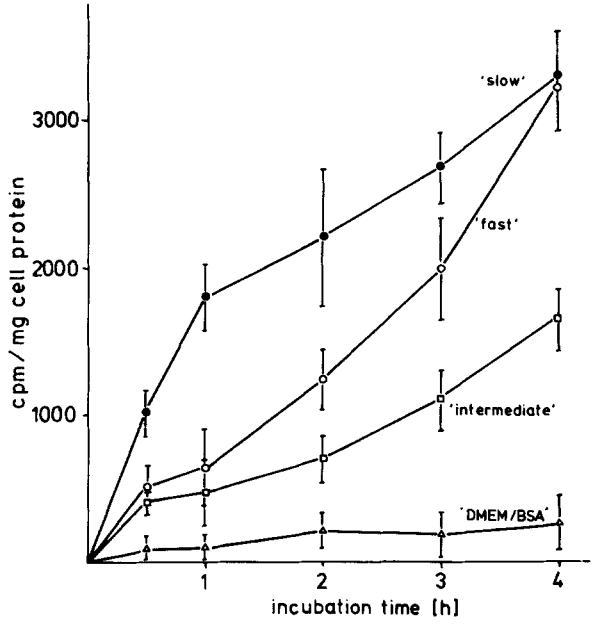

Fig. 11. HDL subfraction-promoted cholesterol efflux from mouse peritoneal macrophages. The cells were preincubated for 18 h DMEM containing 50 µg/ml [^{3}H]cholesteryl oleate-labeled acetyl-LDL. The loaded cells were washed and incubated for 1 h at 37°C with culture medium and then for the indicated times with 20 µg/ml of HDL subfractions. At the end of the incubation period the medium was removed and aliquots were taken to measure total medium ^{3}H activity and for thin-layer chromatography to separate between cholesterol and cholesteryl esters. The open circles represent the fast-migrating HDL (I), the open squares the intermediate-migrating HDL (II), the closed circles the slow-migrating HDL (III), and the open triangles the blank.

As obvious from the binding analyses, the fast-migrating subfraction (I) expresses a high affinity to HDL binding sites (K_D = 7.71 μg/ml; B_{max} = 245.6 ng), the fraction with intermediate mobility (II) interacts with HDL binding sites with lower affinity (K_D = 17.7 μg/ml, B_{max} = 198.4 ng), and the slow-migrating fraction (III) expressed a very high nonspecific binding and contained a small amount of particles that interacted with HDL binding sites with high affinity (K_D = 7.3 μg/ml; B_{max} = 95.9 ng). In all experiments the particles were added to the culture medium on the basis of their total protein content. The differences in the binding properties might be a result of the different apoA-I content in isolated subfractions, since apoA-I is apparently the major ligand of the HDL receptor (13-20). Our observation that the subfraction (I) isolated from postprandial serum had a higher apoA-I content and expressed a higher proportion of specific binding underlines this hypothesis. It might be possible that apoA-IV is involved in specific interactions of the subfraction III (56-58), but we did not observe higher binding properties of the subfraction III isolated from postprandial serum where higher apoA-IV contents appeared. However, total apolipoprotein composition as well as lipid composition of the subfraction could influence the binding properties. The nonspecific interactions could be caused by a defined lipid and apolipoprotein composition of the particles as suggested by Phillips, Johnson, and Rothblat (59) and apoA-IV might be involved in this process. Further investigation is needed to understand the origin of the observed differences in the binding properties of the individual HDL subfractions in more detail.

Since there is evidence that HDL particles are taken up by cells such as rat adrenocortical cells (60), pig hepatocytes (61), rat sinusoidal liver cells (62, 63), human HepG2 cells (64), rat peritoneal macrophages (65), and human monocyte-derived macrophages (66), we performed additional experiments with mouse peritoneal macrophages to measure 37°C trypsin-resistant cell-associated activity of isolated HDL subfractions. Using the trypsin treatment assay, we can estimate the internalization of HDL particles. Thus, while trypsin-resistant cell-associated activity is assumed to represent the internalized ligands, the trypsin-sensitive cell-associated radioactivity is assumed to represent cell surface-associated ligands. A difference between trypsin-resistant activity measured after 4°C and 37°C binding experiments indicates that significant internalization occurs. In our experiments, when binding performed at 4°C was followed by trypsin treatment, about 20% of cell-associated activity was trypsin-resistant and no differences were observed between the analyzed subpopulations. When the cells were incubated at 37°C with identical amounts of HDL protein and treated with trypsin, significant differences in trypsin-resistant activity occurred between the analyzed HDL subpopulations. We observed a higher cell-associated activity for subfraction I compared to the other subfractions. This indicates a higher uptake of the fast-migrating subpopulations. Previous studies from our laboratory, in which gold-labeled and RITC-labeled HDL were used (15, 16), and results published by Takata et al. (63), Takahashi et al. (65), and Alam et al. (66) provide evidence that internalization of HDL particles occur. Our current data are consistent with these results and suggest that there exist HDL subpopulations that are internalized. However, Oram, Johnson, and Brown (42) reported that HDL are not internalized despite the fact that they also found a high trypsin-resistant cell-associated activity. Their experiments were performed with HDL_3 instead of specific apoA-I-rich HDL subpopulations and the observed lack of internalization could be due to the low sensitivity of the trypsin treatment method when ultracentrifugally isolated HDL subfractions are used. Further investigation with other methods is needed to clarify the observed differences.

In addition to binding and uptake properties, we monitored for differences between the isolated subfractions in promoting cholesterol release from cells. The fast-migrating HDL subpopulation (I) rich in apoA-I and the slow-migrating subfraction (III) rich in apoA-IV seem to be good promoters for cholesterol removal. The differences in the time course of cholesterol efflux between fraction I and III within the first 2 h suggest that these particle populations differ in their mode of cellular interaction (Fig. 10). Based on previous studies from our laboratory (15, 16), results reported by other authors (63, 65), and experiments reported in this paper that have shown that the fast-migrating subpopulation (I) is taken up by cells at a higher rate than the slow-migrating subpopulation (III), it can be hypothesized that fast apoA-I-rich particles are internalized, enriched in intracellular cholesterol, and resecreted, while the apoA-IV-/LCAT-rich particles (III) can directly promote cholesterol efflux from the cell surface by the action of LCAT and may stimulate cholesterol translocation from intracellular pools to the plasma membrane. This might be an explanation for the higher cholesterol efflux within the first 1-2 h (Fig. 10) caused by the slow-migrating HDL subpopulation. In addition, our recent observation that HDL deficiency with planar xanthomas (67) is caused by a selective defect in apoA-I synthesis and the inability of the macrophages in these patients to secrete cytoplasmic cholesteryl esters in the absence of apoA-I underlines our hypothesis that apoA-I-containing HDL particles acting via HDL-receptors effectively promote cholesterol release from cytoplasmic lipid droplets (21, 68). However, there are still sufficient conflicting results concerning HDL internalization and it cannot be excluded that fraction I particles are involved in receptor-mediated stimulation of cholesterol translocation from intracellular sites to the plasma membrane as suggested by other authors (69). Further investigation is needed to understand the observed differences.

The capacity of the slow-migrating subfraction (III) to enhance cholesterol release from cells is even higher under postprandial conditions. Enhanced concentrations of apoA-IV and apoE are also observed postprandially. In previous

studies from other laboratories, an increase in the plasma apoA-IV concentration was recognized after feeding of a high cholesterol diet (70) and synthetic apoA-VI- and apoE-containing particles were found more effective in cholesterol removal from cells than apoA-I-containing particles (22).

It was reported that there exists an apoA-I-rich HDL subpopulation with pre-β mobility (71–73) with which LCAT and CETP activities are associated and which plays a major role in the transport, esterification, and transfer of cell-derived cholesterol. On the basis of those results from other laboratories and our current data, we suggest that the pre-β-migrating HDL subpopulation could resemble our slow-migrating HDL subpopulation which contains apoA-I, and the majority of the HDL-associated LCAT activity, and is a good acceptor for cell-derived cholesterol. In addition, in a collaborating study with Dr. Dieplinger (Innsbruck, Austria) we have found the majority of HDL-associated CETP with the slow-migrating particles. However, in our slow-migrating subfractions the majority of HDL-associated apoA-IV is found while no apoA-IV was found in pre-β-migrating HDL. Further studies are needed to clarify the latter discrepancy with pre-β-migrating HDL.

The intermediate-migrating particles (II) rich in apoA-II, apoE, C-apolipoproteins, cholesteryl esters, and sphingomyelin do not seem to contain potent promoters for cholesterol efflux but may be rather responsible for cholesterol delivery. The high content of glycosylated apoC-III and the enhanced uptake and degradation of these particles upon neuraminidase treatment further support this idea. It is consistent with results obtained by Barbaras et al. (17) who showed that apoA-I but not apoA-I/A-II-containing particles are good promoters for cholesterol efflux.

Based on the results presented here and on data already published (21, 63, 65, 68) we hypothesize that two major mechanisms exist, a receptor-mediated transport and a physicochemical transfer for the release of cellular cholesterol and that different HDL subfractions are involved in this process. The removal of cellular cholesterol by fast-migrating HDL particles (I) may be predominantly caused by high affinity binding to HDL receptor sites, subsequent internalization, and resecretion (15, 65). The fast-migrating subpopulation (I) may contain HDL precursor particles that are transformed to mature HDL enriched in cholesteryl esters and apoE. The cholesterol release by slow-migrating particles may be predominantly mediated by nonspecific interactions via physicochemical transfer.The nonspecific interaction could be mediated by apoA-IV-rich particles significantly associated with LCAT activity. This type of cellular release of cholesterol may be associated with the lysosomal route (21, 68). However, we cannot claim that each subfraction strictly participates in only one of these mechanisms. Particles occurring in the subfraction I may also remove cholesterol to some extent by a nonspecific interaction mediated by their lipid composition, and particles in the subfraction (III) may be in part involved in the specific interaction mediated by apoA-I.

Numerous methods have been used for the separation and analysis of plasma lipoproteins and their subclasses. Here we have presented preparative and analytical capillary isotachophoresis as a reproducible and specific method that can be used for lipoprotein fractionation directly from whole plasma and serum. The analytical capillary isotachophoretic pattern of normal whole serum/plasma lipoproteins consists of 14 subfractions. Six of them belong to high density lipoproteins, and the others represent the apoB-containing lipoproteins. The peaks separated in the HDL mobility range, as shown in the present paper, correspond to particles of different lipid and apolipoprotein composition and different biological functions. Moreover, the data for the lymph lipoproteins show that the apoE-/A-II-/C-III-rich HDL fractions migrating in peak 3 are almost absent in lymph lipoproteins. Therefore, it is likely that these particles originate from the liver, while the apoA-I-rich HDL creating peaks 1 + 2 and the apoA-IV-rich HDL migrating in peaks 4 + 5 are major lymph HDL particles that orginate from intestinal mucosa cells.

The efficacy of the technique presented here for lipoprotein separation awaits further confirmation by analysis of plasma or serum from patients. We think that, based on the characterization of HDL subpopulations separated by isotachophoresis, analytical capillary ITP may be a helpful tool for the analysis of HDL subfractions with different functional properties directly in patient samples.

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