Protein heterogeneity of lipoprotein particles containing apolipoprotein A-I without apolipoprotein A-II and apolipoprotein A-I with apolipoprotein A-II isolated from human plasma

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Abstract The protein heterogeneity of fractions isolated by immunoaffinity chromatography on anti-apolipoprotein A-I and anti-apolipoprotein A-II affinity columns was analyzed by high resolution two-dimensional gel electrophoresis. The two-dimensional gel electrophoresis profiles of the fractions were analyzed and automatically compared by the computer system MELANIE. Fractions containing apolipoproteins A-I + A-II and only A-I as the major protein components have been isolated from plasma and from high density lipoproteins prepared by ultracentrifugation. Similarities between the profiles of the fractions, as indicated by two-dimensional gel electrophoresis, suggested that those derived from plasma were equivalent to those from high density lipoproteins (HDL), which are particulate in nature. The established apolipoproteins (A-I, A-II, A-IV, C, D, and E) were visible and enriched in fractions from both plasma and HDL. However, plasma-derived fractions showed a much greater degree of protein heterogeneity due largely to enrichment in bands corresponding to six additional proteins. They were present in trace amounts in fractions isolated from HDL and certain of the proteins were visible in two-dimensional gel electrophoresis profiles of the plasma. These proteins are considered to be specifically associated with the immunoaffinity-isolated particles. They have been characterized in terms of M_r and pI. Computer-assisted measurements of protein spot-staining intensities suggest an asymmetric distribution of the proteins (as well as the established apolipoproteins), with four showing greater prominence in particles containing apolipoprotein A-I but no apolipoprotein A-II. —**James, R.W., D. Hochstrasser, J-D. Tissot, M. Funk, R. Appel, F. Barja, C. Pellegrini, A.F. Muller, and D. Pometta.** Protein heterogeneity of lipoprotein particles containing apolipoprotein A-I without apolipoprotein A-II and apolipoprotein A-I with apolipoprotein A-II isolated from human plasma. *J. Lipid Res.* 1988. **29:** 1557–1571.

Supplementary key words apoA-I • apoA-II • high density lipoproteins • two-dimensional electrophoresis • affinity chromatography • immunoblots

Investigations of the apolipoprotein (apo) components of lipoproteins have greatly enhanced the knowledge of lipid metabolism over the last decade. The studies have emphasized the enormous influence that apolipoproteins exert over lipoprotein metabolism; they provide the structural basis for the assembly of lipoproteins (1–3), modulate the activities of enzymes involved in lipid metabolism (4, 5), and mediate lipoprotein uptake by providing binding sites specifically recognized by cell surface receptors (6–9). In addition, their potential as cardiovascular disease risk factors has been evoked (10–12).

Ultracentrifugation has been an invaluable tool for subfractionation of lipoprotein particles with a view to investigating lipid metabolism. It has been the basis for the vast majority of studies in the area. However, an acknowledged problem of this approach is that the prolonged centrifugation times and high salt concentrations can lead to modification of lipoprotein structure with redistribution or even loss of lipoprotein-associated proteins (13–15). A second limitation of ultracentrifugation has become evident in recent years, concordant with growing appreciation of the influence of apolipoproteins on lipid metabolism and studies underlining the metabolic heterogeneity of the classical lipoprotein subfractions VLDL (very low density lipoproteins), LDL (low

Abbreviations: apo, apolipoprotein; VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; 2DGE, two-dimensional polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; CNBr, cyanogen bromide; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; LCAT, lecithin:cholesterol acyltransferase.

density lipoproteins), and HDL (high density lipoproteins) (16–20). It is logical to assume that such heterogeneity has its basis to a large extent in the apolipoprotein composition of the lipoproteins. Yet protein heterogeneity is largely ignored by ultracentrifugal separation where the lipid component of lipoproteins is the dominant factor.

Immunoaffinity chromatography offers an elegant approach to lipoprotein fractionation by using monospecific antibodies to target individual apolipoproteins, thus allowing lipoprotein particle isolation based on pre-selected criteria of apolipoprotein composition. Recent innovations in the preparation of apolipoprotein-specific antibodies (21) and studies using apoA-I, A-II, B, and E immunoaffinity columns provide encouraging support for the validity of the methodology (15, 21, 22–25).

In this report we have used immunoaffinity chromatography and two-dimensional electrophoresis (2DGE) allied to computerized analysis of the gels to investigate the protein heterogeneity of lipoprotein particles isolated according to their content of A-II and/or apoA-I as the major apolipoproteins. In addition to the established apolipoproteins, we have identified and partially characterized six additional proteins that we consider to be HDL-associated. The newly identified proteins are distinct from established apolipoproteins by M_r, pI, and immunoblot criteria. There appears to be an asymmetric distribution of established and newly identified proteins between the two types of particles, i.e., particles containing apoA-I plus A-II (AIwAII) and particles containing apoA-I but no A-II (AIw/oAII). Furthermore, the results emphasize the simplification of particle composition that occurs during ultracentrifugational manipulation of lipoproteins.

MATERIALS AND METHODS

Preparation and purification of monospecific antibodies

ApoA-I and A-II were isolated from delipidated HDL by gel filtration chromatography (26). Purity was assured by repeated gel filtration until only single bands were visible on polyacrylamide gels (SDS-PAGE). The purified apolipoproteins were used to raise antisera in rabbits by conventional immunization procedures. An IgG-enriched fraction was separated from antisera by ammonium sulfate precipitation (45% saturation), redissolved in phosphate-buffered saline (PBS:NaCl, 0.14 M; KCl, 2.7 mM; phosphate buffer, 10 mM, pH 7.4) and stored at −70°C. Monospecific antibodies were isolated by affinity chromatography. Affinity gels were prepared by covalently coupling either purified apoA-I or apoA-II to CNBr-activated Sepharose 4B (8 mg/ml gel) according to the manufacturer's instructions (Pharmacia). The IgG fraction was passed through the relevant column (5 ml/hr; 4°C) which was subsequently thoroughly washed (PBS containing 0.5 M NaCl) to remove nonspecifically bound protein. Specifically bound antibody was eluted with acetic acid (0.1 M, pH 3.0) into Tris (1.0 M, pH 7.4) then dialyzed against PBS containing sodium azide (0.02% w/v). As a final step to eliminate any cross-contamination, antibodies purified on the apoA-I column were passed through the apoA-II column and anti-apoA-II antibodies were chromatographed on the apoA-I affinity column. In both cases the nonbound fractions were retained.

Immunoaffinity chromatography of lipoproteins

Affinity columns containing monospecific antibodies (4 ml of gel; 7.5 mg antibody/ml gel) either to apoA-I or apoA-II were prepared according to the manufacturer's recommendations (Pharmacia). Lipoproteins containing either apoA-I plus A-II or A-I without A-II were then isolated from HDL (0.5 mg) or plasma (0.25 ml) in the following way.

All manipulations were performed at 4°C. The sample was passed onto the anti-apoA-II column and washed through with PBS (5 ml). The eluant was collected and repassed twice through the column. It was then transferred to the anti-apoA-I column and the operation was repeated (three passages through the column). Preliminary studies had established that these conditions were adequate for complete adsorption of apoA-II- and/or apoA-I-containing lipoproteins. Both columns were washed with PBS containing 0.5 M NaCl (at least 10 column volumes) until no protein could be detected in the eluant (absorption at 280 nm).

Elution of specifically bound lipoprotein particles was effected with thiocyanate (3 M; 10 ml/column) The eluants were rapidly transferred for dialysis either against PBS containing sodium azide (0.02% w/v) or against distilled water for subsequent lyophilization. Under these conditions the bound lipoproteins were in contact with the elution buffer for less than 30 min before dialysis. In this study the fraction isolated on the anti-apoA-II column has been termed Lp(AIwAII) and that purified on the anti-apoA-I column Lp(AIw/oAII). These are prefixed with the source of the sample, i.e., either plasma or HDL.

Preparation of HDL

Blood samples were obtained from fasting male subjects and centrifuged to remove blood cells. VLDL and LDL were removed by adjusting the density to 1.063 g/ml and ultracentrifuging (22 hr, 40,000 rpm, 16°C; Kontron 45.6 rotor). Total HDL (d 1.063–1.21 g/ml), HDL_2 (d 1.063–1.125 g/ml), and HDL_3 (d 1.125–1.21 g/ml) were isolated by centrifuging at 40,000 rpm for 48 hr. They were than dialyzed against PBS containing EDTA (1 mM) and azide (0.02% w/v) and stored at 4°C after sterile filtration.

Electrophoretic analysis

Single dimension. Slab gel SDS-PAGE was carried out according to Laemmli (27), with a running gel of acrylamide (12.5% w/v) and bis (0.33% w/v) and a stacking gel of acrylamide (3.75% w/v) and bis (0.1% w/v). Lyophilyzed samples were redissolved in Tris (0.125 M, pH 6.8) containing SDS (4% w/v), urea (5 M) and β-mercaptoethanol (10% v/v). Electrophoresis was performed for approximately 5 hr at a constant current of 25 mA/slab gel (18 × 16 × 0.15 cm). The gel was fixed in trichloracetic acid (10% w/v) containing sulfosalicylic acid (3.5% w/v), washed thoroughly in methanol–acetic acid–water 3:1:6 and stained in Coomassie G250 (0.14% w/v, in methanol–acetic acid–water, 5:1:4) or with the silver stain technique (28).

Two-dimensional. 2DGE analyses of samples were performed as described previously (29), except for the use of predominantly carrier ampholyte pH gradients. The first dimension was performed in capillary gels (16.5 × 0.15 cm) of acrylamide (6% w/v), bis (0.13% w/v), urea (9.2 M), CHAPS (1.7% w/v), Nonidet (0.6% v/v), Ampholines LKB pH 5–7 (0.4% v/v), pH 3.5–10 (0.9% v/v) and Servalyte (Serva) pH 3–10 (0.9% v/v). The second dimension was adapted from that of Tracy and Young (30). Electrophoresis was performed in slab gels (17 × 23 × 0.15 cm) of acrylamide (13% w/v) and bis (0.35% w/v) in Tris (1.5 M, pH 8.8).

Samples were dissolved by heating (95°C, 5 min) in SDS (1.0% w/v), dithioerythritol (0.23%, w/v) then diluted into sample buffer (urea 9 M; Ampholines LKB pH 3.5–10, 2% v/v; CHAPS 3% w/v; dithioerythritol 1% w/v) and isoelectrofocused (700 V, constant voltage overnight, then 3000 V for 2 hr). The gel was removed from the capillary tubing, positioned on top of the slab gel, and covered with equilibration buffer (200 μl: Tris, 60 mM, pH 6.8; SDS, 2.5% w/v; bromophenol blue, 0.01% w/v) then agarose (0.8% w/v in Tris, 25 mM–glycine, 0.19 M, containing SDS (0.1% w/v)) to hold the capillary gel in place. Electrophoresis was continued for approximately 4.5 hr at a constant 0.04 A/gel. Gels were fixed and silver-stained as described previously (28) with the modifications proposed by Wiederkehr, Ogilvie, and Vonderschmitt (31). Particular attention was paid to step 5 (28) of the staining procedure (3 min exposure) to ensure a reproducible staining intensity.

Electron microscopic analysis of samples

Samples were prepared for electron microscopy in the following way (32). They were dialyzed against buffer (pH 7.4) containing ammonium acetate (0.125 M), ammonium carbonate (2.6 mM), and EDTA (0.26 mM). Copper grids (300 mesh) were covered with a thin Formvar film prior to carbon-coating in a vacuum evaporater (Siemens). Equal volumes of sodium phosphotungstate (2% w/v, pH 7.4) and sample were mixed in a conical tube and a small aliquot (10 μl) was applied to the grid. After 30–60 sec, excess fluid was absorbed with filter paper, the grid was air-dried and immediately examined in the electron microscope (Philips 400). Particle diameter was determined by morphometric analysis (33) of electron micrographs in which the values were computed after electronically tracing the circumferences of the particles.

Immunoblot analyses of 2DGE gels

Western blotting analyses (34) were performed on immunoaffinity isolated particles fractionated by 2DGE as described above. Briefly, immediately after electrophoresis, the 2DGE profiles of particles were electrophoretically transferred to nitrocellulose sheets (Bio-Rad Transblot Cell Unit; 3 hr at constant voltage (70 V) in a buffer of Tris, 25 mM; glycine, 192 mM, pH 8.3). Excess absorption sites were masked by overnight incubation (room temperature, gentle agitation) with blocking buffer [3% (w/v) solution of powdered milk in wash buffer (Tris, 10 mM, pH 7.4, containing NaCl, 150 mM)]. The nitrocellulose sheets were subsequently incubated with the first antibody (in blocking buffer) by gentle overnight agitation at room temperature, then rinsed (4 × 15 min) with wash buffer. When a second antibody was used, the above procedure was repeated. Finally the sheets were dried by gently pressing between filter papers. Radioactively labeled antibodies were visualized by exposure (−70°C) to X-ray film (Kodak Ortho G film). For peroxidase-labeled antibodies, the nitrocellulose sheets were rinsed in wash buffer (pH 6.8) then incubated with freshly prepared substrate solution (0.3% w/v 4-chloro-1-naphthol in wash buffer (pH 6.8) containing 20% (v/v) methanol and 0.01% (v/v) hydrogen peroxide). The reaction was stopped with distilled water.

ApoA-I, A-II, and E were detected with ^{125}I-labeled monoclonal antibodies raised in our laboratory. Polyclonal antibodies to apoA-IV, B, C-III, D, and H, and LCAT and cholesteryl ester transfer protein were revealed with the appropriate, ^{125}I-labeled second antibody. Monoclonal IgM antibodies against LCAT were revealed with peroxidase-labeled rabbit anti-mouse IgM (Sigma).

Lipid and protein analyses

Lipids were measured by automated enzymatic procedures, as described previously (35). Total protein was measured by the method of Lowry et al. (36) using BSA (Sigma) as standard. Apolipoproteins were quantified by electroimmunodiffusion (37). To test for cross-contamination of anti-apoA-I and apoA-II antibodies, an ELISA procedure was used (38). Wells of microtitre plates were coated with purified apoA-I or apoA-II and incubated with affinity-purified antibodies. Bound antibody was revealed with goat anti-rabbit IgG antibody coupled to alkaline phosphatase (Sigma).

Radiolabeling of HDL

HDL and antibodies were radiolabeled by the iodine monochloride technique (39) and separated from free ^{125}I by gel filtration (Pharmacia PD 10 columns). Greater than 95% of the radioactivity was protein-associated, as evidenced by precipitation with trichloracetic acid (10% w/v).

Computer analysis of two-dimensional gels

Stained gels were scanned by a high precision CCD scanner, 1024 × 1024 pixels with 4096 grey levels. Accumulated data were analyzed by the MELANIE program (40). The program permits: *i*) protein bands on different gels to be matched; *ii*) relative and absolute staining intensities of individual protein bands to be measured; and *iii*) molecular weights and isoelectric points to be attributed to individual proteins. The latter were achieved by constructing calibration curves based on the known values for plasma proteins as given by Anderson, Tracy, and Anderson (41).

RESULTS

Antibody specificity

The monospecificity of the affinity-purified antibodies was confirmed by Ouchterlony immunodiffusion and the more sensitive ELISA procedure. In neither analysis did purified anti-apoA-I or anti-apoA-II antibodies react with apoA-II or apoA-I, respectively, and there was no reactivity towards other apolipoproteins or albumin.

Validation of the immunoaffinity procedures

A second series of control experiments was undertaken to confirm the validity and specificity of the immunoaffinity procedure. The first batch of experiments complemented the studies described above. An IgG column was prepared by covalently coupling neutral rabbit IgG to Sepharose 4B. Human plasma was processed on the column as described in Methods. After passage of the eluting buffer, the whole eluant was dialyzed, lyophilized, and analyzed by SDS-PAGE. Using sensitive silver staining we could find no evidence of nonspecific binding of lipoproteins or plasma proteins. In a series of analogous studies, plasma previously depleted of apoA-I- and A-II-containing lipoproteins was repassed through the anti-apoA-I and anti-apoA-II immunoaffinity columns. Analysis of the eluants as described above was unable to show retention of plasma proteins. In the final set of experiments in this section, A-I particles devoid of A-II (i.e., purified on the anti-apoA-I column after prior passage through the anti-apoA-II column) were processed on the apoA-II column. No bound apoA-I could be detected by SDS-PAGE analysis of the thiocyanate eluant.

The second series of studies analyzed the integrity of particles isolated by the immunoaffinity procedure. HDL separated by ultracentrifugation was radiolabeled and fractionated on the immunoaffinity columns to give ^{125}I-labeled HDL-Lp(AIw/oAII) and ^{125}I-labeled HDL-Lp(AIwAII). ^{125}I-Labeled Lp(AIwAII) particles were then investigated for binding to both immunoaffinity columns. The anti-apoA-II and apoA-I columns retained over 96% and 98% of the radioactivity, respectively.

In a second approach, fractions isolated from HDL by immunoaffinity chromatography were investigated by electron microscopy. The results are shown in **Fig. 1.** The average particle diameter of HDL was 8.58 ± 0.23 nm (SEM; n=120) with a range of 5.1–16.0 nm. The HDL-Lp(AIw/oAII) particles averaged 9.98 ± 0.16 nm (SEM; n=149) with a range of 7.9–15.3 nm, compared to 7.65 ± 0.17 nm (SEM; n=188; range 5.1–11.6 nm) for the HDL-Lp(AIwAII) particles. A more detailed examination of the electron micrographs showed that for HDL-

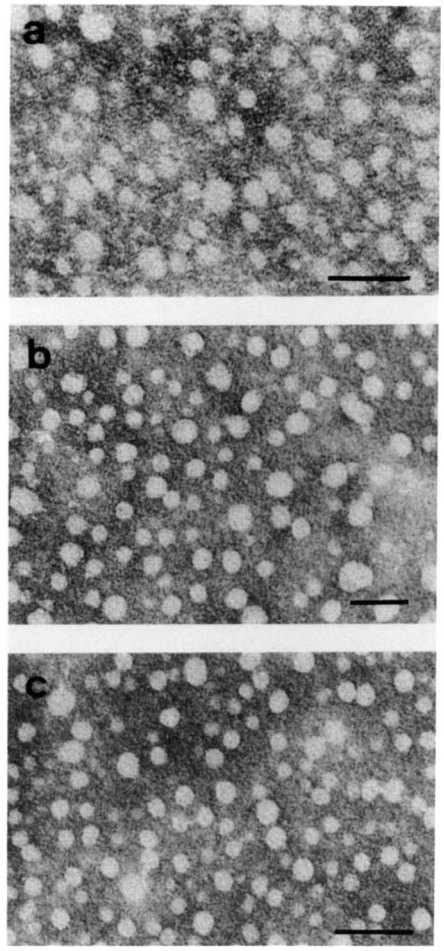

Fig. 1. Electron micrographs of a) HDL; b) HDL-Lp(AIw/oAII) particles; c) HDL-Lp(AIwAII) particles. The particles were isolated from the HDL of a normolipemic male subject by immunoaffinity chromatography. Bars represent 25 nm.

Lp(AIw/oAII), particles with diameters of 9.8 nm and 11.8 nm predominated, while almost 50% of particles HDL-Lp(AIwAII) were found to have diameters of 6.3 nm or 7.3 nm.

Unidimensional gel analysis of immunoaffinity isolated particles

Small-scale, analytical columns capable of processing 0.5 mg of HDL or 0.25 ml of plasma were used for the duration of the study. They were re-used over 100 times (with an occasional thorough wash of 0.1 M glycine, pH 2.8) without appreciable diminution of their binding capacities.

SDS-PAGE analysis of fractions (50 μg protein), from a normolipemic male subject, isolated by passage through anti-apoA-II then anti-apoA-I affinity columns is shown in **Fig 2A.** The anti-apoA-II column gave subfractions from plasma (plasma-Lp(AIwAII), lane 1) and HDL (HDL-Lp(AIwAII), lane 2) with a major band at a molecular weight of 27,000 and a minor band at 8,500 (Fig. 2A; lane 2). The subfraction from the anti-apoA-I column, of which plasma-Lp(AIw/oAII) is given as an example (lane 3), displayed a major band at 27,000; the minor band was absent. Immunoblots with monospecific antibodies (results not shown) confirmed the major band to be apoA-I and the minor band to be apoA-II. Coomassie blue staining also showed the presence of numerous faintly stained bands, notably in the particles eluted from the anti-apoA-I column. This was confirmed by the more sensitive silver stain (Fig. 2B; lanes 2 and 3), which brought to light a series of protein bands in both subfractions. However, even with the sensitive silver stain, no apoA-II could be detected in the eluant from the anti-apoA-I column.

Qualitatively equivalent results were obtained by repetitive analyses of HDL from six normolipemic male subjects and three hyperlipemic subjects and by analysis of fractions isolated directly from the plasma of four subjects.

Two-dimensional gel electrophoresis

A technique of far higher resolution, 2DGE, was used to try to identify the proteins in samples isolated by immunoaffinity chromatography, and to determine whether they were specifically associated with the different subfractions. Fractions isolated from the plasmas of three normolipemic male subjects were processed and analyzed by the 2DGE system. These gave qualitatively similar results and thus the data derived from one subject are discussed in detail below. We have adopted the following convention for deciding whether protein bands identified in different fractions are specifically associated with those fractions. It is based on the comparisons of the electrophoretic pattern for plasma (see Fig. 3) with the pat-

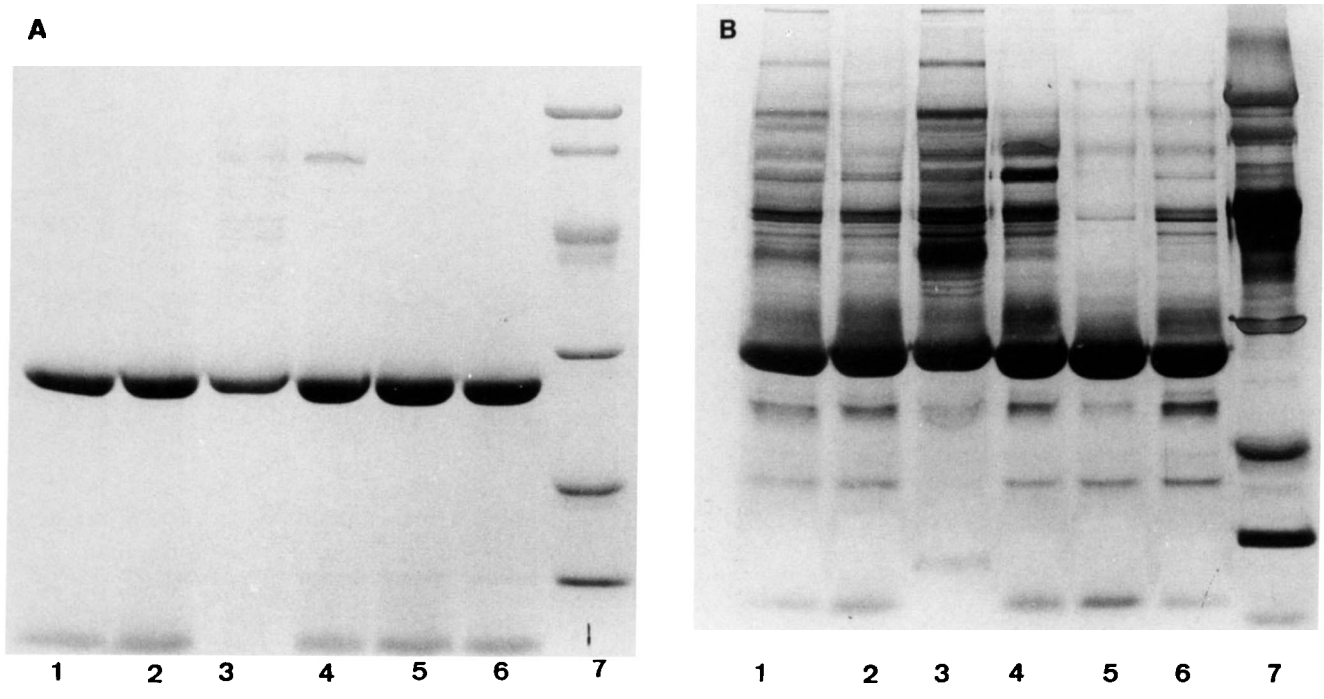

Fig. 2. SDS-PAGE analysis of fractions (50 μg protein) isolated by immunoaffinity chromatography from a normolipemic male subject. The gels were stained with Coomassie G250 (Fig. 2A) or silver reagent (Fig. 2B). Lane 1, plasma-Lp(AIwAII); lane 2, HDL-Lp(AIwAII); lane 3, plasma-Lp(AIw/oAII); lane 4, nonfractionated HDL; lane 5, HDL-2; lane 6, HDL-3; lane 7, Bio-Rad low M_r standards, respectively, 14400, 21500, 31000, 45000, 66200, 92500.

terns for the different samples (see Figs. 4–6) where equivalent concentrations of protein (20 μg) were analyzed. When protein bands are more intensely stained in isolated fractions, as compared to plasma, it suggests an enrichment of these proteins which we interpret as being due to a specific association with the relevant fraction. The interpretations are greatly facilitated by computer-assisted analysis of the gels. This not only permits measurements of the staining intensities of each protein band, but also matching of protein bands in different gels. Thus it allows a semi-quantitative, comparative analysis of the same protein band in different samples, even though the non-quantitative nature of the silver-staining technique precludes a quantitative comparison of different proteins within the same gel.

The electrophoretic pattern of plasma from a normolipemic subject is shown in **Fig. 3.** Clearly distinguishable are the quantitatively major plasma proteins, notably albumin, heavy and light IgG chains, α-antitrypsin, α,β and γ-fibrinogen chains, and transferrin. Several established apolipoproteins—whose identification was confirmed by comparison of this gel with that of HDL (Fig. 4)—such as apoA-I, C, D, and E are also evident (apoB being excluded due to its high M_r, while apoC-I (pI 8.0) lies outside the pH range used in this study). Also indicated within the boxes are regions of the gels (identified by M_r and pI coordinates) occupied by newly identified protein bands associated with Lp(AIw/oAII) and Lp(AIwAII) particles and more easily visible in immunoaffinity-isolated fractions from HDL and plasma (see Figs. 4–6).

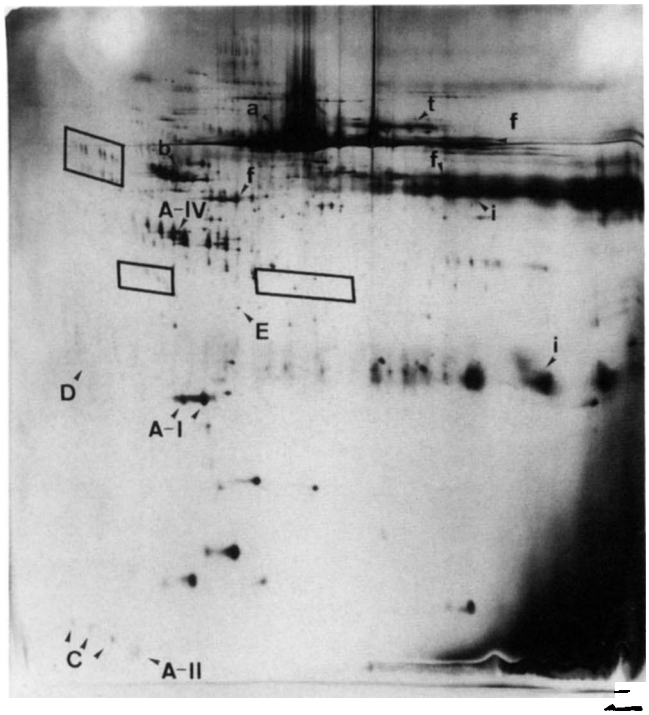

Fig. 3. Plasma (20 μg protein) from a normolipemic male subject was analyzed by 2DGE and protein bands were visualized by silver staining. The boxes indicate regions of the gel where newly identified proteins were found in immunoaffinity-isolated particles (Figs. 5 and 6); a, albumin; b, α-antitrypsin; f, fibrinogen; i, immunoglobulin chains; t, transferrin; A-I, apoA-I; A-IV, apoA-IV; C, apoCs; D, apoD; E, apoE.

In **Fig. 4** is shown the electrophoretic pattern for HDL isolated by ultracentrifugation. Established apolipoproteins were identified according to their M_r and pI coordinates (41, 42) and identification was confirmed by immunoblotting (see below). There was a marked enrichment in the established apolipoproteins, notably apoA-I, A-II, C, D, and E, as defined by the criterion outlined above. Conversely, visible and classified as contaminants by the same criterion are albumin and α-antitrypsin. The HDL fraction also appears to be enriched in a constellation of protein bands whose molecular weights are inferior to that of apoA-I. Prominent amongst these bands are apoA-II and C.

Immunoaffinity fractionation of HDL

When HDL was passed successively over antibody columns against apoA-II then apoA-I, two fractions were isolated. These were termed HDL-Lp(AIwAII) and HDL-Lp(AIw/oAII), respectively. Their analysis by 2DGE is shown in **Figs. 5A and B.** The first observation is that none of the apolipoproteins considered to be specifically associated with the fractions were lost (i.e., apoA-I, A-IV, C, D, and E in HDL-Lp(AIw/oAII) (Fig. 5A) and the same apolipoproteins plus apoA-II in HDL-Lp(AIwAII) (Fig. 5B)) while plasma protein contamination was reduced, as compared to nonfractionated HDL. This allowed protein bands that were barely visible in the total HDL fraction (Fig. 4) to become more prominent, given that a fixed quantity of protein (20 μg) was used for all 2DGE analyses. These bands were present in both subfractions and are considered to be Lp(AIwAII)- and Lp(AIw/oAII)-associated proteins. To facilitate discussion of these proteins and their identification on gels, they have been designated NA1–6 as indicated in Fig. 5. Several of the established apolipoproteins appear to be asymmetrically distributed between the two HDL subfractions in that, for an equivalent concentration of protein, they are more intensely stained in one subfraction than the other. Thus apoA-II and apoE are more prominent in HDL-Lp(AIwAII) whereas apoA-IV is more prominent in HDL-Lp(AIw/oAII). This proposal is examined in greater detail in the following section.

Immunoaffinity fractionation of plasma

Two-dimensional gels of retained fractions from plasma are shown in **Figs. 6A and B.** The protein content of plasma-Lp(AIwAII) was almost twice that of plasma-Lp(AIw/oAII) (ratio 1.9 ± 0.1 (n = 3)), with 33.4 ± 2.0% (n = 3) of plasma apoA-I being associated with the latter

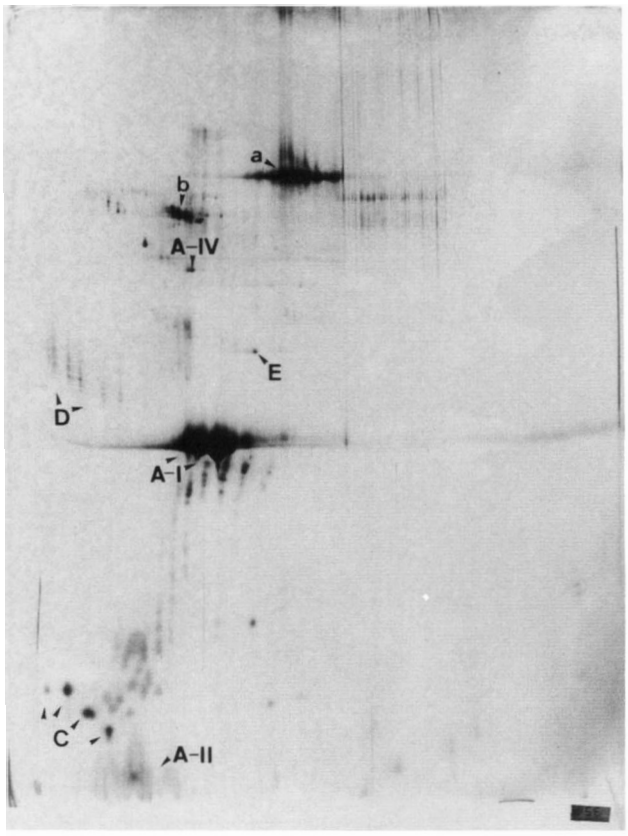

Fig. 4. 2DGE analysis of HDL (20 μg protein) prepared by ultracentrifugation from a normolipemic male subject; a, albumin; b, α-antitrypsin; A-I, apoA-I; A-II, apoA-II; A-IV, apoA-IV; C, apoCs; D, apoD; E, apoE.

particles. The molar ratio of apoA-II–apoA-I in the plasma-Lp(AIwAII) particles was 1.50 ± 0.1 (n = 3). In the molecular weight range above that of apoA-I, the known apolipoproteins i.e., apoA-IV, D, and E are clearly visible, in both plasma-Lp(AIwAII) and plasma-Lp(AIw/oAII). Below apoA-I, the apoC-II and C-III isoforms are also visible in both plasma subfractions, while the reduced form of apoA-II is present only in the plasma-Lp(AIwAII) particles. Apart from the latter apolipoproteins, there exists a constellation of protein bands with molecular weights lower than that of apoA-I, as was observed for the HDL subfractions. The level of contamination (less than 1% of total protein) of plasma-derived particles by plasma proteins was similar to the extent to which they contaminated HDL subfractions (Figs. 5A and B) apart from α and β-fibrinogen which are more prominent in the plasma-derived particles. The M_r and pI values for the apolipoproteins, as determined from 2DGE analyses, are given in **Table 1.** In order to confirm the identity of apolipoproteins as established by M_r and pI coordinates, immunoblotting was performed using specific monoclonal and polyclonal antibodies. A composite picture is presented in **Fig. 7** and **Fig. 8** where silver-stained gels are shown together with immunoblots performed on duplicate gels electrophoresed at the same time. Films derived from individual immunoblotting experiments are overlaid to allow easier comparison with the silver-stained gel profile. For Fig. 7 the gel region encompassing apoA-I to NA-6 demonstrates lack of cross-reactivity of antibodies against established apolipoproteins with the newly identified proteins. In Fig. 8 the gel area corresponding to the apoC/AII region is shown; no cross-reactivity of these antibodies with proteins NA-1 to 6 was observed. These studies also confirm the identities attributed to apoA-I, A-II, A-IV, C-III, D, and E.

In addition to the established apolipoproteins, six additional protein bands fulfill the criterion by which we define specific association with isolated particles. Their molecular weights and isoelectric points, as determined from computer analysis of the gels, are given in **Table 2.** Each consists of a series of protein spots that we consider to be isoforms of the same protein due to the regularity of the spacing (corresponding to M_r and negative charge increments) between each spot. They are present in both plasma-derived fractions (confirmed by computer matching of the gels) and, to a lesser extent, in the fractions isolated from HDL by immunoaffinity (Figs. 5A and B). Two of these (NA-1 and 2) have similar molecular weights and isoelectric points, and it is difficult to discriminate between them in the plasma subfractions. However, in the HDL subfractions a difference in staining properties is discernable. Confirmation of the presence of two distinct proteins was achieved by 2DGE in a narrower pH range using Immobiline® gels as the first dimension (29). As shown in **Fig. 9,** two distinct series of protein bands are visible due to evident differences in staining properties. As mentioned above, none of these proteins cross-reacted in immunoblotting studies with antibodies to established apolipoproteins (apoA-I, A-II, A-IV, B, C-III, D, E, or H).

The asymmetric distribution of proteins specifically associated with plasma-Lp(AIwAII) and (AIw/oAII) was analyzed by comparing the staining intensities of computer-matched bands from the two gels (Figs. 6A and B). The results are shown in **Table 3.** Of the established apolipoproteins, apoE and, to a lesser extent, apoC and D are relatively more important in the plasma-Lp(AIwAII) particles; conversely, apoA-IV is more prominent in the plasma-Lp(AIw/oAII) particles. With respect to the newly identified protein bands, four (NA-2,3,5, and 6) are more prominent in the plasma-Lp(AIw/oAII) particles. NA-1 and 4, as judged by the ratio of staining intensities in the gels (Table 3), appear equally well represented in both particles. These ratios should be compared to those bands considered to be contaminants, such as albumin (0.78), α-fibrinogen (0.88), α-antitrypsin (1.0), and γ-fibrinogen (1.13).

As visual confirmation of these conclusions, the computer was programmed to plot comparisons of the 2DGE

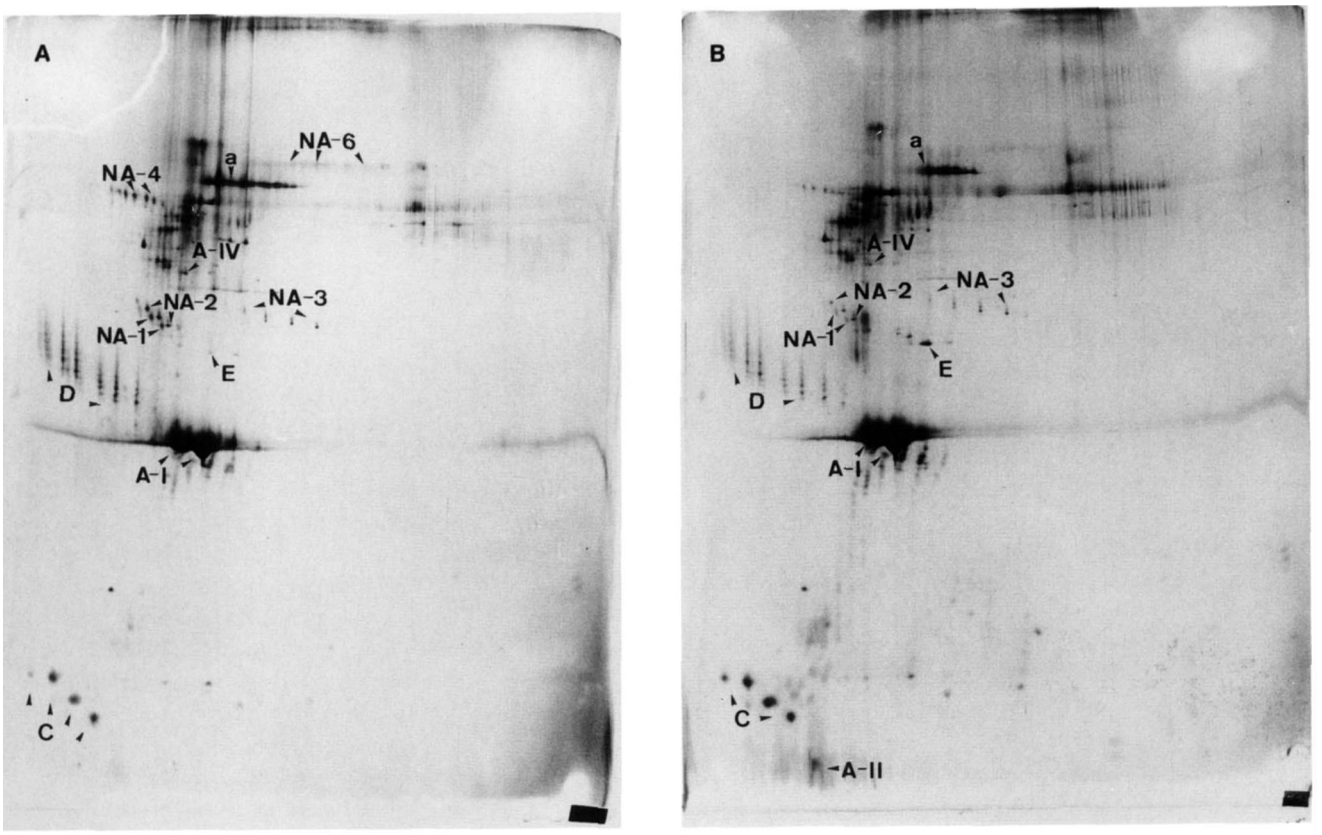

Fig. 5. 2DGE of particles (20 μg protein) isolated by immunoaffinity chromatography from HDL; 5A) HDL-Lp(AIw/oAII); 5B) HDL-Lp(AIwAII); a, albumin; A-I, apoA-I; A-II, apoA-II; A-IV, apoA-IV; C, apoCs; D, apoD; E, apoE.

gels of the particles derived from plasma and HDL. **Fig. 10** is given as an example, where a combination of the screened gels of plasma-derived particles (Figs. 6A and B) was compared to a similar combination of HDL-derived particles (Figs. 5A and B) such that only particle-associated bands with greater staining intensities in the plasma-derived particles were retained. As can be seen, this removes the majority of the established apolipoproteins, except apoA-IV, while all the newly identified proteins are retained.

DISCUSSION

Our aim in this series of studies was to investigate the protein heterogeneity of particles containing as their quantitatively major apolipoproteins apoA-I, and apoA-I plus apoA-II, isolated by immunoaffinity chromatography from plasma and HDL. We have identified and partially characterized, by molecular weight, pI, and relative distribution in plasma subfractions, six proteins which we believe to be associated with lipoproteins floating in the HDL density range (see below). However, before discussing these proteins, there are several points to be considered with respect to the immunochemical approach used in this study.

The immunoisolation procedure does not appear to have greatly modified the subfractions, as indicated by virtually complete retention of rechromatographed ^{125}I-labeled HDL-Lp(AIwAII) by both affinity columns. Moreover, the 2DGE profiles of total HDL and immunoaffinity-isolated subfractions are qualitatively similar (Figs. 4–6). Further, electron microscopic images (Fig. 1) and measurements of particle diameters (which are consistent with literature values for HDL (21, 43)), suggest that there has been no substantial modification of these parameters by immunoaffinity chromatography.

A notable feature of the HDL subfractions, as revealed by the electron microscope, is the net difference in the average particle diameters, with the Lp(AIwAII) particles being smaller (7.65 nm) than the Lp(AIw/oAII) particles (9.98 nm). The isolated particles also seem to show less size heterogeneity, as compared to HDL, judging from the reductions in the SEM values. These observations correspond to previous reports that there is a size difference between (AIw/oAII) and (AIwAII) particles (22, 44). Suggestions of Atmeh, Shepherd, and Packard (45) that the (AIw/oAII) particles are mainly associated with HDL_2

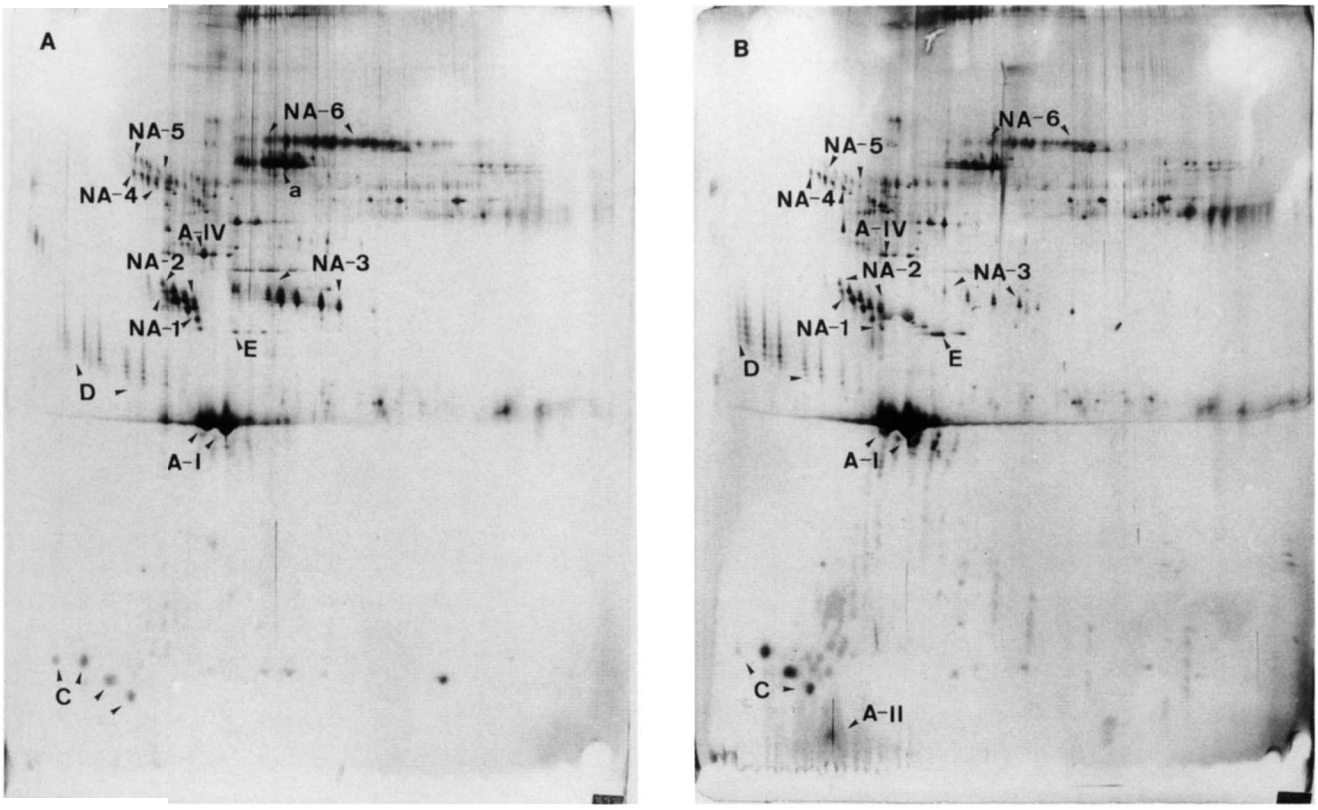

Fig. 6. 2DGE of particles (20 μg protein) isolated by immunoaffinity chromatography from plasma; 6A) plasma-Lp(AIw/oAII); 6B, plasma-Lp(AIwAII); a, albumin; A-I, apoA-I; A-II, apoA-II; A-IV, apoA-IV; C, apoCs; D, apoD; E, apoE.

while the (AIwAII) particles predominate in HDL_3 also agree with the size differences we observe in our affinity-isolated particles. The size heterogeneity we found within the two HDL-derived fractions should be interpreted with caution because of the potential deleterious effects of ultracentrifugation on HDL. The present study, together with previous publications (13–15), indicates that centrifugation modifies the apolipoprotein complement of HDL and its behavior during PAGE (21, 46, 47). Nevertheless, the results agree with suggestions by Cheung and Albers (22) and Nestruck et al. (46) that the (AIw/oAII) and (AIwAII) particles are heterogeneous in size.

The second question of particular importance to the study is whether the newly identified proteins are truly associated with immunoaffinity-isolated particles, or are contaminants. This consideration is complicated by the fact that apoA-I and A-II comprise the bulk of HDL protein; other apolipoproteins are present in relatively minor quantities which, if further reduced by techniques employed to isolate lipoproteins, would render them virtually undetectable. However, we believe the proteins in question to be specifically associated with lipoprotein particles for the following reasons.

1) Using the criterion outlined in the Results section there is no misclassification of established apolipoproteins or the major, nonlipoprotein plasma proteins. ApoA-I, A-II, A-IV, C, D, and E (see Figs. 3–6) are enriched in ultracentrifuged HDL and the immunoaffinity-derived particles; conversely, the quantitatively major plasma pro-

TABLE 1. M_r and pI of established apolipoproteins as determined by 2DGE

Protein	M_r	pI
ApoA-I	24000 (27000)	5.4–5.6 (5.6)
ApoA-IV	43000 (46000)	5.4–5.5 (5.5)
$ApoC\text{-}II_0$	7000	4.9
$ApoC\text{-}II_1$	9000	4.5 (4.7)
$ApoC\text{-}III_1$	8000 (8750)	4.7 (4.9)
$ApoC\text{-}III_2$	9000	4.3
ApoD	27–32000 (32000)	4.3–5.1 (5.3)
ApoE	32000 (35000)	5.6–5.7 (5.9)

Standard curves for M_r and pI were constructed from gel positions (see Fig. 3) of the plasma proteins transferrin, albumin, IgG heavy and light chains, α-antitrypsin, and retinol binding protein, using the values cited in ref. 41. The values in brackets are those currently attributed to the apolipoproteins (42).

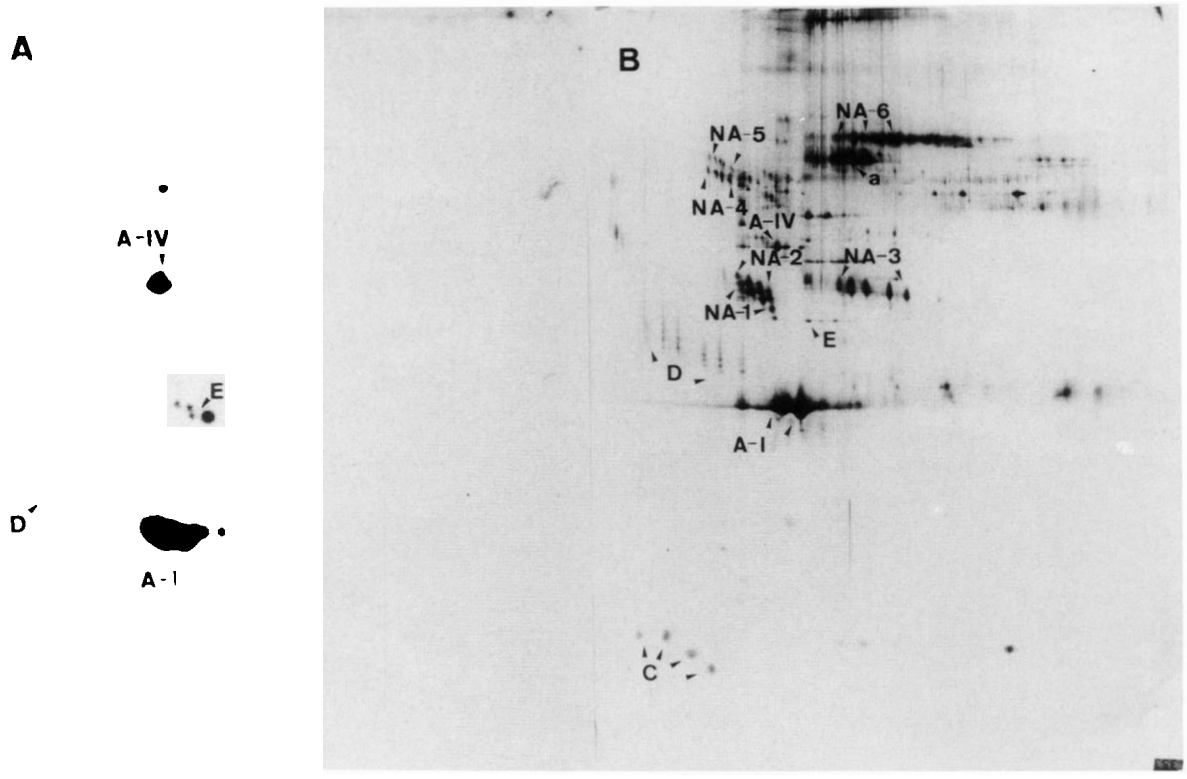

Fig. 7. Immunoblots using monospecific anti-apolipoprotein antibodies. ApoA-I and E were visualized using ^{125}I-labeled monoclonal antibodies. Polyclonal antibodies against apoA-IV and D were visualized with affinity-purified, ^{125}I-labeled goat anti-rabbit IgG. A) Films developed from individual experiments with specific antibodies were overlaid to give a composite picture. The area from just below the apoA-I band to the top of the 2DGE gel (see B) is shown. B) Silver-stained 2DGE gel of plasma-Lp(AIw/oAII).

teins, of which traces are present in all fractions, are clearly identified as contaminants. The presence of contaminating proteins in 2DGE profiles of isolated subfractions would not appear to be a consequence of direct binding of these proteins to the affinity gel (see point 3 below), but may be due to nonspecific association with bound lipoprotein particles.

2) The protein bands are visible on HDL prior to further subfractionation (Fig. 4). This suggests that they are not artifacts of the immunoisolation process. However, they are present in such trace quantities that they could easily be considered as contaminants. It required the high resolution of 2DGE to establish their presence, as they occupy an area of single dimension SDS-PAGE gels (Fig. 2) where established apolipoproteins are to be found. An alternative explanation would be to consider the proteins as contaminants that are more efficiently removed by ultracentrifugational manipulations. If such is the case, it does not explain why the immunoaffinity wash procedure successfully removed the bulk of the quantitatively more important plasma proteins.

3) No bound protein could be detected by SDS-PAGE either when the unbound fraction was passed through the affinity columns a second time or when plasma was processed on a neutral IgG column.

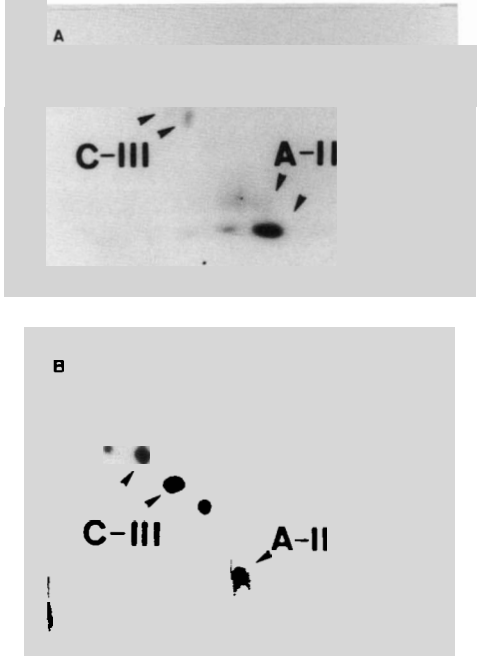

Fig. 8. Immunoblots using monospecific anti-apolipoprotein antibodies. ApoA-II was visualized using ^{125}I-labeled monoclonal antibody and apoC-III with ^{125}I-labeled polyclonal antibodies. A) Composite picture of immunoblots (see legend Fig. 7A) encompassing the apoC-III/A-II region of the 2DGE gel. B) Silver-stained 2DGE gel plasma-Lp(AIwAII) showing the apoC-III/A-II region.

TABLE 2. M_r and pI of proteins associated with particles isolated by immunoaffinity chromatography

Protein	M_r	pI
NA-1	34–38000	5.0–5.3
NA-2	35–39000	4.9–5.3
NA-3	36–37000	5.7–6.4
NA-4	59–62000	4.8–5.0
NA-5	58–66000	4.8–5.2
NA-6	75000	5.4–5.5

Standard curves for M_r and pI were constructed from gel positions (see Fig. 3) of the plasma proteins transferrin, albumin, IgG heavy and light chains, α-antitrypsin, and retinol binding protein, using the values for these proteins cited in ref. 41.

Evidently the criterion chosen to define specificity of association depends on the ability to compare protein bands in isolated fractions with those in plasma, where the much more complex 2DGE profiles could lead to masking of protein bands. However, the gel scanning and comparison procedure that was used greatly facilitates matching of spots in different gels. Indeed, the specificity criterion is quite conservative in that lipoprotein-associated proteins having the same M_r and pI coordinates as nonlipoprotein-associated plasma proteins would tend to be eliminated when the staining intensities are compared.

We believe that the particles isolated from plasma are equivalent to the HDL-derived particles. Not only is almost all apoA-I and A-II found in the HDL density range in normolipemics but, as mentioned above, the 2DGE patterns for fractions derived from HDL and plasma are qualitatively similar, although the proteins NA-1 to 6 are present in trace quantities in the former. A very minor percentage of apoA-I and A-II can be detected in the VLDL density range. However, examination of VLDL isolated by ultracentrifugation showed no trace of any of the six additional protein bands (results not shown). It is considered unlikely that the relatively mild conditions (compared to HDL) necessary for isolating VLDL would have completely removed these proteins. Thus, although we cannot eliminate the possibility that they could also be associated with VLDL, it is most probable that the major proportion of these proteins resides in the HDL range.

The pI values for the established apolipoproteins determined from the 2DGE gels are in excellent agreement with published values (see Table 1). However, the molecular weight estimates are 2000–3000 lower than presently accepted values (Table 1; see ref. 41). It appears to be a consequence of using nonapolipoprotein plasma proteins to construct the calibration curve. Indeed, the values we quote agree extremely well with those given by Anderson et al. (41) for 2DGE studies of plasma proteins. Thus it should be kept in mind that the molecular weights quoted for the newly identified proteins may slightly underestimate their true values. Indeed, construction of a calibration curve based on the molecular weights of the established apolipoproteins (apoC to A-IV, 41) gave estimates (NA-1, 35–40000; NA-2, 38–41000; NA-3, 37–40000) that were 2000–3000 higher than those quoted in Table 2. As the remaining proteins fell outside the range covered by the calibration curve, they were not considered.

It is possible that these proteins correspond to other proteins thought to be associated with HDL. Several are listed in **Table 4,** and are identified by their molecular weights and pI, where available. Of these, the lipid transfer proteins and LCAT appear to be of particular interest as they cover pI and M_r ranges similar to those of proteins NA-4, 5, and 6. It is known that the association of lipid transfer activity with HDL is susceptible to prolonged ultracentrifugation (48, 49) which would correlate with the presence of these proteins in the plasma-derived (Fig. 6A and B) rather than HDL-derived (Fig. 5A and B) particles. With respect to cholesteryl ester transfer activity, the suggestion that it is associated with apoA-I-rich HDL (48) has recently been corroborated by Cheung et al. (50) who localized transfer activity to HDL particles containing apoA-I but not A-II. These authors propose M_r and pI coordinates for the transfer protein that would correspond to NA-4 and 5; from our data we would suggest NA-5 which is more susceptible to ultracentrifugal manipulation (compare Figs. 5A and B to 6A and B) and is more prominent in Lp(AIw/oAII). In a very recent publication, Jarnagin, Kohr, and Fielding (51) claim that cholesteryl ester transfer activity is, in fact, associated with a protein of M_r 74000 and pI 5.2. This corresponds to the coordinates for NA-6 which is also particularly sensitive to ultracentrifugation (Figs. 5 and 6) and preferentially associated with Lp(AIw/oAII) (Figs. 6A and B). We have been unable, however, to confirm the identity of NA-4 to 6 as either LCAT or lipid transfer proteins using

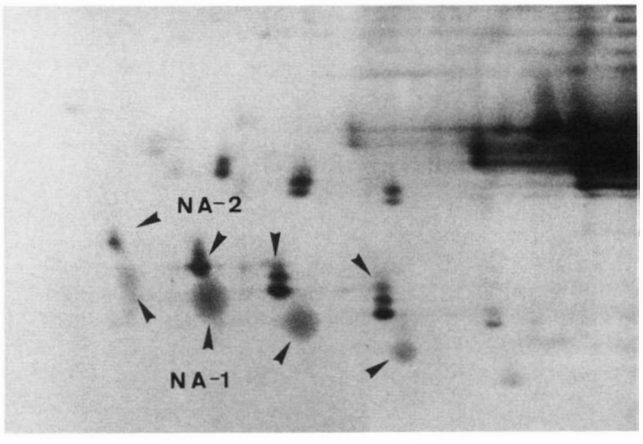

Fig. 9. 2DGE of plasma-Lp(AIwAII) particles (20 μg protein) isolated from plasma by immunoaffinity chromatography, The first dimension was performed in a narrow pH gradient (pH 4.0–7.0). The area of the gel corresponding to proteins NA-1 and NA-2 is shown.

TABLE 3. Comparison of the staining intensities of protein bands in 2DGE gels of plasma-LP(AIw/oAII) and plasma-Lp(AIwAII) particles

Protein	Ratio 1	Ratio 2
ApoA-IV	0.58	0.52
ApoCs	1.73	1.54
ApoD	1.61	1.44
ApoE	3.72	3.32
NA-1	0.82	0.73
NA-2	0.66	0.59
NA-3	0.36	0.32
NA-4	0.87	0.78
NA-5	0.50	0.45
NA-6	0.61	0.54

Comparisons were made using the MELANIE program (40) based on scans of the gels shown in Figs. 6A and 6B. The results (from one set of gels) are expressed as the ratio (Lp(AIwAII):Lp(AIw/oAII)) of the staining intensities for the computer-matched bands corresponding to each protein. In column 1 absolute staining intensities are compared; in column 2 the staining intensity of each band was first standardized to that of the apoA-I band in each gel.

either polyclonal or monoclonal antibodies to these proteins.

Proteins NA-1 and NA-2 of Table 2 appear to have been previously identified in 2DGE of whole plasma. In studies by Anderson and co-workers (41, 52) they are referred to as PLS:29 (M_r 35800) and PLS:30 (M_r 37400). There is, however, no mention of the possible association of these proteins with lipoproteins.

An attempt has been made to characterize the proteins by their relative distributions in the plasma-Lp(AIwAII) and plasma-Lp(AIw/oAII) particles. Unfortunately, due to the low concentrations of apolipoproteins other than apoA-I and A-II, we were obliged to use the more sensitive silver stain rather than other stains which would have allowed a quantitative comparison of different bands within the same subfraction. The problem cannot be overcome simply by increasing the quantity of material analyzed, as the large apoA-I band leads to distortions during electrophoresis. However, by comparing the same protein band in two different subfractions, aided by measurements of the staining intensities of the bands, one can obtain a qualitative estimation of the distribution of the protein between the fractions. The validity of this approach is supported by studies of Kuick et al. (53) who have demonstrated that quantitative data can be derived from silver-stained 2DGE gels when the same protein is examined. Thus, given that the same quantity of total protein was loaded onto each gel, apoE appears to be a more important component of plasma-Lp(AIwAII) as opposed to plasma-Lp(AIw/oAII) (Figs. 6A and B). The same is true of the apoE contribution to HDL-Lp(AIwAII) and (AIw/oAII) (Figs. 5A and B). This is in agreement with observations made by Cheung and Albers (22) who reported a greater concentration of apoE in their Lp(AI with AII) particles. Nestruck et al. (46) also found that in their fractions separated by chromatofocusing, apoE tended to be associated with fractions containing both apoA-I and A-II. In our hands, apoA-IV is a relatively more important component of the plasma-Lp(AIw/oAII) particles. This is a novel observation and correlates with studies by Bisgaier et al. (54) showing that human apoA-IV is associated with a small, dense HDL particles rich in apoA-I.

The staining intensities (Table 3) of the apoC and D bands indicate a preference for the plasma-Lp(AIwAII) particles (although, in the particles derived from HDL, the relative staining intensity (AIwAII):(AIw/oAII) of 1.01 for apoD suggests an equivalent distribution between the two particles). Cheung and Albers (22) also noted a preferential association of apoD with their Lp(AI with AII) particles derived from plasma. Thus, overall the distribution of established apolipoproteins appears quite similar

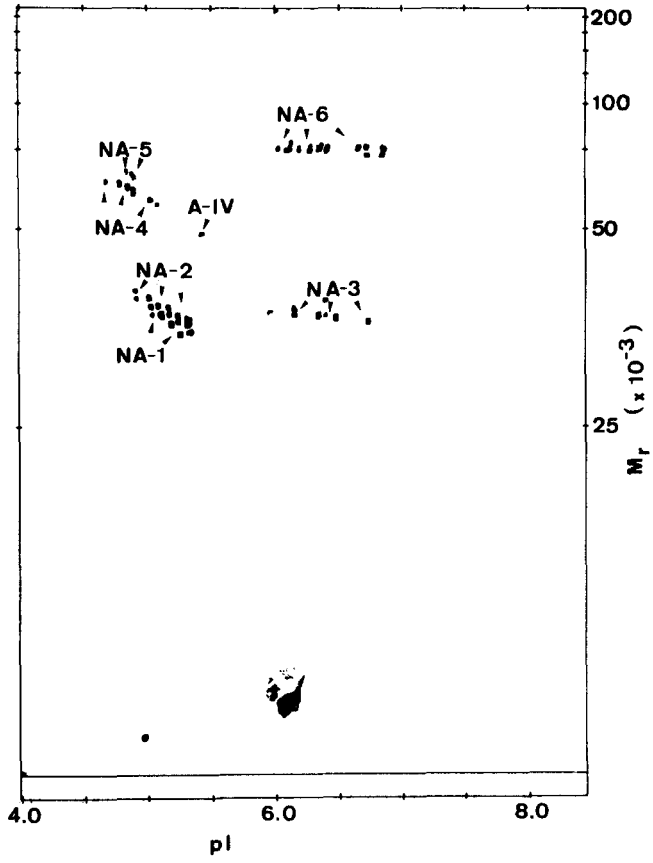

Fig. 10. A comparison of the staining intensities of bands present in particles immunoisolated from plasma (Figs. 6A and 6B) with the staining intensities of computer-matched bands present in particles immunoisolated from HDL (Figs. 5A and 5B). Comparison was made by means of the MELANIE system (40) such that only particle-associated bands of greater staining intensity in the plasma-derived particles were retained.

TABLE 4. M_r and pI of proteins that can associate with HDL

Protein	M_r	pI
ApoF	26-32000	3.7 (65)
ApoG	72000	-(63)
ApoH	46000	5.6-6.4 (63)
LCAT	66000	5.1-55 (66)
Cholesteryl ester transfer protein	58-63000	4.2-5.0 (67, 68)
	74000	5.2 (49)
Phospholipid transfer protein	41000	-(48)
SAA	11-14000	5.7-6.5 (69)
Elastase	28-31000	6.1-6.6 (70)
Platelet activating factor acetylhydrolase	45000	-(71)
Acid phosphatases	54000	4.9-5.2 (72)

The numbers in parentheses are references.

to that observed in fractions immunoaffinity-isolated by Cheung and Albers (22). Moreover, there is a certain concordance with HDL subfractions isolated by Nestruck et al. (46) who found not only apoE but also apoC and D associated with subfractions containing both apoA-I and apoA-II.

With respect to the newly identified proteins (Table 3), four show a net predominance in the plasma-Lp(AIw/oAII) particles; NA-1 and 4 appear to make an equivalent contribution to both particles (Figs. 6A and B). It would be interesting to know whether these proteins are distributed across the HDL density spectrum or are associated with HDL subpopulations. It is in this direction that potential functions for the proteins should be sought, if they are at all associated with lipid transport/metabolism. There is substantial evidence that the minor proteins associated with HDL are not uniformly distributed across its density spectrum. It applies not only to apolipoproteins such as apoA-I and A-II (46, 55, 56), D (22, 57) and E (20, 58), but also to lipid transfer proteins (59, 60) and LCAT (61, 62). Taken together with the abundant evidence of size heterogeneity within the HDL spectrum, it suggests the existence of subpopulations with particular functions e.g., apoE-enriched HDL or the LCAT-HDL complex. In this light it is possible that the proteins have roles to play as auxillary proteins of the lipid transport system (63). It is interesting to note that they appear to be particularly sensitive to ultracentrifugal manipulations (see Figs. 4-6).

Our results reinforce suggestions that lipoprotein composition is substantially modified by ultracentrifugation. It is our contention that this has led to loss of the newly identified proteins from HDL, and receives support from similar observations with established lipoproteins. Thus, judging by its staining intensity, apoA-IV is a relatively more important component of plasma-Lp(AIw/oAII) than of HDL-Lp(AIw/oAII) or HDL-Lp(AIwAII) (see Figs. 4-6). This agrees with recent studies by Bisgaier et al. (54) who concluded that human apoA-IV was redistributed during ultracentrifugation. ApoE distribution is also modified by ultracentrifugation (15, 24). Similarly, our results show the apoE band to be more important in the plasma-Lp(AIwAII) particles than in HDL-derived subfractions (Figs. 4-6). The possibility that ultracentrifugation modifies the HDL content of apoA-IV and E is of particular relevance to the recognition of lipoproteins by receptors; it is well established that apoE binds to at least two distinct receptors (8) and recent studies have implicated apoA-IV as the ligand mediating receptor binding of rat HDL (64).

In summary, we have demonstrated that Lp(AIw/oAII) and Lp(AIwAII) isolated from plasma show a far greater degree of protein heterogeneity than their HDL homologues; it is due in part to the identification of six protein bands which we consider to be specifically associated with the particles, and in part due to greater retention of established apolipoproteins. We believe that the immunoaffinity-isolated particles are a more accurate reflection of HDL composition in plasma and thus of great importance for differentiating and defining subpopulations of potential metabolic significance. The newly identified proteins associated with immunoaffinity-isolated fractions have been characterized in terms of molecular weight and pI, and four appear to be asymmetrically distributed between the two types of particles.

Immunoaffinity chromatography permits the isolation directly from plasma of lipoprotein particles of physiological relevance. The ability to target individual apolipoproteins offers an attractive rationale for lipoprotein fractionation with regard to the HDL density range. In particular, strategies for the identification of metabolically distinct, though minor, HDL particles can now be considered.

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