Protein polymorphism of a human plasma apolipoprotein D antigenic epitope

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Abstract Based on our previous observation that monoclonal antibody anti-apoD-4E11 reacted with several HDL proteins we studied them further with three questions in mind: *i*) is there common protein polymorphism in healthy individuals? *ii*) how many proteins are present and what are their characteristics? *iii*) are they all apolipoproteins and do they have the same lipoprotein distribution as apoD? Isolated, delipidated apoD was used as a standard for radioimmunometric assay of plasma with antibody 4E11. The antigen varied from 3 to 11 μmol-equivalents of apoD per liter of plasma (equivalent to 5-20 mg apoD/dl plasma) with means of 6.1 and 6.8 μmol/l in men and women, respectively. Two-dimensional electrophoresis of plasma found up to eight 4E11-antigenic-proteins of different M_r, each heterogeneous in pI. All plasmas tested contained apoD and an M_r 38,000 antigen, the latter being the most immunoreactive. Six proteins of M_r 70,000-94,000 were found, but the number varied between subjects. Eighty nine percent of the plasma antigen was associated with lipoproteins: 83% with HDL and VHDL, 5% with LDL and VLDL. Lipoproteins of all sizes, separated by polyacrylamide gradient gel electrophoresis, contained the antigen. ApoD was almost the only 4E11-antigen in LDL, and was in two states: the one free, the other an apoD–apoB mixed disulfide complex. The apparent proportions of higher M_r antigens increased with increasing lipoprotein density, and the proportion of apoD decreased reciprocally. None of these 4E11-antigenic-proteins cross-reacted with antiserum to retinol-binding protein. —**Camato, R., Y. L. Marcel, R. W. Milne, S. Lussier-Cacan, and P. K. Weech.** Protein polymorphism of a human plasma apolipoprotein D antigenic epitope. *J. Lipid Res.* 1989. **30:** 865-875.

Supplementary key words HDL • apolipoprotein B • monoclonal antibody • radioimmunoassay

Human plasma apolipoprotein D (apoD) was originally purified and described by McConathy and Alaupovic (1, 2). They used criteria of biochemical characterization and specificity of antisera to show that apoD was distinct from the other well-characterized apolipoproteins known at that time, and that it corresponded to the "thin-line polypeptide." A large part of this information has been summarized (3, 4). Drayna et al. (5, 6) recently determined the amino acid sequence of apoD, the nucleotide sequence of its cDNA and gene, and concluded that apoD has no significant homology of primary structure with any of the recognized apolipoproteins. It does, however, have amino acid sequences that are similar to some in plasma retinol-binding protein, and the disposition of introns in its gene resembles that of the $\alpha_2\mu$-Globulin superfamily.

We have produced monoclonal antibodies 4E11 and 5G10 that reacted with apoD and with other proteins of high molecular weight (4) as did an antiserum (4). The antigenic sites recognized by both antibodies appeared to be composed of the polypeptide and not the oligosaccharides of apoD (4). On replicas of SDS-PAGE of HDL the reduction of the proteins with mercaptoethanol decreased the immunoreaction with 4E11 but increased the immunoreaction of antibody 5G10 with apoD and the higher M_r proteins (4). This protein heterogeneity was not expected, given the background of results mentioned above. Therefore, as a first step towards characterizing these proteins and their relationship to apoD we raised the following three questions: *i*) is there common polymorphism of apoD antigens in healthy individuals? *ii*) in what ways are apoD and the cross-reacting proteins polymorphic within individuals? and *iii*) do all lipoprotein classes contain the polymorphic antigens? To address these questions we analyzed the apoD antigens in plasma and lipoprotein fractions by immunometric assay, two-dimensional and nondenaturing, gradient gel electrophoresis. We report here the characterization of both the 4E11-antigenic proteins and apoD as being apolipopro-

Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PAGGE, polyacrylamide gradient gel electrophoresis; VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; VHDL, very high density lipoproteins; 19,304 was taken as the molecular weight of the apoD polypeptide (5).

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teins, their pI and M_r values, and show that they were polymorphic in all healthy subjects studied, altogether indicating that the function of each of them is tied to lipoprotein metabolism. In the context of the present report, apoD refers only to the protein having the properties described by McConathy and Alaupovic (1, 2), Albers et al. (7), and Drayna et al. (5); "apoD antigens" refers to all proteins that react with our antibodies against apoD (4).

MATERIALS AND METHODS

The sources of supply of chemicals and apparatus for electrophoresis and radioimmunometric assay have been listed previously (8, 9). Polyacrylamide gradient gels PA 2/16, PA 4/30, and Agarose C were purchased from Pharmacia (Uppsala, Sweden), phenylmethylsulfonylfluoride (PMSF) and Ponceau S from Sigma Chemical Company (St. Louis, MO), and Nonidet NP-40 from BDH Chemicals (Montreal, Canada). Rabbit antiserum to human plasma retinol-binding protein was obtained from Behring Diagnostics, Calbiochem (La Jolla, CA).

Subjects and plasma

Plasma samples for radioimmunometric assay and two-dimensional gel electrophoresis were obtained from 90 healthy adults, aged 20–59 years, working for a major utility company, and consecutively admitted into a large population study. Exclusion criteria were clinical manifestations of cardiovascular disease, presence of metabolic disturbances or of chronic disorders requiring medication, and abnormal routine biochemistry including liver function tests. Plasma samples were supplemented with 1 mM EDTA, 1 mM PMSF, and 0.02% NaN_3 and when necessary were stored frozen at −70°C. Plasma samples for ultracentrifugation and gradient gel electrophoresis were obtained from normolipidemic laboratory volunteers. Delipidation of plasma was performed as described by Cham and Knowles (10) using butanol–diisopropyl ether 40:60 (v/v). Lipids were measured as described (11).

Ultracentrifugation and gradient gel electrophoresis

Lipoproteins were isolated by a modification (12) of the technique of Terpstra, Woodward, and Sanchez-Muniz (13) without prestaining of the sample. By this technique VLDL and IDL are coisolated, and the VHDL described here is the clear, colorless layer below the obvious HDL and above the plasma protein fraction. All fractions from ultracentrifugation were dialyzed overnight against 10 mM NH_4HCO_3, 1 mM EDTA, 0.02% NaN_3. Gradient gel electrophoresis was performed under nondenaturing conditions (14) and replicas of these gels were transferred electrophoretically to nitrocellulose in a buffer of the same composition as was used for initial electrophoresis.

Two-dimensional gel electrophoresis

Plasma or lipoprotein samples were diluted 1:15 with 10 mM Tris-HCl, 9.5 M urea, pH 6.8, containing 2% Nonidet NP-40, and separated by isoelectric focusing (15) using pH 4–6 ampholines, at 400 V for 16 h and 500 V for 1 h at 4°C in a 0.75-mm-thick slab gel. Slices of this gel were gently shaken in three changes of the cathode buffer of SDS-PAGE (16) containing 1% SDS. Each slice of isoelectric focusing gel was laid on a 1.5-mm-thick gel for SDS-PAGE (16) and sealed in place with 1% agarose in the 1% SDS cathode buffer.

Following electrophoresis, a replica of each gel was transferred to a nitrocellulose sheet (4, 17). This sheet was then stained for 5 min in a solution of 0.2% (w/v) Ponceau S in 3% trichloroacetic acid, and washed rapidly in water. The outline of the gel and the distances of migration of apoA-I, albumin, and the molecular weight standards were marked at the edges of the sheet with a ball-point pen for reference. The replicas were completely destained in 10 mM Tris, 0.15 M NaCl buffer, pH 7.4, and processed for antibody reaction with monoclonal antibody 4E11 (9). Autoradiographs of the antibody reactions were made as before (9).

In some experiments with LDL we performed two-dimensional electrophoresis in which SDS-PAGE (16) was used for both the first and the second separation. The first gel was made with 4% acrylamide but the second gel was made with 15% acrylamide, in order to resolve apoB in the first dimension and low M_r proteins such as apoD in the second dimension.

Radioimmunometric assay

Quantitative analysis of apoD antigen in plasma and lipoprotein fractions was made by a solid-phase technique using monoclonal antibody 4E11 (4) as follows. Immulon II Removawells (Dynatech Laboratories Inc., Alexandria, VA) were coated overnight at room temperature with 50 µl of the antigen (2 µg protein/ml) dissolved in 5 mM glycine, pH 9.2. The antigen contained apoD, and was the fraction eluted from wheat germ agglutinin with 0.08 M N-acetylglucosamine as described before (4); however, pure apoD was an equal substitute for coating. The wells were washed ten times with 0.25% Tween-20 (v/v) in PBS (0.154 M NaCl, 10 mM $Na_2HPO_4 \cdot 7H_2O$, 5 mM NaH_2PO_4, 0.02% NaN_3, pH 7.2), twice with PBS, and then saturated with 300 µl 1% bovine serum albumin (BSA) in PBS for 1 h at room temperature. This solution was then discarded and the wells were refilled with 100 µl of a mixture of antibody and test plasma, which was incubated there for 5 h. The appropriate dilution of antibody had been determined in previous titration experiments, and in this case was 1/128,000 of the ascites fluid 4E11. To make the mixture of test plasma and antibody we mixed equal volumes of diluted ascitic fluid and diluted plasma in 1%

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BSA-PBS, and incubated them overnight at 4°C before addition to the saturated Removawells. After 5 h incubation in the Removawells, the contents were discarded and the wells were again washed with Tween-PBS buffer as before. The Removawells were then refilled with 100 μl ^{125}I-labeled rabbit anti-mouse IgG (18), 2×10^5 cpm, diluted in 1% BSA-PBS, and incubated overnight at room temperature. The Removawells were washed as before and radioactivity was counted. The assay was calibrated using pure apoD as the standard. Radioimmunometric assay of apoA-I was performed as described (19).

RESULTS

Conditions for measurement of apoD "antigens" in plasma

The results of experiments shown in **Table 1** showed that there was no change in the immunoreactivity of plasma following freezing and thawing (three times at –70°C), heating (3 h at 55°C), or addition of 1% Triton X-100. Delipidation with butanol–diisopropyl ether (10) decreased the immuoreactivity by an average of 25% in five samples. Denaturation with urea also decreased the immunoreaction. Plasma samples that had been stored at –70°C gave values that were not lower than fresh plasma (6.81 and 7.59 μmol/l vs. 6.55 μmol/l) but lower values were observed after storing these plasmas at 4°C (5.77 and 5.75 μmol/l). These results indicated that the antigenic site recognized by antibody 4E11 was fully expressed in fresh plasma.

Antigen and lipid concentration in plasma

The frequency distribution of plasma apoD antigen measured in a population of healthy subjects is shown in

TABLE 1. Effect of storage and denaturation of plasma on its immunoreactivity with monoclonal antibody apoD 4E11

Storage	ApoD Antigen	
	μmol/l	
Fresh	6.55	(0.67)
Delipidated	3.87	(0.39)
Frozen–thawed 3 ×	6.04	(0.31)
1% Triton X-100	7.46	(0.41)
Heated 3 h 55°C	5.94	(0.29)
8 M Urea	4.92	(0.25)
1 Month 4°C[a]	5.77	(0.54)
3 Months 4°C[a,b]	5.75	(0.45)
1 Month –70°C[a]	6.81	(0.66)
3 Months –70°C[a,b]	7.59	(0.64)

All plasma samples came from the same subject. Standard error of the estimate is shown in parentheses.
[a]A different sample from that used in the denaturation tests.
[b]A separate assay from the other tests.

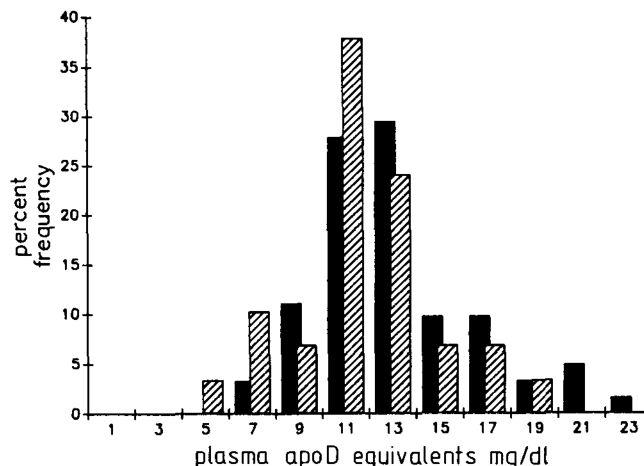

Fig. 1. Frequency distribution of apoD antigen concentration in 61 healthy men (solid bars) and 29 healthy women (hatched bars) measured by radioimmunometric assay with monoclonal antibody 4E11.

Fig. 1, with means and standard deviations in **Table 2**. The mean antigen concentration in men (6.84 μmol apoD equivalents/l, equal to 13.2 mg/dl of the apoD standard) was 12% greater than that in women (6.11 μmol equivalents/l, 11.9 mg/dl), but there were no significant differences (P 0.05) between men and women revealed by either the Student's t test or the Mann-Whitney-U test. Although we found that the 4E11 antigenic site was fully expressed in fresh plasma, it remains to be proven whether or not each of the different cross-reacting proteins has the same or a different affinity for the antibody. Therefore, these results give a first approximation to the amount of antigen in plasma. There were no significant differences between these men and women in their mean plasma cholesterol or triacylglycerol concentrations, but the mean HDL cholesterol concentration in women was higher than in men ($P<0.01$, Table 2). The apoD antigen concentration was significantly correlated with HDL cholesterol concentration in the 61 men ($r = 0.409$, $P<0.01$) but not correlated in women or the group as a whole, or with triacylglycerols.

Detection of antigen on replicas of two-dimensional IEF-SDS-PAGE

We established the conditions for maximum detection of the apoD antigens on replicas of SDS-PAGE. **Fig. 2** shows the effect of storage and delipidation on fresh plasma. In lane 1 MAB 4E11 detected two strong antigens among the total plasma proteins, and a weaker band corresponding to the M_r 29×10^3 apoD (this latter being the apoD described by McConathy and Alaupovic (1, 2)). When this plasma was freshly delipidated with butanol-diisopropyl ether (Fig. 2, lane 2), higher M_r antigenic proteins were detected, which were not seen in lane 1.

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TABLE 2. Mean concentration of lipids and apolipoprotein D antigen in healthy subjects

Fraction	Men (n = 61)	Women (n = 29)
Triacylglycerol (mg/100 ml)	103.9 (50.9)	88.9 (46.2) (ns)
Cholesterol (mg/100 ml)	179.9 (34.3)	177.0 (32.1) (ns)
HDL cholesterol (mg/100 ml)	41.0 (9.5)	54.3 (11.1)*
ApoB antigen (μmol/l)	6.84 (1.81)	6.11 (1.66) (ns)

Values are given as means with standard deviation in parentheses; ns, not significantly different ($P > 0.05$) between men and women; *, significant difference ($P < 0.01$) between men and women.

Different patterns, probably caused by degradation, were observed with plasma stored for 1 or 2 months at 4°C (lanes 3, 5), and most of the immunoreactions were lost from plasma that was delipidated 5 days before being analyzed (Fig. 2, lane 4). However when plasma was stored at −70°C for 2 months and freshly delipidated, no difference from fresh plasma was observed. Antigens of similar M_r were detected in plasma following two-dimensional IEF-SDS-PAGE (results not shown) as were seen following SDS-PAGE alone (Fig. 2), but once again the autoradiographic spots of the higher M_r antigens were more intense in solvent-delipidated plasma. This result was reproduced in a parallel experiment (not shown), and suggested that prior removal of lipids facilitated the electrophoresis of these proteins.

All of the antigens fell within the narrow range of pH 4.71-5.03, and many proteins with different M_r had isoforms with the same pI. Thus, the two-dimensional electrophoresis was essential for complete resolution of these proteins. The most intense spots seen on autoradiography of plasma were invariably of the M_r 38,000 antigen.

Comparison of the pattern of antigens in different subjects

From the set of subjects whose data are shown in Fig. 1 we selected plasmas having extremely high, average, or extremely low concentrations of apoD antigens. The antigens from whole plasma were separated by isoelectric focusing-SDS-PAGE and detected on nitrocellulose replicas using antibody 4E11. Autoradiographs of six plasmas are shown in **Fig. 3**, two of each (one male and one female) having high (A, B), average (C, D), or low (E, F) antigen concentration. In every plasma analyzed the major immunodetectable antigen had M_r 38 × 10^3; apoD M_r 29 × 10^3 was present in all plasmas but less intense on autoradiography. Both of these antigens had at least six isoforms in every plasma. Anti-apoD antigens of M_r between 70 × 10^3 and 94 × 10^3 varied among subjects. In some samples with high levels we detected up to five proteins differing in M_r, each one showing from two to four isoforms (Fig. 3B). Their M_r values have been estimated at 94, 91, 86.5, 84.5, and 75.6 × 10^3. In Fig. 3A, another spot of M_r 71.5 × 10^3 was present. These proteins are numbered 8 to 3, in order of decreasing M_r, in Fig. 3. However, not all plasma samples with high antigen concentrations contained all of these high M_r proteins. In the samples from both subjects with average concentration (Fig. 3C, D) we observed only two of the antigens of higher M_r and barely a third one. In the samples with low concentrations (Fig. 3E, F) we detected only one high M_r antigen, and in one of the two subjects analyzed, barely another antigen, despite the fact that twice as much plasma was electrophoresed to compensate for the low level of apoD antigens (2.85 μmol apoD equivalents/l). We verified that the M_r and pI of the antigens were reproducible by repeating the analyses of some samples.

Density gradient ultracentrifugation

We measured the concentration of apoA-I and apoD antigens and total protein in every fraction of plasma obtained by density gradient ultracentrifugation (**Fig. 4**). After 20 h ultracentrifugation (Fig. 4 A) about 85% of the apoD antigen was present in a single major peak, between d 1.137 and 1.198 g/ml and with a maximum at 1.167 g/ml. The majority, about 90%, of the apoD antigens had floated with the lipoproteins out of the sample application layer. Only 10% of the apoD antigens were detected in the fraction d > 1.21 g/ml, together with the majority of plasma proteins. Three small peaks of protein, not more

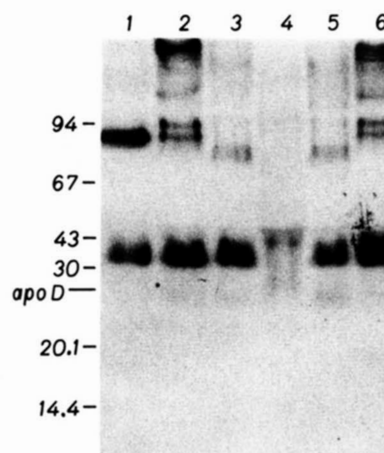

Fig. 2. Effect of storage and delipidation of plasma on its reaction with monoclonal antibody 4E11. An autoradiograph of the reaction of a replica of SDS-PAGE of plasma (10 μl per lane) is shown. Lane 1, fresh plasma; lane 2, fresh plasma, delipidated before electrophoresis; lane 3, plasma stored 1 month at 4°C, delipidated immediately before electrophoresis; lane 4, plasma stored 1 month at 4°C, delipidated 5 days befrore electrophoresis; lane 5, plasma stored 2 months at 4°C, delipidated immediately before electrophoresis; lane 6, plasma stored 2 months at −70°C, delipidated immediately before electrophoresis. The migration of M_r standards is shown at the left.

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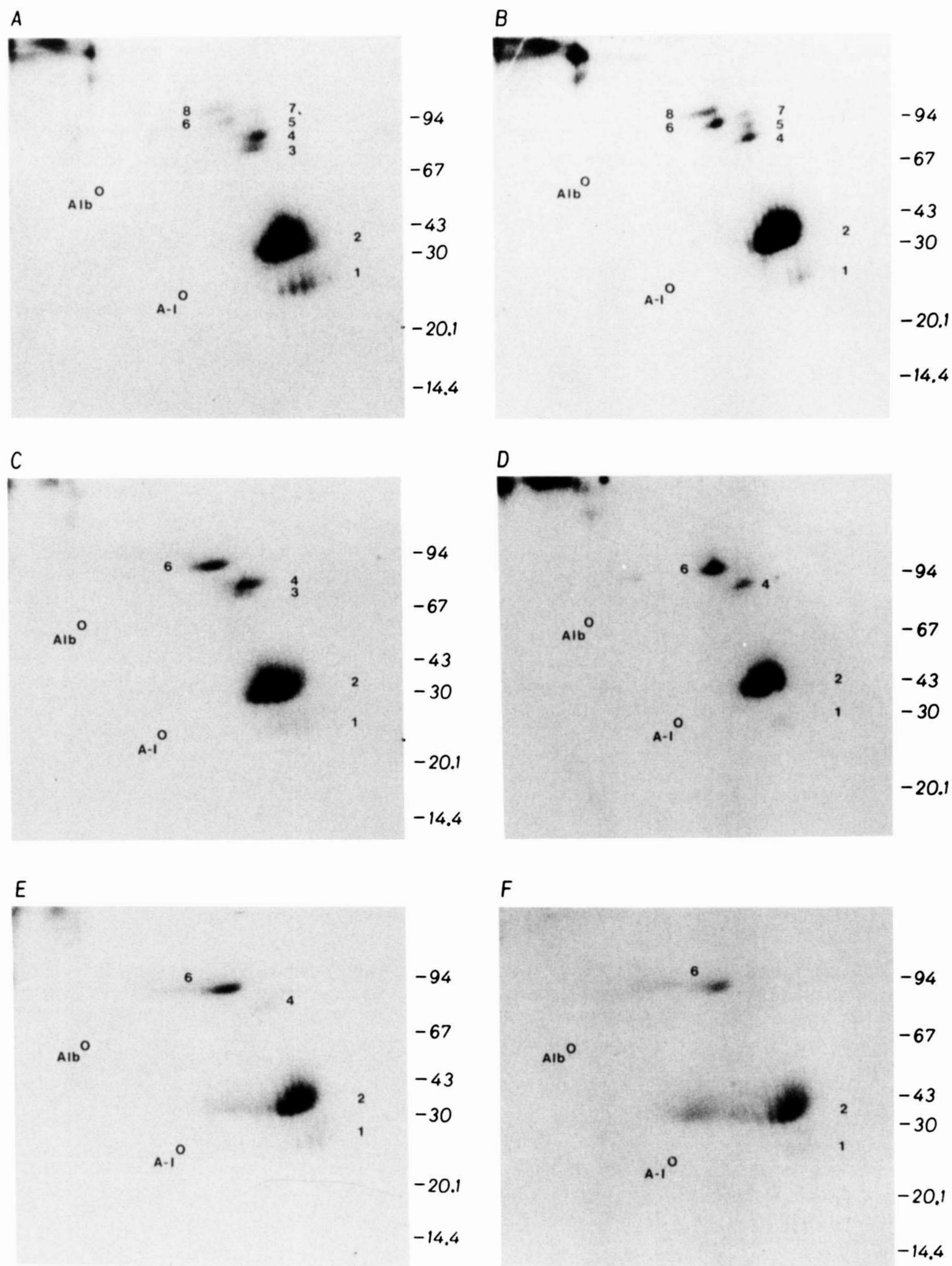

Fig. 3. Plasma from six different individuals was separated by two-dimensional isoelectric focusing SDS-PAGE and replicas were transferred to nitrocellulose sheets and reacted with monoclonal antibody 4E11. The autoradiographs are shown. Panels A, C, E were from males, panels B, D, F were from females. By radioimmunometric assay plasmas contained 8.75, 10.5, 6.11, 6.27, 3.37, 2.90 μmol apoD equivalents/l, in panels A–F respectively. Ten μl plasma was used for panels A–D, 20 μl for panels E, F. The positions of the major forms of albumin and apoA-I were found by staining. ApoD and the 4E11 cross-reacting proteins are numbered 1 and 2–8, respectively. The pH gradient was from left to right (high to low pH) and the immunoreactive proteins were resolved at pH 4.71–5.03.

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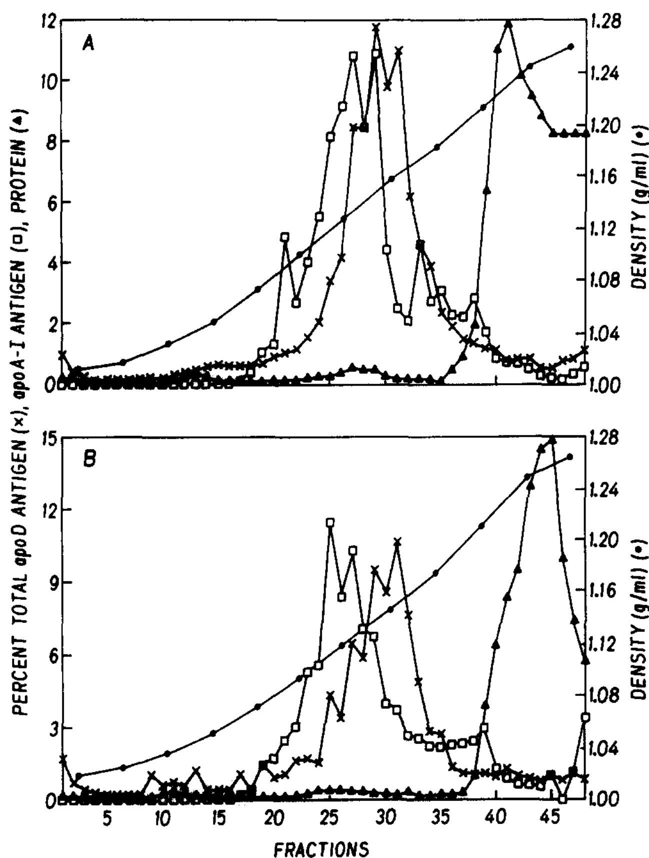

Fig. 4. Radioimmunometric assay of plasma fractions separated by density gradient ultracentrifugation. Panel A shows the results following 20 h ultracentrifugation, panel B shows the results after 40 h. The subject had 6.37 μmol apoD equivalents/l plasma. Fractions of 250 μl from the top of the gradient were analyzed by radioimmunometric assay with monoclonal antibody 4E11 for apoD, and 4H1 for apoA-I. Each fraction is expressed as a percent of the total.

than 1%, were measured at the VLDL, LDL, and HDL fractions. The peak of protein in the HDL coincided with the peak of apoA-I at a density of 1.157 g/ml within a range of 1.117-1.180 g/ml. The same result was found in a sample centrifuged for 40 h (Fig. 4 B), the only difference being a slightly greater separation between the profile of apoA-I and apoD antigen-containing particles.

We found the same distribution of apoD antigens in plasmas from two individuals having widely different total concentrations: 3.99 and 10.5 μmol apoD equivalents/l (**Table 3**). Of the total apoD antigens only 3-6% was found in the VLDL and LDL, and 9-13% in the plasma protein fraction. Forty percent was found in the visible HDL with 43% in the clear layer (VHDL) below the HDL but above the plasma proteins. The recovery of apoD antigens from the density gradient was 96-98%.

Qualitative distribution of apoD antigens in lipoproteins

Gradient gel electrophoresis of lipoproteins. Plasma and lipoprotein fractions from density gradient ultracentrifugation were electrophoresed through polyacrylamide gradient gels (PAGGE) under nondenaturating conditions; **Fig. 5A, C** shows proteins stained in the gels. The VLDL, LDL, and HDL were distinct one from another; lipoprotein size decreased with increasing density; and there was no apparent overlap between the density fractions. The VHDL, however, contained stained bands with the same size as the smallest HDL and other bands including albumin and smaller proteins which were common to the plasma protein fraction. Autoradiography of apoD antigens displayed a heterogeneity of lipoprotein size in each fraction and, in contrast to the stained fractions, showed common bands in the LDL, HDL, and VHDL.

We saw eleven different bands containing apoD antigens in whole plasma (Fig. 5 B, D). Only two bands (bands 5 and 8) were detected in the plasma protein fraction of d > 1.21 g/ml despite equal amounts (492 μg) of plasma and protein fraction being analyzed.

Thus, most of the immunoreactive bands in total plasma were associated with the lipoprotein fractions. Strong immunoreactions were seen in lipoproteins of the HDL and VHDL (Fig. 5 B, D) in accord with the major peak quantitated by RIA of the density gradient (Fig. 4). Comparing HDL and VHDL, the HDL fraction (Fig. 5 B) was enriched in lipoprotein particles of greater size (bands 6, 7, 8, and 9) containing apoD antigens. On a replica from a 2-16% PAGGE, the HDL density class (Fig. 5 D) showed a better separation of this particular group of lipoproteins. However, bands 3, 4, and 5 were enriched in the VHDL density class compared with the HDL, which showed little of the band 4 and none of bands 3 or 5 (Fig. 5 B). Band 4 was almost exclusively enriched in the VHDL fraction.

A high molecular weight band (band 10), which reacted strongly with antibody 4E11, was seen in plasma and LDL at the position of the latter (Fig. 5 A, B). The LDL preparation also contained another immunoreaction

TABLE 3. Percentage of total apoD antigen in lipoprotein fractions separated by density gradient ultracentrifugation

Subject	VLDL[a]	LDL	HDL	VHDL[b]	Plasma Proteins	Whole Plasma
			%			μmol/l
1	0.5	2.6	39.6	43.7	12.6	3.99
2a	2.4	2.1	37.5	46.1	11.9	6.53
2b	1.6[c]	2.6	32.0	51.0	9.0	6.53
3	2.8	3.5	40.0	43.0	10.0	10.52
Mean	1.8	2.7	37.3	46.0	10.9	

[a]VLDL includes the IDL fraction.
[b]VHDL is defined here as the clear colorless layer between the HDL and the plasma protein fraction.
[c]A second sample from the same subject, but collected as 48 fractions from the density gradient ultracentrifugation, with the data pooled after radioimmunoassay.

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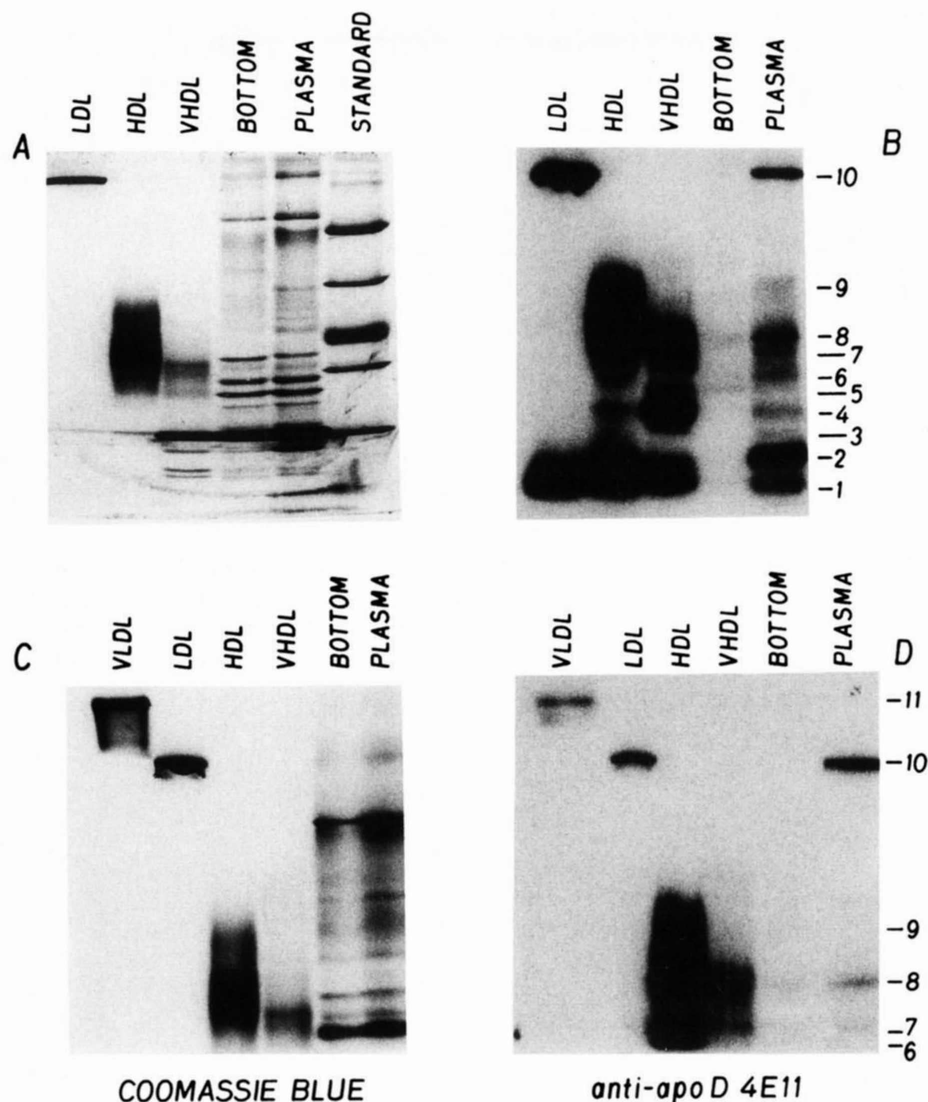

Fig. 5. Polyacrylamide gradient gel electrophoresis of plasma and lipoprotein fractions. Panels A and B show 4-30% acrylamide gels; panels C and D show 2-16% acrylamide. Panels A and C show Coomassie Blue-stained proteins; panels B and D show autoradiographs of the reaction of antibody 4E11. The immunoreactive bands are identified by numbers at the right: 1-11. The amount of protein (μg) applied in each lane was VLDL: 50, 60 (C, D); LDL: 25, 50, 25, 50 (A-D); HDL: 36, 50, 35, 50 (A-D); VHDL: 36, 50, 35, 50 (A-D); bottom (plasma protein fraction): 80, 492, 100, 492 (A-D); plasma: 80, 492, 100, 492 (A-D).

which had the lowest M_r (band 1). When VLDL and LDL were compared on a 2-16% PAGGE the VLDL also reacted with antibody 4E11 (band 11) but band 1 was not seen in either LDL or VLDL, presumably because it migrated out of the gel (Fig. 5 C, and D).

Molecular weight of apoD antigens in lipoprotein classes. Having found that polymorphsim of apoD antigens was common to all of the plasma samples that we examined, we compared the lipoprotein fractions from density gradient ultracentrifugation using IEF-SDS-PAGE replicas reacted with antibody 4E11. These experiments were to determine which antigens were associated with each lipoprotein class. **Fig. 6 A-D** shows, respectively, autoradiographs of the HDL, VHDL, LDL, and plasma protein fractions. The VHDL (Fig. 6 A, B) contained each of the antigenic proteins seen in plasma (Fig. 3). The distribution of the antigens in HDL and VHDL was different: VHDL was always enriched in the higher M_r proteins (M_r 70,000-94,000) in contrast to HDL which was richer in the M_r 38,000 protein and apoD (M_r 29,000). This difference has been observed repeatedly. The plasma protein fraction (d > 1.21 g/ml, Fig. 6 D) contained only one detectable antigenic protein, having M_r 94,000, and no apoD was detected. In contrast, the LDL contained prin-

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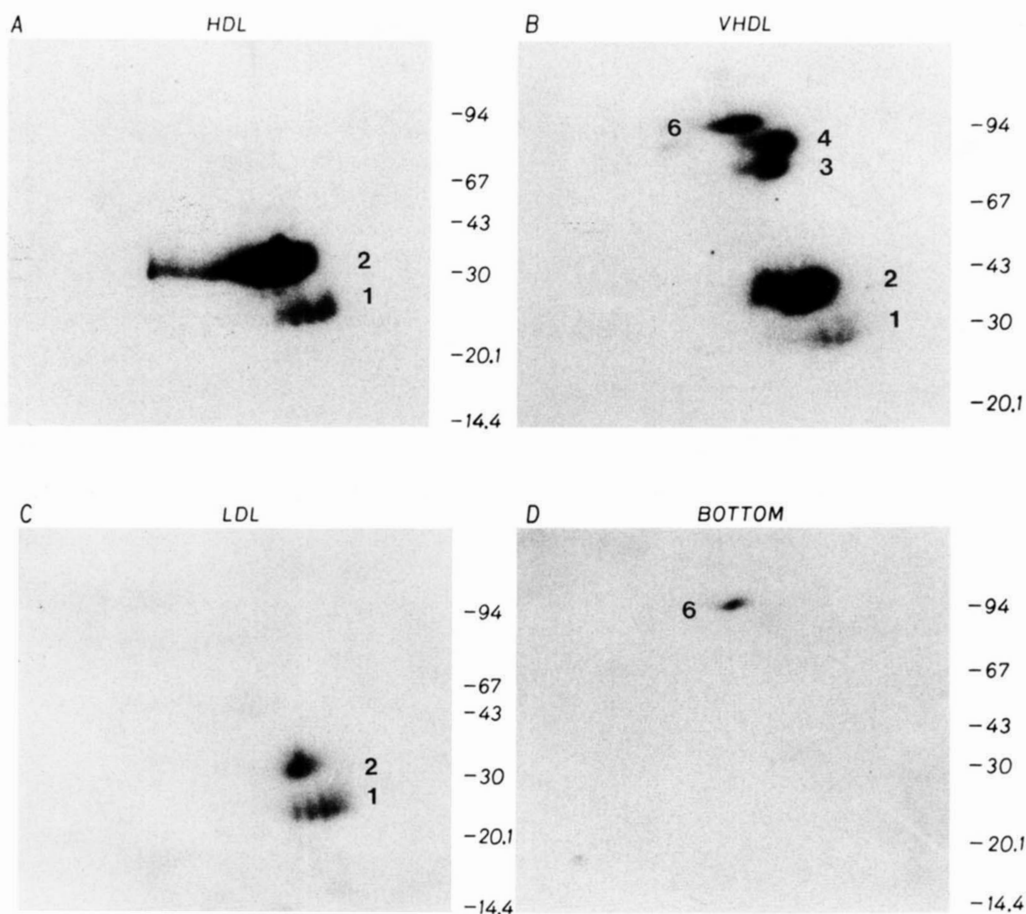

Fig. 6. Autoradiograph of the anti-apoD 4E11 immunoreactive proteins present in LDL (300 μg), HDL (150 μg), VHDL (150 μg), and the bottom (plasma protein, 1.5 mg) fraction, separated by two-dimensional isoelectric focusing SDS-PAGE. The low pH is to the right, and the migration of M_r standards is shown at the right.

cipally apoD (M_r 29,000), some less intense immunoreactions of the M_r 38,000 protein, but none of the components with M_r 70,000–94,000 were detected. On some replicas of SDS-PAGE, antibody 4E11 reacted with proteins that barely entered a 15% polyacrylamide separating gel. The immunoreaction of VLDL was similar to that of LDL but usually less intense on autoradiography.

We investigated the high M_r reactions in LDL further. On replicas of SDS-PAGE (4% acrylamide) of LDL we repeatedly found that the high M_r band identified as apoB-100 by its reaction with anti-apoB 1D1 also reacted with anti-apoD 4E11. In one sample we identified another band as apoB-74 by its reaction with anti-apoB 4G3 (20) and found that this too reacted with anti-apoD 4E11. In addition, each LDL contained a lower M_r band at the electrophoretic front that reacted with 4E11 and corresponded to the apoD seen in Fig. 6.

To study the nature of the apoB-100 band that cross-reacted with antibody 4E11 we performed the experiment shown in **Fig. 7.** Fig. 7 B shows the reaction of unreduced apoLDL with anti-ApoD 4E11 after migration in 4% acrylamide from left to right. The intense band at the left is typical of the band that cross-reacted with anti-apoB 1D1. The less intense band at the right is free apoD at the electrophoretic front. Fig. 7 C shows a similar electrophoresis, but reduced apoLDL was applied to the gel and reaction was with anti-apoD 5G10 (which reacts better with reduced than with nonreduced antigen). Here the major reaction was at the electrophoretic front with a low M_r protein.

The separation between the two antigens of high and low M_r can be seen in Fig. 7 A, not reduced and reacted with 4E11. ApoLDL was separated by a first dimension electrophoresis as in Fig. 7 B; then a strip was applied to a 15% acrylamide gel for migration at 90° to the first dimension. The strong reaction with high M_r at the interface of the stacking gel (s.g.) and the weaker reactions with M_r of apoD and the M_r 38,000 protein can be seen clearly in Fig. 7 A.

Finally, we repeated the experiment shown in Fig. 7 A but this time we incubated the strip of gel with 5% mercaptoethanol after the first dimension electrophoresis and before the second dimension. The immunoreaction was

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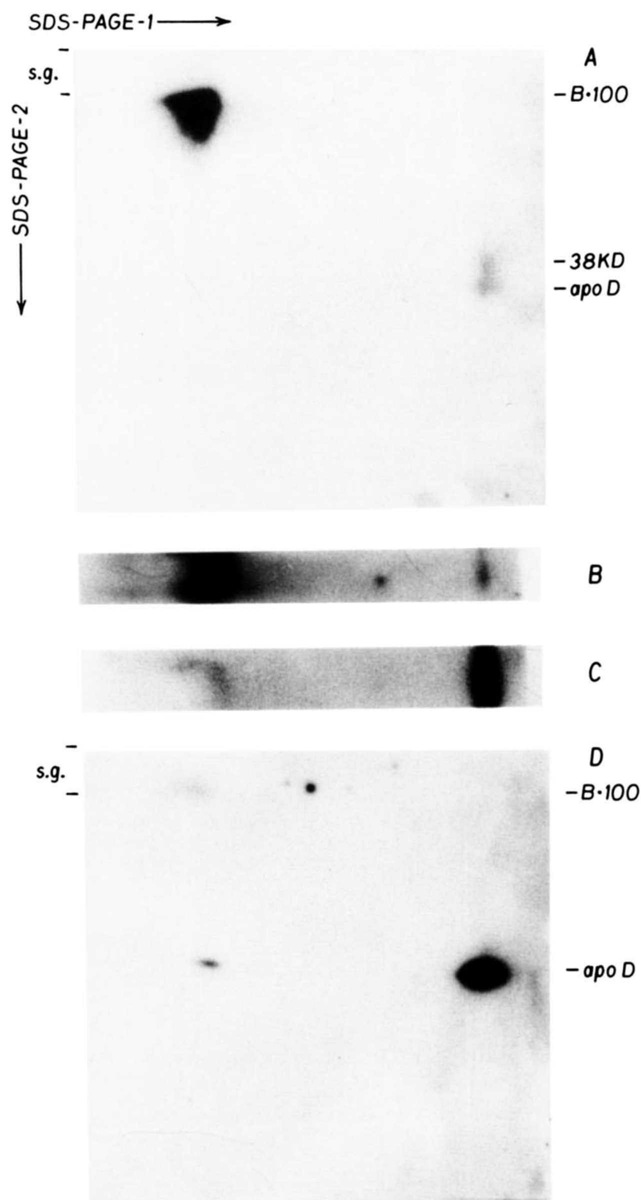

Fig. 7. Delipidated LDL was electrophoresed in a 4% acrylamide SDS-PAGE from left to right and a replica of this first dimension reacted with 4E11 is shown in panel B. A similar strip of gel was subjected to a second dimension SDS-PAGE in 15% acrylamide (at 90° to the first direction) and its reaction with 4E11 is shown in panel A. Panel C shows a replica of a different first dimension gel of mercaptoethanol-reduced LDL, reacted with anti-apoD 5G10. Panel D shows a replica of a two-dimensional SDS-PAGE (4%/15% acrylamide as in 7 A): the proteins were not reduced for the first dimension (which resembled panel B) but were reduced with 5% mercaptoethanol between the first and second dimension SDS-PAGE while in the strip of gel which was being reequilibrated for the second SDS-PAGE. The immunoreaction was with anti-apoD 5G10 to detect reduced apoD. The position of the stacking gel of the second SDS-PAGE is indicated by s. g.

made with anti-apoD 5G10. The effect of this reduction was to produce a new immunoreactive spot, with the M_r of apoD (Fig. 7 D), below the apoB–D spot that was seen before. Only a trace of immunoreaction was seen with anti-apoD 5G10 at high M_r after reduction. Thus, this immunoreaction at high M_r was due to a mixed disulfide dimer of apoD and apoB. The other effect of reduction was to greatly increase the immunoreaction of 5G10 with the apoD that had migrated as a free protein at the electrophoretic front of the first dimension, and was seen in the same position at the right in Fig. 7 D as in Fig. 7 A. We do not know why anti-apoD 4E11 reacted so strongly with the apoB-100–apoD complex (Fig. 7 A) when 5G10 reacted much more weakly with the apoD released from this complex by reduction with mercaptoethanol (Fig. 7 D). It may be due to differences in the efficiency of electrotransfer or trapping of the transferred proteins on the nitrocellulose.

Retinol-binding protein

Three samples of plasma and one sample of HDL were subjected to SDS-PAGE, transferred to nitrocellulose sheets, and incubated with anti-apoD 4E11 and rabbit anti-human retinol-binding protein followed by the appropriate ^{125}I-labeled anti-IgG. By autoradiography we found three bands of 4E11-antigen in all samples as expected. Retinol-binding protein was not detected in HDL, but was detected as a single band of M_r 19,800 in plasma (results not shown). Thus, there was no cross-reaction between anti-retinol-binding protein antibodies and any of the 4E11-antigenic proteins, and retinol-binding protein was not found in the HDL, which is rich in 4E11-antigenic proteins.

DISCUSSION

We have detected apoD antigens by radioimmunometric assay in the plasma of each of 90 healthy subjects, in concentrations varying across a fourfold range from 3 to 11 μmol equivalents of apoD/l (Fig. 1, Table 2). The antigenic site recognized by the monoclonal antibody 4E11 was entirely expressed in native plasma, as were the antigenic sites recognized by the antisera used by other investigators (7,21). No treatment produced a large increase in apparent plasma antigen concentration but delipidation produced a 25% decrease. Albers et al. (7) showed that 17% of apoD was lost, presumably precipitated, during delipidation. The average values that we measured in plasma (equivalent to 13.2 and 11.8 mg/dl for men and women, respectively) were similar to the values of 12–14 mg/dl found by three independent laboratories (21–23), but almost twice the value measured by the other groups (7, 24). The reason for this is not obvious, but it may reflect a difference in the specificity of antisera, as has been noted for apoA-I. Our finding of a greater average apoD antigen concentration in men than in women confirms previous reports (7, 25) although only once was it statistically significant.

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In each plasma that we tested by IEF-SDS-PAGE there was a heterogeneity in the proteins carrying the 4E11 apoD antigenic site. This included men and women and plasmas with low, average, or high apparent concentrations of antigen. While we found some individuals with a simple pattern of antigenic proteins (resembling Fig. 3 E, F) despite a high apparent concentration of antigen in plasma, the most complex patterns of antigen on IEF-SDS-PAGE, with up to eight proteins resolved (Fig. 3 A, B), were found in subjects with high concentrations. The apparent difference between plasmas from different subjects lay in the presence or absence of the antigenic proteins with M_r 70,000–94,000. ApoD and the M_r 38,000 cross-reacting proteins were seen in each plasma tested; the immunoreaction of the M_r 38,000 antigen was always the strongest one seen. Sprecher, Taam, and Brewer (26) noted the presence of six isoforms of apoD in plasma at a pI similar to those we describe here, but they did not comment on finding any other antigenic proteins. Thus, the concentration of apoD that we measured was the same as that measured by other laboratories, but we have demonstrated that in this measurement our monoclonal antibody recognized a polymorphic set of proteins in each subject, rather than only apoD. This is the first report of an extensive test of the qualitative specificity of anti-apoD with whole plasma.

The concept of polymorphism or heterogeneity can be extended to include the distribution or partition of these 4E11-antigenic proteins among the fractions of plasma lipoproteins and plasma proteins. Is the distribution the same for each of them and identical to that of apoD, or are there different polymorphic profiles of distribution of each protein? Immunometric assay of fractions from density gradient ultracentrifugation and immunoreaction of replicas of PAGGE showed that essentially all of most of the antigenic proteins were bound to lipoproteins, as was apoD. Although apoD was present in all lipoprotein density classes, the M_r 38,000 antigenic protein was found mainly in the HDL and VHDL, and the M_r 70,000–94,000 antigenic proteins were confined to the HDL and VHDL. Thus, each different M_r antigen appeared to have an individual distribution among the lipoprotein density classes.

The 4E11-antigenic lipoproteins were heterogeneous in size and we observed a small M_r band in each of the lipoprotein fractions, seemingly separate from the major bands of lipoproteins and so far unnoticed and not characterized in the literature. It is conceivable that this small M_r band was composed of apoD or the M_r 38,000 protein which was stripped off its parent lipoprotein during nondenaturing PAGGE, having remained in the LDL, HDL, and VHDL during the period of force of ultracentrifugation. This band was seen in plasma, so we cannot dismiss it as a simple artefact of centrifugation. Either it was an artefact of PAGGE or it represents lipoprotein having LDL, HDL, or VHDL properties. We found that 90% of the apoD antigen floated out of the sample during ultracentrifugation, in agreement with James et al.(23). The earlier results of Curry, McConathy, and Alaupovic (21) showed that only 64% of apoD was in the HDL. We explain this discrepancy as follows: The majority of apoD antigen is distributed in a single, large peak which spans the HDL and the clear layer between the HDL and plasma protein fraction (Fig. 4). Following sequential ultracentrifugation we also found that the analogous same clear layer contained cross-reacting proteins of the same type as reported above (Weech, P. K., Y. L. Marcel, and R. W. Milne, unpublished observations). By conventional wisdom the floating yellow-colored layer would be taken as lipoprotein and the clear layer below would be pooled with the plasma proteins. Thus a major part of the major peak of apoD antigen would be assigned to the plasma protein fraction, although as we have shown, there is a unimodal major distribution of apoD and cross-reacting proteins centered over a minor part of the HDL. We did find about 10% of the apoD antigen in the plasma protein fraction, and we cannot exclude the possibility that this was detached from lipoproteins during centrifugation. However, this 10% was composed entirely of only one cross-reacting protein corresponding to the highest M_r 4E11 antigen present in the lipoprotein fractions. Finding almost all of the apoD antigens concentrated in the smallest densest HDL indicates that they all possess lipid or lipoprotein-binding properties.

Other authors have noted that trace amounts of apoD were found in the VLDL and LDL (7,21). We have shown that this antigen is in two states, the one free, but the other associated to apoB-100 or B-74 by an intermolecular disulfide bond. This observation is in some ways analogous to the description of Lp[a] as a mixed disulfide complex of apolipoprotein [a] with apoB-LDL (27–29). Our previous experiments with the HDL (4) were unable to show that mixed disulfide complexes were responsible for the anti-apoD cross-reacting proteins.

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