Radioimmunoassay of Human Arginine-rich Apolipoprotein, Apoprotein E

CONCENTRATION IN BLOOD PLASMA AND LIPOPROTEINS AS AFFECTED BY APOPROTEIN E-3 DEFICIENCY

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ABSTRACT A radioimmunoassay for apolipoprotein E in human blood serum has been developed that measures equally the major polymorphic species of the protein (apolipoproteins E-1, E-2, E-3, and E-4) and the apo E in the dimer of apolipoproteins E and A-II. The assay is specific and yields values for apolipoprotein E in very low density lipoproteins that agree closely with those obtained by a quantitative electrophoretic method. Apolipoprotein E is also present in at least one species of high density lipoprotein, but the content of apolipoprotein E in the lipoprotein fractions of plasma is uncertain owing to dissociation during ultracentrifugation. The concentration of apolipoprotein E is higher in serum of normolipidemic, premenopausal women than in men of comparable age and is a direct function of the serum triglyceride level. Apolipoprotein E levels are increased out of proportion to triglyceride levels in hyperlipidemic patients with familial dysbetalipoproteinemia (homozygotes for lack of apolipoprotein E-3). Heterozygous relatives of homozygotes have significantly higher apolipoprotein E levels in serum than unaffected relatives. The concentration of partially degraded (remnant) triglyceride-rich lipoproteins also appears to be increased in heterozygotes, who comprise about 15% of the population.

INTRODUCTION

The arginine-rich apolipoprotein of human plasma lipoproteins (apo E)[1] accumulates in the abnormal beta-migrating very low density lipoproteins of patients with familial dysbetalipoproteinemia (1) in which two of the commonly occurring isoforms are lacking (apo-E-3 and apo E-4) (2). Utermann and his associates (3) have shown that ~1% of all individuals (who have dysbetalipoproteinemia but rarely hyperlipidemia) are homozygous for a gene that specifies lack of apo E-3 and apo E-4; about 15% of all individuals have one gene that specifies this state. This observation suggests that (*a*) the distinctive protein polymorphism is responsible for dysbetalipoproteinemia and (*b*) apo E has a central role in the normal metabolism of "remnants" of triglyceride-rich lipoproteins (4). In addition, Mahley and Innerarity (5) have found that apo E from various mammals, like apolipoprotein B, is recognized by the low density receptor system in cultured human fibroblasts.

We have developed a radioimmunoassay for human apo E, based upon a previously reported radioimmunoassay for this protein in the rat (6). This assay recognizes each of the major isoforms of human apo E equally and has been validated for apo E of very low density lipoproteins by comparison with a quantitative electrophoretic technique (7). Application of the assay to individuals with different phenotypes shows that abnormalities in the concentration and distribution of apo E in plasma lipoproteins are expressed both in individuals with familial dysbetalipoproteinemia and their heterozygous relatives.

METHODS

Sources of blood serum. A subset of serum samples, frozen at −20°C, from an epidemiological survey of an industrial

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[1] *Abbreviations used in this paper:* apo A-I, II, apoprotein A-I, II; apo E, apoprotein E; HDL, high density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; SDS, sodium dodecyl sulfate; TMU, tetramethylurea; VLDL, very low density lipoprotein.

population (8) was selected to obtain normative data on serum apo E concentration. Normolipidemic laboratory personnel and hyperlipidemic patients from the University of California Lipid Clinic were selected for analysis of apo E in serum and lipoprotein fractions. Serum was also obtained from all available first-degree relatives of 10 patients with familial dysbetalipoproteinemia. All blood samples were obtained from antecubital veins of subjects who had fasted 9–15 h.

Materials. Tetramethylurea (TMU), obtained from Burdick & Jackson Laboratories, Inc., Muskegon, Mich. was redistilled to give a pH of 6–7, when diluted with an equal volume of water. Human serum albumin was from Schwarz/Mann, Div., Becton, Dickinson & Co., Orangeburg, N. Y. and bovine serum albumin was from Research Products International Corp., Elk Grove Village, Ill. Pooled serum standard (Sercal No. 5) was from the Clinical Chemistry Division of the Bureau of Laboratories of the Communicable Disease Center, Altanta, Ga.

Preparation of lipoproteins. Lipoproteins were separated from serum by sequential preparative ultracentrifugation (9) or by gel filtration at 7°C through 2 × 90-cm columns of Sephadex G-200 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.), equilibrated with 0.15 M NaCl, 0.004 M disodium EDTA, and 0.002% NaN_3, pH 7.4.

Lipid and protein analysis. Concentration of cholesterol and triglyceride in serum and ultracentrifugal fractions was determined by an automated method (10). Concentration of cholesterol in fractions of serum obtained by gel filtration was determined by an enzymatic procedure (11). For analysis of lipoprotein-proteins by chemical and electrophoretic methods, lipoproteins were purified and concentrated by recentrifugation at the upper density limit. Low density lipoproteins (LDL), high density lipoproteins (HDL), and the 1.21-g/ml infranatant solution of serum proteins were dialyzed at 7°C against 0.15 M NaCl containing 0.004 M disodium EDTA and 0.002% NaN_3, pH 7.4. Concentration of protein in lipoprotein fractions was determined by the Lowry method (12), with bovine serum albumin as standard, and concentration of apo E in apoproteins of very low density lipoproteins (VLDL) obtained by delipidating VLDL with No. 1 ethanol/ether, 3:1 (13) was determined by quantitative densitometric analysis of polyacrylamide gel electrophoretograms stained with Amidoschwarz (7).

Distribution of isoforms of apo E was determined by analytical isoelectric focussing polyacrylamide gel electrophoresis, with a pH gradient of 3.5–7.0, as described (14). In some samples, disulfide dimers and larger complexes of apo E in apo-VLDL were reduced by incubating ~20 μg of protein in a volume of 50 μl 0.01 M Tris–HCl, pH 8.2, containing 1% sodium decyl sulfate, with 5 μl of 2-mercaptoethanol for 30 min. Up to 5 μg of each isoform applied to the gel yielded linear densitometric response to the Coomassie Blue-stained gels. Sodium dodecyl sulfate (SDS) gel electrophoresis in 10% polyacrylamide was performed by the method of Weber and Osborne (15).

Preparation of apo E and its isoforms. VLDL, obtained from patients with endogenous hyperlipemia or familial dysbetalipoproteinemia, were purified by a second ultracentrifugation under the original conditions. The VLDL were delipidated with ethanol/ether, 3:1 (13) or with TMU. For the latter, VLDL containing 1.5–2.0 mg protein/ml were mixed vigorously with an equal volume of TMU and incubated at 37°C for 30 min. The insoluble lipid-protein matrix was removed by centrifugation at 1,000 *g* for 30 min at room temperature. 2 vol of diethyl ether were added to the soluble proteins, mixed gently, and the phases were then separated by low speed centrifugation. The TMU partitions preferentially into the ether phase. Addition of ether was repeated several times until the original aqueous volume was restored (at this point further extraction with ether removes residual traces of TMU and apo E tends to precipitate at the interface). Solid urea was added to a final concentration of 6 M and the sample was concentrated by ultrafiltration with a microporous membrane (UM-2, Amicon Corp., Scientific Sys. Div., Lexington, Mass.) to a vol of about 5 ml. This method of delipidation proved to be rapid and did not lead to selective loss of isoforms of apo E, as judged from densitometric scans of isoelectric focussing gel electrophoretograms. The concentrated protein solution, delipidated by either method, was applied to a 2 × 90-cm column of Sephadex G-200 equilibrated with 0.015 M Tris HCl, pH 8.2, in 6 M urea. The column was run at a rate of about 10 ml/h and fractions of 3 ml were collected. Apoprotein B and aggregated apo E eluted in or close to the void volume. Purity of the fractions was evaluated by SDS and isoelectric focusing gel electrophoresis. The pooled fractions containing monomeric apo E were usually concentrated by ultrafiltration and then dialyzed against 0.05 M phosphate, pH 7.8. Sodium decyl sulfate was added to give a concentration of 0.1 M. Alternatively, the pooled fractions were dialyzed against distilled water, lyophilized, and the protein was dissolved in 0.1 M sodium decyl sulfate in 0.05 M phosphate, pH 7.8. All preparations were stored at −70°C. Purity of the protein and its concentration were determined by amino acid analysis (16).

Isoforms of apo E were prepared from VLDL of patients whose apo E included apo E-4 (variant trait [17]). The VLDL were delipidated with ethanol/ether 3:1 (13) and the protein pellet was dissolved in a small volume of 0.5% sodium decyl sulfate, 0.01 M Tris HCl, pH 8.3 containing 0.1 vol of beta-mercaptoethanol to give a concentration of 12–15 mg/ml. Isoforms were obtained by preparative isoelectric focusing electrophoresis of 10–12 mg of apo VLDL on a flat bed of Ultradex (LKB, Produkter, Bromma, Sweden) with 1.6% Ampholines, pH 5.0–7.0 and 1% beta-mercaptoethanol, as described (18). The purity of each component, eluted with the decyl sulfate buffer, was verified by analytical isoelectric focussing gel electrophoresis. Ampholines were removed by precipitation with trichloroacetic acid (18) or by passing the protein solution through a column of Sephadex G-50. Precipitation with trichloroacetic acid had no effect upon the isoelectric point of the protein components in analytical focussing gels. Protein concentration was determined by amino acid analysis (16). Cysteine was determined by the method of Hirs (19). NH_2-terminal amino acids were determined by dansylation. After a 5-h hydrolysis at 105°C in 5.9 N HCl the dansyl amino acids were identified by chromatography on polyamide sheets using two solvent systems (water/formic acid, 100:1.5; ethyl acetate/methanol/acetic acid, 20:1:1).

Preparation of antisera. Antisera were prepared in rabbits with a schedule described previously (20). Specificity was evaluated by double immunodiffusion (21), and by radioimmunoassay. Goat anti-rabbit IgG was prepared as described (20).

Preparation of ^{125}I-labeled apo E. Phosphate-buffered decyl sulfate solutions of apo E were iodinated by the chloramine T method as described (6). Aggregated or degraded protein and free ^{125}I were separated from the labeled apo E on a 0.9 × 50-cm column of Sephadex G-200, equilibrated with phosphate-buffered decyl sulfate containing 0.1% bovine serum albumin. Antigen-binding was determined (6) with material eluting with the major protein peak. Labeled apo E was kept for up to 1 mo, but was repurified weekly by column chromatography.

Radioimmunoassay procedure. The procedure was the same as that described for radioimmunoassay of rat apo E (6), but with a final concentration of decyl sulfate of 9 mM.

Statistical methods. Significance of differences between means was determined by Student's *t* test (unpaired), with

log transformation of skewed data or by the rank-sum test. Comparisons requiring adjustment of related variables were evaluated by analysis of covariance.

RESULTS

Apo E. Chromatographic preparations of apo E from hyperlipemic subjects with differing apo E phenotypes appeared as a single protein band in SDS gel electrophoretograms and had indistinguishable amino acid compositions (Table I). The four major isoforms of apo E from subjects with the variant trait migrated as single bands in isoelectric focussing gels (Fig. 1) and each had a similar amino acid composition (Table I). Content of cysteine was 0.6–0.7 mol % in each isoform, indicating a cysteine content of 2 mol/mol protein, assuming a molecular weight for apo E of 33,000 (22). Lysine was identified as the NH_2-terminal amino acid in each of the isoforms and in apo E from a patient with familial dysbetalipoproteinemia.

Qualitative evaluation of antisera. Preparations of apo E from subjects with familial dysbetalipoproteinemia, with the common apo E pattern and with the variant apo E trait, which contained two, three, and four major apo E components, respectively, on analytical isoelectric focussing gels, each reacted with identity by double immunodiffusion against antisera prepared against apo E from subjects with each of these apo E phenotypes. Isolated isoforms E-1, E-2, E-3, and E-4 also reacted with a single precipitin line of identity against antibodies to mixed forms of apo E

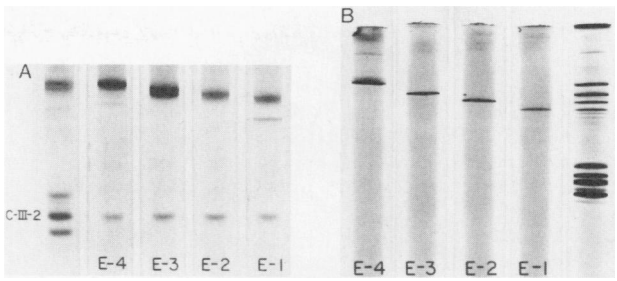

FIGURE 1 Polyacrylamide gel electrophoretograms of apo VLDL and isoforms of apo E from a hypertriglyceridemic individual with the variant apo E trait. (A) Group of five gels was run in 6 M urea at pH 8.6; apo C-III-2 was added to samples of isoforms to define precisely their differing mobilities (R_f values relative to apo C-III-2 for apo E-4, E-3, E-2, and E-1 were 0.205, 0.235, 0.265 and 0.285, respectively). (B) Group of five gels run in an isoelectric focussing system, pH 3.5–7.0; beta-mercaptoethanol was added to the apo VLDL applied to these gels to eliminate disulfide-multimers of apo E and apo E-A-II dimer.

TABLE I
Amino Acid Composition of Apo E

Source	Unfractionated Apo E			Isoforms‖			
	Homozygous normal* (n = 2)	Familial dysbetalipoproteinemia‡ (n = 7)	Variant trait§ (n = 2)	E-4 (n = 3)	E-3 (n = 3)	E-2 (n = 3)	E-1 (n = 2)
				mol/10³ mol			
Lys	49.0±0.0	46.0±1.7	46.5±2.1	44.7±2.1	41.3±0.6	45.3±2.3	45.0±1.4
His	9.0±1.4	7.1±0.7	8.5±0.7	9.0±1.0	8.0±0.0	9.3±0.6	8.0±0.0
Arg	106.0±8.4	104.3±7.5	105.5±0.7	112.7±0.6	111.7±0.6	110.0±4.6	108.0±2.0
Asp	48.5±2.1	46.9±5.3	45.5±2.1	50.3±1.2	48.7±2.1	55.3±5.5	55.0±1.4
Thr	40.5±2.1	40.9±3.3	41.0±1.4	40.0±0.0	38.7±2.1	40.3±2.5	41.5±2.1
Ser	50.0±2.8	50.7±3.5	54.0±2.8	52.3±4.6	49.6±4.1	51.7±3.2	51.5±3.5
Glu	231.5±4.9	239.3±9.4	228.5±16.0	224.0±3.5	233.0±8.2	218.0±13.0	223.5±3.5
Pro	29.0±1.4	29.7±2.6	30.5±4.9	29.0±1.0	29.0±0.0	31.3±1.5	30.0±2.8
Gly	61.0±1.4	60.7±4.3	64.5±4.9	64.7±2.3	62.0±1.7	67.0±2.6	65.5±6.3
Ala	113.5±0.7	119.1±4.9	118.0±1.4	117.0±4.0	122.3±4.0	115.7±2.5	117.0±2.8
Val	68.0±4.2	68.4±4.1	65.0±1.4	66.7±4.2	69.0±2.6	64.3±9.3	68.5±0.5
Ile	11.5±0.7	7.4±1.7	9.5±0.7	9.3±0.6	8.3±1.2	11.0±1.0	8.5±2.1
Leu	122.5±3.5	123.6±5.9	122.0±4.2	121.7±1.5	122.3±4.7	120.3±4.7	121.5±0.7
Tyr	16.0±0.0	15.0±1.4	16.5±0.7	15.3±0.6	14.3±0.6	15.6±1.5	14.5±0.7
Phe	15.5±0.7	14.3±3.0	16.5±3.5	15.3±0.6	13.7±0.6	16.0±1.7	14.5±0.7

Values are mean±SD. Contents of methionine and cysteine are assumed to be 22 and 6 mol/10³ mol, respectively, based upon single analyses of each of the four isoforms, which agreed within ±8%.

* Apo E-3: apo E-2 > 1.15 (see footnote 2).

‡ Apo E-3: apo E-2 < 0.20 (apo E-3 usually undetectable).

§ Distinct apo E-4 component present.

‖ Prepared from hyperlipemic patients with variant trait.

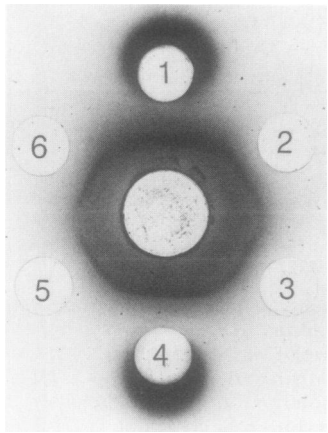

FIGURE 2 Double immunodiffusion of apoproteins against rabbit anti apo E from a patient with familial dysbetalipoproteinemia (center well). Other wells contained: (1) apo VLDL with four major apo E components (variant trait) from a hypertriglyceridemic individual; (2) apo E-4; (3) apo E-1; (4) apo VLDL from a patient with familial dysbetalipoproteinemia; (5) apo E-3; and (6) apo E-2.

(Fig. 2). All antisera gave a single precipitin line against apo VLDL.

Antigen-binding. Antisera to apo E routinely had maximal binding capacities of 70–90%, when tested in 9 mM decyl sulfate (Fig. 3). Low concentrations of decyl sulfate resulted in reduced maximal binding and concentrations exceeding 9 mM inhibited binding.

Standard displacement curve. In the standard immunoassay, optimal sensitivity was obtained with 9 mM decyl sulfate. Over a period of 15 mo, with 10 preparations of ^{125}I-apo E, the mean value for B/B_0 = 0.5 was 0.66±0.077 (SD) μg (Fig. 4). Human serum albumin, apoprotein A-I (apo A-I) and apoprotein A-II (apo A-II) had virtually no detectable displacement capacity up to an excess of 100 times the amount of apo E that gave 50% displacement (Fig. 4).

Reactivity of lipoproteins and serum. Serum, VLDL, HDL, and the 1.21-g/ml infranatant fraction of serum proteins all had identical curves of displacement capacity and were parallel to those obtained with apo E (Fig. 4). Reactivity of serum and lipoproteins delipidated with ethanol-ether 3:1 was almost identical to that of the undelipidated materials. The mean concentration of apo E in 19 samples of apo VLDL was 4.3±6.1% lower than that of undelipidated VLDL. Addition to apo E of emulsified triglycerides in the form of Intralipid had no effect at mass ratios of triglyceride/apo E up to 500:1, but ratios of 5,000:1 or greater inhibited recognition of the protein by up to 50%.

Reactivity of apo E in VLDL was further evaluated by comparison of radioimmunoassayable apo E with estimation of apo E by quantitative polyacrylamide gel electrophoresis in samples of VLDL from 25 subjects, including 14 with apo E-3 but no apo E-4 (of whom 3 here heterozygous for lack of apo E-3), 7 with apo E-4, and 4 who lacked apo E-3 and apo E-4 (familial dysbetalipoproteinemia). As shown in Fig. 5, there was good agreement between apo E concentrations determined by these two methods, with no evident difference in subjects with differing apo E phenotypes.

Reactivity of isoforms of apo E and apo E-apo A-II dimer. Fig. 6 shows the displacement of ^{125}I-apo E in the radioimmunoassay by the four isoforms of the protein and by unfractionated apo E with antisera against apo E from a subject with the variant trait and apo E-3. Reactivities of all forms of apo E were virtually identical with each antiserum. Apo E from subjects with familial dysbetalipoproteinemia usually contains appreciable amounts of material that focusses near and below E-1 and is dissociated in part by treatment of apo VLDL with beta-mercaptoethanol (Fig. 7). Purified apo E (prepared without beta-mercaptoethanol) from a patient with familial dysbetalipoproteinemia that contained an unusually large amount of this material reacted with anti-apo A-II in double immunodiffusion, after reduction of the apo E preparation with beta-mercaptoethanol. However, reactivity of this preparation of apo E in the radioimmunoassay was identical to that of other apo E standards. Fractions of this preparation of apo E from a column of Sephadex G-200 were evaluated by SDS-gel electrophoresis and by radioimmunoassay. All fractions

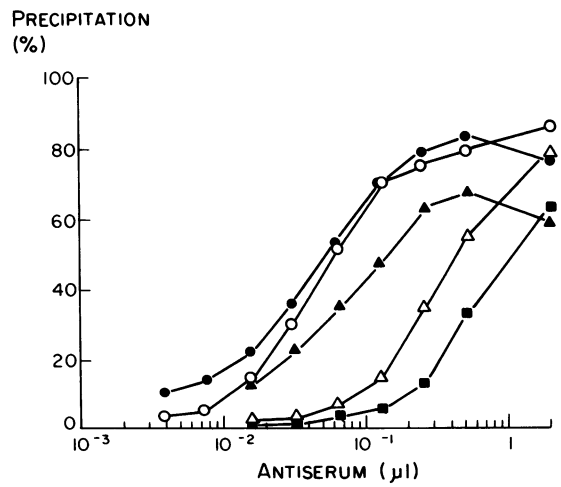

FIGURE 3 Precipitation of ^{125}I-apo E by rabbit antiserum to apo E from a patient with the apo E variant trait in the presence of different concentrations of sodium decyl sulfate: ○, 9.0 mM; △, 13.6 mM; ●, 4.5 mM; ■, 18.0 mM; ▲, 2.3 mM. Ordinate values represent the percentage of radioactivity of washed precipitates related to the total amount of radioactivity introduced into each tube (nonspecific precipitation by normal rabbit serum, amounting to 2–3%, was subtracted in all cases).

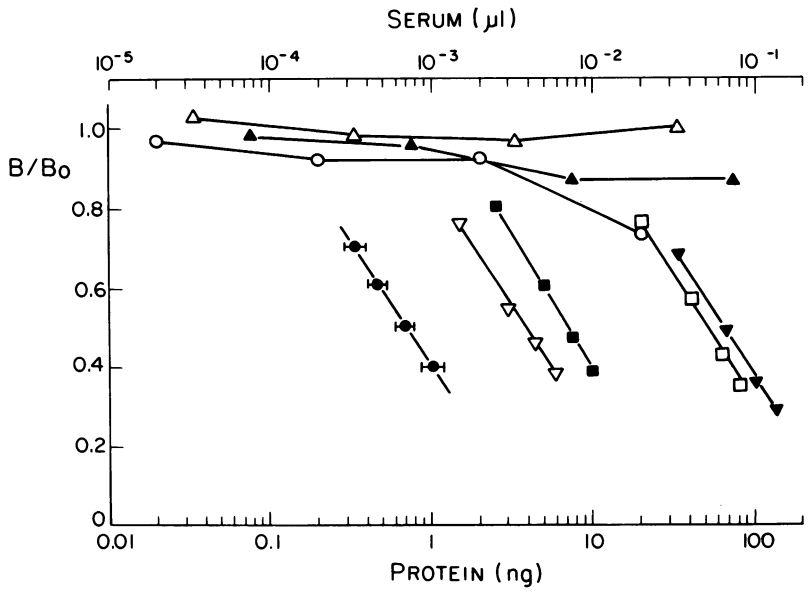

FIGURE 4 Competitive displacement of ^{125}I-labeled apo E from anti-apo E, in the presence of 9 mM sodium decyl sulfate, by unlabeled apo E (●) (bars indicate SD for 10 preparations of ^{125}I-apo E, measured over a period of 15 mo); whole serum (■); VLDL (▽); HDL (□); 1.21 g/ml density infranatant fraction of serum (▼); apo A-I (▲); apo A-II (○); human serum albumin (△). Proteins other than apo E were determined by Lowry's method; apo E standard was determined by analysis of amino acyl mass.

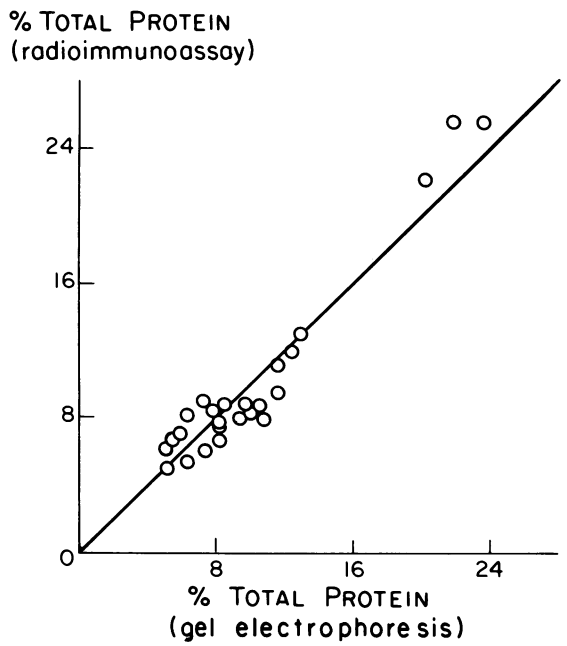

FIGURE 5 Comparison of measurements of apo E in VLDL by quantitative electrophoresis in polyacrylamide gels containing 6 M urea, pH 8.6 (abscissa) and by radioimmunoassay (ordinate). The diagonal line has a slope of 1.0. VLDL were from normotriglyceridemic and hypertriglyceridemic persons (the three samples at the upper right part of the figure were from patients with familial dysbetalipoproteinemia). $r = 0.96$.

gave displacement curves parallel to that of standard apo E. Displacement by protein that eluted in the early part of the protein peak, which appeared to contain about 40% dimer as judged by SDS-gel electrophoresis (Fig. 8) was 7% lower per milligram protein, estimated by the Lowry method, than that of material eluting later in the peak, which contained only traces of the dimer. Based on the molecular weights of apo E and apo A-II, the fraction containing the bulk of the dimer should have contained about 7% apo A-II by mass, indicating that the immunoreactive site of apo E is fully reactive in the dimer.

Reproducibility of the immunoassay. In 32 assays, performed over a period of 16 mo, a sample of a frozen serum pool (Sercal) was included. The mean (±SD) value for apo E in this serum 8.37±1.23 mg/dl. No trend in this value with time was observed.

Concentration of apo E in serum and serum lipoprotein fractions. Frozen serum from subjects who participated in an epidemiological survey of industrial employees in the San Francisco Bay region from 1974 to 1976 was analyzed to provide normative data for apo E concentration. In a randomized sample (172 individuals) mean apo E values were significantly higher in women than men (Table II). In a sample of 51 men whose serum triglyceride level exceeded 200 mg/dl, the concentration of apo E was significantly increased (Table II). Serum apo E increased with serum triglyceride level in both sexes. The slope of the regres-

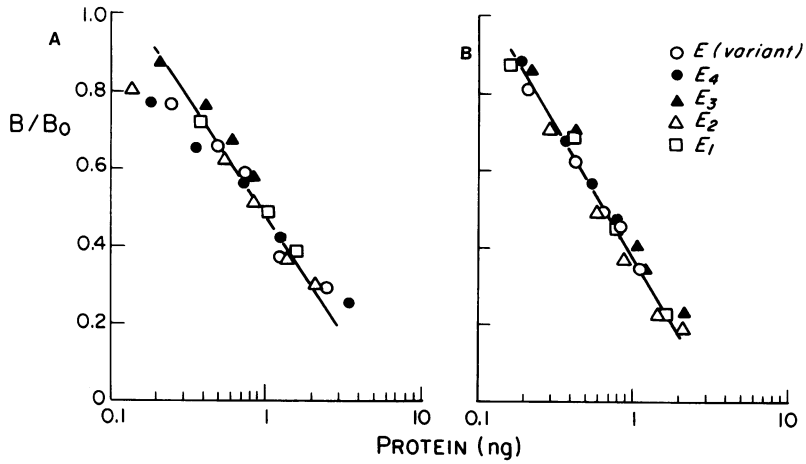

FIGURE 6 Competitive displacement of ^{125}I-apo E from an individual with the variant apo E trait by unlabeled apo E and its four major isoforms. (A) Antiserum against apo E-3. (B) Antiserum against apo E from a subject with the variant trait.

sion of apo E upon triglyceride level common to women and both groups of men was 0.0134 (SE = 0.0009). When adjusted for serum triglyceride level by analysis of covariance, the serum apo E levels of the group of men selected for hypertriglyceridemia were comparable to those of unselected men, but the mean difference between women and men increased to 1.24 mg/dl (SE = 0.32).

In five normolipidemic subjects, apo E was found in appreciable amounts in all lipoprotein fractions separated by sequential preparative ultracentrifugation (Table III). Based upon estimated mean concentrations of total protein in these lipoproteins, apo E comprised approximately 17, 8.1, 1.0, and 0.33% of the apopro-

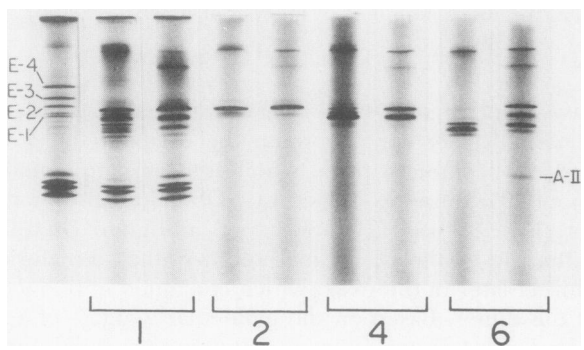

FIGURE 7 Isoelectric focussing gel electrophoretograms of apo VLDL and of components of apo E from a patient with familial dysbetalipoproteinemia, with and without reduction with beta-mercaptoethanol. Components (not exposed to beta-mercaptoethanol) were eluted from a preparative isoelectric focussing gel. Protein applied to the gel on the far left and to the right member of each pair of numbered gels was incubated with beta-mercaptoethanol as described in Methods. Gel on left, reference apo VLDL from an individual with the apo E variant trait; (1) apo VLDL from the patient with familial dysbetalipoproteinemia; (2) apo E-2 (slightly contaminated by apo E-1); (4) apo E-1, contaminated by apo E-2 and multimeric forms; and (6) mainly multimeric material (single band well below apo E components in gel on far right probably represents apo A-11, which was identified in the apo VLDL of this patient, after reduction with beta-mercaptoethanol, by double immunodiffusion against anti apo A-II). The protein bands cathodic to apo E in all gels probably represent oligomeric apo E, as such bands are prominent in apo E of high apparent molecular weight separated from apo VLDL on columns of Sephadex G-200 and are virtually absent in monomeric apo E eluted from such columns (Fig. 8). Note that the appearance of these bands is also altered by exposure to beta-mercaptoethanol.

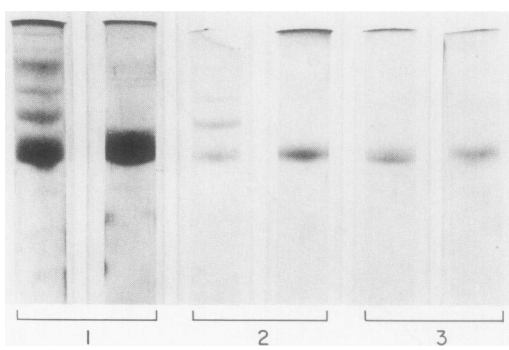

FIGURE 8 SDS-polyacrylamide gel electrophoretograms of fractions of apo E from a patient with familial dysbetalipoproteinemia, obtained from eluates of a column of Sephadex G-200. Protein applied to the right member of each pair of gels was incubated with beta-mercaptoethanol. (1) Original preparation of apo E contains apparent multimers that are dissociated by beta-mercaptoethanol. The highly stained band in the gel on the left has an apparent mol wt of 35,000; in ascending order, the bands above it have apparent mol wt of 49,000, 68,000, and 96,000, respectively. (2) Material from the early portion of the peak containing apo E contains a larger proportion of multimers. (3) Material from the late portion of the peak containing apo E contains no detectable multimeric forms.

TABLE II

Concentration of Apo E in Blood Serum of White Industrial Workers

Sex	Number	Age	Total cholesterol	Triglycerides	Apo E
		yr		*mg/dl (SD)*	
Unselected sample					
Men	86	20–64	209 (39.6)	116 (74.0)	4.94 (1.71)
Women*	86	20–64	209 (41.1)	84 (48.0)	5.89‡ (2.37)
Selected sample§					
Men	51	20–64	257 (52.0)	416 (190.0)	9.67‡ (4.33)

* Not taking exogenous sex hormones.

‡ Significantly different from unselected men ($P < 0.01$).

§ Selected for serum triglycerides exceeding 200 mg/dl.

tein of VLDL, intermediate-density lipoprotein (IDL), LDL and HDL, respectively. In four normotriglyceridemic subjects in which HDL were purified by recentrifugation and total protein was measured directly, apo E comprised 0.97±0.58% of the total apoprotein; for HDL from four hypertriglyceridemic subjects this value was 0.73±0.49%. Apo E was found consistently in the ultracentrifugal fraction of $d > 1.21$ g/ml, which contains the residual plasma proteins. When this fraction was obtained directly by ultracentrifugation of serum at a density of 1.21 g/ml, it contained 18% of the total serum apo E (Table III). In five hypertriglyceridemic subjects, whose mean serum concentration of apo E was 19 mg/dl, 17–48% (mean, 19%) of the apo E was in the $d > 1.21$ g/ml fraction, obtained by direct ultracentrifugation of serum.

In rats, a substantial fraction of apo E dissociates from VLDL and HDL during ultracentrifugal isolation, and is found in the $d > 1.21$-g/ml fraction (6). To determine whether a similar phenomenon occurs in humans, lipoproteins were separated from the bulk of serum proteins by chromatography on Sephadex G-200. Lipoproteins were also separated by filtration through various agarose gels, but recoveries of apo E from these columns were poor with losses of 25–50%. Less than 1% of the apo E in serum of two normolipidemic subjects eluted from Sephadex columns in the region of free apo E (Fig. 9). Comparison of the apo E content of the combined VLDL-IDL-LDL fraction that eluted in the void volume from such columns with content of apo E in the $d < 1.063$-g/ml lipoproteins indicated that most of the apo E in the $d > 1.21$-g/ml fraction was derived from these lipoproteins rather than from HDL. Elution of the included fraction of apo E from columns of Sephadex G-200 preceded that of HDL cholesterol, indicating that this apo E is associated with particles larger than the bulk of HDL, but smaller than LDL (Fig. 9).

The highest concentrations of apo E were observed in the serum of patients with familial dysbetalipoproteinemia (Table IV), greatly exceeding values predicted from the slope of the regression of apo E upon serum triglyceride level in the general population. Serum apo E levels of first degree relatives who were considered to be heterozygous for lack of apo E-3 (apo E-3:apo E-2 = 0.30–1.15) were significantly

TABLE III

Distribution of Apo E in Lipoprotein Fractions of Five Normolipidemic Adults

Fraction	Total cholesterol	Triglycerides	Apo E
		mg/dl	
Sequential ultracentrifugation			
VLDL ($d < 1.006$)*		34 (14)‡	1.15 (0.62)
IDL ($1.06 < d < 1.019$)	7.6 (5.3)		0.35 (0.21)
LDL ($1.019 < d < 1.063$)	94 (16)		0.56 (0.16)
HDL ($1.063 < d < 1.21$)	45 (9)		0.59 (0.37)
Single ultracentrifugation			
$d < 1.21$	186 (20)		3.25 (0.52)
$d > 1.21$			0.74 (0.50)
Serum	204 (13)	79 (17)	4.16 (1.31)

* Density in grams per milliliter.

‡ Mean (SD).

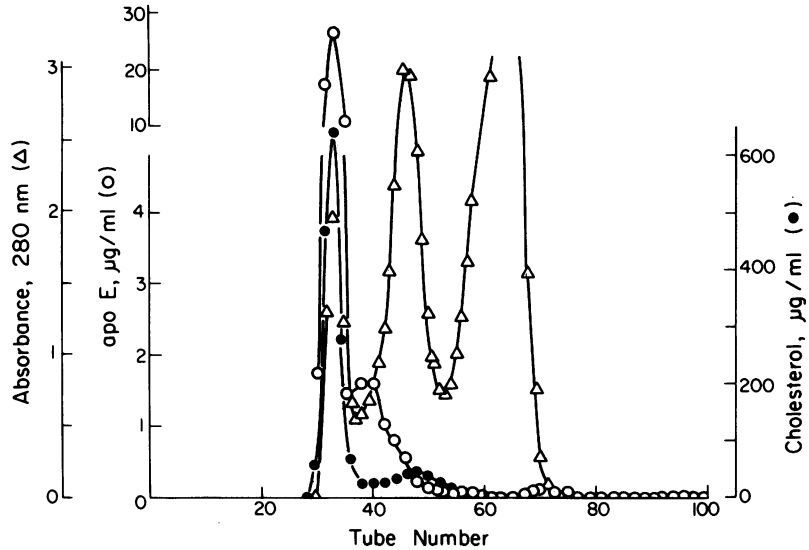

FIGURE 9 Gel filtration of a sample of normal human serum (4 ml) on a column of Sephadex G-200. VLDL, IDL, and LDL elute in the void volume of the column, as indicated by the symmetrical peak of total cholesterol; HDL elutes in a symmetrical peak in the included volume. The initial peak of optical density represents mainly light scattering from VLDL; the second peak represents mainly macroglobulins and the third peak represents other serum proteins, mainly albumin. The majority of the apo E elutes in the void volume and almost all of the remainder elutes in a region of the included volume that precedes most of HDL cholesterol. Only a trace (<1%) of the apo E elutes in the region of the monomeric protein. Recovery of apo E applied was 107%.

higher than those of first degree relatives whose apo-3:apo E-2 ratio in apo VLDL exceeded the discriminating value of 1.15[2] (Table III). When adjusted for both serum triglycerides and sex, the mean difference between heterozygotes and normals in serum apo E level was 2.65 mg. This is only slightly less than the observed (unadjusted) mean difference, owing to compensating effects (heterozygotes had higher serum triglyceride levels than normals, but a larger fraction of heterozygotes were men). Apo E levels in VLDL, LDL, and HDL were also higher in heterozygotes (significantly so in LDL) and heterozygotes had cholesterol-enriched VLDL and triglyceride-enriched LDL (Table V). The cholesterol concentration in VLDL, adjusted for that of VLDL-triglyceride by analysis of covariance, was significantly higher in heterozygotes than in the normal relatives ($P < 0.01$). As expected from this finding (14), a larger proportion of heterozygotes (6 of 20) had slowly migrating VLDL ($R_f < 0.45$ relative to albumin) in agarose gel electrophoretograms than the normal relatives (1 of 11) ($0.10 > P > 0.05$).

DISCUSSION

Our radioimmunoassay for human apo E was developed essentially from the assay for the rat protein, with the exception that we found 9 mM, rather than 4.5 mM sodium decyl sulfate, to give optimal antigen binding. The sensitivity (to 0.3 μg) and precision of the two assays are similar. Investigation of the concentration in plasma and distribution in lipoproteins of apo E in relation to genetic variation of the isoforms of apo E was a major reason for developing the assay. It was therefore essential to evaluate the reactivity of the isoforms. Further, as apo E evidently exists as dimers with apo A-II (23) and probably as dimers and higher multimers that contain only apo E (24; Fig. 8), and such complexing is often more pronounced in familial dysbetalipoproteinemia, the immunoreactivity of these complexes also became relevant to our interests. Our results show that the isoforms had equal immunoreactivity with antibodies raised against apo E from individuals whose apo E included apo E-4. Furthermore, an antibody raised against apo E from such an individual gave identical results with apo E standards from subjects with the

[2] The frequency distribution of apo E-3:apo E-2 ratios in apo VLDL of 67 individuals with heterozygous familial hypercholesterolemia was bimodal, with 16.4% of values <1.15, consistent with the heterozygote frequency of 15% reported by Utermann (3) for 490 blood donors. None of six parents, none of 22 children, and 4 of 11 siblings of 14 propositi with familial dysbetalipoproteinemia had apo E-3:apo E-2 ratios greater than 1.15 (P. Tun, R. J. Havel, J. P. Kane, and M. Malloy. Unpublished observations).

TABLE IV

Concentration of Serum Lipids and Apo E in Patients with Familial Dysbetalipoproteinemia and Their Relatives

Group	Number	Male	Female	Age*	Total cholesterol‡	Triglycerides	Apoprotein E
				yr		*mg/dl*	
Propositi E-3:E-2 = 0	10	7	3	55* (39–75)	292‡ (112)	334 (240)	27.18 (12.04)
Heterozygotes E-3:E-2 < 1.15‖	21	10	11	34 (11–80)	188 (44)	122 (85)	9.69§ (3.22)
Normals E-3:E-2 > 1.15¶	11	4	7	32 (14–78)	169 (46)	80 (30)	6.94 (3.53)

* Mean (range).
‡ Mean (SD).
§ Significantly different from normals by *t* test and rank-sum test, one tail ($P < 0.02$).
‖ Mean and SD = 0.80 ±0.22.
¶ Mean and SD = 1.62 ±0.44.

various apo E phenotypes. Thus, it appears that neither reactivity of our antibodies nor antigens is a function of apo E polymorphism. Our results also indicate that the immunoreactivity of apo E present as the dimer with apo A-II is the same as that of apo E monomer. We did not investigate directly the immunoreactivity of other complexes of apo E, but comparison of the estimated mass of apo E in isolated VLDL by radioimmunoassay and quantitative gel electrophoresis gave no indication that reactivity of apo E from patients who lack apo E-3 (who usually have more apo E in complexes) differed from that of individuals with other apo E phenotypes.

The immunoreactivity of apo E in isolated VLDL was identical with that of standard apo E, as judged from comparison with quantitative gel electrophoresis. The immunoreactivity of apo E in serum and VLDL was unaffected by delipidation, when sodium decyl sulfate was present. This suggests, but does not prove, that the immunoreactivity of apo E in LDL and HDL is the same as that of isolated apo E used as standard.

Others have reported quantitative immunoassays for apo E in human serum, one with an immunoelectrophoretic (25) and the other with a radial immunodiffusion technique (26). Our values for apo E in serum of normolipidemic subjects are about one-half of those reported with the former and one-fourth of those reported with the latter assay. The reasons for these

TABLE V

Concentration of Lipoprotein Lipids and Apo E in Patients with Familial Dysbetalipoproteinemia and Their Relatives

	VLDL ($d < 1.006$ g/ml)			LDL ($1.006 < d < 1.063$ g/ml)			HDL ($d > 1.063$ g/ml)		
	Total cholesterol	Total triglycerides	Apo E	Total cholesterol	Total triglycerides	Apo E	Total cholesterol	Total triglycerides	Apo E
					*mg/dl**				
Propositi ($n = 10$)	148 (106)	256 (169)	10.68 (7.85)	102 (51)	67 (83)	6.19 (2.97)	39 (12)	14 (4)	6.24 (2.64)
Heterozygotes ($n = 19$)‡	26§ (28)	77 (80)	1.99 (1.78)	104 (36)	34 (17)	2.25‖ (1.62)	50 (15)	17 (9)	4.82 (2.32)
Normals ($n = 11$)	10 (7)	44 (25)	0.94 (0.40)	106 (40)	23 (9)	1.35 (1.15)	48 (10)	13 (6)	3.75 (1.91)

* Mean (SD).
‡ For LDL and HDL, $n = 16$.
§ Log total cholesterol significantly different from normals by *t* test (one tail) ($P < 0.02$).
‖ Significantly different from normals by rank-sum test, one tail ($P < 0.05$).

discrepancies are not apparent. Results with the immunoelectrophoretic assay were said to agree with those by radial immunodiffusion (25). We have obtained somewhat lower values with a radial immunodiffusion assay than with our radioimmunoassay (3.68 ±1.76 vs. 4.95±2.32 mg/dl; $n = 6$). The reported radial immunodiffusion assay was said to provide results for apo E in VLDL that closely approximated those obtained by densitometric scanning of polyacrylamide gel electrophoretograms (26). This is in apparent conflict with our results inasmuch as the values reported by radial immunodiffusion for VLDL of normolipidemic subjects greatly exceeded those found by us. Given the values found by these authors, we estimate that apo E would have to comprise approximately one-half of the tetramethylurea-soluble proteins of VLDL.

As in the rat (27), apo E dissociates from human lipoproteins during ultracentrifugal isolation. Our results suggest that the dissociation is more pronounced for VLDL than HDL. As the content of apo E in VLDL may influence the metabolism of this lipoprotein (28), and as reported values (7) evidently underestimate, to a variable degree, the amount of apo E associated with VLDL in vivo, the relationship of observed values in ultracentrifugally isolated VLDL to metabolic events in vivo must be interpreted with caution. Furthermore, the metabolism of intravenously injected VLDL could be influenced not only by the extent of dissociation but perhaps by the availability of apo E in the plasma of recipients as well.

Among the major lipoprotein classes in serum, only in VLDL does apo E comprise a substantial fraction of the protein component. Our observation that the concentration of apo E in serum is a function of the concentration of VLDL is therefore not surprising. Although apo E is also found in all other major lipoprotein fractions, its recovery in these fractions could result from ultracentrifugal dissociation from VLDL. The results of our studies of the distribution of apo E in lipoprotein fractions separated directly from serum by gel filtration suggest that a discrete fraction of apo E exists in HDL. This apo E appears to be associated with particles larger than the bulk of HDL, as observed earlier in the rat (27). In the rat, however, much more apo E exists in this fraction of HDL than in humans. During alimentary lipemia in humans, this apo E appears to transfer to triglyceride-rich lipoproteins (Vigne, J.-L. and Havel, R. J., unpublished observations), in a manner similar to that demonstrated for the C apoproteins (29). Thus, it is to be expected that, as with the C apoproteins (30, 31), the fraction of apo E in serum associated with triglyceride-rich lipoproteins is a direct function of their concentration. The higher concentration of serum apo E that we found in women than men cannot be explained on this basis. We have not determined whether the distribution of apo E among lipoproteins differs between the sexes.

As expected from the increased concentration of remnant-like VLDL and IDL enriched in apo E in hyperlipidemic persons with familial dysbetalipoproteinemia (1), the concentration of apo E in serum, VLDL and the IDL + LDL fraction was found to be substantially increased. The concentration of apo E at any level of serum triglycerides is considerably higher in familial dysbetalipoproteinemia than in normolipidemic persons or persons with other forms of hyperlipidemia (compare Tables II and IV). Of greater interest is our observation that the level of apo E in serum and the IDL + LDL fraction is higher in relatives of persons with familial dysbetalipoproteinemia who are heterozygous for lack of apo E-3 than in those relatives whose apo E contains a normal proportion of apo E-3. The increased concentration of apo E in heterozygotes probably results from an increased concentration of remnant-like lipoproteins, reflected in cholesterol-enriched VLDL and a triglyceride-enrichment of the IDL + LDL fraction (Table V). Our findings extend and confirm those reported by Utermann and associates (32) for heterozygotes. They studied various phenotypic groups including families in which the dyslipoproteinemic proband was not hyperlipidemic, whereas our probands all had hyperlipidemia. Utermann and associates (33) have concluded that severe hyperlipidemia associated with lack of apo E-3 reflects the presence of other genes for monogenic or polygenic hyperlipidemia, occurring in families independently of apo E phenotype. In our sample of families in which the proband with dysbetalipoproteinemia was hyperlipidemic, triglyceride levels exceeded 180 mg/dl in 6 of the 21 heterozygotes, but in none of the 11 individuals whose apo E contained a normal complement of apo E-3.

Recent observations indicate that apo E is recognized by a receptor on the surface of hepatic parenchymal cells of rats (28, 34, 35), which may participate in the uptake of remnant lipoproteins by the liver. This hepatic receptor effectively recognizes phospholipid complexes of human apo E from normal individuals whereas recognition of such complexes prepared with apo E from persons with familial dysbetalipoproteinemia is limited (35). Our present results suggest that the interaction of remnant lipoproteins with a lipoprotein receptor in human liver is a function of the amount of apo E-3 (and apo E-4), either absolute or relative to poorly reactive isoforms of protein. The apparent effect of gene dosage is thus similar to that in familial hypercholesterolemia, in which a lipoprotein receptor rather than the recognized apoprotein is responsible for defective catabolism. As 15% of the population have one allele that specifies poorly reactive

isoforms of apo E, the altered metabolism of lipoproteins could have an important effect upon the expression of hyperlipidemia, as suggested by our observations of relatives of hyperlipidemic probands with dysbetalipoproteinemia. The consequences of this deficiency for the development of atherosclerotic vascular disease remain to be determined, but in a preliminary study the serum concentration of apo E was not found to be increased in a group of patients with angiographically demonstrated coronary atherosclerosis (36).

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