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Research Article

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Familial Apolipoprotein AI and Apolipoprotein CIII Deficiency

Subclass Distribution, Composition, and Morphology of Lipoproteins in a Disorder Associated with Premature Atherosclerosis

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Abstract. Lipoprotein classes isolated from the plasma of two patients with apolipoprotein AI (apo AI) and apolipoprotein CIII (apo CIII) deficiency were characterized and compared with those of healthy, age- and sex-matched controls. The plasma triglyceride values for patients 1 and 2 were 31 and 51 mg/dl, respectively, and their cholesterol values were 130 and 122 mg/dl, respectively; the patients, however, had no measurable high density lipoprotein (HDL)-cholesterol. Analytic ultracentrifugation showed that patients' S_f° 0–20 lipoproteins possess a single peak with S_f° rates of 7.4 and 7.6 for patients 1 and 2, respectively, which is similar to that of the controls. The concentration of low density lipoprotein (LDL) (S_f° 0–12) particles, although within normal range (331 and 343 mg/dl for patients 1 and 2, respectively), was 35% greater than that of controls. Intermediate density lipoproteins (IDL) and very low density lipoproteins (VLDL) (S_f° 20–400) were extremely low in the patients. HDL in the patients had a calculated mass of 15.4 and 11.8 mg/dl for patients 1 and 2, respectively. No HDL could be detected by analytic ultracentrifugation, but polyacrylamide gradient gel electrophoresis (gge) revealed that patients possessed two major HDL subclasses: $(HDL_{2b})_{gge}$ at 11.0 nm and $(HDL_{3b})_{gge}$ at 7.8 nm. The major peak in the controls, $(HDL_{3a})_{gge}$, was lacking in the patients. Gradient gel analysis of LDL indicated that patients' LDL possessed two peaks: a major one at 27 nm and a minor one at 26 nm. The electron microscopic structure of patients' lipoprotein fractions was indistinguishable from controls. Patients' HDL were spherical and contained a cholesteryl ester core, which suggests that lecithin/cholesterol acyltransferase was functional in the absence of apo AI. The effects of postprandial lipemia (100-g fat meal) were studied in patient 1. The major changes were the appearance of a 33-nm particle in the LDL density region of 1.036–1.041 g/ml and the presence of discoidal particles (12% of total particles) in the HDL region. The latter suggests that transformation of discs to spheres may be delayed in the patient.

The simultaneous deficiency of apo AI and apo CIII suggests a dual defect in lipoprotein metabolism: one in triglyceride-rich lipoproteins and the other in HDL. The absence of apo CIII may result in accelerated catabolism of triglyceride-rich particles and an increased rate of LDL formation. Additionally, absence of apo CIII would favor rapid uptake of apo E-containing remnants by liver and peripheral cells. Excess cellular cholesterol would not be removed by the reverse cholesterol transport mechanism since HDL levels are exceedingly low and thus premature atherosclerosis occurs.

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Introduction

Epidemiologic studies have shown that risk of coronary heart disease varies inversely with plasma high density lipoprotein (HDL) concentration. This relationship has recently been emphasized in a report by Norum et al. (1) on two sisters with extraordinarily low levels of HDL cholesterol, which is associated with severe premature atherosclerosis, xanthomas, and corneal clouding. Both patients had a severe deficiency of plasma apolipoprotein AI (apo AI)[1] and apolipoprotein CIII

1. *Abbreviations used in this paper:* apo AI, AII, B, CI, CII, CIII, E, apolipoproteins AI, AII, B, CI, CII, CIII, E, respectively; LCAT, lecithin/cholesterol acyltransferase; SDS-PAGE, 10% polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate.

(apo CIII) as well as decreased levels of apolipoproteins AII, E, CI, and CII (apo AII, E, CI, and CII, respectively). The abnormality of these patients appears to be inherited as an autosomal-recessive trait (1, 2). The apo AI and apo CIII genes have been shown to be closely linked and convergently transcribed (2, 3). A 6.5-kilobase DNA insert into the apo AI gene has been found (2), and recent evidence suggests that the inserted sequence is deleted from its normal position in the promoter region for the apo CIII gene (4). This mutation could account for the absence of both apolipoproteins in these patients.

Disorders involving HDL deficiency are frequently associated with abnormalities in the physical and chemical properties of lipoprotein subclasses as well as with unusual electron microscopic morphologies. Abnormal structures including discoidal particles and small spherical particles are found in HDL from familial lecithin/cholesterol acyltransferase (LCAT)-deficient patients (5) where HDL subclass distribution as determined by gradient gel electrophoresis is also abnormal (6). LCAT-deficient patients also possess unusual, large vesicular particles in the low density lipoprotein (LDL) region and irregularly shaped very low density lipoproteins (VLDL) (5, 7). In Tangier disease, the HDL fraction contains small spherical particles together with large vesicular ones (8). The VLDL and LDL in Tangier disease are morphologically normal, although the latter have an overall smaller mean diameter than control LDL (9). Fish eye disease is a disorder in which HDL deficiency is associated with an abnormal HDL subclass distribution and three morphologically distinct entities (discs, spheres, and vesicles) in the HDL region (10). The LDL in this disease contain a few large vesicular structures. Recently, Schaefer et al. (11, 12) described a patient with severe coronary disease and the absence of both apo AI and apo CIII; this patient possessed morphologically abnormal VLDL and HDL particles interconnected by filamentous material but normal LDL. The apo AI_{Milano} abnormality, which is characterized by aberrant apo AI, does not appear to be associated with abnormal particle morphologies (13); however, the HDL subpopulation distribution has been reported to be abnormal (14). The particle size distribution and physico-chemical properties, including electron microscopic structure, of lipoproteins in familial apo AI and CIII deficiency have not been described and are the subject of this report. In addition, changes in lipoprotein subpopulation distribution and morphology were examined in one of the patients after a fat meal.

Methods

Subjects. The patients studied in this investigation were two sisters previously reported by Norum et al. (1), aged 34 and 32. Clinical features of the patients are described elsewhere (1). Daily medications taken by patient 1 were digoxin, 0.25 mg; furosemide, 40 mg; nitroglycerin, 10 mg; and propranolol, 120 mg. Daily medications taken by patient 2 were digoxin, 0.25 mg and nadolol, 20 mg. Both patients followed a low cholesterol diet. Three age-matched normolipemic females aged 31–37 were used as controls. Blood was collected into EDTA (1 mg/ml) after an overnight fast, and plasma was immediately separated. Streptomycin (0.05 mg/ml) and penicillin (50 g/ml) were added to the plasma. All blood samples were obtained with informed consent. The plasma triglyceride, total cholesterol, LDL-cholesterol, and HDL-cholesterol values for all subjects are summarized in Table I.

Fat-feeding study. To assess the effect of an acute intake of fat on lipoprotein distribution, patient 1 in a single study consumed a 100-g liquid fat meal containing 50% cream and 50% safflower oil with a polyunsaturated to saturated fatty acid ratio of 1. This study was carried out 10 mo after the original sampling. Because of gall bladder disease, patient 2 was not given a high fat meal. Blood samples were drawn at 0 (basal, fasting time point), 2, 4, 6, and 8 h. Blood was drawn into EDTA, and 2 mM diethyl *p*-nitrophenyl phosphate was added to each of the plasma samples.

Preparation of lipoproteins. Lipoproteins were separated by standard sequential preparative ultracentrifugal techniques at 15°C (15). All separations were carried out in the Beckman 40.3 rotor (Beckman Instruments, Inc., Fullerton, CA) at 40,000 rpm. The $d < 1.006$ g/ml fraction was removed after 18 h centrifugation. The intermediate density lipoproteins (IDL) (d 1.006–1.019 g/ml) and LDL (d 1.019–1.063 g/ml) were removed after 24 h centrifugation. HDL (d 1.063–1.21 g/ml) were obtained after 48 h centrifugation. The patients' HDL fractions were further concentrated 12- to 24-fold by an additional centrifugation step for 24 h. To ascertain that the isolation and concentration procedures did not alter lipoprotein recovery or particle size distribution, a separate experiment was carried out on human cord blood plasma, which has low HDL levels and a gradient gel electrophoretic pattern somewhat similar to that of the patients. In this experiment, 24-fold diluted cord blood plasma and undiluted plasma were subjected to similar ultracentrifugal methods and the diluted HDL was concentrated 24-fold. Recovery of the 24-fold concentrated HDL was 102% of undiluted, and analysis of particle size distribution showed no perturbation in the gradient gel electrophoretic pattern. To analyze patients' HDL apolipoprotein distribution as a function of density, an aliquot of HDL was dialyzed against d 1.110 g/ml solution, and subfractionated according to the density gradient method of Anderson et al. (16). Low density lipoproteins were subfractionated by the method of Shen et al. (17).

Analytical ultracentrifugation. Analytical ultracentrifugation and computer-derived schlieren pattern analysis were carried out according to the procedures described by Lindgren (18).

Electrophoresis. Lipoprotein fractions were analyzed by gradient gel electrophoresis in order to determine size distribution of the lipoproteins. This is a nondenaturing electrophoretic system that shows good reproducibility of particle size; the coefficient of variation with 4–30% gels is 0.5–1.7% (14), while that for 2–16% gels is 0.8–1.0%

Table I. Plasma Lipid Values of Subjects

Subject	Age	Triglyceride	Total cholesterol	LDL cholesterol	HDL cholesterol
		mg/dl	*mg/dl*	*mg/dl*	*mg/dl*
Patient 1	34	31	130	124	0
Patient 2	32	51	122	112	0
Control 1	33	34	121	83	31
Control 2	31	61	123	68	43
Control 3	37	38	123	76	39

(19). Total pattern area and the relative distribution of area among the subpopulations is reproducible, on the average, to within ±10%. Precast Pharmacia 4–30% slab gels (Pharmacia Fine Chemicals, Piscataway, NJ) were used to analyze HDL fractions according to the procedure described by Nichols et al. (14). Reference proteins used to determine particle diameters consisted of thyroglobulin, apoferritin, lactate dehydrogenase, and bovine serum albumin. Gels were stained with Coomassie G250 to identify protein bands.

LDL size distribution was determined on 2–16% Pharmacia slab gradient gels (Pharmacia Fine Chemicals) according to the method of Krauss and Burke (19). Carboxylated latex beads (The Dow Chemical Co., Indianapolis, IN), thyroglobulin, and apoferritin were used as standards. Gels were stained either for protein, using Coomassie G250, or for lipid, with oil red O. The latter procedure was essentially that described by Bautovich et al. (20), except that gels were stained for 18–24 h at 60°C, and then rinsed and rehydrated in 5% acetic acid.

10% polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate (SDS-PAGE) was used to determine molecular weights of apolipoproteins; the procedures used were essentially those of Weber and Osborn (21). Protein standards consisting of bovine serum albumin (66,000 mol wt), apo AI (28,000 mol wt), apo E (34,000 mol wt), and egg lysozyme (14,000 mol wt) were co-electrophoresed for determinations of molecular weights. Apolipoprotein from LDL was electrophoresed on 3% polyacrylamide gels according to the method of Kane et al. (22). All SDS-PAGE gels were stained with Coomassie R250.

Densitometric scans of gradient gels and polyacrylamide tube gels were obtained with a Transidyne RFT densitometer (Transidyne General Corp., Ann Arbor, MI). Gels stained for protein were scanned at 603 nm wavelength, while oil red O-stained gels were scanned at 480 nm.

Chemical analyses. Protein and phospholipid concentrations were obtained by the methods of Markwell et al. (23) and Bartlett (24), respectively. Unesterified cholesterol and cholesteryl ester were determined by gas-liquid chromatography according to Hindriks et al. (25). Triglyceride was measured with the enzymatic reagent kit from Gilford Diagnostics, Cleveland, OH.

Electron microscopy. The procedures used for negative staining and sizing lipoprotein particles were those previously described (7). A minimum of 200 free-standing particles per sample was measured.

Results

Analytic ultracentrifugal data. The analytic ultracentrifugal profiles of patients and controls are shown in Fig. 1, and lipoprotein concentrations and peak flotation rates are summarized in Table II. Control subjects show normal distribution patterns for HDL (flotation interval, $F^{\circ}_{1.20}$ 0–9) with varying levels of HDL_2 and HDL_3 ($F^{\circ}_{1.20}$ 3.5–9 and 0–3.5, respectively); however, no lipoproteins are detected in the $F^{\circ}_{1.20}$ 0–9 interval of either patient. The patients have very low levels of VLDL (S_f° 20–400); however, similar levels are noted in two of the control subjects. The patients' S_f° 0–20 lipoproteins differ from control (Fig. 1) in that IDL (S_f° 12–20) material is negligible, and the concentration of LDL (S_f° 0–12), although within normal range, is 35% greater than the average for the three controls (Table II). It is noteworthy, however, that the peak flotation rates of patient and control LDL (Table II) are similar.

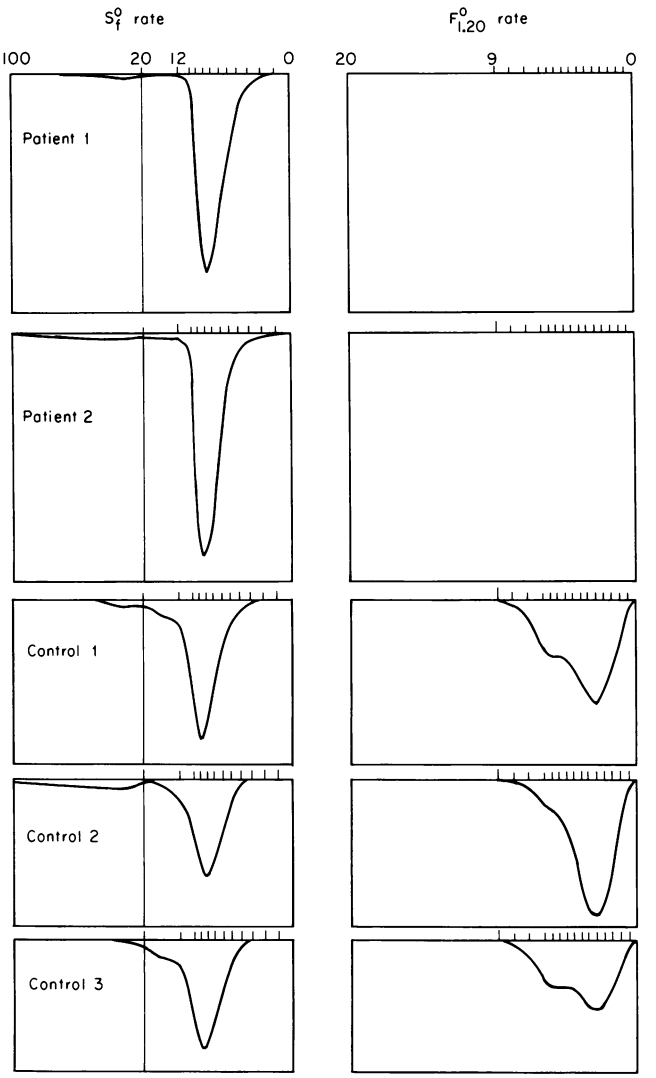

Figure 1. Analytic ultracentrifugal profile for patients and controls. The S_f° rate refers to lipoproteins of $d < 1.063$ g/ml, where S_f° 0–12 = LDL, S_f° 12–20 = IDL, and S_f° 20–100 = VLDL. The $F^{\circ}_{1.20}$ rate refers to the HDL; note that no HDL are detectable in the patients' plasma.

Gradient gel electrophoresis. Since no lipoproteins were observed in patients' $F^{\circ}_{1.20}$ 0–9 region, the HDL fraction was examined by gradient gel electrophoresis, which has greater sensitivity and resolution. The HDL fractions from each patient were concentrated 24-fold by ultracentrifugation and applied to 4–30% gradient polyacrylamide gels. The densitometric patterns of Coomassie-stained gels of the patients' HDL, together with those of three controls, are seen in Fig. 2 *A*. The HDL subpopulation distribution patterns of both patients are similar to one another but deviate markedly from those of controls. The terms $(HDL_{3a})_{gge}$, $(HDL_{3b})_{gge}$, and $(HDL_{3c})_{gge}$ designate major particle size subpopulations observed by gradient gel electrophoresis (gge) within the ultracentrifugal HDL_3

Table II. Flotation Rates and Concentrations of Lipoproteins from Patients and Controls as Determined by Analytic Ultracentrifugation

Subject	S_f^* 20–400	S_f^* 12–20	S_f^* 0–12	$F^*_{1.20}$ 0–3.5	$F^*_{1.20}$ 3.5–9	Flotation rate of peak S_f^* 0–20	Flotation rate of peak $F^*_{1.20}$
	mg/dl	*mg/dl*	*mg/dl*	*mg/dl*	*mg/dl*		
Patient 1	3	0	331	0	0	7.4	—
Patient 2	20	6	343	0	0	7.6	—
Control 1	11	27	250	211	155	7.7	2.4
Control 2	50	12	182	107	265	7.0	2.4
Control 3	5	34	224	132	146	7.1	2.4

fraction (*d* 1.125–1.20 g/ml). Major particle size distributions observed by gradient gel electrophoresis within the HDL_2 fraction (*d* 1.063–1.125 g/ml) have been designated $(HDL_{2b})_{gge}$ and $(HDL_{2a})_{gge}$. Particle size intervals defining these subpopulations are: 12.9–9.7 nm, $(HDL_{2b})_{gge}$; 9.7–8.8 nm, $(HDL_{2a})_{gge}$; 8.8–8.2 nm, $(HDL_{3a})_{gge}$; 8.2–7.8 nm $(HDL_{3b})_{gge}$; and 7.8–7.2 nm $(HDL_{3c})_{gge}$. Patients' HDL are characterized by a major peak at 11.0 nm in the size range corresponding to the normal plasma $(HDL_{2b})_{gge}$ subpopulation and another sharp peak at 7.6–7.8 nm, which corresponds to the $(HDL_{3b-3c})_{gge}$ subpopulation interval. The material in the $(HDL_{2b})_{gge}$ subpopulation interval in both patients is very broad (accounts for 60% of the Coomassie staining area) and includes particles that are larger than normally occurring HDL_{2b}. Both patients have relatively small amounts of material in the $(HDL_{2a})_{gge}$ region (13% of the total area) and a valley in the $(HDL_{3a})_{gge}$ region. In contrast, control HDL, shown in Fig. 2, are characterized by a major HDL peak in the $(HDL_{3a})_{gge}$ region. In controls, a well-defined peak is also seen in the $(HDL_{2b})_{gge}$ region. Control HDL typically have a shoulder in the $(HDL_{3b})_{gge}$ subpopulation and little or no detectable material in the $(HDL_{3c})_{gge}$ interval; this is in contrast to the patients, who have a sharp peak in the $(HDL_{3b})_{gge}$ region.

The subpopulations of LDL (*d* 1.006–1.063 g/ml) were determined from densitometric scans of Coomassie-stained 2–16% gradient gels shown in Fig. 2 *B*. Patients 1 and 2 have very similar patterns consisting of two sharp peaks: a major peak corresponding to particles of 27.2–27.8-nm diam and a minor one with particles of 25.8–26.5-nm diam. The number of peaks in LDL patterns of the control subjects is variable; however, the major lipoprotein constituents have approximately the same particle diameters (26.9–27.6 nm) as the major peaks in the patients. The latter probably accounts for the similar flotation rate values of S_f° 0–12 peaks for controls and patients seen in Table II.

Electron microscopic structure of lipoproteins. The morphology and size of lipoprotein fractions from fasting patients and controls are seen in Table III and Fig. 3. The negatively stained images obtained from one control subject and one patient are representative for each group. The VLDL in both patients and controls are extremely heterogeneous and consist of round particles that readily deform. The size and morphology of LDL from the patients are similar to those of controls and again reflect the similarities seen in analytic ultracentrifugal patterns and gradient gel electrophoresis. The HDL fraction from both patients and controls consists primarily of small, heterogeneous (size range, 6.0–13.0-nm diam), round particles. The mean particle diameter for the patients is somewhat larger than that obtained for controls (9.7 vs. 8.7 nm). However, this larger particle diameter is consistent with the gradient gel data, which show that patients' patterns are skewed toward larger particles.

Composition and apolipoprotein distribution. The percent composition of each of the patients' lipoprotein classes as compared with that of controls is summarized in Table IV. The VLDL contain ~85% lipid and 15% protein for patients and controls. Overall, the lipid composition is comparable for patients and controls, but the content of polar lipids (phospholipid and unesterified cholesterol) is ~20% greater in controls. The percent protein and percent lipid composition in LDL from patients and controls are similar (Table IV); however, the percent cholesterol and cholesteryl ester in patient 1 is slightly lower than controls, while triglyceride is slightly elevated. The HDL fractions from the patients follow the same trend as the other lipoprotein fractions in that there is no difference in percent lipid and protein from that seen in controls; however, the percent total cholesterol is 15–23% higher in the patients than in the controls. On the basis of the lipid and protein mass data for isolated and concentrated HDL fractions from the patients, the estimated plasma level of this lipoprotein class is 15.4 mg/dl for patient 1 and 11.8 mg/dl for patient 2; this is only 3–5% the concentration seen in the control subjects.

Fig. 4 shows the apolipoprotein composition in HDL and LDL fractions from patients and controls as determined by 10% SDS-PAGE. There is a marked difference in the apolipoprotein composition of HDL, which is to be expected since the patients are deficient in apo AI and apo CIII. Fig. 4 clearly demonstrates that apo AII is the major apolipoprotein in the patient; assuming equal chromogenicity, it accounts for >60% of the total Coomassie-staining material. By comparison, 70–75% of the staining protein in control HDL corresponds to apo AI. In the patients, apo E accounts for ~17% of the total HDL protein; moreover, inspection of reduced vs. nonreduced samples indicates that most of the apo E is in the reducible

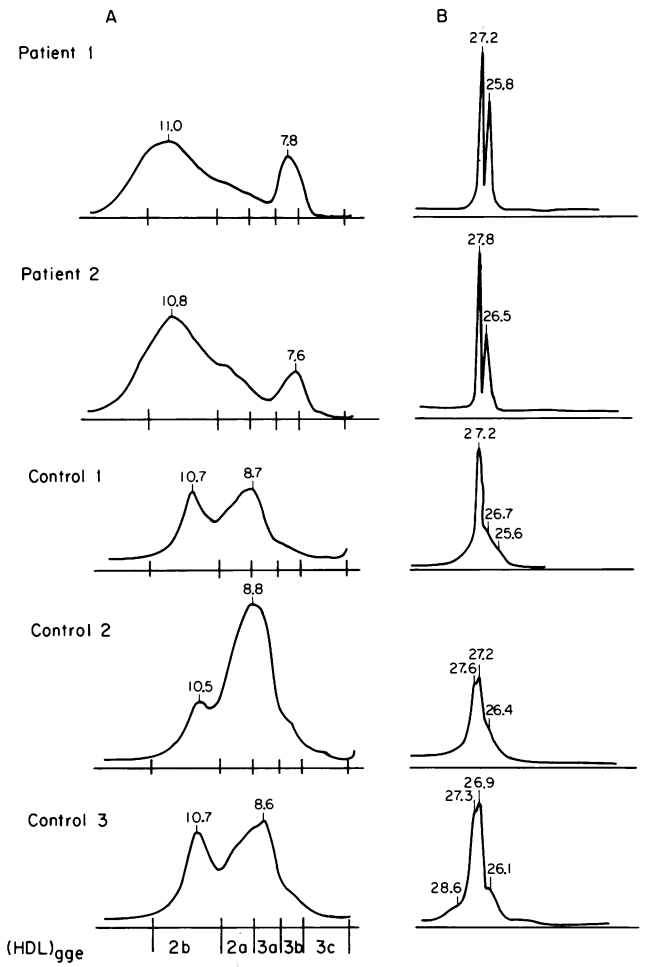

Figure 2. Gradient gel electrophoresis patterns: (*A*) *d* 1.063–1.21 g/ml lipoproteins on 4–30% gels and (*B*) *d* 1.006–1.063 g/ml lipoproteins on 2–16% gels. The HDL from the patients were concentrated 24-fold and ~25 μl of sample was applied to each well; for controls, samples were concentrated twofold, and 6–8 μl was applied. In the patients, the broad peak in the $(HDL_2)_{gge}$ region accounts for 73% of the total staining area, and the sharp peak in the $(HDL_3)_{gge}$ region accounts for 27% of the area. All gels were stained with Coomassie G250. Spectrophotometric scans of the gels show the relative distribution of the various lipoprotein subpopulations. The numbers above the peaks indicate particle diameters in nanometers. The designations $(HDL)_{gge}$ are described in reference 14.

apo (E-AII) complex form. Apo E, however, was not identifiable in any of the control HDL; in the latter, apo AII was the second most abundant apolipoprotein. The apo C proteins appear to be minor constituents in patient and control HDL. The LDL fractions of the patients possess primarily apolipoprotein B (apo B) protein, which remains at the top of the 10% gels; in addition, trace amounts of apo E and apo (E-AII) are also present. LDL from controls contained mainly apo B and also small quantities of apo E and apo AI.

Table III. Lipoprotein Size Determined by Electron Microscopy

	Diameter		
Subject	VLDL	LDL	HDL
	nm±SD	*nm±SD*	*nm±SD*
Patient 1	36.2±8.5	26.3±2.7	9.7±1.8
Patient 2	37.3±12.8	25.3±2.8	9.6±1.7
Controls*	41.3±4.7	26.2±2.7	8.7±1.4

* Values represent mean of three control subjects.

Since the gradient gel electrophoresis data showed that the patients' HDL consisted of two distinct types of particles, one large and one small, we subfractioned these HDL by density gradient centrifugation in order to examine the apolipoprotein distribution. Fig. 5 shows the apolipoprotein distribution of unreduced samples in the five ultracentrifugal fractions from one of the patients. It is readily apparent that apo E is present in all fractions; moreover, the dominant form of apo E is the complex, apo (E-AII). The relative quantity of uncomplexed apo E clearly decreases with increasing density, and the same is true for apo C, which also decreases with increasing density. The least dense fraction that corresponds with the large HDL particles contains contaminating apo B material, which accounted for ~20% of the total Coomassie staining material in this fraction.

Effect of a fat meal on lipoprotein distribution in patient 1. It is well known that HDL serve as a transient reservoir for apolipoproteins and lipids generated by lipolysis. To study the effect of in vivo lipolysis on HDL, as well as LDL, subpopulation distribution, patient 1 was given a 100-g fat meal, and plasma samples were analyzed at 0, 2, 4, 6, and 8 h after the meal. Changes in plasma lipid levels after fat feeding are summarized in Table V. Total triglyceride increases to a maximum of 76 mg/dl at 4 h, and then declines. Cholesterol, however, increases and remains elevated even at 8 h. No HDL-cholesterol is detectable at 0 h, but extremely small amounts are detectable after the fat meal.

The analytic ultracentrifugal data before and after fat feeding in patient 1 is summarized in Table VI. The patient's basal schlieren profile of the S_f° 0–20 class is similar to that obtained 10 mo earlier, although the total concentration and peak S_f° rate are decreased slightly. 2 and 4 h after the fat-rich meal, a small amount of material (27–20 mg/dl) appeared in the VLDL region (S_f° 20–100); by electron microscopy the VLDL had a mean particle size of 41.1±18.5 nm, which was larger than that of basal particles (34.9±8.6 nm). The particle size range after the fat meal was extremely broad (20–135 nm vs. 20–69 nm for the basal point), suggesting that small chylomicrons were present. Although the majority of particles after the fat meal have normal morphology, a small number of notched or flattened particles (see Fig. 6, *A* and *B*) are

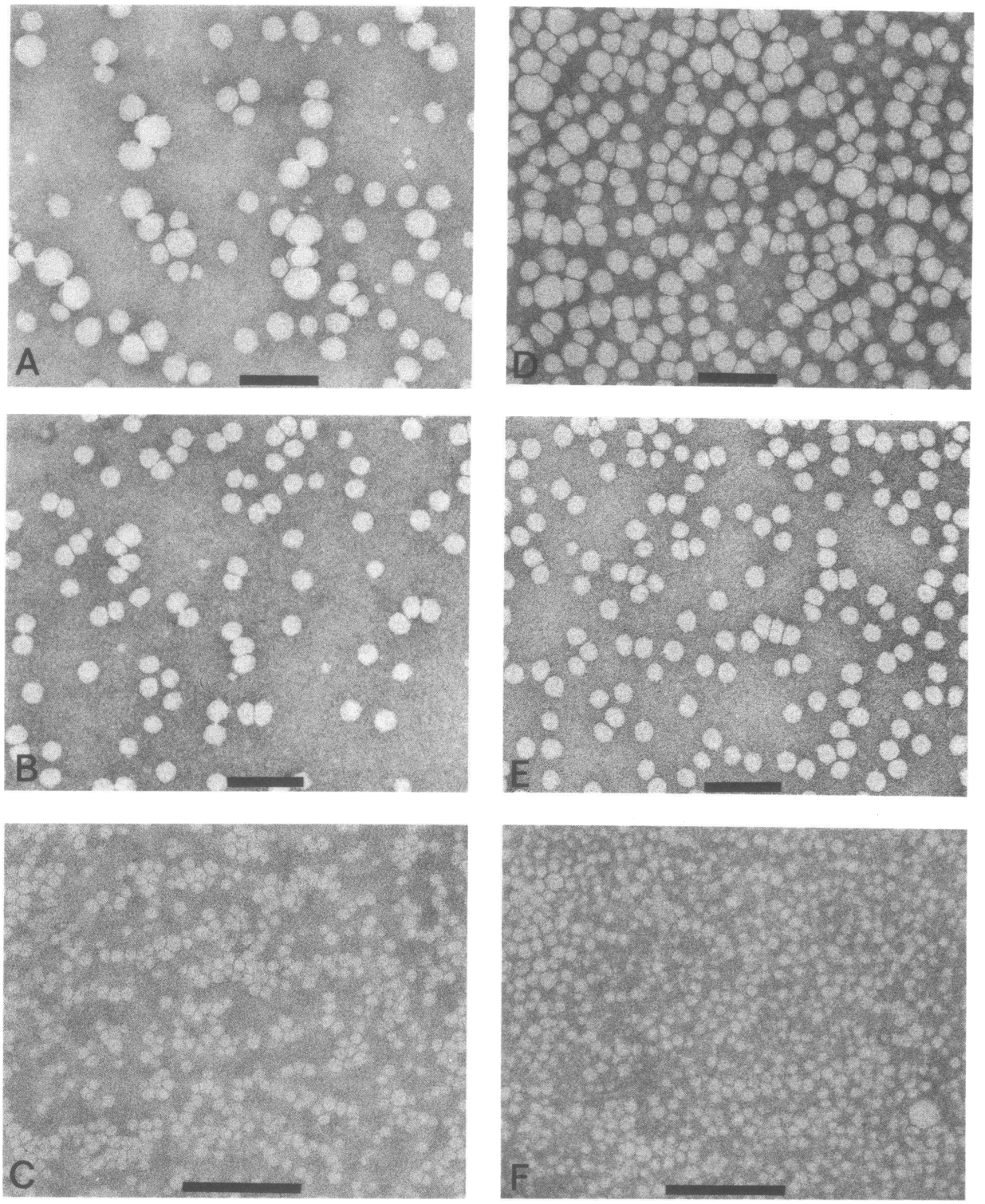

Figure 3. Electron micrographs of negatively stained lipoproteins from control subject and patient 1. *A, B, C* are VLDL, LDL, and HDL, respectively, of the control subject; and *D, E,* and *F* are VLDL, LDL, and HDL, respectively, of the patient. Bars, 100 nm.

Table IV. Composition of the Major Lipoprotein Classes

Lipoprotein	Subject	% Total weight				
		Protein	Phospholipid	Unesterified cholesterol	Cholesteryl ester	Triglyceride
VLDL	Patient 1	13.8	36.4	4.0	11.8	34.2
	Patient 2	15.0	37.9	6.7	18.9	21.5
	Controls*	13.1±0.5	48.9±11.3	3.5±1.0	12.2±0.3	22.2±10.6
LDL	Patient 1	23.7	22.1	7.9	37.7	8.4
	Patient 2	21.5	21.1	8.3	43.0	6.2
	Controls*	21.0±1.5	19.9±4.2	10.3±1.1	43.4±1.1	5.3±0.6
HDL	Patient 1	41.1	31.9	3.9	21.2	2.0
	Patient 2	44.4	26.0	3.7	24.1	2.0
	Controls*	41.8±5.0	34.9±6.9	2.5±0.4	18.8±1.8	2.2±0.1

* Represents the mean±SD of the three controls.

present in postprandial samples. Similar notched and flattened structures have been observed in normal subjects 4 h after a fat meal (Forte, T. M., and R. M. Krauss, unpublished observation). A small amount of material appears in the S_f^o 12–20 region after a fat meal; however, both IDL and VLDL mass declined noticeably toward base-line values in 6–8 h.

After a fat meal, no lipoproteins were detected by analytic ultracentrifugation in the HDL region at any time point. Gradient gel electrophoretic patterns of HDL subpopulation distributions, as a function of time after the fat meal, are seen in Fig. 7 *A*. The HDL distribution of the fasting time point indicates that, in the 10-mo period between our initial and prefat meal analysis of patient 1's HDL, there is an apparent change in relative proportions of HDL subpopulations; the component in the $(HDL_{2a-2b})_{gge}$ region is now a minor peak (30% of the Coomassie staining area instead of 73% noted 10

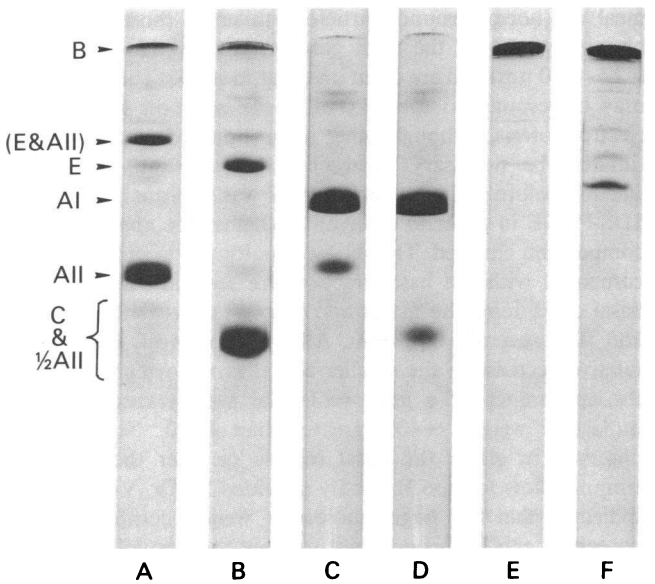

Figure 4. 10% SDS-PAGE gels of patient and control HDL and LDL. (*A*) Patient HDL, unreduced. (*B*) Patient HDL reduced with β-mercaptoethanol. (*C*) Control HDL, unreduced. (*D*) Control HDL reduced with β-mercaptoethanol. (*E*) Patient LDL, unreduced. (*F*) Control LDL, unreduced. Positions of the apolipoproteins are indicated on the left side of figure; 1/2 AII signifies monomeric AII. Proteins applied to gels consisted of 25–30 μg for HDL and 60 μg for LDL.

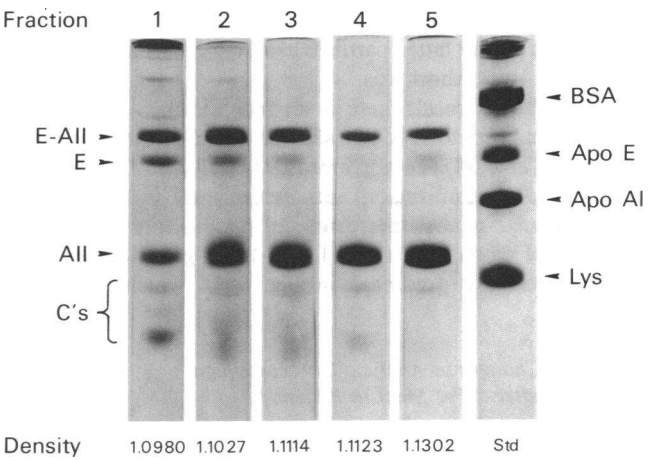

Figure 5. 10% SDS-PAGE gel patterns of HDL gradient subfractions of patient 1; although not shown, the patterns for patient 2 are identical to those shown. The average density of each of the subfractions is indicated below the gel. 30 μg of protein was applied to each gel, and samples were unreduced. The protein standards (Std) are: bovine serum albumin (BSA), Apo E, Apo AI, and egg lysozyme (Lys). The apolipoprotein bands in the patient's subfractions are identified on the left-hand side.

Table V. Plasma Lipid Values after the Fat Meal

Time after meal	Triglyceride	Cholesterol	HDL-cholesterol
	mg/dl	*mg/dl*	*mg/dl*
0	29	89	0
2	56	123	4
4	76	127	2
6	67	128	4
8	51	125	1

mo previously), and the peak at 8.0 nm ($HDL_{3b})_{gge}$ is the major one. Peak positions of the two HDL components in the most recent sample (10.8 and 8.0 nm) are similar to the earlier ones (11.3 and 7.8 nm); additionally, a small peak is apparent in the $(HDL_{3c})_{gge}$ region of the most recent sample. The change in relative peak intensities noted in patient 1's fasting HDL after a 10-mo interval is undoubtedly the result of complex factors, including physiological, environmental, dietary, and drug (e.g., propranolol) effects (26). The gradient gel patterns after a fat meal show no pronounced changes in HDL subpopulations; there appears to be some redistribution of material within the $(HDL_{3c})_{gge}$ interval after fat feeding. Apolipoprotein content as determined by 10% SDS-PAGE (data not shown) was not altered by fat feeding, and at each time point (0, 4, 6, and 8 h), apo E accounted for ~7% of the total Coomassie staining material. Electron microscopy of the postprandial samples revealed that although small round particles (7.6±1.5 nm diam) were the primary structures, a small fraction of the particles (~12%) are discoidal in shape (Fig. 6 *D*). The dimensions of the latter particles are 16.2±2.9 nm, long axis, and 4.1±0.4 nm, short axis.

After the fat meal, there is an increase in S_f° 0–20 peak flotation rate (6.5 to 7.4); this shift persisted throughout the 8-h period (Table VI). Total concentration of the LDL plus IDL (*d* 1.006–1.063 g/ml region) increases slightly and is maximal at 4 h, similar to the time course of plasma triglyceride. The gradient gel patterns of the *d* 1.006–1.063 g/ml fraction after the fat meal are presented in Fig. 7 *B*, which shows the fractions stained separately for protein and lipid. The gradient gel electrophoresis pattern of the 0 h (fasting) time point (Fig. 7 *B*) is similar to that recorded 10 mo previously (see Fig. 2 *B*) and consists of two sharp peaks, one at 26.8 nm and the other at 26.0 nm. The 26.0 nm-peak, however, is less pronounced than the equivalent peak in the earlier study. Coomassie-stained gels indicate that, after consumption of a fat meal, a peak begins to appear in the 33.0-nm region; this peak is most pronounced at 6 h. Oil red O staining indicates that the 33.0-nm peak is probably present in the fasting state in extremely small quantities; however, material in this peak clearly increases in area with time after the meal and is maximal at 6 h. The particle diameter of the 33.0 nm particle suggested that this component might be IDL. Hence, the 6- and 8-h samples (*d* 1.006–1.063 g/ml) were pooled and ultracentrifugally separated into IDL (*d* 1.006–1.019 g/ml) and LDL (*d* 1.019–1.063 g/ml) fractions; however, reassessment of the isolated fractions by gradient gel electrophoresis indicated that the large particles reisolated with the *d* 1.019–1.063 g/ml lipoproteins. The LDL fraction was then subjected to equilibrium density-gradient ultracentrifugation (15) and fractions were harvested; the 33.0-nm peak co-separated in the same fraction as the main LDL peak (data not shown), i.e., in density fractions 1.0357 and 1.0414 g/ml. To determine whether the 33.0-nm peak exhibited any unusual lipoprotein structures, the LDL fraction was examined by electron microscopy. As shown in Fig. 6 *B*, the majority of LDL particles after a fat meal are normal round particles similar to those in Fig. 3; however, ~5% of the total particles are considerably larger (42.5–62.0 nm) and are often irregular in shape. These particles may correspond to the 33.0-nm peak seen on gradient gel electrophoresis, although other separation techniques will ultimately be necessary to resolve this question. The LDL fraction before and after the fat meal was also analyzed on 3% SDS-PAGE in order to determine whether the apolipoprotein composition changed. The gel patterns for postprandial samples compared with the basal fraction are seen in Fig. 8. Under basal conditions, the major apo B species was B100, but B74 and B26 were also apparent. After the fat meal, there was a relative decrease in the smaller apo B forms, which paralleled the appearance of a large molecular weight species with a molecular weight ~40% greater than B100. No B48 was observed in either the basal sample or after the fat meal. Immunoblots for apo B, kindly provided by Dr. Virgie Shore, indicated that the larger molecular weight component also contained apo B; this does not rule out the possibility that the larger molecular weight species is a mixed dimer of apo B and Lp(a) lipoprotein, since Utermann and Weber (27) recently showed that apo B and Lp(a) form a complex of 700,000 D.

Table VI. Changes in Analytic Ultracentrifugal Properties after a Fat Meal in Patient 1

Time	Peak S_f°	S_f° 20–100	S_f° 0–20	S_f° 0–12	S_f° 12–20
h		*mg/dl*	*mg/dl*	*mg/dl*	*mg/dl*
0	6.5	0	307	307	0
2	7.4	27	322	315	7
4	7.4	21	342	330	11
6	7.4	3	326	318	9
8	7.4	3	295	289	6

Discussion

Previous studies by Norum et al. (1) on two sisters with familial apo AI and apo CIII deficiency showed that plasma HDL levels were extremely reduced, but plasma triglyceride

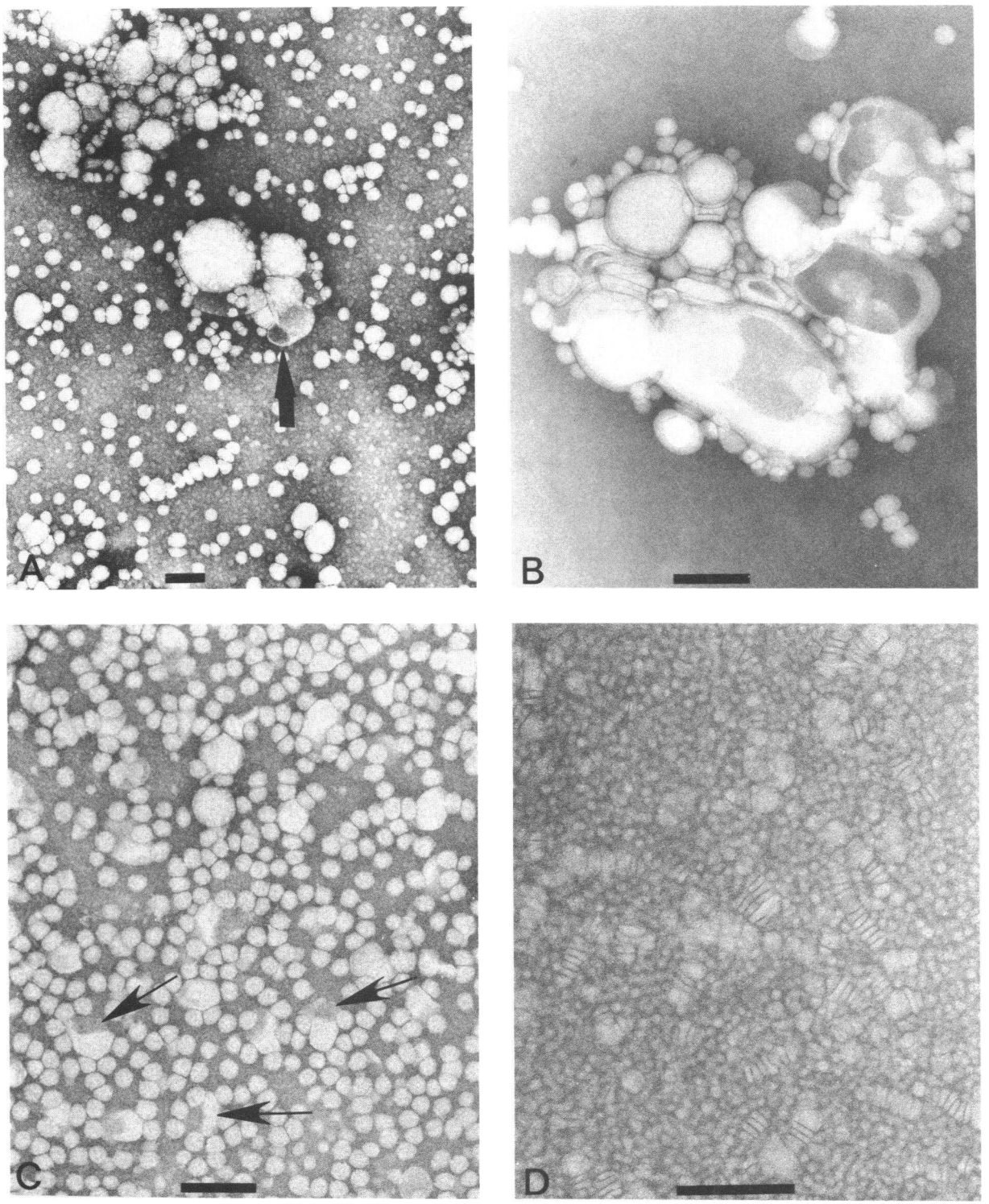

Figure 6. Electron micrographs of negatively stained lipoproteins of patient 1, 4 h after a fat meal. (*A*) VLDL, note extreme heterogeneity of particle size and notched appearance of occasional particles (arrow). The fine, pebbly appearance of background is artifact. (*B*) Higher magnification of patient's VLDL showing the irregular shape of particles. (*C*) LDL, the majority of LDL are normal, but some irregularly shaped structures (arrows) are also present. (*D*) HDL, small round structures form the majority of the particles, but short stacks of discoidal particles are also present. Bar, 100 nm.

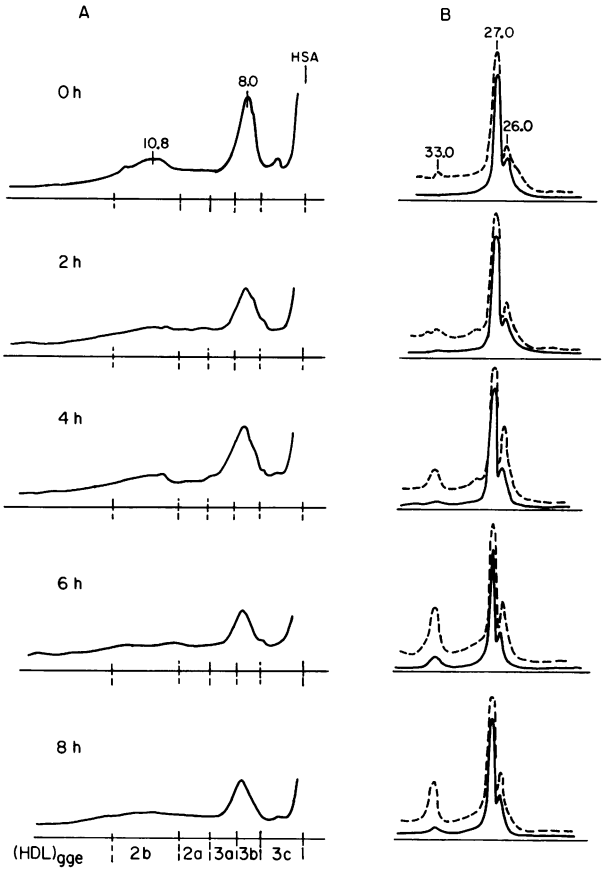

Figure 7. Gradient gel electrophoresis patterns of *d* 1.063–1.21 g/ml (*A*) and *d* 1.006–1.063 g/ml lipoproteins (*B*). The lipoproteins were isolated from patient 1 after a fat meal at the time points indicated on the left side. The spectrophotometric scans of the stained gels show relative distribution of the lipoprotein subpopulations. The patients' HDL possess a broad peak in the $(HDL_{2a-2b})_{ggc}$ region and a sharp peak in the $(HDL_{3b})_{ggc}$ region (8.0 nm); at all time points, the broad peak accounts for ~30% of the staining lipoproteins, while the material in the $(HDL_3)_{ggc}$ region accounts for 70%. HSA (human serum albumin) indicates the position of albumin. *B* shows the LDL electrophoresed on 2–16% gels and stained with Coomassie (solid line) and oil red O (dashed line). The major peak positions are indicated by the numbers above the peaks (diameter of particles in nanometers). The Coomassie and oil red O peaks coincide; however, the peak at 33.0 nm is more pronounced with oil red O staining, suggesting that this subpopulation contained relatively more lipid. The amplitude of the 33.0-nm peak appears to increase with time and is maximal at 6 h.

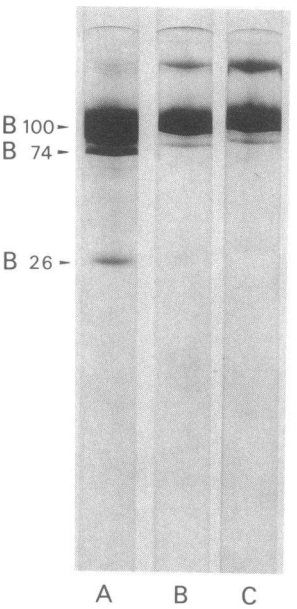

Figure 8. 3% SDS-PAGE of LDL from patient 1 at 0 h (*A*), 4 h (*B*), and 6 and 8 h (*C*) after a fat meal. The apo B variants as described by Kane et al. (22) are designated B100, B74, and B26. After a fat meal, a larger molecular weight component is seen above the B100 band. 40 μg of protein was applied to each tube.

and cholesterol levels were near normal. In addition to a deficiency of apo AI and apo CIII, they found that other apolipoproteins were present in plasma at reduced levels (apo AII, 50%; apo E, 50%; apo CI, 40%; and apo CII, 5–16% of normal) with the exception of apo B, which was normal (1).

Extremely reduced levels of HDL have been found in other disorders, including the apo AI- and apo CIII-deficient subject described by Schaefer et al. (11, 12), familial LCAT deficiency (5), Tangier disease (8), apo AI_{Milano} abnormality (13), fish eye disease (28), and hypoalphalipoproteinemia, described by Toth and Koenig (29). In most disorders where HDL concentration is severely depressed, plasma triglyceride levels are elevated above normal; however, the apo AI- and apo CIII-deficient patients described by Norum et al. (1) and the HDL-deficient patient of Schaefer et al. (11) are remarkable in that plasma triglyceride levels are low. The common factor in all the above disorders is a low HDL cholesterol; however, they are not all associated with premature vascular disease. The notable exceptions are the apo AI_{Milano} abnormality, Tangier disease, and fish eye disease.

In the present study, we have isolated and characterized the lipoproteins from the two sisters with apo AI and apo CIII deficiency. Fasting plasma total cholesterol and triglyceride values of the patients were similar to those of three healthy female controls. Analytic ultracentrifugation revealed that the patients' lipids are transported almost exclusively in the S_f° 0–12 flotation interval. The distribution and flotation characteristics of the patients' $d < 1.063$ g/ml lipoproteins are similar to those described by Schaefer et al. (11) for their HDL-deficient patient. The latter patient, however, had less total LDL mass.

The morphology of VLDL from our patients was normal and did not possess the interconnecting filaments described by Schaefer et al. (11) for their patient. The patient who consumed a 100-g fat meal showed a modest increase in plasma triglyceride levels. This increase was, however, much less than that reported for normal subjects in other studies involving a similar fat load (30, Krauss, R. M., unpublished observation). The increase in the patient's triglyceride was also reflected by increases in S_f° 20–100 mass and by increases in VLDL diameters. The

presence of notched or irregular particles after the fat meal suggests that lipolysis has occurred, since similar structures have been seen by others after in vivo and in vitro lipolysis of lipoproteins (31, 32).

In the present study, patients' and control fasting LDL are alike in flotation rate and electron microscopic structure. This similarity was also borne out in the chemical composition of LDL in the two groups. On the basis of gradient gel electrophoresis, the patients' LDL are not monodisperse, as suggested by analytic ultracentrifugation and electron microscopy, but contain at least two discrete components; moreover, the major peak in the patients was very similar in position (27 nm) to the major peak in the controls. This peak position, based on the work of Krauss and associates (19, 33), is in the LDL-II density class (*d* 1.032–1.035 g/ml) and has an S_f° peak of 7–8, which in normal subjects has a strong positive correlation with HDL_2 levels and a negative one with VLDL and IDL. Although this relationship is consistent with the results in our control subjects, an anomaly occurs in the patients, where the LDL-II density class is associated with a near absence of HDL.

One of the purposes of the fat-feeding study was to determine whether the LDL subpopulations in the patient could be altered by the influx of dietary fat. The data from gradient gels suggested (Fig. 7 *B*) that the major LDL peak at 27 nm was not affected by dietary fat; however, an additional component of apparent larger size (33 nm) appeared and increased in area with time after fat intake. The apparent discrepancy between the size by gradient gel electrophoresis of the latter particle (which is in the size range of IDL) and its density (isolated between 1.0357 and 1.0414 g/ml) suggests that the particle may not be spherical. The latter was supported by electron microscopy, which showed that large, irregularly shaped particles were present in the LDL fraction. The appearance of the 33.0-nm peak was also paralleled by the appearance of a band on 3% SDS-PAGE which was ~40% larger in molecular weight than B100. It is tempting to speculate that this high molecular weight band is associated with the unusual particle, but clearly such a premise awaits further investigation. Since immunoblotting revealed that the large molecular weight band contained apo B, this product may represent an aggregated form of apo B; however, we cannot rule out the presence of an apo B-Lp(a) mixed dimer. Ginsberg et al. (34) recently reported that both patients injected with autologous ^{125}I-VLDL had fractional catabolic rates three to five times greater than normal individuals. Since catabolism of VLDL, and very likely chylomicrons as well, is accelerated in these patients, the 33.0-nm particles may represent abnormal lipoprotein remnants. Accelerated metabolism of chylomicrons may also account for the finding that triglyceride levels after the fat meal were considerably lower than that reported for subjects fed similar amounts of fat in previous studies (30). Fat-feeding studies (70–100 g fat) carried out in 20 normal and one hypertriglyceridemic subject did not generate a peak at 33.0 nm in the LDL fraction (Krauss, R. M., unpublished observation), which adds further support to the hypothesis that the peak is related to abnormal metabolism of triglyceride-rich lipoproteins.

In agreement with the previous investigations by Norum et al. (1), HDL concentrations in the patients were extremely low, i.e., ~3–5% the level in the controls. Although HDL levels were drastically reduced, their compositions were similar to that of normal HDL. Like normal HDL, cholesteryl ester is the major core constituent of the HDL in these patients and probably accounts for the spherical morphology of the particles. The presence of the cholesteryl ester core further suggests that apo AI, which normally activates LCAT, is not an absolute requirement for LCAT activity and that apo CI, which is present at ~40% of normal levels (1), may assume the role of activator, as suggested by early studies of Soutar et al. (35). After a fat meal, a small percentage (12%) of the HDL particles were discoidal. These discoidal particles probably represented surface constituents generated during hydrolysis of triglyceride-rich particles, as suggested by the studies of others (36, 37). However, we have found no such structures in HDL from normal subjects after a fat meal (Forte, T. M., and R. M. Krauss, unpublished observation), which would indicate that there is delayed transformation of discs to spheres in the patient. The latter may be due to the unusual apolipoprotein composition of patients' HDL as well as decreased LCAT activity.

The subpopulation distribution of apo AI- and apo CIII-deficient HDL as determined by gradient gel electrophoresis is abnormal. Two major components, one with large diameters (11.0 nm) and one with small diameters (7.8 nm), are characteristic in this abnormality, along with an absence of $(HDL_{3a})_{ggc}$. The large HDL particles, which were isolated in a density gradient at *d* 1.0980 g/ml, were unique in that they possessed apo E and apo AII as major apolipoprotein constituents. Normally, this density range, which has been termed HDL_1 or HDL_2-with E, contains apo AI particles enriched in apo E (38, 39). Apo AII in HDL of normal subjects is mainly associated with smaller, denser HDL particles (40).

Apo E in our patients is present primarily as a mixed disulfide complex, apo (E-AII). The relative abundance of the apo (E-AII) complex may provide the mechanism by which the patients' HDL particles persist in the plasma even though their HDL turnover rate is twofold greater than normal (1). Generally, apo E is removed via high affinity apo B-E receptors; however, Innerarity et al. (41) have shown that complexed apo (E-AII) is inactive and requires reduction and alkylation for uptake and degradation. Since in our patients much of the apo E in HDL is in the apparently inactive form, a subpopulation of HDL may have a longer residence time in the plasma and may thus accumulate a larger lipid load than ordinarily encountered in normal HDL.

In addition to the lipid-rich 11.0-nm particles, the patients also possess a subpopulation of small HDL in the size range of $(HDL_{3b})_{ggc}$ described by Nichols et al. (14). This subpopulation is greatly diminished in normal individuals where $(HDL_{3a})_{ggc}$ is the major component. A pronounced peak in

the size range of small HDL $(HDL_{3b-3c})_{gge}$ also appears in several other dyslipoproteinemias associated with diminished levels of HDL, including familial LCAT deficiency (6), acute obstructive jaundice (14), and fish eye disease (10). The association between elevated $(HDL_{3b-3c})_{gge}$ subpopulations and HDL deficiency is not related to the absence of apo AI since in the abovementioned defects, plasma apo AI, although low, is present.

The lack of a peak in the $(HDL_{3a})_{gge}$ region is a distinctive feature in apo AI and apo CIII deficiency. A similar absence has also been noted in human umbilical cord blood HDL (42). Like our patients, cord blood HDL possess pronounced components in the $(HDL_{2b})_{gge}$ and $(HDL_{3b-3c})_{gge}$ subpopulations. In both instances, triglyceride-rich lipoprotein levels are extremely low, which suggested the possibility that $(HDL_{3a})_{gge}$ metabolism may be related to that of chylomicron and/or VLDL metabolism. Normal catabolism of VLDL may generate an intermediate-sized HDL particle such as $(HDL_{3a})_{gge}$ by transfer of triglyceride into HDL and its subsequent hydrolysis by lipoprotein lipase, as suggested by the scheme of Deckelbaum et al. (43). Where triglyceride-rich particles are limiting, the intermediate-sized particles may not be generated, and less dense and more dense HDL particles accumulate. The fat-feeding study was carried out to determine whether $(HDL_{3a})_{gge}$ was linked with catabolism of triglyceride-rich particles; however, clearance of such particles in the patient did not produce a shift of material into the $(HDL_{3a})_{gge}$ region. Hence, in these patients, it is unlikely that the $(HDL_{3a})_{gge}$ subpopulation is related to triglyceride-rich lipoprotein metabolism.

Previous studies on these patients suggest that a single gene mutation is responsible for the defect in synthesis of apo AI and apo CIII (2–4). HDL and apo AI play an essential role in removal of excess cholesterol from cells and tissues (44), while apo CIII is thought to inhibit or modulate lipolytic activity (34, 45) and to inhibit cellular uptake of apo E-containing chylomicron remnants (46, 47). Reduction of HDL levels is not sufficient in itself, however, to account for premature atherosclerosis, since there are several instances of hypoalphalipoproteinemia not associated with premature vascular disease (8, 10, 13). The critical link in our patients may be in the simultaneous deficiency of apo AI and apo CIII, which suggests a dual defect in lipoprotein metabolism: one in triglyceride-rich lipoprotein metabolism and the other in HDL metabolism. Firstly, the absence of apo CIII may result in accelerated catabolism of triglyceride-rich particles and an increased rate of LDL formation, as suggested by the apo B turnover studies in these patients (34). Secondly, the absence of apo CIII would favor unusually rapid uptake of apo E-containing chylomicron remnants by liver and peripheral cells. Both these abnormalities would predispose the patients to accumulate excess cellular and arterial cholesterol. Normally, excess tissue cholesterol could be taken up by HDL, but with the near absence of these particles in this disorder, excess tissue and arterial cholesterol accumulates, which leads to premature atherosclerosis.

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