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Research Article

Familial dysbetalipoproteinemia (or type III hyperlipoproteinemia) is characterized by the presence of abnormal, cholesteryl ester-rich beta-very low density lipoproteins (beta-VLDL) in the plasma. Subjects with typical dysbetalipoproteinemia are homozygous for an amino acid substitution in apolipoprotein (apo-) E at residue 158 and have defective apo-E-mediated binding of both pre-beta-VLDL and beta-VLDL to apo-B,E(LDL) (or LDL) receptors (1988. Chappell, D.A., *J. Clin. Invest.* 82:628-639). To understand the effect of substitutions in apo-E at sites other than residue 158, nine dysbetalipoproteinemic (dys-beta) subjects who were either homozygous or heterozygous for substitutions in apo-E at atypical sites were studied. These substitutions occurred at residue 142 (n = 6), 145 (n = 2), or 146 (n = 1) and are known to cause less defective binding than does the 158 substitution. The chemical composition and electrophoretic mobility of pre-beta-VLDL and beta-VLDL from atypical and typical dys-beta subjects were indistinguishable. However, lipoproteins from atypical and typical dys-beta subjects differed in their affinity for the apo-B,E(LDL) receptor on cultured human fibroblasts. The pre-beta-VLDL and beta-VLDL from atypical dys-beta subjects had 640- or 17-fold higher affinity, respectively, than did corresponding lipoproteins from typical dys-beta subjects. The higher binding affinity of lipoproteins from atypical dys-beta subjects was associated with a higher ratio of apo-E to total apo-C. Since higher binding affinity should cause more rapid receptor-mediated clearance of [...]

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High Receptor Binding Affinity of Lipoproteins in Atypical Dysbetalipoproteinemia (Type III Hyperlipoproteinemia)

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Abstract

Familial dysbetalipoproteinemia (or type III hyperlipoproteinemia) is characterized by the presence of abnormal, cholesteryl ester-rich β -very low density lipoproteins (β -VLDL) in the plasma. Subjects with typical dysbetalipoproteinemia are homozygous for an amino acid substitution in apolipoprotein (apo-) E at residue 158 and have defective apo-E-mediated binding of both pre- β -VLDL and β -VLDL to apo-B,E(LDL) (or LDL) receptors (1988. Chappell, D. A., *J. Clin. Invest.* 82:628-639). To understand the effect of substitutions in apo-E at sites other than residue 158, nine dysbetalipoproteinemic (dys- β) subjects who were either homozygous or heterozygous for substitutions in apo-E at atypical sites were studied. These substitutions occurred at residue 142 ($n = 6$), 145 ($n = 2$), or 146 ($n = 1$) and are known to cause less defective binding than does the 158 substitution. The chemical composition and electrophoretic mobility of pre- β -VLDL and β -VLDL from atypical and typical dys- β subjects were indistinguishable. However, lipoproteins from atypical and typical dys- β subjects differed in their affinity for the apo-B,E(LDL) receptor on cultured human fibroblasts. The pre- β -VLDL and β -VLDL from atypical dys- β subjects had 640- or 17-fold higher affinity, respectively, than did corresponding lipoproteins from typical dys- β subjects. The higher binding affinity of lipoproteins from atypical dys- β subjects was associated with a higher ratio of apo-E to total apo-C. Since higher binding affinity should cause more rapid receptor-mediated clearance of β -VLDL in atypical than in typical dys- β subjects in vivo, the mechanism of β -VLDL accumulation may differ in these two groups.

Introduction

Familial dysbetalipoproteinemia (or type III hyperlipoproteinemia) is characterized by the presence of abnormal, cholesteryl ester-rich β -migrating very low density lipoproteins (β -VLDL) in the plasma (1). Apolipoprotein (apo-) E was first implicated in the pathogenesis of dysbetalipoproteinemia when Havel and Kane reported the abundance of apo-E in

β -VLDL isolated from dysbetalipoproteinemic (dys- β) subjects (2). Later, by isoelectric focusing, Utermann et al. showed that apo-E from dys- β subjects was abnormal (3). There are three common isoforms of apo-E, (E2, E3, and E4), which differ in isoelectric focusing positions as a result of substitutions involving charged amino acids (4, 5). Apolipoprotein E4 differs from normal apo-E3 by the substitution of arginine for cysteine at residue 112 (Cys₁₁₂ \rightarrow Arg), whereas apo-E2 differs from apo-E3 by the substitution of cysteine for arginine at residue 158 (Arg₁₅₈ \rightarrow Cys) (5, 6). Most dys- β subjects are homozygous for the substitution at residue 158 (Arg₁₅₈ \rightarrow Cys) and have an E2/2 phenotype (6, 7). These subjects are said to have typical dysbetalipoproteinemia, which is inherited as an autosomal recessive trait. Heterozygous individuals with the E3/2 phenotype rarely develop dysbetalipoproteinemia. However, dys- β subjects with E3/3, E3/2, or E2/1 phenotypes have been described (8-12). In addition, a variety of amino acid substitutions may cause the same phenotype.

Apolipoprotein E binds to apo-B,E(LDL) (or LDL) receptors with high affinity. All of the known substitutions in apo-E that are associated with dysbetalipoproteinemia disrupt this binding to some extent (for review, see reference 1). Schneider et al. showed that apo-E isolated from dys- β subjects may have either very defective or nearly normal receptor binding compared to the binding of apo-E from normal individuals (13). Normal apo-E3, purified and reconstituted into phospholipid complexes, has 50-, 5-, 2.5-, or 2-fold higher affinity than does apo-E with a substitution at residue 158, 142, 146, or 145, respectively (1, 5, 8).

The severity of the binding defect caused by the various substitutions in apo-E does not correlate with the severity of dysbetalipoproteinemia (6, 13). The vast majority of individuals homozygous for the most defective form of apo-E do not develop clinically significant hyperlipidemia. These individuals have β -VLDL in their plasma but have normal or even low cholesterol concentrations (6, 14-16). Clearly, factors other than defective binding of apo-E to apo-B,E(LDL) receptors are important in the expression of this disease. These factors include obesity, the inheritance of hypertriglyceridemia or familial combined hyperlipidemia, and the effects of estrogen or thyroid hormones (17-23).

The mechanism of β -VLDL formation is partially understood. Pre- β -VLDL appear to be precursors of β -VLDL. Pre- β -VLDL are larger, more triglyceride-rich particles with higher flotation rates than β -VLDL and may be converted to

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1. *Abbreviations used in this paper:* dys- β , familial dysbetalipoproteinemic; EC₅₀, the 50% effective concentration of competition for ¹²⁵I-LDL binding to receptors; pre- β -VLDL, pre- β -migrating very low density lipoproteins.

β -VLDL during lipolysis (24–28). Accelerated lipolysis induced by intravenous heparin causes the formation of β -VLDL and a decrease in pre- β -VLDL in subjects with either normal or abnormal apo-E (25–28). Recently, I showed that pre- β -VLDL from typical dys- β subjects had extremely low affinity for apo-B,E(LDL) receptors (27). In these subjects, defective binding of pre- β -VLDL to receptors could result in delayed receptor-mediated clearance and increased conversion to β -VLDL during lipolysis. Their β -VLDL had 68-fold higher binding affinity than their pre- β -VLDL (27). However, the binding of β -VLDL from the typical dys- β subjects was defective when compared with heparin-induced β -VLDL from subjects homozygous for normal apo-E3 (27). These data suggest that defective binding of both pre- β -VLDL and β -VLDL to receptors may be involved in the formation and accumulation of β -VLDL in typical dys- β subjects.

In this study, dys- β subjects who were either heterozygous or homozygous for amino acid substitutions in apo-E at atypical sites (residue 142, 145, or 146) are defined as having atypical dysbetalipoproteinemia. To understand the role of atypical apo-E mutations in the pathogenesis of dysbetalipoproteinemia, the chemical composition, apolipoprotein content, and receptor binding affinities of their $d < 1.006$ g/ml lipoproteins were determined. In addition, cholesterol-fed rabbits and cholesterol-fed dogs, animal models in which there is β -VLDL accumulation (29–31), were studied to see whether their $d < 1.006$ g/ml lipoproteins resembled those from typical or those from atypical dys- β subjects.

Methods

Human subjects

The subjects studied are described in Table I. 10 subjects were members of a single family (family A.) in which dysbetalipoproteinemia was inherited as an autosomal dominant trait through four generations (9, 10). All family members had an E3/3 phenotype. However, family members with dysbetalipoproteinemia were heterozygous for normal apo-E3 and a double mutation in apo-E, that resulted in no net change in the charge (Arg₁₄₂ → Cys, Cys₁₁₂ → Arg) (10). Family members who did not have dysbetalipoproteinemia had a double pre- β pattern on agarose electrophoretograms of their VLDL (9). Subject 3 was taking gemfibrozil (600 mg twice daily) at the time of this study. The other dys- β subjects were not taking medication. Subjects 11 and 12 had an E2/2 phenotype and were homozygous for the Arg₁₄₅ → Cys substitution in apo-E (5, 32). Subject 13 had an E3/2 phenotype and had had a dys- β profile (8); however, at the time of this study he had a type V lipoprotein phenotype as determined by agarose electrophoresis and analytical ultracentrifugation (data not shown). All subjects gave written informed consent for the study; the study was approved by the Human Research Committee at University of California, San Francisco.

Lipoprotein preparation

Subjects fasted for 14 h before blood sampling. Blood samples were adjusted to contain 1 mg EDTA (sodium salt)/ml and were immediately placed on ice. The plasma was separated from the cells by centrifugation at 2,000 rpm for 15 min at 4°C. To prevent proteolytic degradation, the plasma was adjusted to contain 10,000 U of aprotinin/liter (Mobray Chemical Corp., New York), 1 mM benzamide, 1 mM PMSF, and 80 mg of gentamicin/liter (Sigma Chemical Co., St. Louis, MO) (33).

Isolation of lipoproteins by ultracentrifugation. The $d < 1.006$ g/ml lipoproteins and LDL were isolated from the plasma by ultracentrifugation (34). The $S_r > 100$, $S_r 60$ –100, and $S_r 20$ –60 flotation fractions were isolated according to the method of Lossow et al. (35).

Pevikon block electrophoresis. Pre- β -VLDL and β -VLDL were isolated by Pevikon block electrophoresis of the total $d < 1.006$ g/ml lipoproteins (Mercer Consolidated Corp., Yonkers, NY) (36, 37). Human LDL were used as a reference for β -electrophoretic mobility to aid in determining the fractionation of pre- β -migrating and β -migrating lipoproteins on each Pevikon block (27).

Lipoprotein and apolipoprotein characterization. Measurement of protein, cholesterol, triglyceride, phospholipid, and cholesteryl ester concentrations was done in duplicate or triplicate and using different aliquot sizes. Protein concentration was determined by the method of Lowry et al. (38); bovine albumin was used as the standard. Cholesterol and triglyceride concentrations were measured by using a colorimetric enzymatic assay (Boehringer-Mannheim Corp., Indianapolis, IN). Phospholipid concentration was estimated from the phosphorus content (39). Cholesteryl ester concentration was estimated by using gas-liquid chromatography (27). The relative apolipoprotein content of lipoproteins was determined using electrophoresis on 5–20% SDS-polyacrylamide gradient gels stained with Coomassie blue R-250 (27, 40). Coomassie blue-stained gels were scanned at 600 nm on a CS-930 scanner (Shimadzu, Kyoto, Japan). Silver staining was performed on some gels (41). The apo-E phenotypes were determined as described (42). The molecular weights of lipoproteins were estimated from their average molecular volume assessed by electron microscopy (43) and by the partial specific volumes of the chemical constituents (27, 44). The electrophoretic mobility of each fraction was determined by 1% agarose electrophoresis (Corning, Palo Alto, CA) (27).

Fibroblast binding assays

Normal human fibroblasts were grown in 35-mm petri dishes (45). Lipoproteins were iodinated (sp act 200–500 cpm/ng) by the iodomonochloride method (46). The ability of $d < 1.006$ g/ml fractions at various concentrations to compete for the binding of ¹²⁵I-LDL at 2 μ g of protein/ml at 4°C was determined in duplicate (45). Binding data were analyzed by nonlinear least-squares curve fitting (allfit) (27, 47, 48).

Monoclonal antibodies that specifically inhibit binding by apo-E (1D7) or apo-B100 (4G3) to the apo-B,E(LDL) receptor were generously provided by Dr. Y. Marcel and Dr. R. Milne, Clinical Research Institute of Montreal (49, 50). The monoclonal antibody MB47, which specifically inhibits the binding of apo-B100, was generously provided by Dr. S. Young, of the Gladstone Foundation Laboratories (51). The ability of 1D7, 4G3, or MB47 to inhibit the binding of ¹²⁵I-VLDL fractions to cultured human fibroblasts at 4°C was determined by incubating medium containing 5–20 μ g of antibody/ml and 1 μ g of ¹²⁵I-VLDL/ml for 1 h at 25°C before use in the binding assays (27, 52). Nonspecific binding was defined as the amount bound in the presence of 100-fold excess of the corresponding unlabeled lipoprotein fraction. All measurements were performed in duplicate.

Animal studies. Rabbits and foxhounds were fed a high-cholesterol, high-saturated-fat diet (31). The plasma cholesterol concentration in these animals was > 1,000 mg/dl. Pre- β -VLDL and β -VLDL were isolated by using the same method described above for human lipoproteins.

Results

The dys- β subjects studied were 0–45% above their ideal body weight (Table I). Members of family A. who did not have dysbetalipoproteinemia had a double pre- β pattern; furthermore, three were hypercholesterolemic. The plasma cholesterol and triglyceride concentrations in the dys- β subjects ranged from minimally to markedly elevated.

Characterization of $d < 1.006$ -g/ml lipoproteins. Lipoproteins were isolated by Pevikon block electrophoresis of the total $d < 1.006$ -g/ml fraction and by sequential ultracentrifugal flotation of whole plasma. The relative content of pre- β -VLDL and β -VLDL in the $d < 1.006$ -g/ml fractions as as-

Table I. Characteristics of Subjects Studied

Subject*	Age	Sex	%IBW [‡]	C [§]	TG [§]	Lipoprotein phenotype	Apo-E phenotype	Apo-E genotype [¶]
<i>yr</i>								
Family A								
1	72	F	110	259	222	Dys- β (type III)	E3/3	N/Arg ₁₄₂ \rightarrow Cys, Cys ₁₁₂ \rightarrow Arg
2	51	F	124	298	129	Dys- β (type III)	E3/3	N/Arg ₁₄₂ \rightarrow Cys, Cys ₁₁₂ \rightarrow Arg
3	26	M	116	697	852	Dys- β (type III)	E3/3	N/Arg ₁₄₂ \rightarrow Cys, Cys ₁₁₂ \rightarrow Arg
4	23	F	145	285	264	Dys- β (type III)	E3/3	N/Arg ₁₄₂ \rightarrow Cys, Cys ₁₁₂ \rightarrow Arg
5	22	F	110	525	316	Dys- β (type III)	E3/3	N/Arg ₁₄₂ \rightarrow Cys, Cys ₁₁₂ \rightarrow Arg
6	6	M	100	414	233	Dys- β (type III)	E3/3	N/Arg ₁₄₂ \rightarrow Cys, Cys ₁₁₂ \rightarrow Arg
7	51	M	136	216	119	Double pre- β	E3/3	N/N
8	27	F	133	284	90	Double pre- β	E3/3	N/N
9	18	M	118	361	118	Double pre- β	E3/3	N/N
10	7	F	119	290	67	Double pre- β	E3/3	N/N
Other dys- β subjects								
11	58	M	110	224	223	Dys- β (type III)	E2/2	Arg ₁₄₅ \rightarrow Cys/Arg ₁₄₅ \rightarrow Cys
12	44	M	122	235	169	Dys- β (type III)	E2/2	Arg ₁₄₅ \rightarrow Cys/Arg ₁₄₅ \rightarrow Cys
13	56	M	112	474	1480	Type V	E3/2	N/Lys ₁₄₆ \rightarrow Gln

* Subjects 1-5, 7-9, and 11-13 have been described previously (see references 5, 8-10, and 32). [‡]%IBW, percent of ideal body weight from New York Metropolitan Life tables. [§]C and TG, plasma cholesterol and triglycerides in mg/dl, respectively. ^{||}As determined by 1% agarose electrophoresis of the $d < 1.006$ g/ml fraction. [¶]Each allele is indicated; N, normal apo-E3.

essed by agarose electrophoresis (data now shown) was similar to that of corresponding fractions from the typical dys- β subjects studied previously (27). The total $d < 1.006$ g/ml fractions in all dys- β subjects contained a prominent β -migrating band and a less prominent pre- β -migrating band. The pre- β -VLDL predominated in the $S_f > 100$ fraction, whereas the β -VLDL predominated in the $S_f 20-60$ fraction. Using agarose electrophoresis, the $d < 1.006$ -g/ml lipoproteins from atypical

and typical dys- β subjects were indistinguishable. Subject 13 was included in the study because he had had a dys- β profile. However, at the time of this study, he had a type V profile, as determined by agarose electrophoresis and analytical ultracentrifugation (data not shown).

The chemical compositions and calculated average molecular weights of the $d < 1.006$ g/ml lipoproteins are listed in Table II. These data are similar to those for corresponding

Table II. Composition* and Size of $d < 1.006$ g/ml Fractions

Subject	Sample	Protein	Free cholesterol	Cholesteryl ester	Triglyceride	Phospholipid	Diameter [‡]	Molecular weight
							<i>nm</i>	$\times 10^6$
Apo-E substitution - Arg ₁₄₂ \rightarrow Cys								
3	β -VLDL	6.33 \pm 1.0	10.5	32.6	35.9	14.7 \pm 0.58	29 \pm 5.2	7.8
3	Pre- β -VLDL	7.45 \pm 2.0	11.4	13.9	50.3	17.0 \pm 2.2	48 \pm 12	34
4	$S_f 20-60$	8.80 \pm 0.42	13.6	26.8	27.8 \pm 1.4	23.0	31 \pm 3.8	9.3
4	$S_f 60-100$	5.94 \pm 0.16	11.4	22.1	41.1 \pm 1.3	19.4 \pm 3.8	41 \pm 3.1	22
4	$S_f > 100$	3.71 \pm 0.2	7.74	25.2	50.3 \pm 6.9	13.0 \pm 1.3	54 \pm 7.4	47
Apo-E substitution - Arg ₁₄₅ \rightarrow Cys								
12	β -VLDL	8.25 \pm 0.77	15.3	20.2	36.0 \pm 1.1	20.3 \pm 3.4	31 \pm 6.3	9.7
12	Pre- β -VLDL	6.28 \pm 0.23	4.24	12.6	65.7 \pm 2.9	11.2	45 \pm 11	28
12	$S_f 20-60$	10.4 \pm 0.27	9.70	26.6	35.0 \pm 3.0	18.3 \pm 2.2	30 \pm 3.3	8.8
12	$S_f 60-100$	7.75 \pm 0.28	8.22	24.5	43.6 \pm 3.5	15.9 \pm 1.9	42 \pm 5.1	23
12	$S_f > 100$	6.94 \pm 0.11	7.55	25.7	46.2 \pm 2.9	13.6 \pm 1.8	49 \pm 8.7	37
Apo-E substitution - Lys ₁₄₆ \rightarrow Gln [§]								
13	β -fraction	6.50 \pm 0.00	8.47	11.6	54.5 \pm 2.9	18.9 \pm 5.9	47 \pm 8.1	32
13	Pre- β -fraction	6.00 \pm 0.37	6.06	7.37	69.4 \pm 7.3	11.1 \pm 2.8	52 \pm 10	43

* Percent dry weight of two or three measurements \pm SD. [‡]Diameter of 100 particles \pm SD. [§]Since no distinct β -VLDL were found in this subject, the more β -migrating fraction and more pre- β -migrating fractions isolated by Pevikon block electrophoresis were studied.

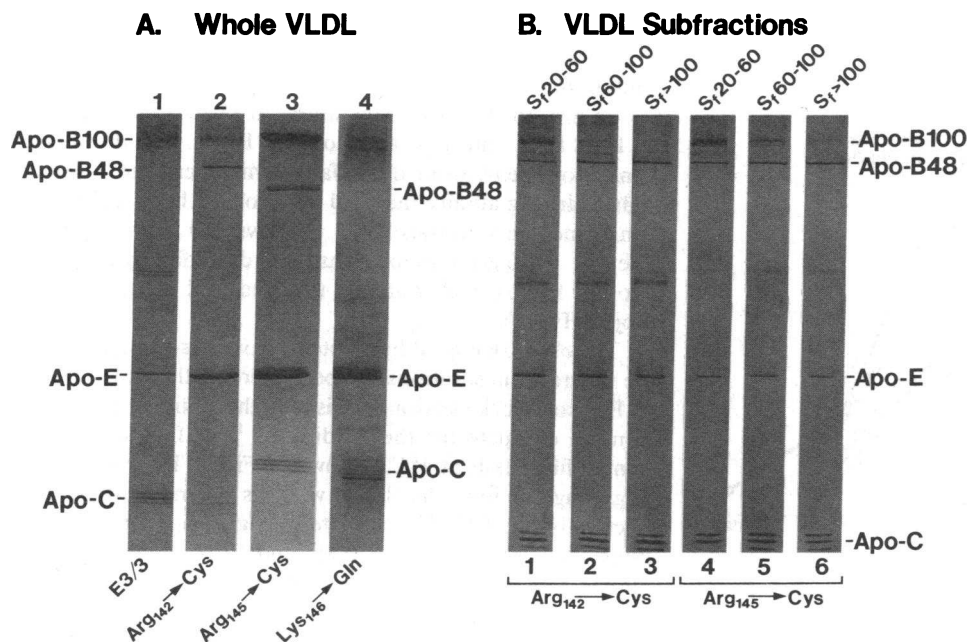


Figure 1. 5–20% SDS-PAGE of $d < 1.006$ g/ml fractions. Samples were reduced with 0.05 M DTT. (A, lanes 1–4) The Coomassie blue-stained total $d < 1.006$ g/ml lipoproteins from subject 7 (homozygous for normal apo-E3), dys- β subject 3 (heterozygous for the Arg₁₄₂ → Cys substitution in apo-E), dys- β subject 11 (homozygous for the Arg₁₄₅ → Cys substitution in apo-E), and dys- β subject 13 (heterozygous for the Lys₁₄₆ → Gln substitution in apo-E), respectively. (B, lanes 1, 2 and 3) The silver-stained S_f 20–60, S_f 60–100, and S_f > 100 fractions, respectively, from dys- β subject 3 (heterozygous for the Arg₁₄₂ → Cys substitution in apo-E). Lanes 4, 5, and 6 are the silver-stained S_f 20–60, S_f 60–100, and S_f > 100 fractions, respectively, from subject 12 (homozygous for the Arg₁₄₅ → Cys substitution in apo-E).

lipoproteins from typical dys- β subjects reported previously (27). The pre- β -VLDL were larger and more triglyceride-rich than the smaller, more cholesteryl ester-rich β -VLDL. The average calculated density of the pre- β -VLDL (0.977 g/ml) was less than that of the β -VLDL (0.994 g/ml). The chemical compositions and molecular weights of the S_f > 100, S_f 60–100, and S_f 20–60 fractions reflected their content of pre- β -VLDL and β -VLDL.

The relative apolipoprotein content of the $d < 1.006$ g/ml fractions was estimated by scanning densitometry of SDS-

polyacrylamide gel electrophoretograms (Fig. 1 and Table III). In Fig. 1 A, the $d < 1.006$ g/ml fraction from representative dys- β subjects had relatively more apo-E and apo-B48 (lanes 2 and 3) than did members of family A, without dysbetalipoproteinemia (lane 1). The S_f 20–60, S_f 60–100, and S_f > 100 fractions from representative dys- β subjects also had increased amounts of apo-E and apo-B48 (Fig. 1 B and Table III) relative to the unaffected subjects. The $d < 1.006$ g/ml lipoprotein fractions from atypical dys- β subjects had higher apo-E to total apo-C (E:C) ratios than those found in corresponding lipopro-

Table III. Scanning Densitometry of SDS-PAGE (Coomassie Blue-Stained)*

Subject	Apo-E [‡]	Sample	E:C ratio [‡]	B:E + C ratio	B48:B100 ratio [‡]
Atypical dys-β					
3	Arg ₁₄₂ → Cys	$d < 1.006$ g/ml	6.7±2.9	0.98±0.33	0.15±0.09
3	Arg ₁₄₂ → Cys	S _f >100	3.9	0.28	0.58
3	Arg ₁₄₂ → Cys	S _f 60–100	2.5	0.39	0.15
3	Arg ₁₄₂ → Cys	S _f 20–60	6.6	0.90	0.05
11	Arg ₁₄₅ → Cys	$d < 1.006$ g/ml	6.5	1.5	0.15
11	Arg ₁₄₅ → Cys	S _f >100	11	0.36	1.3
11	Arg ₁₄₅ → Cys	S _f 60–100	6.7	0.57	0.33
11	Arg ₁₄₅ → Cys	S _f 20–60	7.7	1.9	0.10
13	Lys ₁₄₆ → Gln	$d < 1.006$ g/ml	3.1	0.76	—
Family members without dys-β					
7–8	Normal	$d < 1.006$ g/ml	0.61	0.98	—
Typical dys-β					
n = 3	158	$d < 1.006$ g/ml	2.1±1.6	1.2±0.52	0.06±0.01
Cholesterol-fed rabbit					
n = 1	—	$d < 1.006$ g/ml	12	0.76	0.08

* SDS-PAGE of lipoproteins from typical dys- β subjects that were previously reported (27) were scanned again. The same instrument was used to obtain all of the ratios shown. Ratios are given ±SD where appropriate. [‡] Amino acid substitution in apo-E. [§] Ratio of apo-E to total apo-C. ^{||} Ratio of total apo-B (apo-B48 and apo-B100) to apo-E and apo-C. [¶] No value is given if the ratio of apo-B48 to apo-B100 was too low to measure.

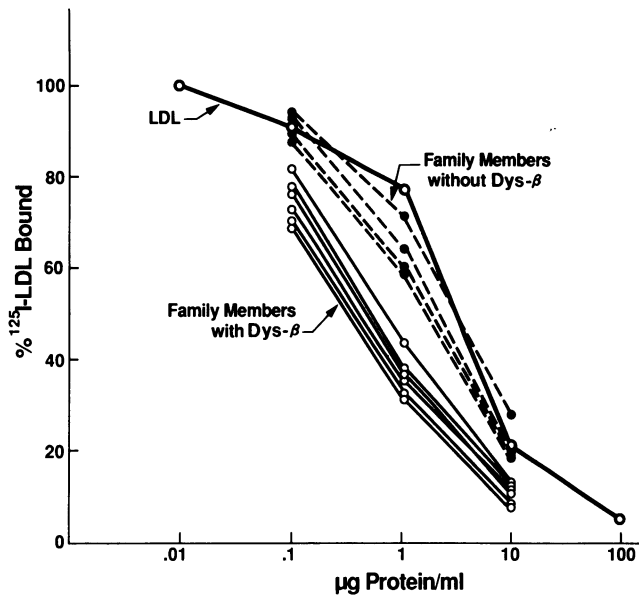


Figure 2. Competition of $d < 1.006$ g/ml lipoproteins for ^{125}I -LDL binding to cultured human fibroblasts at 4°C . The ability of lipoproteins at various concentrations to compete for the binding of ^{125}I -LDL at $2\ \mu\text{g}$ of protein/ml was determined in duplicate. Fibroblasts were prepared as described under Methods. (Dashed line, closed circles) $d < 1.006$ g/ml lipoproteins from members of family A without dys- β (subjects 7–10). (Solid line, open circles) Data from members of family A, with dys- β who had a substitution in apo-E at residue 142 (subjects 1–6). (Bold line, open circles) LDL from a normal subject.

teins from typical dys- β subjects (Table III). A band with the same apparent molecular weight as did albumin was present in some samples (Fig. 1).

Binding of $d < 1.006$ g/ml fractions to the apo-B,E(LDL) receptor. The affinity of the $d < 1.006$ g/ml fractions for the apo-B,E(LDL) receptor on human fibroblasts was determined by the ability of various concentrations of unlabeled lipoproteins to compete for the binding of ^{125}I -LDL at 4°C . As a

control for interassay variation, the affinity of unlabeled human LDL was measured in each assay. The concentration of unlabeled LDL that resulted in 50% competition for the binding of ^{125}I -LDL (EC_{50}) was $2.58 \pm 0.15\ \mu\text{g}$ of protein/ml ($\sim 4.5\ \text{nM}$) for 12 LDL preparations in 18 separate assays.

Data from family A, are shown in Fig. 2. The $d < 1.006$ g/ml lipoproteins from dys- β family members had fourfold higher binding affinity than did lipoproteins from unaffected family members (average $\text{EC}_{50} = 0.5$ vs. $2.0\ \mu\text{g}$ protein/ml). The $d < 1.006$ g/ml lipoproteins from dys- β family members also had fivefold higher affinity than did LDL from a normal subject (Fig. 2).

The $d < 1.006$ g/ml lipoproteins from dys- β subjects with the different substitutions in apo-E were further fractionated by Pevikon block electrophoresis, and the ability of these fractions to compete for the binding of ^{125}I -LDL to cultured human fibroblasts at 4°C is shown in Fig. 3. The most pre- β -migrating fraction is labeled 4, whereas the most β -migrating fraction is labeled 1. The cholesterol/triglyceride ratios of fractions 1–4 were 1.21, 0.82, 0.35, and 0.19, respectively. Although minor differences are apparent in Fig. 3 A, each of these fractions had higher affinity for apo-B,E(LDL) receptors than did normal LDL. Similarly, pre- β -migrating and β -migrating fractions isolated by Pevikon block electrophoresis of $d < 1.006$ -g/ml lipoproteins from subjects with the Arg $_{145} \rightarrow$ Cys or Lys $_{146} \rightarrow$ Gln substitution in apo-E also had higher affinity than did LDL for apo-B,E(LDL) receptors (Fig. 3, B and C, respectively).

The ability of the $S_f > 100$, $S_f 60$ – 100 , and $S_f 20$ – 60 fractions to compete for the binding of ^{125}I -LDL to fibroblasts at 4°C is shown in Fig. 4. Each of these fractions, whether isolated from subjects with an amino acid substitution in apo-E at residue 142, 145, or 146, had higher affinity for apo-B,E(LDL) receptors than did LDL from normal subjects (Fig. 4, A–C, respectively). Thus, all of the $d < 1.006$ g/ml lipoproteins from atypical dys- β subjects (fractionated by either Pevikon block electrophoresis or sequential ultracentrifugal flotation) had higher receptor binding affinity.

The binding affinities of $d < 1.006$ g/ml fractions to receptors on fibroblasts are summarized in Table IV. Subjects 1–10

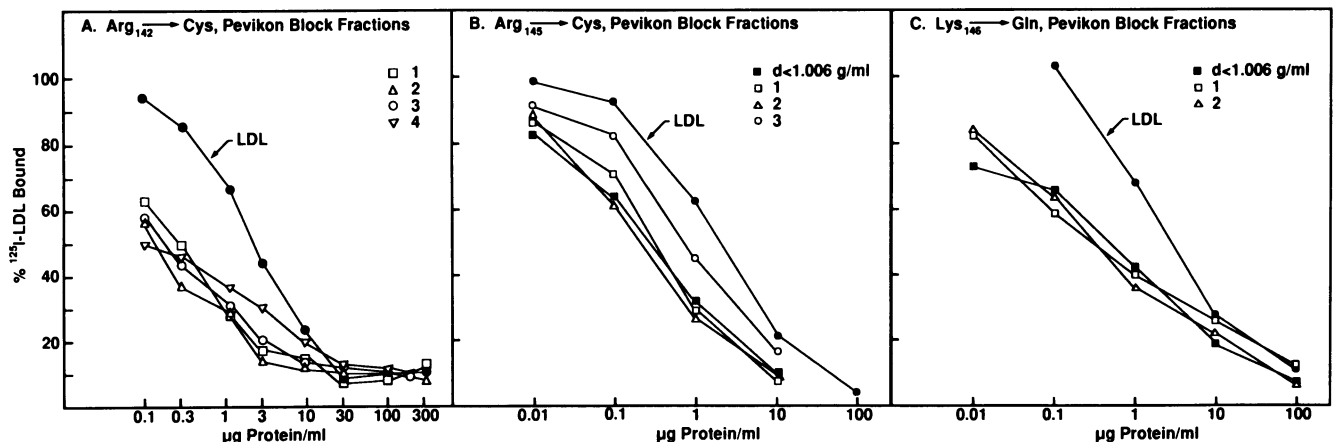


Figure 3. Competition of Pevikon block fractions of the $d < 1.006$ g/ml lipoproteins for the binding of ^{125}I -LDL to cultured human fibroblasts at 4°C . The most β -migrating fraction is labeled 1 and more pre- β -migrating fractions are labeled 2, 3, and 4, respectively. (A, B, and C) Pevikon block fractions from dys- β subjects 3, 11, and 13, respectively, whose apo-E had a substitution at residue 142, 145, or 146, respectively. (B and C) The binding of the total $d < 1.006$ g/ml fraction is also shown. The competition of LDL from a normal donor is shown in each panel.

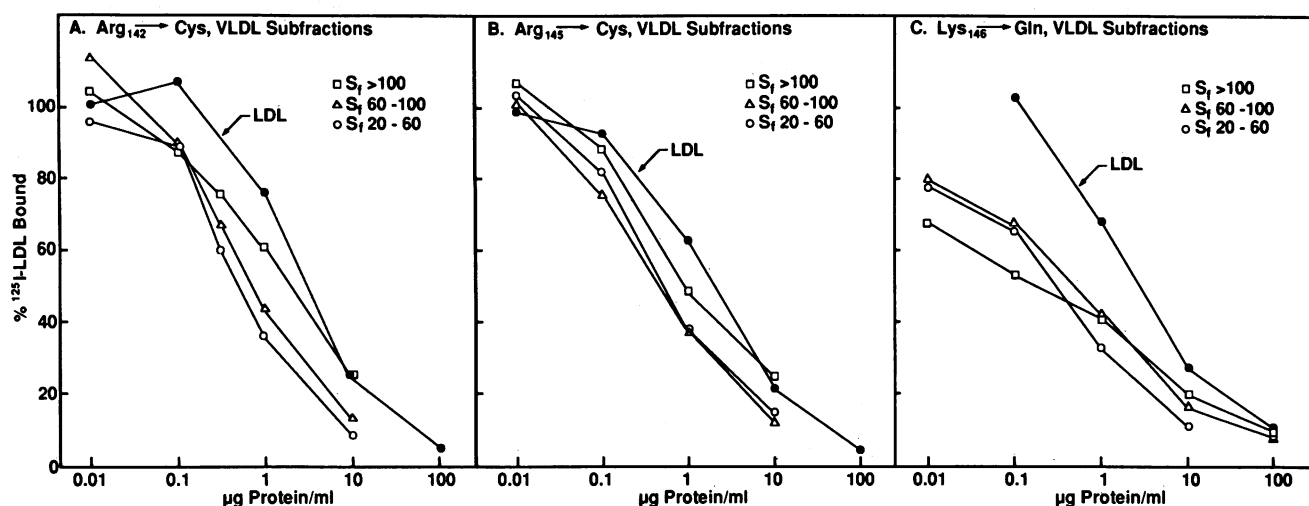


Figure 4. Competition of $S_f > 100$, $S_f 60-100$, and $S_f 20-60$ fractions for the binding of ^{125}I -LDL to cultured human fibroblasts at 4°C . (A, B, and C) Competition of these lipoproteins from dys- β subjects 4, 11, and 13, respectively, whose apo-E had a substitution at residue 142, 145, or 146, respectively.

were studied on two to four separate occasions; the plasma from the other subjects was obtained once. Pre- β -VLDL had slightly lower affinity than did β -VLDL (average $\text{EC}_{50} = 0.76$ versus $0.46 \mu\text{g}$ of protein/ml, respectively). Likewise, the $S_f > 100$ fraction, which was relatively enriched in pre- β -VLDL, had lower binding affinity than did the $S_f 20-60$ fraction, in which β -VLDL predominated (average $\text{EC}_{50} = 1.1$ vs. $0.44 \mu\text{g}$ of protein/ml). In members of family A, who did not have dysbetalipoproteinemia (subjects 7-10), the more β -migrating

fraction of the $d < 1.006 \text{ g/ml}$ lipoproteins had fourfold higher affinity than the more pre- β -migrating fraction (average $\text{EC}_{50} = 1.1$ vs. $4.3 \mu\text{g}$ of protein/ml). When binding affinities were expressed on a molar basis using molecular weights from Table II, the average EC_{50} of lipoproteins from representative atypical dys- β subjects was 0.4 nM for pre- β -VLDL and 0.5 nM for β -VLDL. The EC_{50} of $S_f > 100$, $S_f 60-100$, and $S_f 20-60$ fractions from dys- β subjects with a substitution in apo-E at residue 142 or 145 was $0.4-0.6 \text{ nM}$. Thus, the high affinity of

Table IV. Receptor Binding Affinities* of $d < 1.006 \text{ g/ml}$ Fractions

Subject	Apo-E [‡]	$d < 1.006 \text{ g/ml}$	Pre- β -VLDL	β -VLDL	$S_f > 100$	$S_f 60-100$	$S_f 20-60$
Atypical dys- β							
1-6	142	0.50 ± 0.14 ($n = 14$)	0.47 ± 0.19 ($n = 6$)	0.42 ± 0.16 ($n = 6$)	1.4 ± 0.15 ($n = 6$)	0.75 ± 0.11 ($n = 6$)	0.48 ± 0.11 ($n = 4$)
11-12	145	0.44 ± 0.05 ($n = 4$)	1.60 ± 0.24 ($n = 4$)	0.41 ± 0.04 ($n = 4$)	1.2 ± 0.17 ($n = 4$)	0.58 ± 0.14 ($n = 4$)	0.43 ± 0.12 ($n = 4$)
13 [§]	146	0.43 ($n = 2$)	0.71 ($n = 2$)	0.72 ($n = 2$)	0.64 ($n = 2$)	0.61 ($n = 2$)	0.41 ($n = 2$)
Mean		0.48 ± 0.13 ($n = 20$)	0.76 ± 0.23 ($n = 12$)	0.46 ± 0.15 ($n = 12$)	1.1 ± 0.16 ($n = 12$)	0.67 ± 0.12 ($n = 12$)	0.44 ± 0.11 ($n = 10$)
Family A, members without dys- β [§]							
7-10	Normal	2.0 ± 0.14 ($n = 8$)	4.3 ± 0.15 ($n = 4$)	1.1 ± 0.12 ($n = 4$)			
Typical dys- β							
	158		490 ± 200 ($n = 10$)	8.0 ± 3.3 ($n = 18$)	370 ± 150 ($n = 3$)	62 ± 16 ($n = 5$)	7.8 ± 3.5 ($n = 5$)
Cholesterol-fed animals							
Rabbit	—	0.42 ($n = 1$)	0.38 ($n = 1$)	2.1 ($n = 1$)			
Dog	—	0.27 ($n = 2$)	0.05 ($n = 2$)	0.36 ($n = 2$)			

* EC_{50} expressed as μg protein/ml \pm SD. Values in parentheses are the number of separate binding assays. [‡] Site of amino acid substitution in apo-E. [§] Since no definite β -VLDL were found, values shown are for the more pre- β -migrating and more β -migrating fractions isolated by Pevikon block electrophoresis. ^{||} Values shown from typical dys- β subjects have been reported previously (27).

these lipoproteins compared with that of LDL ($EC_{50} = 4.5 \text{ nM}$) was apparent when binding affinity was expressed on a molar basis.

In an effort to avoid artifacts associated with the isolation of lipoproteins, receptor binding studies using whole plasma were performed (Fig. 5). Fresh whole plasma was studied on two consecutive days. Whole plasma from atypical dys- β subjects 3 or 4 had, on average, sevenfold higher receptor binding affinity than did the whole plasma from two normolipidemic E3/3 subjects; $EC_{50} = 14$ vs. $100 \mu\text{g protein/ml}$ (average of two experiments). These data reflect the average receptor binding affinity of all lipoproteins in the whole plasma and showed that the average affinity of lipoproteins in atypical dys- β plasma was higher than that in normal plasma.

Apolipoprotein E was responsible for the majority of the binding of all $d < 1.006 \text{ g/ml}$ lipoprotein fractions to the apo-B,E(LDL) receptor. Monoclonal antibodies 4G3 and MB47, which specifically block apo-B100-mediated binding, inhibited 0–35% of the binding of the ^{125}I -VLDL subfractions studied (i.e., pre- β -VLDL, β -VLDL, and the $S_f > 100$, S_f 60–100, and S_f 20–60 fractions). However, these antibodies inhibited $> 90\%$ of ^{125}I -LDL binding. In contrast, monoclonal antibody 1D7, which specifically inhibits apo-E-mediated binding, inhibited 60–90% of the binding of the ^{125}I -VLDL subfractions. Comparison of $S_f > 100$ and S_f 20–60 fractions showed no shift from apo-E-mediated to apo-B100-mediated binding. These studies were performed on two or more separate occasions and used pre- β -VLDL, β -VLDL, $S_f > 100$, S_f 60–100, or S_f 20–60 fractions from subjects 3, 4, and 12 who had a substitution in their apo-E at residue 142 or 145.

Studies in cholesterol-fed rabbits and cholesterol-fed dogs. Cholesterol-fed rabbits and cholesterol-fed dogs are animal models in which there is β -VLDL accumulation. However, they do not have a mutation in apo-E. The animals studied had plasma cholesterol concentrations of over 1,000 mg/dl. The binding of Pevikon block fractions of $d < 1.006 \text{ g/ml}$

lipoproteins from cholesterol-fed rabbits and cholesterol-fed dogs is shown in Fig. 6, A and B, respectively. Rabbit Pevikon block fractions 1–3 had cholesterol/triglyceride ratios of 6.4, 3.5, and 0.76, respectively, whereas the corresponding canine fractions had cholesterol/triglyceride ratios of 20, 15, and 8, respectively. Thus, $d < 1.006\text{-g/ml}$ lipoproteins from cholesterol-fed animals were, on the whole, much more cholesterol-rich than those from human dys- β subjects. They were also rich in apo-E as shown by their E:C ratio (Table III). All Pevikon block fractions had higher binding affinity for human apo-B,E(LDL) receptors than did human LDL and were similar in binding affinity to corresponding fractions from human atypical dys- β subjects (Table IV).

Discussion

The association of familial dysbetalipoproteinemia (type III hyperlipoproteinemia) with amino acid substitutions in apo-E that cause defective binding to apo-B,E(LDL) receptors is well established (1, 5, 13). It has been known for several years that the degree of defective receptor binding of the various apo-E mutants may be nearly normal or severely defective and does not correlate with the severity of dysbetalipoproteinemia (1, 6, 13). Nevertheless, since all amino acid substitutions in apo-E that are associated with dysbetalipoproteinemia cause at least some decreased binding to receptors, it was reasonable to suspect that native lipoproteins containing these apo-E mutants would have defective binding as well. Therefore, the finding that $d < 1.006 \text{ g/ml}$ lipoproteins from atypical dys- β subjects had higher binding affinity than did $d < 1.006 \text{ g/ml}$ lipoproteins from normal subjects was surprising. On the other hand, since apo-E from the atypical dys- β subjects had higher affinity than apo-E from typical dys- β subjects, it was not surprising that their $d < 1.006 \text{ g/ml}$ lipoproteins also had higher binding affinity.

In the current study, dys- β subjects with amino acid substitutions in apo-E at atypical sites (residues 142, 145, or 146) were found to have several features in common with typical dys- β subjects. In all dys- β subjects, the pre- β -VLDL were larger, had higher flotation rates, and were more triglyceride-rich than the smaller, more cholesteryl ester-rich β -VLDL. The higher B48:B100 ratio in pre- β -VLDL than in β -VLDL agrees with other studies in which lipoproteins from the intestine were found to be larger and have higher flotation rates than those from the liver (27, 53–55).

A major difference between typical and atypical dys- β subjects was the marked increase in receptor binding affinity of the atypical dys- β $d < 1.006 \text{ g/ml}$ lipoproteins. The receptor binding affinities of pre- β -VLDL and β -VLDL from atypical dys- β subjects were 640- and 17-fold higher, respectively, than corresponding lipoproteins from typical dys- β subjects (Table IV). Another difference was that pre- β -VLDL and β -VLDL from atypical dys- β subjects had similar binding affinities, whereas β -VLDL from typical dys- β subjects had over 68-fold higher affinity than did their pre- β -VLDL (27). Since both pre- β -VLDL and β -VLDL from atypical dys- β subjects had high binding affinities, decreased binding affinity of pre- β -VLDL is not a major factor in the formation of β -VLDL as may be the case in typical dys- β subjects (27).

The $d < 1.006 \text{ g/ml}$ fractions from atypical dys- β subjects had higher affinity than did the $d < 1.006 \text{ g/ml}$ fractions from normolipidemic or hyperlipidemic subjects (27) or from unaf-

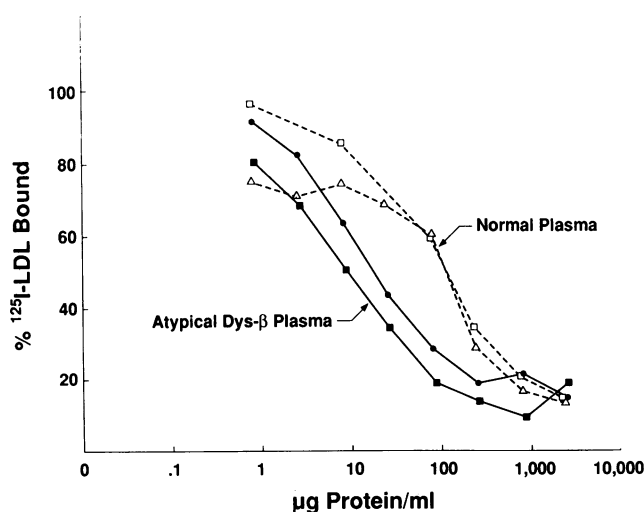


Figure 5. Competition of whole plasma for the binding of human ^{125}I -LDL to fibroblasts at 4°C . Fresh whole plasma (containing 1 mg/ml EDTA, sodium salt) from atypical dys- β subject 3 (\blacksquare , $EC_{50} = 7.5 \mu\text{g protein/ml}$), atypical dys- β subject 4 (\bullet , $EC_{50} = 12.6 \mu\text{g protein/ml}$), or normolipidemic E3/3 subjects (\triangle , $EC_{50} = 83 \mu\text{g protein/ml}$) or \square , $EC_{50} = 97 \mu\text{g protein/ml}$) was used in the concentrations indicated.

ected members of family A. who were homozygous for normal apo-E3. In addition, the receptor binding studies performed using the whole plasma indicate that the average affinity of lipoproteins in atypical dys- β subjects is sevenfold higher than in normal subjects (Fig. 5). Thus, it is difficult to conclude that defective interaction of atypical dys- β lipoproteins with apo-B,E(LDL) receptors results in delayed clearance in vivo. When these lipoproteins were studied in vivo in cholesterol-fed rabbits, the clearance of ^{125}I -labeled apo-B in pre- β -VLDL and β -VLDL from an atypical dys- β subject was much faster than that of ^{125}I -labeled apo-B in corresponding lipoproteins from a typical dys- β subject (unpublished observations). However, the clearance of these lipoproteins from the plasma of rabbits may not be the same as the clearance in humans.

What is the mechanism of the high binding affinity of $d < 1.006$ -g/ml lipoproteins from atypical dys- β subjects, and why do lipoproteins with high binding affinity accumulate in subjects with this disorder? First, the amino acid substitutions in apo-E from atypical dys- β subjects cause less defective binding to receptors than does the typical substitution at residue 158 (1, 5, 10). Second, apo-E on native lipoproteins from subjects who were heterozygous for a substitution in apo-E at residue 142 or 146 was a mixture of both abnormal and normal apo-E. Finally, all $d < 1.006$ g/ml lipoproteins from the atypical dys- β subjects contained relatively more apo-E than did corresponding lipoproteins from typical dys- β subjects (Table III). The enrichment of these lipoproteins in apo-E probably increased their affinity for apo-B,E(LDL) receptors and may be due to differences in how atypical apo-E isoforms partition among lipoproteins. In other studies, when normal apo-E3 was added to $d < 1.006$ g/ml lipoproteins from subjects with the Arg₁₄₂ \rightarrow Cys substitution in vitro, these particles acquired a higher binding affinity (56). This suggests that lipoproteins from atypical dys- β subjects, despite their high receptor binding affinity, have lower affinity than they would if only normal apo-E3 was present. More detailed analysis is needed to estimate the number of apo-E molecules per particle in both atypical and typical dys- β subjects. However, gross differences in lipid composition do not account for the differences in receptor binding affinity observed.

In general, the affinity of lipoproteins for apo-B,E(LDL) receptors on fibroblasts correlates well with their rate of clearance from the plasma (57–60) and therefore cannot explain the accumulation of lipoproteins with high binding affinity in atypical dysbetalipoproteinemia. At least three possible mechanisms could contribute to the accumulation of lipoproteins with high binding affinity in atypical dys- β subjects. First, defective apo-B,E(LDL) receptors could cause decreased clearance of lipoproteins despite high binding affinity. This seems unlikely, however, because atypical dys- β subjects with the Arg₁₄₂ \rightarrow Cys substitution in apo-E, in whom receptors on skin fibroblasts have been measured, have no defect in their receptors (10). Second, atypical mutations in apo-E may disrupt some function of apo-E other than that measured by the fibroblast binding assay. The interaction of apo-E with other hepatic lipoprotein receptors or with lipolytic or other modifying enzymes may be important for the normal metabolism of $d < 1.006$ g/ml lipoproteins. In fact, hepatic lipase deficiency alone has been reported to cause severe dysbetalipoproteinemia (61). However, the atypical dys- β family members with the Arg₁₄₂ \rightarrow Cys apo-E substitution have normal postheparin lipase activity (9). A third possibility is that overproduction of

lipoproteins and downregulation of apo-B,E(LDL) receptors could cause accumulation of lipoproteins that have high binding affinity. The studies reported here using cholesterol-fed rabbits and cholesterol-fed dogs support this possibility. Apparently, in cholesterol-fed animals, a high-cholesterol and high-fat diet alone is sufficient to overwhelm the ability of apo-E to clear lipoproteins thereby causing the accumulation of β -VLDL despite the high receptor binding affinity of the diet-induced lipoproteins (Fig. 6 and Table IV).

The only human in vivo study in which atypical and typical dys- β subjects have been compared shows that the clearance of apo-B in $d < 1.006$ -g/ml lipoproteins from dys- β subjects with an amino acid substitution in apo-E at 158 or 145 was similarly delayed (32). However, this study did not discriminate between the three mechanisms for β -VLDL accumulation described above. In the future, studies of in vivo apo-B kinetics of pre- β -VLDL and β -VLDL from atypical dys- β subjects may be useful to measure the rate of production of these lipoproteins. It is reasonable to speculate that the clearance of LDL in atypical dys- β subjects may be slower than it is in typical dys- β subjects due to competition for receptor binding by $d < 1.006$ g/ml lipoproteins with higher affinity than that of LDL or due to downregulation of apo-B,E(LDL) receptors. Direct measurement of the interaction between pre- β -VLDL or β -VLDL and hepatic tissue may reveal binding sites other than the apo-B,E(LDL) receptor and may also shed light on the pathogenesis of atypical dysbetalipoproteinemia.

A fascinating and unexplained feature of atypical dysbetalipoproteinemia is its apparent inheritance as an autosomal dominant trait in some families (9–11, 62). Members of family A. in this study who did not have dysbetalipoproteinemia had a double pre- β pattern, and some had hypercholesterolemia (Table I). The trait or traits that caused the double pre- β pattern and hypercholesterolemia may have contributed to the expression of dysbetalipoproteinemia. Atypical dys- β subjects might have more than one genetic defect; other hyperlipidemic factors might act in combination with abnormal apo-E to cause a dys- β profile. If this is so, normolipidemic subjects with atypical mutations in apo-E should exist. Environmental or acquired factors might also affect atypical and typical dys- β

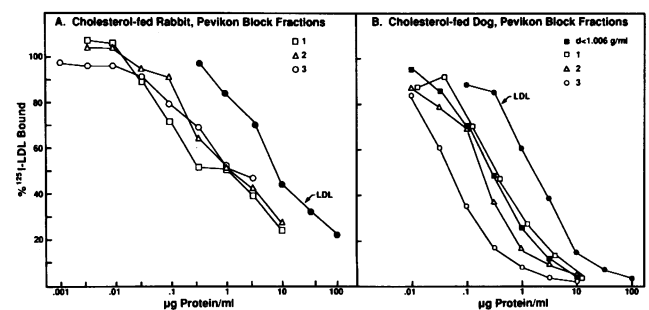


Figure 6. Competition of the Pevikon block fractions of the $d < 1.006$ g/ml lipoproteins from a cholesterol-fed rabbit (A) or cholesterol-fed dog (B) for the binding of ^{125}I -LDL to cultured human fibroblasts at 4°C. The most β -migrating fraction is labeled 1 and more pre- β -migrating subfractions are labeled 2 and 3, respectively. (B) The binding of the total $d < 1.006$ g/ml fraction is also shown. The competition of LDL from a normal human subject is shown in each panel.

subjects differently. More studies are needed to address these issues.

In summary, the major finding in this study is that $d < 1.006$ g/ml lipoproteins from atypical dys- β subjects have paradoxically high affinity for apo-B,E(LDL) receptors. The accumulation of $d < 1.006$ g/ml lipoproteins in atypical dys- β subjects cannot be ascribed simply to low affinity for apo-B,E(LDL) receptors, as happens in typical dys- β subjects. Another explanation for the pathogenesis of atypical dysbetaipoproteinemia must be found. Although cholesterol-fed animals do not have a mutation in apo-E, they may serve as useful models for atypical dysbetaipoproteinemia because they also accumulate β -VLDL by a mechanism other than decreased affinity of lipoproteins for apo-B,E(LDL) receptors. Since overproduction of $d < 1.006$ g/ml lipoproteins and downregulation of apo-B,E(LDL) receptors in these animals appear to cause the formation of β -VLDL, a similar mechanism may be important in the pathogenesis of human atypical dysbetaipoproteinemia.

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