

Structure, Immunology, and Cell Reactivity of Low Density Lipoprotein from Umbilical Vein of a Newborn Type II Homozygote

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ABSTRACT In this report we compare the cord blood lipoproteins of a newborn boy homozygote who has low density lipoprotein (LDL) receptor-defective familial hypercholesterolemia (FH) with the lipoproteins from cord blood of normal newborns. Plasma LDL-cholesterol and apoprotein (Apo)B were 612 and 233 mg/dl (vs. 31 ± 16 and 24 ± 12 mg/dl, respectively, for normals, $n = 21$). LDL-cholesterol/ApoB ratio was 2.6 vs. 1.4 ± 0.5 . Levels of ApoA-I, ApoA-II, and HDL-cholesterol were similar to normal cord plasma. Thus, the lipoprotein abnormality is apparent at birth and is definitely present in LDL. Abnormalities in other lipoprotein, lipid, and in plasma apoprotein levels were not detected. On zonal ultracentrifugation, FH LDL was comprised of two populations (LDL_a and LDL_b), both faster floating than normal cord LDL (LDL_c). This difference was due to the larger diameters of the particles on electron microscopy (LDL_a = $276 \text{ \AA} \pm 32$ and LDL_b = $260 \text{ \AA} \pm 38$ vs. LDL_c = $237 \text{ \AA} \pm 26$, $n = 200$ each, mean ± 1 SD), and their higher contents of lipids relative to protein (86 and 82% vs. 74%, LDL_a, LDL_b, and LDL_c, respectively). More than 94% of the protein in both the FH and the normal preparations consisted of ApoB. FH LDL were more effective than control LDL in competing with ^{125}I -LDL (adult) for limiting amounts of anti-LDL antibodies in radioimmunoassay. FH LDL also competed more effectively for binding to LDL receptors on cultured fibroblasts at 4°C, and FH LDL also delivered more cholesterol into the cells. Cells grown in lipoprotein-deficient serum contained $44 \pm 2 \mu\text{g}$ cholesterol/mg cell protein, incubation of cells for 18 h at 37°C in $5 \mu\text{g/ml}$ FH LDL (protein) or in normal LDL raised cellular cholesterol levels to 75 ± 2 and $60 \pm 2 \mu\text{g/mg}$, respectively.

LDL isolated from the FH patient's plasma at 6 mo of age and from his brother's plasma (a 5-yr-old boy FH homozygote) were similar to LDL isolated from normolipemic subjects in flotation properties, chemical composition, and immunochemical and cell reactivity. The fact that differences between normal cord LDL and FH cord LDL were present at birth, but that the differences between control and FH LDL were no longer present postnatally suggests that the altered immunologic and cell interactive properties of FH cord LDL were probably related to its unusually high contents of core lipids.

INTRODUCTION

Familial hypercholesterolemia (FH)¹ is an autosomal dominant disease in which the disorder is more severe in individuals who inherit two doses of the gene (homozygotes) than those who inherit one dose (heterozygotes) (1). Three distinct mutations of the gene that regulates the function of the cellular low density lipoprotein (LDL) receptor have been described in affected individuals. These mutations interfere with the cellular binding and/or internalization of LDL which leads to the accumulation of LDL in the plasma (2). FH heterozygotes can be detected at the time of birth by measuring LDL-cholesterol in the cord blood of babies born to parents known to carry the FH gene (3), and FH homozygosity has been detected in a fetus (4). Recently, we had the opportunity of studying the umbilical cord plasma of a newborn FH homozygote. We report on the levels of lipoprotein lipids and apoproteins, and on several characteristics of the LDL

¹Abbreviations used in this paper: FH, familial hypercholesterolemia; HDL, high density lipoprotein; LDL, low density lipoprotein; LPDS, lipoprotein-deficient serum; RIA, radioimmunoassay; TG, triglyceride; TMU, tetramethylurea; VLDL, very low density lipoprotein.

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TABLE I
Levels of Plasma Lipids, Apolipoproteins, and Lipoprotein-Cholesterol in the Cord Plasma of the Homozygote and Normal Controls*

	Chol†	TG	ApoA-I	ApoA-II	ApoB	VLDL-Chol	LDL-Chol	HDL-Chol	LDL-Chol/ApoB
FH Homozygote	660	84	65	18	233	2	612	44	2.63
Normal controls	67±25	34±14	74±14	26±9	24±12	3±3	31±16	31±10	1.44±0.5

* Results are given in milligrams per deciliter and represent mean ± 1 SD, *n* = 21.

† Chol, cholesterol.

isolated from FH cord blood. FH cord LDL differed from normal cord blood LDL in structure, composition, immunoreactivity, and in its interactions with cultured human fibroblasts.

METHODS

The patient S.L. is the brother of R.L., a known FH homozygote (5). S.L.'s cells were receptor-defective, as were those of his brother. Plasma (1 mg/ml EDTA) was separated from 15 ml of cord blood or from forearm venous blood by low-speed centrifugation. Lipids and lipoprotein cholesterol levels were determined by standard procedures (6). Levels of apoprotein (Apo)A-I, ApoA-II, and ApoB were determined by radioimmunoassay (RIA) (7-9). Plasma lipoproteins were further analyzed by zonal ultracentrifugation (10).

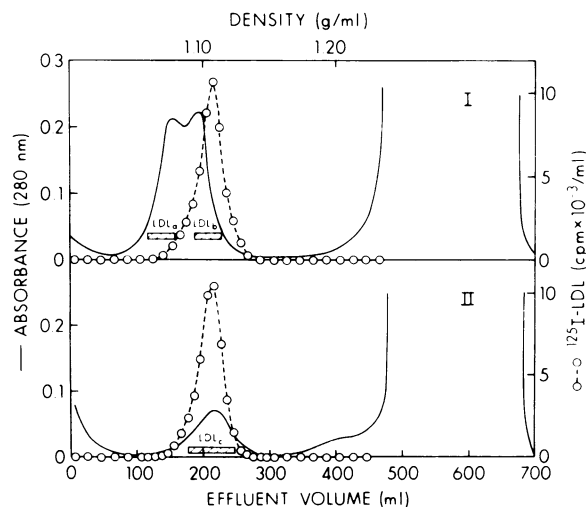


FIGURE 1 Characterization of the LDL region by zonal ultracentrifugation. On top (I), 2.5 ml of the cord plasma of S.L., the FH homozygote, and 5 μ l of 125 I-LDL (normal adult) were subjected to zonal ultracentrifugation (9). Bottom (II), 16 ml of pooled normal cord plasma, consisting of eight 2-ml aliquots of different subjects, and 5 μ l of 125 I-LDL were centrifuged. 125 I-LDL was prepared from a pool of adult plasma and iodinated with iodine monochloride (7). Centrifugation conditions were 140 min at 42,000 rpm, 15°C, using a linear NaBr density gradient in the density range of 1.00 to 1.30 g/ml. VLDL elute at the center of the rotor (effluent volume 0-120 ml), HDL and residual plasma protein between 420 and 700 ml. Lipoprotein material in tubes indicated by bars LDL_a, LDL_b, LDL_c was pooled and used for chemical analyses and electron microscopy.

In some zonal ultracentrifugal experiments, 125 I-LDL, isolated from a normal adult plasma pool and labeled with iodine monochloride (11, 12), was used as internal standard. Lipoprotein fractions were analyzed for chemical composition in triplicate across the zonal profile as described (13). The diameter of LDL was determined by electron microscopy after negative staining (14, 15). Immunoreactivity of the ApoB of LDL was evaluated by RIA using two different antisera (9, 16). R-161 was produced by immunization with ApoB isolated from very low density lipoprotein (VLDL) by column chromatography and R-198 was obtained from rabbits immunized with LDL. These antisera differ from each other in their specificities vis-a-vis VLDL-ApoB (16) and also vis-a-vis normal LDL modified in their lysine residues (unpublished observations). The ApoB content of LDL was also measured by the method of Kane et al. (17) using tetramethylurea (TMU) (Burdick and Jackson Laboratories, Muskegon, Mich.). The interaction of LDL with cells was studied by using cultured normal fibroblasts (18, 19). These cells were grown for 5 d in modified Eagle's medium (Gibco Diagnostics, Chagrin Falls, Ohio) containing 10% fetal calf serum and then allowed to grow for 2 d in medium containing 10% lipoprotein-deficient serum (LPDS). Binding of LDL to the fibroblast LDL receptors was assessed in competitive assays. The media contained 5 μ g 125 I-LDL (prepared from adult plasma) and 0-50 μ g of unlabeled cord plasma LDL (expressed in terms of LDL protein). Cells were incubated in 30-mm dishes at 4°C for 4 h. Cell associated counts, which represent 125 I-LDL bound to the receptor, were determined as described (18, 19). Delivery of cholesterol to cells by cord LDL was assessed on cultures grown as above, but here 60-mm dishes were used, and incubation in the presence of LDL was performed for 18 h at 37°C with 2 ml of LPDS medium containing 10 or 100 μ g of LDL-protein. After washing and harvesting the cells, cellular cholesterol was determined by an enzymatic procedure (20).

RESULTS

As expected, levels of cord plasma LDL-cholesterol and ApoB were greatly elevated in the homozygote (Table I). The ratio of LDL-cholesterol to ApoB was also higher in FH cord plasma suggesting that FH LDL was enriched in cholesterol. No significant differences were observed in levels of ApoA-I, ApoA-II, and high density lipoprotein (HDL)-cholesterol.

Zonal ultracentrifugal analysis (in the presence of a normal adult 125 I-LDL internal standard) revealed two distinct populations, designated LDL_a and LDL_b (Fig. 1, I). Both floated faster than the 125 I-LDL internal standard. By contrast, only one major population of LDL, designated LDL_c (Fig. 1, II), was isolated from a

TABLE II
Composition of LDL in Cord Plasma of the Homozygote and Normal Controls

	Protein	FC	CE	PL	TG	TMU insoluble protein	Diameter
			% dry weight			% of protein*	Å
LDL _a	13.5	15.7	42.1	20.2	8.0	94	276±32‡,§
LDL _b	17.5	14.6	40.3	20.8	6.9	98	260±38‡
LDL _c	26.1±0.5	13±2	19.4±1.6	24.2±2.8	17.4±6	94±3	237±26

LDL_a and LDL_b are isolated from FH cord plasma, LDL_c is isolated from normal cord plasma. LDL_c values (mean±1 SD) are from three pools, each pool consisting of 2-ml aliquots of eight cord plasmas. The cord blood was taken from full-term, normal births and pregnancies. FC, free cholesterol; CE, cholesteryl ester (linoleate was used to convert esterified cholesterol into cholesteryl ester); PL, phospholipids; TMU-soluble protein was measured according to Kane (16). Chemical analyses were carried out in triplicate. Diameter of LDL was measured on negatively stained preparations of lipoproteins viewed under the electron microscope (13, 14), Angstrom units represent mean±1 SD ($n = 200$).

* On sodium dodecyl sulfate polyacrylamide gel electrophoresis (26) of ~100 µg LDL protein very small amounts of protein with molecular weights of >65,000 and 27,000 (probably ApoA-I) were seen, along with a barely detectable band with molecular weight of ~35,000 (probably ApoE).

‡ $P < 0.001$ from LDL_c.

§ $P < 0.001$ from LDL_b.

pool of normal cord plasma, consisting of 2-ml aliquots of eight normal individual cord plasmas. LDL_c was present in greatly reduced amounts (as expected from Table I) but its flotation behavior was similar to the internal standard. Two other pools of LDL_c, each consisting of eight 2-ml aliquots, yielded identical results on zonal ultracentrifugation. Amounts of VLDL were small in both normal and homozygote plasma, as judged from the zonal elution profile. This confirmed the data in Table I. The tubes, indicated by bars in Fig. 1, were pooled for compositional analyses (in triplicate) and electron microscopy. As shown in Table II, LDL_a was larger than LDL_b and had a higher content of lipids. LDL_a and LDL_b had greater diameters and contained more lipids than did LDL_c. When the cholesteryl ester/protein ratios of individual tubes of FH LDL obtained from the zonal rotor were assessed, the ratios from elution volumes 160 to 250 ml fell from 3.1 to 2.3 (Fig. 1, I), confirming the presence of two populations of LDL. In all LDL preparations, ApoB comprised >94% of the protein moiety, when insolubility in 50% TMU was used to assess the ApoB content.

For studies of immunoreactivity and cellular binding other aliquots of LDL_a, LDL_b, and LDL_c were isolated from the appropriate cord sera in the zonal rotor. (To avoid interference with these studies, the ¹²⁵I-LDL internal standard was omitted.) Elution profiles for both the normal (LDL_c) and the homozygote LDL_a and LDL_b fractions were identical to the elution profiles shown in Fig. 1. Immunoreactivity of ApoB was assessed by RIA (Fig. 2). The slopes of the competitive displacement curves and the apparent ApoB contents of LDL_a and LDL_b were indistinguishable from each other. This was true whether R-161 (anti-VLDL-ApoB) (Fig. 2, top) or R-198 (anti-LDL) (Fig. 2, bottom) were used. However, the competitive displacement curve

produced by LDL_c did not parallel either of the curves produced by LDL_a or by LDL_b, with R-198 (Fig. 2, bottom).

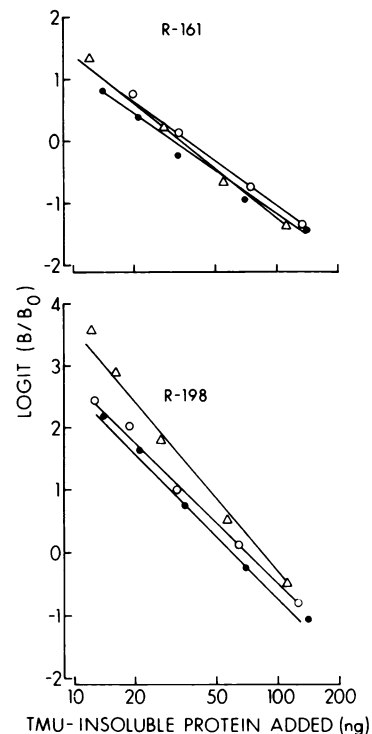


FIGURE 2 Competitive displacement curves produced by LDL_a (○), LDL_b (●), and LDL_c (▲) (see Fig. 1). Two assays are shown, one contained R-161, an anti-ApoB antiserum, and the other R-198, an anti-LDL antiserum. Logit B/B₀ = log B/B₀ - 1 - B/B₀ where B₀ = net counts per minute ¹²⁵I-LDL bound in the absence of LDL and B = net counts per minute ¹²⁵I-LDL bound in the presence of LDL. TMU insoluble protein represents chemically determined ApoB.

We next assessed the abilities of cord plasma LDL to compete with ^{125}I -LDL for binding to the fibroblast LDL receptors (Fig. 3). LDL_a and LDL_b displaced ^{125}I -LDL from the LDL receptor in fibroblast cultures to a similar extent. But LDL_c was less effective than either LDL_a or LDL_b in competing with ^{125}I -LDL for occupancy of the cellular LDL receptor. Next, the ability of cord LDL to deliver cholesterol to fibroblasts was measured (Table III). At low lipoprotein concentrations (10 μg lipoprotein protein/2 ml), LDL_a delivered more cholesterol than did LDL_b . LDL_c delivered the least amount of cholesterol. At high concentrations (100 μg /2 ml) all LDL preparations delivered about the same amount of cholesterol.

The plasma of S.L. was reexamined by zonal ultracentrifugation and by chemical analysis at 1 mo of age and compared with the plasma of his brother R.L., then aged 4 yr. S.L. was doing well and eating a formula diet containing little cholesterol (Similac, Ross Laboratories, Columbus, Ohio, cholesterol 25 mg/d). The brother R.L. has been on a vegetarian diet since birth (cholesterol 25 mg/d). Blood samples were taken in the morning. S.L.'s plasma cholesterol level was 765 mg/dl; LDL-cholesterol, 710 mg/dl; and ApoB, 343 mg/dl. The respective values for R.L. were 960, 920, and 450 mg/dl. LDL-cholesterol ApoB ratios were 2.1 and 2.0. On zonal ultracentrifugation the LDL of the two plasmas appeared to be identical. Only one major LDL-peak, corresponding in flotation rates to LDL_b was found with a shoulder on the leading edge. The plasmas of the two brothers were studied again when S.L. was 6 mo and R.L. 5 yr of age (Fig. 4), when their respective LDL-cholesterol values were 893 and 675 mg/dl. Again, on

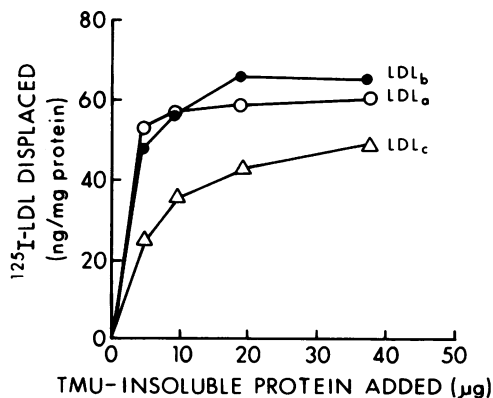


FIGURE 3 Competitive displacement of ^{125}I -LDL (normal adult) by LDL_a , LDL_b , and LDL_c in cultured fibroblast. Displacement of ^{125}I -LDL is expressed as a function of TMU-insoluble protein added per dish. Experiments were done at 4°C , incubation time was 4 h. Values represent the means of triplicate analyses. All points of LDL_b differed from LDL_c . All but the 38- μg point of LDL_a differed from LDL_c ($P < 0.05$ nonpaired t test).

TABLE III
Effect of LDL on the Cholesterol Contents of Fibroblasts

Incubation conditions	Cellular cholesterol	
	10 μg LDL	100 μg LDL
	$\mu\text{g}/\text{mg}$ cell protein	
None	44 \pm 2	44 \pm 2
LDL_a	75 \pm 2*†	93 \pm 11
LDL_b	70 \pm 1*	101 \pm 4
LDL_c	60 \pm 2	96 \pm 11

Cells were grown in 60-mm dishes for 5 d in Eagle's minimal essential medium 10% fetal calf serum and then allowed to grow in 10% LPDS containing medium for 2 d. Cells were then incubated for 18 h at 37°C in 2 ml of Eagle's minimal essential medium-LPDS alone (none) and in Eagle's minimal essential medium-LPDS containing either 10 or 100 μg of LDL (expressed as LDL-protein). The LDL_a , LDL_b , and LDL_c fractions used were described in Fig. 1. Cellular cholesterol content was measured according to Gamble, et al. (20). Results are given as mean \pm 1 SD of triplicate experiments.

* $P < 0.05$ vs. LDL_c .

† $P < 0.05$ vs. LDL_b .

zonal ultracentrifugation, single peaks of LDL with leading shoulders were seen. The normal plasma (plasma cholesterol = 138, triglyceride [TG] = 48,

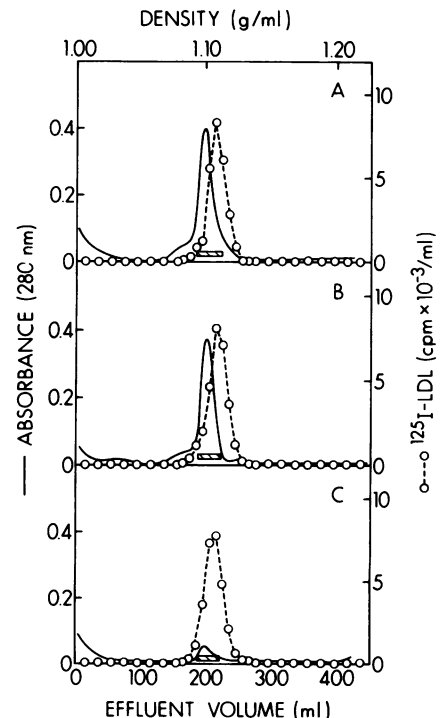


FIGURE 4 Zonal ultracentrifugal elution profile of VLDL and LDL obtained from the FH homozygote at 6 mo of age (A), his FH homozygote brother at 5 yr of age (B), and a control subject (C). 4 ml of plasma was analyzed.

TABLE IV
Composition of LDL in Postnatal Plasmas of Two FH Homozygotes and a Normal Control

	Protein	FC	CE	PL	TG	TMU insoluble protein
		% dry weight				% of protein
LDL _S	20.7	11.3	42.2	22.4	3.1	97
LDL _R	21.1	11.0	42.8	22.3	2.3	97
LDL _N	21.5	9.1	41.4	23.2	4.7	97

LDL_S, LDL isolated from S.L. at age of 6 m. LDL_R, LDL isolated from R.L. at age of 5 yr.
LDL_N, LDL isolated from a normal control subject. See Table II for abbreviations.

LDL-cholesterol = 67 mg/dl) contained a single population of LDL of similar flotation properties, but greatly reduced in amount. (This normal plasma was selected because of its similar LDL flotation rate. Not all normal LDL have identical flotation rates [unpublished observations, see also ref. 21].) FH LDL and normal LDL were indistinguishable from each other in their chemical compositions (Table IV), immunological properties (Fig. 5), and binding to fibroblast LDL receptors (Fig. 6).

DISCUSSION

In this detailed study of lipoproteins in the cord blood of a subject with homozygous FH, the most striking and clear-cut abnormality was in the levels of LDL-cholesterol and ApoB which were 20 and 10 times that of normals, respectively. LDL-cholesterol levels were ~10 times those found in the cord plasmas of FH heterozygotes (3). This indicates that the effect of the

FH gene is fully expressed at the time of birth. Indeed, the trait has been detected in a 20-wk-old fetus (4). The LDL of the homozygote was clearly different from normal cord LDL in several respects: (a) Homozygote LDL was comprised of two populations distinguished by their flotation rates, whereas normal cord blood had only one LDL peak (Fig. 1). Although the flotation behavior of LDL_a was similar to that of intermediate density lipoprotein (22), LDL_a differed from intermediate density lipoprotein in chemical composition and apoprotein content. Therefore, the LDL designation is justified. In any case, both LDL_a and LDL_b differed from LDL_c in flotation properties. (b) There were differences in the composition and sizes of FH LDL and normal LDL which accounted for the altered flotation rates (Table II). (c) The immunoreactivities of ApoB in LDL_a and LDL_b differed from those of LDL_c when the anti-LDL (R-198) but not when the anti-VLDL-ApoB (R-161) antisera were used in the assays (Fig. 2). This suggests that the differences between FH LDL and normal LDL_c were the result of differences in the disposition of ApoB on the LDL surface, rather than due to differences in size. Had size differences alone been responsible both antisera should have given the same results (16). (d) LDL_a and LDL_b were more effective in binding to fibroblasts than LDL_c (Fig. 3) and LDL_a and LDL_b also delivered more cholesterol to the cells at low concentration of LDL (Table III). At a supersaturating

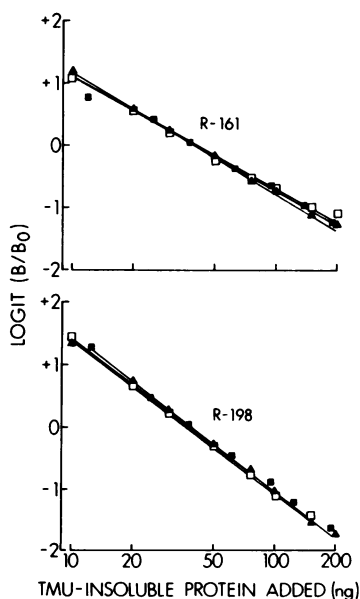


FIGURE 5 RIA competitive displacement curves produced by LDL of S.L. (LDL_S, □; R.L. (LDL_R, ■; and a normal control LDL (LDL_N, ▲) (see legend Fig. 2).

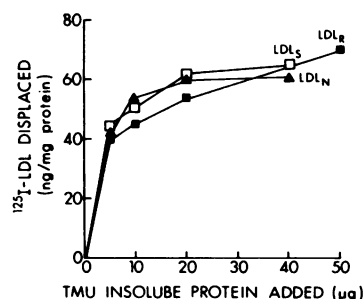


FIGURE 6 Competitive displacement of ¹²⁵I-LDL by LDL isolated from S.L. (LDL_S), R.L. (LDL_R) and LDL obtained from a normal control subject (LDL_N). Experimental conditions were as in Fig. 3.

concentration of the LDL receptor (18) no differences were seen.

The differences in immunologic and cell receptor activities between FH cord and normal cord LDL may be explainable on the basis of the relative TG lack of FH cord LDL which could influence the disposition of ApoB on the surface of the LDL particles. TG-poor VLDL also react more efficiently with the anti-LDL antisera and with cellular LDL receptors than do TG-rich VLDL (16). Thus, it is not necessary to invoke changes in the primary structure of FH ApoB. This is also supported by the observation that postnatal FH, which resembled normal LDL in physical properties and chemical composition, also was indistinguishable from normal LDL with respect to its immunologic and cellular interactions.

We do not have experimental evidence to explain the presence of two populations of LDL or their unique flotation properties in the cord serum of the homozygote. These LDL could have resulted from altered production or intravascular catabolism. Overproduction of cholesterol in homozygotes early in life has been postulated from the LDL receptor model (1), and was indeed found in the patient's brother at the age of 13 mo (5). The production of LDL-ApoB is also increased in FH homozygotes (23). The fact that more cholesterol per ApoB was transported in the LDL of the homozygote suggest that in homozygous FH there may be a discrepancy between cholesterol and ApoB production in the prenatal period. The relative "shortage" of ApoB could result in the formation of larger particles, capable of transporting more core lipids per unit of coat-protein or coat-lipid. A similar discrepancy between lipid and apoprotein production seems to exist when hepatic VLDL production is increased by high carbohydrate diets (24, 25). We cannot explain why LDL appeared in two distinct populations rather than as a continuum of lipoproteins over this density range. Two populations may have been secreted. Alternatively, one LDL population may have been the secretory product and the other could have arisen after transformation in plasma.

The changes in LDL flotation properties and composition which occurred after birth could be explained in at least two ways: (a) LDL_a decreased in quantity because the relative shortage of ApoB was made up by increased hepatic or intestinal production; or (b) the secretion of LDL-cholesterol decreased because of suppression of cholesterol synthesis by non-specific cellular LDL uptake or by any cholesterol absorbed from the diet.

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