REGULATION OF CHOLESTEROL SYNTHESIS BY LOW DENSITY LIPOPROTEIN IN ISOLATED HUMAN LYMPHOCYTES*

Comparison of Cells from Normal Subjects and Patients with Homozygous Familial Hypercholesterolemia and Abetalipoproteinemia

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Rapidly dividing cultured human cells, such as fibroblasts and lymphoblasts, are able to derive all of their required cholesterol from plasma low density lipoprotein (LDL)[1] that is present in the culture medium (reviewed in references 1 and 2). The ability of cultured cells to utilize LDL-cholesterol is dependent on the presence of a high affinity cell surface receptor that binds LDL and thus enhances its rate of cellular uptake and lysosomal degradation (3–7). When grown continuously in the presence of LDL, cultured fibroblasts express a relatively low number of LDL receptors that is just sufficient to supply enough cholesterol to balance cellular sterol losses and to allow for normal cell growth. Under these conditions, the amount of cholesterol derived from the degradation of LDL is sufficient to supply the cell's cholesterol needs and to maintain a suppression of endogenous cholesterol synthesis (8–12).

When rapidly dividing cells are deprived of LDL, they simultaneously develop both an enhanced number of LDL receptors and an enhanced rate of cholesterol synthesis, the latter mediated through an increase in the activity of a rate-controlling enzyme, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA reductase) (1, 8, 11, 12). Under these conditions, the subsequent exposure of the cells to LDL is followed by a rapid uptake and degradation of the lipoprotein through the receptor mechanism, which leads initially to a suppression of cholesterol synthesis and subsequently to a reduction in the number of LDL receptors (1, 8, 11, 12). Cultured fibroblasts and lymphoblasts from subjects with homozygous familial hypercholesterolemia (FH) lack LDL receptors and are therefore unable to bind, take up, and degrade the lipoprotein with high affinity (3–7, 13). Thus, when grown in the presence of LDL, the FH homozygote cells, in contrast to

* This research was supported by grants from the National Foundation – March of Dimes (6-76-138) and the National Institutes of Health (HL 16024, GM 19258, and 5 M0I-RR-00633).

‡ Recipient of a National Institutes of Health Postdoctoral Fellowship T 22-GM-0021.

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‖ Recipient of a U. S. Public Health Service Research Career Development Award (GM 70,277).

[1] *Abbreviations used in this paper:* FH, familial hypercholesterolemia; HDL, high density lipoprotein; HMG CoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase; LDL, low density lipoprotein; LPDS, lipoprotein-deficient serum.

normal cells, derive the cholesterol that they require for cell growth not from plasma lipoproteins but from endogenous synthesis (1, 8, 9).

The pattern of regulation of cholesterol synthesis is altered after cultured fibroblasts have become confluent and ceased to divide. When incubated in the absence of lipoproteins, confluent fibroblasts from both normal subjects and FH homozygotes exhibit relatively low rates of HMG CoA reductase activity and cholesterol synthesis as compared with growing cells (8). Moreover, when deprived of lipoproteins, normal confluent fibroblasts express only a relatively small number of LDL receptors (4). Both of these observations suggest that the sterol requirements of confluent cells are lower than those of dividing cells. Consistent with this conclusion is the additional finding that HMG CoA reductase activity can be enhanced in confluent fibroblasts from normal subjects and FH homozygotes by addition to the culture medium of agents such as lipoprotein-deficient serum (LPDS) and high density lipoprotein (HDL) that are believed to remove cholesterol from cells and hence increase their sterol requirement (8).

To begin to study directly how the LDL receptor functions in certain nondividing cells in the body, we have recently turned to human lymphocytes freshly isolated from the bloodstream. We previously showed that freshly isolated lymphocytes expressed an LDL receptor with properties similar to that of cultured fibroblasts and lymphoblasts (14). As expected from the findings in cultured cells (1, 11), the number of LDL receptors on lymphocytes was relatively low immediately after their removal from the bloodstream where they had been exposed to plasma LDL (14). However, when lymphocytes were incubated in medium devoid of LDL, the number of LDL receptors increased and hence the ability of the cells to take up and degrade LDL rose by 10–20-fold over a 48- to 72-h period (14). This increase in the number of LDL receptors could be prevented by inclusion of LDL, but not HDL, in the initial incubation medium (14). Freshly isolated lymphocytes from patients with homozygous FH exhibited less than 5% of normal LDL receptor activity, even after incubation for 72 h in the absence of lipoproteins (14).

In the present studies, we have used the lymphocyte model to determine whether the LDL receptor plays a role in the regulation of cholesterol synthesis in nondividing cells that have been freshly isolated from the body. The results indicate that: (*a*) sterol synthesis in fresh lymphocytes is subject to feedback suppression mediated specifically by LDL, (*b*) this regulation is achieved at least in part through an interaction of LDL with the LDL receptor, and (*c*) this regulation resembles the pattern observed in confluent, nondividing fibroblasts in that the requirement for cholesterol is low and is enhanced by agents that are believed to remove cholesterol from cells.

Materials and Methods

Materials. [2-^{14}C]Acetic acid, sodium salt (59 mCi/mmol) was purchased from Amersham/Searle Corp. (Arlington Heights, Ill.) Tissue culture supplies and chemicals were obtained from sources as previously reported (8, 14).

Subjects. Normal subjects were healthy individuals who had total plasma cholesterol levels less than 220 mg/dl and no family history of FH. The four patients with homozygous FH and the two patients with abetalipoproteinemia had the typical features of the indicated disorder (15, 16). Their clinical and biochemical data are summarized in Table I. Informed written consent was obtained from each subject or from the parents.

Lipoproteins and Serum. Human LDL (density 1.019–1.063 g/ml), human HDL (density 1.085–1.215 g/ml), and human LPDS (density >1.215 g/ml) were obtained from the plasma of individual healthy subjects and prepared by differential ultracentrifugation (8). The concentration of LDL and HDL is expressed in terms of their protein content. The mass ratio of total cholesterol to protein in LDL and HDL was 1.6:1 and 1:3, respectively. LDL was radiolabeled with ^{125}I as previously described (3).

TABLE I
Clinical and Biochemical Data on Patients with Homozygous FH and Abetalipoproteinemia

Patient	Age	Sex	Plasma cholesterol*		Plasma triglycerides*	Phenotype of cultured fibroblasts‡	Drug therapy
			Total	LDL			
			mg/dl		*mg/dl*		
Homozygous FH							
M. C.§	8	F	625	590	112	Receptor-negative	Inderal, nicotinic acid
K. W.	13	F	582	549	81	Receptor-negative	Ferrous gluconate
D. R.	15	F	545	512	119	Receptor-defective	Ferrous gluconate
B. H.	21	M	723	685	202	Receptor-negative	Ferrous gluconate
Abetalipoproteinemia							
M. W.	14	M	35	0	3	–	Vitamins A and E
A. C.	34	M	40	0	6	–	Vitamins A and E

* Measured by previously described methods (38).
‡ Determined in cultured fibroblasts by previously described criteria (27).
§ An end-to-side portacaval shunt was performed 19 mo before the current studies (38).

Isolation and Prior Incubation of Lymphocytes. Peripheral blood lymphocytes were isolated under sterile conditions by a modification (14) of the method of Boyüm (17). Each washed cell pellet containing 10–15 × 10^6 mononuclear cells from 10 ml of venous blood was resuspended in medium A (RPMI-1640 medium [Grand Island Biological Co., Grand Island, N. Y.] with penicillin [100 U/ml] and streptomycin [100 μg/ml]) containing 10% (vol/vol) human LPDS (final protein concentration, 5 mg/ml). The cell suspensions were then combined and divided into portions of 2 ml (each containing 3–5 × 10^6 cells) which were transferred to 30-ml plastic culture flasks.

Cell viability, as assessed by erythrosin B exclusion, was routinely determined for each lymphocyte preparation and was greater than 90% under all experimental conditions. Purity of the cell preparations was assessed after staining smears with Wright-Giemsa. Differential counts revealed that 90–95% of the mononuclear cells both before and after incubation were morphologically identifiable as lymphocytes. By the functional criterion of latex particle ingestion (18), about 5% of the mononuclear cells were identified as monocytes. When these monocytes were quantitatively removed from the preparations by adherence to plastic, the rate of cholesterol synthesis in the mononuclear preparation was not altered either at zero time or after incubation in LPDS and the response to LDL was not affected in either the FH homozygote or the normal cells. Hence, in most experiments, the small number of contaminating monocytes was not removed.

Incorporation of [2-^{14}C]Acetate into Cholesterol by Intact Lymphocytes. Cholesterol synthesis from [2-^{14}C]acetate was measured by either of two methods. Method 1—Lymphocytes from each culture flask were harvested by centrifugation (1,000 rpm, 10 min, 24°C) and washed once with 15 ml of medium A. The cells from several flasks were pooled together and resuspended in 0.24 ml of medium A at a final concentration of about 20–60 × 10^6 cells/ml (1–3 mg cellular protein/ml). Duplicate portions (10 μl) of the lymphocyte suspension were removed for measurement of cellular protein content and another set of duplicate aliquots (0.1 ml) was incubated in glass tubes containing 1 ml of medium A supplemented with 10% LPDS and 2.5 mM [2-^{14}C]acetate (58 cpm/pmol). The tubes were gassed with 95% O_2: 5% CO_2, capped, and incubated with shaking at 37°C for 4 or 5 h. The incubations were terminated by the addition of 0.25 ml of 50% KOH, 0.5 ml of ethanol, and 10^5 cpm of [1,2-^{3}H]cholesterol (50 Ci/mmol) as an internal standard. Each sample was then autoclaved (15 pounds/inch^2, 240°F, 30 min), after which 1 ml of ethanol was added. The nonsaponifiable lipids were then extracted with three 10-ml portions of petroleum ether, and the pooled ether extracts were backwashed with 2 ml of 0.1-N sodium hydroxide followed by 2 ml of distilled water. The [^{14}C]squalene (R_f = 0.71), [^{14}C]lanosterol, and other 4,4-dimethyl sterols (R_f = 0.44), and [^{14}C]cholesterol (R_f = 0.31) were isolated by thin-layer chromatography and quantified (19). The recovery of the added [^{3}H]cholesterol, which averaged 90%, was used to correct for procedural losses of synthesized [^{14}C]cholesterol. Method 2—This method differs from Method 1 primarily in that the [^{14}C]acetate was added directly to the flask in which the cells had been previously incubated, thus avoiding the necessity for centrifuging the cells immediately before the isotope incorporation was studied. Each flask of lymphocytes containing 180–400 μg of cell protein in 2 ml of medium A and 10% LPDS received 50 μl of [2-^{14}C]acetate (final concentration, 2.5 mM; sp act, 29–58 cpm/pmol). The cells were incubated at 37°C in a humidified incubator (5% CO_2 in air) for 2

or 4 h. One portion of the cells and medium from each flask (0.2 ml) was removed, transferred to a 400-μl microfuge tube, and centrifuged (12,000 rpm, 2 min, 4°C), and the resulting cell pellet was used for measurement of protein content (14). Incubations were terminated by the addition of 0.5 ml of 50% KOH, 1 ml of ethanol, and 10^5 cpm of [1, 2-^{3}H] cholesterol (50 Ci/mmol) as an internal standard. The content of [^{14}C]cholesterol was measured as described for Method 1 except for appropriate volume adjustments for solvents. For all experiments, blank reactions were routinely conducted in which cells were omitted from the incubation mixture.

Incorporation of [1-^{14}C]Oleate into Lipids by Intact Lymphocytes. After prior incubation at 37°C in medium A containing 10% LPDS and the indicated additions, each flask of cells received 50 μl of [^{14}C]oleate bound to albumin (final concentration and sp act, 0.1 mM and 12,000 cpm/nmol, respectively) (20). The cells were incubated at 37°C in a humidified incubator (5% CO_2 in air) for the indicated time, after which the cells and medium were separated by centrifugation (1,000 rpm, 5 min, 4°C). Each cell pellet was washed three times with 5 ml of phosphate-buffered saline, after which the ^{14}C-labeled lipids were isolated by thin-layer chromatography and quantified (20).

Measurement of Cellular Content of Free and Esterified Cholesterol in Lymphocytes. Lymphocytes were isolated and washed twice in medium A as described above. The cell pellet was washed once more in 5 ml of phosphate-buffered saline. The final cell pellet was resuspended in 0.24 ml of water. Duplicate portions (10 μl) were removed for determination of protein content, and 0.2 ml of the remaining suspension was extracted with chloroform:methanol (21). The free and esterified cholesterol fractions were separated on silicic acid:celite columns, and the cholesterol content in each fraction was measured by gas–liquid chromatography as described for fibroblasts (9).

Other Assays. Protein was determined by a modification of the method of Lowry et al. (22), with bovine serum albumin as a standard. The total proteolytic degradation of ^{125}I-LDL by intact lymphocytes was determined as previously described (14).

Results

When normal human lymphocytes were incubated for 48 h in the absence of lipoproteins and were then exposed to [^{14}C]acetate, the isotope was incorporated into nonsaponifiable lipids. Fractionation of the nonsaponifiable lipids by thin-layer chromatography showed three radiolabeled products that corresponded in mobility to authentic samples of squalene, lanosterol, and cholesterol. The time-course in Fig. 1 shows that initially the rate of [^{14}C]acetate incorporation into [^{14}C]lanosterol exceeded the rate of incorporation into [^{14}C]cholesterol. However, after 1–2 h, the rate of accumulation of [^{14}C]lanosterol began to decline whereas [^{14}C]cholesterol continued to accumulate, a finding that is consistent with the known precursor-product relationship of these two sterols (23). Validation experiments indicated that the rate of incorporation of [^{14}C]acetate into [^{14}C]cholesterol was linear with time for at least 6 h, linear with respect to the mass of cells in the incubation medium up to 3 mg of cellular protein, and saturating with respect to [^{14}C]acetate at concentrations above 2 mM.

When normal lymphocytes were freshly isolated from the bloodstream, the rate of [^{14}C]acetate incorporation into [^{14}C]cholesterol was low (Fig. 2A). However, when the cells were incubated in medium containing LPDS, the rate of [^{14}C]acetate incorporation into [^{14}C]cholesterol increased by more than sevenfold over a 56-h interval. (In other experiments in which lymphocytes from different subjects were studied, the rate of cholesterol synthesis from [^{14}C]acetate increased 5–15-fold after incubation for 48–72 h in the absence of lipoproteins.) The addition of LDL to the incubation medium at 56 h led to a 75% reduction in the rate of cholesterol synthesis when measured 9 h later (Fig. 2A). A pronounced inhibition was also achieved by addition of a mixture of 25-hydroxycholesterol plus cholesterol, a combination of sterols that also suppresses cholesterol

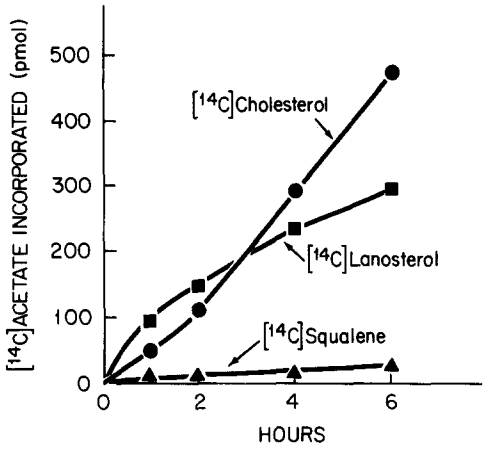

FIG. 1. Time-course of incorporation of [^{14}C]acetate into nonsaponifiable lipids in normal lymphocytes. Lymphocytes from a healthy 24-yr-old female (M. S.) were incubated at 37°C in medium A containing 10% lipoprotein-deficient serum for 48 h, after which the incorporation of [^{14}C]acetate into the indicated ^{14}C-labeled intermediates was measured by Method 1. The results are expressed as the picomoles of [^{14}C]acetate incorporated into the indicated lipid per incubation tube. Each tube contained 100 μg of total cell protein.

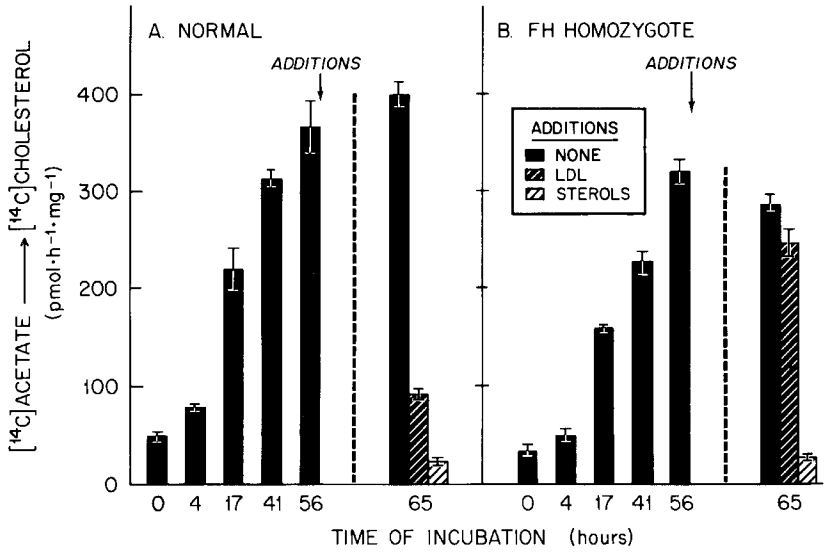

FIG. 2. Regulation of cholesterol synthesis from [^{14}C]acetate in normal (A) and FH homozygote (B) lymphocytes after incubation in the absence of lipoproteins. Lymphocytes from a healthy 22-yr-old male (J. C.) and a 13-yr-old female with homozygous FH (K. W.) were incubated at 37°C in flasks containing medium A with 10% lipoprotein-deficient serum. The incorporation of [^{14}C]acetate into [^{14}C]cholesterol was measured in duplicate flasks at the indicated interval by Method 2 with a 4-h labeling period. After incubation for 56 h in the absence of lipoproteins, duplicate sets of flasks received the following additions: none; 20 μg protein/ml of LDL; or a mixture of 1.2 μg/ml of 25-hydroxycholesterol and 15 μg/ml of cholesterol added in 5 μl of ethanol. The incubations were continued for 9 h, after which the incorporation of [^{14}C]acetate into [^{14}C]cholesterol was measured by Method 2 with a 4-h labeling period. Each bar represents the average and range of the duplicate incubations.

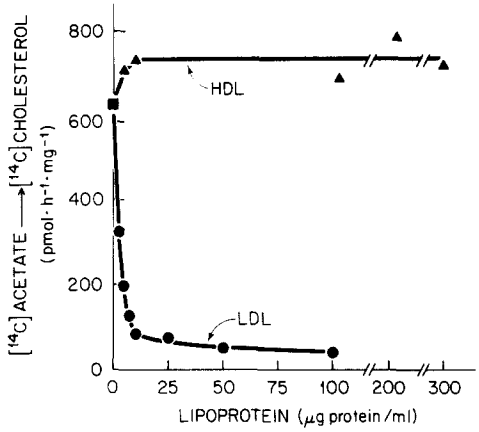

FIG. 3. Ability of plasma lipoproteins to suppress cholesterol synthesis from [^{14}C]acetate in normal lymphocytes that had been subjected to prior incubation in the absence of lipoproteins. Lymphocytes from a healthy 23-yr-old male (R. H.) were incubated at 37°C in medium A containing 10% lipoprotein-deficient serum. After 48 h, each flask received the indicated final concentration of one of the following lipoproteins: ■, none; ▲, HDL; or ●, LDL. The incubations were continued for an additional 24 h, after which the incorporation of [^{14}C]acetate into [^{14}C]cholesterol was measured by Method 1 with a 5-h labeling period. Each value represents either the average of triplicate incubations (■) or single incubations (▲, ●).

synthesis in fibroblasts (24) and in other cell types (25) (Fig. 2A). Fig. 3 shows that HDL, in contrast to LDL, did not suppress cholesterol synthesis when added to normal lymphocytes that had first been incubated for 48 h in the absence of lipoproteins. As with other cell systems tested to date (8, 10, 26), suppression of cholesterol synthesis by LDL in normal human lymphocytes was associated with a parallel decrease in the activity of HMG CoA reductase as measured in cell-free extracts (data not shown).

To determine whether the suppression of cholesterol synthesis by LDL in lymphocytes required the action of the LDL receptor, we studied the ability of LDL to suppress sterol synthesis in FH homozygote cells when LDL was added after sterol synthesis had been elevated by prior incubation of the cells in LPDS. Fig. 2B shows that when lymphocytes from one FH homozygote were isolated from her bloodstream, the rate of [^{14}C]acetate incorporation into [^{14}C]cholesterol was similar to that in normal cells. Moreover, just as in normal cells, the rate of sterol synthesis rose markedly when the cells were incubated for 56 h in LPDS. However, in contrast to normal cells, the subsequent addition of LDL to the FH homozygote cells did not produce a significant suppression of sterol synthesis. On the other hand, these mutant cells responded normally to the addition of 25-hydroxycholesterol plus cholesterol (Fig. 2B). A similar time-course of induction of cholesterol synthesis from [^{14}C]acetate was observed when the experiment in Fig. 2 was repeated with lymphocytes from two different normal subjects and two different FH homozygotes.

Fig. 4 shows that the resistance of FH homozygote lymphocytes to the action of LDL persisted even at levels of LDL that were 20-fold higher than the level of LDL needed to achieve 50% suppression of cholesterol synthesis in normal lymphocytes (5 μg protein/ml). When freshly isolated from the bloodstream,

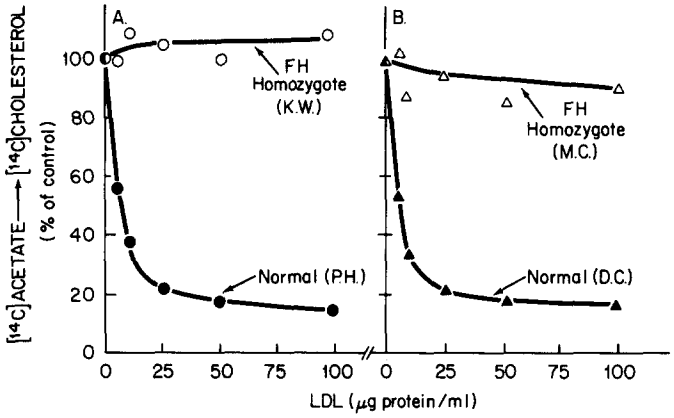

FIG. 4. Effect of LDL on cholesterol synthesis from [^{14}C]acetate in normal (●, ▲) and FH homozygote (○, △) lymphocytes that had been subjected to prior incubation in the absence of lipoproteins. Lymphocytes were isolated in experiment A from a healthy 23-yr-old female (P. H.) and a 13-yr-old female with homozygous FH (K. W.) and in experiment B from a healthy 11-yr-old male (D. C.) and an 8-yr-old female with homozygous FH (M. C.). After the cells had incubated at 37°C for 55 h in the absence of lipoproteins, each flask received the indicated final concentration of LDL. The incubations were continued for an additional 9 h, at which time the incorporation of [^{14}C]acetate into [^{14}C]cholesterol was measured by Method 2 with a 4-h labeling period. Each value represents the average of duplicate incubations. The 100% of control values for P. H., K. W., D. C., and M. C. were 370, 195, 392, and 1,171 pmol of [^{14}C]acetate converted to [^{14}C]cholesterol/h per mg protein, respectively.

normal and FH homozygote lymphocytes showed a similar rate of incorporation of [^{14}C]acetate into saponifiable lipids (including phospholipids and triglycerides) and this rate was unaffected by incubation for three days in the absence of lipoproteins or by the addition of LDL.

In cultured human fibroblasts (20) and suspended lymphoid cells (10), the uptake and degradation of LDL through the receptor mechanism leads to the release of free cholesterol and this not only suppresses cholesterol synthesis but it also increases the rate at which the cells incorporate exogenous [^{14}C]oleate into cholesteryl [^{14}C]oleate. Stimulation of cholesteryl ester formation can also be achieved by exposing these cultured cells to a mixture of 25-hydroxycholesterol plus cholesterol, but not to HDL (20, 27). To determine whether a similar phenomenon occurred in isolated lymphocytes, normal cells were incubated for 67 h in the absence of lipoproteins, after which they were exposed to [^{14}C]oleate in the presence of LDL, HDL, or the sterol mixture. In experiments not shown, LDL and the sterol mixture, but not HDL, caused a marked increase in the incorporation of [^{14}C]oleate into cholesteryl [^{14}C]esters. On the other hand, none of these agents had a significant effect on the incorporation of [^{14}C]oleate into phospholipids or triglycerides. That the stimulation of cholesteryl ester formation induced by addition of LDL required the LDL receptor is indicated by the data in Fig. 5A showing that the FH homozygote lymphocytes failed to develop an enhanced rate of incorporation of [^{14}C]oleate into cholesteryl [^{14}C]oleate when incubated with LDL. The data in Fig. 5B show that LDL had no effect on the incorporation of [^{14}C]oleate into triglycerides in either the normal or FH homozygote cells.

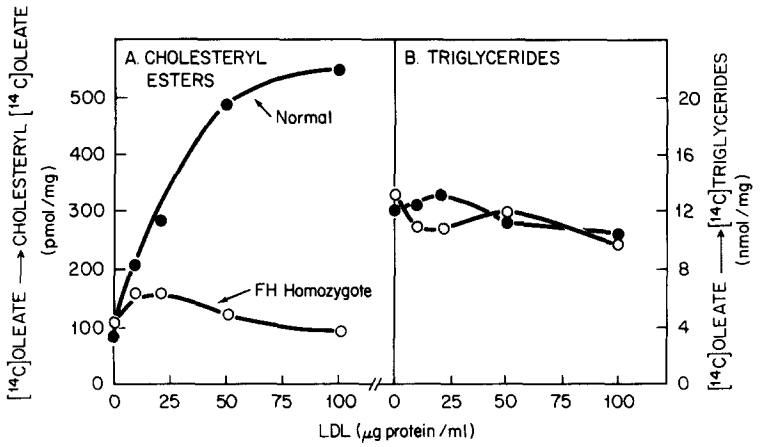

FIG. 5. Effect of LDL on the incorporation of [^{14}C]oleate into cholesteryl esters (A) and triglycerides (B) in normal (●) and FH homozygote (○) lymphocytes that had been subjected to prior incubation in the absence of lipoproteins. Lymphocytes from a healthy 27-yr-old female (S. D.) and a 13-yr-old female with homozygous FH (K. W.) were incubated for 66 h in flasks containing medium A with 10% lipoprotein-deficient serum, after which each flask received 0.1 mM [^{14}C]oleate and the indicated final concentration of LDL. The incubations were continued for an additional 8 h, at which time the cells were harvested for measurement of the cellular content of cholesteryl [^{14}C]esters (A) and [^{14}C]triglycerides (B). Each value represents a single incubation.

In contrast to cultured fibroblasts from FH homozygotes, which manifest enhanced cholesterol synthesis in the presence of whole serum containing LDL (8, 28, 29), lymphocytes from FH homozygotes, when isolated fresh from the bloodstream, showed a normal rate of cholesterol synthesis (Fig. 2B). To document this finding, a series of screening experiments was conducted in which the rate of [^{14}C]acetate incorporation into [^{14}C]cholesterol was measured in freshly isolated lymphocytes from 17 normal subjects, 4 FH homozygotes, and 2 patients with abetalipoproteinemia. Whereas the rates of cholesterol synthesis in lymphocytes from all four FH homozygotes were within the normal range, the rates in both patients with abetalipoproteinemia were above the normal range (Fig. 6). One of these patients, M. W., had a cholesterol synthesis rate that was fourfold above the mean rate of the normal subjects and the other patient, A. C., exhibited a rate that was twofold above the normal mean. The results were similar whether the incubations were conducted for 2 h in an atmosphere of 20% oxygen (open symbols in Fig. 6) or for 5 h in an atmosphere of 95% oxygen (closed symbols in Fig. 6).

Table II shows that the content of free and esterified cholesterol in lymphocytes as measured by gas–liquid chromatography was similar whether the cells were removed from patients with abetalipoproteinemia (serum cholesterol below 40 mg/dl), normal subjects (serum cholesterol of about 200 mg/dl), or FH homozygotes (serum cholesterol above 600 mg/dl).

The preceding experiments on suppression of cholesterol synthesis by LDL were conducted after the lymphocytes had first been incubated for 48–72 h in the absence of lipoproteins under conditions in which the cells had developed a maximal number of LDL receptors (14) as well as an enhanced rate of sterol

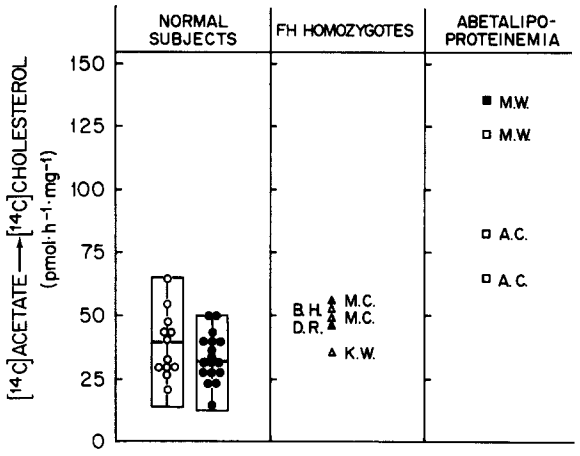

FIG. 6. Cholesterol synthesis from [^{14}C]acetate in freshly isolated lymphocytes from normal subjects, patients with homozygous FH, and patients with abetalipoproteinemia. Lymphocytes were isolated from 10 ml of venous blood obtained from each of the indicated subjects. The incorporation of [^{14}C]acetate into [^{14}C]cholesterol was measured by one of the two procedures described in Methods. Closed symbols refer to Method 1 with a 5-h incubation period. Open symbols refer to Method 2 with a 2-h incubation period. Each value represents the average of duplicate incubations corresponding to the lymphocytes from 5 ml of venous blood. For the normal subjects, each closed symbol represents a different subject (n = 17). The 17 normal subjects consisted of 8 females and 9 males whose mean age was 23 yr (range, 11–32 yr). 12 of the normal subjects who were studied by Method 1 were also studied by Method 2 (open symbols). The clinical data on the patients with homozygous FH and abetalipoproteinemia are provided in Table I. For the normal subjects, the boxed-in areas indicate 2 SD above and below the mean value.

synthesis. Immediately after their isolation from the bloodstream, normal lymphocytes possess only a small number of LDL receptors (14). Moreover, if LDL is included in the incubation medium from the time at which the cells are isolated, the subsequent development of an enhanced number of LDL receptors is prevented (14). In view of these observations, we performed a series of experiments to determine whether the addition of LDL to freshly isolated lymphocytes at a time when the cells expressed only a small number of LDL receptors would suppress the subsequent induction of cholesterol synthesis. The data in Fig. 7 show that the addition of low concentrations of LDL to normal lymphocytes at zero time did suppress the induction of cholesterol synthesis that otherwise occurred when the cells were incubated for 48 h in the absence of lipoproteins. On the other hand, the addition of HDL at zero time failed to suppress the induction of cholesterol synthesis.

The experiment in Fig. 8A shows that LDL added to lymphocytes from an FH homozygote at zero time had a significant ability to suppress the induction of cholesterol synthesis despite the fact that these mutant cells have a marked deficiency in LDL receptors. The data in Fig. 8B show that in the same experiment lymphocytes from the same FH homozygote failed to show any LDL-mediated suppression of cholesterol synthesis when the lipoprotein was added after the cells had been incubated for 56 h in the absence of lipoproteins (Fig. 8B). Similar results were observed in lymphocytes from three other FH homozygotes.

TABLE II

Content of Free and Esterified Cholesterol in Freshly Isolated Lymphocytes Obtained from Normal Subjects and Patients with Abetalipoproteinemia and Homozygous FH

Subject providing cells	Age	Sex	Cholesterol content	
			Free	Esterified
	yr		*μg sterol/mg protein*	
Normal subjects				
D. C.	11	M	21	0.38
J. W.	12	M	37	0.84
L. L.	22	F	35	0.64
C. L.	23	M	31	1.03
S. M.	24	M	46	0.76
M. S.	24	F	22	0.62
G. B.	30	F	44	0.36
M. E.	30	F	32	0.62
S. B.	32	M	27	0.54
Mean ± SEM			33 ± 2.9	0.64 ± 0.07
Abetalipoproteinemia				
M. W.	14	M	37	0.73
A. C.	34	M	49	0.35
FH homozygotes				
M. C.	8	F	29	0.38
D. R.	15	F	37	0.44
B. H.	21	M	28	1.00
Mean ± SEM			31 ± 2.9	0.61 ± 0.19

Lymphocytes were isolated from 10 ml of venous blood obtained from each of the indicated subjects. The cellular content of free and esterified cholesterol in the freshly isolated lymphocytes was determined as described in Methods. Each value represents the average of duplicate determinations.

The LDL-mediated suppression of induction of cholesterol synthesis in the FH homozygote cells occurred under conditions in which the degradation of ^{125}I-LDL was far below normal (Fig. 9). In this experiment, freshly isolated normal and FH homozygote lymphocytes were incubated for 72 h in the presence of ^{125}I-LDL at a concentration of 25 μg protein/ml, a level that is sufficient to suppress the induction of sterol synthesis in both types of cells (compare with Fig. 8). Under these conditions, after 72 h the total amount of ^{125}I-LDL degraded by the normal cells was 10-fold greater than that degraded by the FH homozygote cells. It is important to note that the rate of ^{125}I-LDL degradation in the normal lymphocytes was relatively low and nearly linear from zero time since the concentration of ^{125}I-LDL added (25 μg protein/ml) was sufficient to suppress the 10–20-fold increase in the number of LDL receptors that would have otherwise occurred had LDL not been present from zero time (14).

The data in Fig. 9 indicate that a relatively low rate of LDL degradation in the normal cells, and an even lower rate in the FH homozygote cells, is able to keep cholesterol synthesis suppressed. Thus, the behavior of the isolated lymphocytes resembles that of confluent, nondividing fibroblasts in which the requirement for cholesterol is low enough to be satisfied by a small number of LDL receptors (4, 8). As shown in Fig. 10, the addition of increasing amounts of LPDS

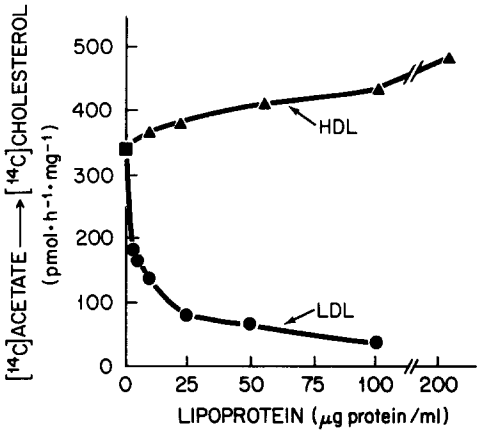

FIG. 7. Suppression of the induction of cholesterol synthesis from [^{14}C]acetate in normal lymphocytes by inclusion of LDL in the prior incubation medium and lack of such prevention by inclusion of HDL. Lymphocytes from a healthy 25-yr-old male (S. A.) were incubated in medium A containing 10% lipoprotein-deficient serum plus the indicated final concentration of one of the following lipoproteins: ■, none; ▲, HDL; or ●, LDL. After incubation at 37°C for 48 h, the incorporation of [^{14}C]acetate into [^{14}C]cholesterol was measured by Method 1 with a 5-h labeling period. Each value represents either the average of triplicate incubations (■) or single (▲, ●) incubations.

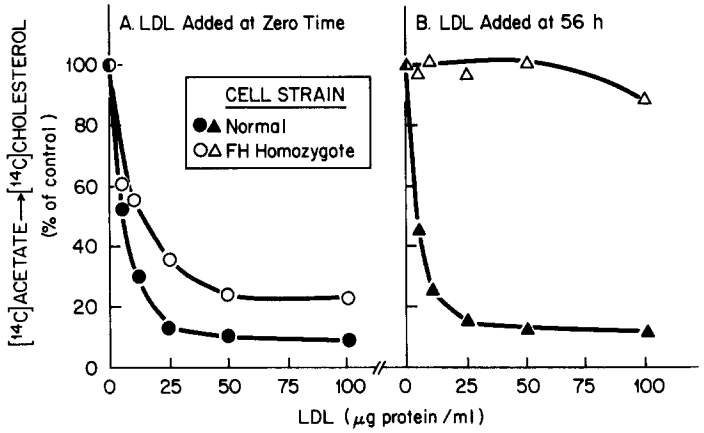

FIG. 8. Effect of LDL added at zero time (experiment A) or after a 56-h incubation (experiment B) on cholesterol synthesis from [^{14}C]acetate in normal (●, ▲) and FH homozygote (○, △) lymphocytes. In experiment A, lymphocytes from a healthy 24-yr-old male (S. M.) and a 21-yr-old male with homozygous FH (B. H.) were incubated for 68 h in medium A containing 10% lipoprotein-deficient serum and the indicated final concentration of LDL, after which the incorporation of [^{14}C]acetate into [^{14}C]cholesterol was measured by Method 2 with a 4-h labeling period. In experiment B, lymphocytes from a healthy 35-yr-old male (D. B.) and the same 21-yr-old male with homozygous FH (B. H.) were incubated for 56 h in medium A containing 10% lipoprotein-deficient serum, after which each flask received the indicated final concentration of LDL. The incubations were continued for an additional 10 h, at which time the incorporation of [^{14}C]acetate into [^{14}C]cholesterol was measured by Method 2 with a 4-h labeling period. The 100% of control values for the normal and FH homozygote cells in experiment A were 266 and 304 pmol of [^{14}C]acetate converted to [^{14}C]cholesterol/h per mg protein, respectively. The 100% of control values for the normal and FH homozygote cells in experiment B were 356 and 603, respectively. Each value represents the average of duplicate incubations.

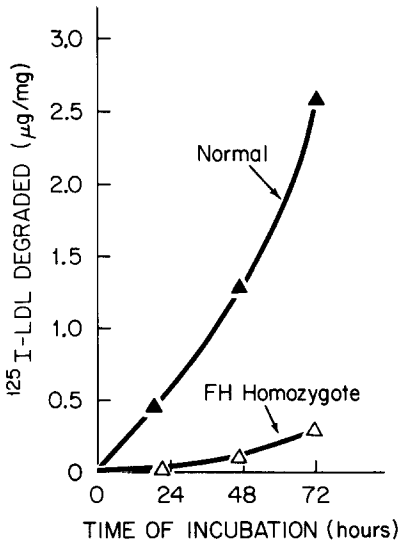

FIG. 9. Time-course of degradation of ^{125}I-LDL in normal (▲) and FH homozygote (△) lymphocytes. Lymphocyte from a healthy 24-yr-old male (S. M.) and a 21-yr-old male with homozygous FH (B. H.) were incubated in 2 ml of medium A containing 10% lipoprotein-deficient serum and 25 μg protein/ml of ^{125}I-LDL (44 cpm/ng). After incubation at 37°C for the indicated time, the content of ^{125}I-labeled acid-soluble material in the medium was determined as described in Materials and Methods. Each value represents the average of duplicate incubations.

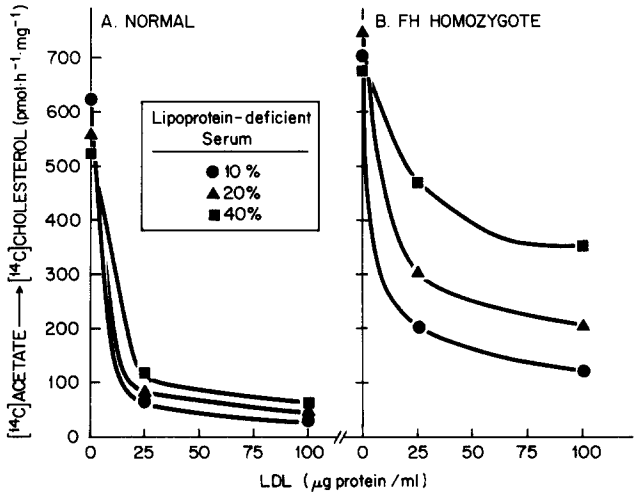

FIG. 10. Effect of varying amounts of lipoprotein-deficient serum on the ability of LDL to suppress the induction of cholesterol synthesis from [^{14}C]acetate in normal (A) and FH homozygote (B) lymphocytes. Lymphocytes from a healthy 22-yr-old male (J. C.) and a 21-yr-old male with homozygous FH (B. H.) were incubated in medium A containing the indicated final concentration of LDL and the indicated final concentration (vol/vol) of lipoprotein-deficient serum that had been dialyzed against medium A. After incubation for 65 h, the incorporation of [^{14}C]acetate into [^{14}C]cholesterol was measured by Method 2 with a 4-h labeling period. Each value represents the average of duplicate incubations.

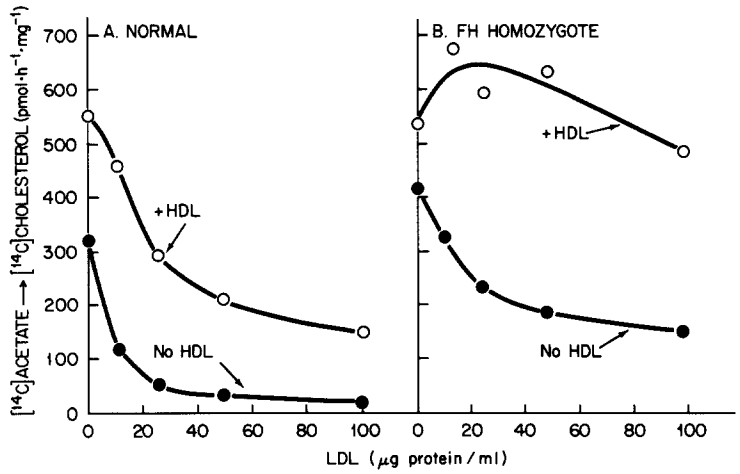

FIG. 11. Effect of HDL on the ability of LDL to suppress the induction of cholesterol synthesis from [^{14}C]acetate in normal (A) and FH homozygote (B) lymphocytes. Lymphocytes from a healthy 22-yr-old male (J. C.) and a 21-yr-old male with homozygous FH (B. H.) were incubated in medium A containing 30% lipoprotein-deficient serum that had been dialyzed against medium A and the indicated concentration of LDL in the presence (○) or absence (●) of 1 mg protein/ml of HDL. After incubation at 37°C for 64 h, the incorporation of [^{14}C]acetate into [^{14}C]cholesterol was measured by Method 2 with a 4-h labeling period. Each value represents the average of duplicate incubations.

stimulated the rate of cholesterol synthesis in the FH homozygote cells but not significantly in the normal cells incubated in the presence of LDL.

Another factor that limited the ability of LDL to suppress the induction of sterol synthesis was HDL. The data in Fig. 11B show that when FH homozygote lymphocytes were incubated for 64 h in the presence of 30% LPDS plus 1 mg protein/ml of HDL, the presence of LDL at levels up to 100 μg protein/ml did not significantly suppress the induction of sterol synthesis. On the other hand, in normal cells the same high level of HDL blunted but did not abolish the LDL-mediated suppression of cholesterol synthesis (Fig. 11A). HDL did not appear to be acting by inhibiting the degradation of LDL. As shown in Fig. 12, freshly isolated normal lymphocytes were incubated with varying concentrations of ^{125}I-LDL in the presence or absence of 1 mg protein/ml of HDL and the amount of ^{125}I-LDL degraded was measured after 6 and 24 h. The results indicate that HDL did not affect the degradation of ^{125}I-LDL either at nonsaturating or saturating levels of ^{125}I-LDL. In other experiments, HDL did not inhibit the degradation of ^{125}I-LDL over a time period as long as 72 h.

A third factor that limited the ability of LDL to suppress the induction of sterol synthesis in the FH homozygote, but not in the normal cells was lecithin. When the FH cells were incubated for 3 days in the presence of 10% LPDS and 100 μg/ml of lecithin, the rate of cholesterol synthesis from [^{14}C]acetate was 638 pmol·h^{-1}·mg^{-1} in cells not receiving LDL and 477 in cells to which 50 μg protein/ml of LDL had been added at zero time (75% of control). In the same experiment, the rate of sterol synthesis in normal cells incubated in the same medium was 1014 pmol·h^{-1}·mg^{-1} in cells not receiving LDL and 109 in cells to which 50 μg protein/ml of LDL had been added at zero time (10% of control).

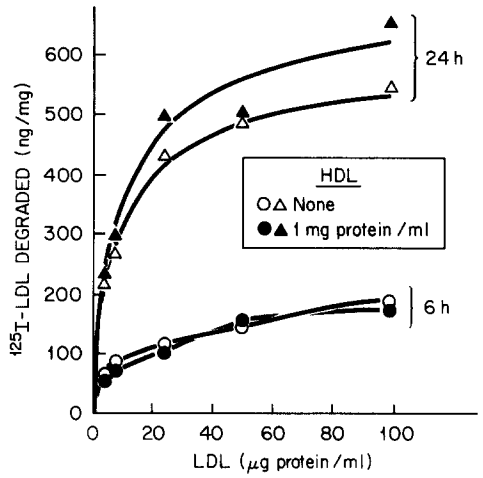

FIG. 12. Failure of HDL to affect the saturation curve for ^{125}I-LDL degradation in normal lymphocytes. Lymphocytes from a healthy 24-yr-old male (S. M.) were incubated in medium A containing 30% lipoprotein-deficient serum that had been dialyzed against medium A and the indicated concentration of ^{125}I-LDL (57 cpm/ng) in the presence (●, ▲) or absence (○, △) of 1 mg protein/ml of HDL. After incubation at 37°C for either 6 h (○, ●) or 24 h (△, ▲), the content of ^{125}I-labeled acid-soluble material in the medium was determined as described in Methods. Each value represents a single incubation.

Discussion

In the current experiments, we have sought to determine whether plasma LDL and its cell surface receptor play a role in the regulation of cholesterol synthesis in freshly isolated human lymphocytes. The results indicate that: (*a*) the rate of sterol synthesis is low immediately after normal lymphocytes are isolated from the bloodstream; (*b*) this rate increases 5–15-fold when the cells are incubated for 48–72 h in LPDS; (*c*) after cholesterol synthesis becomes elevated it can be suppressed by the subsequent addition of LDL, but not HDL, to the incubation medium; and (*d*) LDL, but not HDL, when added to the cells immediately after their isolation from the bloodstream, can prevent the subsequent induction of enhanced sterol synthesis.

Through the use of lymphocytes from FH homozygotes that are severely deficient in LDL receptors (14), we have been able to show that after cholesterol synthesis is elevated by incubation of lymphocytes in the absence of lipoproteins, its subsequent suppression by LDL requires the LDL receptor; hence, such suppression does not occur in lymphocytes from FH homozygotes. In this respect, freshly isolated lymphocytes behave similarly to cultured human fibroblasts and lymphoid cells (2, 7, 8, 10). Moreover, in a further analogy with the behavior of cultured human cells (10, 20), normal lymphocytes were also shown to respond to LDL with an enhancement in the rate of [^{14}C]oleate incorporation into cholesteryl esters, and this response was also lacking in the FH homozygote cells.

On the other hand, it was not possible to demonstrate conclusively that the second action of LDL, namely the suppression of the induction of enhanced cholesterol synthesis when the lipoprotein was added to lymphocytes at zero

time, was totally dependent on the LDL receptor. This was because LDL had a significant ability to suppress the induction of cholesterol synthesis in lymphocytes from FH homozygotes even though these cells were markedly deficient in their ability to bind, take up, and degrade the lipoprotein. Two possible explanations could account for this phenomenon: (*a*) that this action, although specific for LDL and not HDL, is mediated by a mechanism that does not involve the LDL receptor, or (*b*) that this effect requires a small number of LDL receptors that is below the limit of reliable detection by direct measurement of ^{125}I-LDL binding in the FH homozygote cells.

Two lines of indirect evidence suggest that the latter explanation may be the correct one. First, the suppression of induction of cholesterol synthesis was not only specific for LDL but it also occurred at concentrations of the lipoprotein similar to those at which binding to the LDL receptor takes place (14). Thus, although LDL had only a partial effect on the FH homozygote cells, this effect reached a maximum at an LDL concentration of about 50 μg protein/ml (Figs. 8, 10, 11), the concentration at which the LDL receptor in normal lymphocytes is saturated (14). Second, a trace amount of high affinity ^{125}I-LDL degradation (<5% of that observed in normal subjects) has been detected previously in lymphocytes from receptor-negative FH homozygotes incubated for 72 h in the absence of lipoproteins (14). These data suggest the presence of a small number of LDL receptors in lymphocytes from some patients whose fibroblasts appear completely devoid of receptor activity as determined within the sensitivity of present assays.

If nondividing cells such as lymphocytes require only small amounts of cholesterol for structural purposes, it is possible that a small amount of LDL receptor activity and a consequent low rate of LDL degradation in FH homozygote lymphocytes might be capable of supplying sufficient cholesterol to balance cellular losses so long as LDL is maintained continuously for several days in the incubation medium. On the other hand, if lymphocytes are first incubated for several days in the absence of LDL so that they develop an enhanced requirement for cholesterol as evidenced by the development of an enhanced rate of cholesterol synthesis, then the small number of LDL receptors in the FH homozygote cells is insufficient to allow a rapid uptake of cholesterol when LDL is added back to the medium. Consequently, endogenous cholesterol synthesis remains high in the mutant cells as compared to the suppressed level in the normal cells.

Additional evidence supports the hypothesis that a small number of LDL receptors can satisfy the requirement for cholesterol in lymphocytes only when that requirement is minimal. Thus, three agents that are believed to enhance the removal of cholesterol from cells (namely, LPDS, plasma HDL, and lecithin) (8, 30–33) all render FH homozygote cells resistant to the action of LDL in suppressing the induction of cholesterol synthesis. Thus, in the presence of agents that increase the cellular requirement for cholesterol normal cells are able to degrade LDL at a rate sufficient to keep sterol synthesis suppressed. However, the low rate of LDL degradation in FH homozygote cells is unable to supply the cells with sufficient cholesterol under these conditions and hence sterol synthesis is enhanced.

An important finding in the current studies was the observation that the rate of cholesterol synthesis in freshly isolated lymphocytes from 4 different FH homozygotes was the same as that in 17 normal subjects. This normal rate presumably reflects the fact that before their removal from the body these mutant lymphocytes were exposed to plasma LDL-cholesterol levels that were at least 6-fold above normal and approximately 100-fold greater than the level required to maintain a suppression of cholesterol synthesis in nondividing cells in vitro. In contrast to these findings in the FH homozygotes, lymphocytes from two patients with abetalipoproteinemia, which have never been exposed to plasma LDL, showed rates of cholesterol synthesis that were two to fourfold above those in cells of normal subjects immediately after their isolation from the bloodstream. This enhanced rate of cholesterol synthesis did reflect a degree of partial suppression, however, since the rate of cholesterol synthesis in lymphocytes from these abetalipoproteinemia subjects increased an additional three to sixfold when these cells were incubated for 72 h in the absence of lipoproteins (data not shown). The factors responsible for the partial suppression of cholesterol synthesis in abetalipoproteinemia lymphocytes in vivo are not known.

Despite the differences in the plasma level of LDL, in the number of LDL receptors, and in the rate of cholesterol synthesis in lymphocytes from normal subjects, FH homozygotes, and patients with abetalipoproteinemia, the cellular content of free and esterified cholesterol was indistinguishable among the three groups. These data emphasize the ability of the various control mechanisms to adapt to metabolic derangements arising from different mutations affecting LDL metabolism so as to keep the intracellular cholesterol content constant. In abetalipoproteinemia, in which plasma LDL is absent, compensation is achieved at least in part through an enhanced rate of cellular sterol synthesis; whereas in homozygous FH, in which LDL receptors are markedly deficient, compensation is achieved at least in part through an elevation in plasma LDL. Similar findings of elevated cholesterol synthesis in fresh skin slices from two abetalipoproteinemia subjects and a normal cholesterol synthesis rate in skin slices from one FH homozygote have previously been reported (34).

The finding of an induction of enhanced cholesterol synthesis in normal lymphocytes incubated in vitro in the absence of lipoproteins correlates with the previously reported increase in sterol synthesis that occurs after similar incubation of mixed leukocytes (35–37). On the other hand, the results in the current studies differ from those expected on the basis of previous reports that mixed leukocytes from FH heterozygotes showed a more rapid enhancement in the rate of sterol synthesis than did normal leukocytes incubated in delipidized serum (36). In the current studies, lymphocytes from FH homozygotes did not show a more rapid enhancement in the rate of cholesterol synthesis than did the normal cells when incubated in LPDS. Moreover, when lymphocytes were incubated for 72 h in the absence of lipoproteins, no consistent difference was noted in the rate of cholesterol synthesis between the normal and FH homozygote cells, indicating that under the conditions of these experiments the FH homozygote cells did not exhibit an enhanced efflux of cholesterol from the cell as has been proposed to occur in mixed leukocytes isolated from FH heterozygotes (36). Whether the differences in these results reflect the difference in genotype of the FH patients

(heterozygotes vs. homozygotes), a difference in the type of cell preparation used (mixed leukocytes vs. lymphocytes), or a difference in methodology employed (incubation in delipidized serum vs. LPDS) remain to be determined.

Summary

The rate of cholesterol synthesis from [^{14}C]acetate was low in circulating blood lymphocytes freshly isolated from 17 normal subjects and 4 subjects with homozygous FH. On the other hand, the rate of cholesterol synthesis was two to fourfold above normal in freshly isolated lymphocytes from two subjects with abetalipoproteinemia. When the lymphocytes from subjects with all three genotypes were incubated for 48–72 h in the absence of lipoproteins, the rate of cholesterol synthesis increased by 5–15-fold. The subsequent addition of plasma LDL, but not HDL, rapidly suppressed cholesterol synthesis in the lymphocytes from normal subjects. In contrast, lymphocytes from the FH homozygotes, which have been shown previously to be deficient in cell surface LDL receptors, were resistant to LDL-mediated suppression of cholesterol synthesis.

In addition to its ability to suppress cholesterol synthesis after it had been elevated by incubation of the cells in the absence of lipoproteins, LDL was able to suppress the induction of the enhanced rate of sterol synthesis when added to normal lymphocytes immediately after their isolation from the bloodstream. In contrast to the former action of LDL, the latter action of LDL – i.e., the suppression of induction of sterol synthesis – also occurred to a limited extent in lymphocytes from FH homozygotes. However, the FH lymphocytes, but not the normal cells, could be made resistant to this action of LDL by inclusion in the incubation medium of lipoprotein-deficient serum (30%, vol/vol) plus HDL (1 mg protein/ml). Considered together with previous data demonstrating a deficiency of LDL receptors in freshly isolated lymphocytes from FH homozygotes, the current studies provide evidence in support of the hypothesis that the interaction of plasma LDL with its cell surface receptor serves to regulate cholesterol synthesis in human lymphocytes.

Received for publication 3 January 1977.

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