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### Research Article

Circulating human lymphocytes freshly isolated from venous blood of 15 normal subjects exhibited a low capacity to bind, take up, and degrade 125I-labeled low density lipoprotein (LDL). However, when these cells were incubated for 72 h in the absence of lipoproteins, they gradually acquired an increased number of high affinity cell surface receptors for LDL. The increase in the number of LDL receptors was associated with a 16-fold increase in the rate at which the cells were able to take up and degrade the lipoprotein. The LDL binding and degradation processes that developed in normal lymphocytes exhibited the following characteristics; (a) high affinity (saturation was achieved at LDL concentrations below 50 µg protein/ml); (b) specificity (unlabeled LDL was much more effective than human high density lipoprotein or other plasma proteins in competing with 125I-LDL for binding to the LDL receptor); and (c) feedback regulation (the increase in the number of LDL receptors that appeared after incubation of freshly isolated lymphocytes in lipoprotein-deficient medium was prevented by exposure of the cells to either LDL or a mixture of 25-hydroxycholesterol plus cholesterol but not to HDL). Freshly isolated lymphocytes obtained from three subjects with the homozygous form of familial hypercholesterolemia failed to develop normal amounts of LDL receptor activity when incubated in medium devoid of lipoproteins. The current data indicate: (a) that the [...]

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# Regulation of Low Density Lipoprotein Receptor Activity in Freshly Isolated Human Lymphocytes

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**ABSTRACT** Circulating human lymphocytes freshly isolated from venous blood of 15 normal subjects exhibited a low capacity to bind, take up, and degrade  $^{125}\text{I}$ -labeled low density lipoprotein (LDL). However, when these cells were incubated for 72 h in the absence of lipoproteins, they gradually acquired an increased number of high affinity cell surface receptors for LDL. The increase in the number of LDL receptors was associated with a 16-fold increase in the rate at which the cells were able to take up and degrade the lipoprotein. The LDL binding and degradation processes that developed in normal lymphocytes exhibited the following characteristics: (a) high affinity (saturation was achieved at LDL concentrations below  $50\text{ }\mu\text{g protein/ml}$ ); (b) specificity (unlabeled LDL was much more effective than human high density lipoprotein or other plasma proteins in competing with  $^{125}\text{I}$ -LDL for binding to the LDL receptor); and (c) feedback regulation (the increase in the number of LDL receptors that appeared after incubation of freshly isolated lymphocytes in lipoprotein-deficient medium was prevented by exposure of the cells to either LDL or a mixture of 25-hydroxy-cholesterol plus cholesterol but not to HDL).

Freshly isolated lymphocytes obtained from three subjects with the homozygous form of familial hypercholesterolemia failed to develop normal amounts of LDL receptor activity when incubated in medium devoid of lipoproteins. The current data indicate: (a) that the LDL receptors that appear on the surface of cholesterol-deprived, normal human lymphocytes are genetically identical to the previously char-

acterized LDL receptors of cultured human fibroblasts and long-term lymphoid cells and (b) that at least one cell type in the human body, the circulating human lymphocyte, has the capacity to produce a high affinity LDL receptor that mediates the cellular uptake and degradation of plasma LDL.

## INTRODUCTION

Recent studies in cultured human fibroblasts have led to the hypothesis that nonhepatic cells in the body utilize a cell surface receptor to derive cholesterol for membrane synthesis from the major cholesterol-carrying protein in human plasma, low density lipoprotein (LDL)<sup>1</sup> (1). In fibroblasts this receptor shows high affinity and specificity for LDL (2-5). Once bound to the receptor, LDL is internalized and delivered to lysosomes where both its protein and cholesteryl ester components are hydrolyzed by acid hydrolases (3, 6-8). The resultant free cholesterol is then able to leave the lysosome and gain access to the extralysosomal cellular compartment where it is utilized for membrane synthesis (9, 10). The emergence of this LDL-derived cholesterol from the lysosome regulates three events in cholesterol metabolism: (a) it reduces endogenous cholesterol synthesis by suppressing the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA reductase) (11); (b) it stimulates its own reesterification and storage as cholesteryl esters by activating an acyl-CoA:cholesterol acyltransferase (12, 13); and (c) it prevents an excessive uptake of LDL-cholesterol by suppressing the synthesis of the cell surface LDL receptor (14). In addition to cultured fibroblasts, this pathway for LDL metabolism has recently been shown

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<sup>1</sup>Abbreviations used in this paper: DMPA, N,N-dimethyl-1,3-propanediamine; FH, familial hypercholesterolemia; HDL, high density lipoprotein; HMG CoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase; LDL, low density lipoprotein.

**TABLE I**  
*Clinical and Biochemical Data on Patients with Homozygous Familial Hypercholesterolemia*

Patient	Age	Sex	Plasma cholesterol*		Plasma triglycerides*	Phenotype of cultured fibroblasts†	Drug therapy
			Total	LDL			
			mg/dl		mg/dl		
M. C.§	8	F	625	590	112	Receptor-negative	Inderal, nicotinic acid
K. W.	13	F	582	549	81	Receptor-negative	None
D. R.	15	F	545	512	119	Receptor-defective	None

\* Measured by previously described methods (31).

† Determined in cultured fibroblasts by previously described criteria (17, 30).

§ An end-to-side portacaval shunt was performed 19 mo before the current studies (31).

to be present in cultured long-term human lymphoid cells (15, 16).

In both fibroblasts (2–5, 17) and lymphoid cells (16) cultured from patients with the receptor-negative form of homozygous familial hypercholesterolemia (FH), the cell surface LDL receptor is functionally absent. As a result, these mutant cells are unable to bind, internalize, or degrade LDL with high affinity, they cannot utilize the lipoprotein's cholesterol for membrane synthesis, and they are therefore resistant to the regulatory actions of LDL on cholesterol metabolism (1, 17).

The delineation of the LDL pathway in cultured fibroblasts and cultured lymphoid cells depended on the ability to induce maximal synthesis of the LDL receptor by growing the cells in the absence of an exogenous source of cholesterol so that they develop an enhanced requirement for the sterol (1, 14). On the other hand, when fibroblasts and lymphoid cells are exposed continuously to LDL, as when grown in the presence of whole serum, these cells establish a steady state in which they exhibit a low number of LDL receptors that is just sufficient to provide the cells with enough cholesterol to balance cellular losses that occur as a result of membrane turnover (1, 9, 14). In general, this steady-state number of LDL receptors is less than 20% of the maximal number that the cells are capable of synthesizing after they have been deprived of cholesterol for an extended period (14). Since most cells in the body are continuously exposed to LDL and are hence never deprived of cholesterol (18, 19), it seems likely that in the steady state cells in the body will manifest only a small fraction of their maximal number of LDL receptors (1). Consistent with this hypothesis are the experiments of Reichl et al. who were unable to demonstrate high affinity binding, uptake, or degradation of <sup>125</sup>I-LDL in lymphocytes freshly isolated from the blood of normal human subjects (20).

The studies in cultured cells predict, however, that freshly isolated lymphocytes might develop high

affinity LDL receptors if the cells were incubated for a time in the absence of lipoproteins or other exogenous sources of cholesterol. The current studies were designed to test this hypothesis. The results indicate that circulating human lymphocytes obtained from normal subjects do indeed acquire high affinity LDL receptors when incubated in vitro in the absence of lipoproteins. The appearance of these receptors endows the cells with an enhanced ability to degrade LDL with high affinity. In contrast, lymphocytes isolated from FH homozygotes fail to develop significant LDL receptor activity when incubated in the absence of lipoproteins.

## METHODS

**Materials.** <sup>125</sup>I-Sodium iodide (carrier free in 0.05 N NaOH) was purchased from Schwarz Mann Div., Becton, Dickinson and Co., Orangeburg, N. Y. RPMI 1640 medium (no. 187G), Dulbecco's phosphate-buffered saline (no. 419), and Hank's balanced salt solution (no. 402) were purchased from GIBCO, Grand Island, N. Y. Lymphoprep, a mixture of 9.6% (wt/vol) sodium metrizoate and 5.6% (wt/vol) Ficoll (final density, 1.077 g/ml), was obtained from Gallard-Schlesinger Chemical Mfg. Corp., Mineola, N. Y. Sodium heparin (1,000 U/ml) was purchased from Upjohn Co., Kalamazoo, Mich. Erythrosin B was obtained from Fisher Scientific Co., Pittsburgh, Pa. Bovine serum albumin (1x crystallized, no. A 4378) was purchased from Sigma. 400 µl polyethylene microfuge tubes were obtained from Beckman Instruments, Inc., Palo Alto, Calif. Tissue culture flasks and 50-ml plastic conical culture tubes (no. 2070) were obtained from Falcon Plastics, Division of BioQuest, Oxnard, Calif. Reagents for assays were obtained from sources as previously reported (10, 11).

**Subjects.** The age and sex of each of the 15 normal subjects are given in the legends. All normal subjects were healthy individuals who had total plasma cholesterol levels of less than 220 mg/dl and who had no family history of FH. Each of the three patients with the homozygous form of FH had the typical clinical, genetic, and biochemical features of the disorder (21). Their clinical and biochemical data are summarized in Table I. Before obtaining blood for lymphocyte studies, informed written consent was obtained from each subject or from the parents.

**Lipoproteins.** Human LDL (density 1.019–1.063 g/ml), human HDL (density 1.085–1.215 g/ml), and human lipo-

protein-deficient serum (density > 1.215 g/ml) were obtained from the plasma of healthy subjects and prepared by differential ultracentrifugation (11). 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.  $^{125}\text{I}$ -labeled LDL (200–400 cpm/ng of protein) was prepared as previously described (2). The synthesis of cationized  $^{125}\text{I}$ -LDL ( $^{125}\text{I}$ -DMPA-LDL) and of cationized  $^{125}\text{I}$ -albumin ( $^{125}\text{I}$ -DMPA-albumin) was performed as previously described with N,N-dimethyl-1,3-propanediamine (DMPA) as the tertiary amine derivative (22).

**Isolation of lymphocytes.** Peripheral blood lymphocytes were isolated under sterile conditions at room temperature by a modification of the method of Boyüm (23). Venous blood from each subject (20–100 ml) was obtained after an overnight fast and was drawn into a heparin solution (100 U heparin/10 ml blood). The blood was immediately diluted with an equal volume of 0.15 M NaCl, and each 20 ml of the resulting blood-NaCl mixture was layered onto 10 ml of Lymphoprep contained within one 50 ml plastic conical tube. The tubes were centrifuged at 1,400 rpm for 40 min. The interphase material containing the mononuclear cells (6–8 ml/tube) was collected with a Pasteur pipette, mixed with 30 ml of Hank's balanced salt solution, and centrifuged at 1,400 rpm for 10 min. The resulting cell pellet from each tube was resuspended in 20 ml of medium A (RPMI 1640 medium containing penicillin [100 U/ml] and streptomycin [100 µg/ml]) and washed by centrifugation at 1,000 rpm for 10 min. Each cell pellet was again resuspended in 20 ml of medium A, and washed a second time. The washed cell pellets from two tubes, containing about 20–30 × 10<sup>6</sup> lymphocytes and representing the mononuclear cells contained in 20 ml of venous blood, were resuspended in 12 ml of medium A containing 10% (vol/vol) human lipoprotein-deficient serum and placed in a 250-ml plastic culture flask. These stock flasks were maintained in a humidified incubator (5% CO<sub>2</sub>) at 37°C. Aliquots were removed from the stock flasks either at zero time or after incubation for the indicated intervals and used for the assays described below.

Cell viability, as assessed by erythrosin B exclusion, was routinely determined for each lymphocyte preparation and was always greater than 90% both before and after incubation for 72 h in both the normal and FH homozygote cells. Purity of the cell preparations was assessed after staining smears with Wright-Giemsa. Differential counts revealed that 90–95% of the cells that had been incubated for 72 h were morphologically identifiable as lymphocytes. Only an occasional platelet was seen on examination of the smears.

**Binding of  $^{125}\text{I}$ -LDL by intact lymphocytes at 4°C.** Lymphocytes from the stock flasks were harvested by centrifugation (1,200 rpm, 10 min, 4°C), washed once in 6 ml of buffer B (Dulbecco's phosphate-buffered saline containing 50 µM CaCl<sub>2</sub> and 20 mg/ml bovine serum albumin), and resuspended in ice-cold buffer B at a final concentration of about 50 × 10<sup>6</sup> cells/ml. Aliquots of this cell suspension (75 µl containing about 4 × 10<sup>6</sup> cells and 160–280 µg total cell protein) were placed in a 400 µl polyethylene microfuge tube in a final volume of 100 µl of buffer B. The indicated concentration of freshly filtered  $^{125}\text{I}$ -LDL (16) was added in the presence or absence of excess unlabeled LDL, and the cells were incubated for 30 min at 4°C, after which the cells were collected by centrifugation, washed as previously described for cultured human long-term lymphoid cells, and the total amount of  $^{125}\text{I}$ -LDL bound to the cells was determined (16). All of the above additions

and centrifugations were carried out in a 4°C cold room. Total binding of  $^{125}\text{I}$ -LDL represents the amount of  $^{125}\text{I}$ -LDL bound to the cells in the absence of excess unlabeled LDL. High affinity binding is that component of the total that was inhibited competitively by the presence of an excess of unlabeled LDL.

**Cellular accumulation and proteolytic degradation of  $^{125}\text{I}$ -LDL by intact lymphocytes at 37°C.** Aliquots of lymphocytes from the stock flasks (2 ml of medium containing about 4 × 10<sup>6</sup> cells) were transferred to 30-ml flasks.  $^{125}\text{I}$ -LDL was added in the presence or absence of excess unlabeled LDL and the cells were incubated at 37°C for the indicated interval, after which the cells and medium were separated by centrifugation (1700 rpm, 5 min, 4°C). The medium was then treated with trichloroacetic acid to a final concentration of 10% (vol/vol), after which the precipitate was removed by centrifugation and the supernate was treated with hydrogen peroxide and extracted with chloroform to remove free iodine (24). An aliquot of the aqueous phase was counted to determine the amount of  $^{125}\text{I}$ -labeled acid-soluble material formed by the cells and released to the medium (3). Degradation activity is expressed as nanograms of  $^{125}\text{I}$ -LDL protein degraded to acid-soluble material per milligram of cell protein. A blank value due to the presence of small amounts of acid-soluble material (<0.1% of total added radioactivity) in the  $^{125}\text{I}$ -LDL preparation was routinely determined at the appropriate concentration of the lipoprotein by incubation at 37°C in medium containing no cells (3, 16). Total degradation of  $^{125}\text{I}$ -LDL represents the amount of  $^{125}\text{I}$ -LDL degraded in the absence of excess unlabeled LDL. High affinity degradation is that component of the total that was inhibited competitively by the presence of an excess of unlabeled LDL. The total cellular content of  $^{125}\text{I}$ -LDL (i.e., the  $^{125}\text{I}$ -LDL contained both at the receptor site on the cell surface and within the cell) was determined from the cell pellet with the washing procedure previously described for cultured human long-term lymphoid cells (16). The cellular content of  $^{125}\text{I}$ -LDL is expressed as the nanograms of  $^{125}\text{I}$ -LDL protein bound to the cell per milligram of total cell protein.

**Other assay.** The content of protein was determined by a modification of the method of Lowry et al. (25), by using bovine serum albumin as a standard.

## RESULTS

To assay for high affinity cell surface LDL binding activity in human lymphocytes, the cells were incubated at 4°C with low concentrations of  $^{125}\text{I}$ -LDL either in the absence or presence of a 25-fold excess of unlabeled LDL. The amount of  $^{125}\text{I}$ -LDL bound to the cell membrane was then measured by using a washing technique that was previously developed for assay of  $^{125}\text{I}$ -LDL binding in human lymphoid cells maintained in long-term culture (16). In this type of competition assay, the high affinity binding is defined as the amount of  $^{125}\text{I}$ -LDL that is prevented from binding to the cell membrane by the excess of unlabeled LDL (i.e., the difference between the  $^{125}\text{I}$ -LDL binding obtained in the absence and presence of unlabeled LDL). When freshly isolated human lymphocytes obtained from a normal subject were incubated with  $^{125}\text{I}$ -LDL at 4°C, relatively little high affinity binding was observed (Fig. 1, zero time).

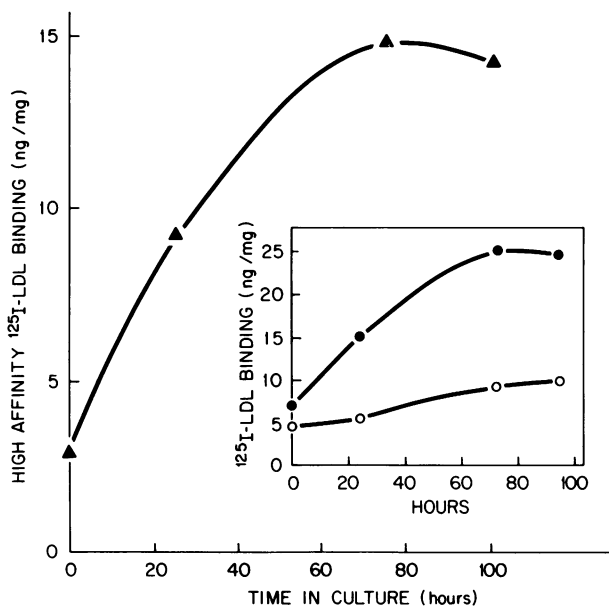


FIGURE 1 Increase in high affinity binding activity for  $^{125}\text{I}$ -LDL in normal lymphocytes during incubation in the absence of lipoproteins. Lymphocytes were isolated from 80 ml of venous blood obtained from a healthy 24 yr-old male (T.B.), and four replicate stock flasks were prepared as described in Methods. The cells were incubated at  $37^\circ\text{C}$  in medium A containing 10% lipoprotein-deficient serum. After the indicated interval, the lymphocytes from one stock flask were harvested, washed, and resuspended in ice-cold buffer B for assay of  $^{125}\text{I}$ -LDL binding as described in Methods. Each binding assay was conducted in  $100\ \mu\text{l}$  of buffer B containing about  $4 \times 10^6$  intact cells ( $160\text{--}220\ \mu\text{g}$  of total cell protein),  $10\ \mu\text{g}$  protein/ml of  $^{125}\text{I}$ -LDL ( $188\ \text{cpm/ng}$ ) either in the presence (○) or absence (●) of  $250\ \mu\text{g}$  protein/ml of unlabeled LDL. The cells were incubated at  $4^\circ\text{C}$  for 30 min, after which the total amount of  $^{125}\text{I}$ -LDL bound to the cells (●,○) was determined as described under Methods. Each value represents the mean of triplicate assays. The values for high affinity  $^{125}\text{I}$ -LDL binding (▲) represent the difference between the values for  $^{125}\text{I}$ -LDL bound in the absence (●) and presence (○) of unlabeled LDL.

However, incubation of such lymphocytes at  $37^\circ\text{C}$  in tissue culture medium deficient in lipoproteins led to a progressive increase in their ability to bind  $^{125}\text{I}$ -LDL as determined by repeated binding assays over a 96-h interval. An identical pattern of appearance of high affinity  $^{125}\text{I}$ -LDL binding activity was obtained when the experiment in Fig. 1 was repeated by using lymphocytes isolated from a different normal subject (E.S., a 29-yr-old female). During the 96-h incubation, the number of lymphocytes in each flask remained constant, and there was no change in the morphologic appearance of the cells.

Fig. 2 shows that the high affinity binding activity that developed in normal lymphocytes after incubation for 72 h in medium devoid of lipoprotein was relatively specific for LDL. Thus, when pre-

incubated lymphocytes were incubated with  $^{125}\text{I}$ -LDL at a concentration of  $15\ \mu\text{g}$  protein/ml at  $4^\circ\text{C}$  in the presence of increasing concentrations of unlabeled LDL, a 50% reduction in the binding of  $^{125}\text{I}$ -LDL occurred at a concentration of  $15\ \mu\text{g}$  protein/ml of unlabeled LDL. On the other hand, HDL was much less effective in competitively inhibiting the binding of  $^{125}\text{I}$ -LDL to the lymphocyte membrane, 50% inhibition requiring a concentration of more than  $240\ \mu\text{g}$  protein/ml of HDL. Further evidence for the specificity of the LDL binding was the fact that  $^{125}\text{I}$ -LDL binding occurred when the lipoprotein was present at only  $15\ \mu\text{g}$  protein/ml even though a 15,000-fold molar excess of bovine serum albumin ( $20\ \text{mg/ml}$ ) was present in the incubation medium.

Previous studies have demonstrated that binding of LDL to its high affinity receptor in cultured human fibroblasts (3, 4) and in cultured human lymphoid cells (16) leads to internalization and degradation of the lipoprotein. Fig. 3 shows that freshly isolated lymphocytes obtained from a normal subject, which exhibited a relatively low number of high affinity LDL binding sites (Fig. 1, zero time), also exhibited a relatively low ability to degrade  $^{125}\text{I}$ -LDL. How-

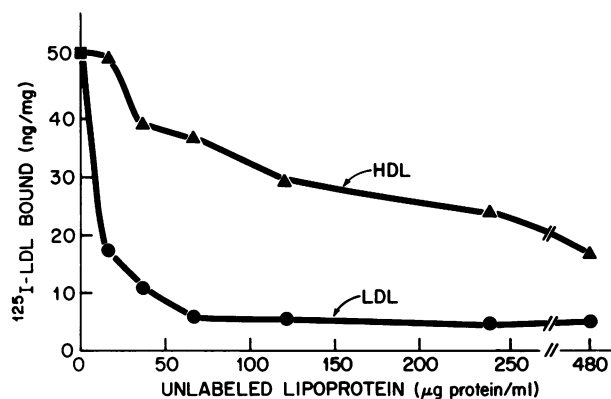
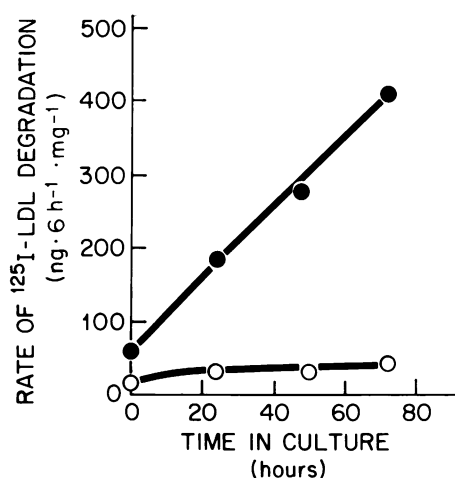


FIGURE 2 Effect of unlabeled plasma lipoproteins on the binding of  $^{125}\text{I}$ -LDL to normal lymphocytes at  $4^\circ\text{C}$ . Lymphocytes were isolated from 60 ml of venous blood obtained from a healthy 25 yr-old male (V.C.), and replicate stock flasks were prepared as described in Methods. The cells were incubated for 72 h at  $37^\circ\text{C}$  in medium A containing 10% lipoprotein-deficient serum, after which the cells from all the flasks were combined and the lymphocytes were harvested, washed, and resuspended in ice-cold buffer B for assay of  $^{125}\text{I}$ -LDL binding as described in Methods. Each binding assay was conducted in  $100\ \mu\text{l}$  of buffer B containing about  $4 \times 10^6$  intact cells ( $200\text{--}220\ \mu\text{g}$  of total cell protein),  $15\ \mu\text{g}$  protein/ml of  $^{125}\text{I}$ -LDL ( $163\ \text{cpm/ng}$ ), and the indicated concentration of either unlabeled HDL (▲) or unlabeled LDL (●). The cells were incubated at  $4^\circ\text{C}$  for 30 min, after which the total amount of  $^{125}\text{I}$ -LDL bound to the cells was determined as described in Methods. The value for  $^{125}\text{I}$ -LDL binding when no unlabeled lipoprotein was added (■) represents the mean of triplicate assays. All other values represent single assays.

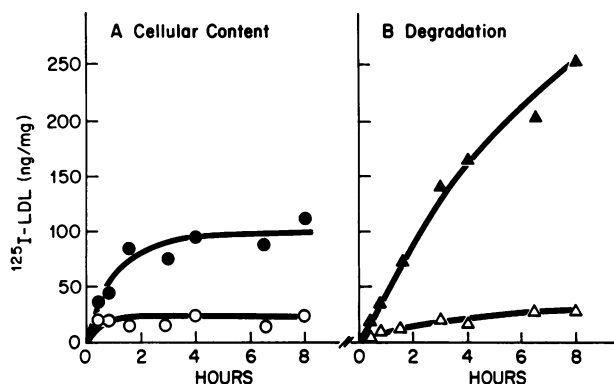
ever, during incubation at 37°C for 72 h in medium devoid of lipoproteins, when the cells were acquiring increased high affinity LDL binding activity (Fig. 1), they also were acquiring an increased ability to degrade  $^{125}\text{I}$ -LDL (Fig. 3).

Fig. 4 shows the time course of metabolism of  $^{125}\text{I}$ -LDL at 37°C by the high affinity process in normal lymphocytes that had been incubated for 72 h in medium devoid of lipoproteins. As previously observed for cultured human fibroblasts (3, 4) and cultured human lymphoid cells (16), the cellular content of  $^{125}\text{I}$ -LDL rose and reached a steady-state plateau at about 2 h (Fig. 4A), while the rate of degradation of  $^{125}\text{I}$ -LDL continued in a nearly linear fashion for 8 h (Fig. 4B). In the presence of excess unlabeled LDL, both the uptake of  $^{125}\text{I}$ -LDL and its rate of degradation were reduced proportionately.

The saturation curve for  $^{125}\text{I}$ -LDL degradation is shown in Fig. 5. As previously observed in cultured fibroblasts (3, 7), the LDL concentration curve in normal lymphocytes consisted of two components, a high affinity component that reached saturation at an LDL concentration of about 50  $\mu\text{g}$  protein/ml and



**FIGURE 3** Increase in the ability of normal lymphocytes to degrade  $^{125}\text{I}$ -LDL after incubation in the absence of lipoproteins. Lymphocytes were isolated from 80 ml of venous blood obtained from a healthy 32 yr-old female (V. J.), and four replicate stock flasks were prepared as described in Methods. The cells were incubated at 37°C in medium A containing 10% lipoprotein-deficient serum. At each indicated interval, the cells and medium from one flask were divided into six 2-ml aliquots. Each 2 ml aliquot was transferred to a 30-ml flask, after which was added 10  $\mu\text{g}$  protein/ml of  $^{125}\text{I}$ -LDL (265 cpm/ng) either in the presence (○) or absence (●) of 250  $\mu\text{g}$  protein/ml of unlabeled LDL. The flasks were incubated at 37°C for 6 h, after which the cells were removed by centrifugation and the content of  $^{125}\text{I}$ -labeled acid-soluble material in the medium was determined as described under Methods. The content of cellular protein in each flask averaged 160  $\mu\text{g}$ . Each value represents the mean of triplicate incubations.



**FIGURE 4** Time course of metabolism of  $^{125}\text{I}$ -LDL at 37°C in normal lymphocytes subjected to prior incubation in the absence of lipoproteins. Lymphocytes were isolated from 100 ml of venous blood obtained from a healthy 25 yr-old male (J. L.), and replicate stock flasks were prepared as described in Methods. After the cells had incubated at 37°C for 72 h in medium A containing 10% lipoprotein-deficient serum, the cells from all of the flasks were combined and divided into 21 2-ml aliquots. Each 2 ml aliquot was transferred to a 30-ml flask, after which was added 10  $\mu\text{g}$  protein/ml of  $^{125}\text{I}$ -LDL (189 cpm/ng) either in the presence (○, △) or absence (●, ▲) of 250  $\mu\text{g}$  protein/ml of unlabeled LDL. After incubation at 37°C for the indicated time, the medium and cells from each flask were separated by centrifugation, after which the content of  $^{125}\text{I}$ -labeled acid-soluble material in the medium (▲, △) and the cellular content of  $^{125}\text{I}$ -LDL (●, ○) were determined as described under Methods. The content of cellular protein in each flask averaged 280  $\mu\text{g}$ . Each value for the closed symbols (●, ▲) represents the average of duplicate incubations. Each value for the open symbols (○, △) represent single incubations.

an apparently nonsaturable component that caused the rate of degradation to increase slightly in a linear fashion at LDL concentrations above 50  $\mu\text{g}$  protein/ml. As with high affinity  $^{125}\text{I}$ -LDL binding at 4°C (Fig. 2), the rate of degradation of  $^{125}\text{I}$ -LDL at 37°C was competitively inhibited by LDL, whereas HDL was much less effective (Fig. 6).

Considered together, the data in Figs. 1–6 suggest that circulating normal lymphocytes incubated in the absence of lipoproteins acquire an increased number of specific, high affinity LDL receptors and that these receptors facilitate the uptake and degradation of LDL in a manner similar to that previously observed in cultured human fibroblasts and cultured human lymphoid cells. In human fibroblasts the induction of LDL receptors has been shown to be a consequence of the removal of the cells from an exogenous source of cellular cholesterol (14). That a similar mechanism was also responsible for the induction of LDL receptors in circulating lymphocytes is indicated by the data in Table II. Inclusion of LDL in the medium during the 72-h incubation prevented the induction of the enhanced receptor-mediated degradation of  $^{125}\text{I}$ -LDL (exp. A and B). This induction was also

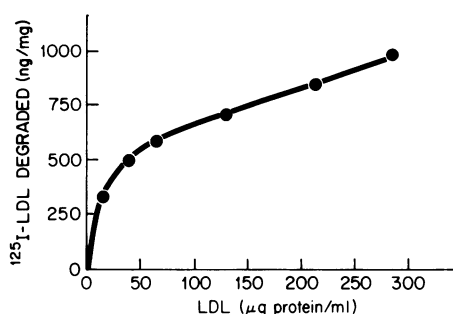


FIGURE 5 Rate of degradation of  $^{125}\text{I}$ -LDL in normal lymphocytes as a function of the  $^{125}\text{I}$ -LDL concentration. Lymphocytes were isolated from 40 ml of venous blood obtained from a healthy 27 yr-old male (F.C.), and replicate stock flasks were prepared as described in Methods. After the cells had incubated for 72 h in medium A containing 10% lipoprotein-deficient serum, the cells from all of the flasks were combined and divided into 12 2-ml aliquots. Each 2 ml aliquot was transferred to a 30-ml flask, after which was added the indicated concentration of  $^{125}\text{I}$ -LDL (392 cpm/ng). After incubation at  $37^\circ\text{C}$  for 6 h, the cells were removed from each flask by centrifugation, after which the content of  $^{125}\text{I}$ -labeled acid-soluble material in the medium was determined as described under Methods. The content of cellular protein in each flask averaged 180  $\mu\text{g}$ . Each value represents the average of duplicate incubations.

prevented by inclusion in the medium of a mixture of 25-hydroxycholesterol and cholesterol (exp. A), a mixture that prevents the induction of LDL receptor activity in human fibroblasts (14). On the other hand, incubation of lymphocytes with HDL at a protein concentration of 100  $\mu\text{g}/\text{ml}$  did not prevent the induction of LDL receptor activity (exp. B).

To test the specificity of the effect of LDL and the mixture of sterols in preventing the enhancement of the receptor-mediated degradation of  $^{125}\text{I}$ -LDL, the ability of the incubated lymphocytes to degrade  $^{125}\text{I}$ -DMPA-albumin was examined. We have previously shown that in fibroblasts  $^{125}\text{I}$ -DMPA-albumin, which has a strong positive charge, binds avidly to negatively-charged sites on the cell surface and is then taken up by cells and degraded in lysosomes (22). Thus,  $^{125}\text{I}$ -DMPA-albumin serves as a good marker for the overall rate of cellular endocytosis and lysosomal proteolysis.<sup>2</sup> As shown in Table II, neither LDL itself nor the mixture of 25-hydroxycholesterol and cholesterol suppressed the degradation of  $^{125}\text{I}$ -DMPA-albumin in incubated lymphocytes under conditions in which the degradation of  $^{125}\text{I}$ -LDL was reduced by more than 10-fold.

In other experiments not shown, normal lymphocytes that had been incubated for 72 h in the absence of lipoproteins degraded  $^{125}\text{I}$ -LDL at a rate that was six-fold faster than that observed in freshly isolated, unin-

cubated cells. In the same cells, however, there was no increase in the rate of degradation of  $^{125}\text{I}$ -DMPA-LDL, a macromolecule that, like  $^{125}\text{I}$ -DMPA-albumin, gains entry to cellular lysosomes by binding non-specifically to negatively-charged sites on the cell surface rather than by binding to the LDL receptor (22).<sup>2</sup>

To determine whether the LDL receptor that appeared in freshly isolated lymphocytes was genetically the same as the LDL receptor previously characterized in cultured fibroblasts and long-term lymphoid cells, we compared the appearance of LDL receptor activity in cells from normal subjects and from patients with the homozygous form of FH. Table III shows that at zero time the total amount of  $^{125}\text{I}$ -LDL binding at  $4^\circ\text{C}$  in lymphocytes from four normal subjects was low and there was little to no competition when an excess of unlabeled LDL was present, indicating that very little, if any, of the observed binding was due to the high affinity LDL receptor. In contrast, when normal lymphocytes were incubated for 72 h in the absence of lipoproteins and then incubated with  $^{125}\text{I}$ -LDL at 10  $\mu\text{g}$  protein/ml, the mean

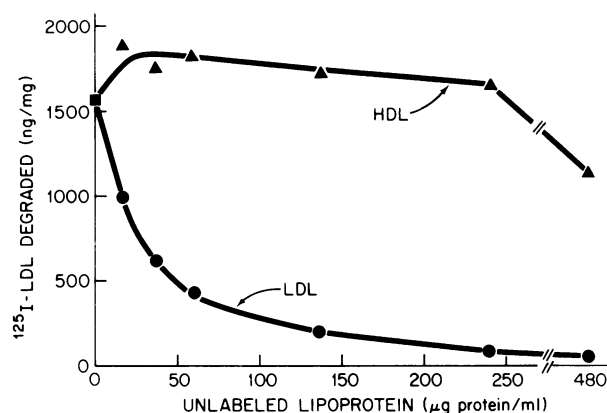


FIGURE 6 Effect of unlabeled plasma lipoproteins on the degradation of  $^{125}\text{I}$ -LDL in normal lymphocytes. Lymphocytes were isolated from 40 ml of venous blood obtained from a healthy 25 yr-old male (V. C.), and two replicate stock flasks were prepared as described in Methods. After the cells had incubated for 72 h at  $37^\circ\text{C}$  in medium A containing 10% lipoprotein-deficient serum, the medium and cells from the two flasks were combined in a final volume of 34 ml of medium A containing 10% human lipoprotein-deficient serum. The resulting mixture was then divided into 17 2-ml aliquots. Each 2 ml aliquot was transferred to a 30-ml flask, after which was added 15  $\mu\text{g}$  protein/ml of  $^{125}\text{I}$ -LDL (218 cpm/ng) and the indicated concentration of either unlabeled HDL ( $\blacktriangle$ ) or unlabeled LDL ( $\bullet$ ). After incubation for 6 h at  $37^\circ\text{C}$ , the cells from each flask were removed by centrifugation, and the content of  $^{125}\text{I}$ -labeled acid-soluble material in the medium was determined as described under Methods. The content of cellular protein in each flask averaged 110  $\mu\text{g}$ . The value for  $^{125}\text{I}$ -LDL degradation when no unlabeled lipoprotein was added ( $\blacksquare$ ) represents the mean of triplicate incubations. All other values represent single incubations.

<sup>2</sup> Basu, S. K., R. G. W. Anderson, J. L. Goldstein, and M. S. Brown. 1976. Manuscript to be submitted for publication.

**TABLE II**  
*Prevention of the Appearance of Enhanced Receptor-Mediated Degradation of <sup>125</sup>I-LDL in Normal Lymphocytes by Inclusion of LDL or Sterols in the Prior Incubation Medium*

Prior treatment of cells	<sup>125</sup> I-LDL degraded		<sup>125</sup> I-DMPA-albumin degraded
	Total	High affinity	
	ng · 6 h <sup>-1</sup> · mg protein <sup>-1</sup>		ng · 6 h <sup>-1</sup> · mg protein <sup>-1</sup>
<i>Exp. A</i>			
LPDS*	506	498	4,540
LPDS + LDL	6.0	4.9	3,900
LPDS + 25-Hydroxy-cholesterol + Cholesterol	2.0	0.9	4,450
<i>Exp. B</i>			
LPDS*	505	465	4,940
LPDS + LDL	35	34	5,760
LPDS + HDL	1,025	979	4,270

Lymphocytes were isolated from 100 ml of venous blood obtained from a healthy 27-yr-old male (F. C.) and from a healthy 23-yr-old female (J. F.) for Exp. A and B, respectively. For each experiment, three replicate stock flasks were prepared as described in Methods except that each stock flask received cells isolated from 33 ml of venous blood. Each stock flask contained 12 ml of medium A containing 10% lipoprotein-deficient serum plus one of the indicated additions (Exp. A: none, 100 µg protein/ml of unlabeled LDL, or 1 µg/ml of 25-hydroxycholesterol plus 16 µg/ml of cholesterol added in 35 µl of ethanol; Exp. B: none, 50 µg protein/ml of unlabeled LDL, or 100 µg protein/ml of unlabeled HDL). After incubation at 37°C for 72 h, the cells from each flask were harvested by centrifugation (1,000 rpm, 10 min, 24°C), washed in 15 ml of medium A containing 10% human lipoprotein-deficient serum, and recentrifuged. Each cell pellet was then resuspended in 12.5 ml of medium A containing 10% human lipoprotein-deficient serum and the resulting suspension of cells was equally divided into six 2-ml aliquots. Each 2 ml aliquot was transferred to a 30-ml flask, after which was added either 7.3 µg/ml of <sup>125</sup>I-DMPA-albumin (Exp. A, 664 cpm/ng; Exp. B, 559 cpm/ng) or 10 µg protein/ml of <sup>125</sup>I-LDL (Exp. A, 369 cpm/ng; Exp. B, 178 cpm/ng) in the presence or absence of 250 µg protein/ml of unlabeled LDL. After incubation at 37°C for 6 h, the medium and cells from each flask were separated, after which the content of <sup>125</sup>I-labeled acid-soluble material in the medium was determined as described under Methods. Total and high affinity degradation of <sup>125</sup>I-LDL were determined as described under Methods. The content of cellular protein averaged 180 µg/flask (Exp. A) and 120 µg/flask (Exp. B). Each value represents the average of duplicate incubations.

\* LPDS, lipoprotein-deficient serum.

total binding had risen to 20 ng/mg protein. Of this amount, a mean of 14 ng/mg was excluded from the cell surface when the binding assay was carried out in the presence of 250 µg protein/ml of unlabeled

**TABLE III**  
*<sup>125</sup>I-LDL Binding at 4°C in Freshly Isolated Lymphocytes and in Lymphocytes that Had Undergone Prior Incubation in the Absence of Lipoproteins*

Subject providing cells	Duration of prior incubation in absence of lipoproteins	<sup>125</sup> I-LDL Binding	
		Total	High affinity
<i>h</i>		<i>ng/mg protein</i>	
<i>Normal subjects</i>			
J. W. (12 M)	0	3.7	0.2
T. B. (24 M)	0	4.6	0.0
V. C. (25 M)	0	5.3	1.7
J. L. (25 M)	0	7.7	0.0
		Mean	5.3      0.5
D. C. (11 M)	72	13	10
M. S. (24 F)	72	34	24
M. E. (30 F)	72	21	18
F. C. (27 M)	72	21	10
D. B. (35 M)	72	13	10
		Mean	20      14
<i>FH Homozygotes</i>			
M.C. (8 F)	72	3.0	0.9
D. R. (15 F)	72	4.8	1.0
		Mean	3.9      1.0

Lymphocytes were isolated from 20 ml of venous blood obtained from each of the indicated subjects, and a single stock flask was prepared as described in Methods. After incubation at 37°C in medium A containing 10% lipoprotein-deficient serum for the indicated time, the lymphocytes were harvested, washed, and resuspended in ice-cold buffer B as described in Methods. Each binding assay was conducted in 100 µl of buffer B containing 3–4 × 10<sup>6</sup> intact cells (160–280 µg of total cell protein) and 10 µg protein/ml of <sup>125</sup>I-LDL (200–223 cpm/ng) either in the presence or absence of 250 µg protein/ml of unlabeled LDL. The cells were incubated at 4°C for 30 min, after which the total amount of <sup>125</sup>I-LDL bound to the cells was determined as described in Methods. Total and high affinity binding of <sup>125</sup>I-LDL were determined as described under Methods. Each value represents the mean of triplicate (– unlabeled LDL) or duplicate (+ unlabeled LDL) assays.

LDL and thus represented binding to the high affinity saturable LDL receptor site. In cells from two FH homozygotes incubated for 72 h in the absence of lipoproteins, the high affinity component of the total <sup>125</sup>I-LDL binding was 1.0 ng/mg. This 93% reduction in high affinity LDL binding activity at 4°C in the lymphocytes of FH homozygotes was associated with a 96% reduction in the rate of high affinity degradation of <sup>125</sup>I-LDL at 37°C when the cells from three FH homozygotes were compared with those from seven normal subjects after incubation for 72 h in the absence of lipoproteins (Table IV).



**TABLE IV**  
*Degradation of  $^{125}\text{I}$ -LDL at 37°C in Freshly Isolated Lymphocytes and in Lymphocytes That Had Undergone Prior Incubation in the Absence of Lipoproteins*

Subject providing cells	Duration of prior incubation in absence of lipoproteins  <i>h</i>	<sup>125</sup> I-LDL Degraded	
		Total	High affinity
		<i>ng·6 h<sup>-1</sup>·mg protein<sup>-1</sup></i>	
<i>Normal subjects</i>			
J. W. (12 M)	0	40	34
T. B. (24 M)	0	36	35
V. C. (25 M)	0	56	52
		Mean	44      40
D. C. (11 M)	72	403	348
J. W. (12 M)	72	496	460
M. S. (24 F)	72	1295	1203
M. E. (30 F)	72	678	613
F. C. (27 M)	72	749	694
S. B. (32 M)	72	624	583
D. B. (35 M)	72	591	515
		Mean	691      631
<i>FH Homozygotes</i>			
M. C. (8 F)	72	14	12
K. W. (13 F)	72	18	13
D. R. (15 F)	72	73	49
		Mean	35      25

Lymphocytes were isolated from 20 ml of venous blood obtained from each of the indicated subjects, and a single stock flask was prepared as described in Methods. After incubation at 37°C in medium A containing 10% lipoprotein-deficient serum for the indicated time, the medium and cells from each flask were divided into five 2-ml aliquots. Each 2 ml aliquot was transferred to a 30-ml flask, after which was added 10  $\mu\text{g}$  protein/ml of  $^{125}\text{I}$ -LDL (150–223 cpm/ng) either in the presence or absence of 250  $\mu\text{g}$  protein/ml of unlabeled LDL. After incubation for 6 h at 37°C, the medium and cells from each flask were separated by centrifugation, after which the content of  $^{125}\text{I}$ -LDL-labeled acid-soluble material in the medium was determined as described under Methods. Total and high affinity degradation of  $^{125}\text{I}$ -LDL were determined as described under Methods. Each value represents the mean of triplicate (– unlabeled LDL) or duplicate (+ unlabeled LDL) incubations. The content of cellular protein in each flask averaged 250  $\mu\text{g}$ , varying among subjects from 160 to 330  $\mu\text{g}$ /flask.

## DISCUSSION

In the present studies normal lymphocytes were found to possess only a small number of high affinity LDL receptors immediately after they were isolated from the blood stream and hence these cells exhibited a low capacity to take up and degrade  $^{125}\text{I}$ -LDL with high affinity. However, when such cells were incu-

bated in vitro in the absence of an exogenous source of cholesterol, they developed an enhanced number of LDL receptors and thus acquired an increased ability to take up and degrade the lipoprotein with high affinity.

The LDL receptors that were expressed on cholesterol-deprived lymphocytes resembled the previously characterized LDL receptors of cholesterol-deprived cultured human fibroblasts (2–4, 14) and lymphoid cells (16) in the following ways. First, the LDL receptors of the incubated human lymphocytes showed a marked preference for binding LDL as opposed to HDL or other plasma proteins (such as albumin). Second, the binding of  $^{125}\text{I}$ -LDL achieved saturation at LDL concentrations below 50  $\mu\text{g}$  protein/ml. Third, the increase in high affinity binding was temporally coupled to an increase in the high affinity processes for the internalization and proteolytic degradation of the lipoprotein. Fourth, the increase in high affinity LDL degradation in circulating lymphocytes was prevented by exposure of the cells to either LDL or a mixture of 25-hydroxycholesterol plus cholesterol, indicating that it was subject to metabolic regulation. Fifth, LDL receptor activity, as reflected by measurement of either the binding or degradation of  $^{125}\text{I}$ -LDL, was not expressed in lymphocytes from FH homozygotes.

Two lines of evidence excluded the possibility that the low number of LDL receptors in freshly isolated lymphocytes was a result of damage to the cells during their isolation. First, the isolation procedure did not reduce the ability of the cells to degrade cationized LDL (DMPA-LDL). And second, the appearance of receptor sites could be specifically blocked by inclusion of low levels of LDL in the 72-h incubation mixture. It was also possible, at least in theory, that the low level of receptor activity in the freshly isolated lymphocytes was due to occupancy of the receptor sites by LDL to which the cells had been exposed in the blood stream. However, we have previously shown in both cultured human fibroblasts and lymphoid cells that all receptor-bound LDL is internalized within 7 min at 37°C and that this internalization leads to the regeneration of the original number of unoccupied receptor sites (3, 4, 14, 16). In the present studies the degradation experiments were carried out for 6 h at 37°C, an interval that is far in excess of the time required for prebound plasma LDL to be cleared from the receptor sites. This conclusion is supported by the data in Tables III and IV. In the normal cells that had been incubated for 72 h in the absence of lipoproteins, the rate of high affinity degradation of  $^{125}\text{I}$ -LDL was 631  $\text{ng} \cdot \text{mg}^{-1} \cdot 6 \text{ h}^{-1}$ , which is equal to 1.75  $\text{ng} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$  (Table IV). Under these conditions the surface binding of  $^{125}\text{I}$ -LDL was 14 ng/mg (Table III). Thus, in the steady-

state an amount of  $^{125}\text{I}$ -LDL equal to the amount bound to the receptor was degraded every  $14 \div 1.75 = 8.0$  min. This figure is in close agreement with the figure of 6.25 min for the turnover of receptor-bound LDL in fibroblasts (4).

A point worthy of discussion is the difference in sensitivity between the two assays used to measure the function of the LDL receptor in lymphocytes, namely the measurement of high affinity binding at  $4^\circ\text{C}$  and the measurement of the rate of high affinity degradation at  $37^\circ\text{C}$ . The binding assay is stoichiometric. That is, the number of particles of  $^{125}\text{I}$ -LDL bound to the cells is equal to the number of LDL receptors. On the other hand, the degradation assay is equivalent to a catalytic assay for the LDL receptor. That is, the number of  $^{125}\text{I}$ -LDL particles degraded is equal to many times the number of receptor molecules since each receptor internalizes many particles of LDL during the usual 6 h incubation. Because of this amplification effect, the assay for high affinity degradation gives values that are many fold higher than the background. On the other hand, the assay for high affinity surface binding is less discriminative at low receptor levels. Thus, as shown by the data in Table IV, in the freshly isolated lymphocytes obtained from normal subjects the degradation assay was sensitive enough to reveal detectable amounts of high affinity  $^{125}\text{I}$ -LDL degradation, a finding that implies the presence of small amounts of high affinity  $^{125}\text{I}$ -LDL binding and uptake. However, as shown by the data in Table III, this amount of receptor activity in nonincubated cells was not sufficiently large to be reliably distinguished from the blank value when  $^{125}\text{I}$ -LDL binding was measured directly at  $4^\circ\text{C}$ .

The current data indicate that at least one type of cell in the human body, the circulating lymphocyte, has the capacity to produce a high affinity LDL receptor that enables the cell to take up and degrade  $^{125}\text{I}$ -LDL. The data further indicate that this capacity is only partially expressed in the steady state in vivo. Since suppression of LDL receptor activity can be maintained in vitro by a continuous exposure of the lymphocytes to LDL and since in vivo lymphocytes are normally exposed to a high level of LDL in the circulation, it is reasonable to suppose that the suppression of LDL receptor activity in vivo is due to the presence of plasma LDL. By analogy to the situation in cultured human fibroblasts (1, 14), it seems likely that the small number of LDL receptors that are normally present on circulating lymphocytes are sufficient to supply all of the cholesterol requirements of these cells so long as they are continuously exposed to LDL. It is only after lymphocytes are deprived of cholesterol that they develop a sterol deficit and hence begin to synthesize high levels of LDL receptors.

The demonstration that the LDL receptor in freshly

isolated lymphocytes does in fact function to regulate cellular cholesterol homeostasis as in cultured human fibroblasts and long-term lymphoid cells will require further studies relating LDL receptor activity to the processes of cholesterol synthesis and cholesteryl ester formation in these cells. That such a correlation may indeed exist is suggested by studies of Fogelman et al. (26, 27) Higgins et al. (28), and Williams and Avigan (29), who have shown that mixed human leukocytes develop an enhanced rate of cholesterol synthesis when incubated in vitro in the absence of lipoproteins.

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