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Research Article

Human low density lipoprotein (LDL, $d = 1.020-1.050$ g/ml) inhibits mitogen-stimulated T lymphocyte DNA synthesis. Because both LDL and transferrin bind to specific cell surface receptors and enter cells by the similar means of receptor-mediated endocytosis, and because transferrin is necessary for lymphocyte DNA synthesis, we investigated the possibility that LDL may inhibit mitogen-stimulated lymphocyte responses by interfering with transferrin metabolism. LDL inhibited mitogen-stimulated lymphocyte [^3H]thymidine incorporation in a concentration-dependent manner. The degree of inhibition was most marked in serum-free cultures, but was also observed in serum-containing cultures. The addition of transferrin not only augmented mitogen-induced lymphocyte [^3H]thymidine incorporation in serum-free medium but also completely reversed the inhibitory effect of LDL in both serum-free and serum-containing media. Similar results were obtained when lymphocyte proliferation was assayed by counting the number of cells in culture. Transferrin also reversed the inhibition of lymphocyte responses caused by very low density lipoproteins and by cholesterol. The ability of transferrin to reverse the inhibitory effect of lipoproteins was specific, in that native but not denatured transferrin was effective whereas a variety of other proteins were ineffective. These results indicate that LDL inhibits mitogen-stimulated lymphocyte responses by interfering with transferrin metabolism. LDL only inhibited lymphocyte responses after a 48-h incubation if present from the initiation of the culture. By contrast, transferrin reversed inhibition when added after [...]

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Immunoregulation by Low Density Lipoproteins in Man

Inhibition of Mitogen-induced T Lymphocyte Proliferation by Interference with Transferrin Metabolism

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Abstract. Human low density lipoprotein (LDL, $d = 1.020-1.050$ g/ml) inhibits mitogen-stimulated T lymphocyte DNA synthesis. Because both LDL and transferrin bind to specific cell surface receptors and enter cells by the similar means of receptor-mediated endocytosis, and because transferrin is necessary for lymphocyte DNA synthesis, we investigated the possibility that LDL may inhibit mitogen-stimulated lymphocyte responses by interfering with transferrin metabolism. LDL inhibited mitogen-stimulated lymphocyte [3 H]thymidine incorporation in a concentration-dependent manner. The degree of inhibition was most marked in serum-free cultures, but was also observed in serum-containing cultures. The addition of transferrin not only augmented mitogen-induced lymphocyte [3 H]thymidine incorporation in serum-free medium but also completely reversed the inhibitory effect of LDL in both serum-free and serum-containing media. Similar results were obtained when lymphocyte proliferation was assayed by counting the number of cells in culture. Transferrin also reversed the inhibition of lymphocyte responses caused by very low density lipoproteins and by cholesterol. The ability of transferrin to reverse the inhibitory effect of lipoproteins was specific, in that native but not denatured transferrin was effective whereas a variety of other proteins were

ineffective. These results indicate that LDL inhibits mitogen-stimulated lymphocyte responses by interfering with transferrin metabolism. LDL only inhibited lymphocyte responses after a 48-h incubation if present from the initiation of the culture. By contrast, transferrin reversed inhibition when added after 24 h of the 48-h incubation. LDL did not inhibit lymphocyte responses by nonspecifically associating with transferrin. In addition, the acquisition of specific lymphocyte transferrin receptors was not blocked by LDL. Moreover, transferrin did not prevent the binding and uptake of fluorescent-labeled LDL by activated lymphocytes. Furthermore, LDL did not prevent the binding of transferrin to its receptor. Finally, LDL inhibition did not require specific high affinity cell surface receptors for cholesterol transport by LDL because similar inhibition and reversal by transferrin were observed with lymphocytes from a patient with homozygous familial hypercholesterolemia. Thus, LDL alters lymphocyte responses in a non-LDL receptor-mediated way by interfering with transferrin metabolism after specific binding of transferrin to receptors on activated lymphocytes.

Introduction

The inhibitory effect of normal low density lipoprotein (LDL) on antigen- and mitogen-stimulated lymphocyte responses has been well described by a number of investigators (1-5). LDL and other classes of lipoproteins have been shown to suppress lymphocyte [3 H]thymidine incorporation stimulated by both allogeneic cells and mitogens (1-5). However, the mechanism whereby LDL is inhibitory has not been completely elucidated. We have recently demonstrated that LDL does not inhibit initial lymphocyte activation and blast transformation but does inhibit lymphocyte DNA synthesis and subsequent proliferation (5). These observations suggest that suppression of DNA synthesis is the major inhibitory effect of LDL.

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It has been previously shown that transferrin is required for mitogen-induced lymphocyte DNA synthesis (6–8). In addition, mitogenic stimulation results in the appearance of transferrin receptors on activated lymphocytes (9–12). Furthermore, both transferrin and LDL bind to specific cell surface receptors and enter cells by the process of receptor-mediated endocytosis (13). Although LDL-mediated inhibition of lymphocyte responses may not require endocytosis of LDL (4) and may involve receptors that are distinct from the classic high affinity receptors involved in cholesterol metabolism (3, 4), it is possible that LDL inhibits mitogen-induced lymphocyte DNA synthesis by altering transferrin metabolism. The following experiments examined this possibility.

Methods

Isolation of lipoproteins. Human LDL ($d = 1.020$ – 1.050 g/ml) was isolated from plasma of normal fasting adults, using a Beckman preparative ultracentrifuge (Beckman Instruments, Palo Alto, CA) as previously described (5). Very low density lipoprotein (VLDL, $d < 1.006$ g/ml), intermediate density lipoprotein (IDL, $d = 1.006$ – 1.019 g/ml),¹ and a combined VLDL + IDL preparation ($d < 1.020$ g/ml) were isolated in a similar manner. High density lipoprotein (HDL) was isolated as previously detailed (14). In preliminary studies, three different density preparations, $d = 1.050$ – 1.230 g/ml, $d = 1.095$ – 1.230 g/ml, and $d = 1.125$ – 1.230 g/ml were isolated and were found to be similar in their effects on mitogen-stimulated lymphocyte responses. Protein concentrations were determined by the method of Lowry et al. (15) and total cholesterol concentration was measured by the enzymatic method of Roeschlau et al. (16). Each individual LDL preparation is designated by a subscript (LDL_x).

Reagents. Transferrin, nitrilotriacetate, catalase, fatty-acid free bovine serum albumin (BSA), ovalbumin, lactoperoxidase, and hydrogen peroxide were purchased from Sigma Chemical Co., St. Louis, MO. Cholesterol was purchased from Eastman Kodak Co., Rochester, NY. Iodo-Gen (1,3,4,6-tetrachloro-3 α ,6 α -diphenylglycoluril) was obtained from Pierce Chemical Co., Rockford, IL, and chloroform from Mallinkrodt Inc., St. Louis, MO. With the exception of cholesterol, all reagents which were added directly to the cultures were dissolved in Hanks' balanced salt solution. An equal volume of Hanks' solution was added to control cultures. Cholesterol was dissolved in ethanol and added directly to the cultures. An equivalent volume of ethanol (final concentration 0.5%) was added to control cultures. Rabbit anti-human transferrin and anti-human β -lipoprotein antibodies were obtained from Dako Corp., Santa Barbara, CA. Staphylococcal protein A (SPA)-Sepharose was purchased from Pharmacia Fine Chemicals, Piscataway, NJ.

Techniques of cell preparation and culture. Peripheral blood mononuclear cells (PBM) were separated from heparinized venous blood of healthy young adults by centrifugation on a layer of sodium diatrizoate/Ficoll as previously described (17). In some experiments, partially monocyte-depleted T cell-enriched populations were obtained as pre-

viously described (18). Cells were cultured in medium RPMI 1640 (Microbiological Associates, Walkersville, MD), with added L-glutamine (0.3 mg/ml), gentamicin (10 μ g/ml), and penicillin G (200 U/ml). In some experiments the medium was further supplemented with 1% human serum (HS). This concentration has been shown to result in lymphocyte responses similar to those obtained with 10% fetal bovine serum (5). Phytohemagglutinin (PHA, Wellcome Reagents Ltd., Beckenham, England) was used as the stimulus for all cultures. Preliminary experiments demonstrated that the optimal PHA concentration was 0.5 μ g/ml for cells incubated in serum-free medium as well as cultures supplemented with 1% HS.

PBM were incubated in sterile microtiter plates (Costar Div., Data Packaging Corporation, Cambridge, MA) for the assay of lymphocyte [³H]thymidine incorporation and measurement of cell proliferation, as previously described (17, 19). All cultures were incubated in a humidified atmosphere of 5% CO₂ and 95% air.

Assay of lymphocyte responses. Mitogen-induced lymphocyte DNA synthesis was assayed by the measurement of [³H]thymidine incorporation as previously described (17). Briefly described, 1 μ Ci [³H]thymidine (6.7 Ci/mmol, ICN Chemical and Radioisotope Division, Irvine, CA) was added to each microtiter well 18 h before harvesting the cells with a semiautomated microharvesting device. Incorporation was determined by liquid scintillation spectroscopy. Data are expressed as the difference in counts per minute between the means of triplicate mitogen-stimulated and control cultures (Δ cpm). In some experiments data are expressed as percentage change when compared with control, calculated by the following equation: % change = $[(\Delta$ cpm with LDL/ Δ cpm control) - 1] \times 100.

Mitogen-induced lymphocyte proliferation was quantitated by counting the number of cells in triplicate microtiter wells as previously described (19). In brief, cells were dispersed after addition of lysing agent and the resultant nuclei counted using the Coulter Counter (Coulter Electronics, Hialeah, FL). Data are expressed as the number of cells per well or the difference between the means of triplicate mitogen-stimulated and control cultures (Δ cells/well). Statistical analyses were performed using Student's *t* test for paired observations.

Analysis of lymphocyte transferrin receptors by indirect immunofluorescence. The monoclonal antibody 5E9 (kindly provided by Dr. Barton F. Haynes) was used for indirect immunofluorescence analysis of transferrin receptors on activated lymphocytes. This antibody recognizes receptors for transferrin that do not appear on resting lymphocytes but develop on activated T cells. The production and characterization of the antibody has been reported (20). The 5E9 monoclonal antibody detects a transferrin receptor antigen distinct from the transferrin binding site. The technique of staining for fluorescence-activated cell sorter analysis has been described previously (21). Briefly, partially monocyte-depleted lymphocyte populations were incubated for 24 h with or without PHA and LDL. After extensive washing, cells (2×10^7 /ml) were incubated for 20 min at 4°C in a 1:1,000 dilution of ascites fluids containing the monoclonal antibody, or as control, ascites fluids containing monoclonal antibody of the same isotype but irrelevant specificity. The cells were washed and then incubated with fluorescein isothiocyanate conjugated goat anti-mouse IgG. After a final wash, cells were resuspended for analysis, as previously described (21).

Analysis of LDL uptake by lymphocytes. In order to assess binding and uptake of LDL by activated lymphocytes, reconstituted LDL labeled with the fluorescent lipid probe 3,3'-dioctadecylindocarbocyanine (DiI) was used (22). Partially monocyte-depleted lymphocytes were incubated for 8 h at 37°C in serum-free medium with DiI-LDL with or without an excess of unlabeled LDL. The cells were then washed, resuspended,

1. **Abbreviations used in this paper:** DiI, 3,3'-dioctadecylindocarbocyanine; FACS, fluorescence-activated cell sorter; FH, familial hypercholesterolemia; HS, human serum; IDL, intermediate density lipoprotein; PBM, peripheral mononuclear cell; PHA, phytohemagglutinin; SPA, staphylococcal protein A.

and analyzed with the fluorescence-activated cell sorter to quantitate cell-associated fluorescence.

Radiolabeling of transferrin. Human transferrin was labeled with ^{125}I by means of lactoperoxidase (23) or Iodo-Gen (24) as previously described. Briefly, lactoperoxidase-catalyzed radioiodination was carried out using 50 μg of transferrin, 0.5 mCi of carrier-free ^{125}I as NaI (sp act 15 Ci/mg, Amersham Corp., Arlington Heights, IL), 5 μg of lactoperoxidase, and 0.003% hydrogen peroxide as described (23). Iodo-Gen-catalyzed radioiodination was carried out using 50 μg of transferrin and 0.2 mCi of carrier-free ^{125}I as NaI as detailed (24). Unbound ^{125}I was removed by extensive dialysis. The specific activity of the radioiodinated transferrin was approximately 2 $\mu\text{Ci}/\mu\text{g}$ in both cases.

Measurement of LDL binding of ^{125}I -transferrin. A possible association of LDL with ^{125}I -transferrin was quantitated by the following methods. First, LDL (2 mg protein) was incubated with 0.5 μg ^{125}I -transferrin for 1 h and then the density of the preparation was adjusted to 1.095 g/ml with solid KBr. The LDL was reisolated by flotation centrifugation and the radioactivity in the lipoprotein and in the infranant was measured. In another procedure, LDL (180 μg protein) was incubated with 0.5 μg ^{125}I -transferrin for 48 h at 4°C. The sample was then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis as previously described (25). In a final procedure, insoluble undenatured LDL was prepared and the binding of ^{125}I -transferrin was quantitated. SPA-Sepharose was preincubated with rabbit anti-human β -lipoprotein antiserum for 1 h at 4°C. As a control SPA-Sepharose was preincubated with normal rabbit serum or rabbit anti-human transferrin antiserum. An aliquot of each of these was then incubated with LDL for 24 h at 4°C. Quantitation of cholesterol concentration confirmed that LDL (30 μg protein/ml) bound to the rabbit anti- β -lipoprotein SPA-Sepharose but not other antibody-coated SPA-Sepharose preparations. ^{125}I -transferrin (1 $\mu\text{g}/\text{ml}$) was then incubated for 1 h with each preparation and the radioactivity bound to the sepharose was quantitated.

Radioiodinated transferrin binding assays. The binding reactions were carried out in 12 \times 75-mm glass tubes (American Scientific Products Div., American Hospital Supply Corp., McGaw Park, IL) in the presence of 1% BSA. The cell preparations used included both PBM and T cell-enriched populations prepared as described previously by sequential glass adherence and passage over nylon wool columns (18). The cell populations were incubated for 24 h with or without PHA in 17 \times 100-mm polypropylene tubes (Falcon Div., Becton, Dickinson & Co., Cockeysville, MD), with each tube containing 2–4 \times 10⁶ cells in 2 ml of culture medium either supplemented with 0.1% HS or without serum. In some experiments, LDL (200–360 μg protein/ml) was also included in the incubation medium. After extensive washing, the cells were resuspended in RPMI 1640, containing 1% bovine serum albumin and 10 mM Hepes, pH 7.4 at 5–10 \times 10⁶ cells/ml. Viability was >90% as measured by uptake of fluorescein diacetate and exclusion of ethidium bromide (26). Preliminary experiments established that binding assays were reproducible using 1–2 \times 10⁶ cells and incubation for 60 min with the radiolabeled ligand. Unbound ligand was removed by passage of cells through a 200- μl density cushion of dibutyl phthalate and dinonyl phthalate as detailed previously (27). Binding assays were carried out in triplicate and in each experiment an additional assay was run containing a 500-fold excess of unlabeled transferrin to allow correction for nonspecific binding of ligand.

Results

LDL inhibits lymphocyte responses. Initial experiments were carried out to compare the effect of LDL on lymphocyte re-

sponses in serum-free medium with those obtained when cells were cultured in medium supplemented with serum. As seen in Table I, mitogen-induced [^3H]thymidine incorporation in serum-free medium was diminished compared to that observed in cultures containing serum supplementation, but was easily detectable and of a statistically significant magnitude in each experiment. LDL inhibited PHA-induced [^3H]thymidine incorporation in a concentration-dependent manner in both serum-free and serum-containing cultures (Table I). The inhibitory effect of any given concentration of LDL was significantly greater when serum was omitted from the culture medium (Table I). In 11 experiments with various LDL preparations, the concentration of LDL resulting in 50% inhibition of mitogen-stimulated lymphocyte [^3H]thymidine incorporation was 91 \pm 13 μg of protein/ml (mean \pm SEM) in serum-free medium. In medium supplemented with 1% HS, the concentration of the same LDLs required to inhibit responses by 50% was significantly greater at 313 \pm 41 μg of protein/ml ($P < 0.001$). When 1% lipoprotein-poor plasma was used to supplement the cultures, the results were similar to those obtained using 1% HS (data not shown). There was some variability in the concentration of LDL that resulted in 50% inhibition of lymphocyte responses in serum-free medium (mean 75, range 16–171 μg of protein/ml for seven different LDL preparations used in 28 separate experiments). This variability could not be accounted for by differences in cholesterol content of the LDL preparations. Thus, 50% inhibition of lymphocyte responses was observed with a mean of 88 $\mu\text{g}/\text{ml}$ of cholesterol, range 19–198 $\mu\text{g}/\text{ml}$. Similar variability has been described previously for cultures in serum-containing medium (5). These initial studies demonstrated that LDL inhibited lymphocyte responses in a qualitatively similar manner, regardless of the culture medium. However, the inhibition noted in serum-free medium was significantly greater than that observed in serum-containing cultures or those supplemented with

Table I. LDL Inhibits Mitogen-stimulated Lymphocyte Responses

Medium	PHA-induced lymphocyte [^3H]thymidine incorporation* LDL (μg protein/ml)			
	0	90	180	360
	$\Delta\text{cpm} \times 10^{-3}$ (% inhibition)			
Serum-free	19.2 \pm 0.8	10.2 \pm 2.7 (47%)	3.2 \pm 0.1 (83%)	0 (100%)
1% HS	137.2 \pm 8.9	122.4 \pm 4.1 (11%)	105.5 \pm 8.7 (23%)	58.4 \pm 8.4 (57%)

* PBM were incubated in serum-free medium or medium supplemented with 1% HS with or without PHA and varying concentrations of LDL_M as indicated. Lymphocyte DNA synthesis was measured by the incorporation of [^3H]thymidine after 4 d. Results are mean \pm SEM of four separate experiments.

lipoprotein-poor plasma. This suggested that a component of both normal serum and lipoprotein-depleted plasma might prevent LDL-mediated inhibition.

Transferrin prevents LDL-mediated inhibition of lymphocyte DNA synthesis. The effect of transferrin on LDL-mediated inhibition of mitogen-stimulated lymphocyte responses was next examined. As seen in Table II, the addition of transferrin significantly increased ($P < 0.001$) the response of lymphocytes cultured in serum-free medium. Moreover, when transferrin was added to cultures containing LDL, the inhibitory effect of LDL was markedly decreased. At the highest concentration of transferrin (50 $\mu\text{g/ml}$) the inhibition by LDL was completely prevented. Furthermore, the combination of LDL and transferrin (50 $\mu\text{g/ml}$) resulted in significant enhancement of mitogen-induced lymphocyte DNA synthesis over that observed with transferrin alone. Similar results were obtained with all LDL preparations tested. In fact, lymphocyte responses in serum-free medium supplemented with transferrin and LDL were equal to or greater than those observed in serum-containing cultures.

When lymphocytes were cultured in serum-free medium, the concentration of LDL required to inhibit mitogen-stimulated lymphocyte responses by 50% was significantly increased by the addition of as little as 0.5 $\mu\text{g/ml}$ transferrin (Table III). In serum-supplemented cultures (containing ~ 20 $\mu\text{g/ml}$ of transferrin from 1% HS), a higher concentration of LDL_M was required to inhibit lymphocyte responses to a similar degree (Table III) and addition of low concentrations of transferrin had no significant effect. However, in both serum-free medium and cultures supplemented with 1% HS, no inhibition was caused by LDL in the presence of 50 $\mu\text{g/ml}$ of transferrin.

Transferrin reverses inhibition by other lipoproteins and cholesterol. We next examined whether transferrin was able to prevent the inhibitory effects of lipoproteins other than LDL. Both

Table II. LDL Inhibition of PHA-induced Lymphocyte Responses: Reversal by Transferrin

LDL $\mu\text{g protein/ml}$	PHA-induced lymphocyte [³ H]thymidine incorporation* Transferrin ($\mu\text{g/ml}$)			
	0	0.5	5.0	50
	$\Delta\text{cpm} \times 10^{-3}$	$\Delta\text{cpm} \times 10^{-3}$	$\Delta\text{cpm} \times 10^{-3}$	$\Delta\text{cpm} \times 10^{-3}$
LDL _N				
0	34.8 \pm 13.9	61.5 \pm 10.9	71.3 \pm 8.1	68.0 \pm 13.6
35	14.4 \pm 7.6	32.6 \pm 4.9	51.3 \pm 4.4	100.8 \pm 11.2
70	9.3 \pm 4.1	14.4 \pm 2.5	34.7 \pm 2.3	105.5 \pm 12.0
175	2.8 \pm 2.1	4.8 \pm 2.2	9.1 \pm 1.7	117.0 \pm 11.1
350	0.4 \pm 0.1	2.3 \pm 0.9	3.1 \pm 0.9	128.0 \pm 11.0

* PBM were incubated in serum-free medium with or without PHA and varying concentrations of transferrin and LDL_N as indicated. After 4 d, mitogen-stimulated lymphocyte DNA synthesis was measured by the incorporation of [³H]thymidine. Results are mean \pm SEM of four experiments.

Table III. LDL Inhibition of PHA-induced Lymphocyte DNA Synthesis: Reversal by Transferrin

Medium	Addition	Concentration of LDL _M inhibiting PHA-induced lymphocyte [³ H]thymidine incorporation by 50%*	
		Mean $\mu\text{g protein/ml}$	Range $\mu\text{g protein/ml}$
Serum-free	Nil	96	31–136
	Transferrin		
	0.5 $\mu\text{g/ml}$	204	118–269
	5.0 $\mu\text{g/ml}$	213	129–282
	50 $\mu\text{g/ml}$	NI \ddagger	
1% HS	Nil (=transferrin 20 $\mu\text{g/ml}$)	311	209–416
	Transferrin		
	0.5 $\mu\text{g/ml}$	311	191–536
	5.0 $\mu\text{g/ml}$	345	244–541
	50 $\mu\text{g/ml}$	NI	

* PBM were incubated in serum-free medium or medium supplemented with 1% HS (equivalent to transferrin 20 $\mu\text{g/ml}$) with or without PHA and varying concentrations of LDL_M and transferrin. After 4 d, mitogen-induced lymphocyte DNA synthesis was measured by the incorporation of [³H]thymidine and the concentration of LDL_M required to inhibit responses by 50% was calculated. Results represent mean and range of four separate experiments.

\ddagger NI, no inhibition in presence of 360 μg of protein/ml LDL_M.

VLDL and IDL isolated from normal human donors inhibited mitogen-induced lymphocyte [³H]thymidine incorporation in a concentration-dependent manner. Similar results were obtained when a lipoprotein preparation containing both VLDL + IDL ($d < 1.020$ g/ml) was tested (Table IV). The addition of 50 $\mu\text{g/ml}$ transferrin completely reversed the inhibitory effects of these lipoproteins, as seen in Table IV. HDLs of varying densities were also studied for inhibitory effects. When HDL was added in concentrations up to 2 mg of protein/ml, no inhibitory effect was seen. HDLs of different density ranges ($d = 1.050$ – 1.230 , 1.095 – 1.230 , and 1.125 – 1.230 g/ml) were similar in that they failed to inhibit lymphocyte DNA synthesis and often enhanced mitogen-induced lymphocyte responses in serum-free medium. The addition of transferrin (50 $\mu\text{g/ml}$) to cultures containing HDL resulted in a further increase in PHA-stimulated lymphocyte [³H]thymidine incorporation. LDL and VLDL fractions containing IDL are bound and taken up by specific cell surface receptors on lymphocytes and other cultured cells (28–31) whereas similar specific receptors for HDL have not been reported (31, 32). Furthermore, both VLDL fractions containing IDL and LDL provide cholesterol, thereby regulating cholesterol synthesis by the cells (28, 29, 33) whereas HDL

Table IV. Lipoprotein and Cholesterol Inhibition of Lymphocyte Responses: Reversal by Transferrin

Addition	PHA-induced lymphocyte [³ H]thymidine incorporation*	
	Control	Transferrin
	$\Delta cpm \times 10^{-3}$	$\Delta cpm \times 10^{-3}$
Nil	35.0±4.2	76.9±9.9
VLDL + IDL		
9.5 μ g protein/ml	40.2±4.2	114.3±12.9
95 μ g protein/ml	3.1±1.4	151.9±10.5
HDL		
185 μ g protein/ml	68.1±11.7	157.2±10.4
1.85 mg protein/ml	107.5±21.0	155.5±8.1
Nil	35.7±7.5	75.7±8.5
Cholesterol, 50 μ g/ml	16.7±6.4	77.8±6.0

* PBM were incubated in serum-free medium with or without PHA, varying concentrations of lipoprotein (VLDL + IDL, $d = <1.020$ g/ml, HDL $d = 1.050$ – 1.230 g/ml) and transferrin 50 μ g/ml. After 4 d incubation, mitogen-induced lymphocyte DNA synthesis was measured by the incorporation of [³H]thymidine. Results represent mean±SEM of five separate experiments.

removes cholesterol (32). Therefore, we investigated the effect of adding cholesterol alone. The addition of cholesterol (50 μ g/ml) to mitogen-stimulated lymphocytes cultured in serum-free medium resulted in inhibition of lymphocyte [³H]thymidine incorporation (Table IV). Transferrin (50 μ g/ml) also reversed the inhibitory effect of cholesterol. Mevalonate did not reverse the inhibition, indicating that oxygenated sterol contamination was not the cause of the inhibition (17, 19, 34). Cholesterol was not inhibitory when added to mitogen-stimulated lymphocytes cultured in medium supplemented by serum. These data indicate that the inhibitory effect of the lipoproteins and cholesterol was similar in that it was reversed by transferrin.

Transferrin also reverses inhibition of lymphocyte proliferation. Under optimal conditions, mitogenic stimulation of lymphocytes in vitro results in proliferation of the responding cells (5, 19, 35). We have previously demonstrated that LDL inhibits mitogen-induced lymphocyte proliferation (5). It was therefore of interest to determine whether transferrin could also reverse this effect. In these experiments the number of cells per microtiter well was quantified after a 7-d incubation with PHA. As seen in Table V, transferrin completely reversed LDL-mediated inhibition of lymphocyte proliferation in serum-containing medium. Of interest, inhibition of PHA-induced lymphocyte proliferation was evident at concentrations of LDL (90 μ g protein/ml, Table V) which had little or no effect on PHA-induced lymphocyte [³H]thymidine incorporation (Table I). Transferrin

(50 μ g/ml) prevented inhibition of lymphocyte proliferation by all concentrations of LDL examined.

Transferrin also prevented LDL-mediated inhibition of mitogen-stimulated lymphocyte proliferation in serum-free medium (Fig. 1). Without the addition of transferrin to the serum-free medium, there was no detectable increase in cell number after mitogenic stimulation. When transferrin alone was added, a small increase (two to threefold) in cell number was observed, indicating that it had permitted a modest degree of PHA-stimulated lymphocyte proliferation. The smallest concentration of LDL enhanced the proliferative response supported by all concentrations of transferrin with the degree of proliferation observed being dependent on the concentration of transferrin. With the addition of increasing concentrations of LDL, PHA-stimulated lymphocyte proliferation was inhibited in cultures containing low concentrations of transferrin (0.5 \rightarrow 5 μ g/ml). 50 μ g/ml of transferrin completely prevented LDL-mediated inhibition of lymphocyte proliferation. PHA-stimulated lymphocyte proliferation in serum-free cultures containing LDL and 50 μ g/ml transferrin was greater than in cultures supplemented with 1% HS (LDL + transferrin = 406,300±5,100 cells/well after 7 d, 1% HS = 296,000±29,700 cells/well, mean±SEM, $n = 4$, 50,000 cells/well initially cultured).

Native but not denatured transferrin reverses the inhibitory effect of LDL. The studies outlined above demonstrated that transferrin reversed LDL-modulated inhibition. However, they did not establish the specificity of this effect. We next determined whether denatured transferrin was able to prevent the inhibition. In these experiments, an aliquot of transferrin was denatured by heating to 100°C for 60 min. The resultant preparation was unchanged in protein concentration, determined by the method of Lowry et al. (15). However, the protein was no longer identified by a rabbit anti-human transferrin antiserum. Moreover, de-

Table V. Transferrin Prevents LDL-mediated Inhibition of Lymphocyte Proliferation

Stimulus	Addition	PHA-induced lymphocyte proliferation*	
		Control	Transferrin
	μ g protein/ml	cells per well $\times 10^{-3}$	
Nil	—	38.7±2.3	38.5±1.8
PHA	—	334.7±27.9	288.2±20.7
	LDL 90	220.8±8.7	313.1±12.0
	LDL 180	133.4±8.6	316.0±11.8
	LDL 360	63.8±11.1	301.2±10.9

* PBM were incubated for 7 d in medium supplemented with 1% HS, with or without PHA, varying concentrations of LDL_M, and transferrin 50 μ g/ml as indicated. Initial cultures contained 50 $\times 10^3$ cells per well, unstimulated cultures contained 38.7±2.3 $\times 10^3$ cells per well after 7 d and this was unchanged by the additions. Results represent mean±SEM of four separate experiments.

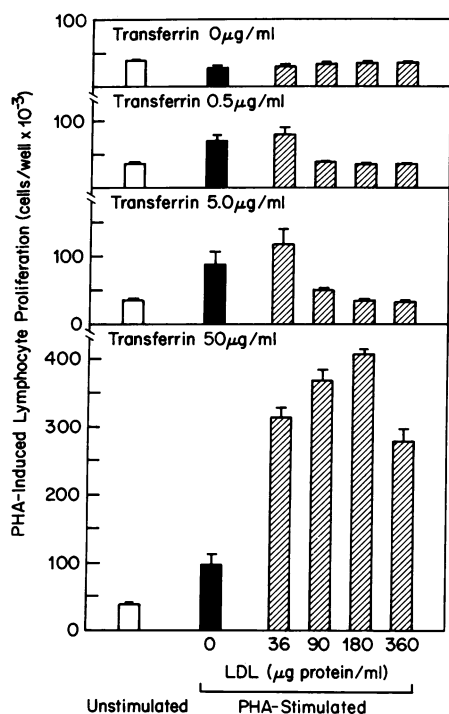


Figure 1. Transferrin reverses LDL-mediated inhibition of PHA-stimulated lymphocyte proliferation. PBM were incubated for 7 d with or without PHA and varying concentrations of LDL_M and transferrin as indicated. Initial cultures contained 50×10^3 cells/well. Data represent mean \pm SEM of four separate experiments.

natured transferrin was unable to enhance the response of lymphocytes cultured in serum-free medium. When compared with native transferrin (Table VI), the denatured transferrin was markedly less effective at preventing inhibition by LDL.

Other proteins do not prevent LDL inhibition. A number of

other different proteins were also examined for their capacity to reverse the inhibitory effect of LDL. BSA and ovalbumin were tested because albumin from various sources has been used to supplement serum-free cultures. Catalase was also tested because it contains iron within heme groups. Although catalase enhanced the response of lymphocytes cultured in serum-free medium, it had no effect on LDL mediated inhibition (Table VII). Neither BSA nor ovalbumin enhanced responses in serum-free cultures nor were they able to reverse LDL-modulated inhibition. These experiments indicate that the capacity of transferrin to prevent inhibition by LDL was not merely the result of supplementation of the culture with protein.

Effect of delayed addition of transferrin and LDL. Addition of transferrin together with LDL prevented LDL-mediated inhibition of mitogen-stimulated lymphocyte responses. We next examined the kinetics of the inhibition and the reversal in order to characterize this effect in greater detail. For these experiments, PHA-induced lymphocyte [³H]thymidine incorporation was measured after a 48-h incubation. As seen in Table VIII, PHA stimulated a small but reproducible increase in the incorporation of [³H]thymidine in serum-free cultures. LDL added at the initiation of the culture inhibited this response in a concentration-dependent manner. In cultures containing both transferrin 50 μg/ml and LDL at the initiation (0 h) there was no inhibitory effect from LDL, and significant enhancement of lymphocyte [³H]thymidine incorporation was observed. When addition of transferrin was delayed for 24 h, similar reversal of LDL inhibition was found except at the highest concentrations of LDL (175–350 μg LDL protein/ml). In contrast, when addition of LDL was delayed until 24 h after the commencement of the culture, no inhibitory effect was observed. LDL could enhance lymphocyte responses in the presence of transferrin when added at 24 h. These experiments indicated that the inhibitory effect of LDL required that it be present from the initiation of culture. On the other hand, transferrin was able to reverse LDL-mediated inhibition, even when added 24 h after the LDL.

Table VI. Native but Not Denatured Transferrin Reverses Inhibition by LDL

LDL μg protein/ml	Transferrin*	PHA-stimulated lymphocyte [³ H]thymidine incorporation‡ Transferrin (μg/ml)			
		0	0.5	5.0	50
Nil	Control	25.8 \pm 3.0	60.7 \pm 3.0	73.4 \pm 0.8	71.3 \pm 0.5
	Heated	25.8 \pm 2.7	23.4 \pm 0.1	23.3 \pm 0.1	43.8 \pm 4.0
27	Control	9.5 \pm 0.4	49.7 \pm 9.4	79.3 \pm 12.4	98.9 \pm 9.9
	Heated	11.0 \pm 0.8	10.0 \pm 0.1	14.7 \pm 0.8	72.2 \pm 10.5
270	Control	0	0.7 \pm 0.6	1.5 \pm 1.4	106.1 \pm 10.1
	Heated	0	0	0	2.9 \pm 1.4

* Native transferrin or transferrin denatured by heating (100°C, 60 min). ‡ PBM were incubated in serum-free medium with or without PHA and varying concentrations of LDL_H and native or denatured transferrin. After 4 d, mitogen-stimulated lymphocyte DNA synthesis was measured by the incorporation of [³H]thymidine.

Table VII. Transferrin but Not Other Proteins Prevent Inhibition by LDL

Addition (50 µg/ml)	PHA-stimulated lymphocyte [³ H]thymidine incorporation* LDL (µg protein/ml)		
	0	90	180
	<i>Δcpm × 10⁻³ (% change)‡</i>		
Nil	19.2±0.8	10.2±2.7 (-47%)	3.2±1.1 (-83%)
Transferrin	74.2±2.1	119.4±5.6 (+61%)	122.7±5.9 (+65%)
BSA	19.0±0.2	6.8±2.2 (-64%)	0.9±0.3 (-95%)
Ovalbumin	19.7±0.7	8.2±2.1 (-58%)	2.7±1.3 (-86%)
Catalase	40.0±6.5	9.5±1.9 (-76%)	6.7±1.0 (-83%)

* PBM were incubated in serum-free medium, with or without PHA and concentrations of LDL_M and other proteins as indicated, for 4 d before measuring [³H]thymidine incorporation. Results are mean±SEM of four separate experiments.

‡ Percentage change from control without LDL.

Lack of a physical association between LDL and transferrin. The mechanism whereby LDL interfered with transferrin metabolism was next investigated. A series of experiments examined the possibility that nonspecific binding of transferrin to LDL occurred, thereby preventing its uptake by activated cells. First, ¹²⁵I-transferrin was incubated with LDL for 1 h and the LDL was reisolated by ultracentrifugation. >99.7% of the radioactivity was recovered in the infranatant, not associated with LDL. Next, ¹²⁵I-transferrin was mixed with LDL and then analyzed after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. <0.5% of the radioactivity remained with the B apoprotein. These results suggested that transferrin did not associate with LDL. However, it remained possible that a noncovalent association may have occurred that could have been disrupted by the ultracentrifugation or sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Therefore, insolubilized undenatured LDL was prepared by incubating it with SPA-Sepharose that had been preincubated with rabbit anti-human β-lipoprotein antiserum. Rabbit serum and anti-human transferrin were used as negative and positive control antibodies, respectively. As shown in Table IX, ¹²⁵I-transferrin only bound to SPA-Sepharose containing anti-human transferrin and not to SPA-Sepharose with anti-β-lipoprotein and LDL attached. None of these experiments indicated that a physical interaction between transferrin and LDL occurred.

Transferrin does not prevent binding and uptake of LDL. The possibility that transferrin may reverse the inhibitory effect

Table VIII. Transferrin Reversal of LDL-mediated Inhibition of Lymphocyte Responses: Effect of Delayed Addition of Transferrin and LDL

Expt.	Addition of LDL	Addition of transferrin	PHA-stimulated lymphocyte [³ H]thymidine incorporation* LDL concentration (µg protein/ml)				
			0	35	70	175	350
			<i>cpm × 10⁻³</i>				
1	0 h	Nil	8.6±0.7	3.3±0.4	3.7±0.6	2.0±0.4	1.9±0.2
		0 h	24.6±0.5	23.2±2.3	29.7±4.3	32.9±4.8	42.3±4.5
		24 h	20.5±2.1	21.9±4.1	26.6±4.1	20.5±2.5	13.1±1.7
	24 h	Nil	7.7±0.5	8.9±1.0	11.5±1.4	8.9±1.7	12.1±1.5
		0 h	22.7±0.4	31.2±1.5	34.7±3.4	34.6±2.1	37.1±1.7
		24 h	20.4±2.8	22.9±2.5	30.7±3.7	26.5±3.8	31.1±0.3
2	0 h	Nil	20.4±1.3	17.0±2.1	15.5±1.6	11.3±0.1	10.6±0.7
		0 h	29.8±1.7	29.6±2.7	38.2±1.2	33.5±3.9	42.3±2.1
		24 h	22.1±3.8	20.0±1.7	22.3±0.8	16.7±0.1	17.5±1.3
	24 h	Nil	26.1±1.4	21.0±1.7	25.6±2.4	25.7±1.8	27.9±0.7
		0 h	34.8±2.1	37.1±3.8	40.3±2.1	31.1±1.0	40.1±0.5
		24 h	27.8±1.4	25.0±2.9	32.8±2.1	29.6±3.1	37.1±1.7

* Transferrin 50 µg/ml and varying concentrations of LDL_N were added to PBM at the initiation of the culture (0 h) or addition was delayed for 24 h. Lymphocyte [³H]thymidine incorporation was measured after 48 h. Results represent mean±SEM of triplicate determinations. In both experiments, unstimulated cells incorporated <0.8 × 10⁻³ cpm.

Table IX. Transferrin Does Not Bind Nonspecifically to LDL

Antibody bound to SPA-Sepharose	LDL preincubation	¹²⁵ I-Transferrin binding*
		% bound
Normal rabbit serum	-	0.6
	+	0.4
Anti-β-lipoprotein	-	1.7
	+	2.0
Anti-transferrin	-	91.0
	+	91.2

* SPA-Sepharose was preincubated with antibody and an aliquot of each preparation was also incubated with LDL. ¹²⁵I-transferrin was then incubated with all preparations, and after washing, the radioactivity bound to the Sepharose was quantitated.

of LDL by altering the binding and uptake of LDL was next examined. To investigate this possibility, LDL receptors were induced by incubating T cell-enriched populations overnight in medium supplemented with lipoprotein-poor plasma (30) in the presence or absence of PHA. Following this initial incubation, the cells were cultured for 8 h with DiI-LDL with or without excess unlabeled LDL. As shown in Fig. 2 A, there was measurable uptake of DiI-LDL in unstimulated cells, which was markedly increased in the PHA-activated cell population. As shown in Fig. 2 B, uptake of DiI-LDL was markedly inhibited by the addition of an excess of unlabeled LDL. By contrast, transferrin (50 μg/ml) had no effect on the uptake of DiI-LDL by the cells (Fig. 2 B). Thus, transferrin did not reverse LDL-mediated inhibition by lymphocyte DNA synthesis by altering the binding and uptake of LDL.

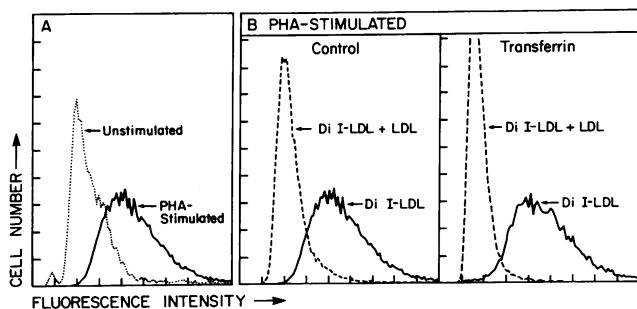


Figure 2. Transferrin does not prevent binding and uptake of LDL. PBM, depleted of monocytes, were incubated with or without PHA in 1% lipoprotein-poor plasma overnight. After extensive washing, the cells were incubated in serum-free medium for 8 h with DiI-LDL (· · · unstimulated, — PHA) with or without transferrin and excess LDL (---). The relative number of cells in each sample with specific fluorescence intensity was analyzed by the fluorescence-activated cell sorter (FACS III, Becton, Dickinson & Co., Sunnyvale, CA).

LDL does not prevent acquisition of transferrin receptors. Both transferrin and LDL can enter cells by binding to specific cell surface receptors. The bound ligand is then internalized by the process of receptor-mediated endocytosis. Resting lymphocytes do not have cell surface receptors for transferrin (9, 10). During the process of activation, specific receptors for transferrin first appear 20–24 h after the commencement of mitogenic stimulation. The monoclonal antibody 5E9 was used to identify transferrin receptor antigen on activated lymphocytes after a 24-h incubation. As shown in Table X, PHA stimulation markedly increased the number of cells that were 5E9 positive, indicating that the cells had acquired transferrin receptor antigen. When LDL was present during the process of activation, there was no effect on the appearance of the specific receptor antigen nor was there a decrease in receptor antigen density (Fig. 2). Parallel experiments examined the effect of the same concentrations of LDL on PHA-induced lymphocyte DNA synthesis measured after 48 h. LDL inhibited DNA synthesis, assayed by the incorporation of [³H]thymidine, by >50%. These data indicate that LDL does not prevent the appearance of transferrin receptor antigen.

LDL does not prevent receptor-mediated binding of transferrin. The aforementioned experiments indicated that LDL did not prevent the development of transferrin receptor antigen on activated lymphocytes. However, in that the monoclonal antibody 5E9 does not recognize the binding site on the transferrin receptor, it remained possible that LDL altered the acquisition of functional transferrin receptors. The possibility that LDL inhibited receptor-mediated transferrin binding was therefore examined. Initial experiments demonstrated that ¹²⁵I-transferrin was specifically bound by activated but not resting lymphocytes with characteristics similar to those previously described (11). To investigate the effect of LDL on the binding of ¹²⁵I-transferrin, cells were incubated for 24 h with or without PHA. LDL was added for 1 h before the binding assay was carried out to allow

Table X. LDL Does Not Inhibit Acquisition of Transferrin Receptors

	Addition	Unstimulated % positive*	PHA-stimulated % positive*
Control ascites	Nil	2.7±0.4	4.0±0.2
	LDL	3.1±0.3	3.7±0.4
5E9	Nil	4.0±0.3	30.1±3.7
	LDL	4.5±0.4	27.8±5.0

* T cell-enriched populations were incubated with or without PHA and one of three different preparations of LDL (250–350 μg of protein/ml of LDL_E, LDL_P, or LDL_Q) as indicated. After washing, the cells were reacted with either the monoclonal antibody 5E9 or a mouse ascites with an irrelevant specificity. The cells were washed, incubated with fluoresceinated goat anti-mouse IgG and then analyzed using the FACS III. The percentage of cells staining with 5E9 was then calculated. The results represent the mean±SEM of six separate experiments.

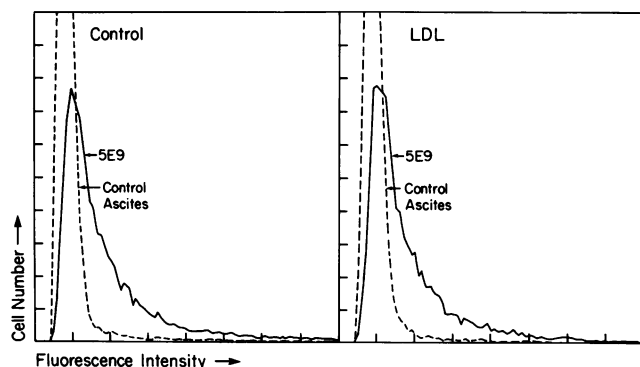


Figure 3. LDL does not prevent acquisition of transferrin receptors after mitogenic stimulation. PBM, depleted of monocytes, were incubated with PHA for 24 h with or without LDL_p (720 µg of protein/ml) as indicated and prepared for fluorescence analysis with the monoclonal antibody 5E9 (—) or with control ascites (---). The relative number of cells in each sample with specific fluorescence intensity was analyzed by the FACS III.

equilibration and remained during the assay. As shown in Table XI, the binding of ¹²⁵I-transferrin to unstimulated cells was minimal. After a 24-h incubation, PHA stimulation resulted in a more than fourfold increase in receptor-mediated binding of ¹²⁵I-transferrin. When LDL was added to the cells after their initial mitogenic stimulation, there was no inhibition of the amount of ¹²⁵I-transferrin bound. Rather, there appeared to be increased binding and uptake of ¹²⁵I-transferrin in the presence of LDL.

The effect of LDL on the acquisition of functional transferrin receptors was also examined. For these experiments, lymphocytes were incubated for 24 h with PHA in the presence or absence of LDL. As can be seen in Table XI, the presence of LDL during the incubation with mitogen had no significant effect on subsequent binding of ¹²⁵I-transferrin assayed at 4°C. It is important to note that the concentrations of LDL employed significantly inhibited mitogen-induced lymphocyte DNA synthesis. When the binding studies were carried out at 37°C, the cells that had been mitogen-activated in the presence of LDL appeared to exhibit decreased ¹²⁵I-transferrin binding (456±168 pg/10⁶ cells compared with 664±216 pg/10⁶ cells with PHA alone) but the difference was not statistically significant. These studies indicate that LDL does not inhibit the development of functional transferrin receptors on mitogen-activated lymphocytes.

LDL-mediated inhibition of mitogen-induced lymphocyte DNA synthesis does not require specific high affinity LDL receptors. The preceding experiments indicated that LDL inhibited lymphocyte DNA synthesis by altering transferrin metabolism. The rationale for these studies was the evidence that cellular uptake of both LDL and transferrin involved the process of receptor-mediated endocytosis. However, previous evidence has suggested that LDL need not be internalized to inhibit lymphocyte activation and that the classic high affinity LDL receptors may not be involved (3, 4). Therefore, the possibility that LDL inhibited lymphocyte DNA synthesis without binding to its spe-

cific high affinity cell-surface receptor was examined. In order to study this question, PBM were isolated from venous blood obtained from a 6-yr-old child with LDL-receptor negative familial hypercholesterolemia (FH). As shown in Table XII, LDL inhibited PHA-stimulated lymphocyte [³H]thymidine incorporation and this inhibition was prevented by transferrin. Similar results were obtained with a number of other preparations of LDL. Of importance, the concentration of LDL causing inhibition of FH lymphocyte DNA synthesis was not higher than that leading to a similar degree of inhibition of normal PBM.

Discussion

Normal plasma lipoproteins have been shown to inhibit both mitogen- and antigen-stimulated lymphocyte responses *in vitro* (1-5). We have previously found that LDL inhibits DNA synthesis in mitogen-stimulated lymphocytes after initial blast transformation (5). Since DNA synthesis by lymphocytes and other cultured cells requires transferrin (6-8), we examined the possibility that the inhibitory effect of LDL may result from an alteration of transferrin metabolism. A number of observations suggest that LDL and transferrin interact with activated lymphocytes in a related manner. First, activated lymphocytes acquire specific receptors for the binding and uptake of transferrin

Table XI. LDL Does Not Inhibit Receptor-mediated Transferrin Binding

Lymphocyte preincubation	Addition during binding assay	¹²⁵ I-transferrin binding*	
		4°C	37°C
		pg/10 ⁶ cells	pg/10 ⁶ cells
Control	Nil	44±3	120±31
PHA	Nil	235±42	475±74
PHA	LDL (20 µg protein/ml)	316±60	716±38
PHA	LDL (200 µg protein/ml)	391±28	810±153
PHA	Nil	224±80	664±216
PHA + LDL (200-360 µg protein/ml)	Nil	208±96	456±168

* PBM or T cell-enriched populations were incubated for 24 h with or without PHA and with or without one of three different LDL preparations, N, Q, and R (200-360 µg protein/ml). Alternatively, LDL_R was added for the final hour of the incubation and was present during the binding assay. For the assay, 100 ng of ¹²⁵I-transferrin was added to triplicate samples, and total and nonspecific binding were measured as outlined in Methods. The results represent mean±SEM of three separate experiments.

Table XII. LDL-mediated Inhibition of Lymphocyte Responses Does Not Require Specific High Affinity Cell Surface Receptors

Addition	PHA-stimulated lymphocyte [³ H]thymidine incorporation*	
	Control <i>cpm</i> × 10 ⁻³	Transferrin <i>cpm</i> × 10 ⁻³
Nil	1.1±0.2	1.6±0.3
PHA	16.4±0.2	72.5±1.8
PHA + LDL (85 μg protein/ml)	8.4±0.5	97.7±0.7

* PBM were obtained from a patient with homozygous FH and incubated in serum-free medium with additions as indicated. Mitogen-induced lymphocyte [³H]thymidine incorporation was determined after 4 d. Results represent mean±SEM of triplicate determinations.

(9–11). Transferrin binds to its specific receptors and enters the cell in coated vesicles by the process of receptor-mediated endocytosis (13, 36). LDL also enters the cells by a similar process (13). Specific receptors for LDL have been demonstrated on cultured cells and on lymphocytes (28–31, 33). These metabolic similarities supported the contention that the lipoprotein may interfere with transferrin metabolism and thus indirectly inhibit lymphocyte DNA synthesis.

Normal human LDL was found to inhibit mitogen-induced lymphocyte responses. The addition of transferrin reversed the inhibitory effect of all LDL preparations. Similar results were obtained when the inhibitory effects of VLDL and IDL were examined. The inhibitory effect of LDL was decreased when cultures were supplemented with serum that contained transferrin. In this circumstance transferrin (50 μg/ml) also reversed the inhibitory effect of LDL. Therefore, transferrin reversed the inhibitory effect of all lipoproteins when lymphocytes were cultured with or without supplemental serum.

Transferrin that was substantially iron-free (~34 μg of iron/g transferrin) was effective in preventing inhibition of LDL and was able to enhance mitogen-stimulated [³H]thymidine incorporation in serum-free medium. The latter finding confirms previous reports (7, 8). In that the medium used in these experiments contains ~160 ng of iron/ml, the possibility exists that transferrin becomes optimally saturated with iron under the culture conditions utilized. The findings, however, that LDL-mediated inhibition was observed despite the presence of free iron in the culture medium and that inhibition was reversed by the addition of essentially iron-free transferrin, indicated that iron alone could not reverse LDL-mediated inhibition. A number of additional findings support this conclusion. First, iron salts such as FeCl₃ and nitrilotriacetate chelates of iron had no effect on inhibition by LDL (unpublished observation). In addition, denatured transferrin, catalase, and denatured catalase also were unable to reverse LDL-mediated inhibition. Finally, ferritin and lactoferrin, two additional iron-containing proteins, were also ineffective at enhancing lymphocyte responses in serum-free medium and at reversing the inhibitory effect of LDL (unpublished observation). These results indicate, therefore, that trans-

ferrin and not free iron or iron-protein complexes specifically prevents LDL-mediated inhibition.

The observation that the inhibition of lymphocyte DNA synthesis caused by LDL was reversed by transferrin could be explained in a number of ways. One explanation could be that transferrin alters the binding or uptake of LDL by lymphocytes and thus prevents inhibition. A number of observations make this unlikely. First, transferrin could reverse LDL-mediated inhibition of lymphocyte DNA synthesis, even if added after the first 24 h of a 48-h incubation, thereby making it unlikely that an alteration of the interaction between LDL and lymphocytes could explain the capacity of transferrin to reverse inhibition. Moreover, transferrin was found to have no effect on the binding and uptake of LDL by mitogen-stimulated lymphocytes. Thus, it appears unlikely that the prevention of LDL-mediated inhibition of lymphocyte DNA synthesis by transferrin can be explained by an alteration in the interaction of LDL with responding lymphocytes. Rather, it appears more reasonable to conclude that LDL inhibits lymphocyte DNA synthesis by altering transferrin metabolism.

The mechanism by which LDL alters transferrin metabolism is still not completely defined. Although both LDL and transferrin can enter cells by initially binding to specific receptors in coated pits, followed by endocytosis of the receptor and its bound ligand, LDL-mediated inhibition of lymphocyte function does not appear to require either endocytosis of the LDL (4) or engagement of the classic high affinity LDL receptor (3, 4). Binding to specific receptors followed by endocytosis does, however, appear to be necessary for transferrin to promote DNA synthesis (13, 36). Specific receptors for transferrin appear on activated lymphocytes after 20–24 h incubation with mitogen. Galbraith and co-workers (12) have reported that inhibition of RNA or protein synthesis prevented the expression of transferrin receptors and also abrogated lymphocyte activation assessed by the appearance of blast cells after 66 h. Thus, inhibition of mitogen responsiveness might be anticipated if LDL prevented transferrin receptor expression. LDL has been claimed to inhibit some aspects of early lymphocyte activation because it was found to suppress mitogen-stimulated phosphatidyl inositol turnover and calcium accumulation (4). However, in the current study LDL did not alter the acquisition of transferrin receptor antigen measured after a 24-h incubation with mitogen. Therefore, inhibition of transferrin receptor expression could not explain the inhibition of DNA synthesis caused by LDL. These data also confirm our previous finding that LDL does not inhibit initial entry of mitogen-activated cells into the cell cycle (5).

The possibility remained that LDL may decrease the binding of transferrin to its specific receptor in that the monoclonal antibody 5E9 does not recognize the binding site of the transferrin receptor. This was initially examined functionally by delaying the addition of either LDL or transferrin. Addition of transferrin 24 h after the addition of LDL resulted in enhancement of responses and reversal of LDL-mediated inhibition. This finding suggested that LDL did not prevent receptor-mediated binding and uptake of functionally active transferrin. Moreover, the lack of LDL-mediated inhibition, when addition was delayed, also

indicated that simple competition for receptor binding is not the explanation for the inhibitory effect. The conclusion that LDL does not prevent the development of functional transferrin receptors was substantiated with binding studies. Binding of radiolabeled transferrin at 4°C and binding and uptake of transferrin at 37°C were not significantly inhibited by LDL whether present during initial activation or during the binding assay. These results indicate that LDL inhibits transferrin metabolism after the appearance of specific functionally active membrane receptors and without inhibiting the binding of transferrin to its specific receptor.

Further insight into the mechanism whereby LDL inhibits lymphocyte responses was provided by the experiments utilizing cells lacking specific high affinity receptors for LDL. These studies clearly establish that the inhibitory effect of LDL does not require binding to high affinity cell surface receptors specific for cholesterol transport by native LDL. Harmony and Hui (4) have reported that lymphocytes from patients with receptor-negative familial hypercholesterolemia have normal binding of LDL to "immunosuppression receptors." The nature and specificity of such putative receptors remain unclear. Moreover, it has not been established that "immunosuppression receptors" reside within coated pits. Regardless, the finding implies that LDL would be expected to exert a similar degree of suppression when tested on lymphocytes from patients with FH. This expectation was confirmed by the current studies. Harmony and Hui (4) also observed that internalization of LDL was not required for inhibition of mitogen-stimulated lymphocyte responses. Surface-associated LDL may alter lymphocyte plasma membrane function perhaps by exchange of lipid moieties such as cholesterol and thereby interfere with lymphocyte responses. The current studies suggest that cholesterol is the lipid that is most likely to explain the inhibitory effect of LDL. The reports by other investigators that lipid-depleted LDL may also be inhibitory suggest the possibility that inhibitory mechanisms other than interference with transferrin metabolism may be involved. Because we have not found any native LDL preparations whose inhibitory effects were not reversed by transferrin, it is possible that the process of lipid depletion may introduce additional inhibitory effects not shared by native LDL. Moreover, the possibility that apoproteins found associated with higher density lipoproteins may contribute to the inhibitory effects reported by others deserves consideration. In this regard, we have found in preliminary experiments that purified apoprotein E peptides (kindly provided by Dr. K. Weisgraber) inhibit mitogen-stimulated lymphocyte DNA synthesis but that the effect is not reversed by transferrin.

There are a number of mechanisms whereby LDL may inhibit transferrin metabolism without altering receptor expression or initial receptor binding. Recent reports indicate that both transferrin and its receptor are recycled after endocytosis (36, 37). LDL may interfere with the recycling process for either the transferrin receptor or for transferrin itself. The means by which this is accomplished could involve transfer of LDL cholesterol to the lymphocyte membrane. Changes in plasma membrane cholesterol have been shown to alter both membrane fluidity and transferrin binding (38, 39). For example, cholesterol

depletion has been found to increase both membrane fluidity and the binding of transferrin to cell (38) and model membranes (39). We have demonstrated that cholesterol alone inhibits PHA-induced lymphocyte [³H]thymidine incorporation in serum-free medium and the inhibitory effect is prevented by transferrin. This result supports the conclusion that changes in membrane cholesterol may be involved in the mechanism of the inhibition.

Whereas transferrin may be required for DNA synthesis, it may also be necessary for cell division (40). Inhibition of this transferrin-requiring function by LDL would result in failure of lymphocyte cell division and inhibition of proliferation. We have previously demonstrated that concentrations of LDL that completely inhibit lymphocyte proliferation may have no effect on mitogen-stimulated DNA synthesis during the first cell cycle (5). Furthermore, we have presented data herein demonstrating that LDL, in concentrations that had no significant effect on mitogen-stimulated lymphocyte [³H]thymidine incorporation after 96 h (Table I), inhibited lymphocyte proliferation by nearly 40% ($P < 0.02$, Table V). The possibility remains that LDL exerts an additional inhibitory effect by blocking transferrin-dependent cell division, thereby preventing continuing proliferation of responding lymphocytes.

In summary, the results indicate that LDL inhibits mitogen-stimulated lymphocyte DNA synthesis and proliferation. In addition, they indicate that the mechanism whereby LDL is inhibitory is by interference with transferrin metabolism. This model system should be useful in dissecting the mechanism whereby LDL modulates growth of stimulated lymphocytes.

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