Immunoregulation by low density lipoproteins in man: low density lipoprotein inhibits mitogen-stimulated human lymphocyte proliferation after initial activation

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Abstract Low density lipoprotein (LDL, d 1.020–1.050 g/ml), isolated from normal human plasma by ultracentrifugation, inhibited mitogen-stimulated proliferation of human lymphocytes in a concentration-dependent manner. In order to characterize the inhibition more fully, the effect of LDL on initial lymphocyte activation and subsequent DNA synthesis was investigated. LDL had no effect on lymphocyte blast transformation assessed by quantitating the change in volume of the stimulated cells after a 24-hour incubation. Moreover, initial lymphocyte activation assessed by mitogen-stimulated RNA or protein synthesis was not inhibited by LDL. Finally, the acquisition of transferrin or T cell growth factor receptors by the activated lymphocytes was not affected by LDL. DNA synthesis, evaluated by measuring the incorporation of [^{3}H]thymidine between 30 and 48 hours of culture was inhibited by LDL in a concentration-dependent manner. The DNA content of individual mitogen-stimulated cells was analyzed by flow cytometry after mithramycin staining. These studies confirmed that LDL inhibited DNA synthesis in the initially activated lymphocytes. In addition, LDL in concentrations that did not inhibit initial DNA synthesis did suppress cell division and lymphocyte proliferation. These results indicate that LDL inhibits lymphocyte responses by exerting inhibitory effects on DNA synthesis, cell division, and subsequent growth of the activated cells rather than by altering initial lymphocyte blast transformation. LDL thus may play a role in regulating clonal expansion of activated lymphocytes during immune responses.—**Cuthbert, J. A., and P. E. Lipsky.** Immunoregulation by low density lipoproteins in man: low density lipoprotein inhibits mitogen-stimulated human lymphocyte proliferation after initial activation. *J. Lipid Res.* 1983. **24:** 1512–1524.

Supplementary key words DNA synthesis • immune responses

Plasma lipoproteins transport cholesterol, phospholipids, and triglycerides in the blood, facilitating the movement of lipids between various tissues and regulating lipid synthesis and catabolism (1). Besides these functions, it has been suggested that lipoproteins may be important in regulating the immune response (2–15). Human lipoproteins isolated from normal individuals and from patients with hyperlipidemia have been shown to inhibit both mitogen- and antigen-stimulated lymphocyte responses in vitro (9, 10). In these studies, chylomicrons and lipoproteins of the lighter density fractions, very low density lipoproteins (VLDL), intermediate density lipoprotein (IDL), and low density lipoprotein (LDL), were found to be potent inhibitors of lymphocyte proliferation stimulated by allogeneic cells and mitogens, whereas high density lipoprotein (HDL) had little inhibitory effect (9).

In addition to normal plasma lipoproteins, a number of subspecies of LDL have also been described that alter lymphocyte function (2–8, 11). A specific LDL fraction, isolated from the serum of patients with hepatitis, has been shown to suppress the ability of thymus-derived lymphocytes to express the receptor for the binding of sheep erythrocytes (11). Another species of lipoprotein, designated LDL-inhibitor (LDL-In) has been shown to suppress lymphocyte responses to specific antigens and nonspecific mitogens, both in vitro and in vivo (2–8).

The mechanism whereby lipoproteins inhibit lymphocyte responses has not been fully elucidated. Curtiss and Edgington (2, 6, 7) demonstrated that LDL-In was not cytotoxic and suggested that it might alter metabolic processes occurring during the initial in vitro activation of lymphocytes, since no inhibition was observed when LDL-In was added 24 hr after the stimulus. They also showed that LDL-In suppressed the in vitro generation of cytolytic T cells but did not affect the cytotoxic potential of differentiated killer cells (4). Furthermore, the induction of a primary humoral immune response in vivo was suppressed by LDL-In, although antibody secretion was not

Abbreviations: LDL, low density lipoproteins; PBM, peripheral blood mononuclear cells; PHA, phytohemagglutinin; HS, human serum.

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altered (3, 8). These findings suggested that LDL-In inhibited the initiation of lymphocyte proliferation in response to antigenic stimulation, but had little effect on the function of the differentiated cells generated during such responses (4, 8). Further in vitro studies of mitogen-stimulated human immunoglobulin synthesis supported the conclusion that LDL-In preferentially inhibited lymphocyte responses that were dependent on cell division for their expression, but had a lesser effect on responses that did not require lymphocyte proliferation (6). Thus, suppressor T cell function which requires cell proliferation (16, 17) was more sensitive to inhibition by LDL-In than helper T cell activity, which does not (16–18).

The inhibition produced by unfractionated LDL has not been characterized as well as the inhibitory effect of LDL-In. In particular, the ability of LDL to inhibit lymphocyte activation, although suggested, has not been established (12–15). Hui and Harmony (12–14), for example, have demonstrated that early events occurring after mitogenic stimulation such as phosphatidylinositol turnover and uptake of ionic calcium are suppressed by LDL. However, the relationship between these early metabolic processes and subsequent progress of lymphocytes through the cell cycle has not been completely defined.

Proliferation of lymphocytes stimulated by mitogens and antigens is the end result of a linked sequence of biochemical and morphologic events (19, 20) during which resting or quiescent G_0 lymphocytes are triggered to enter the cell cycle (20). Initial activation is followed by orderly progression through the cell cycle and mitosis. The daughter cells generated may then re-enter the cell cycle (20, 21). Lipoproteins could therefore inhibit lymphocyte proliferation by an effect on the initial activation process or by an action on the progress of activated lymphocytes through the first or subsequent cell cycles. The following studies were undertaken to examine in greater detail the nature of the inhibitory effect of LDL on human lymphocyte proliferation.

MATERIALS AND METHODS

Isolation of lipoproteins

Human LDL (d 1.020–1.050 g/ml) was isolated from plasma obtained from individual fasting normal adults, using a Beckman preparative ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA) and solid KBr for adjustment of density, as previously described (22). Protein concentrations were determined by the method of Lowry et al. (23). Total cholesterol concentrations were measured by the enzymatic method of Roeschlau, Bernt, and Gruber (24). The purity of lipoprotein fractions was confirmed by electrophoresis on agarose (22). Each individual LDL preparation is designated by a subscript (LDL_x).

Techniques of cell preparation and culture

Peripheral blood mononuclear cells (PBM) were separated from heparinized venous blood, obtained from healthy young adults, by centrifugation on a layer of sodium diatrizoate/Ficoll (Isolymph®, Teva Ltd., Jerusalem) as previously described (25). Cells were cultured in medium RPMI 1640 (Microbiological Associates, Walkersville, MD) to which was added L-glutamine (0.3 mg/ml), gentamicin (10 μg/ml), and penicillin G (200 units/ml). The medium was further supplemented with pooled human serum (HS). Phytohemagglutinin (PHA, Wellcome Reagents Ltd., Beckenham, England) was used as the stimulus for all cultures. In preliminary experiments, supplementation of medium with 1% HS was found to be adequate to support maximal PHA responses (Δcpm = 138,400 ± 6,600 in 1% HS, Δcpm = 103,200 ± 10,000 in 10% fetal bovine serum, mean ± SEM, n = 12).

PBM were incubated in sterile U-well microtiter plates (Dynatech Laboratories, Inc., Alexandria, VA) for the assay of [^{3}H]uridine, [^{3}H]leucine, or [^{3}H]thymidine incorporation, as previously described (25). For measurement of cell volume, cellular DNA content, and analysis of lymphocyte surface receptors, cells were cultured in 17 × 100 mm polypropylene tubes (Falcon Div., Becton Dickinson Co., Cockeysville, MD) and for measurement of cell proliferation, PBM were incubated in sterile flat-well microtiter plates (Falcon Div., Becton Dickinson Co.) as detailed previously (26). All cultures were incubated in a humidified atmosphere of 5% CO_2 and 95% air with a previously determined optimal PHA concentration (0.5 μg/ml) dissolved in Hanks' balanced salt solution (HBSS) or an equal volume of HBSS as control.

Assays of lymphocyte activation

Measurement of cell volume. Cell volume was measured using a Coulter channelyzer® (Curtin Matheson Scientific, Inc., Houston, TX). In these experiments, PBM were partially depleted of larger sized monocytes by glass adherence, using previously described techniques (27). After incubation for 24 hr, the cells were resuspended and cell volume was measured with the channelyzer. The number of cells in each of the 100 channels of 7.5 μm^3 was obtained, as described previously (26).

Measurement of lymphocyte RNA and protein synthesis. Incorporation of [^{3}H]uridine and [^{3}H]leucine was used to assay mitogen-induced RNA and protein synthesis, respectively, as previously reported (25). One μCi of [^{3}H]uridine (2 to 10 Ci/mmol) or 2 μCi of [^{3}H]leucine

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(5 Ci/mmol) (New England Nuclear Co., Boston, MA) was added to each well 18 hr before harvesting onto glass-fiber filter paper. Incorporation was determined by liquid scintillation spectroscopy.

Analysis of lymphocyte receptors by indirect immunofluorescence. Anti-Tac (a gift of Dr. Thomas A. Waldmann) and 5E9 (kindly provided by Dr. Barton F. Haynes) monoclonal antibodies were used for indirect immunofluorescence analysis of activated lymphocytes. These antibodies recognize receptors for T cell growth factor (anti-Tac) and transferrin (5E9) that do not appear on resting lymphocytes but develop on activated T cells. Their production and characterization has been previously reported (28–31). The technique of staining for fluorescence-activated cell sorter analysis has been described previously (32). Briefly, monocyte-depleted lymphocyte populations were obtained as detailed elsewhere (33) and the cells were incubated for 24 hr with or without PHA and LDL. After extensive washing, cells (2×10^7/ml) were incubated for 20 min at 4°C in a 1:1000 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 (32).

Assays of lymphocyte DNA synthesis

Measurement of cellular DNA content. Fluorescent staining of DNA with mithramycin was used to assay cellular DNA content (34). The resultant cell-associated fluorescence was quantitated by flow cytofluorimetry. After 48 hr incubation, cells were washed and resuspended in an aqueous solution of 25% ethanol with 100 μg/ml mithramycin and 15 mM $MgCl_2$ as previously described (26). A minimum of 10,000 cells per sample was analyzed for cell-associated fluorescence with a fluorescence-activated cell sorter (F.A.C.S. III, Becton Dickinson Co.). In this assay, fluorescence intensity is directly proportional to cellular DNA content. Vinblastine (0.05 μM) was added to cultures after 24 hr to prevent mitosis and allow quantitation of all initially activated cells. Preliminary experiments demonstrated that this concentration of vinblastine resulted in accumulation of cells with a mithramycin/DNA fluorescence intensity characteristic of the G_2 + M phases of the cell cycle and had no inhibitory effect on cellular DNA synthesis.

Measurement of [^{3}H]thymidine incorporation. This was used to assay mitogen-induced DNA synthesis as previously described (25). Briefly, 1 μCi of tritiated thymidine (6.7 Ci/mmol) was added 18 hr before harvesting the cells with a semiautomated microharvesting device and incorporation was determined by liquid scintillation spectrometry. Data are expressed as the difference in cpm between the means of triplicate mitogen-stimulated and control cultures (Δcpm). In some experiments, data are expressed as percentage inhibition of control, calculated according to the following equation:

$$\% \text{ inhibition} = [1 - (\Delta\text{cpm with LDL}/\Delta\text{cpm control})] \times 100$$

Measurement of lymphocyte proliferation

For determination of the number of cells in culture, PBM were incubated in flat-bottom microtiter wells. After incubation for 6 days, an aliquot of lysing agent (Zap-Isoton®) was added to the cultures to disrupt the plasma membrane, as previously described (26). Free nuclei were counted using a Coulter counter and the total number of cells in each microtiter well was calculated. In some experiments, data are expressed as percentage inhibition of control, calculated as above.

RESULTS

LDL inhibits mitogen-stimulated lymphocyte growth and [^{3}H]thymidine incorporation

Stimulation by a mitogenic lectin such as PHA causes multiple waves of lymphocyte cell division with subsequent increase in the number of cells in the culture (26). As shown in the three experiments depicted in **Fig. 1,** after a 6-day incubation, PHA stimulation resulted in a marked increase in the number of cells. In the first two experiments (LDL_A and LDL_B), the number of cells increased from 100,000 per well initially cultured to more than 400,000 per well, and in the third experiment (LDL_E) there was a sevenfold increase, from 50,000 cells initially to 350,000 cells. The effects of three different lipoprotein preparations, LDL_A, LDL_B, and LDL_E, are shown. When LDL was added, there was a concentration-dependent inhibition of mitogen-stimulated lymphocyte growth. At the highest concentrations of LDL added (LDL_A 920 μg of protein/ml; LDL_B, 220 μg of protein/ml; LDL_E, 500 μg of protein/ml) lymphocyte proliferation was completely inhibited. There was only a minimal effect on unstimulated cultures. When LDL_A and LDL_B were tested on cells obtained from five additional individuals, the mean concentration of lipoprotein required to inhibit PHA-stimulated lymphocyte growth by 50% was 334 μg of protein/ml for LDL_A (range 179–551 μg of protein/ml) and 74 μg of protein/ml for LDL_B (range 54–106

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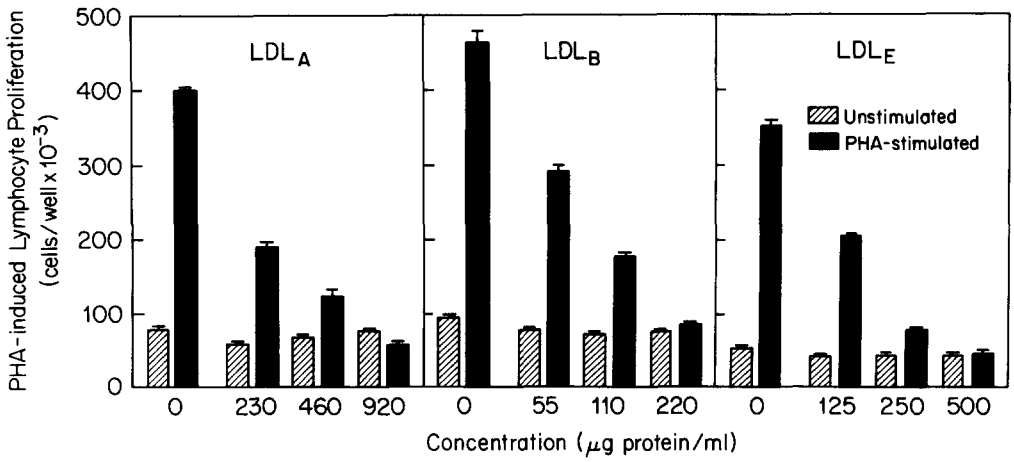

Fig. 1. LDL inhibits lymphocyte growth in a concentration-dependent manner. PBM were incubated for 6 days with or without PHA and varying concentrations of LDL as indicated. Initial cultures contained 100 × 10^3 cells (LDL_A and LDL_B) or 50 × 10^3 cells (LDL_E). Data represent mean ± SEM of triplicate determinations.

μg of protein/ml). Additional LDL preparations differed in the absolute concentration required to inhibit proliferation but the overall effects were always similar. In 12 experiments using four other samples of LDL, the concentration required for 50% inhibition ranged from 42 μg of protein/ml to 376 μg of protein/ml.

LDL was also found to inhibit mitogen-stimulated lymphocyte DNA synthesis as shown in **Fig. 2.** These experiments examined the effect of various LDL preparations on PHA-induced lymphocyte [^{3}H]thymidine incorporation, assayed after 48 or 96 hr incubation. As can be seen, each LDL preparation caused concentration-dependent inhibition of mitogen-stimulated [^{3}H]thymidine incorporation. Moreover, in each experiment, the degree of inhibition of [^{3}H]thymidine incorporation was greater when the cells were harvested after a 4-day rather than a 2-day incubation. This was particularly striking with lower concentrations of LDL. One explanation for finding increased inhibition after a 4-day as compared to a 2-day incubation was that alteration of lymphocyte function may have required prolonged exposure of the cells to LDL in order to become apparent. In order to test this possibility, cells were preincubated with an inhibitory concentration of LDL for 4 days and then were washed and cultured with PHA in the presence or absence of the same concentration of LDL. As shown in **Table 1,** a 4-day preincubation with LDL did not alter subsequent mitogen responses and had little effect on the susceptibility of the cells to inhibition by LDL. These results indicate that an inhibitory effect does not result from even a prolonged preincubation with LDL. Moreover, preincubation with LDL did not alter the cells such that they were subsequently more sensitive to LDL-mediated inhibition of mitogen responsiveness.

One possible mechanism for the finding of a decrease in the incorporation of [^{3}H]thymidine was that LDL inhibited thymidine transport. This possibility was examined by incubating cells for 72 hr without LDL and then adding the lipoprotein for the final 24 hr of incubation. Two representative experiments are shown in **Fig. 3.** When LDL was present during the entire 4-day incubation period, concentration-dependent inhibition of [^{3}H]thymidine incorporation was observed. However, if LDL was only present for the final 24 hr of the culture, there was no inhibitory effect. Thus, delaying the addition of LDL prevented the inhibition, indicating that thymidine transport was not directly inhibited by LDL.

In order to confirm that LDL exerted an inhibitory action on mitogen-stimulated lymphocyte DNA synthesis, the DNA content of individual cells was measured using flow cytofluorimetry after mithramycin staining (26, 34). This method permits quantitation of the DNA content of single cells by assaying the fluorescence of mithramycin-DNA complexes (34). Experiments with the different LDL preparations are shown in **Fig. 4.** Unstimulated cells in each experiment have a uniform fluorescence intensity corresponding to their diploid DNA content with less than 10% of the cells containing more than diploid DNA content. After 48 hr, 34.5 ± 3.0% (mean ± SEM, n = 4) of the PHA-stimulated cells in control cultures had synthesized new DNA. Each LDL preparation inhibited DNA synthesis in a concentration-dependent manner similar to that observed when [^{3}H]thymidine incorporation was measured.

These experiments demonstrated that LDL inhibited mitogen-stimulated lymphocyte growth and DNA synthesis. Increased inhibition was observed with either higher concentrations of LDL or longer lengths of incubation. Since these experiments assayed lymphocyte DNA synthesis and proliferation, which occurred many hours after the mitogenic stimulation, they could not

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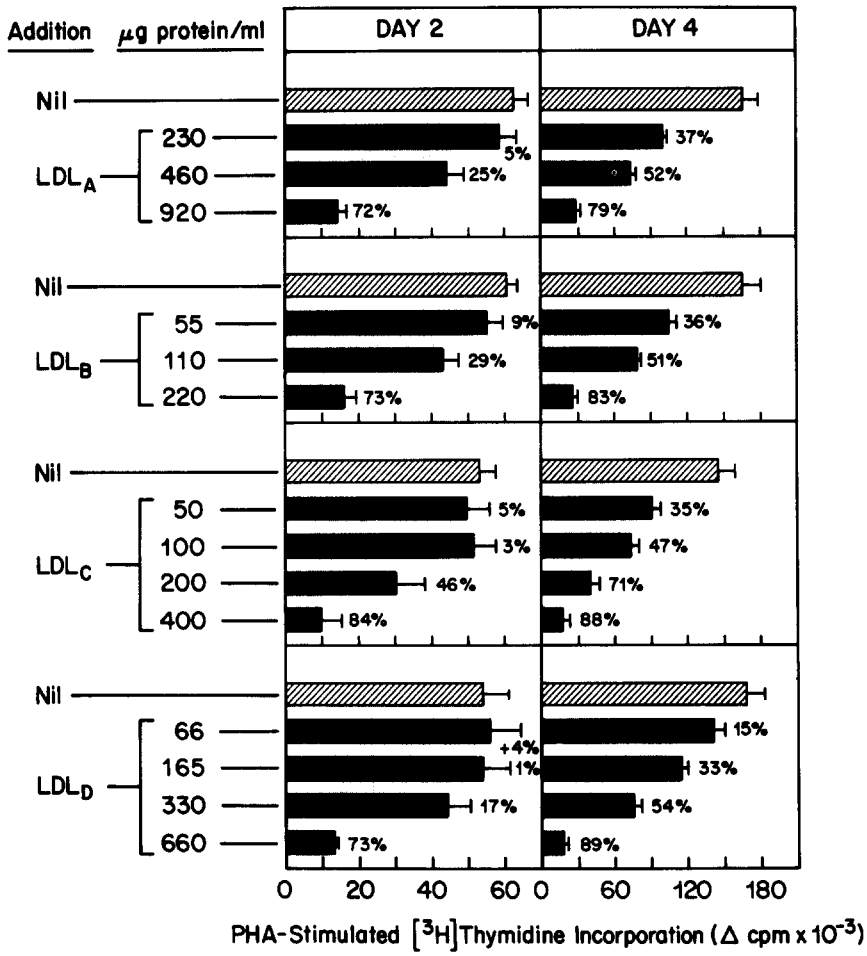

Fig. 2. Effect of LDL preparations on PHA-stimulated lymphocyte [^{3}H]thymidine incorporation after a 4-day incubation. PBM were incubated with PHA and varying concentrations of LDL as indicated. Data represent mean of three to seven experiments, for each LDL preparation. Mean percentage inhibition by the concentration of LDL is given at the end of each bar.

determine whether LDL inhibited initial lymphocyte activation or rather later events in the cell cycle. The following experiments were therefore done to determine whether LDL also inhibited initial lymphocyte activation.

Lack of effect of LDL on lymphocyte blast transformation

Blast transformation in activated lymphocytes occurs within hours of mitogenic stimulation and thus precedes initial DNA synthesis which commences after 30–36 hr. We therefore examined the effect of LDL on blast transformation after a 24-hr incubation, at which time there is no DNA synthesis in the activated lymphocytes. Blast transformation of lymphocytes in response to PHA was quantitated by measuring cell volume using a Coulter channelyzer®. This method permits calculation of the mean volume of the entire cell population and the percentage of cells responding to mitogenic stimulation by

TABLE 1. Effect of preincubation with LDL on mitogenic responses

Preincubation	Addition	PHA-Stimulated [^{3}H]Thymidine Incorporation[a] Expt. 1	Expt. 2
		Δcpm × 10^{-3}	
Nil	Nil	140.1	103.4
	LDL_F		50.9
Medium	Nil	197.2	143.4
	LDL_F	86.8	78.2
LDL_F	Nil	209.5	124.0
	LDL_F	84.3	68.1

[a] PBM were incubated with or without PHA and LDL_F (Expt. 1 = 175 μg of protein/ml, Expt. 2 = 350 μg of protein/ml) and [^{3}H]thymidine incorporation was measured after 4 days. PBM were freshly isolated (preincubation = Nil) or preincubated with medium or LDL_F (same concentrations as above) for 4 days before the incubation with PHA. Data represent mean of triplicate determinations.

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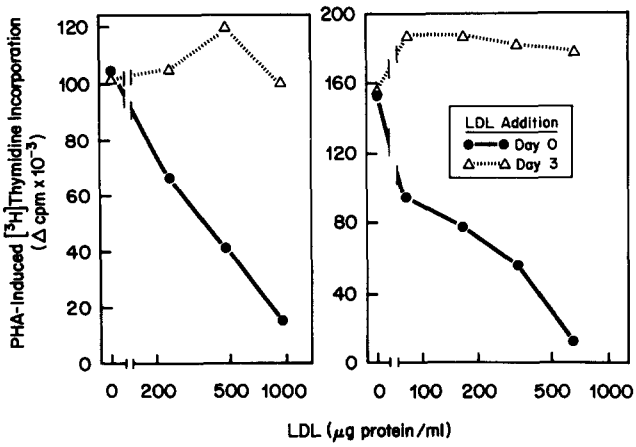

Fig. 3. Effect of delayed addition of LDL on PHA-induced [^{3}H]thymidine incorporation. PHA was added at the initiation of culture (day 0) and LDL_A (left panel) or LDL_F (right panel) at the same time (day 0) or after 72 hr (day 3). [^{3}H]Thymidine incorporation was determined after a 4-day incubation. Data represent the mean of triplicate determinations.

undergoing blast transformation (26). As shown in **Fig. 5,** analysis of cell volume after a 24-hr in vitro incubation indicated that unstimulated cells were distributed in a narrow peak, with a mean volume of less than 200 μm^3. There were few blasts (larger than 250 μm^3). In contrast, after a 24-hr incubation with PHA, the average cell volume increased to 280 μm^3 and 54% of the cells were blasts (larger than 250 μm^3). The addition of LDL at concentrations that inhibited [^{3}H]thymidine incorporation (Fig. 2) and completely prevented lymphocyte proliferation (Fig. 1) did not alter initial lymphocyte activation since equal numbers of larger cells were found (Fig. 5). In five separate experiments, these LDL preparations had no effect on PHA-induced blast transformation (**Table 2**). LDL had no effect on the percent of blast cells or the average volume of the activated cells found in PHA-stimulated cultures. Similar results were obtained with other LDL preparations. Thus, LDL in concentrations that inhibited mitogen-stimulated DNA

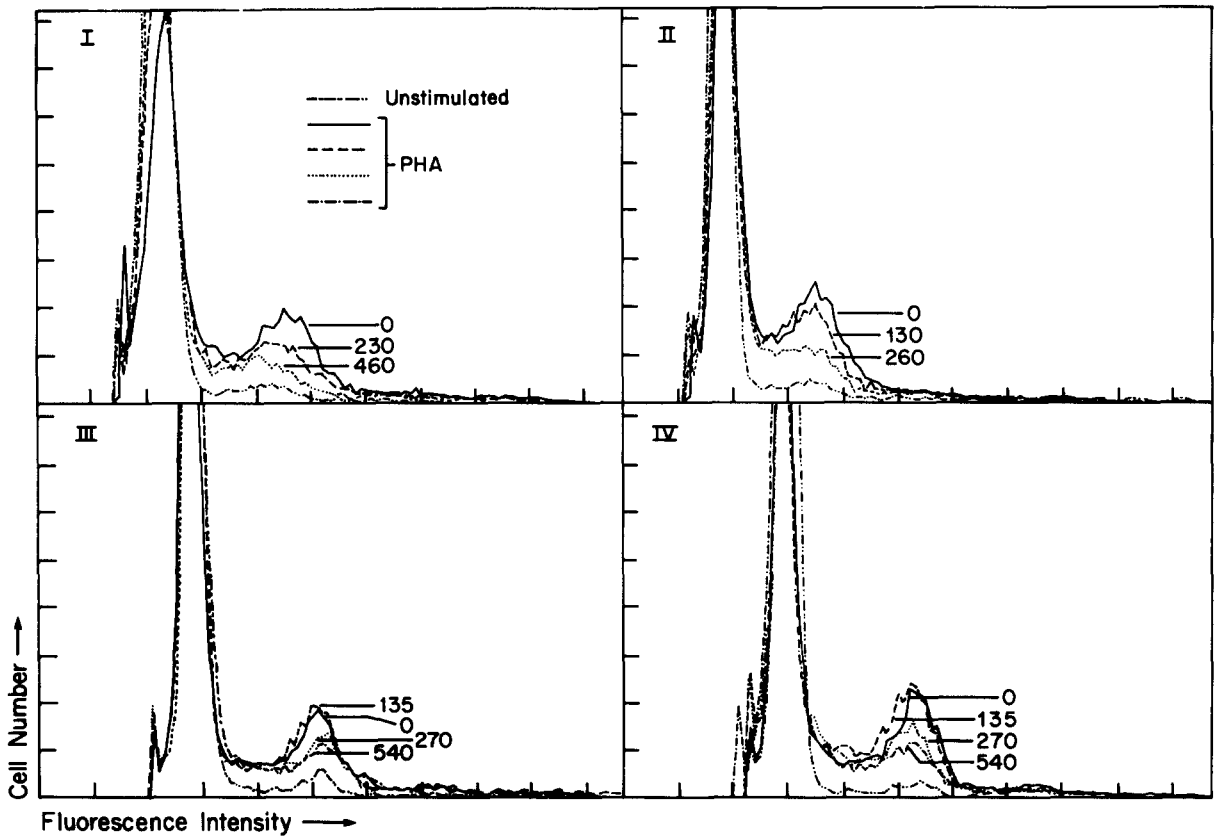

Fig. 4. LDL inhibits lymphocyte DNA synthesis. PBM were incubated with or without PHA and varying concentrations of LDL_A (230, 460 μg of protein/ml, panel 1), LDL_G (130, 260 μg of protein/ml, panel 2), LDL_H (135, 270, 540 μg of protein/ml, panels 3 and 4), as indicated. After a 48-hr incubation the cells were stained with mithramycin. Vinblastine had been added at 36 hr to prevent cell division. DNA content of individual cells is proportional to fluorescence intensity, assessed with the use of the FACS III. Unstimulated cells (–· · –· · –) can be seen to be distributed in a narrow peak corresponding to a diploid DNA content. Cells which have synthesized new DNA are distributed along the x axis further from the origin with cells in the G_2 + M phase of the cell cycle forming a peak with twice the fluorescence intensity of resting cells. LDL and vinblastine had no effect on unstimulated cells.

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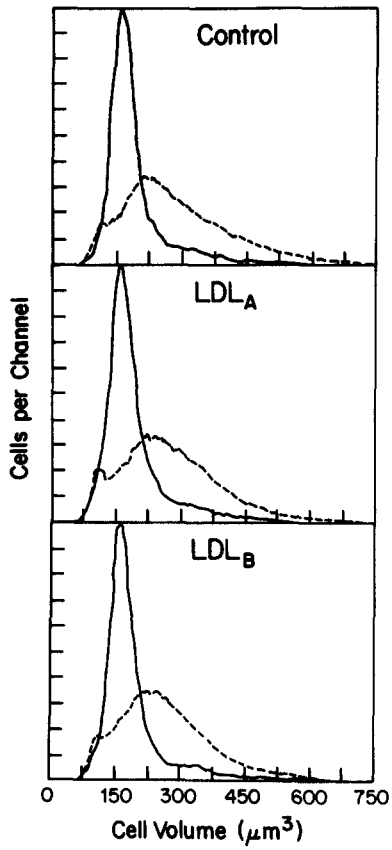

Fig. 5. LDL does not inhibit blast transformation. PBM, partially depleted of monocytes, were incubated for 24 hr with (– – –) or without (——) PHA and LDL_A (920 μg protein/ml) or LDL_B (220 μg of protein/ml) as indicated. Cell volume was measured by using a Coulter channelyzer®, and a size distribution profile was obtained for approximately 50,000 cells.

synthesis and completely prevented PHA-induced lymphocyte proliferation did not affect the proportion of cells initially stimulated by PHA to undergo blast transformation.

LDL does not inhibit RNA and protein synthesis

The observation that LDL had no effect on mitogen-stimulated blast transformation suggested that initial lymphocyte activation was not affected. In order to investigate this possibility in greater detail, the effect of LDL on other aspects of initial lymphocyte activation was examined. LDL was found to have no effect on mitogen-induced RNA and protein synthesis assayed by the incorporation of [^{3}H]uridine and [^{3}H]leucine, respectively, after a 24-hr incubation with PHA (**Table 3**). When DNA synthesis was measured by the incorporation of [^{3}H]thymidine after a 48-hr incubation, LDL at concentrations that had no effect on RNA and protein synthesis at 24 hr was found to be significantly inhibitory (Table 3).

LDL does not prevent appearance of new receptors

The lack of effect of LDL on lymphocyte blast transformation and RNA and protein synthesis supported the conclusion that initial lymphocyte activation was unaffected by LDL. In order to confirm this finding, lymphocyte activation was assessed by measuring the expression of cell surface receptors identified by the monoclonal antibodies anti-Tac and 5E9. These receptors (T cell growth factor (Tac) and transferrin (5E9), respectively) are not found on resting, quiescent lym-

TABLE 2. LDL does not prevent PHA-induced blast transformation

	Lymphocyte Blast Transformation at 24 Hours[a]			
	Large Cells (%)		Mean Volume (μm^3)	
Addition	>250 μm^3	>400 μm^3	Total Cells	Activated Cells
Nil	13 ± 1	3 ± 1	192 ± 3	
PHA	48 ± 2	11 ± 1	263 ± 5	352 ± 4
PHA + LDL_A				
460 μg protein/ml	50 ± 2	11 ± 1	265 ± 4	351 ± 2
920 μg protein/ml	49 ± 2	10 ± 1	261 ± 4	348 ± 2
PHA + LDL_B				
110 μg protein/ml	48 ± 2	10 ± 1	261 ± 3	347 ± 2
220 μg protein/ml	46 ± 2	9 ± 1	256 ± 4	344 ± 1

[a] PBM (partially depleted of monocytes) were incubated with or without PHA and LDL. Cell volume was measured using a Coulter channelyzer® after a 24-hr incubation. The percentage of large cells (blasts > 250 μm^3) and very large cells (>400 μm^3) and the mean volume of the total cell population and of the activated cells (>250 μm^3) were calculated. LDL had no significant effect on unstimulated cells. Data represent mean ± SEM of five separate experiments.

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TABLE 3. LDL does not inhibit RNA and protein synthesis

Addition	PHA-Induced [^{3}H]Precursor Incorporation[a]		
	Uridine	Leucine	Thymidine
	Δcpm × 10^{-3}		
Nil	14.2 ± 1.4	1.6 ± 0.1	46.1 ± 4.4
LDL_J 160 μg protein/ml	15.1 ± 1.0	1.8 ± 0.1	27.4 ± 3.0
Nil	13.7 ± 1.4	1.0 ± 0.1	41.0 ± 4.7
LDL_K 330 μg protein/ml	11.6 ± 1.6	1.3 ± 0.1	25.9 ± 1.4

[a] PHA-induced precursor incorporation was measured after 24 (uridine and leucine) or 48 hr (thymidine) incubation. Data represent mean ± SEM of three separate experiments.

phocytes but appear following activation of T cells by mitogens, soluble antigens, and allogeneic cells (28–31). Analysis with the fluorescence-activated cell sorter demonstrated that unstimulated cells had few positive cells, 1.0 ± 0.4% (mean ± SEM, n = 3), when stained with the anti-Tac antibody, and LDL had no effect on unstimulated cells. More than 60% of lymphocytes expressed the Tac antigen after a 24-hr culture with PHA (**Table 4**). LDL had no significant effect on the acquisition of the Tac antigen (Table 4, **Fig. 6**) although the same concentrations of LDL inhibited [^{3}H]thymidine incorporation by 46.7 ± 4.7% after a 48-hr incubation. Similarly, LDL had no effect on the number of the receptors as evidenced by a similar degree of fluorescence intensity in the positively staining cells (Fig. 6). Comparable results were obtained when the monoclonal antibody 5E9 was used. Thus, more than 25% of PHA-stimulated lymphocytes acquired transferrin receptors as identified by 5E9 and this was not altered by LDL (Table 4). These results all support the conclusion that lymphocyte activation is not altered by LDL.

LDL inhibits lymphocyte responses occurring after initial DNA synthesis

The previous experiments demonstrated that LDL did not inhibit lymphocyte activation but did inhibit mitogen-stimulated DNA synthesis. The possibility that LDL also inhibited responses beyond initial DNA synthesis was then investigated. Under optimal conditions, lymphocytes responding to mitogenic stimulation divide after approximately 48 hr in culture. The newly generated daughter cells may then re-enter the cell cycle. Consequently, [^{3}H]thymidine incorporation measured after 4 days quantitates DNA synthesis in activated daughter cells. In the initial experiments described above, the inhibitory effect of LDL was found to be greater after 4 days than after a 2-day incubation. The greater inhibition of [^{3}H]thymidine incorporation after 4 days may therefore result from inhibition of DNA synthesis in the initially activated cells with resultant inhibition of proliferation. Alternatively, responses occurring after DNA synthesis has been completed may be suppressed. In order to examine these possibilities, several different aspects of lymphocyte responses were measured in the same experiment. As shown in **Table 5,** LDL did not alter initial lymphocyte activation, measured by the mitogen-stimulated increase in lymphocyte RNA synthesis after 24 hr, regardless of the concentration added. LDL did inhibit lymphocyte DNA synthesis in a concentration-dependent manner and the inhibitory effect was greater after 4 days than after 2 days, as previously described. Comparison of the results obtained with different concentrations of LDL demonstrated two different effects. At the lowest concentration of LDL, there was little inhibition of DNA synthesis after a 2-day incubation (9.4 ± 2.1%), however, there was significant inhibition after 4 days (45.5 ± 8.4%) and proliferation was almost completely prevented. These results indicate that LDL at low concentrations inhibits events occurring after initial DNA synthesis. In contrast, with high concentrations of LDL there was substantial inhibition of initial DNA synthesis, measured after 2 days and little additional inhibition with longer incubation. These results indicate that high concentrations of LDL inhibit lymphocyte proliferation by suppressing DNA synthesis in the initially activated cells and lower concentrations inhibit proliferation after initial DNA synthesis is completed.

DISCUSSION

Studies of the effect of lipoproteins on mitogen-stimulated lymphocytes have led a number of investigators

TABLE 4. LDL does not inhibit appearance of new receptors

Antibody	PHA-Induced Lymphocyte Response	
	Control	LDL_E
	% positive[a]	
Control ascites	1.3 ± 0.2	0.8 ± 0.4
Anti-Tac	63.7 ± 4.3	61.8 ± 4.6
5E9	29.1 ± 4.5	26.0 ± 7.2

[a] Monocyte-depleted lymphocyte populations were incubated with or without PHA and LDL_E (250 μg of protein/ml) as indicated. After washing, the cells were reacted with either anti-Tac, 5E9, or a mouse ascites fluid 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 anti-Tac was then calculated. Unstimulated cells were 2.4 ± 0.3% positive with control ascites, 1.0 ± 0.4% positive with anti-Tac, and 1.5 ± 0.4% positive with 5E9. The results represent the mean ± SEM of three experiments.

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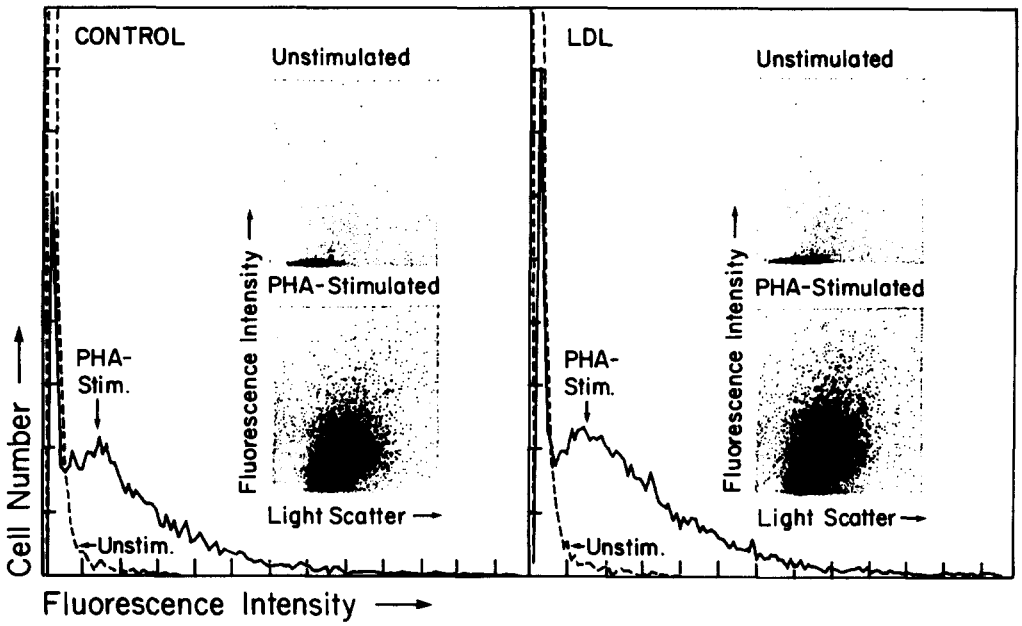

Fig. 6. LDL does not prevent appearance of Tac. PBM, depleted of monocytes, were incubated for 24 hr with (——) or without (– – –) PHA and LDL_E (250 μg of protein/ml) as indicated and prepared for fluorescence analysis with anti-Tac antibody. The relative number of cells in each sample with specific fluorescence intensity was analyzed by the FACS III. The insets depict analysis of 10,000 cells, for light scatter vs. fluorescence intensity. Each dot represents a single cell whose position on the plot is determined by its light scattering properties (size) and its fluorescence (anti-Tac positivity) intensity.

to suggest that LDL may have the capacity to regulate lymphocyte function (2–9, 12–15). Chisari and Edgington (11) originally demonstrated that lipoprotein fractions isolated from normal serum and from patients with hepatitis were able to inhibit the mixed lymphocyte reaction. Later, Waddell, Taunton, and Twomey (10) examined the inhibitory effect of lipoproteinemic plasma. They reported that both VLDL and chylomicrons inhibited lymphocyte [^{3}H]thymidine incorporation stimulated by mitogenic lectins or allogeneic leukocytes. Morse, Witte, and Goodman (9) extended these observations by systematically comparing the effects of the different classes of lipoproteins on lymphocyte responses. All classes of normal plasma lipoproteins were shown to be inhibitory with the lighter density ones (VLDL, IDL, and LDL) causing the most inhibition. It was therefore suggested that lipoproteins may modulate immune responses.

Curtiss and Edgington and their co-workers (2–8) have characterized a specific fraction of lipoprotein, designated LDL-In. They demonstrated that LDL-In suppressed both antigen- and mitogen-induced human T and B lymphocyte responses in vitro (2, 4–8). Additional experiments have shown that LDL-In is also effective in vivo, suppressing the murine primary antibody response (3). Studies of the interaction between

TABLE 5. LDL inhibits lymphocyte responses after initial DNA synthesis

Length of Incubation	Assay	PHA-induced Lymphocyte Response[a] LDL (μg protein/ml)		
		135	270	540
			% inhibition	
1 day	[^{3}H]Uridine incorporation	+3.8 ± 6.2	+10.8 ± 9.6	+1.3 ± 8.6
2 days	[^{3}H]Thymidine incorporation	9.4 ± 2.1	38.0 ± 1.6	73.1 ± 8.8
4 days	[^{3}H]Thymidine incorporation	45.5 ± 8.4	82.9 ± 7.8	97.9 ± 1.9
7 days	Lymphocyte proliferation	93.4 ± 3.7	100	100

[a] PBM were incubated with or without PHA and varying concentrations of LDL_H. RNA synthesis was measured after 24 hr by the incorporation of [^{3}H]uridine (without LDL, Δcpm = 6,500 ± 2,000). DNA synthesis was measured after 2 days and 4 days by the incorporation of [^{3}H]thymidine (without LDL, Δcpm = 44,600 ± 2,200 day 2, 118,700 ± 9,000 day 4). Lymphocyte proliferation was quantitated after 7 days by counting the number of cells per microtiter well (without LDL, 269,200 ± 25,900 Δ cells per well). Results are mean ± SEM of four experiments.

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LDL-In and the lymphocyte plasma membrane have indicated that LDL-In may bind to a specific membrane receptor on the lymphocyte surface, distinct from the receptor for normal LDL (5). It should be pointed out that the relationship between inhibition caused by LDL-In and that resulting from other lipoprotein fractions has not been clearly established.

The mechanism(s) whereby lipoproteins inhibit lymphocyte responses has not been completely defined. Lymphocyte activation by mitogen is accompanied by complex changes in the plasma membrane, cytoplasm, and nucleus (19, 20). During the first few hours there is uptake of calcium and potassium ions and lipid turnover (20). Hui and Harmony (12–15) have examined the effect of LDL on a series of these early events. In their experiments, mitogen-induced changes in calcium and cyclic guanosine 3′,5′-monophosphate (GMP) accumulation and phosphatidylinositol turnover were measured (12–15). LDL inhibited these changes in a concentration-dependent manner even after being depleted of cholesterol. Moreover, LDL bound to Sepharose, and thus unable to be internalized, was also inhibitory (14). These results suggested that the interaction of apoproteins or other nonsterol constituents of LDL with the lymphocyte plasma membrane was sufficient for the inhibitory effect. However, the implications of these findings with regard to the effects of lipoproteins on lymphocyte function remain somewhat conjectural since the relationship between these early biochemical events, that occur within 1–2 hr after mitogenic stimulation, and more complex events, such as blastogenesis and proliferation which develop later in culture, has not been clearly established. For example, the biochemical processes requiring initial calcium uptake and phospholipid turnover have not been identified (20). Similarly, the role of cyclic GMP in lymphocyte activation has not been defined (20). Thus, although these early biochemical changes occur, they may not be essential for lymphocyte growth in culture. Consequently, LDL may inhibit lymphocyte growth by other mechanisms. The studies described herein were undertaken in order to define more clearly the inhibitory action of normal LDL in lymphocyte function and to identify the phase in the cell cycle when lymphocytes were inhibited.

The results presented here demonstrated that normal human LDL inhibited mitogen-induced lymphocyte DNA synthesis and proliferation. The initial studies indicated that LDL prevented or decreased cell proliferation by mitogen-stimulated lymphocytes and did not merely interfere with thymidine transport and incorporation. There was considerable variability in the degree of inhibition caused by different LDL preparations, and also differences in the inhibitory action of the same LDL when tested on cells obtained from different normal individuals. Despite the variability between preparations, the results were qualitatively similar for all preparations of LDL.

The possibility that the resultant inhibitory effects of LDL are caused by a direct cytotoxic effect of the LDL with increased length of incubation appears unlikely for a number of reasons. LDL did not appear to alter cell viability of resting or mitogen-stimulated lymphocytes. Moreover, preincubation of lymphocytes with LDL for as long as 4 days was found to have no effect on their subsequent capacity to respond to mitogens. Finally, additional experiments indicated that LDL had minimal toxic effects on mitogen-stimulated cells. Thus, in experiments not shown, lymphocytes were incubated for 48 hr with PHA and LDL. Tritiated thymidine incorporation was inhibited by 40–60% at 48 hr. The LDL was then removed by washing and the lymphocytes were re-cultured with PHA in fresh medium without LDL. After an additional 72-hr incubation, the cells were reassayed for [^{3}H]thymidine incorporation and found not to differ in the magnitude of their response from control cells that had not been exposed to PHA during the initial 48 hr. These results indicate that LDL had no cytotoxic effect on unstimulated or mitogen-stimulated lymphocytes.

Suppression of mitogen-induced lymphocyte growth can be the end result of inhibition of one or more of a variety of biological events necessary for the activation and growth of cells. In order to define more precisely the processes inhibited by LDL, several different aspects of this response were examined. Mitogen-induced blast transformation, RNA and protein synthesis, and expression of receptors characteristic of activated lymphocytes were not altered by LDL, regardless of the concentration employed. Hui and Harmony (12–15) reported that LDL inhibited a number of metabolic processes that occur more proximate to mitogen exposure, such as changes in cellular calcium accumulation and phosphatidylinositol turnover and suggested that lymphocyte activation was inhibited by LDL. There are a number of possible explanations for the discrepancy between the results of Hui and Harmony and those reported here. Variation in the cultural conditions might have explained the differences. We feel that this is an unlikely explanation. Thus, for example, we have found that mitogen-induced blast transformation occurs in serum-free medium similar to that used in the above report and is unaffected by LDL.[1] However, differences in cell density or other features of the culture system may still have some effect on the results, although in the current

[1] Cuthbert, J. A., and P. E. Lipsky. Unpublished observations.

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experiments it is clear that LDL does not prevent lymphocyte activation. Alternatively, the use of different LDL preparations may have contributed to the discrepancy in conclusions. In order to exclude this possibility, we have examined the effect of LDL provided by Dr. J. A. K. Harmony. The results obtained were similar to the findings reported here, in that lymphocyte activation as measured by blast transformation was unaffected by LDL preparations that markedly inhibited mitogen-induced phosphatidylinositol turnover.[1] Another possibility is that some of the "early events" that result from mitogen stimulation may not be involved in the process of lymphocyte activation. Thus, the difference between the results reported herein and those of Hui and Harmony would be consistent with the conclusion that increased lymphocyte calcium accumulation, cyclic GMP production, and phosphatidylinositol turnover occur after mitogenic stimulation, but are not necessary for lymphocyte blast transformation. An alternative and more intriguing explanation is that some of the early metabolic changes that occur soon after mitogen stimulation, such as calcium accumulation, may not be necessary for initial blast transformation but could be required for later biochemical processes such as lymphocyte DNA synthesis. This conclusion is consistent with the observation that prevention of calcium accumulation with inhibitors other than LDL, such as citrate and EDTA, has been found to block mitogen- and alloantigen-stimulated lymphocyte [^{3}H]thymidine incorporation (35, 36). Preliminary experiments in our own laboratory have also supported this conclusion in that EDTA and EGTA, which prevent calcium accumulation, inhibit PHA-stimulated [^{3}H]thymidine incorporation. However, these chelating agents have no effect on the incorporation of [^{3}H]uridine and [^{3}H]leucine into RNA and protein, respectively, or initial blast transformation as measured with the channelyzer.[1] Regardless of the explanation for the discrepancy, the results presented herein indicate that LDL does not inhibit initial lymphocyte responses including mitogen-stimulated RNA and protein synthesis, blast transformation, and acquisition of new membrane receptor proteins.

LDL-induced inhibition of DNA synthesis, observed by measuring the incorporation of [^{3}H]thymidine, was confirmed by flow cytometric analysis. The mechanism(s) whereby LDL inhibited lymphocyte DNA synthesis is unknown. LDL may directly inhibit the process of DNA synthesis or could be inhibitory by an indirect mechanism such as by altering signals necessary for progression into and through the S phase. Growth of T lymphocytes in culture appears to require factors or signals from other cells as well as uptake of a variety of nutrients from the medium (37–40). One possible explanation, therefore, is that LDL alters the synthesis or degradation of the required factor(s) or prevents their action. Alternatively, the integrity and function of lymphocyte plasma membrane or the expression and orientation of membrane receptors may be affected by LDL through mechanisms such as altering calcium accumulation, phospholipid turnover, or disturbing the cholesterol-phospholipid ratio. The uptake of necessary growth factors or reception of other signals from regulatory cells could then be suppressed.

Lymphocyte responses measured after longer periods of incubation were more inhibited by LDL. Thus, there was greater suppression of [^{3}H]thymidine incorporation after 4 days than after 2 days, and lymphocyte proliferation, quantitated after 6 or 7 days, demonstrated the greatest inhibitory effect of LDL. Responses measured beyond 48 hr are dependent upon activation of newly generated daughter cells, since the initially activated cells have divided. Therefore, measurements of [^{3}H]thymidine incorporation at 4 days and proliferation at 6 days reflect multiple rounds of cell division. The experiments described above indicate that LDL also inhibits events after initial DNA synthesis. This additional inhibitory effect of LDL may explain part or all of the increased inhibition observed with prolonged incubation.

LDL may inhibit both endogenous sterol synthesis and DNA synthesis in a related manner. In resting lymphocytes, exogenous LDL regulates the rate of endogenous synthesis of cholesterol (41). In previous experiments we have shown that lymphocyte DNA synthesis is suppressed by inhibition of the rate-limiting enzyme in cholesterol synthesis, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase with the competitive inhibitors ML-236B or mevinolin (26, 42). The inhibition was similar in character to that reported to occur with other types of cultured cells and resulted from severely limiting the availability of mevalonate, the product of the inhibited enzyme (26, 42–44). However, LDL-mediated inhibition of the activity of HMG-CoA reductase does not appear to be of sufficient magnitude to limit the availability of mevalonate necessary for nonsterol products (45). Furthermore, additional experiments in our laboratory have confirmed the findings of other investigators (7) that mevalonate can not reverse LDL-induced inhibition of lymphocyte DNA synthesis. Therefore, it appears unlikely that LDL and ML-236B inhibit lymphocyte DNA synthesis by the common mechanism of limiting HMG-CoA reductase activity.

All the experiments demonstrated that LDL does not inhibit initial lymphocyte activation at any concentration. The lack of effect of LDL on morphologic blast transformation, RNA and protein synthesis, and on the appearance of receptors for T cell growth factor and transferrin supports this conclusion. The inhibitory ef-

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fect of LDL is first observed as an inhibition of DNA synthesis in mitogen-stimulated lymphocytes and is confirmed by the finding of concentration-dependent inhibition of lymphocyte proliferation. Proliferation of both T lymphocytes and B lymphocytes is necessary for effective immune responses. Clonal expansion of antigen specific T and B lymphocytes involves DNA synthesis and proliferation of the relevant precursor cells. Such clonal expansion is required for the development of maximal humoral and cell-mediated responses as well as the development of immunological memory. Thus, LDL may modulate the primary response to antigenic stimulation by inhibiting proliferation and thereby decreasing the number of cells available to participate in a secondary response upon re-exposure to the antigen. Similarly, LDL may inhibit the proliferation of cytotoxic T lymphocyte precursors and B lymphocyte precursors of antibody-secreting cells. In contrast, the function of the differentiated effector cytotoxic cells or plasma cells, which do not require further proliferation or clonal expansion, would not be affected. Thus, LDL may play an important role in the modulation of normal human immune responses.

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