

Plasma lipoproteins and transferrin regulate the proliferation of a continuous T lymphocyte cell line

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Abstract Lipoproteins of hydrated densities <1.063 g/ml, very low density (VLDL) and low density (LDL) lipoproteins, could both enhance and suppress the proliferation of T lymphocyte cell lines. Enhancement and suppression were dependent on lipoprotein and transferrin concentrations. Enhancement occurred at low lipoprotein and high transferrin; suppression, at high lipoprotein and low transferrin. Lipoprotein suppression required a constituent of cell-conditioned medium as evidenced by the fact that lipoproteins did not suppress the replicative response of the IL-2-dependent murine cell line CTLL-2 to purified IL-2 but could suppress the response to cell-conditioned medium IL-2. For lipoprotein suppression and its relief by transferrin, both growth-regulating factors were required early in the cell cycle, suggesting that events important to progression through G1 are influenced. ■ The data establish that the interplay between plasma lipoproteins, transferrin, and an unknown constituent of cell-conditioned medium can regulate the proliferation of T lymphocytes.—McCarthy, B. M., Y. Okano, T. Nakayasu, M. Macy, S. R. Watson, and J. A. K. Harmony. Plasma lipoproteins and transferrin regulate the proliferation of a continuous T lymphocyte cell line. *J. Lipid Res.* 1987. 28: 1067–1077.

Supplementary key words LDL • VLDL • DNA synthesis • murine cell line CTLL-2

Polyclonal T lymphocyte mitogens, such as phytohemagglutinin (PHA) and neuraminidase-galactose oxidase, activate nonproliferating peripheral blood T cells to divide in vitro. The entry of lymphocytes into the cell cycle (complete activation), however, requires accessory cells (e.g., monocytes or dendritic cells) and contributions from T cell subsets. Mitogen alone has been proposed to induce the expression of lymphocyte receptors for T cell growth factor interleukin 2 (IL-2) (1, 2), although considerable evidence points to the necessity for accessory cells in this activation process (3–5). Accessory cells plus mitogen are required to elicit IL-2 production by lymphocytes (5). Subsequent proliferation of T cells is limited by the availability of IL-2 (6), and requires other growth factors such as transferrin that are normally provided by the

serum (7). Plasma lipoproteins, particularly those containing apolipoproteins B and E, can suppress mitogen-primed activation and subsequent proliferation of peripheral blood T lymphocytes (for a review, see ref. 8). Lipoprotein suppression of mitogen-activated peripheral blood T lymphocytes, when assessed in vitro, can occur at three distinct states: mitogen priming (9–14), delivery of a signal from accessory cells to the primed lymphocytes (15–17), and possibly the proliferation of fully activated (mitogen plus accessory cell signals) lymphocytes.

Since lymphocyte activation is a complex process, it is difficult to elucidate the mechanism(s) of lipoprotein suppression. To avoid the complications inherent in a multi-signal, multi-cell activation system, we have explored the possibility that lipoproteins can suppress the proliferation of an IL-2-dependent T cell line, CTLL-2 (18). In cell cycle terms, CTLL-2 cells, which do not require mitogen or accessory cells to progress through the cell cycle, resemble fully activated peripheral blood T lymphocytes in that their proliferation is limited by IL-2 availability. This investigation is therefore based on the assumption that lipoprotein effects on CTLL-2 proliferation are represent-

Abbreviations: BSA, bovine serum albumin; conA, concanavalin A; FBS, fetal bovine serum; HBSS, Hanks' Balanced Salt Solution; HDL, high density lipoproteins; IL-2, interleukin 2; LDL, low density lipoproteins; MO, monocytes; PBM, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PDB, plasma density buffer; PHA, phytohemagglutinin; SF, spleen factor; SFM, serum-free medium; TdR, [methyl-³H]thymidine; VLDL, very low density lipoproteins.

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ative of their effects on fully activated peripheral blood T lymphocytes. We demonstrate here that very low (VLDL) and low (LDL) density lipoproteins can both enhance and suppress the progression of IL-2-fed CTLL-2 cells through the cell cycle. Suppression but not enhancement requires a component of cell-conditioned medium. Transferrin can augment lipoprotein enhancement and can completely ablate lipoprotein suppression. Lipoproteins also influence DNA replication in other T cell lines: gibbon MLA-144 and human HUT-102B2 cells. Since HUT-102B2 cells do not require IL-2 for growth, the mechanism by which lipoproteins influence cell proliferation seems to be independent of a requirement for the T cell growth factor. Consistent with this interpretation is the inability of purified IL-2 to ablate lipoprotein suppression.

MATERIALS AND METHODS

Reagents

Isolymp (d 1.077 g/ml) was purchased from Gallard-Schlesinger Chemical Mfg. Co. Phytohemagglutinin (PHA, type V) purified from *Phaseolus vulgaris*, concanavalin A (conA), and bovine serum albumin (BSA, fraction V) were obtained from Sigma Chemical Co. Interleukin 2 (IL-2) was obtained from Collaborative Research and from Cetus; it was active as determined by bioassay (18). Human transferrin was purchased from Sigma Chemical Co. and from Calbiochem. Penicillin and streptomycin were obtained from Gibco Labs. RPMI 1640, Hanks' Balanced Salt Solution (HBSS), phosphate-buffered saline (PBS), and fetal bovine serum (FBS) were purchased from KC Biological, Inc. ICN Pharmaceuticals, Inc. was the source of [methyl-³H]thymidine (TdR, 6.7 Ci/mM). Scintillation cocktail, 4a20, was obtained from Research Products International.

Complete medium consisted of RPMI 1640 supplemented with 100 units/ml of penicillin, 100 µg/ml of streptomycin, 2 mM L-glutamine, and 5% FBS. MM medium was RPMI 1640 buffered with 25 mM HEPES and sodium bicarbonate and supplemented with 100 units/ml of penicillin, 100 µg/ml of streptomycin, 2 mM L-glutamine, and 5×10^{-5} M β -mercaptoethanol. Serum-free medium (SFM) was RPMI 1640 supplemented with 15 µg/ml of insulin, 10 µg/ml of transferrin except when indicated otherwise, 0.5 µg/ml of linoleic acid, 5×10^{-5} M β -mercaptoethanol, 5 µg/ml of catalase, and 0.1–2 mg/ml of BSA (as specified). Murine CTLL-2 cells were obtained from Dr. Diane Eardley; gibbon lymphoma MLA-144 cells were from Dr. Harvey Rabin; and human HUT-102B2 cells were from Drs. Thomas Waldmann and Warner Greene.

Isolation of plasma lipoproteins

Plasma lipoproteins were isolated from freshly collected plasma of fasted human volunteers by sequential ultracentrifugal flotation in KBr (19), using a Beckman 50.2Ti rotor. VLDL were obtained by centrifugation at plasma density for 18 hr at 300,000 g. LDL were isolated between d 1.019 and 1.063 g/ml by ultracentrifugation for 18 hr at 300,000 g. HDL₃ were isolated between d 1.12 and 1.25 g/ml by centrifugation for 24 hr at 300,000 g. All lipoproteins were stored at 4°C under N₂ in plasma density buffer (PDB; 10 mM Tris-HCl, pH 7.4, containing 1 mM EDTA, 0.9% NaCl, and 0.01% NaN₃). The purity of each lipoprotein sample was assessed by electrophoresis on agarose (1%, pH 8.6); the chemical composition of the lipoproteins was determined by standard methods (9). Lipoproteins were dialyzed against 10 mM HEPES, pH 7.4, and sterile-filtered immediately prior to their addition to cell cultures.

Isolation of peripheral blood T lymphocytes and monocytes (MO)

Heparinized venous blood was obtained from healthy subjects. Blood donors were requested to restrict intake of methylxanthine-containing foods and beverages for 12 hr. One volume of blood was diluted with three volumes of PBS, and centrifuged through Isolymp (20). Peripheral blood mononuclear cells (PBM) were isolated at the interface of the gradient and washed three times with PBS. Washed cells were resuspended in complete medium.

Accessory cells (>85% MO) were isolated from PBM by glass adherence. PBM were suspended in complete medium, and 20-ml aliquots containing 4×10^7 cells were dispensed in glass Petri dishes. After incubation for 1 hr at 37°C, nonadherent cells were decanted and suspended in fresh medium for further separation. Adherent cells remaining on the Petri dishes were washed with warm HBSS and PBS to remove loosely adhered cells. The cells were then covered with cold PBS and further incubated for 1 hr at 4°C. Adherent cells were recovered from the plates by gentle scraping with a rubber policeman. Accessory cell populations were treated with mitomycin C (40 µg/ml) for 45 min at 37°C, washed, and suspended in fresh medium for use in cell cultures.

Nonadherent cells were separated into T cell-enriched and B cell-enriched populations by rosetting with neuraminidase-treated sheep red blood cells (21) and centrifuging the rosettes over Isolymp. For rosetting, 2.5×10^6 cells/ml were suspended in complete medium and incubated with erythrocytes (4% hematocrit) and additional FBS at a ratio of 2:1:1 (volume) for 10 min at 37°C. The mixture was centrifuged 10 min at 100 g, and further incubated for >2 hr at 4°C. After separation by centrifugation through Isolymp, the pelleted rosetted cells were treated with NH₄Cl to lyse the erythrocytes.

The lymphocytes were allowed to adhere to a warm nylon wool column for 30 min at 37°C before the nonadherent T lymphocytes were slowly eluted with complete medium. The T cells were washed and resuspended in complete medium for further use.

Preparation of conditioned medium

Conditioned medium was obtained from conA-stimulated rat spleen cells. Spleens from Lewis rats were ground into a single-cell suspension using a sterile frosted-glass slide. To remove residual tissue mass, the suspension was allowed to stand in a 50-ml conical tube for 1 min, and then was decanted into a new tube. The cells were washed three times with HBSS and resuspended in MM medium. FBS was omitted from the medium for some experiments and included in others as indicated. Cells, at a density of 5×10^6 /ml viable cells, were incubated with conA (5 µg/ml) for 48 hr at 37°C in an atmosphere of 5% CO₂/air. After incubation, the cells were removed by centrifugation and α-methylmannoside was added to the supernatant at a final concentration of 20 mg/ml. The conditioned medium (termed spleen factor, SF) was filter-sterilized and stored at -20°C.

Cell cultures

CTLL-2 lymphocytes were grown in MM medium routinely supplemented with 10% FBS and 10% rat spleen factor (SF). The cells doubled within 12–24 hr when plated at a density of $0.1\text{--}1.5 \times 10^5$ cells/ml. MLA-144 cells and HUT-102B2 cells were propagated in MM medium without β-mercaptoethanol; the amount of FBS was 10%.

Cell characterization and viability

Routinely, all cell preparations were characterized by morphologic criteria. Cell smears were prepared using a cytocentrifuge (Cystospin 2, Shandon), and the cells were stained with a modified Wright-Giemsa stain (Harleco Diff-Quik, Dade Diagnostics, Inc.) Cell viability was determined by staining with Trypan blue or with fluorescein diacetate and ethidium bromide (22).

DNA synthesis assay

DNA synthesis was measured by the incorporation of TdR into cellular DNA. CTLL-2, MLA-144, and HUT-102B2 cells were collected by centrifugation at 200 *g* for 10 min, and were washed twice in HBSS or HEPES-buffered RPMI 1640. Cells were resuspended in medium containing the amount of serum indicated and aliquoted into 96-well flat-bottom microtiter plates. The medium was either MM medium or SFM. CTLL-2 culture medium was always supplemented with at least 50 ng/ml catalase. Additives were prepared and diluted in the culture medium. Except as indicated, lipoproteins were

added immediately after the cells were dispensed into microtiter plates and at the same time as the conditioned medium. Two to four hr (as indicated) before harvest, TdR (2 µCi/well) was added. The amount of TdR incorporated into DNA by "control" cells varied, depending on the length of the TdR pulse, the length of the experiment, and the preparation of conditioned medium or IL-2.

Human peripheral blood T lymphocyte activation was also assayed by the amount of TdR incorporated into DNA. Triplicate cultures containing T cells (10^5) and mitomycin C-treated accessory cells (10^4) were established in microtiter plates with flat-bottom wells (final volume 0.24 ml of modified complete medium containing 5% FBS). For lectin stimulation studies, the cultures were preincubated with or without the lipoproteins for 1–2 hr. The cells were then incubated at 37°C in 95% air–5% CO₂ with PHA (3 µg/ml) in HBSS or with an equal volume of HBSS as control. At 2–12 hr (as specified) before harvesting at 48, 72, or 96 hr, 1 µCi of TdR was added to each well.

All cells were harvested onto glass-fiber filter papers with a multiple sample automated harvester (mini-MASH, M. A. Bioproducts). TdR incorporation (percent error ≤ 10%) was determined by liquid scintillation counting in 4a20. The amount of inhibition or activation by lipoproteins was calculated using Equation 1,

$$\frac{[(\Delta\text{cpm} + \text{lipoproteins}) - \Delta\text{cpm} - \text{lipoproteins}] - 1}{\Delta\text{cpm} - \text{lipoproteins}} \times 100 = \text{percent change relative to control} \quad \text{Eq. 1}$$

where Δcpm represents the difference in amount of TdR incorporated in the presence and absence of PHA or conditioned medium as appropriate. All reported data are for single experiments (triplicate analyses) but are representative of at least three separate experiments. Statistical analysis was performed in two ways: the method of the Student's *t*-test for two-group hypothesis testing and the method of analysis of variance for multiple (> two)-group hypothesis testing. Newman-Keuls and/or Duncan's multiple range tests were applied to the analysis of variance results to assess the specific two-group comparisons. The analyses were conducted on the CLINFO Computer System of the General Clinical Research Center at the University of Cincinnati.

RESULTS

A comparison of lipoprotein suppression of DNA replication in IL-2-dependent murine T lymphocytes and in mitogen-activated peripheral blood T lymphocytes

Lipoproteins of $d \leq 1.063$ g/ml suppressed the DNA synthesis of CTLL-2 cells that was induced by the IL-2 in spleen cell-conditioned medium (SF). Over the lipo-

protein concentration range tested, the lipoproteins were not cytotoxic. HDL₃ had no influence on CTLL-2 cell proliferation (data not shown). As is indicated in **Table 1**, the suppressive potencies of VLDL and LDL preparations varied considerably. The most suppressive VLDL and LDL reduced DNA synthesis by 50% when present at concentrations of about 10 μg of protein/ml; the least suppressive lipoproteins, at concentrations greater than 100 μg of protein/ml. Furthermore, lipoproteins isolated from the same donor at different times varied significantly in their suppressive potencies. Compare, for example, the half-maximum suppressive concentrations of VLDL isolated from DS (samples 1-3), which ranged from 17 to 200 μg of protein/ml, and of LDL isolated from JJ (samples 1-3), which ranged from 70 to 360 μg of protein/ml. The origin of the difference in suppressive potencies did not appear to be due to differences in apolipoprotein constituents. Analysis of the apolipoproteins of the VLDL and LDL preparations by gel electrophoresis in SDS-urea (15) revealed no obvious

TABLE 1. Plasma lipoproteins suppress DNA replication in activated lymphocytes^a

Lipoprotein	Amount of Lipoprotein Required for 50% Inhibition	
	CTLL-2 cells	T cells + MO
	$\mu\text{g/ml protein}$	
VLDL		
RM ₁	9	5
RM _r	NT ^b	10
DT	13	3
AC	8	2
DS ₁	17	2
DS ₂	150	15
DS ₃	200	10
BC	8	3
AS	70	6
PG	80	10
HH	100	3
AD	100	25
TD	10	2
DL	9	5
LDL		
RJ	13	8
TW	10	8
MS	10	8
LT	1000	90
PI	400	150
JJ ₁	250	25
JJ ₂	360	100
JJ ₃	70	50

^aHuman peripheral blood T cells (10^5) and accessory MO (10^4) in complete medium or mouse CTLL-2 cells (4×10^5) in MM medium containing a 1/25 dilution of SF and 2% FBS were incubated in microtiter wells without and with VLDL or LDL as outlined in Materials and Methods. The peripheral blood cells were harvested at 72 hr and the CTLL-2 cells at 24 hr. The concentration of lipoproteins required to reduce DNA synthesis by 50% was estimated from a plot of percent suppression versus log (lipoprotein concentration).

^bNT, not tested.

correlation between apolipoprotein profile and suppressive potency (data not shown). Very suppressive and essentially nonsuppressive lipoproteins could contain apoE, apoB₄₈, apoB₁₀₀, and proteolytic cleavage fragments of apoB₁₀₀ (apoB₇₄ and apoB₂₆).

The lipoproteins that suppressed CTLL-2 cell proliferation also suppressed the activation of peripheral blood T cells. The effectiveness of lipoprotein suppression of PHA-induced T cell activation and of IL-2-induced CTLL-2 cell DNA replication was qualitatively different, however. In general, VLDL and LDL were more suppressive when tested with peripheral blood T lymphocytes relative to CTLL-2 cells. The differences in concentrations of lipoproteins required for half-maximum suppression in the two systems varied from no difference (e.g., LDL-RJ, -TW, and -MS) to 4-fold (e.g., VLDL-DT and -AC) to 33-fold (e.g., VLDL-HH).

Lipoproteins influence the proliferation of other lymphocyte cell lines

We next asked whether lipoproteins could suppress DNA replication in two other T lymphocyte cell lines, human HUT-102B2 cells and gibbon MLA-144 cells (**Table 2**). Depending on the VLDL concentration, DNA synthesis in MLA-144 cells could be either enhanced or suppressed by VLDL. Enhancement occurred when the amount of VLDL was low (5 μg of protein/ml). VLDL at 20-100 μg of protein/ml, however, suppressed DNA replication. In the case of HUT-102B2 cells, no signifi-

TABLE 2. VLDL influence DNA replication in MLA-144 and HUT-102B2 cells^a

Cell Line	VLDL	Percent Change in DNA Synthesis (Relative to Control) ^b	
		FBS (Lot No. 300276)	FBS (Lot No. 300176)
	$\mu\text{g protein/ml}$		
MLA-144	5	+18.7 \pm 2.9	+13.9 \pm 5.5
MLA-144	20	-3.1 \pm 12.3	-89.8 \pm 1.0 ^{2c}
MLA-144	100	-42.0 \pm 5.7	-98.5 \pm 0.8 ^{2c}
HUT-102B2	5	+1.2 \pm 7.6	-10.3 \pm 6.9
HUT-102B2	20	-35.0 \pm 3.7	-80.1 \pm 7.3 ^{3c}
HUT-102B2	100	-56.8 \pm 13.1	-98.7 \pm 0.7 ^{1c}

^aMLA-144 (5×10^4 cells/well) and HUT-102B2 (2×10^4 cells/well) were cultured in MM medium and microtiter plates as described in Materials and Methods. The medium also contained 10% FBS (MLA-144) or 2% FBS (HUT-102B2). The cultures were pulsed with TdR (2 μCi /well) at $t = 23$ hr and harvested at $t = 26$ hr. The amount of TdR incorporated into DNA by control cells incubated in the absence of VLDL was: MLA-144, 11010 \pm 990 (FBS Lot No. 300276) and 11825 \pm 1010 (FBS Lot No. 300176); HUT-102B2, 22740 \pm 1350 (FBS Lot No. 300276) and 28920 \pm 1170 (FBS Lot No. 300176).

^bThe control is cell proliferation in the absence of VLDL. A positive value indicates that the amount of radiolabeled thymidine incorporated by cells incubated with VLDL was greater than that by control cells. A negative value indicates suppression of DNA synthesis by VLDL.

^cSignificance of the difference in extents of suppression obtained in the two sera: 1, $P = 0.025$; 2, $P = 0.01$; 3, $P = 0.005$.

cant enhancement of DNA replication occurred at low concentrations of VLDL. In other experiments, VLDL concentrations as low as 0.5 μg of protein/ml failed to enhance the cells' proliferative response. VLDL were, however, capable of suppressing DNA replication in HUT-102B2 cells. VLDL, at the concentrations used, did not affect cell viability during incubation period.

Interestingly, the extent of suppression depended on the source of FBS added to the medium: the VLDL were substantially more suppressive when the cells were cultured in FBS lot 300176 than in lot 300276. The influence of the serum on the extent of lipoprotein suppression was particularly interesting in view of the fact that DNA synthesis in the absence of lipoproteins was unaffected by serum lot. The extent of suppression by VLDL of DNA synthesis in CTLL-2 cells was also influenced by the serum source (data not shown).

Lipoprotein suppression of the CTLL-2 response increases with increasing amounts of cell-conditioned medium

In the case of the CTLL-2 cells, the amount of lymphocyte growth-promoting factors can be manipulated. We therefore probed the relationship between the amount of conditioned medium and the extent of lipoprotein suppression of DNA replication. Because the serum source could influence the experimental outcome (Table 2), the cells were cultured in SFM and in medium containing 1% FBS. The results, summarized in Table 3, indicate that the extent of suppression by VLDL was determined by the amount of conditioned medium present. The suppression was greatest when the conditioned medium was concentrated, and was substantially less when the conditioned medium was diluted. This result occurred in the absence or presence of FBS. In SFM at the conditioned medium dilutions of 1/72 and 1/144, the VLDL at 10–20 μg of protein/ml were not suppressive, but rather augmented the cellular proliferative response. These same concentrations of VLDL suppressed DNA replication in the presence of 1/24 and 1/6 dilutions of conditioned medium. When the identical cultures were established in 1% FBS, the lipoproteins were suppressive at each concentration tested. Thus, lipoprotein suppression was favored when the CTLL-2 cells were cultured in medium containing serum and concentrated conditioned medium. In our initial experiments, reported in Table 1, the cells were cultured under conditions that allowed only the expression of lipoprotein suppression.

Lipoprotein regulation of CTLL-2 cell proliferation is independent of IL-2

Since increasing the amount of IL-2-containing conditioned medium in the culture increased the extent to which CTLL-2 cell proliferation was suppressed by lipoproteins, we questioned whether purified IL-2 influenced

TABLE 3. The influence of VLDL on CTLL-2 cell replication depends on the amount of conditioned medium^a

Culture Medium	VLDL	Percent Change in DNA Synthesis ^b SF (SFM) dilution			
		1/144	1/72	1/24	1/6
	$\mu\text{g protein/ml}$				
SFM	10	+ 17	+ 7	- 57	- 94
SFM	20	+ 4	+ 1	- 72	- 99
SFM	50	- 30	- 20	- 82	- 99
SFM + 1% FBS	10	- 31	- 27	- 46	- 59
SFM + 1% FBS	20	- 56	- 47	- 65	- 95
SFM + 1% FBS	50	- 77	- 74	- 91	- 99

^aCTLL-2 cells, 4×10^3 cells/well, were incubated at 37°C for 26 hr in SFM (300 $\mu\text{g/ml}$ of BSA) in the absence or presence of serum as indicated. Four hr prior to harvest, TdR (2 $\mu\text{Ci/well}$) was added. The amounts of incorporated TdR in the absence of VLDL were: 29820 ± 3990 for SFM and 33590 ± 3660 for SFM + 1% FBS. The DNA synthetic response of the CTLL-2 cells in the absence of VLDL was independent, within experimental error, of the dilution of conditioned medium in this experiment.

^bA positive value indicates that the amount of radiolabeled thymidine incorporated by cells incubated with VLDL was greater than that by control cells (enhancement). A negative value indicates suppression of DNA synthesis by VLDL (suppression).

lipoprotein inhibition. The ingredient in the conditioned medium that influenced the extent of lipoprotein suppression was not IL-2 since the addition of purified IL-2 to cultures containing conditioned medium at the 1/8 and 1/32 dilutions did not exacerbate or relieve VLDL suppression (Fig. 1). Similar results were obtained when genetically engineered IL-2 was used. Furthermore, when the CTLL-2 cells were induced to proliferate with purified IL-2, lipoproteins were not inhibitory but enhanced the cellular response as is shown in Fig. 2. Enhancement was independent of lipoprotein concentration over the concentration range tested, 50–200 μg of VLDL-protein/ml, but increased as the amount of IL-2 increased.

Transferrin can relieve lipoprotein suppression and augment lipoprotein enhancement of DNA replication

The data presented in Table 2 suggest that lipoprotein suppression is influenced by a constituent of serum. Transferrin, the iron transport protein present in high quantities in adult serum but in low and variable quantities in fetal serum, is reported (23) to modulate lipoprotein effects on activated peripheral blood T lymphocytes. We therefore investigated transferrin's effect on lipoprotein suppression and augmentation of CTLL-2 cell proliferation. To define the transferrin effect, the amount of conditioned medium was varied and transferrin was added at two different concentrations. Transferrin profoundly influenced the extent to which CTLL-2 cells were sensitive to lipoproteins, as is illustrated in Fig. 3.

In SFM containing dilute conditioned medium and a low amount of transferrin, 10 $\mu\text{g/ml}$ (panel A), VLDL

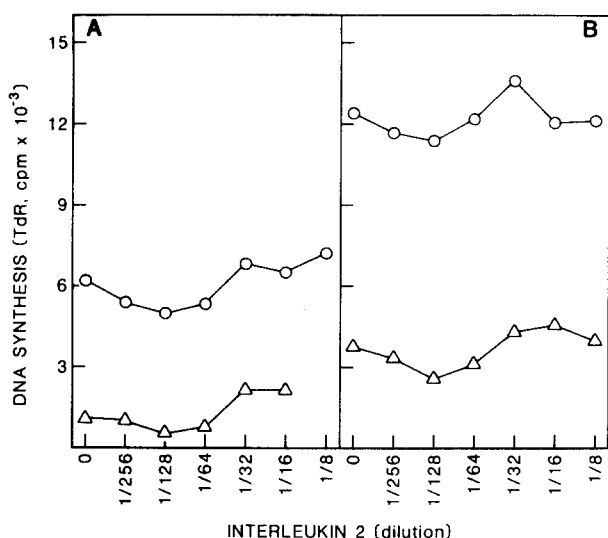


Fig. 1. Interleukin 2 does not influence the lipoprotein suppression of CTLL-2 cell proliferation. CTLL-2 cells (4×10^3 /well) were cultured for 26 hr at 37°C without (\circ) and with (Δ) VLDL ($150 \mu\text{g}$ of protein/ml) in complete medium supplemented with SF(SFM) at a final dilution of (A) $1/8$ or (B) $1/32$ with additional genetically engineered IL-2 at the indicated dilutions. Four hr prior to harvest, TdR ($2 \mu\text{Ci}$ /well) was added. The size of the symbols is representative of the limits of uncertainty of the data.

augmented the DNA synthetic response to IL-2. The enhancement of the cellular response by VLDL was not dependent on the concentration of VLDL from 50 – $200 \mu\text{g}/\text{ml}$ of VLDL-protein. This VLDL effect was similar to the VLDL effect on the cellular response induced by purified IL-2 (Fig. 2). As the amount of conditioned medium was increased, VLDL suppressed DNA replication, and the extent of suppression was dependent on the concentration of VLDL, being greater at the higher concentration of VLDL tested, $200 \mu\text{g}$ of VLDL-protein/ml. When the amount of transferrin was $800 \mu\text{g}/\text{ml}$ (panel B), the augmentation by VLDL of the cellular response that occurred in dilute conditioned medium was amplified, whereas the suppression by VLDL that occurred in concentrated conditioned medium was relieved. The amplification of the VLDL-enhanced response remained independent of the concentration of VLDL over the range of 50 – $200 \mu\text{g}/\text{ml}$. However, the ability of transferrin to ablate VLDL suppression was dependent on the amount of VLDL in the culture. Transferrin not only shifted the VLDL enhancement/VLDL suppression transition to higher concentrations of conditioned medium, but relieved the lipoprotein suppression entirely at VLDL concentrations as high as $50 \mu\text{g}$ of VLDL-protein/ml. At the $1/48$ dilution of conditioned medium, $200 \mu\text{g}$ of VLDL-protein/ml suppressed the DNA synthetic response by about 40% at $10 \mu\text{g}$ of transferrin/ml but augmented the response by twofold at $800 \mu\text{g}$ of transferrin/ml. In the absence of lipoproteins, transferrin had little effect on the replicative response of the cells.

Relief of lipoprotein suppression was not achieved by the addition of catalase (heme iron), ovalbumin, or albumin (Table 4). Albumin, could, in some experiments and with particular preparations of lipoproteins, reduce suppression partially, but the mechanism for this reduction was different from that of transferrin (McCarthy, B. M., and J. A. K. Harmony, unpublished data). High concentrations of ferritin killed the CTLL-2 cells. VLDL or LDL preincubated with high concentrations of transferrin and reisolated retained the suppressive potency of the unincubated control lipoproteins when assayed both with CTLL-2 cells (data not shown) and with mitogen-stimulated peripheral blood T cells plus accessory cells (Scupham, D. W., B. M. McCarthy, and J. A. K. Harmony, unpublished data). This result suggests that transferrin is not acting directly on the lipoproteins.

Lipoproteins suppress progression of cells through G_1

It was of interest to determine whether lipoproteins acted to suppress the CTLL-2 cells at a specific stage in the cell cycle. A growth experiment was performed to ascertain whether the CTLL-2 cells responded synchronously to IL-2 induction. The cells did respond reasonably synchronously, with most of the cells in S phase every 24 – 26 hr (Fig. 4), allowing a test of the dependence of VLDL suppression on the timing of VLDL addition. CTLL-2 cells could be incubated in the absence of IL-2 containing medium at 37°C for as long as 8 hr with no loss in their ability to respond to IL-2. The effect of adding VLDL 4 hr prior to SF addition to as late as 24 hr following SF addition is shown in Table 5. VLDL

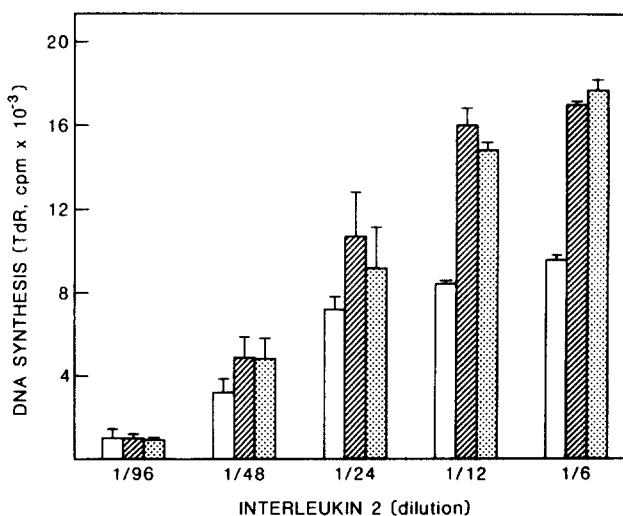


Fig. 2. Lipoproteins do not suppress CTLL-2 cell proliferation when purified IL-2 is the growth factor. CTLL-2 cells (4×10^3 /well) were cultured for 26 hr at 37°C without (\square) and with VLDL at (\square) 50 or (\blacksquare) $200 \mu\text{g}$ of protein/ml in SFM containing $0.5 \text{ mg}/\text{ml}$ of BSA. Four hr prior to harvest TdR ($2 \mu\text{Ci}$ /well) was added. The data are presented as mean \pm SEM.

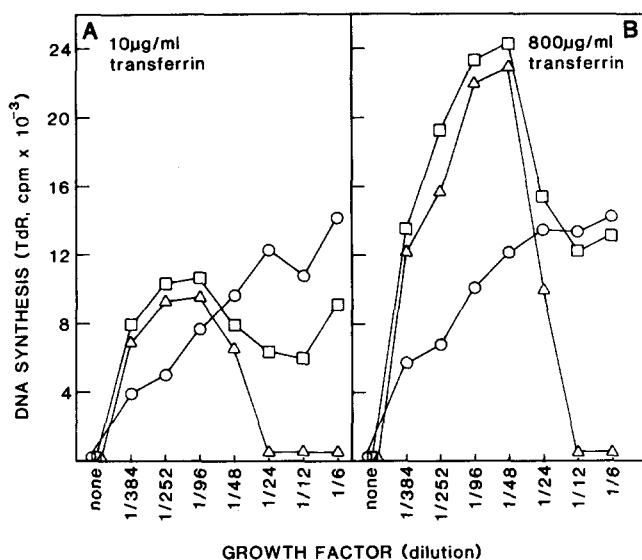


Fig. 3. The influence of lipoproteins on CTLL-2 cell proliferation is determined by the amount of transferrin. CTLL-2 cells (4×10^3 /well) were cultured for 26 hr at 37°C without (○) and with VLDL at (□) 50 $\mu\text{g}/\text{ml}$ (Δ) 200 $\mu\text{g}/\text{ml}$ of VLDL-protein in SFM medium supplemented with SF(SFM) at the indicated final dilution, human transferrin at (A) 10 or (B) 800 $\mu\text{g}/\text{ml}$, and 0.5 mg/ml of BSA. Four hr prior to harvest, TdR (2 $\mu\text{Ci}/\text{well}$) was added. The size of the symbols is representative of the limits of uncertainty of the data.

were most suppressive when added before or at the same time as the conditioned medium. The extent of suppression was somewhat reduced when VLDL were added between 2 and 12 hr following induction. By 20 hr following addition of conditioned medium, the cells were refractory to inhibition by VLDL, even when the concentration of VLDL was high, 50 μg of protein/ml VLDL. These data suggest that VLDL exerted its suppressive effect before the cells reached the G_1/S boundary. Preliminary data using propidium iodine and flow cytometry supported this conclusion (McCarthy, B. M., and J. A. K. Harmony, unpublished data).

TABLE 4. Transferrin relieves VLDL suppression^a

Added Protein	Percent Suppression of DNA Synthesis	
	10 $\mu\text{g}/\text{ml}$	800 $\mu\text{g}/\text{ml}$
Transferrin	82.1	25.4
Catalase	97.2	98.3
Ferritin	95.3	0 ^b
Ovalbumin	98.5	98.4
Albumin	96.3	98.7

^aCTLL-2 cells, 4×10^3 cells/well, were incubated in SFM containing a $\frac{1}{4}$ dilution of SF(SFM), 10 $\mu\text{g}/\text{ml}$ of transferrin, and 100 $\mu\text{g}/\text{ml}$ of albumin. Proteins were added, at final concentrations of 10 and 800 $\mu\text{g}/\text{ml}$, at the beginning of the incubation. TdR (2 μCi) was added at $t = 22$ hr, and the cells were harvested at $t = 26$ hr. The amounts of TdR incorporated by control cells (no VLDL) varied from 9320 to 12710 cpm.

^bCells were dead after 26 hr.

Ablation of lipoprotein suppression by transferrin depends on the time at which transferrin is added

The importance of the timing of transferrin addition was similarly tested. CTLL-2 cells were cultured with 10 $\mu\text{g}/\text{ml}$ of transferrin and in the absence and presence of VLDL. At various times after SF(SFM) addition, 800 $\mu\text{g}/\text{ml}$ of transferrin was added. Relief of lipoprotein suppression occurred when the transferrin was added at the same time as the VLDL and 1 hr later, but not 3 hr after VLDL addition (Table 6). The addition of 10 $\mu\text{g}/\text{ml}$ of transferrin at any time did not relieve suppression.

DISCUSSION

The data presented here establish that plasma lipoproteins can influence the progression of T lymphocyte cell lines through the cell cycle. DNA synthesis and proliferation of T lymphocyte cell lines could be either enhanced or suppressed by lipoproteins. Regulation of lymphocyte proliferation by lipoproteins is not species-specific (8), a finding confirmed by this investigation since human, murine, and gibbon lymphocytes were susceptible to regulation by human lipoproteins. Low concentrations of lipoproteins could enhance whereas high concentrations

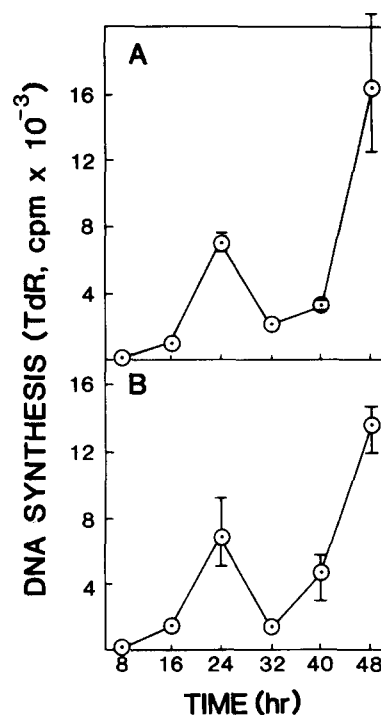


Fig. 4. The response of CTLL-2 cells to IL-2 is reasonably synchronous. CTLL-2 cells (4×10^3 /well) were cultured for the indicated times at 37°C in MM medium supplemented with 50 ng/ml of catalase and 8% FBS. SF(FBS) was added at a final dilution of (A) 1/12 or (B) 1/48. Two hr before harvest, TdR (1 $\mu\text{Ci}/\text{well}$) was added.

TABLE 5. The influence of VLDL on IL-2-induced CTLL-2 cell proliferation depends on the time of VLDL addition^a

Time of VLDL Addition ^b	TdR Incorporated by Control Cells	Percent Change in DNA Synthesis by VLDL ($\mu\text{g protein/ml}$) ^c		
		10	20	50
hr	cpm			
-4	53700 \pm 6305	-37	-54	-84
-2	53020 \pm 3020	-30	-47	-74
0	52470 \pm 2230	-34	-52	-69
+2	52955 \pm 3705	-29	-38	-57
+4	54440 \pm 3310	-28	-34	-52*
+8	62175 \pm 3185	-23*	-32*	-42*
+12	59790 \pm 6385	-27	-32*	-36*
+20	59025 \pm 4090	-4*	-16*	-7*
+24	55415 \pm 1005	+8*	+4*	+1*

^aCTLL-2 cells, 4×10^3 cells/well, were incubated at 37°C in MM medium supplemented with 2% FBS and 50 ng/ml of catalase. The source of IL-2 was SF(FBS) added at a final dilution of 1/30. Four hr after the cells were plated, SF(FBS) was added ($t = 0$ for the reference point). TdR (2 $\mu\text{Ci/well}$) was added at $t = 22$ hr, and the cells were harvested at $t = 26$ hr. The asterisk indicates that the difference of the value from that obtained when VLDL were added at $t = 0$ is significant by the Student's t -test ($P < 0.05$).

^bTimes of VLDL addition are indicated relative to the time of addition of SF(FBS), which was added at $t = 0$.

^cA negative number indicates VLDL suppression of the cellular response; a positive number, enhancement.

could suppress cellular proliferation. Lipoproteins of $d \leq 1.063$ g/ml had the most profound bioregulatory potency. HDL were not suppressive; their potential as growth enhancers was not tested. Cell viability was not altered by incubation with lipoproteins.

The pattern of lipoprotein augmentation-suppression of the murine CTLL-2 line was influenced by the amount of transferrin added to the cultures. As the transferrin concentration increased, growth promotion by the $d \leq 1.063$ g/ml lipoproteins increased and suppression of growth decreased. The transferrin dependence of the lipoproteins' bioregulatory capacity may account for the effect of serum lot on the lipoprotein effect since the amount of transferrin in serum preparations varies. Lipoprotein suppression required that the suppressors be present during early G_1 , and transferrin exerted its antisuppressive effect during the same period. These results suggest that the processes sensitive to lipoproteins and to transferrin are important in determining whether the cells progress through G_1 . The molecular basis of the antagonistic interaction between transferrin and lipoproteins is unknown. In the CTLL-2 system, other proteins did not influence lipoprotein regulation, and transferrin did not remove or inactivate a suppressive lipoprotein constituent during a preincubation period. Transferrin, the iron transport system in the body's circulatory compartments, delivers essential iron to cultured cells (24). Cuthbert and Lipsky (23) have suggested that lipoprotein suppression is due to interference with transferrin utilization, an effect

that can be overcome by increasing the concentration of transferrin in the cultures. Certainly, iron deprivation prevents cells from entering the S phase of the cell cycle (25, 26), reportedly by inhibiting ribonucleotide reductase (27, 28). In the absence of lipoproteins, 10 $\mu\text{g/ml}$ of transferrin was sufficient for optimal CTLL-2 cell proliferation; in contrast, in the presence of lipoproteins, 800 $\mu\text{g/ml}$ was optimal. Our results are, therefore, not inconsistent with the hypothesis of Cuthbert and Lipsky (23), although the experiments did not specifically address it. Transferrin is reported to be directly mitogenic in some systems (29), raising the alternative possibility that the combination of lipoproteins and transferrin allows the expression of a unique growth-promoting capacity of transferrin.

An additional important contribution of this work is the finding that the pattern of lipoprotein influence, at constant transferrin, was also influenced by the amount of conditioned medium added. When the CTLL-2 cells were incubated in dilute conditioned medium, the growth-promoting influence of the lipoproteins was expressed. In contrast, in concentrated conditioned medium, the growth-suppressing activity of the lipoproteins was expressed. The constituent in conditioned medium that determines whether lipoproteins can suppress CTLL-2 cell proliferation is not identified by this work. However, three lines of evidence indicate that the constituent is not IL-2. First, the extent of lipoprotein suppression that occurred in the presence of high concentrations of cell-conditioned medium was uninfluenced by additional purified IL-2. Second, only lipoprotein enhancement of DNA replication occurred when the CTLL-2 cells were induced to divide with purified IL-2. Third, dependence on an exogenous source of IL-2 was not a requirement for suppression by VLDL of DNA syn-

TABLE 6. The antisuppressive effect of transferrin occurs early in the cell cycle^a

Time of Tf Addition	TdR Incorporated by Control Cells		% Suppression by VLDL	
	Transferrin		Transferrin	
	10 $\mu\text{g/ml}$	800 $\mu\text{g/ml}$	10 $\mu\text{g/ml}$	800 $\mu\text{g/ml}$
hr	cpm \pm SEM		%	
0	14965 \pm 165	15220 \pm 715	67.6	27.0
1	12545 \pm 1150	14425 \pm 505	64.2	8.7
3	11535 \pm 1220	15610 \pm 1060	78.5	51.9
4	13355 \pm 180	16010 \pm 1100	74.0	68.8
6	8800 \pm 135	12800 \pm 870	69.9	60.7
10	6915 \pm 370	10510 \pm 215	61.7	75.0

^aCTLL-2 cells, 4×10^3 cells/well, were incubated in the absence and presence of VLDL (150 μg of protein/ml) for 26 hr in SFM (10 $\mu\text{g/ml}$ of transferrin and 100 $\mu\text{g/ml}$ of BSA) and a $\frac{1}{4}$ dilution of SF (SFM). At the indicated times, additional transferrin was added to the cultures. TdR (2 $\mu\text{Ci/well}$) was added 4 hr prior to harvest. VLDL and SF(SFM) were added at $t = 0$.

thesis in lymphocyte cell lines. Murine CTLL-2 cells require an exogenous source of IL-2 (18). Gibbon MLA-144 cells produce IL-2 constitutively, and appear to require it for growth (30). However, HUT-102B2 cells do not require IL-2 for proliferation as evidenced by the failure of antiTAC, a monoclonal antibody that binds to the IL-2 receptor, to inhibit their proliferation and by the failure of exogenous IL-2 to enhance their proliferation (31, 32). Yet DNA replication in all three cell lines could be suppressed by lipoproteins. The intriguing possibility that IL-2 is important for the lipoproteins' enhancement of lymphoid cell growth, on the other hand, is suggested by the result that DNA replication in HUT-102B2 cells was not increased when the cells were cultured with low concentrations of plasma lipoprotein. Additional cell lines must be tested before this suggestion can be confirmed.


Suppression by lipoproteins may be mediated by their initial interaction with cell surface receptors. Unstimulated peripheral blood T lymphocytes have membrane receptors, distinct from the classic LDL receptors (33), which bind lipoproteins of the $d \leq 1.063$ g/ml density classes (9). The lymphocyte receptors (termed immunoregulatory receptors), like the LDL receptors, recognize apoB and apoE of the lipoproteins (9). While this study did not directly assess the possibility that fully activated T lymphocytes also bear immunoregulatory receptors, their presence is indirectly indicated by the fact that only apoB- and/or apoE-containing plasma lipoproteins could suppress their proliferation. Assuming the existence of both immunoregulatory receptors and the high-affinity LDL receptors on CTLL-2 lymphocytes, the factor in cell-conditioned medium that is required for lipoprotein suppression may switch the binding of the lipoproteins from the LDL receptors (enhancement) to the immunoregulatory receptors (suppression). Fogelman et al. (34) have reported that a lymphokine in the conditioned medium of activated lymphocytes can decrease the utilization of LDL via monocyte-macrophages' LDL receptors. That lipoprotein enhancement is mediated by the classic LDL receptors is suggested by the inability of HDL₃ to enhance CTLL-2 proliferation. Further support is provided by Cuthbert and Lipsky (35) who reported that LDL do not enhance the mitogen-induced activation of peripheral blood T cells obtained from a donor with the homozygous form of familial hypercholesterolemia. These possibilities are currently under investigation in the CTLL-2 system.

The lipoprotein suppression of CTLL-2 cell DNA replication shares some features with the lipoprotein suppression of mitogen-induced T cell proliferation, although higher concentrations of lipoproteins were required to suppress DNA synthesis in the CTLL-2 cells relative to that in the peripheral blood lymphocytes (Table 1). The polyclonal mitogen-primed activation of peripheral blood T lymphocytes was suppressed by the same lipoproteins

that suppressed DNA replication in CTLL-2 cells and in other lymphocyte cell lines. Suppression in both peripheral blood T cell and CTLL-2 cell systems was alleviated by transferrin (23 and Scupham, D. W., B. M. McCarthy, and J. A. K. Harmony, unpublished data), and, in fact, in both systems lipoproteins could enhance the cellular responses. The concentration of transferrin at which the lipoprotein suppression-enhancement switch occurred in both systems was about 100 μ g/ml. These similarities exist in spite of the cell cycle differences: peripheral blood T cells exist out of the cell cycle in a kinetically defined G₀ (non-cycling) stage while IL-2-deprived CTLL-2 cells arrest in the absence of IL-2 in the G₁ stage of the cell cycle.

Variability in lipoprotein potency occurred in both the CTLL-2 cell system and the peripheral blood T cell system. Proliferation may be interdependently regulated by individual lipoprotein constituents and by the intact lipoprotein particles themselves, allowing for at least two possible explanations of variable bioregulatory potency: the amount of the bioregulatory constituents differed and/or the effectiveness of the intact lipoproteins differed. The fact that lipoproteins isolated at different times from the same donor, e.g., VLDL(DS) and LDL(JJ), had substantially different suppressive capabilities argues that suppression may be dependent on both a lipoprotein-cell interaction and the presence of a suppressive lipoprotein constituent, possibly a lipid. It is intriguing that apoE can completely prevent the mitogen-induced activation (at the stage of early biochemical changes), and therefore the subsequent cell cycle progression, of peripheral blood T cells (36, 37) but had no influence on IL-2-induced CTLL-2 cell proliferation (McCarthy, B. M., and J. A. K. Harmony, unpublished data), arguing for a suppressive lipoprotein-lipid constituent which plays an important role in suppressing the progression of fully activated lymphocytes through the cell cycle.

In summary, this is the first demonstration that plasma lipoproteins of $d \leq 1.063$ g/ml can either enhance or suppress the replication of continuous lymphocyte cell lines such as the murine line CTLL-2. Suppression was contingent on the presence of an as yet unidentified factor in cell-conditioned medium; both enhancement and suppression depended upon the concentration of transferrin. The IL-2-dependent CTLL-2 cell line is, therefore, a good model with which to elucidate the molecular basis of the complex and interrelated bioregulation by lipoproteins, by cell-derived growth mediators, and by transferrin since cell proliferation can be regulated by the delivery of IL-2 and since the cellular response to IL-2 is reasonably synchronous. Lipoprotein suppression could be completely ablated by transferrin but not by IL-2. Based on an analogy with the activation of peripheral blood lymphocyte system, this work identifies and isolates one window of lipoproteins' and transferrin's effects to be

beyond the requirement for antigen-accessory cells but early in the progression of T cells through G₁. 

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