Role of lipid peroxidation in the inhibition of mononuclear cell proliferation by normal lipoproteins

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Abstract Stimulated peripheral blood mononuclear cells (PBMC) can oxidize normal lipoproteins, and sufficiently oxidized lipoproteins are cytotoxic. However, the role of lipid peroxidation in the inhibition of mitogen-stimulated PBMC proliferation by physiologic concentrations of normal lipoproteins is unclear. In the present investigation, normal low density lipoprotein (LDL) and very low density lipoprotein (VLDL) suppressed [^{3}H]thymidine incorporation and gamma interferon production in concanavalin A-stimulated PBMC without causing cell death. This suppression was accompanied by parallel increases in lipid peroxidation products measured as thiobarbituric acid reactive substances (TBARS). In contrast, high density lipoprotein (HDL) failed to inhibit PBMC and TBARS remains low. Differences between the PBMC suppression from LDL, VLDL, and HDL were best accounted for by normalizing the lipoprotein concentrations by their total lipid content. Moreover, the antioxidants superoxide dismutase and butylated hydroxytoluene each substantially ameliorated the inhibition of PBMC caused by LDL, and reduced the levels of lipid peroxidation products that were generated. Altogether, these results suggest that reactive oxygen species generated by stimulated PMBC may cause oxidative alterations of normal lipoproteins that may, in turn, account for much of the previously reported inhibition of PBMC by normal lipoproteins. —**Kasiske, B. L., and W. F. Keane.** Role of lipid peroxidation in the inhibition of mononuclear cell proliferation by normal lipoproteins. *J. Lipid Res.* 1991. **32:** 775–781.

Supplementary key words thiobarbituric acid reactive substances (TBARS) • reactive oxygen species • lymphoproliferation • LDL • VLDL • HDL

Plasma and tissue lipoproteins may have important effects on immune system activity. Concentrations of low density lipoprotein (LDL), comparable to those normally found in human plasma have been shown to inhibit the proliferation of peripheral blood mononuclear cells (PBMC) stimulated with alloantigens or mitogens (1–12). A large number of investigations have been carried out to define the relative inhibitory effects of the major lipoprotein classes (1–4), the specific lipoprotein component(s) involved (4–8), and the role of interactions between lipoproteins and growth factors, such as transferrin (3).

Recently, a number of studies have demonstrated that reactive oxygen species generated by macrophages and neutrophils can oxidize normal lipoproteins (13–15). Moreover, oxidatively modified lipoproteins are cytotoxic (14–16). Schuh, Novogradsky, and Haschemeyer (17) added low concentrations of LDL (0–12.5 mg protein/dl), oxidized by prolonged room air exposure, to PBMC. The autoxidized LDL caused cell death and, not surprisingly, inhibited mitogen-stimulated proliferation. In contrast, low concentrations of normal LDL had little effect on PBMC (17), and the possibility that PBMC-induced oxidation could explain the inhibition of PBMC by normal lipoproteins was not examined. Other investigators demonstrated that concentrations of LDL similar to those normally found in plasma inhibited PBMC proliferation (1–12). Although none of these studies used antioxidants, the inhibitory effects of LDL were never attributed to oxidation products (1–12). Thus, experiments testing the role of PBMC lipoprotein oxidation in the well-known inhibition of PBMC by normal concentrations of lipoproteins have never been carried out. In addition, the impact of altered lipoproteins on the production of important PBMC activating cytokines, such as gamma interferon, has not previously been reported.

We stimulated PBMC in the presence of increasing concentrations of normal LDL, very low density lipoprotein (VLDL), and high density lipoprotein (HDL). Two separate measures of PBMC proliferation, [^{3}H]thymidine incorporation and gamma interferon production, were

Abbreviations: PBMC, peripheral blood mononuclear cells; TBARS, thiobarbituric acid reactive substances; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; LPO, lipid peroxidation; SOD, superoxide dismutase.

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used in these experiments. Not only was proliferation inhibited by LDL and VLDL, but the inhibition was associated with increased levels of lipid peroxidation products, measured using two independent assays. Moreover, the inhibitory effects of LDL were partially blocked by the addition of antioxidants to the culture medium, suggesting that peroxidation of the lipoproteins had a major role in the inhibition of the PBMC proliferation.

METHODS

Lipoprotein isolation

Pooled plasma, obtained from normal, fasting volunteers, was supplemented with 0.1 mM EDTA, 0.01 mM butylated hydroxytoluene (BHT), and 0.01% NaN_3. The VLDL (d 1.006 g/ml), LDL (d 1.019–1.063 g/ml), and HDL (d 1.063–1.210 g/ml) were isolated, washed, and concentrated using sequential ultracentrifugation with a Beckman 50.2-Ti rotor. Solid KBr was used to adjust the density, and centrifugation was carried out at 40,000 rpm and 4°C for 15 h (VLDL), or 18 h (LDL and HDL). Concentrated fractions were dialyzed at 4°C in phosphate buffer for 1 h × 2 changes, overnight, and 1 h in Hank's balanced salt solution. Dialyzed lipoproteins were filtered using 0.45-μm and 0.22-μm filters, and stored under N_2 at 20°C until used. The VLDL was used within 7 days of isolation, while HDL and LDL were used within 30 days of isolation. Protein concentrations were determined using the method of Lowry et al. (18).

Peripheral blood mononuclear cell isolation and culture

Heparinized blood (10 ml) was mixed with 3 ml of Macrodex (Pharmacea, Medical Products Division, Piscataway, NJ). The tubes rested for 45 min at a 45 degree angle, then remained upright for 15 min. The red cell-poor layer (8 ml) was removed and added to 5 ml of Lymphocyte Separation Medium (Organon Teknika, West Chester, PA) and centrifuged for 30 min at room temperature. Differential counts obtained on a thin layer of cell using a Wright stain revealed that approximately 32% of the cells were monocytes, 51% were lymphocytes, and 17% were red blood cells. After washing three times in Hank's balanced salt solution, 2×10^5 cells and 200 μl of media were added to each well in flat-bottom, 96-well plates (Falcon, Lincoln Park, NJ). Media contained RPMI-1640, 100 U penicillin/ml, 100 μg streptomycin/ml, 10 μg/ml Conconavalin A (Con A, Sigma, St. Louis, MO), 5% pooled human serum, and varying lipoprotein concentrations. Plates were incubated for 48 h at 37°C with 5% CO_2. Then [^{3}H]thymidine, 1 μCi/ml, was added to the cells and incubation was continued for 24 h. Supernatants were frozen (– 70°C) for subsequent gamma interferon, thiobarbituric acid reactive substances (TBARS), and lipid peroxidation (LPO) assays. Cells were used immediately to determine [^{3}H]thymidine uptake.

Cell viability was assessed by examining the number of nonadherent cells in the wells at the end of the 72°C incubations. In addition, trypan blue was added to supernatants and to wells to determine the number of cells that failed to exclude the dye, an index of cell viability.

Three separate sets of experiments were carried out. In the first set of experiments, PBMC were stimulated with Con A and incubated with increasing concentrations of LDL, VLDL, and HDL. In the second set of experiments, stimulated PBMC were incubated with LDL and either vehicle, superoxide dismutase (SOD), 50 μg/ml, or BHT, 20 mM. Finally, stimulated PBMC were also incubated with increasing concentrations of LDL in the presence and absence of HDL.

Gamma interferon assay

Levels of gamma interferon were measured using a radioimmunoassay (Centocor, Inc., Malvern, PA). Supernatant (200 μl diluted 1:50) was added to polystyrene beads coated with murine monoclonal antibody to human gamma interferon. The beads were incubated at room temperature for 2 h, and washed three times with distilled water. Antibody labeled with ^{125}I was added to the beads and incubated for 2 h. The amount of ^{125}I (cpm) bound to the beads was determined using the Crystal Multi Detector Gamma System (United Technologies, Packard Instrument Co., Downers Grove, IL).

Determination of lipid peroxidation products

The TBARS were measured using an adaptation of previously described techniques (19). Briefly, dilutions of tetraethoxypropane were used to generate standards. The incubation media (2 ml) contained three parts 1% H_3PO_4 and one part 0.6% thiobarbituric acid. Blank controls, standards, and experimental samples were mixed with incubation reagent and incubated at 95°C for 30 min. After cooling, each tube was filled with n-butanol (2 ml), vortexed, and spun at 2200 rpm for 15 min. The top butanol layer was read on a fluorescence spectrophotometer using an excitation wavelength of 515 nm and an emission wavelength of 550 nm.

The LPO assay has previously been described in detail (20). This assay makes use of the oxidative capacity of lipid peroxides to convert iodide to iodine, which can then be measured photometrically using a color reagent. The color reagent was made up of H_2KPO_4, 0.20 M, KI, 0.12 M; NaN_3, 0.15 mM; H_2MoO_4, 10 μM; Triton X-100, 2 g/l; and benzalkonium chloride, 0.10 g/l. Samples (100 μl) and blanks were added to 1 ml of the color reagent, mixed, and then read on a spectrophotometer at 365 nm. A standard curve was generated with these methods using cumene hydroperoxide.

Statistical analysis

The statistical significance of the differences between group means was tested using analysis of variance. Correlations between two variables were examined with linear regression using least-squares and Pearson *r*-values. The relationship between PBMC proliferation and lipoprotein concentrations, expressed according to the different lipoprotein compositional components, was examined using multivariate linear regression analysis. Variables that were not normally distributed, or had unequal variances, were corrected with a logarithmic transformation before analysis. Results are expressed as mean ± SEM. Differences were considered significant for $P < 0.05$. All analyses were carried out using the Statistical Package for the Social Sciences (21).

RESULTS

Effects of the major lipoprotein classes on PBMC proliferation

The addition of HDL caused little inhibition of PBMC [^{3}H]thymidine incorporation (**Fig. 1**). In contrast, both LDL and VLDL caused a dose-dependent inhibition of PBMC proliferation. This inhibition of PBMC proliferation was not the result of cell death. Indeed, after incubation at 72°C with maximal concentrations of LDL, virtually all cells were still adherent. In addition, there were almost no cells that failed to exclude trypan blue.

Due to the heterogeneity between lipoprotein classes, differences in the apparent effects of LDL, VLDL, and HDL on PBMC proliferation were dependent on the compositional elements used to express lipoprotein concentrations. Thus, the relative differences in effects of LDL, VLDL, and HDL on PBMC [^{3}H]thymidine incorporation appeared to be greater when lipoprotein concentrations were expressed as mg protein/dl, than when the same comparison was made with lipoprotein concentrations expressed as mg cholesterol/dl (Fig. 1).

To further define the lipoprotein compositional characteristics that best explained differences in the effects of the major lipoprotein classes on PBMC, univariate and multivariant regression analyses were carried out. Of all the normal lipoprotein compositional characteristics examined, none explained differences in the effects on PBMC [^{3}H]thymidine incorporation better than the percent of the lipoprotein made up of total lipid (**Table 1**). When multivariate linear regression analysis was used to examine the independent correlations between the various lipoprotein characteristics and their impact on PBMC, total lipid and protein composition had equal, and opposite, independent effects: Log [^{3}H]Thymidine Incorporation = $-0.0061(\pm 0.0004) \times$ Lipoprotein Total Lipid + $0.0060(\pm 0.0001) \times$ Lipoprotein Total Protein, $P < 0.0001$. Thus, the relative proportion of lipoprotein made up of lipid was associated with a greater degree of inhibition of [^{3}H]thymidine incorporation, while the amount of protein in the lipoproteins was independently associated with less inhibition. Other compositional characteristics had no independent impact on the PBMC once total lipid and protein content were taken into account.

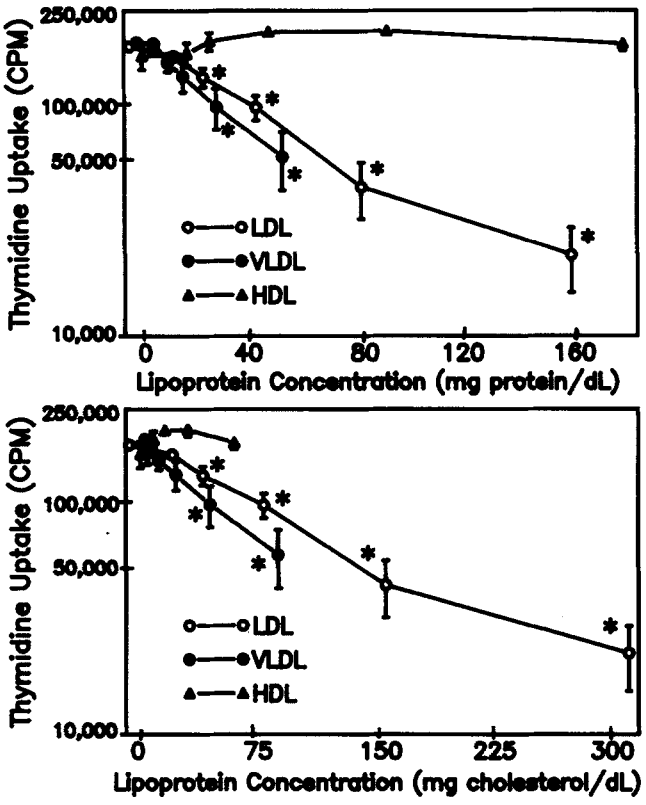

Fig. 1. The effects of increasing concentrations of LDL, VLDL, and HDL on [^{3}H]thymidine incorporation by PBMC stimulated with Con A. For the LDL experiments each point represents n = 12, for VLDL n = 18, and for HDL n = 9; mean ± SEM. The concentrations of lipoproteins are expressed as mg protein/dl (top panel) and as mg cholesterol/dl (bottom panel). To convert mg protein/dl to μg protein/l, multiply values by 10. *$P < 0.05$ versus no lipoprotein.

The effects of the different lipoprotein classes on PBMC gamma interferon production were similar to those seen with [^{3}H]thymidine incorporation (**Fig. 2**). Moreover, the inhibition of PBMC proliferation both by LDL and by VLDL was associated with increased TBARS (Fig. 2). In contrast, very little TBARS were detected in the supernatants of PBMC incubated with increasing concentrations of HDL.

Effects of antioxidants on lipoprotein-induced PBMC inhibition

In a separate set of experiments, SOD and BHT both ameliorated the degree of inhibition caused by increasing concentrations of LDL (**Fig. 3**). In addition, the salutary

TABLE 1. The relationship between normal lipoprotein composition and the inhibition of peripheral blood mononuclear cell proliferation[a]

Lipoprotein Component	Normal Lipoprotein Composition			Correlation (r-values) with [³H]Thymidine Incorporation
	LDL	VLDL	HDL	
Total protein (%)	22	10	54	- 0.22
Total lipid (%)	78	90	46	- 0.66
Phospholipid (%)	27	18	25	- 0.55
Cholesterol (%)	41	17	16	- 0.55
Triglyceride (%)	10	55	5	- 0.55
ApoA I (mg/100 mg protein)	0.8	0.9	62	0.22
ApoB (mg/100 mg protein)	95	25	1	- 0.48
ApoC (mg/100 mg protein)	2	55	8	- 0.35
ApoE (mg/100 mg protein)	3	15	2	- 0.58

[a]The values for normal lipoprotein composition were adapted from reference 26. All correlations (r-values) were statistically significant ($P < 0.01$).

effects of the antioxidants were associated with reductions in TBARS (Fig. 3). The amelioration in [³H]thymidine incorporation and the reduction in TBARS caused by the antioxidants were not complete. However, within all three experimental groups, there was a strong inverse correlation between [³H]thymidine incorporation and TBARS (**Fig. 4**). The correlation between TBARS and LPO levels was also high ($r = 0.64$, $P < 0.001$). Thus, as was the case for TBARS, there was a strong inverse relationship between [³H]thymidine incorporation and LPO levels ($r = -0.74$, $P < 0.001$).

Effects of HDL on LDL-inhibition of PBMC proliferation

Since HDL may have antioxidant properties (22), we hypothesized that HDL could ameliorate the LDL-inhibition of PBMC proliferation. However, the dose-dependent inhibition of LDL on the [³H]thymidine incorporation of stimulated PBMC was not inhibited by normal (50 mg/dl) or high 100 mg/dl) concentrations of HDL (**Fig. 5**).

DISCUSSION

In the present investigation lipoprotein inhibition of PBMC proliferation was caused, at least in part, by oxidative modification of the lipoproteins. Although these results do not exclude other mechanisms for the inhibitory effects of normal lipoproteins on PBMC, a number of observations suggest that lipid peroxidation may, indeed, play a major role. First, the relative degree of inhibition of PBMC proliferation by the different lipoprotein classes was proportional to the percent of the lipoprotein that was made up of total lipid. If lipid peroxidation was important in the inhibitory effects of the lipoproteins, a greater effect would be expected in the presence of more lipid substrate. Moreover, these results are consistent with those of Schuh et al. (17) who reported that the inhibitory factor in autoxidized LDL was lipid-extractable. Second, the lipoprotein-induced inhibition of PBMC was uniformly accompanied by increases in lipid peroxidation products. This was documented using two different assay systems. Finally, two structurally distinct antioxidants were effec-

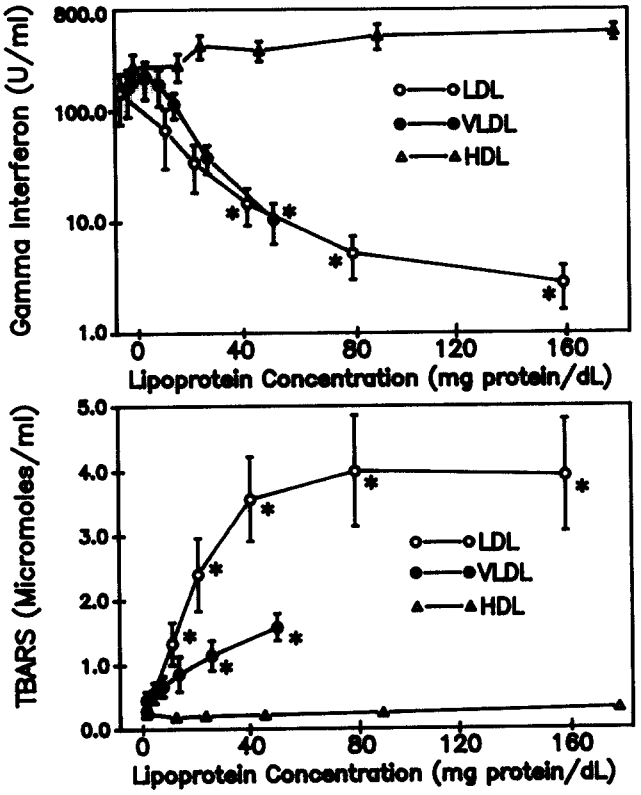

Fig. 2. The effects of increasing concentrations of LDL, VLDL, and HDL on gamma interferon production (top panel) and TBARS (bottom panel) generated by PBMC stimulated with Con A. For the LDL experiments each point represents n = 12, for VLDL n = 18, and for HDL n = 9; mean ± SEM. *$P < 0.05$ versus no lipoprotein.

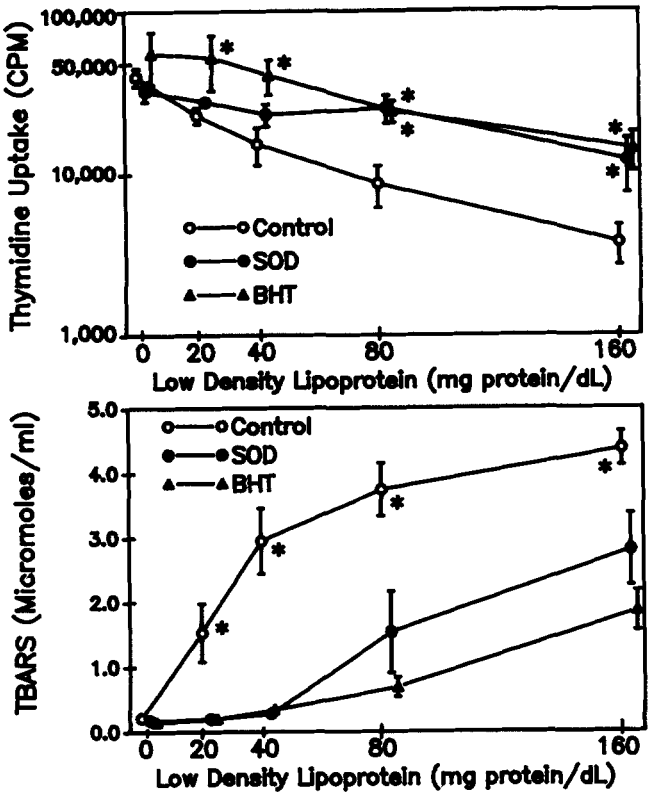

Fig. 3. The effects of superoxide dismutase (SOD) and butylated hydroxytoluene (BHT) on [^{3}H]thymidine incorporation (top panel) and TBARS (bottom panel) produced by stimulated PBMC incubated with LDL. For the control group each point represents n = 8, for SOD n = 4, and for BHT n = 4; mean ± SEM *$P < 0.05$ versus controls at the same LDL concentration.

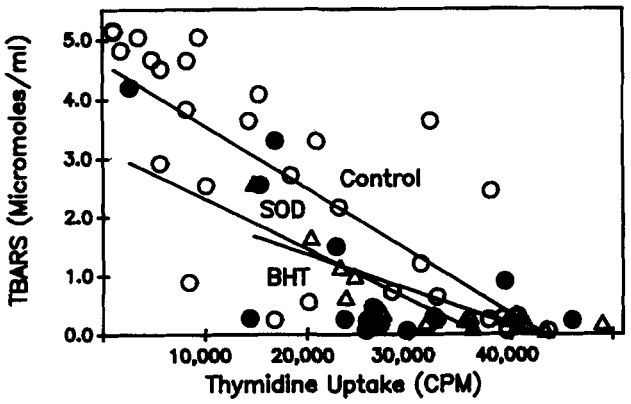

Fig. 4. The correlation between TBARS and [^{3}H]thymidine incorporation in PBMC incubated with LDL and vehicle (open circles), superoxide dismutase (SOD, filled circles), and butylated hydroxytoluene (BHT, open triangles). The least-squares-fitted linear regression lines in the three groups are shown: control [^{3}H]thymidine = -10.7×10^{-3} (TBARS) + 4.62, $r = 0.78$, $P < 0.01$; SOD [^{3}H]thymidine = -8.6×10^{-3} (TBARS) + 3.16, $r = 0.69$, $P < 0.01$; BHT [^{3}H]thymidine = -6.0×10^{-3} (TBARS) + 2.55, $r = 0.83$, $P < 0.01$.

tive in partially blocking the inhibitory effects of lipoproteins on PBMC, and the degree of antioxidant amelioration was proportional to the reduction in levels of peroxidation products.

Most of the differences in the inhibition of PBMC by the major lipoprotein classes could be explained by the total amount of lipid contained in the lipoproteins. Specifically, normal HDL is relatively lipid-poor and caused minimal suppression of PBMC compared to lipid-rich VLDL and LDL. However, the protein content of the lipoproteins (relatively high for HDL) also had a positive effect on PBMC proliferation that was independent of the relative content of total lipid. Indeed, for comparable amounts of lipoprotein total lipid, the [^{3}H]thymidine incorporation and gamma interferon production by PBMC incubated with HDL were higher than those seen for LDL and VLDL (data not shown). Thus, properties of the lipoproteins that are unrelated to lipid content may also be important in the differences in PBMC inhibition between lipoprotein classes.

In this regard, HDL was recently found to have antioxidant properties. Indeed, HDL and its major apolipoprotein, apoA-I, caused significant reductions in the oxidation of LDL induced by incubation with $CuSO_4$ (22). It is interesting to speculate that the lack of HDL-suppression of PBMC proliferation, despite the presence of a small but significant amount of lipid, could, in part, be due to antioxidant effects of HDL proteins. However, HDL failed to ameliorate the inhibition of PBMC proliferation caused by LDL. Why HDL reduced the $CuSO_4$-induced oxidation of LDL, but did not reduce the LDL-inhibition of PBMC is unclear.

Although LDL and VLDL caused a similar amount of inhibition of PBMC, TBARS appeared to be higher in LDL than VLDL. Why the higher TBARS in LDL versus VLDL were not associated with a greater degree of in-

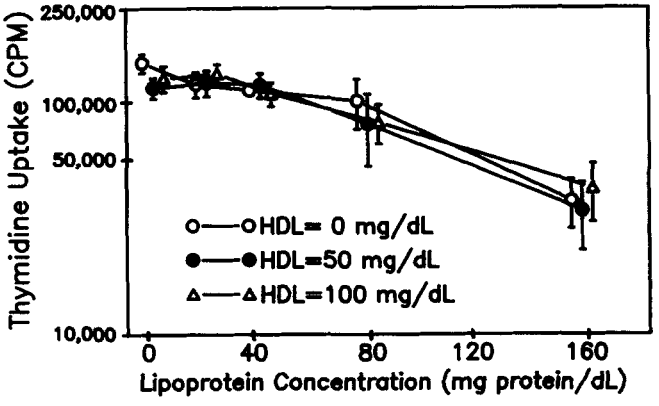

Fig. 5. The effects of HDL on LDL-suppression of [^{3}H]thymidine incorporation by PBMC stimulated with Con A. The LDL concentrations are indicated on the x-axis. Each point represents the mean of six determinations ± SEM. There were no differences between 0, 50, or 100 mg/dl HDL at any of the LDL concentrations tested.

hibition is unclear. However, it is possible that differences unrelated to lipid peroxidation per se could have accounted for differences in the inhibitory effects of VLDL compared to LDL.

The results of the present investigation do not necessarily contradict the results of others who have investigated the role of other specific lipoprotein components in the inhibition of PBMC proliferation. For example, the apoE contained in lipoproteins could have effects on PBMC proliferation that are independent of the effects of lipoprotein peroxidation seen in the present studies (6). In still other investigations, lipoprotein inhibition of cell proliferation was abolished by adding transferrin to the culture media (3). The 5% pooled human serum used in the present experiments should have provided enough transferrin to overcome any lipoprotein inhibition of transferrin-induced proliferation (3). Moreover, additional transferrin did not alter the observed lipoprotein-induced inhibition of PBMC (data not shown). Thus, in the present experiments, the inhibitory effects of lipoproteins on PBMC appeared to be independent of transferrin-dependent cell proliferation.

In the present investigation, lipoprotein-induced inhibition of PBMC [^{3}H]thymidine incorporation was accompanied by decreased gamma interferon production. Gamma interferon is involved early in the cascade of events that lead to PBMC proliferation. Thus, these results confirm those of others who have shown that lipoprotein suppression of PBMC involves the inhibition of an early phase in PBMC proliferation (11, 17).

Although the present investigations were carried out in vitro, it is possible that lipid peroxidation products could also modulate immune system activity in vivo. Indeed, a growing body of evidence has indicated that TBARS are often present, and may contribute to the pathogenesis of cell injury, in a number of different clinical settings. For example, several investigations have suggested that lipid peroxidation products are involved in the chemotaxis and migration-inhibition of macrophages in early atherosclerotic lesions (23). The measurement of TBARS in the urine of rejecting renal allografts (24) and in other sites of inflammation (25) indicates that lipid peroxidation often accompanies the immune response. Thus, the principle finding in the present investigation, that the oxidative modification of lipoproteins may explain much of the reported interaction between lipoproteins and PBMC seen in vitro, could have important implications in a number of different clinical settings.

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