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Inhibition of Lymphocyte Mitogenesis by Autoxidized Low-Density Lipoprotein

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SUMMARY

Mitogenic stimulation of lymphocytes is significantly inhibited by addition of human serum low-density lipoprotein that has undergone autoxidation, while no significant effect is seen with non-oxidized lipoprotein. The inhibition is effective for cells stimulated either by the plant lectin phytohemagglutinin or enzymatically by neuraminidase-galactose oxidase treatment. However, it is markedly attenuated when oxidized LDL is added to cells which have been triggered 24 hours earlier. Lipid extracts from oxidized LDL are similarly inhibitory, indicating that the effect is mediated by an oxidized lipid fraction.

INTRODUCTION

Comparative studies on isolated serum lipoproteins have demonstrated that the low-density lipoprotein fraction (LDL) is the one most susceptible to spontaneous autoxidation (1-4). This reaction results in major changes in the overall structure (1-3, 5-8), absorption spectrum (3,6,9), lipid composition (2,8) and apoprotein structure (4) of LDL. In addition, this observed susceptibility to oxidation *in vitro* has led some authors to suggest that LDL oxidation may occur *in vivo* (1,7,8). Consistent with this hypothesis are both the report that plasma lipids contain significant diene conjugation (10), an early manifestation of lipid autoxidation, and the observation from our laboratory that minor protein bands, corresponding to oxidative artifacts, are present in apo-B preparations from fresh serum in spite of safeguards taken against oxidation (4).

Recent studies have reported effects of LDL on isolated lymphocytes, including control of lipoprotein receptor activity (11) and inhibition of

ABBREVIATIONS: LDL, low-density lipoprotein; PHA, phytohemagglutinin; NAGO, neuraminidase-galactose oxidase; TBRS, thiobarbituric reactive substances.

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mitogen stimulation (12,13). In view of the proven susceptibility of LDL to autoxidation *in vitro* and the possibility that this reaction may be initiated *in vivo*, we have examined the influence of oxidized LDL on isolated human lymphocytes.

MATERIALS AND METHODS

Lipoproteins. LDL was prepared by ultracentrifugal flotation as previously described (4). Following dialysis for 24 hours against phosphate-buffered saline at 4°, the samples (2.5 mg protein/ml) were autoxidized by further dialysis against air saturated-buffered saline at 37° for 24 hours, unless otherwise noted.

Lipid Extraction. One volume of lipoprotein solution was added dropwise to 10 volumes of chloroform-methanol (2:1) in a separatory funnel. The mixture was vigorously shaken and then washed with 25 volumes of a 0.5% calcium chloride solution (14). The chloroform phase was separated by centrifugation at 3000 RPM for 5 minutes and subsequently dried under nitrogen before being redissolved.

Isolation of Cells. Human peripheral blood lymphocytes were obtained from healthy, normal subjects by Ficoll-Hypaque gradient centrifugation (15).

Lymphocyte Cultures. Lymphocytes (1×10^6/ml) suspended in RPMI 1640 medium containing heat-inactiviated fetal calf serum (5%) and supplemented with penicillin (100 units/ml) and streptomycin (100 µg/ml) were cultured (0.2 ml aliquots) in flat bottom microwells (Microtest II, Falcon 3040) at 37° in 95% air-5% CO_2 atmosphere for 72 hr. Twenty hours before the termination of the incubation, 2 µCi (in 50 µl of medium) methyl-[^{3}H]-thymidine (2 Ci/mmole) were added to each culture well and its incorporation into DNA was measured (16). [^{3}H]-Thymidine incorporation is expressed as the mean of duplicate cultures.

Mitogenic Stimulation. PHA from *Phaseolus vulgaris* (purified HA 16) was obtained from Wellcome Research Laboratories, Beckenham, England. Cells were incubated with PHA at a concentration of 2 µg per ml. Neuraminidase (NA) from *Vibrio comma* was obtained from Grand Island Biological Company as a solution containing 500 units/ml (1 unit releases 1 µg N-acetyl-neuraminic acid glycoprotein at 37° in 15 min at pH 5.5). Galactose oxidase (GO) was obtained from Worthington Biochemical Corp., Freehold, N.J. The preparation contained 30 to 80 units/mg. One unit is the quantity of the enzyme that yields an absorbance of 1.0 at 420 nm by the peroxidase chromogen (17). Cells (10 to 20 x 10^6/ml) suspended in phosphate-buffered saline at pH 7.2 (18) were treated with NA (50 units/ml) and GO (5 units/ml) at 37° for 30 min with shaking, followed by two washings with phosphate-buffered saline to remove excess reagent.

Miscellaneous Procedures. Lipid oxidation was determined colorimetrically by the presence of thiobarbituric reactive substances (TBRS) having a peak absorbance at 532 nm (4). Protein was determined by the method of Lowry *et al* (19) using bovine serum albumin as a standard. Cytotoxicity was determined by the trypan blue exclusion test.

RESULTS AND DISCUSSION

Lymphocytes can be triggered to proliferate and divide after interaction with a number of agents, including antigens and mitogens (20,21). The degree

of stimulation can be measured by the incorporation of [^{3}H]-thymidine into cellular DNA. Addition of autoxidized LDL to lymphocytes just prior to stimulation by the plant lectin PHA results in a significant inhibition of the proliferative response at concentrations greater than 60 μg protein/ml, while no significant effect is found for non-oxidized LDL (Fig. 1-A). The possibility that the inhibition by the oxidized fraction is due to oxidized lipid acting on the lectin (e.g., crosslinking, scission) is eliminated by the observation that oxidized LDL also inhibits cells stimulated enzymatically (Fig. 1-B) by treatment with neuraminidase and galactose oxidase (NAGO) (22). In experiments using LDL and lymphocytes from different donors, it was found that the inhibition of mitogenesis showed a quantitative variation beyond the expected experimental error although results were qualitatively reproducible. This variability may be due to differences in either the physiological state of the cells or the lipoprotein lipid composition (23).

The dependence of the inhibitory effect on the oxidation state of the LDL is shown in Fig. 2. Lipid oxidation was measured by the thiobarbituric acid test (24). The decrease in thiobarbituric reactive substances (TBRS) at longer periods of oxidation has been reported by others and is believed to be due to further chemical changes in the TBRS occurring with continued oxidation (25).

Inhibition of mitogenesis is also dependent on the time at which the oxidized LDL is added (Fig. 3). At concentrations of oxidized LDL that decrease DNA synthesis to 50% of the control value only a 5% decrease is found when the lipoprotein is added to cells which have been triggered 24 hours earlier. This decrease in the inhibitory effect suggests that some early transient phase of mitogenesis is effected. Inhibition of lectin induced lymphocyte transformation by competing saccharides (26,27), depletion of Ca^{++} (28) or increased cyclic AMP levels (29) are also confined to an early phase of stimulation. Alternatively, pre-stimulated cells may have acquired a pro-

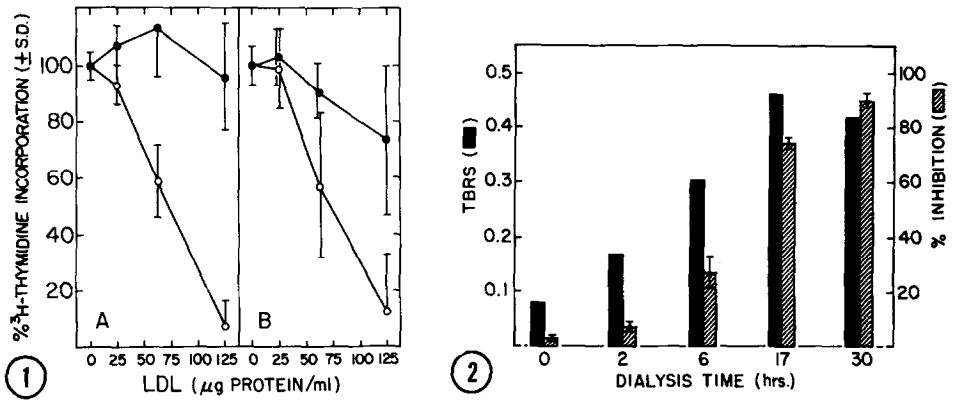


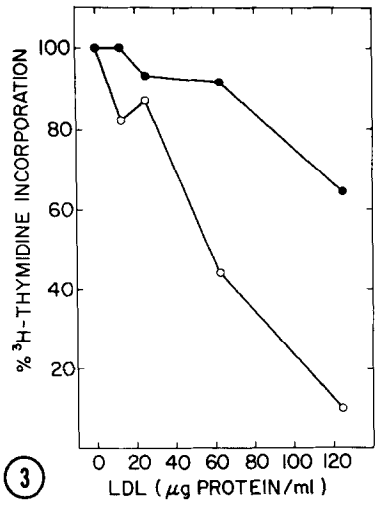
Fig. 1 The effect of oxidized (-O-) and non-oxidized (-●-) LDL on cells stimulated by: (A) PHA, (B) NAGO treatment. Data points represent the mean from seven (A) or nine (B) different experiments using duplicate cultures. The mean of the control incorporations, taken as 100%, were 189,000 cpm (A) and 134,000 cpm (B). Error bars represent the standard deviation.

Fig. 2 Relationship of lipoprotein oxidation state to lipoprotein inhibition of PHA stimulated incorporation of [^{3}H]-thymidine. LDL was oxidized for various time periods at 37°. The lipoprotein was then added at a final concentration of 125 μg protein per ml. TBRS is expressed as the absorbance at 532 nm when 0.5 ml of LDL (2.5 mg protein/ml) is reacted with thiobarbituric acid (4).

tective factor such as the lipid soluble antioxidant recently reported in mouse neuroblastoma cells that have been transformed (30,31).

Inhibition of mitogenesis by oxidized LDL is at least in part accompanied by a cytotoxic effect. The total number of viable cells present with either PHA or NAGO treated lymphocytes decreased by about 75% at concentrations of LDL which totally inhibited thymidine incorporation. After 72 hours of incubation total cell recovery was about half of the control value.

In order to characterize the chemical nature of the inhibitory factor produced by oxidized LDL the lipoprotein lipid was extracted, dried under nitrogen and resuspended in buffer. The results, shown in Fig. 4, indicate that the inhibitory factor is present in the lipid of oxidized LDL. The inhibition seen for the extract is also similar to that seen for oxidized LDL in that it effects cells stimulated by NAGO treatment and has a reduced effect when added to cells that have been stimulated 24 hours earlier.



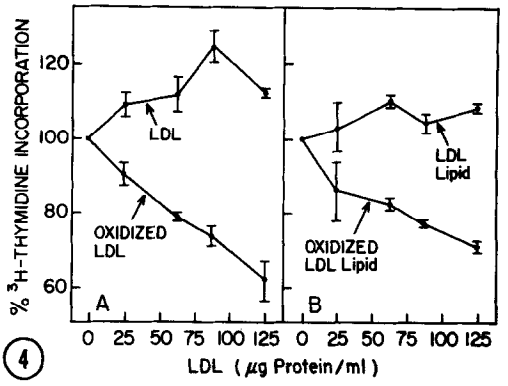


Fig. 3 Comparison of inhibitory effect of oxidized LDL on cells stimulated by PHA 24 hours prior to addition of lipoprotein (-●-) and 20 minutes after addition of lipoprotein (-○-). Data points are the mean of duplicate cultures, and have an average error of ± 3% of the control incorporation.

Fig. 4 Effect of oxidized and non-oxidized LDL (A), and their respective lipid extracts (B), on thymidine incorporation into lymphocytes stimulated by PHA.

In related studies others have reported inhibition of lectin induced lymphocyte mitogenesis by a low-density lipoprotein fraction (12,13). However comparisons with our results are unwarranted due to differences in the methods used.

The data presented here suggest that inhibition of mitogenesis by autoxidized LDL is due to a lipid fraction acting on the lymphocyte at an early phase of stimulation in a manner that also results in cytotoxicity.

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