EFFECT OF PHOSPHATIDYLCHOLINE LIPOSOMES ON THE MITOGEN-STIMULATED LYMPHOCYTE ACTIVATION

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Summary: The effect of phosphatidylcholine liposomes on the mitogen-stimulated lymphocyte activation was examined in vitro in an attempt to determine whether liposomes influence the cell growth. Phosphatidylcholine liposomes reduced the cellular cholesterol level and effectively inhibited lymphocyte activation. On the other hand, phosphatidylcholine-cholesterol liposomes (molar ratio 1:1) increased the cellular cholesterol level and was relatively ineffective in the inhibition. After phosphatidylcholine treatment, the addition of high-density lipoprotein to the medium reversed the inhibition of lymphocyte activation. It is concluded that the inhibition was related to the attraction and association of cellular cholesterol with liposomes. This is consistent with the notion that cholesterol is required for successful blast transformation.

Introduction: Liposomes are aqueous dispersions of phospholipids. The potential use of liposomes as carriers of drugs and enzymes for transport into cells is now well recognized (1). Understanding the interactions of the lipid components of liposomes with cells is essential for the successful utilization of liposomes as a tool in research and therapy. In an attempt to determine whether liposomes influence the control of cell growth and the immune response, we have investigated the effect of liposomes on the mitogen-stimulated lymphocyte activation. Phosphatidylcholine is frequently utilized as a component in the preparation of liposomes. One of the effects which result from the interactions of phosphatidylcholine liposomes with cells in culture is an increase in the rate of efflux of cell cholesterol into the medium (2). It has been suggested that cholesterol, a major constituent of cell membranes, plays an important role in the regulation of cell growth (3). Our results suggest that phosphatidylcholine liposomes inhibit lymphocyte

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activation, and that the inhibition is related to the alterations in the cholesterol metabolism.

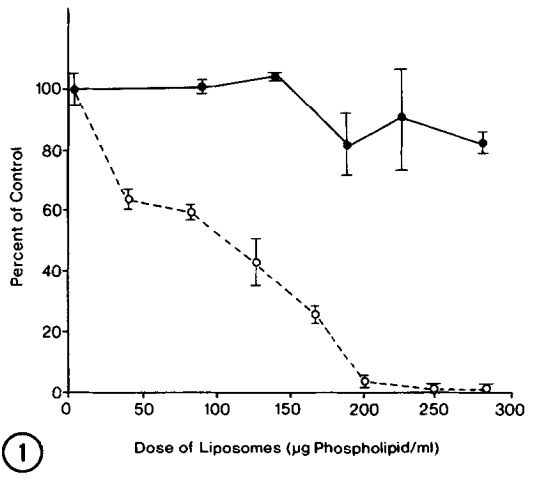
Materials and Methods: Preparation of Liposomes and Lipoproteins. Liposomes were prepared by sonication for 15 min. of 50 mg egg phosphatidylcholine or a mixture of phosphatidylcholine (50 mg) and cholesterol (26.5 mg) in 10 ml of RPMI-1640 culture medium as described by Edwards (2). Lipoproteins were isolated by ultracentrifugation according to the method of Havel et al (4) in a Spinco Ultracentrifuge from pooled human serum containing 1 mg/ml of EDTA. VLDL (density <1.006), LDL-1 (density 1.006 - 1.019), LDL-2 (density 1.019 - 1.063) and HDL (density 1.063 - 1.21) were dialyzed exhaustively against 0.9% NaCl/0.01% EDTA solution (pH 7.4) initially and against RPMI-1640 medium finally. Lipoproteins were sterilized by filtration (millipore HA) and kept at 4°C until used. The cholesterol to protein ratio in each fraction was 0.25, 0.32, 1.00 and 0.31 respectively for VLDL, LDL-1, LDL-2 and HDL.

Assay of Lymphocyte Activation. Peripheral blood lymphocytes were isolated from heparinized venous blood obtained from healthy donors by the Ficoll-Hypaque method (5). Lymphocyte activation was assessed in a microculture system. Cells were adjusted to a concentration of $5x10^5$ cells per ml in complete media (RPMI-1640 supplemented with gentamicin, 1% glutamine and 15% fetal calf serum) and 0.20 ml was distributed to each well. Liposomes in 0.05 ml of medium, or medium alone, were added and cultures were incubated at 37°C under humidified 5% CO_2 in air. After 24 hours of incubation a dose of Con. A. (concanavalin A, Sigma Co., St. Louis, Mo.), 10 µg per ml, was added to the well. The cultures were incubated for an additional 48 hours. To each well was added 0.5 µCi of ^{3}H-methyl thymidine (2.0 Ci/mM, New England Nuclear, Boston, Mass.). Lymphocytes were harvested 18 hours later with a Multiple Automated Sample Harvester (MASH-II, Microbiological Associates, Washington, D.C.). After drying for two hours at 80°C, the filter paper disks were solubilized with 0.5 ml of soluene-350 (Packard Co.) overnight, and were counted for β emission in 15 ml of Liquifluor (New England Nuclear).

Binding of [^{3}H]-labeled Con. A. Two million lymphocytes were cultured in 2 ml of RPMI medium with or without phosphatidylcholine liposomes (276 µg). After 24 hours, 20 µg of [^{3}H]-labeled Con. A (New England Nuclear, Boston, Mass., specific activity adjusted to 5.3 Ci/mM) was added and incubation continued for one more hour at 37°C. The fraction of [^{3}H]-labeled Con. A bound to lymphocytes was determined with a filtration method as described by Yahara and Edelman (6).

Determination of Cellular Cholesterol, Protein and Phospholipids. Liposomes were added in the beginning of the culture. After 24 hours, duplicate cultures were combined to give $2x10^6$ cells per tube and washed free of original medium with 10 ml of phosphate-buffered saline (PBS) for 5 times. The cell pellet was suspended in 0.5 ml of PBS for the determination of total cellular cholesterol, protein and phospholipids as described by Stein et al (7).

Results: Lymphocyte Activation. In this series of experiments liposomes were added in the beginning of lymphocyte culture, 24 hours before mitogenic stimulation. Fig. 1 shows the effect of various concentration of liposomes on Con. A-stimulated lymphocyte activation. Phosphatidylcholine liposomes effectively inhibited DNA synthesis. Complete inhibition of lymphocyte



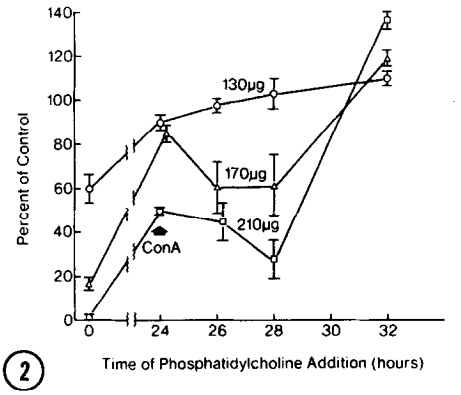


Figure 1. Inhibition of Lymphocyte Activation by phosphatidylcholine liposomes (—o—) and by phosphatidylcholine-cholesterol liposomes (—●—). The dose of liposomes was expressed as µg phospholipid per ml of medium. Lymphocyte activation was measured by uptake of tritiated thymidine and was expressed as percent of the control (100 x cpm test culture/cpm control culture). Points and range represent mean ± SEM obtained from triplicate assays.

Figure 2. Effect of phosphatidylcholine liposomes on lymphocyte activation as a function of phosphatidylcholine concentration and the time of addition. The cultures were established at time 0 and liposomes added at times indicated on the abscissa. All cultures received Con. A at 24 hours, were labeled with ^{3}H-thymidine at 72 hours, and harvested at 90 hours. The following doses of liposomes were tested: o——o 130 µg lipid per ml, Δ——Δ 170 µg lipid per ml, and □—□ 210 µg lipid per ml. Expression of results is the same as Figure 1.

activation was generally obtained at phosphatidylcholine concentrations above 200 µg per ml of medium. The dose of 50% inhibition for Con. A-stimulation was 99 ± 10 µg/ml (mean ± SEM obtained from six experiments). On the other hand, phosphatidylcholine-cholesterol liposomes (molar ratio 1:1) was relatively ineffective in inhibition, compared to phosphatidylcholine. A slight degree of inhibition was observed only when a relatively large dose was used. Inhibition of DNA synthesis by liposomes did not appear to be due to toxicity resulting in impaired viability. We have evaluated the cytoxicity over a period of four days and no apparent differences between the control and the experimental in viability were observed with trypan blue exclusion. Fig. 2

shows that the inhibition of lymphocyte activation by phosphatidylcholine was related to the dose and the duration of incubating lymphocytes with liposomes. Phosphatidylcholine liposomes effectively inhibited mitogen-induced lymphocyte activation even when it was added to the culture at the same time as mitogen or a few hours later. Approximately 8 hours after mitogenic stimulation, lymphocytes became completely refractive to the inhibitory action of liposomes, even when a relatively large dose of liposomes was added.

Binding of Con. A. The binding of [^{3}H]-labeled Con. A by the control and phosphatidylcholine liposome-treated lymphocytes is shown in Table 1. There was no significant difference between these groups. Thus, the inhibitory effect of phosphatidylcholine liposomes on lymphocyte activation must result from events occuring after the binding of mitogen.

Cellular Cholesterol Levels. Based on the assumption that the effect of liposomes on the lymphocyte activation is related to their effect on the cholesterol metabolism, the cellular cholesterol levels were measured. Table 1 shows that human peripheral blood lymphocytes contained about 48 μg of total cholesterol per mg of cellular protein. After incubation with phosphatidylcholine liposomes for 24 hours the level of cellular cholesterol decreased by about 16%. On the other hand, phosphatidylcholine-cholesterol liposomes (molar ratio 1:1) induced a 15% increase in the cellular cholesterol level. There were no significant differences between the control and the liposomes-treated groups in the content of cellular protein and phospholipids.

Influence of Serum Lipoproteins. Since the concentration of serum lipoproteins in the medium influences cholesterol flux (8), the possibility of using serum lipoproteins to reverse the alterations induced by liposomes was examined. In these experiments lymphocytes were incubated with phosphatidylcholine liposomes (170 μg phospholipid per ml.) for one day before the addition of Con. A and lipoproteins. HDL was effective in reversing the inhibition from 18% of tritiated thymidine uptake of the control to 83% of the control (Fig. 3). VLDL, LDL-1 and LDL-2 reversed the inhibition only

Table 1. Effect of Liposomes on Cellular Cholesterol Protein Phospholipid and Binding of [^{3}H]-Con. A.[a]

Liposomes in Medium	Total Cholesterol in Cells µg/mg Protein	Protein in Cells µg/2x10^6 Cells	Phospholipid in Cells µg/2x10^6 Cells	Binding of [^{3}H]-Con.A. % of Added Dose
none	48.4±1.1(4)	144±2(4)	17.1	1.41±0.14(3)
phosphatidylcholine	40.6±1.3(4)[b]	150±3(4)	17.2	1.24±0.07(3)
phosphatidylcholine-cholesterol	55.7±1.2(4)[b]	143±2(4)		

a. Lymphocytes were cultured with or without liposomes in the medium for 24 hours and assayed as described in Methods. Each value represents Mean ± SEM (No. of determinations) except for phospholipid values, which is the average of duplicate determinations.

b. $P<0.01$ (Significance of Difference)

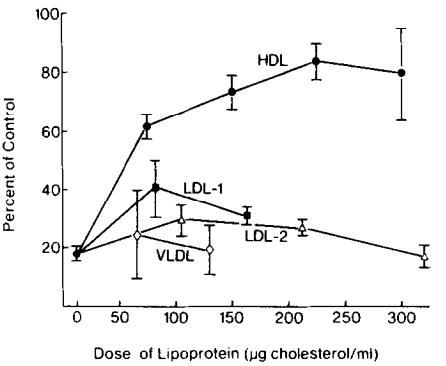


Figure 3. Influence of serum lipoprotein on the inhibition of lymphocyte activation by phosphatidylcholine liposomes. Procedures were the same as those in Figure 2, except that all test cultures received phosphatidylcholine liposomes (170 µg/ml) at zero time and the lipoprotein (the dose expressed as µg cholesterol per ml of medium) at 24 hours. Lymphocyte activation of liposome-treated cultures was expressed as percent of the control, which received RPMI medium instead of liposomes.

slightly. The addition of similar doses of lipoprotein fractions to control cultures, which received RPMI medium instead of liposomes, did not significantly enhance the uptake of tritiated thymidine (data not shown). The reversibility of the inhibition is another indication that the inhibition is not due to the impairment of cell viability.

Discussion: A prominent effect of adding phosphatidylcholine liposomes to the cell culture is in the increased rate of efflux of cell cholesterol into the medium and the decreased level of cell cholesterol (2, 9). The inhibition of lymphocyte activation by liposomes may be related to this effect. It has been suggested that the synthesis of cholesterol is an essential preprequisite for successful initiation and completion of the cell cycle in lymphocytes after mitogenic stimulation (10). In the early stage of stimulation the addition of sterol synthesis inhibitor to the culture can abolish the mitogenic response. The requirement for cholesterol presumably is for generating new membranes. In phosphatidylcholine-treated lymphocytes the level of available cell cholesterol for blastogenesis probably is too low to initiate or complete the process. The finding that phosphatidylcholine-

cholesterol liposomes (molar ration 1/1) are relatively ineffective in inhibition supports this interpretation. Phosphatidylcholine-cholesterol liposomes do not increase the rate of cholesterol efflux. Their presence in the culture actually raised the level of cell cholesterol (Table 1).

Plasma lipoproteins are effective in reversing the inhibition of lymphocyte activation induced by phosphatidylcholine. The effect may be due to the ability of plasma lipoproteins to alter either the rate or the direction of cholesterol flux (8). This is another indication that a deficiency in the available cell cholesterol may be the cause of inhibition. It should be noted that LDL is not as effective as HDL in reversing the inhibition, although LDL is generally recognized as a cholesterol carrier. The reason for this difference is presently unclear. It may be related to the differences between these two lipoproteins in the efficiency of providing cells with cholesterol. The utilization of LDL-cholesterol by lymphocytes in our assay system might be impaired by either liposomes or Con. A. The latter agent indeed has been shown to inhibit the proteolytic degradation of LDL in fibroblasts (11). A direct facilitating role of HDL in the mitogen-stimulated lymphocyte activation appears unlikely, since HDL did not influence the lymphocyte activation of the control cultures.

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