Drug Development and Industrial Pharmacy, 25(4), 517–521 (1999)

COMMUNICATION

The In Vivo Effect of Liposomes on Hematopoiesis

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ABSTRACT

The influence of liposome structure on hematopoiesis in vivo was assessed in relation to the different contents and origins of phospholipids that make up their membrane structures. Changes within different hematopoietic cells and serum tumor necrosis factor alpha (TNF-α) levels were estimated up to 14 days following intravenous administration of liposomes made of either pure egg yolk phosphatidylcholine (L_{EY}) or a soybean phospholipid preparation (L_{SB}) into normal CBA mice. In peripheral blood, only transient changes within white blood cells were observed. In bone marrow, a persistent decline in the number of mature granulocytes, monocytes, and lymphocytes was found. The changes within femoral granulocytic proliferative compartments in various stages of differentiation and a maturation compartment pointed out that, parallel with the depletion of the granulocyte-storage pool, stimulation of de novo production of granulocytic cells occurred. Although both types of tested liposomes induced similar cellular changes, only liposomes made of pure egg yolk phosphatidylcholine induced a transient increase in serum TNF-α levels.

INTRODUCTION

Liposomes are biocompatible membranelike vesicles consisting of phospholipid bilayers surrounding an aqueous compartment. Since their discovery, they have been widely used, from basic research as models for biological membranes, to delivery systems with controlled-release mechanisms for bioactive compounds (1–3). Despite the

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cumulative evidence, both in experimental animals and clinically, concerning the fate and behavior of liposomes in vivo, the mechanisms involved in the effects following lipid/liposome administration remain mostly unclear since the body's response includes coordinating action of different cell types and regulatory molecules.

The aim of this study was to evaluate the influence of liposome structure on hematopoiesis in vivo in relation to the different contents and origins of phospholipids making up the membrane structures. The effect of liposomes made of either pure egg yolk phosphatidylcholine (L_{EY}) or a soybean phospholipid preparation (L_{SB}) on different hematopoietic cells and serum levels of tumor necrosis factor alpha (TNF-α) was assessed in normal mice.

MATERIALS AND METHODS

Materials

Egg yolk phosphatidylcholine (approximately 99%), cholesterol (cell culture tested), and DL-α-tocopherol (cell culture tested) were obtained from Sigma Chemical Company (St. Louis, MO). A soybean, Epicuron 145 V, (97% total phospholipids: 45% phosphatidylcholine, 10% phosphatidylethanolamine, and 42% other phospholipids) was kindly provided by Lucas Meyer GmbH, Germany. Limulus amoebocyte lysate (LAL) was purchased from Haemachem, Incorporated (St. Louis, MO). All other chemicals and solvents were p.a. grade or high-performance liquid chromatography (HPLC) grade.

Preparation of Liposomes

Liposomal vesicles were freshly prepared using pure L_{EY} or L_{SB}, cholesterol, and α-tocopherol in a molar ratio 1:1:0.2 by the dry film method and sonication (4). The precise diameter and size distribution of liposomes were determined by laser scattering (Cilas, Alcatel, France). As the average diameter of the obtained vesicles was 0.43 μm, to get a narrow diameter, liposomes were repeatedly extruded through a Millipore filtration unit 0.22 μm (Millipore Corp., Bedford, MA) into sterile vials. Endotoxin levels, assessed by LAL test, were not more than 10 EU for both types of liposomes prepared. Suspended in a sterile, pyrogen-free physiological solution at a concentration of 7% w/w, the liposomes were further used for animal testing within 48 hr.

Experimental Protocol

Normal male CBA mice, weighing 20–22 g, were injected intravenously with 0.2 ml of L_{EY} or L_{SB} liposomal dispersion. The animals were sacrificed on days 1, 2, 3, 4, and 14 after treatment. The experiments were repeated twice, and 4–6 mice were used per group for each time point. The nontreated mice were used as controls. Blood samples were collected for determination of the serum TNF-α levels. In each animal, the following hematological parameters were estimated: in bone marrow, the number of granulocyte-macrophage committed stem cells (CFU-GM), the total number of nucleated cells, and the differential count of morphologically recognizable cells; in peripheral blood, the total number and differential count of white blood cells, platelets, and erythrocytes, as well as hematocrit.

The number of CFU-GM-derived colonies was determined using a methylcellulose cell-culture system with murine lung-conditioned medium derived colony-stimulating factor (CSF) (5,6). Morphologically recognizable bone marrow cells were estimated on femoral cell smears stained by the May-Grunwald-Giemsa procedure, and 1000 nucleated cells were differentiated and divided into the following compartments: proliferative granulocytes (PG), metamyelocytes (META), mature granulocytes (GRAN), monocytes (MONO), lymphocytes (LYMPHO), erythroblasts (ERBL), orthochromatic blasts (ORTHO), and other cells, including nonidentified cells. Differential counts of peripheral blood nucleated cells were made on 100 counted cells on blood smears stained by the May-Grunwald-Giemsa procedure, and cells were divided into the following compartments: metamyelocytes (META), mature granulocytes (GRAN), monocytes (MONO), and lymphocytes (LYMPHO). TNF-α activity was determined in serum samples by a bioassay of in vitro cytotoxicity against the murine L929 fibroblast cell line (7).

Statistical Analysis

Statistics were performed employing Student's *t* test. *P* values are expressed for liposome-treated against nontreated (control) mice.

RESULTS AND DISCUSSION

In peripheral blood, both types of liposomes studied induced some transient decrease in the total number of white blood cells 24 hr after application due to the decrease in the number of granulocytes and lymphocytes (Figs. 1A, 2A). A significant increase in the number of monocytes was observed only 3 days after L_{SB} liposome injection (Fig. 2B). Liposome-treated mice exhibited no

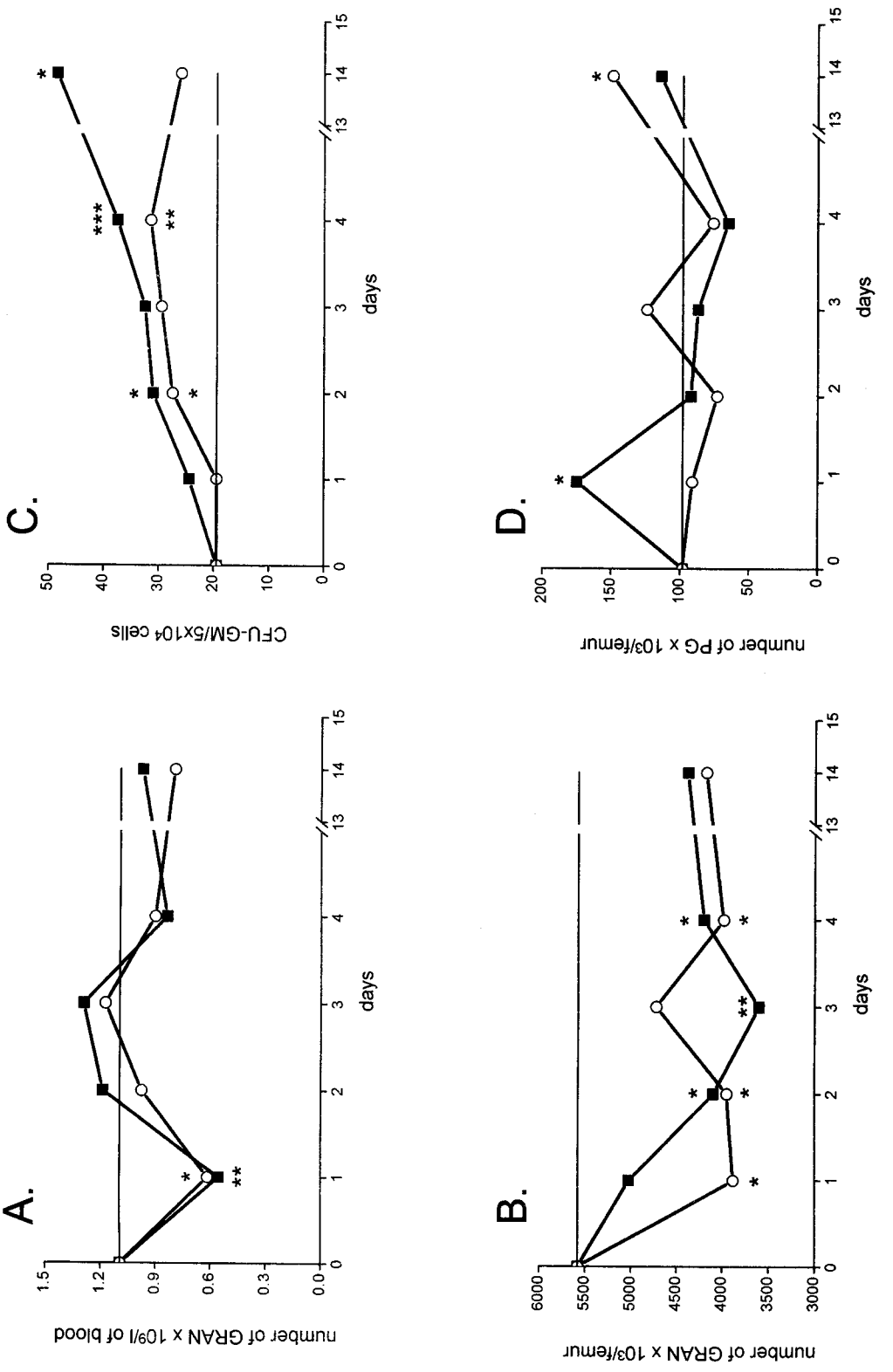

Figure 1. The effect of liposomes made of either pure egg yolk phosphatidylcholine (L_{EY}) or soybean phospholipid preparation (L_{SB}) on (A) peripheral blood granulocytes; (B) bone marrow granulocytes; (C) bone marrow CFU-GM; and (D) bone marrow morphologically recognizable proliferative granulocytes at different time intervals after intravenous administration in normal mice; —, controls, nontreated mice; —○—, L_{EY}; —■—, L_{SB}. Significant difference from control by *t* test: $*p < .05$; $**p < .01$; $***p < .001$.

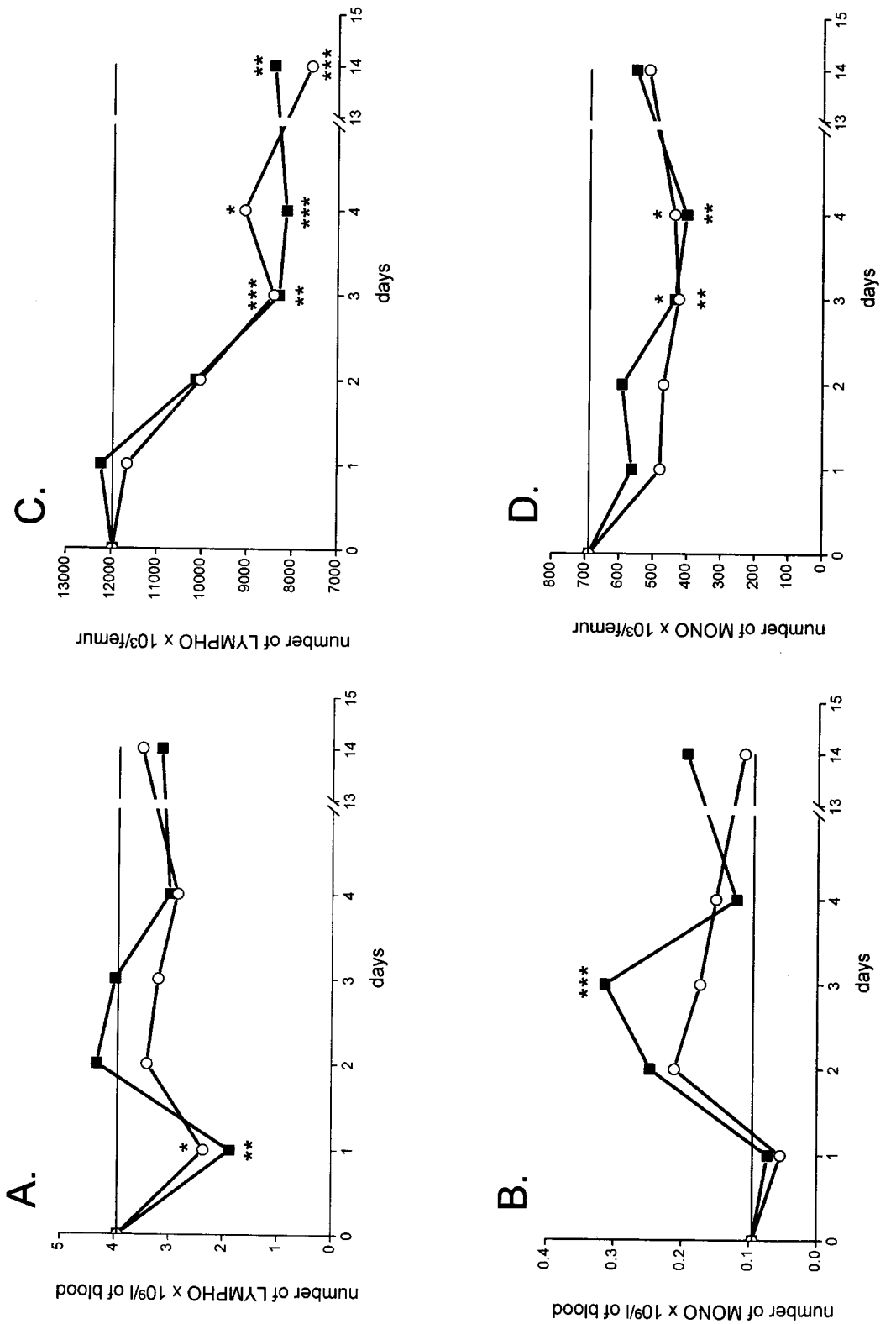

Figure 2. The effect of liposomes made of either pure egg yolk phosphatidylcholine (L_{EY}) or soybean phospholipid preparation (L_{SB}) on (A) peripheral blood lymphocytes; (B) peripheral blood monocytes; (C) bone marrow lymphocytes; and (D) bone marrow monocytes at different time intervals after intravenous administration in normal mice; —, controls, nontreated mice; —○—, L_{EY}; (—■—, L_{SB}. Significant difference from control by *t* test: $*p < .05$; $**p < .01$; $***p < .001$.

overall changes in the number of erythrocytes and platelets, as well as hematocrit (data not shown). The observed changes within white blood cells might be the consequence of the mobilization and migration of these cells to the sites of liposome distribution.

In bone marrow, the administration of a single intravenous L_{SB} or L_{EY} liposome injection significantly influenced the cells of granulocyte-macrophage, as well as lymphocyte, lineage, while the cells of erythroid lineage remained unchanged during all observed time points (data not shown). A statistically significant decrease in the number of mature granulocytes was observed starting as early as 24 hr after liposome treatment (Fig. 1B). A persistent decline in the numbers of monocytes and lymphocytes was also seen, with a statistically significant decrease found from day 3 after liposome injection onward (Figs. 2C, 2D). These persistent cellular changes suggested that liposomes induced mobilization of mature cells from the bone marrow. The analysis of changes within different granulocytic cell compartments demonstrated the increase in the number of CFU-GM (Fig. 1C) and oscillating changes in the number of morphologically recognizable proliferative granulocytes (Fig. 1D), accompanied by the decrease in the number of metamyelocytes (data not shown). These changes pointed out that, parallel with delivery of granulocytes from the bone marrow storage pool, stimulation of de novo production of granulocytic cells took place. The occurrence of enhanced granulopoiesis, manifested by the involvement of all granulocytic cell line compartments, including the most immature cells, CFU-GM, pointed to the extent and importance of granulocytic participation in the organism's response to liposomal treatment. Thus, when liposomal treatment is undertaken, involvement of mature granulocytes should be taken into consideration since these cells are recognized not only as cells engaged in phagocytosis, but also as cells that have the ability to synthesize and release immunoregulatory cytokines (8).

Simultaneous with the assessment of cellular changes, serum levels of TNF-α, an important mediator of the immune and inflammatory response, were determined. A transient increase in the level of circulating TNF-α was seen following L_{EY} administration, with peak activities of 73.3–164.5 pg/ml TNF-α seen in samples collected from day 1 to day 4 after injection (vs. controls, 25.5 pg/ml, $p < .001$) and a decrease to 20.2 pg/ml by day 14. In the sera of L_{SB}-injected mice, no significant changes in the amount of TNF-α were found compared to the control mice (values varied from 11.3 pg/ml on day 1 to 36.9 pg/ml on day 14). This finding implies that the secretion of TNF-α in vivo was related to the content and/or origin of the phospholipids used in liposome preparation. Our results are in agreement with other reports that describe different effects of various phospholipids on TNF levels in mice treated with empty liposomes (9–11), demonstrating that TNF release varies widely with the lipid(s) used and suggesting that the type of phospholipid might be the important factor in inducing/depressing cytokine release. Due to the complexity of the interconnected cytokine network, it is highly likely that the levels of other cytokines, such as interleukin-1 (IL-1), IL-6, IL-8, and colony-stimulating factors (CSFs), could also have been influenced. Thus, the modulating effects of different types of phospholipids used for liposome formulation have to be considered when these structures are intended for use for delivery of bioactive materials since, through cytokine secretion, they can influence the direction and evolution of an organism's reaction.

ACKNOWLEDGMENT

The assistance and helpful comments of Dr. S. Siler, as well as the excellent technical assistance of Mrs. K. Bozanic and Mrs. S. Markovic, are appreciated.

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