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LYSOPHOSPHATIDYLCHOLINE IN LIPOSOMAL MEMBRANES

ENHANCED PERMEABILITY BUT LITTLE EFFECT ON TRANSFER OF A WATER-SOLUBLE FLUORESCENT MARKER INTO HUMAN LYMPHOCYTES

EVELYN RALSTON *,[a], ROBERT BLUMENTHAL [a], JOHN N. WEINSTEIN [a]
SUSAN O. SHARROW [b] and PIERRE HENKART [b]

[a] *Laboratory of Theoretical Biology and* [b] *Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD 20205 (U.S.A.)*

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Summary

In an attempt to enhance delivery of liposome contents into cells, we tested the effect of lysophosphatidylcholine on transfer of the fluorescent dye, carboxyfluorescein, from small unilamellar and large multilamellar vesicles to human lymphocytes. Dioleoyl phosphatidylcholine and dioleoyl phosphatidylcholine-lysophosphatidylcholine small unilamellar vesicles with varying lipid ratios were prepared and characterized. In the presence of lysophosphatidylcholine, small unilamellar vesicles were slightly smaller and more leaky than those made without lysophosphatidylcholine. Lysophosphatidylcholine induced less leakage in large multilamellar vesicles. It did not show any appreciable effect on transfer of liposome contents, whether included as part of the liposomal bilayer (of unilamellar or multilamellar vesicles) or added exogenously together with small unilamellar dioleoyl phosphatidylcholine vesicles.

Much of the recent interest in liposomes (for recent reviews see Refs. 1 and 2) has been motivated by their potential use for introducing foreign material into cells, either to answer cell biological questions or for clinical purposes. However, most such applications have been limited by inefficiency of delivery,

* On leave from the Université Libre de Bruxelles (Belgium).

Abbreviations: DOPC, L-α-dioleoyl phosphatidylcholine; Hepes, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid. The word 'liposome' will be used as a general term for both small unilamellar vesicles and large multilamellar vesicles.

especially when the target cells are non-phagocytic. This state of affairs has stimulated a search for ways to enhance delivery, and the most popular suggestion has been inclusion of lysophosphatidylcholine in the system.

Lysophosphatidylcholine directly added to the cell growth medium has been shown to enhance or induce cell-cell fusion [3—5]. This is why it was suggested that small amounts of lysophosphatidylcholine in natural cell membranes play a role in cell membrane fusion in vivo [6]. Further work has shown that the capacity of small unilamellar vesicles to induce cell-cell fusion is increased when lysophosphatidylcholine is introduced into the vesicle bilayer [7,8]. Finally, lysophosphatidylcholine introduced in large multilamellar vesicles was reported to induce vesicle-vesicle fusion [9] and to increase the capacity of these vesicles to transfer their contents into cells by cell-vesicle fusion [10,11]. Hence the interest in lysophosphatidylcholine for enhancing delivery of liposome contents into cells.

However, the action of lysophosphatidylcholine as a 'fusogen' is not absolutely consistent. In one study [10], transfer of liposome contents into non-phagocytic cells was increased in only two of three cell lines by inclusion of lysophosphatidylcholine in the liposomes. In another instance [12] lysophosphatidylcholine reversibly inhibited natural cell fusion in monolayer cultures of muscle cells.

Lysophosphatidylcholine has also been reported to induce leakage of multilamellar liposome contents [10,13,14]. Therefore, if lysophosphatidylcholine were to be used in a liposome-cell system, a delicate balance would have to be achieved to enhance fusion without simultaneously losing liposome contents. But, here again, there is no agreement in the literature on the correlation between the amount of lysophosphatidylcholine introduced in the bilayer and the degree of leakage. In addition, no data could be found reporting leakage induced by lysophosphatidylcholine from small unilamellar vesicles.

The uncertainties on both qualitative and quantitative aspects of lysophosphatidylcholine's effects on large multilamellar vesicles, and the lack of data concerning its effects on the permeability of small unilamellar vesicles and on transfer of small unilamellar vesicle contents into cells prompted us to undertake a careful study of these issues. We used a system well-characterized in our laboratory [15], consisting of small unilamellar vesicles of dioleoyl phosphatidylcholine (DOPC) and of a non-phagocytic cell, the human peripheral blood lymphocyte. We have shown previously [15] that the transfer of a highly water-soluble fluorescent dye, carboxyfluorescein, from these DOPC liposomes to the lymphocytes consists of two components, one of which saturates at high vesicle concentrations. At V, only 0.01% of the presented material is transferred during a 10 min incubation with $4 \cdot 10^6$ cells/ml. This low efficiency illustrates the need for an enhancing factor.

We first characterized DOPC-lysophosphatidylcholine unilamellar vesicles (up to 20% lysophosphatidylcholine) by gel chromatography on Sepharose 4B and by measurement of their permeability to carboxyfluorescein; we then tested the effect of lysophosphatidylcholine on the transfer of liposome-encapsulated carboxyfluorescein to lymphocytes, either using DOPC-lysophosphatidylcholine (94 : 6) liposomes or using DOPC liposomes while adding lysophosphatidylcholine exogenously. We also measured the permeability to

carboxyfluorescein of large multilamellar vesicles made of DOPC-lysophosphatidylcholine (94 : 6 and 80 : 20) and measured the transfer of carboxyfluorescein from these vesicles into lymphocytes.

Our methods for preparing human peripheral blood lymphocytes and small unilamellar (probe-sonicated) vesicles are described elsewhere [15,16]. For large multilamellar vesicles, sonication and millipore filtration steps were omitted. The assay used to follow transfer of liposome contents to cells is based on the self-quenching properties of the fluorescent dye carboxyfluorescein and has also been described in detail [15—18]. That carboxyfluorescein is a good marker of the liposomal aqueous compartment and does not associate appreciably with fluid, uncharged, liposomal bilayers is substantiated, among others, by the following indications (Weinstein et al., unpublished results): (i) if 'empty' vesicles are exposed to a high concentration of carboxyfluorescein and then quickly passed over a Sephadex column at 0°C, little dye remains associated with them; (ii) leakage of carboxyfluorescein from vesicles can be followed to the point at which very little dye remains associated with them; (iii) when small unilamellar vesicles are prepared with carboxyfluorescein and another small marker (inulin, sucrose or methotrexate) the fraction of carboxyfluorescein encapsulated is identical to the fraction of the other marker. Carboxyfluorescein does not associate with cellular membranes either, since lymphocytes incubated with carboxyfluorescein show under the fluorescence microscope an evely distributed fluorescence pattern indicating free diffusion of carboxyfluorescein in the cell cytoplasm. The possibility that a small amount of carboxyfluorescein might be membrane-associated cannot be totally excluded but it would be a small fraction of the encapsulated carboxyfluorescein and in loose association. Furthermore, human lymphocytes do not induce significant leakage of liposome contents as do some other cell types [19].

Incubations proceeded essentially as described previously [15] but with preincubations of 10 min at 37°C and with $2 \cdot 10^6$ cells per tube in a total incubation volume of 500 μl. Population distributions of cell fluorescence and the average number of molecules of carboxyfluorescein incorporated into each viable cell were determined by flow microfluorometry using the Fluorescence-Activated Cell Sorter (Becton-Dickinson Electronics, Mountain View, CA) as described in Ref. 15. To measure liposome leakage, polystyrene tubes containing 1 ml of 168 mM NaCl/6.8 mM KCl/10 mM Hepes (Medium 1) were brought to 37°C. At time zero, 200 μl of liposome preparation was added to each tube. At different times of incubation (1.5, 30 and 60 min), 50 μl aliquots were removed and immediately diluted into 1 ml of ice-cold Medium 1 to stop leakage. The fluorescence was measured on 100 μl aliquots of these dilutions in quartz microcuvettes (optical pathlength 3 mm). Measurements were done at 470 nm excitation wavelength and 520 nm emission wavelength in an Aminco-Bowman spectrophotofluorometer, before (F) and after (F_T) addition of 5 μl of Triton-X100. The percentage of dye remaining inside the vesicles after t min of incubation was calculated as $P(t)(\%) = 100\ (1 - F/F_T)$. No correction was made to take into account relief of intravesicle self-quenching when large amounts of carboxyfluorescein have leaked out. Lipid concentrations were determined by phosphorus analysis [20].

DOPC, egg lysophosphatidylcholine, and L-α-monooleoyl lysophosphatidylcholine were obtained from Avanti Biochemicals, Inc. in benzene solution; 100 μg samples gave single spots on thin-layer chromatography [15]. Carboxyfluorescein was obtained from Eastman (Rochester, N.Y.) and was further purified as described in Ref. 15. Media and buffers were prepared by the NIH media production unit.

Size and permeability of DOPC lysophosphatidylcholine liposomes

Fig. 1 shows the elution profiles of DOPC and DOPC-lysophosphatidylcholine (80 : 20) vesicles on Sepharose 4B. They both have the double-peak pattern characteristic of sonicated vesicles [21]: a small peak (fraction I) containing the residual multilamellar vesicles eluted with the void volume and a broader one (the early and late fractions of which are called IIa and IIb) containing primarily unilamellar vesicles. The larger elution volume required for the DOPC-lysophosphatidylcholine vesicles suggests that these vesicles are somewhat smaller than the DOPC ones. On the figure are also indicated the lipid/carboxyfluorescein ratios for the different fractions. These ratios were determined by comparing phosphorus analysis and fluorescence. We had previously found a lipid/carboxyfluorescein ratio of 31 for DOPC vesicles sonicated with 100 mM carboxyfluorescein and prepared by gel chromatography on Sephadex G-50; this ratio agrees well with that found by Hauser et al. [22] for small unilamellar vesicles. Simple calculations (after Ref. 22) give an approximate idea of the correlation between the lipid/carboxyfluorescein ratio and the dimensions of unilamellar vesicles: assuming an area of 70Å^2 per phosphatidylcholine molecule (both DOPC and lysophosphatidylcholine) and a bilayer thickness of 46Å [22] we found that a ratio of 31 corresponds to

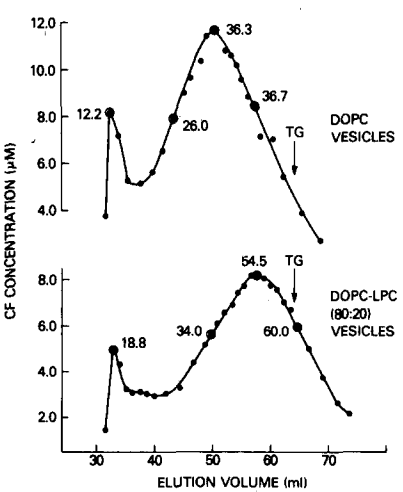

Fig. 1. Elution profiles of DOPC vesicles and DOPC-lysophosphatidylcholine (DOPC-LPC) (80 : 20) unilamellar vesicles on Sepharose 4B (24 · 220 mm column); vesicles were detected by fluorescence of the entrapped carboxyfluorescein (CF) (post-Triton values). Thyroglobulin (TG), used as a marker, was eluted where indicated by the arrow. The numbers written along the curves are the molar lipid/carboxyfluorescein ratios measured for the fractions indicated (•).

vesicles with an outer diameter of 250Å. A ratio of 36 as found for the peak of DOPC vesicles (Fig. 1) corresponds to a diameter of 237Å whereas 54, as found for the peak of DOPC-lysophosphatidylcholine vesicles, corresponds to vesicle diameters of 205Å. Thus, these calculations also indicate a decrease in liposome size in the presence of 20% lysophosphatidylcholine (diameter reduced by 14%, volume by 36%). It seems likely that lysophosphatidylcholine, with only one fatty acyl chain, permits a lower radius of curvature.

In Fig. 2 the rate of leakage of carboxyfluorescein from DOPC and DOPC-lysophosphatidylcholine small unilamellar vesicles at 37°C is shown as a function of time. Incorporation of lysophosphatidylcholine in the liposomal bilayer increases leakage of liposome contents but a reasonable amount of dye still remains inside the vesicles after 1 h until about 10% lysophosphatidylcholine has been incorporated. Similar results were obtained whether egg lysophosphatidylcholine or L-α-monooleoyl lysophosphatidylcholine was used. Leakage from large multilamellar vesicles was smaller: after one hour at 37°C, DOPC large multilamellar vesicles retained 82%, DOPC-lysophosphatidylcholine (94 : 6) 76% and DOPC-lysophosphatidylcholine (80 : 20) 70% of their initial carboxyfluorescein content.

As mentioned earlier, there is little agreement in the literature on the extent

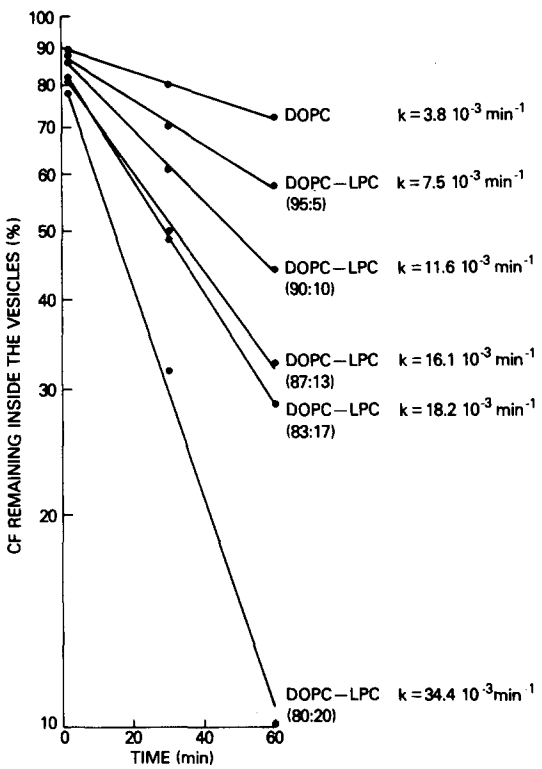

Fig. 2. Leakage of carboxyfluorescein (CF) from DOPC or DOPC-lysophosphatidylcholine (DOPC-LPC) small unilamellar vesicles of varying lysophosphatidylcholine content: fraction of dye (percentage of initial content) remaining inside the vesicles as a function of incubation time at 37°C. Rate constants for leakage are indicated on the figure.

of the leakage increase from large multilamellar vesicles caused by lysophosphatidylcholine; the rates of leakage obtained by each group differ, more than would be expected on the basis of differences in the lipid composition of the vesicles or the nature of the entrapped solute. Mandersloot et al. [13], monitoring the leakage of K^+, observed for egg lecithin-lysophosphatidylcholine (77.5 : 22.5) vesicles retention of about 50% of the entrapped solute after 1 h incubation at 13°C. Weissman et al. [10,11] reported a much higher leakage: 90% of the entrapped solute (CrO_4^{2-} or glucose) was released after 90 min incubation at 37°C from vesicles made of phosphatidylcholine, dicetylphosphate, cholesterol, and just 2% lysophosphatidylcholine. Kitagawa et al. [14] prepared vesicles made of egg lecithin and lysophosphatidylcholine with or without cholesterol and measured the amount of glucose retained inside the vesicles after 90 min at room temperature as a function of the amount of lysophosphatidylcholine incorporated (mol% of total phospholipid). Vesicles containing 20% lysophosphatidylcholine and no cholesterol leaked 50% more than those without lysophosphatidylcholine; in the presence of 23 mol% cholesterol they leaked about 40% more than without lysophosphatidylcholine. Our data show that with liposomes made of DOPC and lysophosphatidylcholine, the latter induces more leakage from small unilamellar vesicles than from large multilamellar vesicles. However, this leakage is still reasonable enough to warrant use of small unilamellar vesicles containing moderate amounts of lysophosphatidylcholine in experiments with relatively short incubation times at 37°C (10 min for example).

Effect of lysophosphatidylcholine on carboxyfluorescein transfer

At the outset of this work we hoped that lysophosphatidylcholine would enhance transfer of liposome contents into lymphocytes. Recent work done in our laboratory (Blumenthal et al., unpublished results) has shown that carboxyfluorescein and other markers of the liposomal aqueous compartment (sucrose and inulin) are taken up commensurately and thus that carboxyfluorescein is a good marker for the study of the transfer of liposome contents into cells (such as lymphocytes) which do not induce major leakage from liposomes. The precise nature of this transfer has been discussed by ourselves [15,23] and by others [24] and has not been fully understood as yet. Fusion is only one of the possible mechanisms. However, regardless of the mechanism of carboxyfluorescein uptake in the absence of lysophosphatidylcholine, this compound would be expected to enhance carboxyfluorescein uptake were it to act as a 'fusogen'. As shown in Fig. 3, though, no enhancement of carboxyfluorescein transfer from small unilamellar vesicles to lymphocytes could be detected, whether using DOPC-lysophosphatidylcholine (94 : 6) vesicles or adding lysophosphatidylcholine (6 mol%) to the incubation medium containing the cells and DOPC vesicles. The curves plotted in Fig. 3 represent the initial rates of carboxyfluorescein transfer from liposomes to lymphocytes as a function of vesicle concentration. The experiment was done a total of three times with similar results. The slight apparent decrease in transfer from DOPC-lysophosphatidylcholine (94 : 6) vesicles is within experimental error, and the small apparent increase in transfer with exogenous addition of lysophosphatidyl-

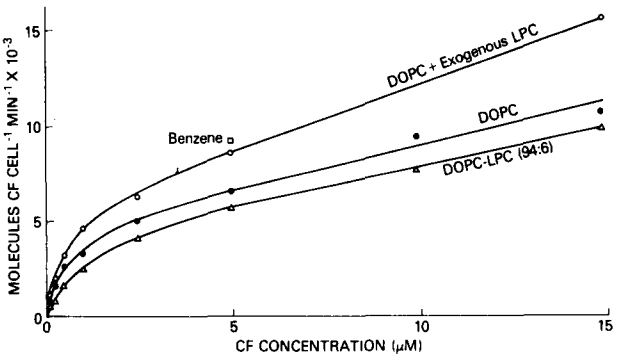

Fig. 3. Initial rate of transfer of carboxyfluorescein from unilamellar vesicles to lymphocytes at 37°C as a function of carboxyfluorescein (CF) concentration (post-Triton values). •, Control: DOPC vesicles; △, DOPC-lysophosphatidylcholine (DOPC-LPC) vesicles; ○, DOPC vesicles + 6% exogenous lysophosphatidylcholine (LPC) added in 5 μl benzene; □, DOPC vesicles + 5 μl benzene.

choline proved to be an artifact: lysophosphatidylcholine had been dissolved in 5 μl of benzene, which itself causes a slight increase (20%) in the rate of liposome-mediated transfer, as shown by the control point (addition of benzene alone). Leakage of carboxyfluorescein from the vesicles during the 10 min incubation was small and could be accounted for by a correction factor of 2% for the control and 4% for the DOPC-lysophosphatidylcholine vesicles. Table I shows that transfer from large multilamellar vesicles is not greatly enhanced either: 6% lysophosphatidylcholine increased transfer by about 10%, 20% lysophosphatidylcholine increased it by 25% (at 10.0 μM carboxyfluorescein) and 45% (at 1.0 μM carboxyfluorescein). This is small compared to the 2—5-fold enhancement of enzyme uptake reported in the literature for just 1% lysophosphatidylcholine [10,11]. We have not studied the effect of lysophosphatidylcholine on transfer of macromolecules. Table I also illustrates that transfer from large multilamellar vesicles is lower than transfer from small unilamellar vesicles. This could simply be due to osmotic equilibration of carboxyfluorescein at high concentration across the large

TABLE I

Initial rate of transfer of carboxyfluorescein (CF) from liposomes to lymphocytes at 37°C: comparison between unilamellar and multilamellar vesicles with or without lysophosphatidylcholine (LPC), at two liposome concentrations (1.0 and 10.0 μM carboxyfluorescein, post-Triton values).

CF (μM)	LPC (%)	Transfer (mol/cell/min) (X 10^{-3})	
		Unilamellar vesicles	Multilamellar vesicles
10.0	0	25.4	11.7
	6	28.0	12.6
	20	26.2	14.8
1.0	0	8.3	2.3
	6	9.1	2.6
	20	9.3	3.3

multilamellar vesicle membrane, but such differences between the two types of vesicles have been seen in other systems [8,25,26].

Although not greatly increasing transfer of liposome contents into the cell, lysophosphatidylcholine might still cause adsorption of intact vesicles to the cells. In that case, high ratios of fluorescence after addition of 5% Triton X-100 (F_T) over fluorescence before addition of the detergent (F) would be obtained, due to the release by Triton of the self-quenched carboxyfluorescein from the adsorbed vesicles. In our experiments the F_T/F ratio was unchanged with lysophosphatidylcholine, for both small unilamellar vesicles and large multilamellar vesicles. Stable adsorption of intact vesicles to the cells, caused by lysophosphatidylcholine, is thus also ruled out. These same data rule out the possibility that, on interaction between the cell and n-bilayer multilamellar vesicles, fusion occurs between the liposomal outer bilayer and the cell membrane with resulting transfer of $(n-1)$-bilayer vesicles (containing self-quenched carboxyfluorescein) inside the cell. No such $(n-1)$ forms are revealed within the cells by fluorescence microscopy either.

In conclusion, we have shown (i) that DOPC-lysophosphatidylcholine vesicles are smaller than DOPC vesicles, (ii) that appreciable amounts of lysophosphatidylcholine can be included in the lipid of either multilamellar or small unilamellar DOPC vesicles without causing a prohibitive leakage of vesicle contents, (iii) that this lysophosphatidylcholine failed to enhance appreciably the transfer of carboxyfluorescein from the vesicles into lymphocytes, and (iv) that lysophosphatidylcholine did not cause stable adsorption of vesicles to the cells.

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