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The Interaction of Liposomes with Cells: The Relation of Cell Specific Toxicity to Lipid Composition.

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Abstract—*Liposomes, which have been proposed as agent carriers, can themselves produce a wide variety of effects on the viability of co-incubated cells.*

In this study we show that the lipid composition of empty liposomes produced varied effects on the viability, as measured by [^{3}H]-thymidine incorporation, of leukemic cells and fibroblasts. Certain liposomal compositions, particularly those involving stearylamine, were highly toxic to both cell types.

It is evident from this investigation that caution must be exercised in the choice of lipid composition if the effect of the liposomes is not to conceal that of the drug, or carried agent, either on the target cells in terms of therapeutic effect, or on normal cells in terms of toxicity.

Normal lysophosphatides, having an acyl bond in sn-1, *can be metabolised by both normal and leukemic cells; however the replacement of the acyl by an alkyl bond in a lysophosphatide requires an O-alkyl cleavage enzyme to be metabolised. Such an enzyme is present in a multifunctional oxygenase in normal cells but appears to be absent or inoperative in certain tumor cells.*

Coincubation of alkyl lysophosphatide containing liposomes is shown to produce selective destruction of L1210 leukemic cells; in addition the liposomal form of such analogs is suggested as being more effective against leukemic cells and less toxic to normal cells than when used in the free form.

INTRODUCTION

THE INTEREST in phospholipid vesicles, liposomes, as convenient models for biological membranes [1] was rapidly augmented by their potential use as carriers of drugs [2] and enzymes [3]. A review of their potential and usage has been given recently by Tyrrell *et al.* [4].

Much of the recent effort in this area has focused on the use of liposomes to deliver their contents directly to the cells, and in attempting to introduce some target cell specificity into this delivery [5]. It has become apparent, however, that often the major effect observed is due to the liposomal complex acting as a controlled release system. This has the advantage of a slower plasma clearance, a concomitantly constant plasma concentration, and possible protection of the agent from metabolic degradation [6].

It is also evident that liposomes themselves can have considerable effects at a cellular level. Evidence has been presented to show that liposomes containing stearylamine may cause intracellular release of lysomal enzymes [7], and when DBA/2 mice with L1210 tumors were injected i.p. with positively charged liposomes and entrapped methotrexate (MTX), significant toxicity was found at MTX concentrations several hundred-fold below the dose of free drug which would be toxic [6]. Negatively charged liposomes have been found to induce the rounding up of fibroblasts [8] whereas liposomes with a po-

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Abbreviations: PC: 3-*sn*-phosphatidycholine; CHOL: cholesterol; SP: sphingomyelin; SA: stearylamine; DCP: dicetyl phosphate: PS: phosphatidylserine: LL: 2-lysophosphatidylcholine; LPE: 2-lysophosphatidylethanolamine; LLA: 2-lysophosphatidylcholine analog; PBS: phosphate buffered saline; DMEM: Dulbecco's modified Eagles medium; FCS: foetal calf serum.

sitive charge have been reported to agglutinate and hemolyse red blood cells [9].

Thus we decided to investigate the effect of liposomes of different lipid composition interacting with fibroblasts and leukemic cells since such a coincubation appeared to offer the possibility of selectively affecting or destroying cells, either by introducing leaky patches into the plasma membrane, and/or by supplying lipids which could not be metabolized by the target cells.

In terms of the therapeutic use of liposomes as carriers one is interested in two aspects; firstly that the carrier system distinguishes between normal and target cells and kills or affects only the latter and secondly that it is the carrier–drug complex that causes the effect and not the carrier itself. In addition we report here that the coincubation of liposomes containing certain analogs of lysophosphatidylcholine can produce cell specific effects thus demonstrating their chemotherapeutic potential.

MATERIALS AND METHODS

3-*sn*-Phosphatidylcholine, phosphatidylserine, 2-lysophosphatidylcholine, lysophosphatidylethanolamine and sphingomyelin were obtained from Lipid Products, Nutfield Ridge, U.K.; stearylamine from Koch Light Labs Ltd., Colnbrook, U.K.; and dicetylphosphate, cholesterol and mitomycin from Sigma Chemical Co., St. Louis, U.S.A. All lipids were routinely assayed for purity by thin layer chromatography before use. 6-[^{3}H]-Thymidine (5 Ci/mmole sp. act.) was purchased from Radiochemical Centre, Amersham, U.K. The 2-lysophosphatidylcholine analogs were synthesized as described elsewhere [10]. The structure of natural 2-lysophosphatidylcholine modified by replacing the acyl link by an alkyl link in *sn*-1, altering the number of carbon atoms in the aliphatic side chain, or substituting the -OH group in *sn*-2. The analogs were described as follows [10], the first symbol ES or ET denoting an acyl or alkyl link, the second symbol the number of carbon atoms in the aliphatic side chain, and the third denoting the functional group replacing the -OH. In this report two analogs were used, ET-18-OCH_3, and ET-18-H, in addition to natural 2-lysophosphatidylcholine and 2-lysophosphatidylethanolamine.

Liposome preparation

Chloroform or chloroform–methanol solutions of the lipid mixtures were evaporated to dryness under reduced pressure in a rotary evaporator. Aqueous medium, physiologically iso-osmotic phosphate buffered saline (PBS) (0.8% w/v NaCl, 27 mM KCl, 7.9 mM $Na_2HPO_4.2H_2O$, 1.47 mM KH_2PO_4, pH 7.3) was added and the dried lipid film agitated off the walls of the evaporator flask. These multilamellar vesicles (10 ml) were then sonicated in an N_2 atmosphere at 25°C, using a Branson Sonifier (75 W, 20 kHz, 27.5 mm probe). For each of the various lipid mixtures sonication was carried out for such a time (6–10 min) that after resealing under N_2 at 25°C (at least 4 hr) the multilamellar liposomes all had the same or very similar absorbance at 215 nm. Light scattering measurements indicated a diameter of 80 ± 5 nm suggesting that these liposomes were small vesicles bounded by a few bilayers; the liposomes were routinely examined for lipid oxidation by the method of Klein [11], none was detected within the sensitivity limits of the method (0.01%).

Cell cultures

L1210 mouse leukemia cells (2×10^5) were incubated (10% humified CO_2 atmosphere) in 2 ml of Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum (Gibco Batch K982301S), in Linbro tissue culture trays (FB 16 24 TC, Linbro Scientific Co., Hamden, Conn., U.S.A.) with appropriate aliquots of liposomes. For measuring the viability of the cells after incubation with liposomes, 0.2 ml of the cell suspension was transferred to microtiter trays (Microtest II, Falcon) and pulsed with 0.1 μCi [^{3}H]-thymidine. After a 2-hr pulse the cells were harvested with a multiple cell harvester (Skatron, Lierbyen, Norway) on glass-fibre paper (Skatron), and counted in a liquid scintillation counter (Intertechnique) in toluol with butyl-PBD (5 g/l) (New England Nuclear, Boston, U.S.A.) as scintillation fluid. Since the cells were harvested with a water wash and consequently lysed during the harvesting procedure only nucleic acid is adsorbed to the glass-fibre filter and counted. For comparison with leukemic cells, L929 mouse fibroblasts were chosen as a model for normal cells as they are only weakly tumorgenic after repression of the host defense by irradiation. But because they adhere to plastic these cells, in the same concentration as the tumor cells, were mixed with the appropriate dilutions of the liposomes and cultured directly in 0.2 ml aliquots in microtiter trays under the same incubation conditions. For each time point,

one tray was pulsed with [^{3}H]-thymidine (0.1 μCi per well) and then harvested as described for the L1210 cells. All cells used were taken from proliferating cultures and were dividing during the experiments with a doubling time of 13–14 hr.

RESULTS

The aim of this study was to determine the effect of small multilamellar liposomes (i.e., the potential carrier system) on the viability of normal and leukemic cells. The concentration of liposomes in the incubation medium varied from 0.25 to 1000 nmole total lipid/ml. Various combinations of lipids were used for the formation of the liposomes but with the exception of those composed of phosphatidylcholine (PC) and cholesterol only (CHOL), a three component mixture (in a molar ratio of 7:2:1) was used throughout; the predominant lipid being either phosphatidylcholine or sphingomyelin (SP), 20 mole % always being cholesterol (to reduce the fluidity of the membrane) [12], the remaining 10 mole% being either a charged lipid or a lysophosphatide. The mole ratio of 7:2:1 was adopted since it appears to be one of the most common ones chosen for liposomes employed as carriers [13], but this does not necessarily imply that it has optimal properties. The work of Munder *et al.* [10] had shown selective therapeutic effects of 2-lysophosphatidylcholine analogs on tumor bearing mice, and therefore co-incubation with analogs in the free form were carried out at the same concentrations as were contained in the liposomes, i.e., a range of 0.025 to 100 nmole/ml.

Since it has been reported that the adsorption of liposomes onto cells does affect some transport mechanisms, we chose the incorporation of [^{3}H]-thymidine [14] as a parameter of membrane functions and cell viability, as precursor incorporation is an internal measure of metabolic activity in the cells and may record changes in cell viability earlier than other methods. The effect of incubation in the presence of liposomes is expressed as the percentage of [^{3}H]-thymidine incorporation by the cells as compared to that of control cultures incubated under the same conditions but without liposomes. The effects of the co-incubation of liposomes of different composition on the metabolic activity of L1210 leukemic cells and L929 fibroblasts are shown in Figs. 1–5. Co-incubation was carried out for 2, 20 and 40 hr (not shown graphically); I_{50} values (the concentration of lipid causing

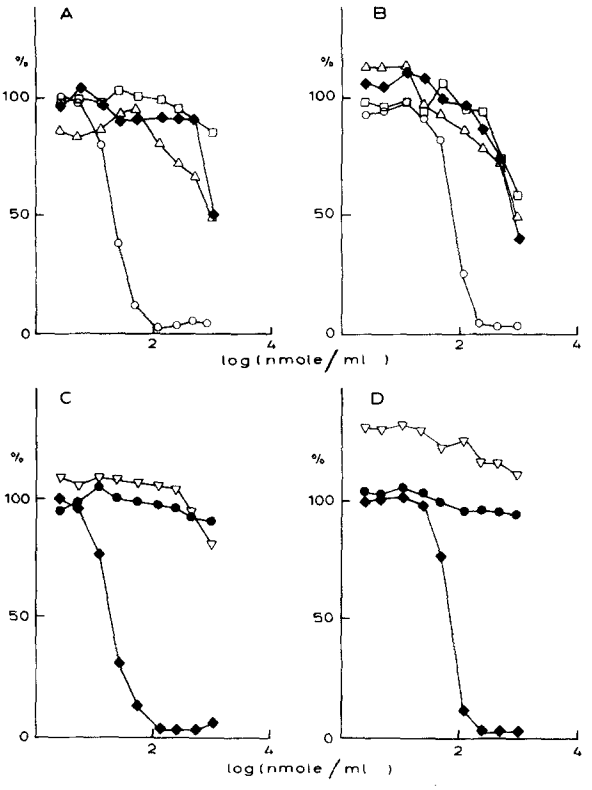

Fig. 1. The effect on cell viability of co-incubation for 2 hr *of (A) and (C) L1210 leukemic cells, and (B) and (D) L929 fibroblasts with liposomes of different composition. In parts A and B liposomes composed of :* (◆—◆) *PC:CHOL* (8:2 mole ratio), (○—○) *PC:CHOL:SA* (7:2:1 mole ratio), (△—△) *PC:CHOL:DCP* (7:2:1 mole ratio), (□—□) *PC:CHOL:PS* (7:2:1: mole ratio), *for parts C and D liposomes composed of* (▽—▽) *PC:CHOL:LL* (7:2:1 mole ratio), (◆—◆) *SP:CHOL:SA* (7:2:1 mole ratio), (●—●) *SP:CHOL:PS* (7:2:1 mole ratio). *The* [^{3}H]-*thymidine incorporation at different total lipid concentrations is expressed as a percentage of the incorporation by control cultures: the incubation conditions being as described in Materials and Methods.*

50% inhibition of [^{3}H]-thymidine incorporation) for different incubation times are given in Tables 1 and 2. I_{50} values were either directly measured or estimated where sufficiently marked inhibition was observed to allow relatively unambiguous extrapolation of the inhibition curve. From these results the effect of co-incubation appears to fall into three categories: (1) inhibition only at high concentrations with little incubation time dependency; (2) strong inhibition showing both concentration and time dependency, and (3) no effect with either variable.

As can be seen from Figs. 1 and 2 and Table 1, liposomes composed of PC:CHOL (8:2) or PC:CHOL:DCP (7:2:1) fit in the first group; after 2 hr incubation inhibition occurs only at high concentration (> 1000 nmole/ml), this inhibition increased after 20 hr incubation, but not so markedly

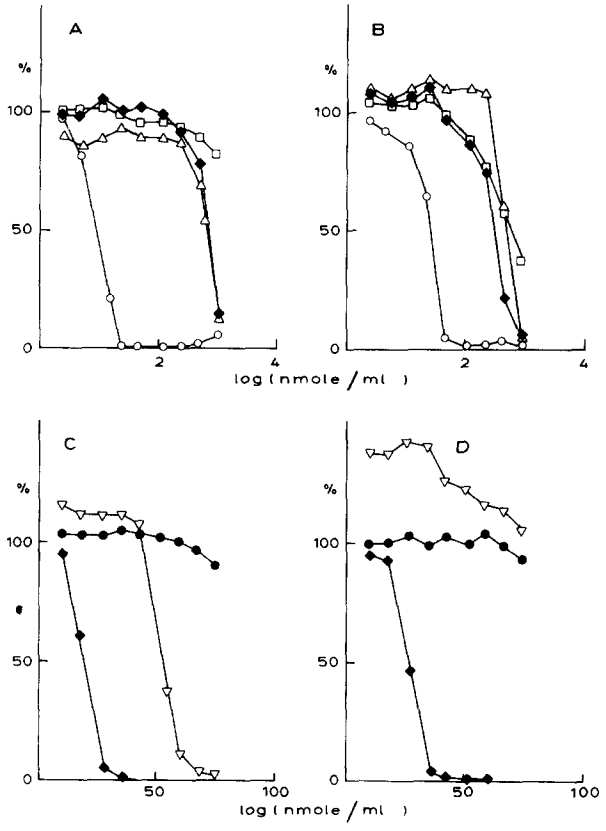

Fig. 2. The effect on cell viability of co-incubation for 20 hr *of (A) and (C) L1210 leukemic cells, and (B) and (D) L929 fibroblasts with liposomes composed of* (◆—◆) *PC:CHOL* (8:2 mole ratio), (○—○) *PC:CHOL:SA* (7:2:1 mole ratio), (△—△) *PC:CHOL:DCP* (7:2:1 mole ratio), (□—□) *PC: CHOL: PS* (7 : 2 : 1 mole ratio), *for parts C and D liposomes composed of* (▽—▽) *PC:CHOL:LL* (7:2:1 mole ratio), (◆—◆) *SP:CHOL:SA* (7:2:1 mole ratio), (●—●) *SP:CHOL:PS* (7:2:1 mole ratio). *The results are presented as described in Fig.* 1, *the incubation conditions being as in Materials and Methods.*

thereafter (Table 1). This probably indicates that the predominant cellular effect, observed at high concentrations, is due to the initial surface adsorption of the liposomes (the doubling time of both cell types is about 12 hr). The possible stimulation of thymidine incorporation observed at low PC:CHOL:DCP liposome concentration after 2 hr incubation is difficult to explain; certainly Ham [15] has shown that the presence of exogenous lipids stimulates thymidine uptake during division, whilst this was observed after 20 hr incubation (one cell division) it was not observed after 40 hr (three cell divisions). Liposomes composed of PC:CHOL:PS (7:2:1) only fit the first category for incubation with L929 fibroblasts, the leukemic cells were virtually unaffected (see Figs. 1 and 2).

When the small amount of negatively charged lipid is replaced by positively charged octadecylamine, the second category effect is

Table 1. *The effect on cell viability of co-incubation of L1210 leukemic cells and L929 fibroblasts with liposomes of different composition*

	I_{50} (nmole lipid/ml)					
	L1210			L929		
Incubation (hr)	2	20	40	2	20	40
Liposomes (mole ratio)						
PC:CHOL (8:2)	954	660	600	851	330	290
PC:CHOL:SA (7:2:1)	20	9	5	75	22	8
PC:CHOL:DCP (7:2:1)	1380*	758	645	1320*	645	456
PC:CHOL:PS (7:2:1)	†	†	†	1318	645	398
PC:CHOL:LL (7:2:1)	†	169	94	†	†	†
SP:CHOL:PS (7:2:1)	†	†	†	†	†	†
SP:CHOL:SA (7:2:1)	18	6	4	69	16	5

I_{50} is defined as the total lipid concentration which reduced [^{3}H] thymidine uptake to 50% of that of the controls.
*Extrapolated values.
†Not estimated but greater than 2000 nmole/ml.

observed, both for PC:CHOL:SA and SP : CHOL : SA liposomes (see Figs. 1 and 2 and Table 1); after only 2 hr incubation, inhibitions is observed at concentrations >80 nmole/ml, and after 40 hr at concentrations >10 nmole/ml. Sphingomyelin was included since Bergelson *et al.* [16] had found by whole cell analysis an increased proportion of this lipid in the plasma and intracellular membranes of neoplastic cells as compared with appropriate non-neoplastic cell type. In contrast liposomes composed of SP:CHOL:PS did not induce any inhibition either at high concentrations or after long incubation (Table 1), i.e., fit the third category. 2-Lysophosphatidylcholine (LL), the lyso derivative of 3-*sn*-phosphatidylcholine, has been reported [17] to enhance cell–liposome fusion, which in turn may aid targeting of drug carrying liposomes. Figures 1 and 2 show the effect on cell viability of incubation with liposomes containing 10 mole % 2-lysophosphatidylcholine; that on leukemic cells fits in the first category, whilst that on fibroblasts does not fit any of the suggested groups, but produces an overall stimulation of [^{3}H]-thymidine incorporation and is thereby in general agreement with the findings of Nilhausen [18]. That the inhibition produced by these liposomes was confined to leukemic

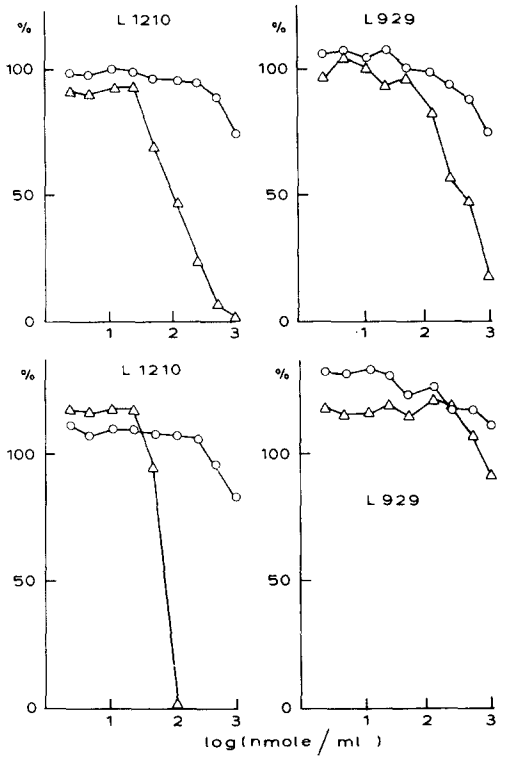

Fig. 3. The effect of co-incubation with liposomes containing 2-lysophosphatidycholine (ES-16, 18-OH) *on the viability of L1210 leukemic cells and L929 fibroblasts;* (*A*) 2 mole % ES-16, 18-OH, (*B*) 10 mole % ES-16, 18-OH. (○—○) 2 hr *co-incubation,* (△—△) 40 hr *co-incubation. The results are presented as described for Fig.* 1, *the incubation conditions being as in Materials and Methods.*

cells warranted further investigation and it was found (see Figs. 3A and B) that the inhibition of thymidine incorporation of leukemic cells and the stimulation observed for fibroblasts was related to the amount of 2-lysophosphatidylcholine present in the liposomes. At 2 mole % of 2-lysophosphatidylcholine there is a greater inhibition of incorporation by L1210 cells compared to that by L929 cells, and this difference is maintained with increasing incubation times. As the percentage of 2-lysophosphatidylcholine is increased then the L929 incorporation of thymidine becomes stimulated whilst that by the L1210 cells remains inhibited to a similar extent (see Table 2).

When the experiments are repeated with liposomes containing the 2-lysophosphatidylcholine analog ET-18-H, rather different effects are observed as shown in Figs. 4A and B. The incorporation by L929 cells is affected by the presence of liposomes to almost the same extent regardless to the amount of analog present or the incubation time (see Table 2). L1210 incorporation appears to be mainly affected after the first cell division (12–14 hr; [15]), but not greatly thereafter. Virtually the same effect was seen at 2 and 5 mole %; only at 10 mole % were there signs of increasing inhibition with incubation time. Thus the

Table 2. I_{50} *values* (nmole/ml)

		L1210			L929	
Time (hr)	2	20	40	2	20	40
Incubated with liposomes						
PC:CHOL:LL	2* >2000	380	104	>2000	692	398
	5 >2000	263	59	>2000	>2000	>2000
	10 >2000	138	70	>2000	>2000	>2000
PC:CHOL:LLA[1]	2* >2000	630	323	>2000	>2000	794
	5 >2000	199	45	>2000	>2000	794
	10 >2000	98	12	>2000	>2000	>2000
PC:CHOL:LLA[2]	2* >2000	398	398	1200†	1200†	>2000
	5 >2000	363	363	1995†	1380†	1380†
	10 831	120	96	1778†	575	520
PC:CHOL:LPE	2* >2000	>2000	>2000	>2000	>2000	>2000
	5 >2000	>2000	>2000	>2000	>2000	>2000
	10 >2000	>2000	>2000	>2000	>2000	>2000
Free analog						
LLA[1]	>2000	8	4	>2000	158†	26
LLA[2]	58	21	16	61	52	52
LL	>2000	>2000	>2000	>2000	200†	200†

LL:ES-16, 18-OH.
LPE:lysophosphatidylethanolamine.
LLA[1]:ET-18-OCH_3.
LLA[2]:ET-18-H.
*Percentage of lysophosphatide in liposome.
†Extrapolated value.

effects on L1210 cells tended to be of the first category, whilst on L929 cells there was a second category effect though showing less inhibition than when compared, for example, to stearylamine containing liposomes.

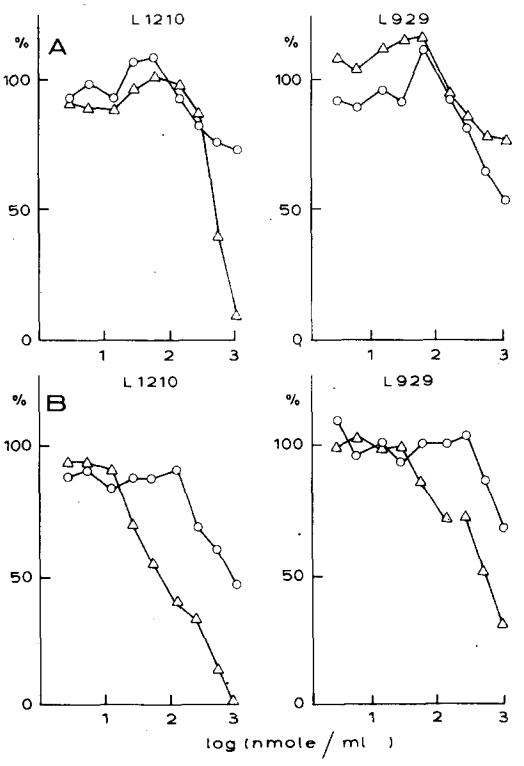

Fig. 4. The effect of co-incubation with liposomes containing the lysophosphatide analog ET-18-OH *on the viability of L1210 leukemic cells and L929 fibroblasts; (A)* 2 mole % ET-18-H, *(B)* 10 mole % ET-18-H. (○—○) 2 hr *co-incubation,* (△—△) 40 hr *co-incubation. The results are presented as described for Fig.* 1, *the incubation conditions being as in Materials and Methods.*

The substitution in the analog of the -OH group by $-OCH_3$ instead of -H, produces much greater effects in the co-incubation experiments. Figures 5A and B show the effect on thymidine incorporation by L1210 and L929 cells of co-incubation with liposomes containing 2 and 10 mole % of the analog ET-18-OCH_3 respectively. Some slight effect on L929 incorporation is seen but this is relatively independent of analog concentration and time. In marked contrast, however, is the effect on L1210 thymidine incorporation, there is inhibition with both analog concentration and incubation time (see Table 2). This is an interesting situation in which the liposomal effect on leukemic cells is of the second category, whilst that on L929 fibroblasts (our model for normal cells) is of the third category.

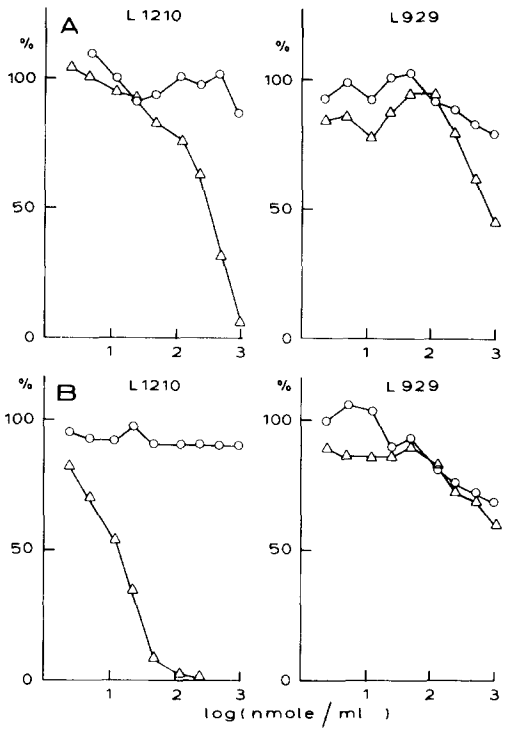

Fig.5. The effect of co-incubation with liposomes containing the lysophosphatide analog ET-18-OCH_3 *on the viability of L1210 leukemic cells and L929 fibroblasts; (A)* 2 mole % ET-18-OCH_3, *(B)* 10 mole % ET-18-OCH_3. (○—○) 2 hr *co-incubation,* (△—△) 40 hr *co-incubation. The results are presented as described for Fig* 1, *the incubation conditions being as in Materials and Methods.*

For comparison of the effect on viability of the natural lysophosphatide, a co-incubation experiment was performed with liposomes containing lysophosphatidylethanolamine. At none of the concentrations or incubation times was there more than 20% inhibition of thymidine incorporation and it was not possible to extrapolate any of the results to give meaningful I_{50} values (Table 2); hence for both cell lines the third category applies.

Co-incubation with the lysophosphatides in the free form, at concentrations equivalent to liposomes containing 10 mole % analog (Fig. 6), indicated that 2-lysophosphatidylcholine had no significant inhibitory effect, whilst both analogs strongly inhibited thymidine incorporation by both cell types, the ET-18-H analog again showed a less time dependent effect. The respective I_{50} values are given in Table 2.

DISCUSSION

In this study the effect of empty liposomes of various lipid composition on the viability, as measured by [^{3}H]-thymidine incorporation, of L929 fibroblasts and L1210 leukemic cells

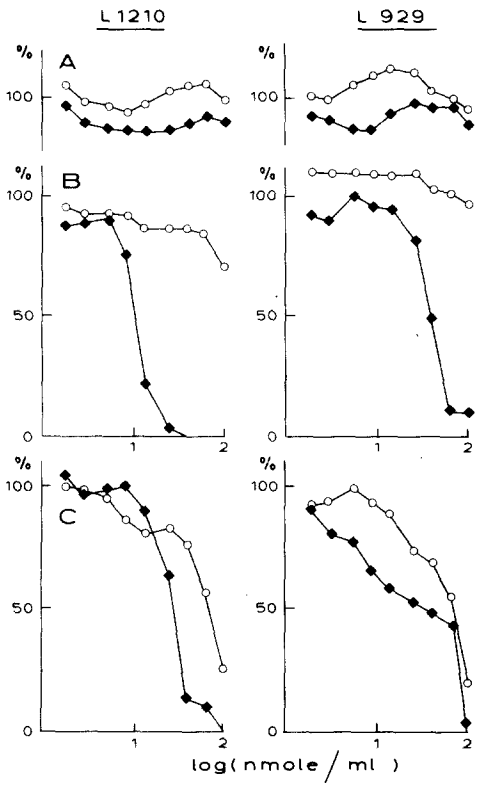

Fig. 6. The effect of co-incubation of lysophosphatides in the free form on the viability of L1210 leukemic cells and L929 fibroblasts; (A) Es-16, 18-OH, *(B)* ET-18-OCH_3, *(C)* ET-18-H. (○—○) 2 hr *incubation*, (◆—◆) 40 hr *incubation. The concentration range of free lysophosphatide was the same as was contained in the respective liposomes at* 10 mole % *lysophosphatide. The results are presented as described for Fig.* 1, *the incubation conditions being as in Materials and Methods.*

was examined. It is evident that many of the lipid compositions used herein had, at high concentrations, an inhibitory effect of [^{3}H]-thymidine incorporation (see Table 1), and that especially those containing stearylamine were highly toxic. Liposomes composed of PC:CHOL:DCP affected both cell types equally, as did those containing sphingomyelin instead of 3-*sn*-phosphatidylcholine (SP:CHOL:DCP, data not shown); whilst liposomes in which PS is substituted for DCP affect fibroblasts only, even high concentrations having little effect on leukemic cell viability. A similar effect was observed for liposomes composed of sphingomyelin, cholesterol and phosphatidylserine. Liposomes containing 2-lysophosphatidylcholine did not affect fibroblast viability, in fact did stimulate [^{3}H]-thymidine uptake over the whole concentration range used, the same liposomes having an inhibitory effect at high concentrations on the leukemic cells.

It is not possible from this initial study to determine exactly which lipids affect different cells viabilities or how they do so when incorporated in liposomes present in the incubation medium. However, certain criteria for the composition of carrier liposomes are evident. Normally the carrier alone should affect normal and target cells to the same extent and as little as possible (as is the case of PC, CHOL, DCP liposomes in this study; see also [19]), but if the potential of the carrier is to deliver its load which in turn is to be detectably effective at low concentrations it is of prime importance to select a carrier composition which has no effect by itself on target cell viability (such as PC, CHOL, PS here). Alternatively it may be considered essential to be able to monitor the slightest effect on non-target cells (as in toxicity studies), then the carrier should be one that leaves such cells unaffected over a wide range of carrier concentrations (about 1000-fold) such as PC, CHOL, LL used herein. Certainly liposomes containing stearylamine are quite likely to mask any effect due to the carried agent and therefore should be avoided.

In this study we also observed that small differences in the chemical structure of the minor lipid component of the liposome could cause considerable and specific changes in the viability of the co-incubated cells. The naturally occurring lysophosphatide (ES-16, 18-OH) tended to stimulate thymidine incorporation by L929 cells but inhibited that of L1210 cells; the inhibition was only partially concentration and time dependent (see Fig. 3 and Table 2). The substitution of an alkyl link in place of an acyl link in *sn*-1 and of the -OH by -H in *sn*-2 caused some changes in the inhibition pattern (Fig. 4), stimulation of the thymidine incorporation by fibroblasts was no longer observed, the inhibition of incorporation by L1210 cells was not greatly altered except that little effect was seen after the first cell division. The most striking differences were seen for co-incubation with liposomes containg the analog ET-18-OCH_3 (Fig. 5), the incorporation by L929 cells showed only inhibition at high lipid concentrations and after long incubation times, but probably this is merely a reflection of a general effect of high lipid concentrations in the medium rather than a specific effect of this analog. Short incubation produced virtually no effect on incorporation by L1210 cells, but each successive division produced a marked inhibition which was concentration dependent. The presence of lysophosphatidyl ethanolamine in the liposomes produced only a small inhibition of thymidine incorporation for either cell line

which was independent of concentration or incubation time (Table 2).

When the lysolecithin analogs were added in free form, in DMEM plus 10% FCS, to the incubation medium (Fig. 6) they caused the greatest inhibition of incorporation for both cell types (Table 2). The comparison between the effectiveness of the analogs in free or liposomal form may be examined in more detail for the most striking case, that of the analog ET-18-OCH_3. The I_{50} values for the liposome form were calculated on the basis of the total amount of lipid present; however since PC/CHOL liposomes produced inhibition only at concentrations above 600 nmole/ml, it may be assumed that the inhibition observed when the analog was presented in the liposome was only due to the analog. The I_{50} values are therefore recalculated on the basis of the actual amount of analog present in the liposomes (Table 3).

Table 3. I_{50} *values* (nmole/ml) *recalculated on the basis of the amount of analog present*

		L1210			L929		
Time (hr)		2	20	40	2	20	40
Liposomes							
PC:CHOL:LLA[1]	2*	>2000	63	32	>2000	>2000	79
	5	>2000	19	4	>2000	>2000	79
	10	>2000	10	1	>2000	>2000	>2000
Free analog							
LLA[1]		>2000	8	4	>2000	158	24

LLA[1], analog ET-18-OCH_3.
*Percentage of analog present in liposome.

From this it would appear that the analog is equally effective in inhibiting thymidine incorporation by L1210 cells in either free or liposomal form, but that in the free form the incorporation by L929 cells was also inhibited.

It was shown by Munder *et al.* [10] that 2-lysophosphatidylcholine analogs were effective in syngeneic tumor graft rejection and these authors considered such analogs to be a new class of immunopotentiators. Arnold *et al.* [20] demonstrated earlier that alkyl analogs of 2-lysophosphatidylcholine had a much higher stability *in vivo* than the natural compounds and that irrespective of administration route these synthetic lipids were incorporated into immunologically relevant organs and remained detectable up to 4 days after single injection. Recent data by Andreesen *et al.* [21] shows that these compounds are not only very active in the animal system but equally effective against human leukemia cells of various types. We consider that the observed effects are linked to the biochemical fate of these compounds once they enter the phospholipid pools that take part in normal cellular metabolism. Well established pathways exist for the metabolism of natural 2-lysophosphatidylcholine [22, 23] and it is evident that both of these cell lines can deal effectively with its presence.

In contrast, the metabolism of the analogs, the alkyl-lysophospholipids, depends upon the activity of a specific oxygen requiring *O*-alkyl cleavage enzyme [24], which is reported to be part of the multifunctional oxygenase system present in normal tissue [25] but absent in certain tumor cells [26]. Hence such 2-lysophosphatidylcholine analogs may be metabolised by normal cells but will be accumulated by these tumor cells. The resulting accumulation may in turn destroy the tumor cells by introducing weak points into the membrane, or by interfering with the acylating–deacylating cycle of cellular phospholipid metabolism, or by activating other oxygenases such as those which metabolise aromatic amino acids [27].

One aim of this study was to compare the interaction of free and liposome form of 2-lysophosphatidylcholine and two of its analogs with cells. It has already been shown that certain analogs of 2-lysophosphatidylcholine have considerable potential in leukemia chemotherapy [10, 20, 21]. We would suggest, however, that the therapeutic effects of such analogs would be greatly augmented if they were administered in liposome form, for the following reasons. The plasma clearance rate of sonicated liposomes can be extremely slow,

$T_{\frac{1}{2}} > 4$ hr [6], whilst that of the free analogs is considerably faster, $T_{\frac{1}{2}}$ calculated from Arnold *et al.* [20] is $\approx$ 1 hr. Furthermore it appears that the initial addition of free 2-lysophosphatidylcholine analogs does cause cell lysis if it is not in the serum bound form [28], and, as can readily be seen from Tables 1 and 2 in this study, there is much less effect on L929 fibroblast incorporation of thymidine by the liposomal form of the analog ET-18-OCH_3 as compared to that of the free form, whereas both forms were effective in inhibiting the incorporation by L1210 leukemic cells. The exact mechanisms of uptake into and action on cells of such analogs are not known; though several have been proposed [29], all will involve the alkyl lysophosphatides in the serum bound form, which in turn may reduce the efficiency of cell interaction. Although liposomes do have associated serum [29, 30], nevertheless they have been shown not only to readily adsorb to the plasma membrane, but suspected of either fusing with it, or being endocytosed [31]. In addition, cell surface adsorption, the intial step in most interactions, is often accompanied by lipid exchange between the liposomal and plasma membranes [32]. Hence the delivery of alkyl lysophosphatides may be more efficient if they are in the liposomal form. Finally, liposomes do, whilst in the circulation, release not only their entrapped contents but some lipid components by virtue of exchange [33]; hence liposomes may additionally provide a better depot for a controlled release of the alkyl-lysophosphatides.

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