Biochimica et Biophysica Acta, 1103 (1992) 185–197

BBAMEM 75514

Recognition of liposomes by cells: in vitro binding and endocytosis mediated by specific lipid headgroups and surface charge density

Kyung-Dall Lee, Keelung Hong and Demetrios Papahadjopoulos

Cancer Research Institute and Department of Pharmacology, University of California, San Francisco, CA (USA)

(Received 12 July 1991)

Key words: Liposome; Endocytosis; Anionic lipid; CV1; J774

We investigated the interaction of liposomes of different surface properties with two mammalian cell lines, CV1, an African green monkey kidney cell line, and J774, a murine macrophage-like cell line. Cell surface binding and endocytosis of liposomes were quantified by fluorometry, using the liposome-encapsulated pH-sensitive fluorescent dye, pyranine, and the lipid marker rhodamine-PE. The liposome uptake was dependent both on the surface properties of the liposomes and on the cell line. Negatively charged phospholipids incorporated into egg phosphatidylcholine (PC)/cholesterol (C) (2:1) liposomes were recognized by the two cell lines to different extents depending on the lipid headgroup and its charge density in the liposome bilayer. Inclusion of 9% phosphatidylserine (PS), phosphatidylglycerol (PG), or phosphatidic acid (PA) promoted the uptake by CV1 cells more than 20-fold. Increasing the content of these negatively charged lipids beyond 9% did not further enhance the uptake. In contrast, 9% monosialoganglioside G_{M1}, phosphatidylinositol (PI), or phosphatidylethanolamine conjugated to poly(ethylene glycol) (PEG-PE) did not promote the uptake. Inclusion of 9% PS, PG, PA or PI in PC/C liposomes did not enhance the uptake by J774 cells, but a drastic enhancement was observed when increasing concentrations of these anionic lipids were incorporated in the liposome bilayer. At least 50% PS, PG, or PI was needed to reach the level of uptake seen with CV1 cells. The uptake of liposomes containing 50% PS by J774 cells was inhibited by poly-anions which are the competing ligands for scavenger receptors, but the uptake by CV1 was not inhibited. Different mechanisms of liposome uptake by these two cell lines are suggested from the different patterns of uptake and the competition with various poly-anions. The differences observed in the uptake rate of liposomes with different lipid compositions seemed to be primarily due to the differences in the binding between liposomes and cell membrane components. The *in vitro* interaction of various liposomes with these cell lines, especially CV1 cells, shows significant similarities to the *in vivo* clearance rates of the liposomes.

Introduction

Liposomes have been suggested as efficient carriers for delivery of drugs or macromolecules into cells in culture [1,2] as well as to specific tissues in the body [3,4]. They can be used as a drug depot for controlled release [5] and for reducing the non-specific toxicity of

Abbreviations: PC, egg phosphatidylcholine; C, cholesterol; PS, phosphatidylserine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PA, phosphatidic acid; G_{M1}, monosialoganglioside G_{M1}; PEG-PE, poly(ethylene glycol) conjugated to distearoylphosphatidylethanolamine; RES, reticuloendothelial system; HPTS, pyranine (1-hydroxypyrene-3,6,8-trisulfonate); Rho-PE, rhodamine phosphatidylethanolamine; PBS, phosphate-buffered saline containing 0.2 g/l KCl, 8.0 g/l NaCl, 0.1 g/l $CaCl_2$, 0.1 g/l $MgCl_2$; DMEM, Dulbecco's Modified Eagle's Medium containing 4.5 g/l glucose, 0.58 g/l glutamine, 3.7 g/l $NaHCO_3$, 0.1 g/l $CaCl_2$, 0.1 g/l $MgCl_2$; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; Poly(I), poly(inosinic acid); Poly(C), poly(cytidylic acid).

Correspondence: K.-D. Lee, Cancer Research Institute Box 0128, University of California, San Francisco, CA 94143, USA.

the drug [6–8]. Several liposome-encapsulated antimicrobial and chemotherapeutic agents are currently under evaluation for use in humans against parasitic and fungal infections [9,10] and neoplastic diseases [11–14]. Liposomes can be naturally targeted to mononuclear phagocytes [15,16], but also have been successfully targeted to specific cells by conjugation to appropriate ligands [17–21]. In spite of all the progress, it is still not possible to describe in detail how various cells respond to different types of liposomes, in terms of recognition of specific headgroups of phospholipids and glycolipids.

Early *in vivo* studies, using PS or PG containing liposomes, indicated that liposomes are cleared from the circulation primarily by the reticuloendothelial system (RES), mainly due to the uptake by the resident macrophages of the liver and spleen [3,22–24]. This is a disadvantage when liposomes are used as a drug delivery system to target cells other than liver and spleen macrophages. Recently, it has been reported that a drastic reduction of the liposome uptake by RES and consequent prolongation of liposome's blood circulation time can be obtained [25,26]. This has been accomplished by replacing PS or PG by G_{M1} or PI, and also more recently by PEG-PE [27–29]. However, the exact molecular role of the headgroup of G_{M1}, PI, or PEG-PE on the liposome surface in determining their behavior *in vivo* and *in vitro* is still not clear. Further advances in the design of liposomes to avoid early clearance from the blood stream is likely to benefit from a detailed understanding of the mechanism by which various relevant cells recognize and take up liposomes. Progress in this area will also enhance our understanding of the removal of foreign particles in general.

The primary mode by which liposomes are incorporated into certain types of cells is known to be endocytosis via the coated pit pathway [30]. The involvement of clathrin-coated vesicles in liposome endocytosis has been confirmed recently by microinjection of anti-clathrin antibody into cells [31]. Once endocytosed, liposomes come in contact with low pH compartments such as endosomes and lysosomes. However, the nature of the membrane component responsible for liposome binding and internalization is unknown at present. Our current approach for investigating the mechanism of liposome uptake is to make specific changes in the lipid composition of liposomes and document the conditions which promote or reduce their uptake by cells. Analytical tools for investigating the mechanism for cellular incorporation of liposomes and their contents have been provided by the recent success in making liposomes containing colloidal gold [32] and the development of a fluorescence assay for monitoring liposome endocytosis [33–35]. The fluorescent probe HPTS has been used with CV1 [34] and J774 [35] cell lines as a good liposomal aqueous content marker with pH-dependence which is useful for differentiating liposomes at neutral pH from the ones in low pH compartments.

In this report, we have examined how various negatively charged liposomes are recognized by endocytic (or phagocytic) cells in culture, primarily in the absence of serum. The reason for excluding serum was to investigate the direct interaction between cells and liposomes and characterize the ability of certain cells to differentiate different negatively charged liposomes. We chose CV1, an African green monkey kidney cell line, as sone of the well characterized cell lines for liposome-cell interaction [2,34,36,37], and J774, a murine macrophage-like cell line, possibly representing mononuclear phagocytes in RES [35,38,39]. The negatively charged lipids used in these experiments include PS, PG, PA, PI, monosialoganglioside G_{M1} and PEG-PE. The modulatory effect of these anionic lipids on the liposome uptake by the two cell lines was investigated, and compared with their clearance rates from blood which have been shown previously to differ drastically [26]. Our findings obtained by a quantitative analysis of binding and endocytosis with each cell line demonstrate that these cells have the capacity to distinguish small differences in the lipid headgroup structure on the bilayer surface of the liposome. We conclude that the rate of liposome uptake is specifically controlled by the headgroup of the lipid rather than simply the net negative charge on the liposome surface. Furthermore, higher surface charge density also promotes uptake, but the concentration of these negatively charged lipids needed for high level of uptake is dependent on the cell type. Different mechanisms or different receptors for liposome uptake are suggested to exist in CV1 and J774 cells.

Materials and Methods

Tissue culture. CV1, an African green monkey kidney cell line, and J774, a murine macrophage-like cell line, were cultured in DMEM H21 media with 10% FBS (Fetal Bovine Serum) supplied by the UCSF cell culture facility. Both cell lines were maintained in monolayer culture and plated at the density of $1 \cdot 10^6$ cells per 60 mm petri dish 24 h before the experiments.

Materials. PS (from bovine brain), PG (trans esterified from PC), PI (from bovine brain), PC (from egg yolk), and Rho-PE were purchased from Avanti Polar Lipids (Pelham, AL), and cholesterol from Calbiochem (San Diego, CA). HPTS (1-hydroxypyrene-3,6,8-trisulfonate) was purchased from Molecular Probes (Eugene, OR). For competition experiments, poly(I), poly(C), dextran-sulfate (500 000 mol. wt.), and fucoidan were purchased from Sigma (St. Louis, MO).

Preparation of liposomes. Liposomes were made according to the reverse phase evaporation method described by Szoka and Papahadjopoulos [40]. They were subsequently extruded [41,42] under high pressure argon through polycarbonate membranes (Nuclepore, Pleasanton, CA) with a final pore size of 0.05 μm. HPTS was encapsulated in the liposomes at 35 mM concentration with 10 mM Hepes buffer, and the unencapsulated HPTS was removed by Sephadex G-75 gel filtration column. HPTS has been proven to be a good marker for endocytosis with the two cell lines [34,35], but in some cases Rho-PE was additionally introduced as a second marker for comparison of the uptake of liposomal lipids versus aqueous contents. The buffer had a final pH of 7.4, and an osmolality of 300 mosmol/kg adjusted by NaCl. The concentration of phospholipid was assayed by the method of Bartlett [43].

Liposome-cell incubation. Cells were washed with fresh media without serum and kept in the same media for 1 h before incubating with liposomes. Liposomes were diluted with PBS to a concentration of 100 μM of total phospholipid and 0.5 ml (total of 50 nmol of lipids) was added to each dish containing 1.5 ml of media. Unless otherwise indicated, the cells were incubated with liposomes for 1 h at 37°C. The non-associated liposomes in solution were washed away at the end of the incubation by rinsing three times with 3 ml of cold PBS. For binding experiments, the cells were prepared the same way except that they were incubated with liposomes at 4°C on ice instead of at 37°C for 1 h. Again, the cells were washed with cold PBS thereafter. Experiments were done either in duplicate or in triplicate, and the maximum deviation from the average value was 15% within each experiment. However, the variance between experiments was larger ($\approx$ 50%), which indicated that the absolute value of liposome uptake varies depending on the preparation of the cells. In each experiment, however, the relative enhancement of uptake in comparison with PC/C liposomes by changing the lipid composition was consistently reproducible.

Fluorescence microscopy. The washed cells were viewed in monolayer state under a Leitz fluorescence microscope, equipped with epifluorescence and a Nikon camera with an automatic exposure meter. The epifluorescence was viewed with two excitation filter sets; the first set that allows excitation in the range 350–410 nm and observation of emission with a long wave pass dichroic mirror and barrier filter at wavelength greater than 455 nm, and the second set that excites in the range 450–490 nm and allows observation of emission at wavelengths greater than 515 nm with a long wave pass dichroic mirror and barrier filter. The first set gives the view of total fluorescence by HPTS (the liposomes at both neutral and lower pH), and the second allows observation of liposomes mostly at neutral pH [34,35].

Fluorimetric measurement of binding and endocytosis. Cells were dislodged by treatment with PBS buffer containing 3 mM EDTA (5 min at 20°C) and adjusted to the concentration of $1 \cdot 10^6$ cells in 1 ml of PBS buffer. For the binding and endocytosis measurements using HPTS containing liposomes, fluorescence excitation spectra (λ_{ex} 395–465 nm) were obtained at 510 nm emission with SPEX Fluorolog2 fluorometer with continuous stirring at controlled (20°C) temperature. The fluorescence intensities at λ_{ex} 403, 413, 450 nm were measured. The intensity at the isosbestic point 413 nm is pH-independent and served as a good measure of total number of cell-associated HPTS containing liposomes regardless of their location along the endocytic pathway. The intensity at 403 and 450 nm relative to 413 are sensitive to the pH where the probe resides [44]. The intensity ratio 403/413 nm and 450/413 nm were calculated to estimate the percentage of liposomes endocytosed according to the method in Straubinger et al. [34] and Daleke et al. [35]. For simultaneous monitoring of the liposomal lipid uptake (monitored by Rho-PE) and the liposomal aqueous contents uptake (monitored by HPTS), the procedure for liposome-cell incubation was the same. In this case, the liposomes contained 0.2 mol% of Rho-PE in the bilayer and 35 mM HPTS encapsulated inside. Under this condition the HPTS fluorescence intensity of the liposomal solution did not change when the liposomes were solubilized by adding detergent, thus the fluorescence resonance energy transfer from HPTS to rhodamine was negligible. An additional excitation spectrum was measured at emission 590 nm and the fluorescence intensity at 570 nm was recorded to monitor the accumulation of Rho-PE. For calibration purpose, the excitation spectra for the liposome suspensions alone were measured and the fluorescence intensity at 413 nm and at 570 nm were taken to document the fluorescence due to HPTS and due to Rho-PE, respectively, per nanomole of lipids. The fluorescence intensity associated with $1 \cdot 10^6$ cells at each peak was converted to comparable nanomoles of phospholipid that had a corresponding intensity of fluorescence in the liposome suspension.

Results

We compared initially the uptake (total cell-association which consists of binding plus endocytosis) of neutral liposomes composed of PC/C (2:1, mole ratio) with and without additional anionic phospholipid. Lipids carrying a net negative charge, PS, PG, PA, PI, G_{M1}, and PEG-PE were included at a certain percentage of total phospholipid.

The effect of lipid headgroups on liposome uptake

The two cell lines, CV1 and J774, showed differences in the pattern of liposome uptake depending on the type of negatively charged lipids incorporated. Fig. 1 shows the total number of liposomes associated with these cells (i.e., uptake) during 1 h incubation at 37°C (expressed as nanomoles of lipid per million cells). The uptake was monitored both by HPTS and by Rho-PE. CV1 cells showed an intriguing sensitivity to the presence of certain anionic lipids; for liposomes containing 9 mol% PS, PG, or PA, there was more than 20-fold higher uptake compared with the neutral PC/C liposomes. However, the negative charge imparted by the inclusion of either 9% PI, G_{M1}, or PEG-PE did not show any enhancement of the uptake over PC/C.

With J774 cells, on the contrary, inclusion of any of the above negatively charged lipids at a concentration of 9 mol% showed no significant enhancement of the uptake over PC/C liposomes. Among the lipid compositions shown in Fig. 1, only the liposomes composed of PS/C (2:1) were avidiy taken up by J774 cells. The effect of increasing concentration of negatively charged lipids will be discussed in next section.

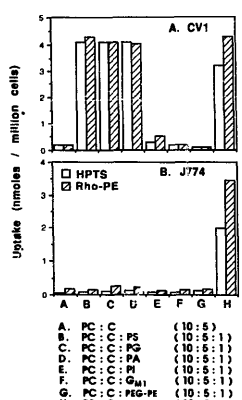

Fig. 1. The headgroup of the negatively charged lipids incorporated into PC/C (2:1) liposomes determines the extent of enhancement in their uptake by CV1 and J774. The anionic phospholipid added was one of the following: PS, PG, PA, PI, G_{M1}, and PEG-PE. Their effect on the uptake by cells was monitored by both HPTS and Rho-PE in comparison with neutral PC/C liposomes. The lipid composition H contained only PS and cholesterol. The experiments were done in duplicate, and the maximum deviation from the mean was within 15% of the mean.

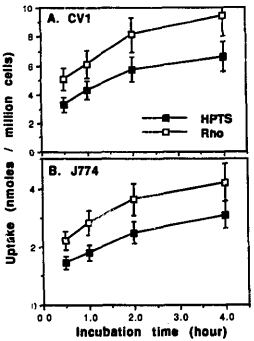

Fig. 2. The increase of fluorescence associated with one million cells due to the accumulation of HPTS (encapsulated inside the liposomes) and rhodamine (incorporated as Rho-PE in the liposome bilayer) as a function of incubation time. The fluorescence was converted to the comparable amount of liposomal lipids that contained the same intensity of fluorescence. PS/PC/C (1:1:1) was used to show the time course of liposome uptake during 4 h of incubation in serum free media in CV1 cells (A) and J774 cells (B). Total 50 nmol of lipids were added to about $2 \cdot 10^6$ cells in 60 mm petri dish.

It is worth noting that the overall pattern of uptake reported by HPTS correlates with that by Rho-PE, even though the absolute values are not the same. The difference in the two values can be due to the possibility that some fraction of encapsulated HPTS leaks upon interacting with cells, thus underestimating the uptake of liposomes. Also it can be partly due to the possible transfer of Rho-PE, which leads to overestimation of the uptake. The values of liposome uptake correlate well when anionic lipid was included at 9% of total phospholipid, while HPTS seems to leak more from the liposomes with more negative charge. However, either probe can be used to compare the relative extent of uptake, as will be discussed later.

The amount of liposomes associated with either cell line increased rather slowly as the incubation time was increased beyond 1 h. A typical kinetic curve of liposome uptake is shown in Fig. 2. The total amount of liposomes (PS/PC/C 1:1:1) associated with CV1 cells monitored by HPTS and Rho-PE is plotted in Fig. 2A as a function of incubation time. The increase in cell-bound liposomes was rapid for the initial 30 min, and progressed slowly afterwards as the incubation time

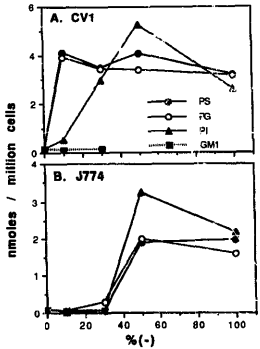

Fig. 3. The effect of increased charge density on liposome uptake by cells. Liposomes of lipid composition x/PC/C (x = PS, PG, PI, or G_{MI}, as percent of total phospholipids) were used with increasing percentage (x). The amount of liposomal HPTS taken up by CV1 cells (A) and J774 cells (B) after 1 h incubation at 37°C is monitored as a function of x. The relative increase in the enhancement of uptake with increased negatively charge lipids over neutral liposomes was consistent with standard deviation less than 15% in each experiment.

increased *. Similar uptake kinetics were seen using Rho-PE or HPTS, but the amount of uptake monitored by Rho-PE was greater than that monitored by HPTS accumulation in cells at all time points. However, the difference in the extent of uptake monitored by the two markers is consistent throughout the time points. Similar uptake kinetics were also observed with J774 cells as shown in Fig. 2B. The kinetics of liposome binding at 4°C, when endocytosis is negligible, was similar to those shown in Fig. 2.

The effect of surface charge density

As shown above, neutral liposomes were taken up minimally by CV1 cells when compared with liposomes containing 9% PS or PG. Increasing the density of these two negatively charged phospholipids beyond 9% did not further increase uptake by CV1 cells (Fig. 3A). In contrast, J774 cells behaved quite differently from CV1 cells with respect to the increase of PS or PG content. PS or PG, if introduced as 9% of phospholipids, had little effect on the uptake by J774 cells, but they induced enhanced uptake as their concentration in the liposome bilayer was increased to more than 30% (Fig. 3B). This enhancement by PS or PG was saturated around 50% concentration.

PI, which showed only a minimal enhancement of uptake in both cell lines when incorporated at 9%, significantly increased uptake in a concentration dependent manner at mole percentages between 9% and 50% (Fig. 3A). It is noteworthy that PI behaved the same as PS or PG in J774 cells, but it was distinct from PS and PG in CV1 cells.

G_{MI} showed the same effect as PI at 9% concentration in both cell lines in the sense that they did not induce any enhancement of liposome uptake. Thus we investigated whether G_{MI} enhances the uptake as its concentration in liposome bilayer increases like PI does. Fig. 3 shows that G_{MI} did not increase the uptake by either cell type even when 30% of PC was replaced by G_{MI} in the bilayer. Unfortunately, the maximum concentration of G_{MI} one can incorporate in the lipid bilayer is approx. 30%. Beyond that concentration, G_{MI} is known to form micelles in equilibrium with the bilayer form [45]. Therefore we could not test whether it starts to be recognized in a similar fashion like PI if it is incorporated into liposome bilayer at more than 30% of PC.

Binding versus endocytosis

In view that the whole process of liposome association with cells (uptake) is a two-step process involving binding and endocytosis, it is important to distinguish these two steps and assess which step controls the overall uptake. Table I compares the total amount of liposomes (expressed as nanomoles of lipid) associated with a million cells after 1 h of incubation at 37°C to that after 1 h of incubation at 4°C. The first value is a measure of the total uptake, while the second is a measure of binding without any endocytosis. In general, the binding constant between ligand and receptor at 37°C is slightly smaller than that at 4°C. The difference however is not significant, and it has been demonstrated previously in the case of liposomes containing 10% PS with isolated rat Kupffer cells by measuring the binding at 37°C with the blockage of endocytosis using metabolic inhibitors [46]. For both CV1 and J774 cells, the higher extent of uptake of a certain type of liposome correlates with a correspondingly higher binding level at 4°C. PC liposomes show minimal uptake during 1 h incubation and also the binding of PC liposomes is minimal. PS (and PG, not shown here) promoted increased binding of the liposomes to CV1 cells and concomitantly higher uptake. The presence of 10% G_{MI} inhibited this promotion by PS very ineffec-

* Under the incubation condition where no stirring is applied, the diffusion constant for the liposome of 80 nm diameter turns out to be very small ($\approx 10^{-8}$ cm^2/s). We estimate that the average distance a liposome travels during 1 h is around 150 μm. Therefore, after the initial interaction between cells and the liposomes within 50 μm from the cell monolayer is complete, the whole process becomes diffusion-limited and the uptake progresses slowly as the time increases.

)

BLE I

ent of liposome association with CV1 and J774 cells at 37°C and at 4°C

: total amount of cell-associated liposomal lipids after 1 h incubation with cells at 37°C or 4°C was calculated from the cell-associated HPTS. er 1 h incubation at 37°C. generally more than 90% of HPTS was at pH lower than 7, and there was no acidification of HPTS after 1 h ibation at 4°C. The values are the means of two independent experiments each consisted of duplicates and the biggest standard deviation was than 15% of the value.

id composition	Cell-associated liposomes (nmoles/10^6 cells)					
	37°C				4°C	
	CV1		J774		CV1	J774
	total	low pH	total	low pH	total	total
/C (10:5)	0.2	0.2 [a]	0.1	0.1 [a]	0.1	0.3
/C/PS (10:5:1)	4.1	3.9	0.1	0.1	4.3	0.3
/C/PS (8:5:2)	3.1	2.8	–	–	–	–
/C/PS (7:5:3)	3.5	3.2	0.1	–	–	0.3
/C/PS (5:5:5)	4.1	3.8	1.9	1.6	3.5	2.0
/C/PS (0:5:10)	3.2	2.1	2.0	1.8	1.9	2.0
/C/G_{M1} (10:5:1)	0.1	–	0.1	–	0.1	0.1
/C/PI (10:5:1)	0.2	–	0.1	–	0.6	0.3
/C/PEG-PE (10:5:1)	0.1	–	0.1	–	0.3	0.3
/C/PS/G_{M1} (8:5:1:1)	3.0	2.8	0.1	0.1	2.0	0.2

he numbers in these columns are the calculated fraction of liposomes at low pH (internalized) among the total cell-associated liposomes. The action endocytosed was obtained by utilizing the pH dependence of HPTS excitation spectrum.

ely in CV1 cells; the liposomes with lipid composi- n PS/G_{M1}/PC/C 1:1:8:5 were as much taken up PS containing liposomes with no G_{M1}. It is also wn in Table I that higher content of PS (and also i, and PI) increased the levels of cell associated osomes by J774 cells both at 4°C and 37°C. Lipo- nes with low content of PS bound less and the total el of cell associated liposomes was less, and lipo- nes with high content of PS bound more and conse- ently higher was the level of uptake by J774 cells.

The fraction of liposomes at low pH (internalized) er 1 h incubation was calculated from the pH-de- ndent excitation spectrum of HPTS and is shown in : column next to the total number of cell-association Table I. Almost 90% of the cell-associated lipo- nes (uptake) after 1 h incubation at 37°C were own to be already endocytosed into the compart- ents with pH lower than 7. This was also visually nfirmed by fluorescence microscopy using two filter ts. Fig. 4 shows two sets of cells after incubation with i liposomes at 37°C (A, B, C) and 4°C (D, E, F). fter incubation at 37°C, the HPTS fluorescence pat- rns in (B) and (C) are markedly different from each her. The differences represent the liposomes at low H already endocytosed, which are the large bright tracellular spots seen only in (B). However, incuba- on at 4°C results in the pattern of HPTS fluorescence (E) almost identical to that in (F), indicating that no gnificant acidification of cell associated liposome con- nts has occurred. The only difference between (E) id (F) is the absolute fluorescent intensity, because e filter in (F) transmits less light than that in (E). Thus, most of HPTS fluorescence in (E) or (F) is due to binding, and the total amount of HPTS in the liposomes associated with cells at 4°C is a good indicator of the initial binding.

The effect of various additives in incubation medium

All the experiments reported above were done in the absence of serum in the incubation medium. We investigated whether the presence of serum proteins in the incubation medium changes the pattern of liposome uptake, as there have been many studies implicating the possibility of adsorption of serum proteins onto the liposomes mediating the uptake of liposomes both *in vitro* and *in vivo* [47,48]. Yet, the relative amount of liposome uptake mediated by opsonizing serum proteins in comparison with the portion which does not involve serum opsonization is not known. Also, the specific components that opsonize liposomes of a certain lipid composition have not yet been identified.

Addition of 10% serum in the media made no difference in the pattern of uptake of liposomes containing 9% of various negatively charged lipids either by CV1 or by J774. The same was true with liposomes bearing higher percentages of negative charges in the case of CV1 cells. However, the extent of liposome uptake by J774 cells was in general higher in the absence of serum. This is more obvious with liposomes containing a high content of negatively charged lipids, as is shown in Fig. 5. The uptake monitored by Rho-PE shows that 50% PS was enough to induce a high uptake in the absence of serum, but 100% PS was

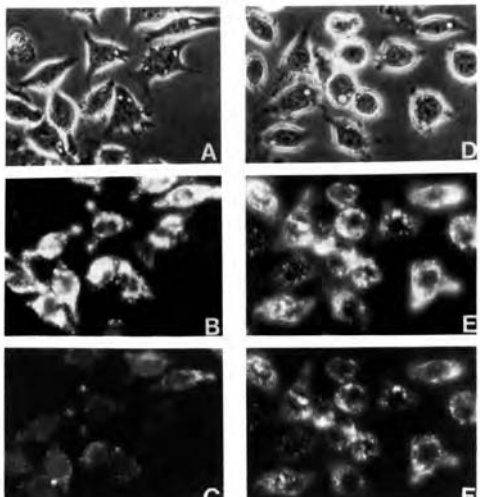

Fig. 4. Phase contrast and fluorescence micrographs of J774 cells which have taken up liposomes containing HPTS (PS/PC/C = 1:1:1) after 1 h incubation at 37°C (A, B, C) and at 4°C (D, E, F). A and D, the phase contrast view of the cells; B and E, total fluorescence from the liposomes associated with cells regardless of their local pH (excitation 350–410 nm; emission > 455 nm); C and F, fluorescence contributed mostly by the liposomes residing in neutral pH environment (excitation 450–490 nm; emission > 515 nm). The difference in the fluorescence pattern in B and C reveals the liposomes in the low pH compartment, which correspond to the liposomes already endocytosed. There is no apparent difference between E and F except the total fluorescence intensity. The filter set in C (or F) transmits less light than the filter in B (or D).

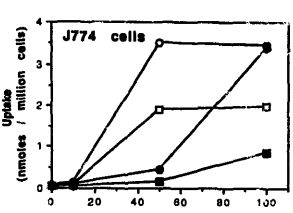

Fig. 5. The effect of serum on the uptake of PS containing liposomes in J774 cells. The uptake of liposomes with increasing concentration of PS content were monitored both by Rho-PE (○) in absence and (●) in presence of 10% serum and by HPTS (□) in absence and (■) in presence of serum.

needed to reach a comparable level in the presence of serum. The results in Fig. 5 also show that a significant amount of HPTS leaks out of the liposomes with high negative charge contents during their interaction with J774 cells in the presence of serum.

We also studied the involvement of divalent cations in liposome binding to the cell surface. It has been reported previously that the interaction of PS containing liposomes with Kupffer cells requires the presence of calcium [49]. The effect of calcium on the initial binding stage in our case could not be addressed due to the technical difficulty in maintaining the cell monolayer in presence of EDTA. Instead, the dissociation of prebound PS liposomes (PS/PC/C 1:1:1) was measured by treating the cells for 10 min at room temperature with PBS containing 3 mM EDTA after washing free liposomes from the cell monolayer. This proce-

dure did not dissociate the majority of liposomes which were associated with cells after 1 h incubation at 37°C, since most of the cell-associated liposomes at 37°C were inside the cells within endosomes and lysosomes as already discussed earlier. However, more than 90% of the cell-associated PS liposomes after the incubation at 4°C were dissociable by this EDTA treatment as cells were dislodged from the plastic dish. Yet, it is not clear whether the depletion of divalent cations induces the dissociation of bound liposomes directly or there are other cellular changes which cause the loss of binding. Also, 50% of the bound PS liposomes were removed by 10 minute trypsin treatment (50 μg/ml) at room temperature. This indicates that divalent cations and cell surface proteins may play a role in the binding of liposomes to the cell surface.

Nishikawa et al. [38] suggested, from the cross competition between modified LDL and negatively charged liposome, that the receptor for negatively charged liposomes on macrophages may be the scavenger receptor [50]. We investigated the involvement of this receptor in the uptake of PS containing liposomes by J774 and CV1 cells via the addition of increasing concentrations of poly(I) and poly(C) in the media during incubation. Poly(I), as well as dextran sulfate and fucoidan, are known to compete for the binding site of the scavenger receptor with oxidized or chemically modified LDL, whereas poly(C) competes ineffectively [51]. Fig. 6 shows that poly(I) reduced the extent of PS liposome uptake by J774 cells in a concentration dependent manner. At 500 μg/ml concentration, it inhibited more than 90% of liposome uptake. Poly(C) also inhibited the uptake, but not as well as poly(I). Both dextran sulfate and fucoidan had the similar effect as poly(I) at similar concentrations. On the contrary, none of poly(I), poly(C), dextran sulfate, and fucoidan, added to the media at concentrations as high as 5 mg/ml, showed any effect on the liposome uptake by CV1 cells. These competition experiments provide only indirect evidence and do not prove the involvement of scavenger receptor in the liposome uptake. However, the result in Fig. 6 demonstrates that the mechanism of PS liposome uptake by these two cell lines is not the same.

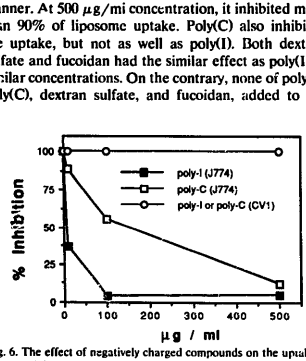

Fig. 6. The effect of negatively charged compounds on the uptake of PS containing liposomes. The uptake of PS/PC/C (1:1:1) liposomes by CV1 and J774 cells in the presence of different concentrations of poly(I) and poly(C) was expressed as percent of the uptake in absence of the compounds. The curve (○) shows that these poly-anions have no effect on the lipsome uptake by CV1 cells, contrary to the case of J774 cells (□, ■).

Discussion

The interaction of liposomes with cultured cells *in vitro* is complex. It involves surface binding, internalization and possible release of liposomal contents. Our investigation focuses on the first two processes. Studies on the fate of aqueous contents after internalization into endocytic compartments and the release into the cytoplasm have been reported and most recently by Brown and Silvius [36] and Chu et al. [52]. An understanding of the cellular parameters involved in the above mentioned processes is critical for designing liposomes which possess specific requirements as a carrier system for drugs or macromolecules. The cellular components involved at each step in the processing of liposomes are not yet known. Here, we have attempted to identify some of the parameters that control the early events in liposome–cell interaction by analyzing the uptake of different types of liposomes with systematically varied surface properties. Relatively short incubation time, 1 h, was used for this reason, contrary to that of longer than 4 h in most of other experiments [38,53].

Generally liposomes containing negatively charged phospholipids exhibited increased binding and endocytosis over neutral liposomes. However, some of our data demonstrate that the negative charge is not a sole determinant for the extent of this increase in binding and endocytosis. The uptake depends on the specific headgroup of the anionic phospholipid (Fig. 1). PS, PG, and PA possess distinct properties when compared with G_{M1}, PI and PEG-PE. No dramatic difference has been observed among PS, PG, and PA in terms of enhancement of liposome uptake, and they all behaved similarly in both cell lines in our study. It has not been carefully investigated whether these phospholipids behave differently in the liposome–cell interaction, although there has been a report by Schroit et al. [54] which showed that PA liposomes are less taken up by macrophages than PS or PG. The inclusion of any of these three anionic lipids into the neutral PC/C liposomes makes a drastic difference in their uptake by CV1 cells. Based on the average diameter of about 80 nm of liposomes extruded through final pore size 0.05 μm, 1 nanomole of lipid taken up per 10^6 cells is comparable to approx. 7500 liposomes per cell. According to this calculation, about 1300 liposomes composed of neutral PC/C liposomes were taken up by

one CV1 cell during 1 h incubation, while 30000 liposomes containing 9% PS were taken up per cell for the same period of incubation.

Earlier *in vitro* studies reported that negatively charged liposomes containing PS or PG are taken up better and deliver drugs or DNA more efficiently than neutral liposomes [37,55–57]. Also, the *in vivo* clearance rate of negatively charged liposomes from the blood circulation is known to be faster than that of neutral liposomes [58–60]. These studies had concluded, on the basis of the behaviors of liposomes containing PS, PG or PA, that simply the negative surface charge of liposomes is the important factor in determining the uptake rate. This notion can not be generalized to all the negatively charged lipids, on the basis of the newer evidence. Thus, the recent *in vivo* studies [25,26] have demonstrated that several negatively charged lipids, such as G_{M1}, PI and PEG-PE, when incorporated as 9% of PC/C liposomes, produce a drastic decrease in the rate of RES uptake compared to similar liposomes composed of 9% PS or PG. This was also seen in our present study with CV1 cells; none of G_{M1}, PI, and PEG-PE showed any enhancement of the uptake as did PS, PG, or PA, even if introduced at the same mol% (Fig. 1). Recently, Allen et al. [53] showed a reduction in the uptake of neutral liposomes in bone-marrow macrophages when G_{M1} or PEG-PE was incorporated in the liposomes. Additionally, G_{M1} and PI have been shown to suppress the complement-mediated enhancement of liposome uptake in peritoneal macrophages [61]. The mechanism of how G_{M1}, PI, and PEG-PE work on the liposome surface has not been determined, but will be discussed below. However, the recognition of PS by cell surface is strong and is not inhibited by the presence of G_{M1}, as shown here in Table 1 and also by Allen et al. [53]. The biological significance as well as the mechanism for this specificity in recognizing certain negatively charged groups and not others is not known at present, although some studies suggest that the presence of PS on the surface of cells or liposomes triggers a strong recognition by macrophages and imply its possible involvement in the clearance of senescent red blood cells [62–64].

The main focus of this report was to compare relative values of liposome uptake with varying lipid composition. Therefore it was critical to show that the HPTS method used in this study for monitoring the liposome uptake was not influencing the result by differential leakage from a certain composition of liposomes compared to others. To ensure the pattern of uptake obtained by monitoring HPTS, Rho-PE was additionally introduced as lipid marker of liposome along with HPTS. Rho-PE, which has its fluorescent reporter moiety on the headgroup of PE, has its limitation due to the possibilities that it may transfer from the liposome membrane to cell membrane upon binding, and may be cleaved off by phospholipase activity. Therefore, Rho-PE may not be a reliable single marker, but is useful in conjunction with an encapsulated aqueous marker, such as HPTS. The concentration of the Rho-PE selected here (0.2 mol% of phospholipid) was chosen to minimize any interference with the liposome–cell interactions due to the presence of rhodamine on the liposome surface. Up to 1 mol% Rho-PE in the liposome membrane was shown to alter only minimally the *in vivo* blood circulation time of G_{M1} containing liposomes in mice compared with the same type of liposomes containing no Rho-PE (unpublished results). The double labeling study adopted here strengthens the reliability of the fluorescence method for the quantitative analysis of liposome uptake. It eliminates the possibility that liposomes with less negative charge density have low extent of uptake because HPTS leaks preferentially in those liposomes. In fact, Fig. 5 shows that HPTS leaks more out of the liposomes with high PS content and the enhancement of uptake by increasing PS content is under-reported by HPTS. The leakage mediated by serum has been reported to increase when more negative charged lipids are included [65].

The comparison between binding at 4°C and total cell-association (uptake) at 37°C (Table I) shows the importance of the binding step in liposome uptake. It is clearly demonstrated that PS and PG are readily recognized by the cell surface, thus leading to strong binding and consequently high total uptake. On the contrary, G_{M1}, PI and PEG-PE do not significantly increase the binding of liposomes to cells, despite the fact that these lipids impart to the liposomes a net negative charge. Under the incubation condition used here, the binding at the cell membrane surface appears to be the step that governs the overall rate of liposome uptake, since the total number of liposomes associated with cells at 37°C for 1 h of interaction is directly proportional to the number of liposomes bound for the same duration of interaction at 4°C. At the end of 1 h incubation at 37°C, only a small fraction of total cell-associated liposomes were found to be at the cell surface, suggesting that the rate of endocytosis following the stable binding is much faster than the rate of binding. Our preliminary data showed an endocytic rate constant of ≈ 0.07/min, which corresponds to approx. 70% of bound liposomes internalized in 10 min [66]. If more liposomes were bound to the cells, they would be endocytosed quickly to show an apparently higher extent of total uptake. Thus, the number of liposomes that can bind to the cells may be controlling the overall uptake. More quantitative analysis of the kinetic rate constant of liposome binding and subsequent endocytosis is under progress to confirm this model.

Calculation of zeta potential has shown that lipo-

somes with 10% G_{M1} are significantly different from liposomes containing 10% PS or PG, but those with 10% PI are not [67,68]. Although this type of physical measurement can not distinguish 10% PS containing liposomes from 10% PI containing liposomes, CV1 cells have the ability to recognize the different surface property of these two types of liposomes and bind differently, as is shown in Table I. This suggests the possibility of recognition of these specific headgroups on the liposome surface by specific components at the cell membrane. The observation that 10% PS is still recognized by CV1 cells in the presence of 10% G_{M1} in the same liposome bilayer (Table I) adds further credence to that possibility. We hypothesize from the comparison of the chemical structures of these negatively charged phospholipids that the common feature which this putative membrane component may recognize and bind to, is the negatively charged phosphate group * at the lipid/water interface. PA has this phosphate group exposed and has a high affinity for the binding site on the cell surface. In the case of PC, the charge of phosphate is neutralized by a positive charge of choline group and is not recognized by this membrane protein. The presence of serine and glycerol group, in the case of PS and PG, respectively, has limited modulatory effect on this high-affinity binding, thus PS and PG are as equally well recognized by cells as PA. On the contrary, inositol ring of PI and bulky poly(ethylene glycol) of PEG-PE strongly reduce the binding constant between the phosphate and the membrane binding site. Yet, we can postulate that even though the binding constant of this binding site for phosphate in PI is weakened, the binding of PI containing liposome can be enhanced by incorporating more PI as explained by the multi-ligand effect. In the case of G_{M1}, the negative charge on the carboxyl of sialic acid is either not recognized by this membrane component, or it is not accessible for direct interaction. The idea of shielded charge in PI and G_{M1} has been proposed earlier [26]. PEG-PE in particular might act as a strong steric hindrance barrier due to the bulky PEG group inhibiting the accessibility of the phosphate charge by the putative cell surface components.

The enhancement of liposome uptake by the negatively charged lipids was shown to be modulated by the 2-dimensional concentration of them in the liposome bilayer. Higher PI density in the liposome bilayer is needed for these liposomes to be recognized by CV1 cells. The same is true with J774 cells. This increased uptake of PI-containing liposomes with PI has been also seen in the *in vivo* clearance rate [69]. However, the extent of the increase in the uptake in response to increased PS or PG differs between the two cell lines. The level of uptake of PC/C liposomes is the same with CV1 and J774 cells. CV1 cells require only 9 mol% PS or PG to achieve the maximum liposome uptake, while J774 cells need up to 50 mol% PS or PG to reach a high uptake level (Fig. 3).

Even though our data do not offer a conclusive model for liposome–cell interaction, they do suggest a few modes of interaction that can explain the different pattern of uptake by CV1 and J774 cells. A simplest explanation for the cell specificity is that CV1 and J774 cells have different binding sites (or receptors) for the negatively charged liposomes. CV1 cells have membrane components that have a high binding constant for PS, PG, or PA, thus avidly bind liposomes which contain low concentration of these lipids. J774 cells do not have the capacity to detect a low concentration of these negatively charged lipids, but have the membrane components that bind highly negatively charged liposomes. The hypothesis that different cells have different liposome-binding sites is supported by the competition experiment with various poly-anions. Fig. 5 demonstrates that CV1 cells take up liposomes by binding sites which are not inhibited by the poly-anions that compete for scavenger receptors; CV1 cells possess a mechanism for liposome uptake that is not through the scavenger receptor. Further experiments are needed to prove the direct involvement of scavenger receptors in the liposome uptake by J774 cells. On the other hand, if we assume that the liposome-binding sites are the same in these two cell lines, the cell surface density of the binding sites in CV1 may be higher than that in J774 cells. In this case, a multi-ligand effect can explain the difference in the threshold charge density for high uptake. The binding of a liposome may require several negatively charged lipids and clustering of one or several types of membrane binding components. This would account for the observation that the uptake by J774 cells can be increased by higher density of negatively charged lipids in the liposomes. Since the liposome uptake is through endocytosis, a complex process which requires cytoplasmic components [31] as well as the binding sites, the machinery involved after the binding may be also critical. Our preliminary results with J774 cells indicate that a significant percentage of liposomes are taken up through smooth pits [70] contrary to the case of CV1 cells which take up liposomes exclusively through coated pits [30], and suggest the possibility of a different route of liposome uptake in J774 cells versus CV1 cells. Obviously, the liposome uptake is more complicated than a pure electrostatic interaction between the liposome surface and the cell membrane surface. In summary, the specificity of the

* Incorporation of cholesterol sulfate or cholesterol hemisuccinate into the bilayer also enhances the liposome uptake by macrophages *in vitro* (Dr. F. Szoka, personal communication). Thus, the specificity for the negative charge that the cell recognizes may be quite broad and not just for phosphate.

binding observed here is not only dependent on the lipid composition of the liposomes but also on the cell type, as shown especially by the comparison between CV1 and J774 using liposomes of the same lipid composition and by the competition with poly(I) and poly(C).

Like other ligands that are taken up by cells, the uptake of liposomes can also be assumed to fall into either one of two categories: nonspecific [71] or receptor-mediated endocytosis [72,73]. The fact that the liposome binding is lipid headgroup-specific and cell type dependent indicates that the liposome-cell interaction shows considerable specificity that may be described as receptor-mediated. Using the term 'receptor-mediated' requires the existence of cell surface proteins that bind a specific chemical structure (ligand) on the liposome surface and mediate endosome formation which contains the ligand-receptor complex (receptosome) [74,75]. The latter mechanism has been documented for many ligands including the LDL receptor-mediated uptake of LDL particles [50]. The binding of anionic liposomes to the cell surface is trypsin sensitive to a large extent, suggesting the involvement of cell surface proteins. Neutral PC liposomes have been shown earlier to bind to trypsin sensitive membrane proteins in fibroblast cell line [76]. We are presently attempting to identify the putative cell surface binding site for the negatively charged liposome.

The discussion in the above sections was based on the *in vitro* observation where no serum was present during the interaction of liposomes with cells. When serum is added to the incubation medium, proteins may be adsorbed to the liposome surface [47,48,77] and change the mechanism of uptake as well as the rate of uptake. Opsonization by IgG or components of complement system induces higher liposome uptake mediated by Fc-receptors [78] or complement-receptors [79,80] in macrophages. There may exist other opsonins that play more important roles than the direct interaction between liposomes and macrophages *in vivo*, and recently organ-specific serum opsonins for liver and spleen macrophages have been described [77]. There are studies which indicate that the binding of certain serum components can be dependent on the lipid composition of liposomes. A recent report showed an enhancement of complement activation by liposomes containing more negative charge given by PS, PG, PA, or PI [81]. Also Wassef et al. showed an intriguing suppressive effect of PI and G_{M1} on the complement mediated enhancement of liposome uptake [61]. Both results resemble the behavior of these liposomes *in vivo*, and may explain the difference in the *in vivo* uptake rate if liposomes are opsonized in the blood and taken up through complement receptors of macrophages. However, more studies are needed in order to systematically investigate the *in vivo* role of these different mechanisms of serum mediated liposome uptake in relation to the direct interaction between cells and liposomes reported here. The differences observed in the uptake rates of liposomes by macrophage-like cells in the presence or absence of serum (Fig. 5) indicate a possible involvement of serum proteins. However, no difference was observed in CV1 cells using the same lipid composition, and this again supports the specificity in the cell type in the liposome–cell interaction. CV1 cells can clearly distinguish the presence of 9% negatively charged lipids in the liposome bilayer in the presence of serum and take them up at the same rate as in the absence of serum.

We have reported here some striking similarities between the specificities of the uptake of liposomes by cells *in vitro* (Fig. 1, Table I) and their blood clearance rates by RES *in vivo* [25,26]. Liposomes that are either neutral or carry a negative charge contributed by either PI or G_{M1} are not recognized avidly by cells *in vitro*, and show a much diminished uptake by the RES cells *in vivo*. On the contrary, as already discussed above, when liposomes contain either PS or PG (and also PA), they are taken up avidly by the cells *in vitro* and also removed rapidly from the blood circulation. We investigated the parameters involved in the direct interaction between cells and liposomes, although it is possible that the direct interaction may not be the sole determining factor in the *in vivo* clearance of liposomes. CV1 cell line mimicked the *in vivo* behavior better than macrophage-like cell line J774, a result which defies our initial expectation. It is not clear whether it is due to some other complicating factors *in vivo* or due to the possibility that J774 cell line may not represent the cells of the mononuclear phagocytic system (RES) which is responsible for the *in vivo* clearance. Numerous studies utilized macrophages in primary culture as well as several macrophage cell lines to mimic the RES uptake of liposomes, but none was in perfect agreement with the rate of liposome clearance *in vivo*. For example, a recent paper by Allen et al. [53] showed that the uptake of PC liposomes by bone marrow macrophages was more than 4-fold reduced when 10% G_{M1} or PEG-PE was incorporated, but the increase in the uptake by incorporating PS was merely 2-fold. Therefore it should be pointed out that the results of the *in vitro* experiments cannot be applied directly to explain the events *in vivo*. Indeed, it is obvious that *in vivo* there are many complicating factors such as the blood dynamics and the interaction with a variety of cell types in addition to the opsonization of liposomes by serum proteins. However, we believe that a better understanding of the liposome–cell interactions *in vitro* based on the information presented in this report will provide us with important clues to the behavior of liposomes *in vivo*.

Acknowledgements

This work was supported by Grants CA25526 and CA35340 from the National Institutes of Health. K.-D.L. was a recipient of an American Heart Association Postdoctoral fellowship, California affiliate. We thank Drs. Leonidas Stamatatos, Frank Szoka and Robert Debs for reading the manuscript.

References

1 Papahadjopoulos, D., Mayhew, E., Poste, G. and Smith, S. (1974) Nature (Lond.) 252, 163–166.
2 Fraley, R.T., Subramani, S., Berg, P. and Papahadjopoulos, D. (1980) J. Biol. Chem. 255, 10431–10435.
3 Gregoriadis, G. and Ryman, B. (1972) Eur. J. Biochem. 24, 485–491.
4 Mayhew, E. and Papahadjopoulos, D. (1983) in Liposomes (Ostro, M.J., ed.), pp. 289–341, Marcel Dekker, New York.
5 Mayhew, E., Papahadjopoulos, D., Rustum, Y.M. and Dave., C. (1976) Cancer Res. 36, 4406–4411.
6 Rahman, A., Kessler, A., More, N., Sikic, B., Rowden, G., Woolley, P. and Schein, P.S. (1980) Cancer Res. 40, 1532–1537.
7 Lopez-Berestein, G., Mehta, R., Hopfer, R., Mills, K., Kasi, L., Mehta, K., Fainstein, V., Hersh, E. and Juliano, R. (1983) J. Infect. Dis. 147, 939–945.
8 Szoka, F., Milholl, D. and Barza, M. (1987) Antimicrob. Agents Chemother. 31, 421–429.
9 Lopez-Berestein, G., Fainstein, V., Hopfer, R., Mehta, K., Sullivan, M.P., Keating, M., Rosenblum, M.G., Mehta, R., Mills, K., Luna, M., Hersh, E.M., Reuben, J., Juliano, R.L. and Bodey, G.P. (1985) J. Infect. Dis. 151, 704–710.
10 Lopez-Berestein, G. and Juliano, R.L. (1987) in Liposomes (Ostro, M.J., ed.), pp. 253–276, Marcel Dekker, New York.
11 Gabizon, A., Peretz, T., Ben-Yosef, R., Catane, R., Biran, S. and Barenholz, Y. (1986) Proc. Am. Soc. Clin. Oncol. 5, 43.
12 Peretz, T., Gabizon, A., Catane, R., Ben-Yosef, R., Biran, S., Drukman, S. and Barenholz, Y. (1987) Proc. Am. Soc. Clin. Oncol. 6, 43.
13 Treat, J., Greenspan, A. and Rahman, A. (1989) in Liposomes in the Therapy of Infectious Diseases and Cancer, Vol. 89, (Lopez-Berestein, G. and Fidler, I.J., eds.) pp. 353–366, Alan R. Liss, New York.
14 Treat, J., Greenspan, A., Frost, D. and Rahman, A. (1988) Breast Cancer Res. Treat 12, 148.
15 Alving, C.R., Steck, E.A., Chapman, Jr., W.L., Waits, V.B., Hendricks, L.D., Swartz, G.M. and Hanson, W.L. (1978) Proc. Natl. Acad. Sci. USA 75, 2959–2963.
16 Fidler (1980) Science 208, 1469–1471.
17 Heath, T.D., Fraley, R.T. and Papahadjopoulos, D. (1980) Science 210, 539–541.
18 Leserman, L.D., Machy, P. and Barbet, J. (1981) Nature (Lond) 293, 226–228.
19 Martin, F., Hubbell, W. and Papahadjopoulos, D. (1981) Biochemistry 20, 4229–4238.
20 Huang, A., Kennel, S.J. and Huang, L. (1983) J. Biol. Chem. 258, 14034–14040.
21 Heath, T.D., Montgomery, J.A., Piper, J.R. and Papahadjopoulos, D. (1983) Proc. Natl. Acad. Sci. USA. 80, 1377–1381.
22 Hunt, A.C., Rustum, Y.M., Mayhew, E. and Papahadjopoulos, D. (1979) Drug Metab. Dispos. 7, 124–128.
23 Fidler, I.J., Raz, A., Fogler, W.E., Kirsh, R., Bugelski, P. and Poste, G. (1980) Cancer Res. 40, 4460–4466.
24 Poste, G., Bucana, C., Raz, A., Bugelski, P., Kirsh, R. and Fidler, I.J. (1982) Cancer Res. 42, 1412–1422.
25 Allen, T.M. and Chonn, A. (1987) FEBS Lett. 223, 42–46.
26 Gabizon, A. and Papahadjopoulos, D. (1988) Proc. Natl. Acad. Sci. USA 85, 6949–6953.
27 Woodle, M.C., Newman, M.S., Collins, L.R. and Martin, F.J. (1990) Biophys. J. 57, 261a.
28 Klibanov, A.L., Maruyama, K., Torchilin, V.P. and Huang, L. (1990) FEBS Lett. 268, 235–237.
29 Blume, G. and Cevc, G. (1990) Biochim. Biophys. Acta 1029, 91–97.
30 Straubinger, R.M., Hong, K., Friend, D.S. and Papahadjopoulos, D. (1983) Cell 32, 1069–1079.
31 Chin, D., Straubinger, R.M., Acton, S., Nathke, I. and Brodsky, F.M. (1989) Proc. Natl. Acad. Sci. USA 86, 9289–9293.
32 Hong, K., Friend, D.S., Glabe, C.G. and Papahadjopoulos, D. (1983) Biochim. Biophys. Acta 732, 320–323.
33 Hong, K., Straubinger, R.M. and Papahadjopoulos, D. (1986) J. Cell Biol. 103, 569a.
34 Straubinger, R.M., Papahadjopoulos, D. and Hong, K. (1990) Biochemistry 29, 4929–4939.
35 Daleke, D.L., Hong, K. and Papahadjopoulos, D. (1990) Biochim. Biophys. Acta 1024, 352–366.
36 Brown, P.M. and Silvius, J.R. (1990) Biochim. Biophys. Acta 1023, 341–351.
37 Heath, T.D., Lopez, N.G. and Papahadjopoulos, D. (1985) Biochim. Biophys. Acta 820, 74–84.
38 Nishikawa, K., Arai, H. and Inoue, K. (1990) J. Biol. Chem., 265, 5226–5231.
39 Stevenson, M., Baillie, A.J. and Richards, R.M.E. (1984) J. Pharm. Pharmacol. 36, 824–830.
40 Szoka, F. and Papahadjopoulos, D. (1978) Proc. Natl. Acad. Sci. USA 75, 4194–4198.
41 Olson, F., Hunt, C.A., Szoka, F.C., Vail, W.J. and Papahadjopoulos, D. (1979) Biochim. Biophys. Acta 557, 9–23.
42 Szoka, F., Olson, F., Heath, T., Vail, W., Mayhew, E. and Papahadjopoulos, D. (1980) Biochim. Biophys. Acta. 601, 559–571.
43 Bartlett, G.R. (1959) J. Biol. Chem. 234, 466–468.
44 Wolfbeis, O.S., Fürlinger, E., Kroneis, H. and Marsoner, H. (1983) Fresenius Z. Anal. Chem. 314, 119–124.
45 Felgner, P.L., Thompson, T.E., Barenholz, Y. and Lichtenberg, D. (1983) Biochemistry 22, 1670–1674.
46 Dijkstra, J., Van Galen, W.J.M., Hulstaert, C.E., Kalicharan, D., Roerdink, F.H. and Scherphof, G.L. (1984) Exp. Cell Res., 150, 161–176.
47 Bonte, F. and Juliano, R.L. (1986) Chem. Phys. Lipids 40, 359–372.
48 Senior, J.H. (1987) Crit. Rev. Ther. Drug Carrier Syst. 3, 123–193.
49 Dijkstra, J., Van Galen, M. and Scherphof, G. (1985) Biochim. Biophys. Acta 813, 287–297.
50 Brown, M.S. and Goldstein, J.L. (1983) Annu. Rev. Biochem. 52, 223–263.
51 Brown, M.S., Basu, S.K., Falck, J.R., Ho, Y.K. and Goldstein, J.L. (1980) J. Supramol. Struct. 13, 67–81.
52 Chu, C.-J., Dijkstra, J., Lai, M.-Z., Hong, K. and Szoka, F.C. (1990) Pharm. Res. 7, 824–834.
53 Allen, T.M., Austin, G.A., Chonn, A., Lin, L. and Lee, K.C. (1991) Biochim. Biophys. Acta 1061, 56–64.
54 Schroit, A.J., Madsen, J. and Nayar, R. (1986) Chem. Phys. Lipids 40, 373–393.
55 Raz, A., Bucana, C., Fogler, W.E., Poste, G. and Fidler, I.J. (1981) Cancer Res. 41, 487–494.
56 Fraley, R.T., Straubinger, R.M., Rule, G., Springer, E.L. and Papahadjopoulos, D. (1981) Biochemistry 20, 6978–6987.
57 Schroit, A.J. and Fidler, I.J. (1982) Cancer Res. 42, 161–167.
58 Gregoriadis, G. and Neerunjun, D.E. (1974) Eur. J. Biochem., 47, 179–185.

59 Juliano, R.L. and Stamp, D. (1975) Biochem. Biophys. Res. Commun. 63, 651–658.
60 Senior, J., Crawley, J.C.W. and Gregoriadis, G. (1985) Biochim. Biophys. Acta 839, 1–8.
61 Wassef, N.M., Matyas, G.R. and Alving, C.R. (1991) Biochem. Biophys. Res. Commun. 176, 866–874.
62 Schroit, A.J., Madsen, J.W. and Tanaka, Y. (1985) J. Biol. Chem. 260, 5131–5138.
63 Allen, T.M., Williamson, P. and Schlegel, R.A. (1988) Proc. Natl. Acad. Sci. USA 85, 8067–8071.
64 Connor, J., Bucana, C., Fidler, I.J. and Schroit, A.J. (1989) Proc. Natl. Acad. Sci. USA 86, 3184–3188.
65 Comiskey, S.J. and Heath, T.D. (1990) Biochemistry 29, 3626–3631.
66 Lee, K.-D., Hong, K. and Papahadjopoulos, D. (1991) Biophys. J. 59, 345a.
67 McDaniel, R.V., Sharp, K., Brooks, D., McLaughlin, A.C., Winiski, A.P., Cafiso, D. and McLaughlin, S. (1986) Biophys. J. 49, 741–752.
68 Langner, M., Cafiso, D., Marcelja, S. and McLaughlin, S. (1990) Biophys. J. 57, 335–349.
69 Gabizon, A. and Papahadjopoulos, D. (1992) Biochim. Biophys. Acta 1103, 94–100.
70 Lee, K.-D., Hong, K., Friend, D.S. and Papahadjopoulos, D. (1990) J. Cell Biol. 111, 191a.
71 Hsu, M.J. and Juliano, R.L. (1982) Biochim. Biophys. Acta 720, 411–419.
72 Stahl, P. and Schwarts, A.L. (1986) J. Clin. Invest. 77, 657–662.
73 Pastan, I. and Willingham, M.C. (1983) Trends Biochem. Sci. 8, 250–254.
74 Pastan, I.H. and Willingham, M.C. (1981) Science 214, 504–509.
75 Helenius, A., Mellman, I., Wall, D. and Hubbard, A. (1983) Trends Biochem. Sci. 8, 245–250.
76 Pagano, R.E. and Takeichi, M. (1977) J. Cell Biol. 74, 531–546.
77 Moghimi, S.M. and Patel, H.M. (1989) Biochim. Biophys. Acta 984, 384–387.
78 Petty, H., Hafeman, D.G. and McConnell, H.M. (1980) J. Immunol. 125, 2391–2396.
79 Roerdink, F., Wassef, N.M., Richardson, E.C. and Alving, C.R. (1983) Biochim. Biophys. Acta 734, 33–39.
80 Wassef, N.M. and Alving, C.R. (1987) Methods Enzymol. 149, 124–134.
81 Chonn, A., Cullis, P.R. and Devine, D.V. (1991) J. Immunol. 146, 4234–4241.