Biochimica et Biophysica Acta, 733 (1983) 65–74
Elsevier

BBA 71804

PLASMA MEMBRANE-MEDIATED LEAKAGE OF LIPOSOMES INDUCED BY INTERACTION WITH MURINE THYMOCYTIC LEUKEMIA CELLS

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(Received March 28th, 1983)

Key words: Membrane fusion; Liposome cell interaction; (Mouse thymocyte)

The interaction of liposomes with BW 5147 murine thymocytic leukemia cells was studied using fluorescent probes (entrapped carboxyfluorescein and fluorescent phosphatidylethanolamine) in conjunction with a Ficoll-Paque discontinous gradient system for rapid separation of liposomes from cells. Reversible liposomal binding to discrete sites on the BW cell surface was found to represent the major form of interaction; uptake of intact liposomal contents by a process such as liposome-BW cell membrane fusion was found to apparently represent a minor pathway of interaction (2%). Liposomal lysis was found to be associated with the process of liposomal binding (perhaps as a result of the binding itself). Lysis was followed by release of the entrapped carboxyfluorescein into the media and its subsequent uptake by the cells. This lysis was shown to be dependent upon discrete membrane-associated sites that have some of the properties of proteins. The results of these studies suggest that liposomal binding to the cells, subsequent lysis of the liposomes and cellular uptake of their contents should be seriously considered in all studies of liposome-cell interactions as an alternate mode of interaction to the four modes (fusion, endocytosis, adsorption and lipid exchange) previously emphasized in the literature.

Introduction

One approach to studying the molecular mechanisms of biological membrane fusion is through the use of model systems. The interactions between phospholipid bilayer vesicles (liposomes) and mammalian cells have been the subject of numerous studies [1–11]. One reason for this interest has been the possibility that these interactions may involve fusion between the liposomes and the surface membranes of the cells in culture; thus the process might be suitable as a model fusion system [12,13]. There is, however, little agreement on the basic mechanisms involved in liposome-cell interactions, since there appears to be more than one pathway for the incorporation of both intact vesicles (endocytosis, adsorption and fusion) and individual phospholipid molecules (contact exchange and monomeric exchange; for a review see Ref. 14). Because of this uncertainty, identification of liposome-cell fusion is a difficult task.

In the present report we present the results of experiments on the interactions of liposomes with murine thymocytic leukemia cells in suspension culture. The liposomes are prepared with fluorescent labels incorporated both in their lipid and aqueous compartments; by monitoring cell and

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Abbreviations: CF, carboxyfluorescein; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine.

non-cell associated fluorescence we are able to characterize the binding of vesicles to the cells. During these studies we utilize a simple but useful technique which allows the quantitative separation of free liposomes from cell associated-liposomes within a matter of seconds in a manner which damages neither the cells nor the liposomes. This approach allows a more quantitative look at the relative significance of fusion than has been possible before.

Materials and Methods

Cell culture and harvesting

Murine thymocytic leukemia cells (BW 5147) were maintained in spinner culture with RPMI 1640 medium containing 10% fetal calf serum and extra glutamine (2 mM). The cells were harvested from log phase growth and washed three times by gentle ($500 \times g$, 10 min) centrifugation and resuspension in Ca^{2+}-Mg^{2+} free Hank's buffer (pH 7.4, buffer A). After the final washing, the cells were adjusted to $1 \cdot 10^7$/ml and were held at 37°C for a maximum of 15 min. Viability was monitored by Trypan blue exclusion.

Lipids and preparation of liposomes

Chromatographically pure lipids (egg PC and phosphatidylserine) in ethanol were obtained from Avanti Biochemicals, Birmingham, AL; fluorescent phosphatidylethanolamine (*N*-4-nitrobenzo-2-oxa-1,3-diazole phosphatidylethanolamine) was also purchased from Avanti Biochemicals. Carboxyfluorescein was purchased from Eastman Chemicals, Rochester, NY and recrystallised.

Ten mg of egg PC (to be referred to subsequently as PC) in ethanol was rotoevaporated to dryness and lyophilized overnight. The dried lipid was rehydrated in 1 ml of buffer A, allowed to swell at room temperature for 20 min and sonicated in a water-jacketed probe sonicator (Branson Model 200) for 4 min under argon atmosphere at 0°C. Thin-layer chromatography was used to monitor for oxidation and/or degradation products. Liposomes have been passed through Sepharose 4B. They are mainly in the included volume, with a Stokes radius of 141 Å.

For liposomes of varying lipid content (PC/PS and PC/fluorescent PE), lipids in organic solvents were mixed in proportions to yield a total of 10 mg lipid and were immediately rotoevaporated. The fluorescent dye carboxyfluorescein (CF) was entrapped in liposomes by sonicating the lipid in buffer A containing 200 mM 6-CF. All sonicated suspensions were spun at 12000 rpm ($R_{av} = 17600 \times g$) for 60 min in a Beckman J-21B centrifuge equipped with a JA 20 fixed angle rotor, and the supernatant was retained.

In all instances, the supernatant from the centrifugation was passed over 1×40 cm Sephadex G-50 column equilibrated with buffer A. The void volume (containing small unilamellar vesicles) was retained. Phosphorus determination were by the method of Fiske and SubbaRow [15].

Incubation of cells and liposomes

In most incubations, 1 volume of washed cells ($1 \cdot 10^7$ cells per ml) was mixed with 1 volume of liposomes (2.5 mg lipid per ml) to yield a suspension containing $5 \cdot 10^6$ cells per ml and 1.25 mg lipid per ml.

All incubations were at 37°C in buffer A without the addition of serum. The reader is referred to the legends of the figures for the specifics of the incubations.

Separation of cells and liposomes

Cells were separated from liposomes by a method described in detail elsewhere [16]. Cells and liposomes were subjected to a brief (60 s) centrifugation at $300 \times g$ on discontinuous Ficoll-Paque gradients. All gradients were prepared in 5 ml cellulose nitrate centrifuge tubes (No. 305050, Beckman Instruments, Palo Alto, CA) and contained a 1 ml cushion of undiluted (5.7% w/v) Ficoll-Paque (Pharmacia, Inc., Piscataway, NJ) and an overlay of 2.5 ml of Ficoll-Paque diluted to 35% (v/v) with buffer A. One ml of a suspension of cells ($5 \cdot 10^6$ cells per ml) and liposomes (1.25 mg/ml) was gently layered over the gradient, and the sample was immediately centrifuged at 1200 rpm ($R_{av} = 300 \times g$) for 60 s in a table-top centrifuge (IEC Model HN-S) equipped with a swinging-bucket rotor. Immediately after centrifugation, the tubes were punctured and fractionated (0.25 ml fractions were collected).

A 1-ml sample containing the cells (which band at the cushion-gradient interface) and a 1.5 ml

sample containing liposomes (which remain at the top of gradient) were held for determinations of fluorescence.

Fluorescence of cells and liposomes

The fluorescence of isolated cell fractions, isolated liposome fractions, and cell-liposome suspensions were determined on an Aminco SPF-500 corrected spectra spectrofluorimeter with $\lambda_{ex} = 472$ nm (bandpass = 4 nm) and $\lambda_{em} = 520$ nm (bandpass = 1 nm). One-ml samples were read in all experiments. For experiments involving lysis of cells and liposomes, 10 μl of 10% Triton X-100 in buffer A was added to give a final concentration of 0.1%. All fluorescent measurements were done in a dequenched state (using an appropriate dilution of liposomes) and the CF concentration determined with a standard curve.

Cell sizing

The mean cell volume of BW thymocytes was obtained by electronic particle counting via an Electrozone/ Celloscope (Particle Data, Inc. Elmhurst, IL), with a 48 μm orifice, $I = 1/2$, gain = 4– 1/4, and calibrated with 6 μm and 10 μm latex spheres. Raw data were fed to a microprocessor programmed with MP-12 4K R.A.M. program (Particle Data, Inc.).

Results

The fluorescence of BW cells incubated with CF entrapped in PC liposomes and with PC/ fluorescent PE (99 : 1, w/w) are shown in Fig. 1. In these experiments, cells were incubated with PC/fluorescent PE liposomes (99 : 1, w/w) or liposomes containing 200 mM CF, and the cell-liposome mixtures were fractionated on discontinuous Ficoll gradient. The uptake of CF and of fluorescent lipid are qualitatively quite similar, demonstrating linearity within the first 30 min and reaching a virtual plateau between 30 and 90 min. When the cells are incubated with CF liposomes and their fluorescence is monitored after Triton X-100 lysis, maximal fluorescence occurs at 30 min; beyond 30 min there is a decrease in cell-associated fluorescence which is susceptible to Triton X-100 lysis.

Using PC/fluorescent PE (99 : 1, w/w) and

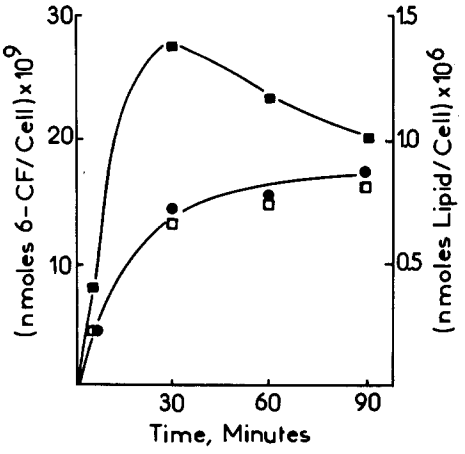

Fig. 1. Fluorescence of cell fractions from Ficoll gradients. Cells were incubated with PC/fluorescent PE liposomes, (99 : 1, w/w) (□———□) and pure PC liposomes containing 200 mM CF (●———●). Cells incubated with PC liposomes were also lysed with Triton X-100, 0.1% final concentration (■———■). All incubations were at 37°C and contained $5 \cdot 10^6$ cells per ml and 1.25 mg lipid per ml.

^{14}C-PC liposomes the uptake was studied as a function of liposomes concentration. It is seen from Fig. 2a that liposomal uptake is saturable; further, the fluorescent and radiolabeled lipids provide uptake curves essentially identical within experimental error. Replotting the liposomal uptake data in double reciprocal form (Fig. 2b) confirms the saturability of the process and the similarity in uptake between fluorescent and radioactive lipid labels. Based on data to be presented later, the major portion of this uptake represents an easily reversible binding of liposomes to the thymocyte surface membrane rather than a less easily reversible process such a fusion, lipid exchange or endocytosis. Thus a K_m calculated from Fig. 2b of 0.16 mM represents the K_m of binding of liposomes to the BW cells [17].

It is possible from the data represented by Figs. 1 and 2a to calculate the number of liposomes bound per cell. The number is $6 \cdot 10^4$ based on measurements of PC/fluorescent PE and ^{14}C-PC liposomes. Thus a maximum of 13% of the available BW cell surface is occupied by bound liposomes at equilibrium. Because these calculations assume the BW cell to be a sphere, an obvious approximation, 13% represents an overestimation;

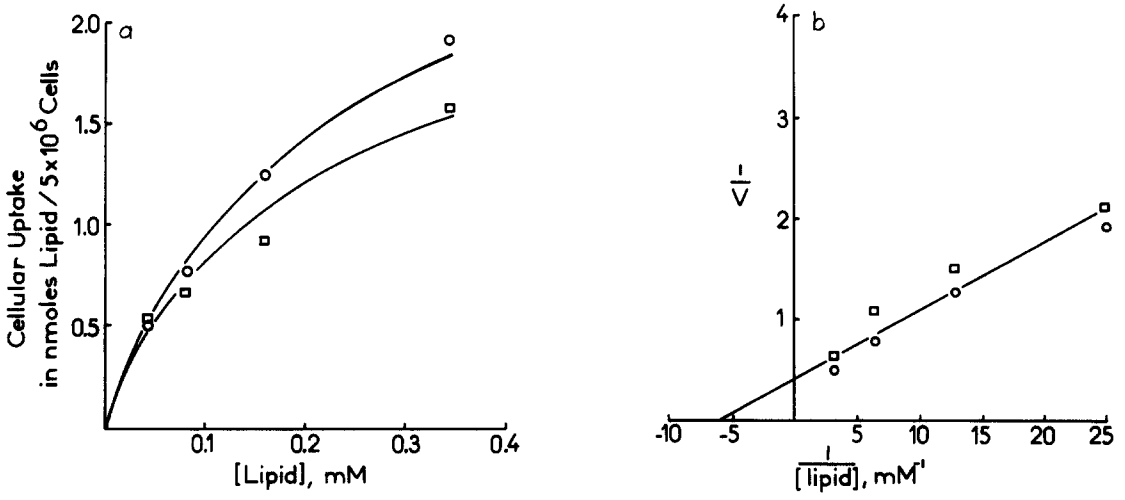

Fig. 2. Uptake of lipids by BW cells after Ficoll-Paque gradient separation following incubation for 30 min at 37°C with a range of concentrations of PC/fluorescent PE (□———□) and ^{14}C-PC (○———○). (a) Molar uptake of lipid per $5 \cdot 10^6$ cells versus lipid concentration. (b) Double reciprocal plot of data in (a).

a sphere has the minimal surface area to volume ratio for geometric solids.

If CF is initially entrapped in liposomes and those vesicles are incubated with cells, then the partitioning of CF between the cytoplasmic compartment and the extracellular milieu can be calculated. If there is a gradient established across the plasma membrane (i.e. concentration of CF inside the cell greater than concentration outside), this would support an uptake process involving internalization of liposomal contents with diffusion into the cytoplasm (fusion and/or endocytosis). However, if there is a substantial leakage of liposomal contents outside the cells followed by passive incorporation of free CF, the concentration of CF inside the cells should not be appreciably greater than that outside. These calculations require data including measurements of the mean cell volume and the concentration of cells, from which a 'dilutional factor' (volume outside/volume inside the cells) is calculated, as well as measurements of the fluorescence of cells versus the fluorescence of the incubation medium (cells are separated from the incubation medium on Ficoll gradient). We also assign two basic routes for the uptake of entrapped CF and express each as a fraction of the total available fluorescence: the dye may escape from the liposomes and may partition into the incubation medium ('leakage') or it may be delivered undiluted to the cytoplasm of the cells ('fusion'). Our calculations reveal that a minimum of 1.7% of the uptake of CF can be assigned to 'fusion'.

We interpret the results of Fig. 1 to indicate binding of liposomes to the cells within the first 30 min. The increase in fluorescence after Triton X-100 lysis we ascribe to detergent disruption of the bound liposomes with dequenching of the previously entrapped CF. Because an apparent equilibrium of bound liposomes is reached after 30 min (Fig. 1), the decrease in total bound CF between 30 and 90 min must be due to liposomal disruption and subsequent CF leakage.

Fig. 3a shows the results of a time-course study of cells preincubated with CF liposomes (15 min), washed by centrifugation and fractionated on Ficoll-Paque gradients [16] at various times. The single washing results in a marked reduction in the cell-associated fluorescence (compare Figs. 1 and 3a), which is consistent with an equilibrium-type binding of liposomes to the cells. In addition, there is a gradual reduction in the fluorescence of the isolated intact cells with time. The increase in fluorescence after Triton X-100 lysis also decrease progressively, which is consistent with a progressive leakage of the contents of the surface bound

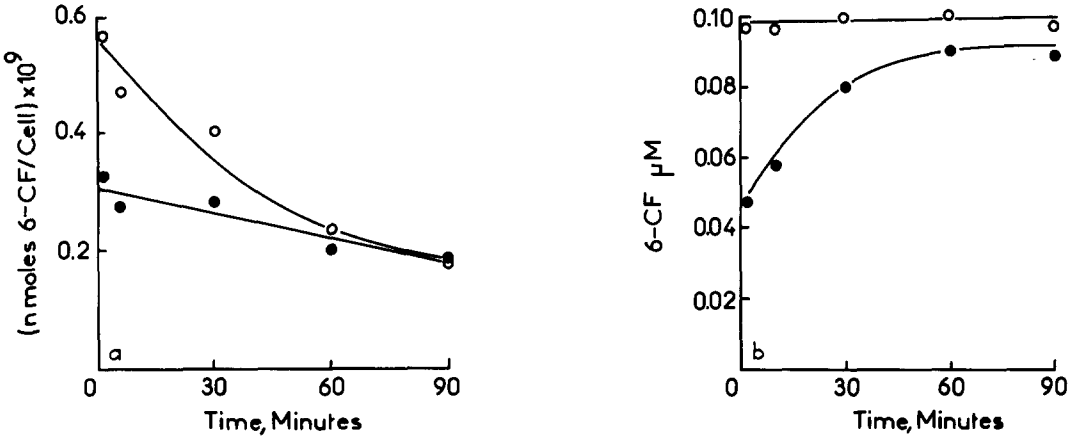

Fig. 3. (a) Fluorescence of cells after one washing by centrifugation followed by fractionation on Ficoll gradients. Cells ($5 \cdot 10^6$/ml) were initially incubated with PC liposomes (1.25 mg lipid per ml) containing 200 mM CF for 15 min at 37°C, diluted 1 : 1 with buffer A (pH 7.4) centrifuged ($R_{av} = 300 \times g$, 10 min), resuspended to the original volume in buffer A, and fractionated on Ficoll gradients. t_0 = immediately after resuspension in buffer A. Fluorescence was measured before (●———●) and after (○———○) lysis with Triton X-100, 0.1% final concentration. (b) Fluorescence of liposome fractions from Ficoll gradients of cells treated as in (a), before (●———●) and after (○———○) lysis with Triton X-100 (0.1% final concentration).

liposomes into the medium.

The decrease in Triton X-100 sensitive cellular fluorescence is accompanied by an increase in fluorescence of the liposome fractions (Fig. 3b), which also is consistent with an increased 'leakiness' of the surface bound liposomes to CF. When the liposome fractions are lysed by Triton X-100, the fluorescence remains relatively constant at all times, indicating that all samples contain essentially the same amounts of CF; the basic difference is therefore an altered partitioning of the CF between the liposomal compartment and the aqueous milieu.

By 60 min Triton X-100 has a minimal effect on both cellular (Fig. 3a) and liposomal (Fig. 3b) fluorescence, indicating that the majority of the cell-associated liposomes are either disrupted or are so altered that they have become extremely leaky.

Since these data indicate a release of significant amounts of free dye into the incubation medium, it was necessary to investigate the cells' ability to incorporate free CF. Cells were incubated in free CF and uptake of the dye measured as a function of time (Fig. 4a). The concentration of free dye (2.1 μM) is equal to the estimated concentration of free dye due to cell-induced liposomal leakage at 30 min in the experiments shown in Fig. 1. The uptake is approximately linear during the first 10 min and reaches a plateau between 20 and 30 min. From the calculations of the final concentration reached inside the cells versus the free dye concentration, at the plateau the CF concentration inside and the CF concentration outside are approximately equal (2.2 μM and 2.1 μM, respectively), indicating approximate equilibrium within 30 min.

Uptake of free dye as a function of concentration in the range of 0.6–4.8 μM is shown in Fig. 4b. The uptake of free CF is linearly dependent upon the concentration of free CF, and the cells are diffusely fluorescent via epifluorescence light microscopy (data not shown). The uptake of free CF is therefore non-specific rather than saturable. The dye is in a dequenched state after it enters the cells, since Triton X-100 lysis causes no further increase in fluorescence.

The induction of leakage therefore appears to be a significant factor in the interaction of liposomes with BW thymocytes. Although fractionating the cell-liposome mixture on Ficoll-Paque gradients is useful for analyzing the cellular and liposomal components of fluorescence, it is not essential to perform this step in all cases, since

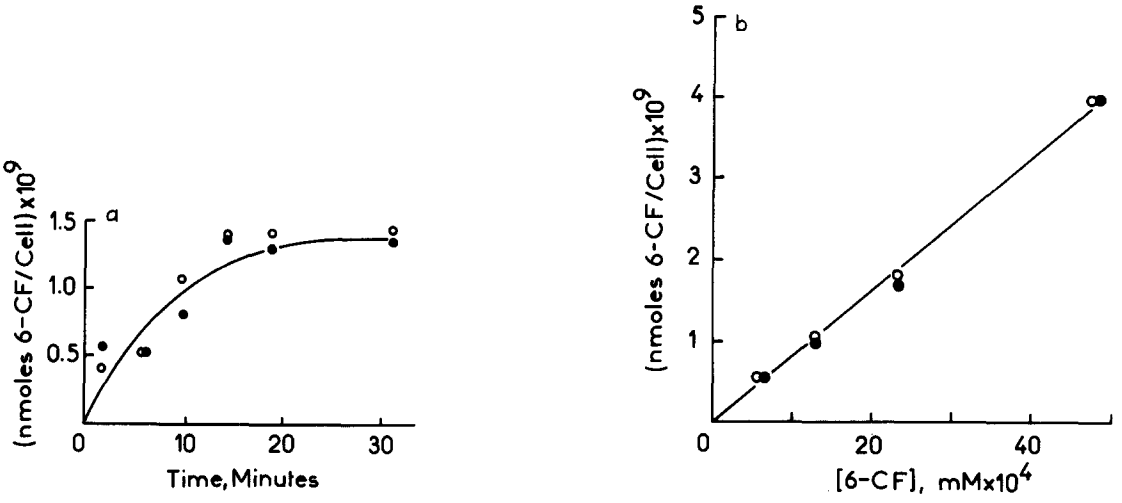

Fig. 4. Fluorescence of cells after incubation with free CF (2.1 μM) at 37°C. Incubations contained $5 \cdot 10^6$ cells per ml. (a) Fluorescence as a function of time (two experiments). (b) Fluorescence as a function of free CF concentration (30 min incubation) (two experiments).

fluorescence observed under these conditions represents essentially the leaked dye; fluorescence from any sequestered dye is quenched and fusion is negligible. When the fluorescence of liposome-cell mixtures are monitored (Fig. 5a), the leakage, measured by fluorescence increases, is essentially linear and only time-dependent in the first hour. The addition of 10% (w/w) PS to the liposomes

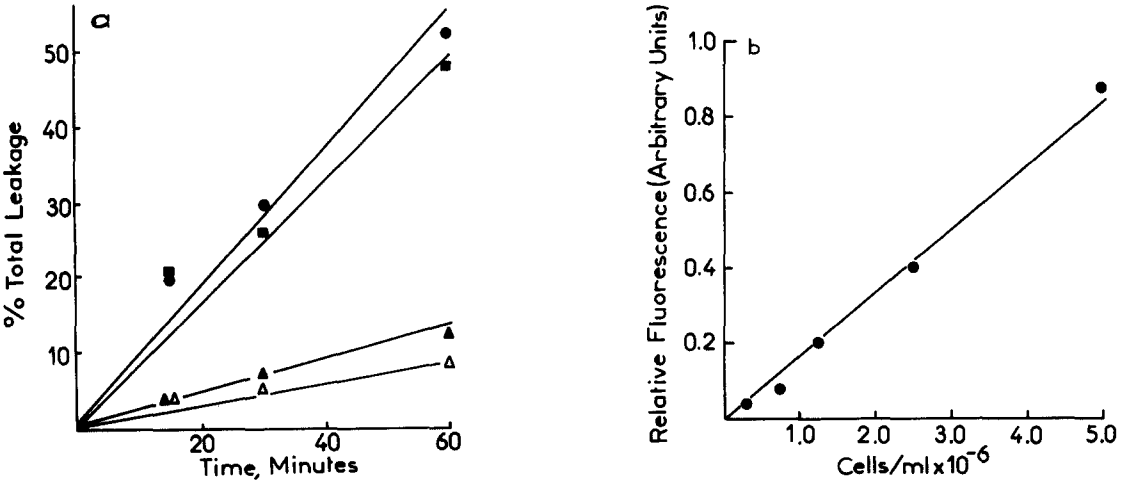

Fig. 5. (a) Fluorescence of cell-liposome mixtures as a function of lipid composition in the liposomes. All incubations contained $5 \cdot 10^6$ cells per ml and 1.25 mg lipid per ml as liposomes containing 200 mM CF. Results are expressed as percent total leakage, i.e. (fluorescence before/after lysis with Triton X-100) $\times 100$. Liposomes were composed of pure PC (●———●), PC/PS (9:1) (■———■) and PC/PS (8:2) (▲———▲). Control PC liposomes (△———△) were incubated under the same conditions without cells. Controls for the PC/PS liposomes were not significantly different from the PC control. (b) Fluorescence of cell-liposome mixtures, incubated at 37°C for 30 min, as a function of cell concentration. Cells at the various concentrations were incubated with identical concentrations (1.25 mg lipid per ml) of PC liposomes containing 200 mM CF. The background fluorescence of controls (liposomes alone) has been subtracted from the readings.

(imparting a net negative charge) does not significantly alter the rate of leakage, although leakage is dramatically reduced to approximately the level of the control by increasing the PS content to 20% (w/w)/(Fig. 5a).

The results of Fig. 5b show that liposomal leakage induced by BW cells is dependent upon cell concentration in the range of $(1-5)\cdot 10^6$ cells per ml. Measuring liposomal leakage induction by thymocyte cells, as a function of liposomal concentration, shows (Fig. 6) that this process is saturable just as liposomal binding to BW cells is. These data, when plotted in the double reciprocal form, confirm that leakage (as opposed to free dye uptake) is saturable and has a K_m of 2.0 mM [25]. The addition of liposomes empty of CF to the liposomes containing CF results in an increase in the slope of the double reciprocal plot and an increase in the K_m of the process of leakage. This suggests that empty liposomes act as 'inhibitors', competing with CF containing liposomes for binding and lysing sites on the thymocyte cell surfaces.

From Fig. 7, it can be seen that the BW cell-induced leakage of liposomes is inhibited at low temperature (0°C) and by pretreatment at high temperature (100°C for 20 min). The leakage activity is confined to the crude membrane pellet isolated from hypotonically lysed BW cells; the water soluble fraction is essentially free of leakage activity. Finally BW cell media exposed to the cells for 30 min is free of leakage activity, indicating that the activity is not some metabolic or secretory product of the BW cells.

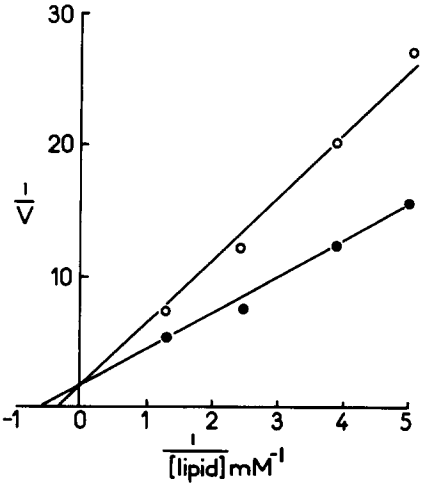

Fig. 6. Double-reciprocal plot of fluorescence induced by BW cells at 45 min versus concentration of liposomes in 37°C incubation mixture. Studies were done at various concentrations of PC liposomes containing 200 mM CF, in the absence of (●———●) and in the presence of empty vesicles, 0.25 mM (○———○) lipid.

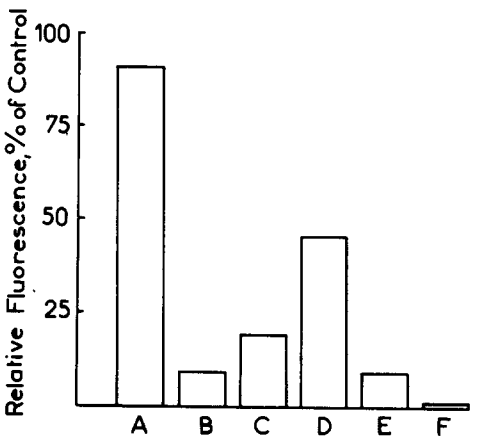

Fig. 7. Fluorescence of cell-liposome mixtures under varying conditions. All readings are expressed as percent of controls ($5\cdot 10^6$ cells per ml, incubated for 60 min with 1.25 mg/ml lipid as liposomes containing CF). A, 25000 × *g* pellet, and B, 25000 × *g* supernatant from hypotonically lysed cells; C, cells incubated with liposomes at 0°C; D, cells boiled at 100°C for 20 min before incubation; E, cells incubated with 80% PC/20% PS liposomes; F, liposomes + buffer A previously incubated with cells for 30 min. All incubations were for 60 min at 37°C.

Discussion

We interpret the results of our studies of the interaction of liposomes with BW 5147 murine thymocytic leukemia cells as follows: Liposomal binding to the thymocyte cell surface represents the major form of interaction; uptake of intact liposomal contents by a process such as liposome-membrane fusion is apparently a minor but definite means of interaction (2% at a minimum). Associated with the process of liposome binding is liposomal lysis, CF release and subsequent uptake of free CF by the cells, presumably by a pinocytotic mechanism. The lysis is dependent upon discrete membrane-associated sites that have some of the properties of protein.

It is likely that a portion of the fluorescent PE

uptake shown in Fig. 1 represents phospholipid exchange with the BW plasma membrane [19]. However, exchange is felt to represent a minor portion of this uptake for the following reasons: First, most of the liposomal uptake measured by CF fluorescence is reversible by dilution (Fig. 3A). This indicates that liposomal binding is a major component of the interaction of liposomes with BW cells. Further, if a significant portion of the uptake of fluorescent PE in Fig. 1 were due to exchange, one would expect that saturation of this process would be unlikely to occur within 90 min. While there does appear to be a minor, relatively unsaturable component of uptake in Fig. 1, the uptake curve of both CF and fluorescent lipid share the same relative degree of unsaturation, thus making it unlikely that this component of uptake represents phospholipid exchange.

The binding of liposomes to the thymocyte cell surface takes up a maximum of 13% of the calculated available cell surface at equilibrium ($6 \cdot 10^4$ liposomes bound per cell). Thus the saturability of this binding process is not due exclusively to steric hindrance. This is compatible with localized binding domains such as surface proteins.

Liposomal lysis appears temporally to lag behind liposomal binding. This is shown by the fact that isolated cell-liposome complexes contain Triton X-100 sensitive liposomes (Fig. 1). However, the fact that at 30 min Triton X-100-induced fluorescence is only twice the untreated fluorescence indicates that many of the cell-associated liposomes are partially or completely emptied of their CF contents (at 200 mM the liposomal entrapped CF is 97% quenched and a 30-fold increase in fluorescence results from lysis). Although this interpretation of Fig. 3 is complicated by cellular uptake of free dye, which also decreases the ratio of Triton X-100 fluorescence, nevertheless lysis must occur rather rapidly after liposomal binding.

While none of our data definitively shows that the liposome binding sites are identical to the lytic sites, the close similarities of the properties of binding and lysis are consistent with this assumption. Lysis is dependent on cell concentration, requires contact with the cell surface, is saturable and empty liposomes compete for lytic sites on the cell. Further the process of liposomal lysis is located on the membranous fraction of the cells.

The facts that the time dependent curves for liposomal binding and uptake of liposomally entrapped CF (Fig. 1) have indistinguishable shapes (i.e. both plateau at approximately the same time) is important. This means that, if the temporal sequence is as we propose (binding, lysis, dye uptake), then the rates of the two subsequent process, lysis and dye uptake, must be faster than liposome binding. Three independent observations support the conclusion that binding is the rate limiting step. First, the thymocyte cells equilibrate with free CF within 20 min; binding requires 90 min for equilibration. Second, the results of Fig. 1 suggest that at 30 min incubation, only a small percent of liposomes bound to isolated cells are intact, suggesting that leakage, as a process, occurs more rapidly than the process of binding. Finally it is useful to look at the kinetics of what we propose to be the process of liposomal-BW cell interaction:

$$(F)+[C] \underset{k_2}{\overset{k_1}{\rightleftharpoons}} (F)[C] \begin{cases} \xrightarrow{k_3} F+[C] \underset{k_6}{\overset{k_5}{\rightleftharpoons}} [FC] \\ \xrightarrow{k_4} (FC) \end{cases} \quad (1)$$

where (F) = liposomally entrapped CF, [C] = thymocyte cell, F = free CF, (FC) = CF incorporated into cells by 'fusion', [FC] = free CF incorporated into cells by equilibration.

The double reciprocal plot of liposome binding gives a K_m (B) of 0.16 mM (Fig. 2b).

From Eqn. 1

$$K_m(B) = \frac{k_2}{k_1} = 0.16 \quad (2)$$

The double reciprocal plot of liposomal leakage gives a K_m (L) of 2.0 mM (Fig. 6).

From Eqn. 1

$$K_m(L) = \frac{k_2 + k_3}{k_1} = 2.0 \quad (3)$$

We have assumed that the lytic step is irreversible and that the fusion step (k_4) is both negligible and irreversible. Given these assumptions, from Eqns. 2 and 3 one can calculate that $k_3 = 1.84\ k_1$, i.e. the rate of binding of liposomes measured by CF uptake is slower than the rate of release of CF by lysis of bound liposomes.

The results shown in Fig. 2a are indirect evidence that the rate contant k_6 (efflux of CF out of the cells) is considerably slower than k_5 (influx of CF into the cells) which would suggest either that internalization of CF occurs by some pathway other than passive diffusion (e.g. pinocytosis) or there are active CF binding sites within the BW cell. We found by calculation that the internal concentration of CF after equilibration of the cells with free dye is equal to the external concentration, this is evidence against the possibility of a large number of internal CF binding sites.

The studies described here illustrates the usefulness of the Ficoll-Paque discontinuous gradient system [16] for studying liposome-cell interactions. The results of these studies strongly suggest that liposome-cell membrane fusion is a minor pathway in the interaction of liposomes with BW-5147 mouse thymocytic leukemia cells; the major pathway appears to be liposomal binding to discrete sites. The process of binding induces liposomal leakage, perhaps as a result the binding itself. It seems more likely that the CF efflux from the liposomes is a slow leak rather than a sudden, complete lysis, because of the continued presence of liposomally entrapped CF with isolated cells containing bound liposomes (Fig. 1).

It is useful to compare our results with several studies reported in the literature. Huang et al. [3] reported studies of the interaction of liposomes with normal mouse thymocytes. They found that adsorption was the major route of uptake for liposomes composed of disaturated phosphatidylcholine. In contrast, unsaturated phosphatidylcholine was found to interact with the tymocytes in such a way that entrapped material, such as CF, was taken up internally by the cells. Further, ^{14}C-PC and entrapped ^{3}H-inulin were taken up at approximately the same rate. There is no indication in the report of these studies that liposomal leakage was monitored. Although our studies were not performed on identical cell types to those used by Huang et al. [3], one possible way to reconcile our results with theirs is to assume that the change induced in liposomes by interaction with thymocytes is a slow leak rather than a complete lysis. Thus ^{3}H-inulin, having a large molecular weight compared to CF, might not leak under these circumstances and would be taken up at the identical rate to ^{14}C-PC.

Pagano and Takeichi [20] have reported that liposomes are bound to specific proteins on the cell surface of Chinese hamster fibroblasts. In contrast to our results with BW cells, this binding was felt to be irreversible.

Finally, Szoka et al. [21,22], have recently reported results similar to those that we report here. They found that in the presence of cells (human peripheral blood lymphocytes and mouse L-1210 leukemia cells) the uptake of 6-CF entrapped in liposomes is less than the uptake of radiolabeled tracer lipid (dipalmitoylphosphatidylcholine, DPPC) of the liposomes. They showed that at least part of this discrepancy is due to leakage of CF into the media. Our results are in good agreement with the 'unstirred layer' model of Blumenthal et al. [23] and with the mechanism of cell-induced leakage reported by Margolis et al. [25].

Increased liposome permeability induced by cell-liposome contact was demonstrated with other types of cells [24,25]. Van Renswoude et al. [26] bring evidence for a vesicle binding mechanism with Zajdela ascites hepatoma cells. They speculate on the existence of two binding sites on the cell surface.

In conclusion, our studies indicates that liposomal binding to cells, subsequent lysis of the liposomes and uptake of the liposomal contents by the cells should be seriously considered in all studies of liposome-cell interactions as an alternate mode of interaction to the four modes (fusion, endocytosis, adsorption and lipid exchange) previously emphasized in the literature [19]. This leakage-uptake pathway is capable of causing confusion during liposome-cell studies when the uptake of liposome contents is monitored. However, parallel analysis of the uptake of liposomal contents and liposomal lipids, in conjuction with the Ficoll-Paque discontinuous gradient system for rapid separation of bound liposomes from unbound liposomes and media, is one way of minimizing this confusion.

Acknowledgements

We would like to thank John N. Weinstein and Robert Blumenthal for helpful discussions. We are indebted to the UAB Comprehensive Cancer Center for providing us with BW-5147 cells. This

work was supported in part by PHS grants CA-22868 and DE-02670.

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