Biochimica et Biophysica Acta, 730 (1983) 313–320
Elsevier Biomedical Press

BBA 71645

SMALL LIPOSOMES ARE BETTER THAN LARGE LIPOSOMES FOR SPECIFIC DRUG DELIVERY IN VITRO

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(Received December 10th, 1982)

Key words: Endocytosis; Fluorescence; Liposome; Methotrexate; Monoclonal antibody

We have compared drug transfer into target cells in vitro from liposomes of different sizes. Liposomes of mean diameter 800 Å, 2000 Å or 4000 Å, containing the folate analogue, methotrexate, and the fluorophore, carboxyfluorescein, were covalently coupled to *Staphylococcus aureus* protein A. Cells of the murine k haplotype were preincubated with an anti-H-2K^k monoclonal antibody. Excess antibody was removed and then cells were incubated with liposomes. The number of cell-bound liposomes was determined by fluorimetry. The drug effect was assayed by the methotrexate-mediated inhibition of radiolabeled deoxyuridine uptake. The drug effect was more important in the case of the 800 Å vesicles than for the larger liposomes, despite the fact that the quantity of drug bound to cells was several-fold greater for large liposomes than for small ones. Since fusion is excluded by the non-proportionality of drug binding and drug effect, the predominant manner of liposome entry seems to be endocytosis. At least for these in vitro studies, the endocytosis by target cells of small liposomes seems to be more efficient than that of large liposomes.

Introduction

Liposomes are able to carry relatively large amounts of encapsulated materials such as drugs in their aqueous spaces (for recent reviews, see Refs. 1–3), and they have been used in some areas of chemotherapy and cellular biology. Liposomes have been used as passive or slow release capsules [4] or, in other models, specific ligands have been attached to liposome surfaces with the aim of promoting drug effects on given cell populations [5–7].

Different techniques are available for the preparation of suitable liposomes [8,9] and as large unilamellar liposomes have a greater encapsulating efficiency than small liposomes, the former type has been used preferentially by some groups. In addition, certain macromolecules which cannot be encapsulated in small liposomes, such as DNA, are easily encapsulated in large liposomes [10]. With smaller molecules such as drugs, large and small liposomes can be employed.

Given the higher encapsulation efficiency of large liposomes, we wondered whether they are also more efficient for specific delivery of their contents to target cells in vitro. In order to answer this question, we used the fluorophore, carboxyfluorescein, and the folic acid analogue, methotrexate, encapsulated in large or small unilamellar vesicles [5,6] to which we covalently coupled protein A from *Staphylococcus aureus* [7]. Protein A has an affinity for several classes of

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Abbreviations: SUV, small unilamellar liposome; DMPC, dimyristoylphosphatidylcholine; DPPS, dipalmitoylphosphatidylserine; DPPE, dipalmitoylphosphatidylethanolamine; SPDP, *N*-hydroxysuccinimyl 3-(2-pyridyldithio)propionate; DPPE-DTP, DPPE modified by SPDP; REV, reverse-phase evaporation vesicles; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; RPMI, Roswell Park Memorial Institute.

mouse antibodies and will specifically bind to cells preincubated with antibodies directed at determinants present on the cell surface [11]. This technique has enabled us to compare large and small liposomes bound to identical determinants on various cell types.

In this paper, we show that small liposomes are superior for in vitro delivery of these drugs, presumably because cells are better able to ingest small liposomes by endocytosis.

Materials and Methods

Preparation of liposomes. Small unilamellar liposomes (SUV) of lipid composition of 54 mol% dimyristoylphosphatidylcholine (DMPC) (Sigma); 10 mol% dipalmitoylphosphatidylserine (DPPS) (available from Avanti Polar Lipids, Birmingham, AL, U.S.A.); 35 mol% cholesterol (Applied Sciences Laboratories) and 1 mol% dipalmitoylphosphatidylethanolamine (DPPE) (Calbiochem) modified with *N*-hydroxysuccinimidyl 3-(2-pyridyldithio)propionate (SPDP) (Pharmacia) (DPPE-DTP) were made by probe sonication at 40°C for 30 min [7,12,13]. The total lipid concentration was 40.4 μmol. Liposomes contained 20 mM methotrexate (Division of Cancer Treatment, National Cancer Institute) and 40 mM recrystallized and Sephadex LH-20-purified [14] carboxyfluorescein (Eastman) in 100 mM $NaHCO_3$ (pH 7.5). Large unilamellar vesicles of the same lipid composition were made according to a modification of the reverse phase evaporation technique of Szoka and Papahadjopoulos [8]. Lipid was dissolved in 1 ml chloroform/2 ml diisopropyl ether (Merck). The aqueous (1 ml containing 40 mM carboxyfluorescein and 20 mM methotrexate) and organic phases were placed in separate gas-tight glass syringes fitted with Luer Locks. The syringes were joined by a metal three-way stopcock. The aqueous phase was injected into the organic phase, then the mixture was passed rapidly 10–20-times from syringe to syringe [15]. The lipid-aqueous emulsion was transferred to a 15 ml Rotovap tube and warmed to 45°C, and the organic phase was removed at 360 mmHg pressure. Reverse-phase evaporation vesicles (REV) were then selected on the basis of size by filtration through 0.4 or 0.4 and 0.2 μm Unipore polycarbonate filters (Bio-Rad) [16].

Dosage of vesicle phospholipids. The concentration of phospholipid in liposomes was made by colorimetric determination with ammonium ferrothiocyanate [17]. The calibration curve was made with different concentrations of DMPC (5 μg to 80 μg/ml). Aliquots of liposomes were dissolved in 2 ml chloroform, mixed for 1 min with 2 ml ammonium ferrothiocyanate and centrifuged at $700 \times g$. The absorption of the organic phase was then measured at 488 nm. Carboxyfluorescein, which absorbs at the same wavelength, partitions into the aqueous (upper) layer and does not interfere.

Coupling reaction between liposomes and S. aureus protein A. SUV and REV were incubated with protein A (Pharmacia), iodinated by the chloramine T method [18] and modified by 10 mol SPDP/mol protein A (final protein concentration, 200 μg/ml) [7,15,19]. The final concentration of lipids was 5 mM. The coupling reaction was performed in buffer A (145 mM NaCl/10 mM Hepes (pH 8)) for 20 h.

Size distribution of liposomes. The size of liposomes was measured by using a 1×34 cm Sephacryl S-1000 superfine column (Pharmacia) [20]. The column was presaturated with 20 μmol lipid (54 mol% DMPC, 10 mol% DPPS, 35 mol% cholesterol, 1 mol% DPPE-DTP) in 1 ml buffer A (pH 7.45) and with 1 ml fetal calf serum to avoid nonspecific adsorption during chromatography. REV, containing carboxyfluorescein, filtered through 0.4 μm Unipore filters, and free carboxyfluorescein were used to calibrate the column (void volume (V_0), 11.5 ml; total volume (V_t), 29.5 ml; flow rate, 7.4 ml/h). Liposomes were monitored by fluorescence and by measurement of ^{125}I-radiolabeled protein A covalently coupled to liposomes.

Cell cultures. Spleen cells from CBA/J (CSEAL-CNRS, Orléans, France) mice were treated with 0.83% NH_4Cl in order to lyse red blood cells. $30 \cdot 10^6$ cells were incubated at 37°C in 7% CO_2 in tissue culture flasks with 20 ml RPMI 1640 medium (Gibco) supplemented with 5% fetal calf serum (Gibco), 2 mM glutamine, $5 \cdot 10^{-5}$ M 2-mercaptoethanol, penicillin and streptomycin. For mitogen-induced proliferation, cells were incubated with 2 mg/l concanavalin A (Miles) as previously described [19,21]. L cells (gift

of F. Lemonnier, Centre d'Immunologie) and RDM4 tumor cells were cultured in RPMI medium supplemented with 7% fetal calf serum.

Measurement of cell-associated carboxyfluorescein. After 40 h incubation, stimulated lymphoblasts were resuspended in supplemented medium without mitogen at $8 \cdot 10^6$ cells/ml. RDM4 cells were resuspended at the same concentration. L cells were detached with 1.5 mM EDTA for 5 min at 37°C, washed and resuspended as the other cells. 50 μl aliquots were placed in conical wells of 96-well microtiter plates (Greiner). Cells were incubated with protein A-bearing liposomes after preincubation of the cells with H100-30/33, an IgG2b, kappa, anti-H-2K^k monoclonal antibody [22] at 40 μg/ml for 30 min at 4°C and one wash with 100 μl buffer A (pH 7.45) followed by centrifugation. Cells were incubated with liposomes at 37°C for 2–3 h and washed four times in buffer A, then centrifuged. The fluorescence of cell-associated carboxyfluorescein was measured as described previously [12,13]. For fluorescence microscopy, cells were placed on glass slides and sealed under coverslips and examined with a Zeiss universal microscope.

Methotrexate-mediated inhibition of d[^{3}H]Urd incorporation. To flat-bottomed wells of microtiter plates containing 10^5 cells was added anti-H-2K^k antibody (H100-30/33) at a final concentration of 20 μg/ml. After 30 min at 4°C, the cells were washed once in 100 μl RPMI medium followed by centrifugation and then resuspended in supplemented medium. Dilutions of free methotrexate or sterilized (on 0.45 μm Millipore filters) protein A-bearing liposomes containing 20 mM methotrexate were added to the cultures. After 3 h incubation at 37°C, 0.5 μCi radiolabeled deoxyuridine (d[^{3}H]Urd) was added to the cultures. After an additional 16 h at 37°C, the cells were collected and the concentration of incorporated d[^{3}H]Urd in DNA was measured [5,6,12,19,21].

Results

Characterization of vesicles

Lipid of the same composition was sonicated to obtain small unilamellar vesicles (SUV) or treated by reverse-phase evaporation to obtain large unilamellar vesicles (REV). Liposomes were made of 54 mol% DMPC, 10 mol% DPPS, 35 mol% cholesterol and 1 mol% DPPE-DTP. This change from our standard lipid composition of 64 mol% dipalmitoylphosphatidylcholine (DPPC), 35 mol% cholesterol and 1 mol% DPPE-DTP [7,19,21] was made necessary by our inability to make consistently good REV in the absence of phosphatidylserine. The REV can pass through polycarbonate membranes to size the vesicles. Fig. 1 shows the elution profile on a Sephacryl S-1000 superfine column of three different preparations of liposomes: SUV, REV filtered through 0.4 μm membranes and sequential passage through 0.4 μm and 0.2 μm membranes, visualized by the dequenched fluorescence after lysis of liposomes by the detergent Triton X-100. The sonicated SUV made with 10 mol% DPPS had a larger diameter

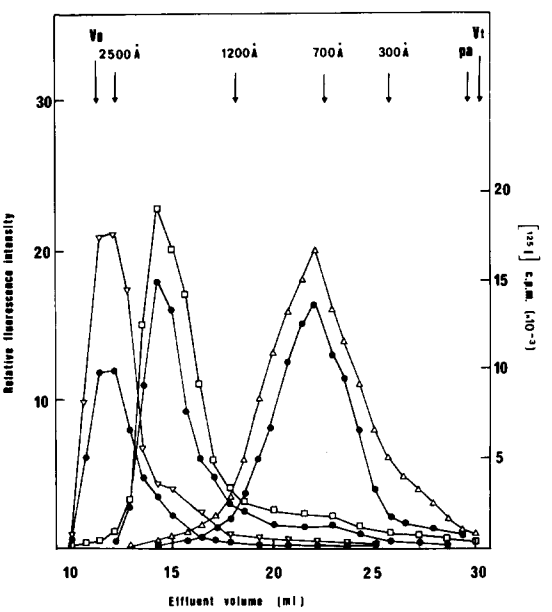

Fig. 1. Chromatography of liposomes on a Sephacryl S-1000 column. Aliquots of liposomes preparations were passed over a Sephacryl S-1000 column. Sample size was 0.7 ml in buffer A (pH 7.45). Aliquots of the effluents were measured for fluorescence after addition of 0.2% Triton X-100 on an Aminco SPF spectrofluorimeter (excitation 488 nm, emission 520 nm), and counted in a gamma counter. The column was calibrated according to Nozaki et al. [20]. (●) ^{125}I protein A coupled to liposomes. (△) SUV; (▽) REV after filtration through 0.4 μm Unipore membranes; (□) REV after sequential passage through 0.4 μm and 0.2 μm Unipore filters. Column markers: PA = protein A; V_t = total volume; V_0 = void volume.

(800 Å as compared to 500–600 Å) than sonicated liposomes lacking this charged phospholipid (data not shown). REV filtered through 0.4 μm and 0.2 μm membranes had an homogeneous size of about 2000 Å in diameter and nominally 4000 Å (void volume) for those passed through 0.4 μm membranes. When the liposomes were coupled to radiolabeled protein A, the position of radioactivity was the same as for carboxyfluorescein encapsulated in liposomes.

The homogeneous distribution of each liposome preparation excludes the aggregation or fusion of liposomes as a consequence of the coupling reaction. When incubated with 10% fetal calf serum we have observed that these charged vesicles leak from 0.3 to 4% of their contents during dialysis overnight, as measured by fluorescence. This leakage is not increased as compared with non-charged liposomes made by sonication. Following this dialysis the liposomes contents remain stably encapsulated for many weeks after their sterilization by filtration through 0.45 μm Millipore filters.

The encapsulated volume of such vesicles, calculated from fluorescence dequenching after lysis of liposomes and phospholipid dosage (see Methods) was 1.5 ± 0.2 l/mol; 6.2 ± 0.5 l/mol, and 9.4 ± 0.2 l/mol of vesicle phospholipids for the SUV, 2000 Å and 4000 Å diameter REV, respectively. These values are in good agreement with published values for a population of predominantly unilamellar liposomes [8,23].

Protein coupling to small and large liposomes

As seen in Fig. 2, the number of protein A molecules bound per liposome was proportional to the size of liposomes. The calculation was based on the percentage of protein A which became bound to liposomes after filtration on a Sepharose 4B column [13]. Accepting published figures [10] of $4.5 \cdot 10^{11}$ 4000 Å vesicles made with cholesterol per μmol of phospholipid, we have obtained 11–13, 33–37 and 96–100 protein A molecules per 800 Å, 2000 Å and 4000 Å diameter liposome, respectively, corresponding to about 560, 290 and 200 molecules per μm^2 of liposome surface. We have observed that small liposomes made without the negatively charged phospholipid DPPS bound about twice as many protein A molecules as those containing DPPS. Theoretically, one would expect the same percentage of protein A fixation on SUV and REV made with the same lipid concentration because the lipid area is identical. In the incubation, performed here the expected number would be 80 and 320 protein A molecules per 2000 Å and 4000 Å diameter liposome.

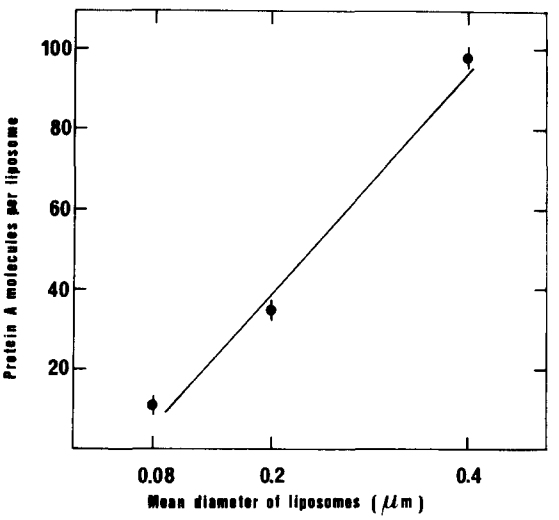

Fig. 2. Number of protein A molecules bound per 800 Å, 2000 Å and 4000 Å sized liposome. The calculation of the number of protein A molecules bound per liposome preparation was made after filtration of liposomes on a Sepharose 4B column to determine the percentage of liposome-associated protein A [13]. Results are mean ± S.D. of three experiments.

Liposome binding studies

In order to evaluate the fixation of liposomes to cells, we have incubated RDM4 tumor cells, L cells and stimulated lymphoblasts with an IgG2b anti-H-2K^k monoclonal antibody prior to incubation with protein A-bearing liposomes. As seen in Table I, the binding of carboxyfluorescein encapsulated in large liposomes was greater than in small liposomes. The values for cell-associated fluorescence were approximately 4.7-, 2- and 2.7-times greater with 2000 Å diameter liposomes as compared to SUV on RDM4 cells, L cells and lymphoblasts, respectively. With 4000 Å diameter liposomes, the values were not increased as compared with 2000 Å REV. Nonspecific binding of liposomes was not observed when incubated with cells without antibody or with an irrelevant anti-

TABLE I

SPECIFIC BINDING OF PROTEIN A-BEARING SUV OR REV TO CELLS PREINCUBATED WITH ANTI-H-2K^k MONOCLONAL ANTIBODY H100-30/33

Results are shown as mean ± S.E. of four experiments for RDM4 cells and L cells and two experiments for lymphoblasts. $4 \cdot 10^5$ cells were incubated with protein A-bearing liposomes containing a total of 212 pmol of carboxyfluorescein. Cells were preincubated with 40 mg/l final of antibody and washed before addition of protein A-bearing liposomes. In the case of 'free protein', before addition of liposomes cells were incubated for 5 minutes with 80 mg/l final protein A. The concentration of liposomes was 82.5 μM for SUV; 23.7 μM for 2000 Å diameter REV, and 15.4 μM for 4000 Å diameter REV expressed as total lipid.

	Antibody	Free protein A	cell-associated carboxyfluorescein (pmol)		
			SUV of mean diameter 800 Å	REV of mean diameter 2000 Å	REV of mean diameter 4000 Å
RDM4 cells	+	−	8.0 ± 1.5	37.6 ± 2.0	35.0 ± 1.5
	+	+	0.2 ± 0.03	0.3 ± 0.1	0.3 ± 1.5
	−	−	0.8 ± 0.2	1.0 ± 0.5	1.2 ± 0.05
L cells	+	−	16.0 ± 2.0	32.0 ± 1.5	26.7 ± 1.2
	+	+	0.5 ± 0.05	0.4 ± 0.1	0.5 ± 0.05
	−	−	0.8 ± 0.4	0.8 ± 0.6	1.3 ± 0.5
Lymphoblasts	+	−	10.5 ± 1.4	28.9 ± 0.7	20.4 ± 2.0
	+	+	0.3 ± 0.05	0.3 ± 0.07	0.5 ± 0.02
	−	−	0.7 ± 0.3	0.8 ± 0.2	1.1 ± 0.3

body (data not shown). The binding of protein A-bearing liposomes was totally inhibited by free protein A in solution at the time of incubation. Considering the mean diameter of a cell to be about 10 μm and assuming cells and liposomes to be spheres, it is theoretically possible to bind to cells $1.56 \cdot 10^4$, $2.5 \cdot 10^3$ and 623 liposomes of 800 Å, 2000 Å and 4000 Å diameter, respectively. The volumes of 2000 Å and 4000 Å liposomes are, respectively, 15.6- and 125-times greater than 800 Å vesicles. Thus the total carboxyfluorescein in 2000 Å liposomes bound per cell would be expected to be 2.5-times greater, and for 4000 Å liposomes 5-times greater, than SUV. Our results show that while the fixation of 2000 Å liposomes was increased as expected, there was no additional increase for 4000 Å REV.

Drug transfer from liposomes to cells

The quantity of liposome contents which became cell-bound was greater for REV than SUV, but the quantity of liposome contents transferred into the cells was greater with SUV than REV (Fig. 3). The inhibition of incorporation of radiolabeled deoxyuridine by the methotrexate encapsulated in liposomes was more important with SUV than REV for all cells tested. Only the more phagocytic L cells were appreciably affected when incubated with REV, but this drug effect was less important than that obtained with SUV. In control experiments (not shown) we have confirmed that methotrexate encapsulated by the reverse-phase evaporation technique was not destroyed by exposure to organic solvent during liposome formation. The data have also been confirmed for antibodies directed at other cell determinants (not shown).

Fluorescence microscopy observations

We have previously suggested that lysosomotropic compounds such as ammonium chloride can inhibit the drug transfer from SUV to the cell via an acidic compartment such as lysosomes [12,21]. This point, together with the differential drug effects seen when liposomes are bound to different determinants, has suggested that liposomes were endocytosed together with the target cell surface molecule. In this way, the absence of effect or

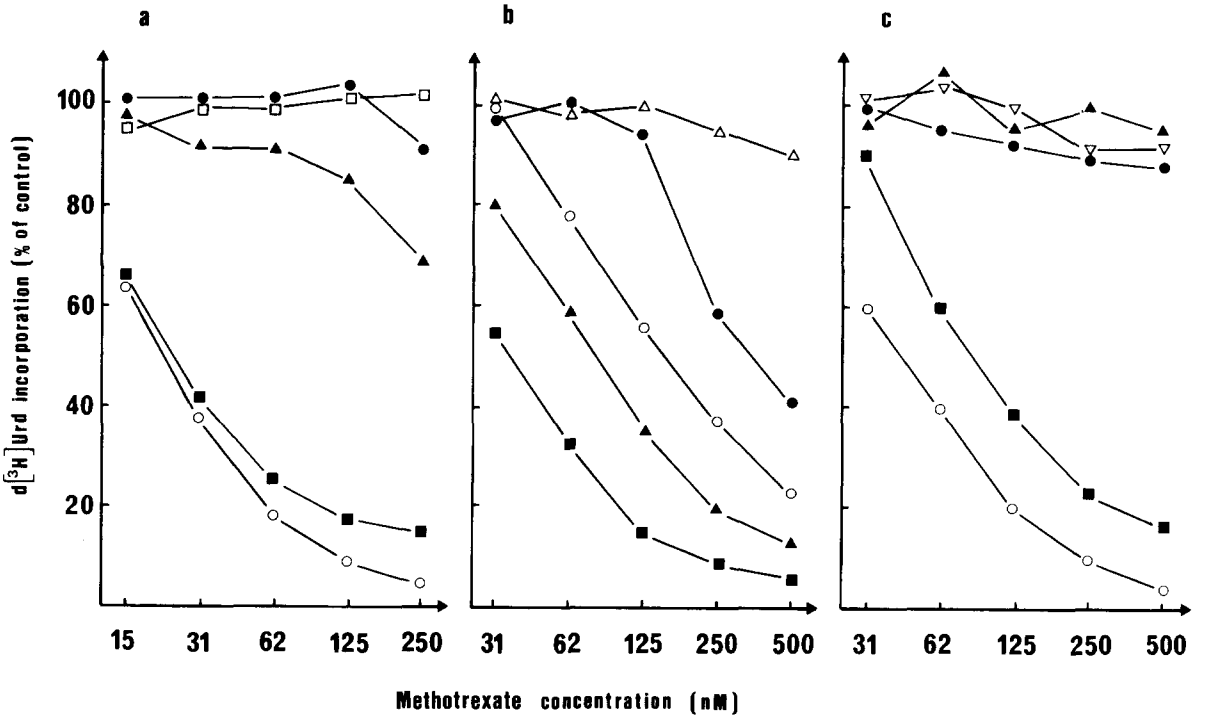

Fig. 3. MTX-mediated inhibition of d[^{3}H]Urd incorporation. Cells (10^5) were preincubated with H100-30/33 monoclonal antibody before addition of SUV or REV coupled to protein A. ○, free methotrexate; ■, methotrexate in protein A-bearing SUV; ▲, methotrexate in protein A-bearing REV of 2000 Å in diameter; ●, methotrexate in protein A-bearing REV of 4000 Å in diameter; □, protein A-bearing SUV without methotrexate; △, methotrexate in protein A-bearing REV of 2000 Å in diameter without preincubation of the cells with the monoclonal antibody or with preincubation with an irrelevant monoclonal antibody; ▽, methotrexate in protein A-bearing SUV without preincubation of the cells with H100-30/33 antibody or with preincubation with an irrelevant antibody. The effect of methotrexate encapsulated in liposomes was totally inhibited by preincubation of the cells with free protein A (50 mg/l) for 5 min before addition of liposomes (data not shown). a. RDM4 tumor cell line: control d[^{3}H]Urd incorporation: 152000 ± 2000 cpm. b. L cells: control d[^{3}H]Urd incorporation: 260000 ± 10000 cpm. c. Lymphoblasts (T cells origin): control d[^{3}H]Urd incorporation: 90000 ± 3000 cpm. Each point plotted corresponds to the average of four experiments for RDM4 cells and L cells and two experiments for lymphoblasts.

reduced effects of the drug encapsulated in specifically bound REV suggested the non-endocytosis of these large liposomes by the cells. In order to confirm this hypothesis, we have observed cells by fluorescence microscopy after a 2–3 h incubation of the cells with specifically bound liposomes at 37°C. L cells and RDM4 cells showed diffuse cytoplasmic fluorescence when incubated with SUV, indicating internalization of liposomal contents. In contrast, when cells were incubated with REV no such fluorescence was seen. We observe, rather, patches on the cell surface, indicating clustering of large liposomes. We have observed that when cells were first incubated with ammonium chloride before incubation with SUV, that the cytoplasmic fluorescence is much reduced and that most carboxyfluorescein seems to be confined to the perinuclear region, presumably in lysosomes (not shown).

Discussion

Liposomes can now be made in a variety of sizes and bearing a number of ligands on their surfaces. If the purpose of the ligand is to mediate the binding of liposomes to a specific target tissue, so that the liposome contents can be introduced into that tissue, then it is apparent that the liposomes must first be of a size capable of having access to the target cells, and secondly, be able to

be taken up by the cells. However, the complexity of behavior of liposomes in vivo makes studies of the mechanisms of liposome uptake difficult to interpret.

We have previously shown that, in vitro, small unilamellar liposomes (mean diameter 600 Å), composed of DPPC, cholesterol and DPPE-DTP covalently coupled to antibodies or protein A, are able to transfer their contents into cells to an extent which depends on both the cell and the target molecule. Thus, the cell surface molecule H-2K^k is an excellent target on murine T cell blasts, RDM4 tumor cells and L cells, but not on B cells, even though all these cells express large amounts of this molecule, bind comparable numbers of liposomes, and B cells are sensitive to methotrexate in liposomes bound to other cell-surface determinants [12,19,21].

In the present paper, we have compared the ability of liposomes of various sizes to deliver methotrexate via this same determinant. The liposome-protein coupling reaction originally described for liposomes made without phosphatidylserine [7] was less effective in the presence of this charged phospholipid. Similarly, the coupling of protein to large liposomes was less efficient than to small liposomes. We were unable to couple more protein to the large liposomes even when they were made with increased DPPE-DTP (2%) and incubated with more protein (data not shown). The mechanism for the reduced fixation of protein on the surface of REV remains to be explained. Despite this reduced protein fixation, the quantity of carboxyfluorescein in liposomes which bound to cells was greater for 2000 Å liposomes than for small (800 Å) liposomes to the extent predicted by the increase in encapsulated volume. The binding of 4000 Å liposomes was not increased as compared to 2000 Å liposomes, perhaps because of the reduced diffusion of these liposomes.

Although there was greater binding to cells of carboxyfluorescein and methotrexate in large liposomes, cells were more sensitive to drug delivered from small than from large liposomes. These results are incompatible with models implying fusion of liposomes with the cell membrane, for which, lipid composition being identical, the drug effect should have been proportional to the amount of drug which became cell bound. The inverse relationship between size and drug delivery, our previously published results on the inhibition of the effect of liposome-encapsulated methotrexate by NH_4Cl [12,21], and the results from fluorescence microscopy presented here all support endocytosis as the principal or exclusive mechanism of liposome entry.

Even cells not normally thought of as phagocytically active must have the capacity to take up relatively large molecules such as low-density lipoprotein (250 Å). These particles are taken up in coated pits, which have a diameter of about 1500 Å [24]. We did not observe differences between DPPC/cholesterol/DPPE-DTP (600 Å) and DMPC/DPPS/cholesterol/DPPE-DTP (800 Å) liposomes with respect to drug delivery. However, 2000 Å liposomes were practically incapable of mediating drug delivery into RDM4 or T cell blasts and the drug effect of large liposomes on L cells was less than that predicted for the quantity of drug which became cell associated. We do not know if the failure of 2000 Å liposome entry reflects the 1500 Å diameter of coated pits: we observed patching of large liposomes on the cell surface. This aggregation, which was not seen for liposomes in solution, could either be the cause or the consequence of the failure of these liposomes to enter the cell.

The other possibility is that the 'zippering' mechanism proposed for endocytosis of antibody-opsonized particles [25] was defective with large liposomes as a consequence of the reduced number of protein A molecules bound per μm^2 of liposome surface as compared to SUV. This seems unlikely, as a density as low as of 25 antibody molecules bound per μm^2 was shown to be sufficient for internalization of red blood cells by phagocytic cells [26] and the density of protein A on even large liposomes was several-fold in excess of this number. Furthermore, this hypothesis would be limited to RDM4 cells and to T blasts, as L cells were capable of internalizing large liposomes, albeit to a lesser extent than small liposomes.

In these studies, protein A-bearing liposomes have been shown to become cell-associated via interaction with antibody bound to a specific surface molecule (H-2K^k). The internalization of molecules by L cells shows the same selectivity as that shown for T and B cells, that is, not all cell

surface determinants mediated drug delivery. Thus, in these studies the H-2K molecule was a good target for liposome entry, but fibronectin was a poor target, even though substantial numbers of liposomes could be bound to this determinant by anti-fibronectin antibody (data not shown).

It is of considerable interest that large liposomes bound to the H-2K molecule were internalized by L cells, at least to some extent. Are endocytic vacuoles larger on L cells? Can a given cell surface molecule be associated with multiple routes of entry into the cell? The association of liposomes and techniques such as electron microscopy will be necessary to respond to these questions.

Acknowledgements

We thank Jacques Barbet and Alemseged Truneh for constructive comments, Geneviève Rougon for her help with fluorescence microscopy, and Geneviève Victorero for technical assistance. This work was supported by Institutional grants from the Institut National de la Santé et de la Recherche Médicale and the Centre National de la Recherche Scientifique, by a contract under the program 'Institut National de la Santé et de la Recherche Médicale-National Cancer Institute Recherches cliniques en Cancérologie', and by a grant from the Association pour le Développement de la Recherche contre le Cancer.

References

1 Gregoriadis, G. and Allison, A.C. (eds.) (1980) Liposomes in Biological Systems, John Wiley, Chichester
2 Knight, C.G. (ed.) (1981) Liposomes: From Physical Structure to Therapeutic Applications, Elsevier/North-Holland Biomedical Press, Amsterdam
3 Gregoriadis, G., Senior, J. and Trouet, A. (eds.) (1982) Targeting of Drugs. N.A.T.O. Advanced Study Institute Series A, Vol. 47, Plenum Press, New York
4 Juliano, R.L. and Stamp, D. (1978) Biochem. Pharmacol. 27, 21–27
5 Leserman, L.D., Weinstein, J.N., Blumenthal, R., Sharrow, S.O. and Terry, W.D. (1979) J. Immunol. 122, 585–591
6 Leserman, L.D., Weinstein, J.N., Blumenthal, R. and Terry, W.D. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 4089–4093
7 Leserman, L.D., Barbet, J., Kourilsky, F. and Weinstein, J.N. (1980) Nature 288, 602–604
8 Szoka, F. and Papahadjopoulos, D. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 4194–4198
9 Szoka, F. and Papahadjopoulos, D. (1981) in Liposomes: From Physical Structure to Therapeutic Applications (Knight, C.G., ed.), pp. 51–82, Elsevier/ North-Holland Biomedical Press, Amsterdam
10 Fraley, R., Subramani, S., Berg, P. and Papahadjopoulos, D. (1980) J. Biol. Chem. 255, 10431–10435
11 Goding, J.W. (1978) J. Immunol. Methods 20, 241–253
12 Leserman, L.D., Machy, P. and Barbet, J. (1981) Nature 293, 226–228
13 Barbet, J., Machy, P. and Leserman, L.D. (1981) J. Supramol. Struct. Cell. Biochem. 16, 243–258
14 Ralston, E., Hjelmeland, L.M., Klausner, R.D., Weinstein, J.N. and Blumenthal, R. (1981) Biochim. Biophys. Acta 649, 133–137
15 Machy, P. and Leserman, L.D. (1983) in Liposome Technology (Gregoriadis, G., ed.), CRC Press, Cleveland, in the press
16 Olson, F., Hunt, C.A., Szoka, F.C., Vail, W.J. and Papahadjopoulos, D. (1979) Biochim. Biophys. Acta 557, 9–23
17 Stewart, J.C.M. (1980) Anal. Biochem. 104, 10–14
18 Hunter, W.M. and Greenwood, F.C. (1962) Nature 194, 495–497
19 Machy, P., Pierres, M., Barbet, J. and Leserman, L.D. (1982) J. Immunol. 129, 2098–2102
20 Nozaki, Y., Lasic, D.D., Tanford, C. and Reynolds, J.A. (1982) Science 217, 366–367
21 Machy, P., Barbet, J. and Leserman, L.D. (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 4148–4152
22 Lemke, H., Hämmerling, G.J. and Hämmerling, U. (1979) Immunol. Rev. 47, 175–206
23 Martin, F.J., Hubbell, W.L. and Papahadjopoulos, D. (1981) Biochemistry 20, 4229–4238
24 Pastan, I.H. and Willingham,. M.C. (1981) Science 214, 504–509
25 Griffin, F.M., Jr., Griffin, J.A., Leider, J.E. and Silverstein, S.C. (1975) J. Exp. Med. 142, 1263–1282
26 Ehlenberger, A.C. and Nussenzweig, V. (1977) J. Exp. Med. 145, 357–371