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Mechanisms of delivery of liposome-encapsulated cytosine arabinoside to CV-1 cells in vitro. Fluorescence-microscopic and cytotoxicity studies

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Fluorescence microscopy and assays of the cytotoxicity of liposome-encapsulated cytosine arabinoside (araC) have been used to examine the interactions of CV-1 cells with pH-sensitive liposomes, combining phosphatidylethanolamine (PE) with oleic acid or with double-chain protonatable amphiphiles, and with pH-insensitive liposomes combining phosphatidylcholine (PC) and phosphatidylglycerol (PG). Fluorescence-microscopic observations indicate that double-chain protonatable amphiphiles remain tightly associated with pH-sensitive liposomes during incubations with CV-1 cell monolayers, and that cellular uptake of liposomes is strongly promoted by transferrin coupled to the liposome surface. Liposome-encapsulated araC showed much greater cytotoxicity toward CV-1 cells than did the free drug at equivalent concentrations under the same conditions. The cytotoxicity of encapsulated araC was strongly enhanced by liposome-conjugated transferrin and was maximal using pH-sensitive liposomes combining PE with the double-chain protonatable amphiphile *N*-(*N'*-oleoyl-2-aminopalmitoyl)serine. However, the drug was also markedly more cytotoxic when encapsulated in other types of transferrin-conjugated liposomes, including pH-insensitive PC/PG/cholesterol liposomes, than in the free form. The cytotoxicity of liposome-encapsulated araC is significantly attenuated by the nucleoside transport inhibitor nitrobenzothioinosine, and fluorescence microscopy using calcein-containing liposomes provides no evidence for efficient fusion between cellular membranes and any of the types of liposomes examined here. Based on these observations, we suggest that the major mechanism for cytoplasmic delivery of liposome-encapsulated araC is the carrier-mediated transport of drug that has been released from liposomes into the endosomal and/or the lysosomal compartments.

Introduction

A variety of different types of liposomes has been developed and evaluated for their potential to deliver encapsulated molecules to mammalian cells in vitro and in vivo [1–4]. One attractive strategy to promote both efficient and selective delivery of liposome-encapsulated materials to mammalian cells is to utilize liposomes that are targeted to specific cell surface determinants by means of liposome-conjugated proteins or other molecules [2,5] and that are stable under the conditions of the extracellular medium but are readily destabilized following uptake by the 'target' cells.

pH-sensitive liposomes, which are stable at neutral or higher pH but which rapidly destabilize at the weakly acidic pH values found in endocytic vesicles, represent one potentially very useful class of differentially stable liposomes of the type described above [2,6–13]. Liposomes of this type, combining PE with single-chain protonatable amphiphiles such as oleic acid or *N*-palmitoyl homocysteine, have been utilized to deliver water-soluble fluorescent markers [9,10] and cytotoxic compounds [11,12] to mammalian cells in vitro. The efficiency and selectivity of cellular delivery of encapsulated material from such liposomes is significantly enhanced by conjugation of the liposomes to proteins that

Abbreviations: 12-CA-OAP, *N*-[12-(((7'-diethylaminocoumarin-3-yl)carbonyl)methylamino)octadecanoyl]-2-aminopalmitic acid; 12-CA-stearic acid, 12-(((7'-diethylaminocoumarin-3-yl)carbonyl)methylamino)octadecanoic acid; araC, cytosine arabinoside; BSA, bovine serum albumin (fraction V); D-MEM, Dulbecco's modified Eagle's medium; DOSG, 1,2-dioleoyl-3-succinylglycerol; EDTA, ethylenediaminetetraacetic acid trisodium salt: EMCS, ε-maleimidocaproic acid *N*-hydroxysuccinimide ester; FBS, fetal bovine serum; HBSS, Hanks' balanced salt solution; HBSS-BSA, Hanks' balanced salt solution plus 1 mg/ml BSA; NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; NBMPR, nitrobenzothioinosine; OA, oleic acid; OAP, *N*-oleoyl-2-aminopalmitic acid; OAP-serine, *N*-(*N'*-oleoyl-2-aminopalmitoyl)serine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; SATA, succinimidyl-*S*-acetylthioacetate; Tes, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid sodium salt.

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bind to cell surface receptors [11,12]. Endocytosis of such liposomes is postulated to promote the subsequent delivery of liposome-encapsulated compounds to their intracellular sites of action, by mechanisms that have been suggested to include liposome-cell membrane fusion or liposome-induced destabilization of endocytic vesicles [10,11]. However, the precise mechanisms by which soluble molecules are delivered from pH-sensitive liposomes to their ultimate intracellular sites of action largely remain to be clarified and may vary depending on the nature of the encapsulated substance.

In this study, we have combined fluorescence microscopy and assays of the cytotoxicity of liposome-encapsulated cytosine arabinoside (araC) to examine the interactions of several types of liposomes with CV-1 (monkey kidney fibroblast) cells in vitro. We have investigated the interactions of these cells both with pH-sensitive liposomes, combining PE with either oleic acid or double-chain protonatable amphiphiles that are less readily exchangeable between membranes [13], and with pH-insensitive phosphatidylcholine/ phosphatidylglycerol liposomes. Our results indicate that liposome-encapsulated araC can be delivered to CV-1 cells with markedly greater efficiency than is the free drug, particularly when the liposomes are conjugated to transferrin to promote liposome-cell binding and endocytosis. Several lines of evidence, obtained from both fluorescence microscopy and araC cytotoxicity assays, suggest that the liposome-mediated delivery of araC may proceed largely by release of encapsulated material from liposomes into the endosomal and/or the lysosomal compartment, followed by carrier-mediated transport of the released araC to the cytoplasm.

Materials and Methods

Materials

Egg phosphatidylcholine (PC) and transphosphatidylated phosphatidylethanolamine (PE) were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). Cholesterol and oleic acid were obtained from Nu-Chek Prep. (Elysian, MN). PG was prepared by enzymatic transphosphatidylation of egg yolk PC with phospholipase D as described previously [14]. SATA-PE, prepared by reacting PE (10 mM in dry $CHCl_3$ containing 1% triethylamine) with a 20% molar excess of SATA for 3 h at room temperature, was purified by silicic acid column chromatography. *N*-Oleoyl-2-aminopalmitic acid (OAP), *N*-(*N'*-oleoyl-2-aminopalmitoyl)-serine (OAP-serine), and 1,2-dioleoyl-3-succinylglycerol (DOSG) were synthesized and purified as described previously [13]. 12-(((7'-Diethylaminocoumarin-3-yl)carbonyl)methylamino)octadecanoic acid (12-CA-stearic acid), and its amide conjugate with 2-aminopalmitic acid (*N*-[12-(((7'-diethylaminocoumarin-3-yl)carbonyl)methylamino)octadecanoyl]-2-aminopalmitic acid (12-CA-OAP), were synthesized and purified using methods described elsewhere [13,15].

ε-Maleimidocaproic acid *N*-hydroxysuccinimide ester (EMCS), apotransferrin, succinimidyl-*S*-acetylthioacetate (SATA), bovine serum albumin (fraction V), calcein, carboxyfluorescein, cytosine arabinoside (araC, free base), chloroquine, nitrobenzothioinosine (NBMPR) and probenecid were obtained from Sigma Chemical Co. (St. Louis, MO). Apotransferrin was converted to holotransferrin as described previously [16]. Calcein and carboxyfluorescein were purified by chromatography on Sephadex LH-20 as described by Ralston et al. [17].

CV-1 cells were obtained from the laboratory of Dr. N. Sonenberg (McGill University). All reagents for cell culture were obtained from Gibco Laboratories (Grand Island, NY). [^{3}H]Thymidine (specific activity 35 Ci/mmol) and Cytoscint were obtained from ICN Biomedicals (Irvine, CA), and [^{3}H]araC was obtained from Amersham (Oakville, Ont.).

Methods

Preparation of liposomes. Reverse-phase evaporation vesicles were prepared and filtered through 0.1 μm Nucleopore membrane filters according to the procedure of Wilschut et al. [18]. All solutions used in vesicle preparation contained 10 mM Tes and 0.1 mM EDTA (pH 7.8) and were adjusted with NaCl to an osmolarity (determined by direct osmometry) equivalent to that of 150 mM NaCl, 10 mM Tes, 0.1 mM EDTA.

Preparation of protein-coupled liposomes. Transferrin was covalently coupled to liposomes using a procedure adapted from that described by Derksen and Scherphof [19,20]. Liposomes, incorporating 4 mol% of SATA-PE, were treated with hydroxylamine (50 mM) in buffer for 10 min at room temperature to expose PE-linked free thiol groups. Maleimido-modified transferrin was prepared by reacting transferrin (16 mg/ml in phosphate-buffered saline) with a 2-fold molar excess of EMCS for 90 minutes at room temperature. Modified and native transferrin were shown to be equivalent in their ability to compete with ^{125}I-labeled transferrin for binding to sheep reticulocyte transferrin receptors, using the assay of Adam et al. [21]. The EMCS-coupled protein was separated from unreacted EMCS on a column of Sephadex G-50 and was immediately added to the deprotected liposomes (normally at 200 μg/ml transferrin and 0.5 mM lipid) for overnight coupling (20 h) at 4 °C. *N*-Ethylmaleimide (1 mM) and β-mercaptoethanol (2.5 mM) were then added sequentially, with a 15-min incubation period after each addition. Protein-coupled liposomes were separated from uncoupled protein by Dextran gradient flotation as described by Heath et al. [22].

Fluorescence microscopy. CV-1 cells, grown on glass cover slips to roughly 50% confluency, were washed

once and incubated for 1 h at 37°C in serum-free medium, then washed with HBSS and cooled to 4°C. The washed cell monolayers were incubated for 20 min at 4°C with one of the following preparations of lipid vesicles (at 50 μM lipid): bath-sonicated vesicles prepared containing either 12-CA-stearic acid/oleic acid/PE or 12-CA-OAP/OAP/PE in 5:20:75 molar proportions, or 75:20:5 PC/PG/SATA-PE large unilamellar vesicles loaded with 45 mM carboxyfluorescein (freed of extraliposomal dye by gel filtration on Sephadex G-75). After the liposome-cell incubation, the medium was replaced with liposome-free HBSS, and the cells were observed by fluorescence microscopy, either immediately or after further incubations at 35°C as indicated. Incubations of cells with calcein-loaded liposomes were carried out using exactly the same conditions as those for the araC cytotoxicity measurements described below.

A Zeiss epifluorescence microscope (model IM-35), 40X Planapo lens, and Kodak T-Max film (ASA 400) were used for photography.

Cytotoxicity assays. Liposomes were prepared containing either 200 mM araC, or (for 'empty' liposomes) an isoosmotic concentration of sucrose, together with 10 mM carboxyfluorescein and/or [^{3}H]araC (7.5 nCi per μl encapsulated volume), which allowed quantitation of the encapsulation and subsequent retention of liposomal contents and, for some experiments, observation of the cellular uptake of the liposomes by fluorescence microscopy. CV-1 cells were plated in 24-well tissue culture plates and grown to 30–40% confluency in D-MEM containing 10% fetal bovine serum. The cells were washed twice in D-MEM without serum, then incubated for 1 h and finally washed once more in the same medium. Various amounts of liposomes (0–250 μM lipid) were added to the cells and incubated for 3 h in SFM at 37°C. The liposome-containing medium was then removed, and the cells were washed with serum-free medium and further cultured for 20–24 h in serum-containing medium supplemented with [^{3}H]thymidine (2 μCi/well). The incorporation of [^{3}H]thymidine was determined by following the procedure of Tandan et al. [23], with the modification that the solubilized cells in 0.2 M NaOH were dispensed directly into Cytoscint, without prior neutralization, for liquid scintillation counting. Cytotoxicity was quantified as a reduction in [^{3}H]thymidine incorporation into liposome-treated as compared to control cells.

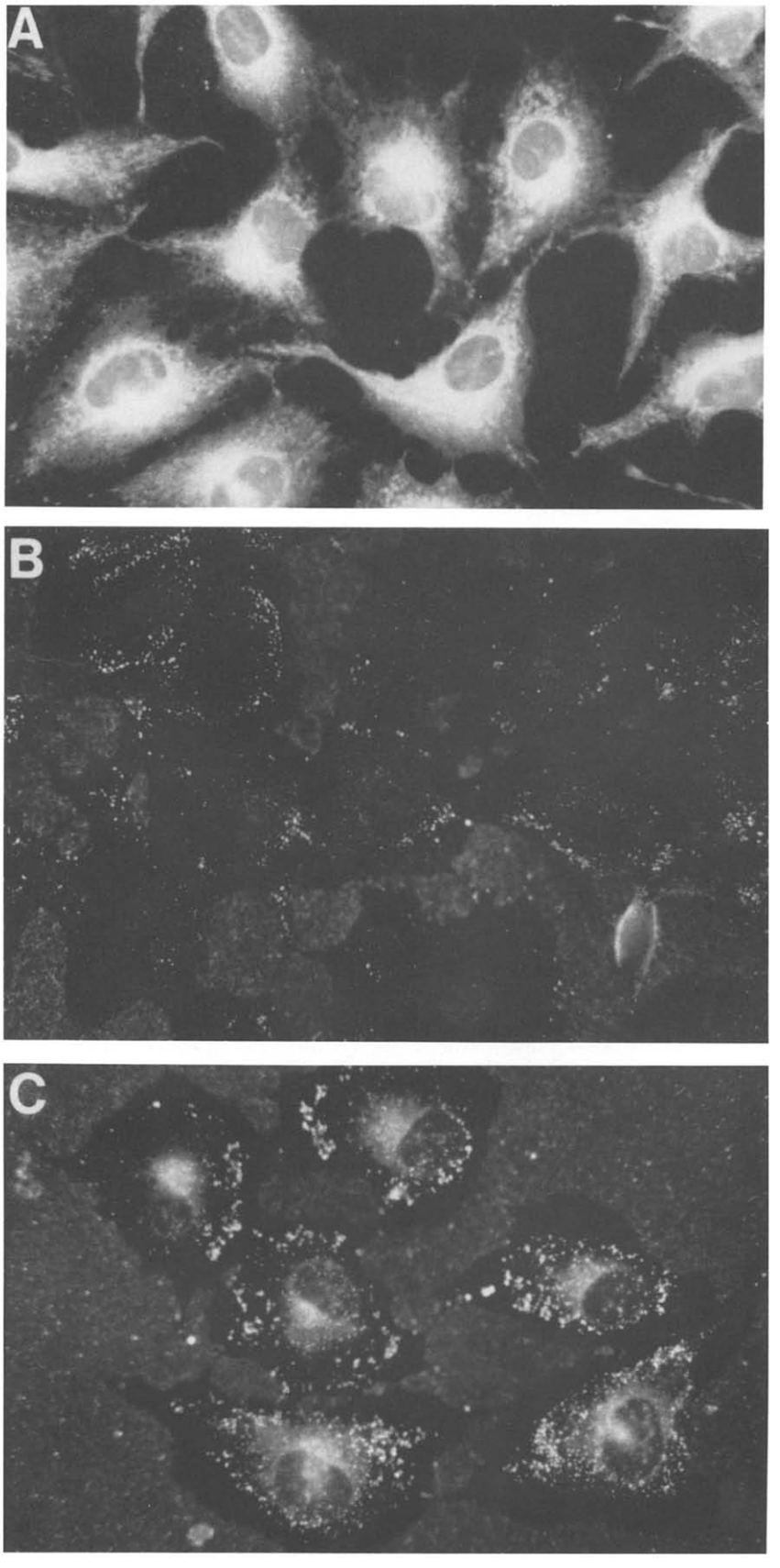
Cytotoxicity assays similar to those described above were also carried out in which cells were treated with free araC, in the presence of absence of 'empty' (sucrose-loaded) liposomes. For experiments in which cells were treated with NBMPR, this agent (100 nM) was added 1 h before liposome addition and was maintained in the incubation medium until 30 min after the liposomes were removed.

Results

The interactions of pH-sensitive liposomes with CV-1 cells were first characterized by fluorescence microscopy, using liposomes containing either fluorescent-labeled lipids or encapsulated water soluble fluorescent dyes, to examine the intracellular distribution of different liposomal components following liposome-cell incubation.

Previous studies have shown that single-chain amphiphiles, such as free fatty acids, exchange rapidly between artificial lipid membranes, while double-chain protonatable amphiphiles such as *N*-oleoylpalmitic acid (OAP) do not [13]. As shown in Fig. 1, a similar difference in behavior is reflected in the interactions of lipid vesicles containing the two different types of protonatable amphiphiles with CV-1 cells. For the experiment shown, small unilamellar vesicles, prepared from 75:20:5 mixtures of PE, protonatable amphiphile (OA or OAP), and the corresponding fluorescent-labeled protonatable amphiphile (12-CA-stearic acid or 12-CA-OAP), were incubated with CV-1 cells for 20 min at 4°C, and the pattern of cell-associated fluorescence was subsequently observed either at 4°C, a temperature at which endocytosis is inhibited [24], or after warming the cells to 25°C to allow endocytosis to resume. As shown in Fig. 1A, rapid and extensive staining of intracellular membranes, apparently by exchange of fluorescent fatty acid molecules, is observed when cells are incubated at 4°C with liposomes containing 12-CA-stearic acid. A parallel double-labeling experiment, using vesicles containing both 12-NBD-methylaminostearic acid and a nonexchangeable coumarin-labeled phosphatidylcholine [25], showed extensive labeling of intracellular membranes with the NBD-labeled fatty acid, but only cell surface labeling with the latter component, at 4°C (not shown). By contrast, cells incubated with vesicles containing the double-chain protonatable amphiphile 12-CA-OAP showed only surface-associated fluorescence at 4°C (Fig. 1B), which gradually became internalized during subsequent incubation of the cells at 25°C (Fig. 1C). In the latter experiment, the intracellular distribution of fluorescence appeared punctate after short periods (e.g., 15 min) at 25°C and gradually appeared to delocalize into various intracellular membrane structures at longer times, as shown in Fig. 1C.

To assess the efficacy of liposome-coupled transferrin in promoting specific liposome–cell interactions, we examined the delivery of carboxyfluorescein to CV-1 cells from transferrin-conjugated and unconjugated PC/PG/SATA-PE (70:25:5) liposomes. Carboxyfluorescein is a weak acid that can readily enter the cell cytoplasm when liposomes are endocytosed and their contents become exposed to the reduced pH values of the cellular endosomal/lysosomal compartments [1,4]. In Figs. 2A and 2B are shown micrographs of CV-1


A
B
C

cells that were incubated for 30 min at 4°C with transferrin-conjugated PC/PG liposomes containing 45 mM carboxyfluorescein, then washed and warmed to 37°C for 1 h to allow the endocytic uptake of cell surface-bound vesicles. Strong diffuse cytoplasmic fluorescence can be observed when the liposome-cell incubations are carried out in the absence of free transferrin (Fig. 2A), while the presence of free transferrin (1 mg/ml) strongly reduces the binding (and subsequent endocytic uptake) of the carboxyfluorescein-containing vesicles (Fig. 2B). Cells incubated under identical conditions with carboxyfluorescein-containing liposomes that are not conjugated to transferrin also show only a low level of cytoplasmic fluorescence, as shown in Fig. 2C. Thus, transferrin conjugated to liposomes by the procedure described in Materials and Methods was able to promote the endocytic uptake of liposomes, apparently by promoting a specific interaction with the transferrin receptor [26,27].

To examine more quantitatively the delivery of a soluble bioactive species from different types of liposomes to CV-1 cells, we evaluated the cytotoxicity toward these cells of cytosine arabinoside (araC) encapsulated in various preparations of transferrin-conjugated and unconjugated liposomes. Cell monolayers were incubated with varying amounts of liposomes containing 200 mM (intraliposomal concentration) araC, and delivery of araC was assessed by measuring the decrease in [^{3}H]thymidine incorporation by liposome-treated cells as compared to untreated cells, as described in Materials and Methods. These experiments employed pH-sensitive liposomes, combining PE with either single- or double-chain amphiphiles, as well as pH-insensitive liposomes combining PC with PG. In preliminary experiments, it was found that retention of aqueous contents by vesicles during the protein-conjugation step in particular was markedly enhanced by the inclusion of cholesterol (25 mol%) and, for PE-containing liposomes, a small proportion of PC (15 mol%) in the vesicle bilayer. All of the liposome preparations described below were therefore of the compositions PE/(protonatable amphiphile)/ PC/ cholesterol/ SATA-PE (42 : 14 : 15 : 25 : 4 molar proportions) or PC/ PG/cholesterol/SATA-PE (61 : 15 : 25 : 4) but will be designated below for brevity simply by their first two components. Vesicles of the former compositions, loaded with the soluble fluorescent dye ANTS and the quencher DPX, were found using the vesicle-destabilization assay of Ellens et al. [28] to show release of contents at pH values below 6.6, 5.8, 6.0 or 6.2, respectively, when the protonatable component was oleic acid, OAP, OAP-serine or DOSG.

The cytotoxicity of araC toward CV-1 cells can be strongly potentiated by encapsulation in lipid vesicles. This point is illustrated by the data shown in Fig. 3A, which present the results of experiments in which cells were treated with araC encapsulated in transferrin-conjugated liposomes combining PE with the double-chain protonatable amphiphile OAP-serine. A 50% inhibition of [^{3}H]thymidine incorporation is observed when the cells are treated with these araC-containing liposomes at an overall araC concentration of approx. 30 μM. Free araC produces much smaller cytotoxic effects at equivalent concentrations; 50% inhibition of [^{3}H]thymidine incorporation into CV-1 cells is observed only at a free araC concentration of approx. 2.5 mM (Fig. 3C). The toxicity of araC encapsulated in these liposomes is significantly reduced if the liposomes are not transferrin-conjugated (Fig. 3A, open circles), although the drug is substantially more cytotoxic when encapsulated even in unconjugated liposomes than in the free form.

Given the markedly greater cytotoxicity of liposome-encapsulated than of free araC, it appears unlikely that the cytotoxic effects of the encapsulated drug can be ascribed to cellular uptake of free drug that has leaked from liposomes. To confirm this point, cytotoxicity assays were also carried out for cells that were incubated with free araC in the presence of empty liposomes, at overall araC and lipid concentrations equivalent to those shown in Fig. 3A. As shown in Figs. 3B (open circles) and 3C, the toxicity of free araC is somewhat greater in the presence than in the absence of empty liposomes, which by themselves show minimal cytotoxicity up to at least 250 μM lipid (Fig. 3B, solid circles). In spite of this potentiating effect of empty liposomes, free araC remains markedly less cytotoxic than the liposome-encapsulated drug even when 'empty' liposomes are present. In other experiments, we observed that at least 85% of the soluble contents of the liposomes were retained (as judged by their co-elution with liposomes on gel filtration) during a 3-h incubation of the liposomes with CV-1 cells under the conditions of the cytotoxicity assays. Taken together, these results indicate clearly that the bulk of the cytotoxic effects of

Fig. 1. Fluorescence micrographs of CV-1 cells incubated with pH-sensitive liposomes (50 μM) containing fluorescent single or double-chain amphiphiles. (A) Cells were incubated with PE/oleic acid/12-CA-stearic acid (75 : 20 : 5, molar proportions) liposomes for 20 min at 4°C. (B) Cells were incubated with PE/OAP/12-CA-OAP (75 : 20 : 5) liposomes for 20 min at 4°C. (C) Cells were treated as in (B), then excess liposomes were removed, and the cells were further incubated for 90 min at 25°C. Details of the liposome-cell incubations and fluorescence-microscopic conditions are described in Materials and Methods; the micrographs shown were obtained using a 2-s exposure for panel A and 16-s exposures for panels B and C. The faint background fluorescence surrounding individual cells in (B) and (C) is a consequence of adsorption of fluorescent-labeled liposomes to the cover slips. Cells treated with large unilamellar vesicles of the above compositions rather than sonicated liposomes gave results similar to those shown, but the level of background fluorescence was higher in this case.

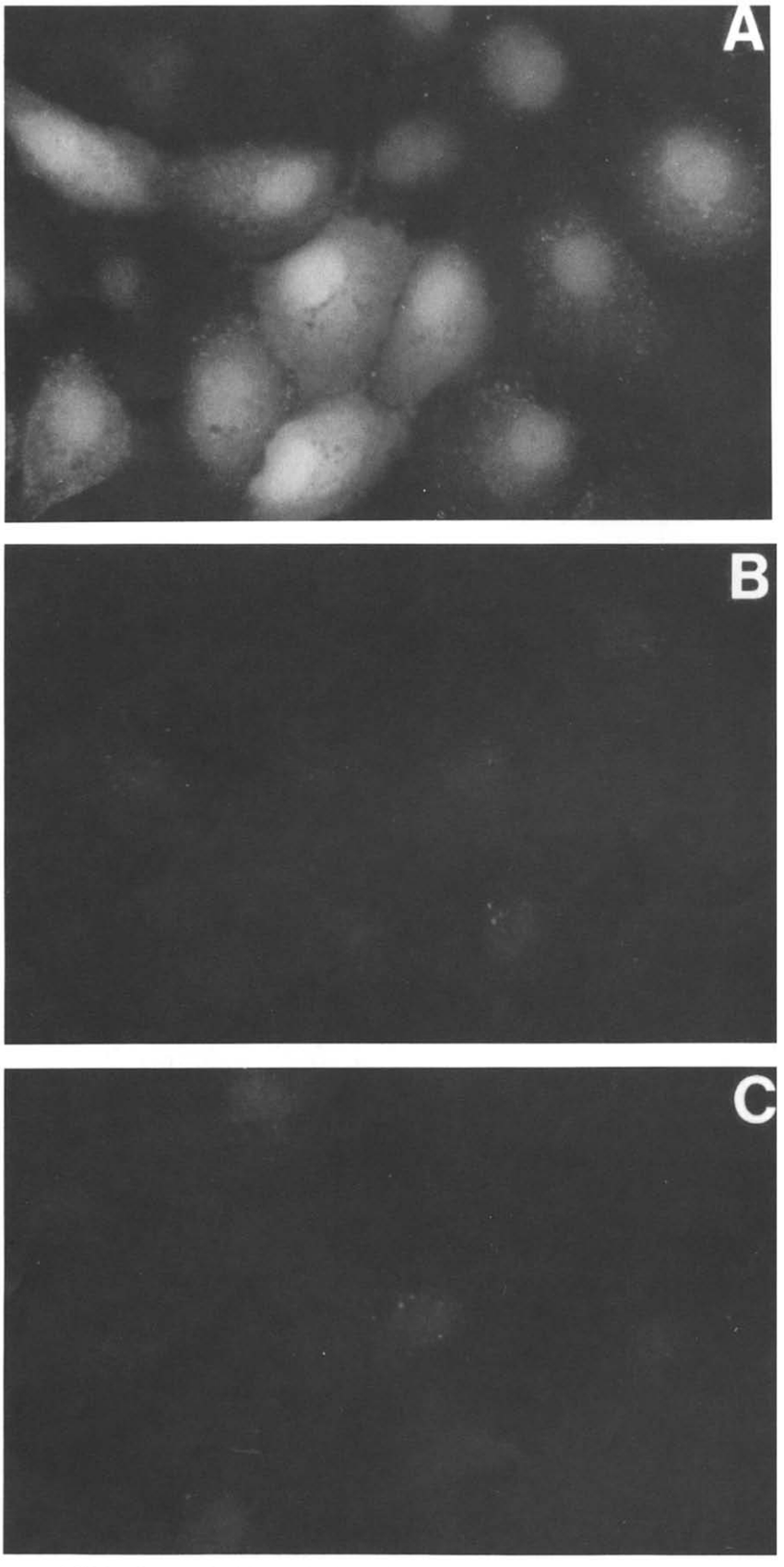


Fig. 2. Fluorescence microscopy of CV-1 cells treated with transferrin-conjugated or unmodified PC/PG liposomes loaded with carboxyfluorescein (45 mM). Cells were incubated as described in Materials and Methods with: (A) transferrin-conjugated liposomes (at lipid and transferrin concentrations of 50 μM and 11 μg/ml, respectively) in the absence of free transferrin; (B) transferrin-conjugated liposomes in the presence of 1 mg/ml free transferrin; or (C) unmodified liposomes in the absence of transferrin. Details of sample incubations and fluorescence-microscopic conditions are given in Materials and Methods.

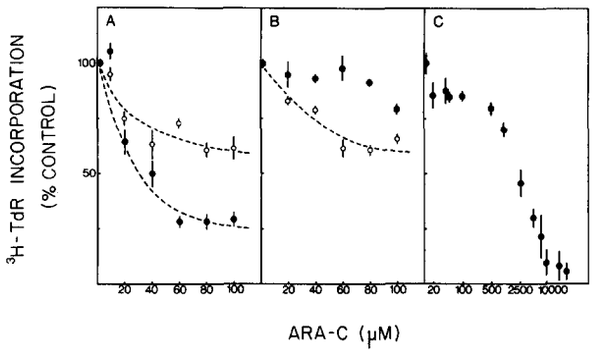


Fig. 3. Cytotoxicity toward CV-1 cells of liposome-encapsulated and free araC. The data shown represent the results of duplicate experiments, using four independently treated samples of cells for each araC concentration; the points shown indicate the mean ± 1 S.D. (A) Cells were incubated with transferrin-conjugated (filled circles) or transferrin-free (open circles) PE/OAP-serine/PC/cholesterol/SATA-PE (42:14:15:25:4) liposomes containing araC (200 mM internal concentration) at the indicated overall concentrations of araC. (B) Open circles, cells were incubated with free araC at the indicated concentrations together with transferrin-conjugated 'empty' liposomes, at a ratio of lipid to araC comparable to that for the samples in panel (A) (3.3 μmol lipid/μmol araC). Closed circles, cells were incubated with empty liposomes alone, at the same lipid concentrations as shown for the empty liposome/free araC incubations. (C) Cells were incubated with free araC in the indicated concentrations in the absence of liposomes. Note the logarithmic x-axis scale, and the higher range of araC concentrations covered, in this panel.

liposome-encapsulated araC cannot be attributed to the action of drug that has been released from lipid vesicles and subsequently taken up by the cells.

In experiments similar to those discussed above, several other types of pH-sensitive liposomes, as well as pH-insensitive PC/PG/cholesterol liposomes, were examined for their abilities to deliver liposome-encapsulated araC to CV-1 cells. As shown in Fig. 4, the cytotoxicity of araC is also significantly enhanced by encapsulation in pH-sensitive liposomes combining PE with either oleic acid or the double-chain protonatable amphiphiles OAP or DOSG. AraC encapsulated in pH-insensitive PC/PG-containing liposomes also shows significantly greater toxicity than does the free drug (Fig. 4D). Control experiments of the type shown in Fig. 3B (not shown) indicated that the cytotoxicities of these preparations of liposome-encapsulated araC could in no case be attributed either to potentiation of the toxicity of free araC by 'empty' liposomes or to toxicity of the liposomes themselves over the range of lipid and drug concentrations covered in Fig. 4.

The above observation that the efficiency of delivery of araC from pH-insensitive PG/PG/cholesterol liposomes to CV-1 cells is comparable to that observed using certain types of pH-sensitive liposomes raises some questions concerning the mechanism by which the liposome-encapsulated material is delivered to the cell cytoplasm. Several types of experiments were carried out to pursue this question further. First, to test whether membrane nucleoside transporters are implicated in the mechanism of araC delivery, measurements of the cytotoxicity of free and liposome-encapsulated araC were carried out in the presence of the nucleoside transport inhibitor NBMPR [29,30]. In preliminary experiments, NBMPR was found to increase substantially the survival of CV-1 cells treated with even high concentrations of free araC. Cell survival after treatment with 8 mM araC, for example, was enhanced from 18% to 50% when the cells were simultaneously exposed to 100 nM NBMPR. As shown in Fig. 4 (open circles), 100 nM NBMPR also significantly reduced the cytotoxicity of araC encapsulated in both pH-sensitive and pH-insensitive liposomes, although in no case was the cytotoxic effect of araC completely abolished by NBMPR at this concentration. Substantially higher levels of NBMPR could not be used in these experiments, nor could NBMPR be maintained in the medium for much longer times after removal of the liposomes from the cells, as this agent itself caused significant toxicity under such conditions.

In a second series of experiments, we used fluorescence microscopy to examine the intracellular disposition of the contents of pH-sensitive liposomes containing the membrane-impermeant fluorescent marker

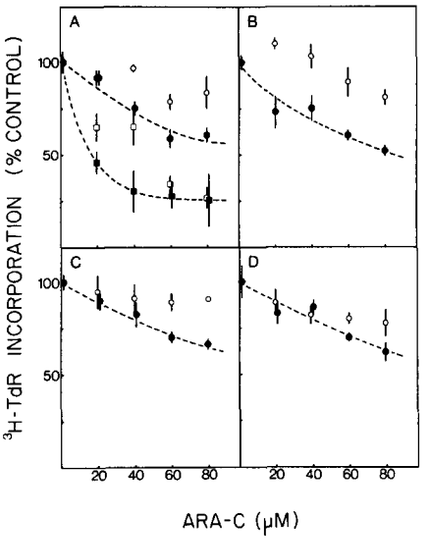


Fig. 4. Cytotoxicity of araC encapsulated in different types of transferrin-conjugated liposomes in the absence (filled circles) and presence (open circles) of 100 nM NBMPR. Panel (A): Squares, PE/OAP-serine/PC/cholesterol/SATA-PE (42:14:15:25:4) liposomes; circles, PE/oleic acid/PC/cholesterol/SATA-PE (42:14:15:25:4) liposomes. Panel (B): PE/DOSG/PC/cholesterol/SATA-PE (42:14:15:25:4) liposomes. Panel (C): PE/OAP/PC/cholesterol/SATA-PE (42:14:15:25:4) liposomes. Panel (D): PC/PG/cholesterol/SATA-PE (56:15:25:4) liposomes. Ratios of araC/lipid and of transferrin/lipid for these liposome preparations fell in the ranges 0.37–0.50 μmol/μmol and 2.4–3.4 nmol/μmol, respectively. Data points shown represent values (mean ± S.D.) from individual experiments, using four independently treated samples for each araC concentration.

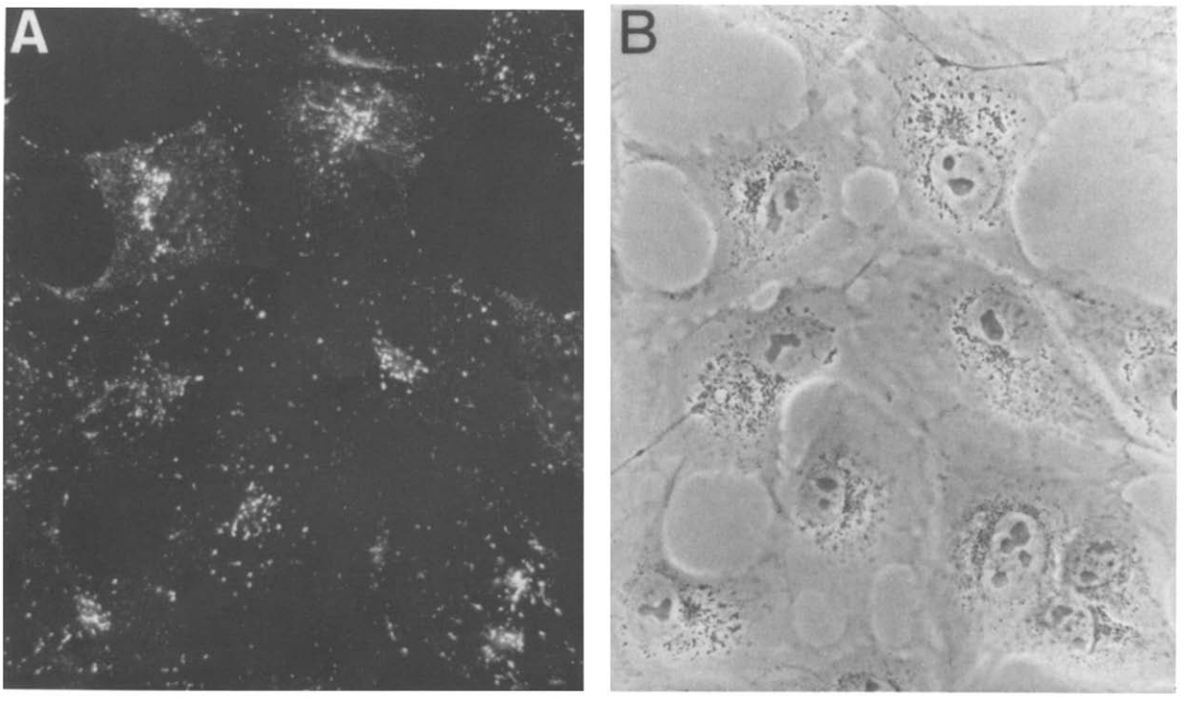


Fig. 5. Microscopy of CV-1 cells incubated with transferrin-conjugated, calcein-containing PE/OAP-serine/PC/cholesterol/SATA-PE (42:14:15:25:4) liposomes. (A) Fluorescence image; (B) phase-contrast image of the same field. Details of liposome incubation and cell-liposome incubation were as described in Materials and Methods.

calcein. Cells were incubated with calcein-containing liposomes, using exactly the same conditions as for the cytotoxicity assays described above, then the cells were washed free of liposomes and examined by fluorescence microscopy. As illustrated in Fig. 5, cells treated in this manner showed strong punctate intracellular fluorescence but no discernible evidence of diffuse cytoplasmic fluorescence. To ensure that possible cytoplasmic delivery of calcein in these experiments was not masked by subsequent resequestration of the dye into intracellular vesicles, parallel incubations were carried out in the presence of probenecid, which has been reported to inhibit such sequestration of organic anions in murine macrophages [31,32]. However, cells treated with probenecid (5 mM) likewise showed no discernible cytoplasmic fluorescence after incubation with the calcein-containing liposomes (not shown). The experiment shown in Fig. 5 was carried out using transferrin-conjugated liposomes combining PE and OAP-serine; similar results were obtained using vesicles of the other lipid compositions described above (not shown).

Discussion

In this study, we have characterized the interactions of transferrin-conjugated liposomes of several compositions with CV-1 cells, using both fluorescence microscopy to assess the intracellular dispositions of the liposomal components and cytotoxicity assays to monitor cytoplasmic delivery of liposome-encapsulated araC. Of particular interest in this study was the potential to use pH-sensitive liposomes, combining PE with either free fatty acids or novel double-chain protonatable amphiphiles [13], to deliver encapsulated molecules to animal cells. pH-sensitive liposomes have attracted considerable interest for their possible ability to fuse with cellular membranes, thereby transferring directly their internal components to the cell cytoplasm, when exposed to the weakly acidic pH of the endosomal compartment [6–13]. Our fluorescence-microscopic observations indicate that pH-sensitive liposomes prepared using protonatable double-chain amphiphiles retain their titratable components during incubation with CV-1 cells, and that the lipid and the encapsulated components of these liposomes are gradually internalized by the cells at 25°C (or at 37°C, not shown) but not at 4°C. The efficiency of this uptake process is markedly enhanced by conjugation of the liposomes to transferrin, whose receptor is a constitutively internalized membrane protein [33]. While transferrin-conjugated liposomes appear to be internalized in a transferrin receptor-dependent (transferrin-inhibitable) manner, further study will be required to determine to what extent these liposomes (and their individual components) follow the normal intracellular trajectory of the transferrin receptor (which normally includes recycling of undegraded receptors [33] after internalization).

The results of cytotoxicity assays indicate that the

delivery of araC to CV-1 cells can be markedly enhanced by encapsulation of the drug in transferrin-conjugated liposomes. Maximal cytotoxicity was observed in this study using araC-containing liposomes incorporating the protonatable double-chain amphiphile OAP-serine; araC encapsulated in such liposomes produced 50% cytotoxicity at a concentration of approx. 30 μM, while approx. 2.5 mM free drug was required to produce a comparable cytotoxic effect. AraC encapsulated in liposomes combining PE with other types of protonatable amphiphiles, or in PC/PG/cholesterol liposomes, also showed significantly greater cytotoxicity in this system than did the free drug. Drug-free ('empty') liposomes of these compositions showed negligible cytotoxicity up to at least 250 μM lipid. However, the presence of empty liposomes was observed in some cases to potentiate significantly, albeit modestly, the cytotoxicity of free araC. While the mechanism of this effect remains unclear, in no case was the extent of this potentiation great enough that a substantial fraction of the cytotoxicity of liposomal araC could be attributed to the action of free drug that had been released from liposomes.

Several previous studies have demonstrated enhanced cytotoxic effects of araC toward proliferating cells, in both in vivo and in vitro experimental systems, when the drug is incorporated into various types of liposomes [11,34–38]. The mechanisms for this enhanced activity of liposomal araC appear to vary depending on the experimental system and the type of liposome employed. The enhanced in vivo cytotoxicity toward model tumor systems of araC encapsulated in untargeted, non-pH-sensitive liposomes [34–38] has been attributed to the combined effects of protection of the drug from deactivation by serum deoxycytidine deaminase [39] and accumulation of the drug in tissues of the reticuloendothelial system in proximity to some types of tumors [38]. Connor and Huang [11] have shown that araC can be delivered to L929 cells in vitro from cell surface-targeted pH-sensitive liposomes, in a largely receptor-specific manner, with efficiencies significantly greater than those observed for the free drug. Bankert et al. [38] have reported that the toxicity of araC toward a murine hybrid B-cell line in vitro is enhanced more than 40-fold, compared to the toxicity of free araC, when the drug is encapsulated in non-pH-sensitive liposomes targeted to an immunoglobulin expressed on the cell surface. While these latter examples suggest that liposome-encapsulated araC can be taken up more efficiently by 'target' cells than is the free drug, the precise mechanism of liposome-mediated delivery of araC in such cases remains uncertain.

While pH-sensitive liposomes such as those examined in this study show efficient fusion with liposomes of like composition at weakly acidic pH [6,7,13], we find little evidence that such liposomes fuse efficiently with either the plasma or the endosomal/lysosomal membranes of CV-1 cells in tissue culture. The membrane-impermeant marker calcein is readily delivered to internal vesicular structures of CV-1 cells from transferrin-conjugated liposomes combining PE with various pH-sensitive amphiphiles, including both oleic acid and nonexchangeable double-chain amphiphiles, but no unambiguous indication is obtained in any case for cytoplasmic delivery of a significant fraction of the dye. This result contrasts with the report of Connor and Huang [10] that calcein can be efficiently delivered from PE/*N*-palmitoylhomocysteine immunoliposomes to the cytoplasm of L929 cells. Our findings more closely resemble those of Straubinger et al. [9], who observed very inefficient delivery of calcein to the cytoplasm of CV-1 cells from pH-sensitive PE/oleic acid liposomes unless the cells were exposed to glycerol shock. However, these authors reported detectable, albeit very limited, cytoplasmic delivery of calcein from such vesicles even without glycerol treatment, while we observed no unambiguous indication for cytoplasmic delivery of calcein from any of the various types of liposomes examined in this paper. It is nonetheless clear that the liposomes examined in this study can deliver araC in cytotoxic doses to CV-1 cells under conditions where direct fusion of cellular and liposomal membranes is undetectable, even for those liposome preparations that give the most efficient cellular delivery of the encapsulated drug.

The results just noted suggest that araC is delivered in cytotoxic concentrations from liposomes to the cytoplasm of CV-1 cells either by the fusion of an undetectably small fraction of the liposomes with cellular membranes, or by alternative mechanisms that do not involve direct fusion of liposomal and cellular membranes. Among plausible mechanisms of the latter type we must consider extracellular release of liposomal araC, which is subsequently taken up into CV-1 cells in the free form [30], and the release of araC from endocytosed liposomes to the endosomal or lysosomal lumen, from whence the drug reaches the cytoplasm by facilitated transmembrane diffusion. The former mechanism, while it may operate to some degree in this system, cannot account for the markedly higher cytotoxicity of liposome-encapsulated than of free araC toward CV-1 cells. Free araC is found to be much less cytotoxic when added to cells in the free form, in the presence or absence of empty liposomes, than are preparations of the liposome-encapsulated drug. Moreover, only a small fraction ($< 15\%$) of liposomal contents was found to be released from different types of liposomes during the standard 3-h incubation with cell monolayers. The observation that araC is substantially more cytotoxic when encapsulated in transferrin-conjugated rather than unconjugated liposomes is also consistent with the conclusion that cytoplasmic delivery of liposome-encapsulated

araC takes place mainly by cellular uptake of drug-containing liposomes rather than by leakage and subsequent cellular uptake of liposomal contents.

We suggest that carrier-mediated diffusion of araC from the lumen of endosomes and/or lysosomes may represent the primary mechanism of delivery of this agent from transferrin-conjugated liposomes to the cytoplasm of CV-1 cells in our experiments. This conclusion rests primarily on three lines of evidence: the lack of indications for substantial fusion-mediated delivery of liposomal contents to CV-1 cells; our inability to account for the cytotoxic effects of liposomal araC on the basis of araC leakage and cellular uptake of the free drug; and the observation that the cytotoxicity of liposomal araC can be significantly attenuated by nitrobenzothioinosine (NBMPR), an inhibitor of nucleoside transport [29]. While to our knowledge no evidence has been reported to date to assess the possible existence of nucleoside carriers in the endosomal compartment, a nucleoside transport system capable of transporting araC has recently been demonstrated in human fibroblast lysosomes [40]. Such carriers could mediate the transfer to the cytoplasm of araC that has been released from endocytosed liposomes either by low pH-induced vesicle destabilization or through the action of lysosomal hydrolases. This mechanism may account for a still larger fraction of the liposome-mediated delivery of araC to the cell cytoplasm than the partial block of liposomal araC cytotoxicity by NBMPR would suggest. Given the observation that NBMPR at nontoxic concentrations blocks only partially the cytotoxicity even of unencapsulated araC, the previous finding that the lysosomal nucleoside carrier system is comparatively insensitive to NBMPR [40], and the possibility that released or liposome-encapsulated araC may remain in the endosomal or lysosomal compartment for some time after both liposomes and NBMPR are removed from the cells, it is possible that the above mechanism alone could account for virtually all of the liposome-mediated uptake of araC into CV-1 cells.

It is not possible at this stage to assess whether the bulk of the liposome-mediated delivery of araC to the cytoplasm of CV-1 cells occurs from the lysosomal or the endosomal compartment. The observation that araC is appreciably cytotoxic even when encapsulated in non-pH-sensitive PC/PG/cholesterol liposomes suggests that delivery from the lysosomal compartment (where such liposomes could be destabilized by lipid hydrolysis) is at least possible. Further study will be required to assess quantitatively how the liposomal lipid and protein composition affect the relative efficiency of delivery of encapsulated nucleoside analogues and other agents (including those potentially labile in the lysosomes) from the endosomal vs. the lysosomal compartments to their final site(s) of action in the cell.

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