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Research Article

Efficiency of Cytoplasmic Delivery by pH-Sensitive Liposomes to Cells in Culture

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The intracellular processing of pH-sensitive liposomes composed of cholesterylhemisuccinate (CHEMS) and dioleoylphosphatidylethanolamine (DOPE) by eukaryotic cell lines has been compared to non-pH-sensitive liposomes made of CHEMS and dioleoylphosphatidylcholine (DOPC). The pH-sensitive liposomes can deliver encapsulated fluorescent molecules [calcein, fluoresceinated dextran, fluoresceinated polypeptide, and diphtheria toxin A chain (DTA)] into the cytoplasm. Cytoplasmic delivery can be blocked in the presence of ammonium chloride or EDTA, indicating that the process requires a low-pH environment and the presence of divalent cations. Inhibition of cellular protein synthesis by DTA delivery from the pH-sensitive liposome is orders of magnitude greater than from the non-pH-sensitive liposome composition. The delivery of DTA into the cytoplasm by pH-sensitive liposomes is at least 0.01% of cell-associated liposomal DTA. There is no significant difference in the degradation rate of bovine serum albumin (BSA) or the rate of acidification of pH-sensitive dye, 8-hydroxy-1,3,6-pyrene-trisulfonate (HPTS), when delivered to cells in pH-sensitive and non-pH-sensitive liposomes. Thus the efficiency of cytoplasmic delivery is less than 10% of the cell-associated liposome contents, which is the smallest difference that can be detected by these two assays. Based upon the various assays used to measure liposome content disposition in the cell, we conclude that the efficiency of cytoplasmic delivery by the CHEMS/DOPE liposomes is greater than 0.01% and less than 10% of the cell-associated liposomal contents.

KEY WORDS: cell culture; drug delivery; fluorescence; fusion; *in vitro;* liposomes; pH sensitive.

INTRODUCTION

A variety of macromolecules that can modulate the physiology and metabolism of cells, such as antibodies (1), DNA (2), antisense oligonucleotides (3), and ribozymes (4), have been proposed as novel therapeutic modalities. These molecules cannot readily cross the plasma membrane, hence a delivery system to introduce them into the cytoplasm is essential for their continued development. One possibility is to use liposomes, which have been widely employed as drug carriers, functioning both as a controlled-release system and to deliver encapsulated compounds into cells (5). However, the majority of liposomes internalized by cells enter through an endocytic pathway (6,7) and the ultimate fate of the liposome is the lysosome. Here enzymatic degradation of the lipids and their contents occur (8,9). Compounds that are degraded in or cannot escape the lysosomal compartment would be inactive when delivered by most liposome compositions described to date.

pH-sensitive liposomes have been developed to circumvent delivery to the lysosome. Such liposomes destabilize membranes or become fusogenic when they are exposed to an acidic environment. In the process of endocytosis the pH is reduced in the endosome, a compartment that precedes the lysosome (10). The appropriately designed pH-sensitive liposome might then transfer its contents into the cytoplasm before the liposome can be conveyed to the lysosomes.

Straubinger and co-workers (11) demonstrated that a liposome composed of oleic acid (OA)[6]/phosphatidylethanolamine (PE) can deliver membrane impermeant calcein and fluoresceinated dextran to the cytoplasm. Huang and colleagues (12–14) incorporated monoclonal antibodies with the

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[6] Abbreviations used: BSA, bovine serum albumin; CHEMS, cholesterylhemisuccinate; DMEM, Dulbecco modified Eagle medium; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; DPX, *N,N'-p*-xylenebis(pyridinium bromide); DTA, diphtheria A chain; EPC, egg phosphatidylcholine; EPE, egg phosphatidylethanolamine; FCS, fetal calf serum; FITC, fluorescein isocyanate; FITC–poly-GL, FITC-labeled poly(D-glutamic acid-D-lysine); FI 450/413, the ratio of fluorescence emission at excitation wavelengths of 450 and 413 nm; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; HPTS, 8-hydroxy-1,3,6-pyrene-trisulfonate; IC_{50}, concentration at 50% inhibition; LysoPC, lysophosphatidylcholine; NH_4Cl, ammonium chloride; OA, oleic acid; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; poly-GL, poly(D-glutamic acid-D-lysine); TCA, trichloroacetic acid.

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fatty acid containing pH-sensitive liposomes to construct pH-sensitive immunoliposomes and were able to deliver various chemotherapeutic agents and DNA to the cytoplasm of target cells. Evidence has also been presented that cytoplasmic delivery can occur *in vivo* (15). In these prior studies the efficiency of cytoplasmic delivery via the pH-sensitive liposomes was not quantitated.

We have demonstrated that cholesterylhemisuccinate (CHEMS) behaves like cholesterol and stabilizes PE vesicles at neutral pH (16,17) and that protonated CHEMS accelerates the destabilization of PE vesicles at low pH (<6.0) by catalyzing the formation of the hexagonal H_{II} phase (16,17). Thus the CHEMS/PE composition is sensitive to the pH change which occurs along the endocytic pathway and liposomes composed of CHEMS/PE may become leaky or fuse with the intracellular membrane by this proton-triggering mechanism after they are endocytosed by cells.

In this report, the intracellular processing of pH-sensitive liposomes has been compared to non-pH-sensitive liposomes using a combination of fluorescent pH-sensitive dyes, radiolabeled albumin, and diphtheria toxin A chain (DTA). We show that pH-sensitive liposomes made of CHEMS/DOPE deliver encapsulated fluorescent molecules and biologically active macromolecules into the cytoplasmic compartment. Cytoplasmic delivery from the pH-sensitive liposome is orders of magnitude greater than from the non-pH-sensitive composition. However, cytoplasmic delivery still accounts for less than 10% of the liposome contents that become cell associated.

MATERIALS AND METHODS

Materials

Dioleoylphosphatidylethanolamine, dioleoylphosphatidylcholine (DOPC), egg phosphatidylcholine (EPC), egg phosphatidylethanolamine (EPE), lysophosphatidylcholine (LysoPC), and oleic acid were obtained from Avanti Polar Lipids Inc. (Birmingham, AL). Ammonium chloride (NH_4Cl), guanidine, dithiothreitol, trichloroacetic acid (TCA), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), Triton X-100, CHEMS, fluorescein isocyanate (FITC), FITC–dextran (MW 4200; 0.0035 mol FITC/mol glucose), and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). Calcein, 8-hydroxy-1,3,6-pyrene-trisulfonate (HPTS), *N,N'-p*-xylenebis-(pyridinium bromide) (DPX) and sulforhodamine 101 were obtained from Molecular Probes (Junction City, OR). Poly(D-glutamic acid-D-lysine) at a 6/4 ratio (poly-GL; MW 69,000) was obtained from Miles (Naperville, IL). Nicked diphtheria toxin was obtained from Calbiochem (San Diego, CA). ^{3}H-Inulin, ^{3}H-leucine, and ^{125}I-NaI were purchased from Amersham (Arlington Heights, IL). ^{125}I-Labeled *p*-hydroxybenzamidine dihexadecylphosphatidylethanolamine was synthesized as described (18). Nuclease-treated rabbit reticulocyte lysate mixture, leucine-deficient amino acid mixture, and Brome mosaic virus RNA were purchased from Promega Biotec Inc. (Madison, WI).

Cell Culture

The macrophage-like cell line RAW 264.7 and P388D1 cells were maintained in Dulbecco modified Eagle medium (DMEM) with 10% heat-inactivated fetal calf serum (FCS) and RPMI 1640 with 10% FCS, respectively. For all the experiments, cells were plated as monolayers in 35-mm or 96-well culture dishes (Costar, Cambridge, MA) 16–20 hr prior to use. Cells were checked and found free from mycoplasma contamination.

Preparations of Liposomes

Liposomes (22 μ*M* lipid) were prepared by the method of reverse-phase evaporation (19) and extruded through 0.2-μm polycarbonate membranes (20). pH-sensitive liposomes were composed of CHEMS/DOPE at molar ratios of 4/6, if not otherwise indicated, while control liposomes were made of CHEMS/DOPC with the corresponding ratio. Compounds to be encapsulated were suspended in an isotonic, pH 7.4 solution with the following concentrations: 50–75 m*M* calcein, 50 m*M* FITC–dextran (FITC/dextran, 0.08/1), 30 m*M* HPTS/50 m*M* DPX, 1 mg/ml BSA/1 m*M* inulin, 0.6 m*M* fluoresceinated poly-GL, and 10 μ*M* DTA. Nonencapsulated calcein, FITC-dextran, and HPTS/DPX were separated by Sephadex G-75 (1 × 20-cm) gel filtration. Nonencapsulated BSA and poly-GL (in 10 m*M* Hepes, 145 m*M* NaCl, pH 7.4 buffer) were separated from encapsulated material by Bio-Gel A 5m (1 × 20-cm) gel filtration. Nonencapsulated DTA was separated from liposome-encapsulated DTA by floating the liposomes through a metrizamide gradient (21). Liposome diameter was determined with a laser light-scattering apparatus (NS-4; Coulter Electronics, Inc., Hialeah, FL). Phospholipid concentration was measured by the method of Bartlett (22).

Stability of Liposomes in Serum

Calcein at 75 m*M*, a self-quenched fluorescence concentration, was encapsulated in liposomes composed of CHEMS/EPE (1/2), CHEMS/EPC (1/2), OA/EPC (3/10), and lysoPC/EPC (3/10). Nonencapsulated calcein was removed from the preparation by column chromatography on Sephadex G-25 and 1 μmol of lipid was placed in 1 ml of a 50% FCS/Hepes, pH 7.4, buffer at 37°C. At intervals, a sample was removed from the incubation mixtures and the percentage of calcein remaining in the liposomes was quantitated from the dequenching of calcein fluorescence (23).

Preparation of FITC–Poly-GL

Poly-GL was labeled with FITC as follows (24). Peptide dissolved in 50 m*M* borate buffer, pH 9, was mixed with FITC in DMSO at a 1/9 (poly-GL/FITC) molar ratio. The reaction mixture was kept in the dark at pH 9, room temperature, with constant stirring for 20 hr. Nonreacted FITC was separated on a Sephadex G/50 (1 × 28-cm) column eluted with water. The FITC–poly-GL complex fractions were lyophilized and redissolved in 10 m*M* Hepes, 145 m*M* NaCl, pH 7.4, at a concentration of 0.6 m*M* before encapsulation. The final product has approximately 6 molecules of FITC conjugated to 1 molecule of poly-GL.

Liposome Uptake by Cells in Culture

Cells (1.5×10^6) in 35-mm culture dishes were rinsed

with FCS-free media before the addition of liposomes. Liposomes containing ^{125}I-*p*-hydroxybenzamidine dihexadecylphosphatidylethanolamine were diluted in serum-free media and incubated with cells for 1 hr at 37°C. At the end of the incubation, cells were washed with cold PBS (6×) and then lysed with 0.5 *N* NaOH. Radioactivity associated with the cell lysate was measured in a Beckman gamma scintillation spectrometer and protein concentrations were assayed by the method of Lowry (25).

Fluorescence Microscopy of Liposomal Cytoplasmic Delivery

P388D1 cells (1×10^6) cultured in 35-mm culture dishes were rinsed with FCS-free media and then incubated with calcein, FITC–dextran or FITC–poly-GL containing liposomes (50–1000 μmol of lipid diluted in FCS-free medium) with or without NH_4Cl (20 m*M*) at 37°C for 1 hr. Cells were then washed three times with 2 ml of phosphate-buffered saline (PBS) (137 m*M* NaCl, 2.7 m*M* KCl, 1.5 m*M* KH_2PO_4, 8.1 m*M* Na_2HPO_4, pH 7.4) and refed with 1 ml of FCS-free medium. A Leitz fluorescence microscope with an excitation filter in the range 450–490 nm (blue band) and a barrier filter for emission fluorescence at wavelengths greater than 515 nm was used to examine cells treated with calcein/FITC liposomes. For cells incubated with HPTS/DPX-containing liposomes, one filter set consisting of a blue-band excitation filter was used to observe the fluorescence from intracellular HPTS in the higher-pH compartment. Fluorescence from HPTS at lower pH (<6) was efficiently filtered using the blue-band filter set. A second set of filters (violet band), excitation 350–410 nm and an emission filter for wavelengths greater than 455 nm, was employed to observe the fluorescence from all HPTS inside of cells. The violet-band filter permitted observation of HPTS in both the low- and the high-pH compartments (26).

To investigate if the endosome/lysosome membrane can be destabilized by the pH-sensitive vesicles, P388D1 cells were incubated with the sulforhodamine 101 (12.5 μg/ml). This fluorescent dye was concentrated in the lysosomes after 3 days of incubation. Empty CHEMS/DOPE liposomes (100 μ*M*) were added to the cell culture in dye-free medium for 4 hr, and then the nonattached vesicles were removed by washing three times with PBS. The cells were examined with a fluorescence microscope using the 530- to 560-nm excitation filters and an emission filter set to pass light at wavelengths greater than 610 nm.

Fluorescence was photographed using Kodak P800/1600 film. Exposure time varied from 5 to 78 sec.

Inhibition of Protein Synthesis by Liposomal DTA

Determination of Encapsulated DTA Concentration

Diphtheria toxin A chain was prepared by a modification of a published method (27). Nicked diphtheria toxin was mixed with dithiothreitol (100 m*M*) and guanidine (500 m*M*) at pH 7.5 for 1 hr at 37°C. Denatured B chain was precipitated by centrifugation at 10,000*g* for 20 min. DTA remaining in the supernatant was dialyzed against 10 m*M* Hepes, 145 m*M* NaCl, pH 7.5, buffer. The DTA-containing liposomes were lysed with 0.4% Triton X-100 and the amount of DTA encapsulated was determined using the reticulocyte lysate assay (28). A standard curve was constructed by assaying known amounts of DTA with lysed empty lipid vesicles. Aliquots (3.5 μl) of lysed liposomes and DTA were mixed with 17.5 μl of reticulocyte lysate mixture, 0.5 μl of 1 m*M* amino acid mixture (minus leucine), 1 μl of Brome mosaic virus RNA (0.5 μg/μl), and 2.5 μl of ^{3}H-leucine (156 mCi/μmol, 1 mCi/ml) and then incubated at 33°C for 1 hr. Amino acid incorporation into protein was assayed in a 3-μl aliquot of the reaction mixture to which 25% TCA and 1% carrier BSA were added. The protein precipitate was collected onto Whatman GF/C filter paper and the radioactivity associated with the filter was measured in a Beckman beta scintillation spectrometer. A standard curve of DTA concentration (10^{-6} to 10^{-9} *M*) versus the percentage inhibition of leucine incorporation to protein by DTA was thus constructed. This standard curve was used to determine the amount of biologically active DTA in the lysate of DTA-containing liposomes.

Inhibition of ^{3}H-Leucine Incorporation to Cellular Protein

P388D1 cells (1×10^5) in a 96-well dish (flat bottom) were incubated with medium, nonencapsulated DTA, empty vesicles, or DTA-encapsulated CHEMS/DOPC (2.5/7.5) or CHEMS/DOPE (2.5/7.5) liposomes for 1 hr at 37°C. In order to obtain a more pronounced effect via pH-sensitive liposomes, the ratio of CHEMS/PE is changed to a lower ratio, which is more likely to become destabilized or fusogenic when encountering the acidic pH (17). After two washes, cells were pulsed with ^{3}H-leucine (1 μCi/well) in fresh medium for 6 hr and then lysed with 20 μl 7 *M* guanidine. Protein precipitated with 100 μl 25% TCA and 50 μl 1% carrier BSA was collected onto glass-fiber disks and washed with 10% TCA using a cell harvester (Skatron Inc., Sterling, VA). The radioactivity associated with the dried disks was determined in a Beckman beta scintillation spectrometer.

Fluorimetry of Cell-Associated HPTS

In order to decrease the fluorescence contributed from noninternalized HPTS-containing liposomes on the cell surface or the background fluorescence from liposomes attached to the culture dish, HPTS was coencapsulated with the collisional quencher DPX at a 1:1.7 ratio (30/50 m*M*) in liposomes. P388D1 cells (1.5×10^6) in 35-mm culture dishes were incubated with vesicles, 50 μ*M* CHEMS/DOPE or 500 μ*M* CHEMS/DOPC, for 1 hr and washed with medium three times. Then incubation was continued in fresh medium without liposomes. At each indicated time point after washing, cells were removed from dishes by exposure to 3 m*M* EDTA/PBS and diluted to $2.5–5 \times 10^5$ cells/ml in PBS. Fluorescence emission was monitored at 510 nm using a SPEX Fluorolog 2 spectrofluorometer (26). Fluorescence intensity associated with the cell suspension irradiated at excitation wavelengths of 413 and 450 nm was measured. The ratio of emitted fluorescence intensity at each excitation wavelength of 450 and 413 nm (Fl 450/413) was calculated.

The excitation spectrum of HPTS is a function of pH. The isobestic point for the excitation wavelength of this pH-dependent phenomenon is 413 nm. When an excitation wavelength of 450 nm is used, the fluorescence signal increases more than 100-fold as the pH increases from 6 to 8.

The ratio of the fluorescence emission for the 450-nm excitation wavelength compared to the 413-nm excitation wavelength (Fl 450/413) can be used as an indicator of the pH of the HPTS solution (29). For instance, a Fl 450/413 of 2.1 indicates that HPTS experiences a neutral pH of 7.4 and a Fl 450/413 of 0.2 represents that HPTS is in an acidic pH (pH < 6) environment. The pH-dependent fluorescent pattern of nonencapsulated HPTS is not affected by the coencapsulation of DPX with HPTS in the liposomes. Since the relationship between the pH and the Fl 450/413 value is almost linear between pH 8 (Fl 450/413 = 3.2) and pH 6.5 (Fl 450/413 = 0.59), this assay permits one to estimate the pH experienced by HPTS molecules when they become cell associated. The measured cell-associated fluorescence included contributions from HPTS in the cytoplasm, HPTS released in acidic vesicles, and to a small extent, HPTS in vesicles that were on the cell surface. The encapsulated HPTS/DPX elicited less than 4% of the maximum unquenched signal at neutral pH, thus the contribution from noninternalized liposomes containing HPTS/DPX to the total cell-associated fluorescence is small. This assumes that there is no leakage of vesicles contents at the cell surface. If 50% of the contents leak, then the residual encapsulated HPTS fluorescence would be 12% of the maximum unquenched signal and the contribution from these HPTS/DPX-containing liposomes on the cell surface is still small. The cell-associated Fl 450/413 would range between 2.1 to 0.2, since the majority of HPTS would be exposed to either an acidic environment in the intracellular compartments or the neutral environment of the cytoplasm, and the ratio would decrease as the fraction of HPTS exposed to an acidic pH increased.

Degradation of Liposomal BSA

Iodinated BSA (300 μCi/mg) was prepared by the chloramine-T method (30) and was coincorporated with ^{3}H-inulin (28 μCi/mg) into liposomes. Mixtures of double-labeled CHEMS/DOPC vesicles (500 μ*M*) plus empty CHEMS/DOPE vesicles (50 μ*M*) and double-labeled CHEMS/DOPE vesicles (50 μ*M*) plus empty CHEMS/DOPC vesicles (500 μ*M*) were incubated with 1.8×10^6 P388D1 cells in serum-free medium for 2 hr at 37°C. Vesicles not associated with cells were removed by washing six times with PBS. Cells were incubated in the fresh medium without FCS or liposomes for the period indicated. At each time point, cells were washed with PBS (six times) and lysed with 0.5 *N* NaOH. Radioactivity (^{125}I or ^{3}H) associated with cell lysates was measured using a Beckman gamma counter and a Beckman beta scintillation spectrometer, respectively. The ratio of cell-associated ^{125}I-BSA/^{3}H-inulin was calculated and normalized to the ratio of ^{125}I-BSA/^{3}H-inulin at the beginning of the experiment.

The iodopeptide product from BSA degradation in the lysosomal compartment is released to the extracellular medium (8). When a nonmetabolizable ^{3}H-labeled compound is encapsulated with the BSA, the ratio of the ^{125}I to ^{3}H is a measure of the metabolism of BSA. Inulin which is not degraded by lysosomal enzymes was selected as the nonmetabolizable marker. The loss of inulin could occur due only to dissociation of vesicles from the cell, release of liposomal contents at the cell surface, and/or regurgitation of lysosomal contents. Thus inulin coencapsulated with BSA serves as an internal standard to normalize the cell-associated liposomal contents and the BSA/inulin ratio is an indicator for the extent of degradation of liposomal BSA in the cells.

RESULTS

Liposome Preparation and Characterization

The charge and head group of phospholipids can influence the physical properties of the resulting liposomes (31). In the case of PE, containing unsaturated acyl chains, a stable liposome cannot be formed at a pH less than 9.0; rather planar sheets of lipid or hexagonal H_{II} phase lipid tubes exist at room temperature. Incorporating a charged lipid with PE, such as CHEMS, results in the formation of liposomes which are stable at pH 7.0 and room temperature but destabilize as the pH is lowered (16).

Liposomes composed of CHEMS/DOPC have a higher encapsulation volume and encapsulation efficiency than pH-sensitive (CHEMS/DOPE) liposomes (Table I). This occurs in spite of their comparable size (diameters, 203 ± 70 nm) and surface charge density. The encapsulation volume is 1.5- to 3-fold higher for the control liposomes than for the pH-sensitive liposomes. This suggests that the pH-sensitive liposomes are oligolamellar.

Table I. Comparison of Control and pH-Sensitive Liposome Preparations

Lipid composition	Encapsulated compound	Encapsulated ratio (μl/μmol lipid)[a]	Encapsulation efficiency (%)
CHEMS/DOPC[b]	Calcein	4.1	ND[c]
CHEMS/DOPE[b]	Calcein	2.8	ND
CHEMS/DOPC[b]	BSA/inulin	5.1	17.7
CHEMS/DOPE[b]	BSA/inulin	3.4	12.5
CHEMS/DOPC[d]	DTA	5.2	20.9
CHEMS/DOPE[d]	DTA	1.8	7.0

[a] Results are values from a single experiment of each preparation. When other preparations were measured, the CHEMS/DOPE encapsulation ratio was within 30% of the given values.
[b] The molar ratio of CHEMS/DOPC or CHEMS/DOPE is 4/6.
[c] Not determined.
[d] The molar ratio of CHEMS/DOPC or CHEMS/DOPE is 2.5/7.5.

Stability of Liposomes in Serum

Incorporation of cholesterol into the lipid bilayer results in a more rigid membrane (32). In the presence of serum, such vesicles are more stable than single-component phospholipid vesicles. Cholesterylhemisuccinate behaves like cholesterol and stabilizes PE liposomes at neutral pH (17). As shown in Table II, the CHEMS/PE composition retains encapsulated calcein as well as the CHEMS/PC formulation and considerably better than formulations that contain oleic acid or lysophospholipid.

Uptake of Liposomes by Cells in Culture

Liposomes composed of CHEMS/DOPE are taken up by P388D1 cells 5–10 times more efficiently than the CHEMS/DOPC vesicles when the same amount of lipid is added to cells in culture (Fig. 1a). The uptake is concentration and time dependent. The kinetics of uptake saturates about 8 hr after the start of incubation (data not shown). The higher uptake of the PE vesicles cannot be due to the negative charge density since the PC vesicles contained the same molar ratio of CHEMS.

Ellens and co-workers showed that the PE/CHEMS vesicles tended to aggregate in the presence of a high concentration of Ca^{2+}/Mg^{2+} (33) and these divalent cations can influence the cellular uptake of certain negatively charged liposomes. The amount of CHEMS/DOPE vesicles taken up by RAW (Fig. 1b) or P388D1 (data not shown) cells is influenced by the Ca^{2+}/Mg^{2+} present in the medium, 1.8 m*M* Ca^{2+}/0.8 m*M* Mg^{2+} in DMEM and 0.5 m*M* Ca^{2+}/0.4 m*M* Mg^{2+} in RPMI, whereas the uptake of CHEMS/DOPC vesicles is not affected by the Ca^{2+} concentration. The reason for the different cell affinities of CHEMS/PE and CHEMS/PC liposomes is not clear. We speculate that the hydration of phospholipids, e.g., PE is less hydrated than the PC, may play a role in the phenomenon observed. Vesicles containing PE are shown to adhere to each other more strongly than do vesicles containing PC because of the difference in hydration (34). This may explain the greater effect of divalent cations, which can promote the dehydration of phospholipid, on the uptake of the CHEMS/PE vesicles than on that of CHEMS/PC vesicles.

The cellular uptake of CHEMS/PE is higher than that of CHEMS/PC vesicles (Fig. 1), and the leakage in serum is comparable, therefore the amount of contents delivered varies accordingly. Based on the data in Fig. 1a, 50 μ*M* CHEMS/DOPE and 500 μ*M* CHEMS/DOPC were selected for the experiments reported below. These respective concentrations result in comparable amounts of cell-associated content delivery by the two liposome compositions.

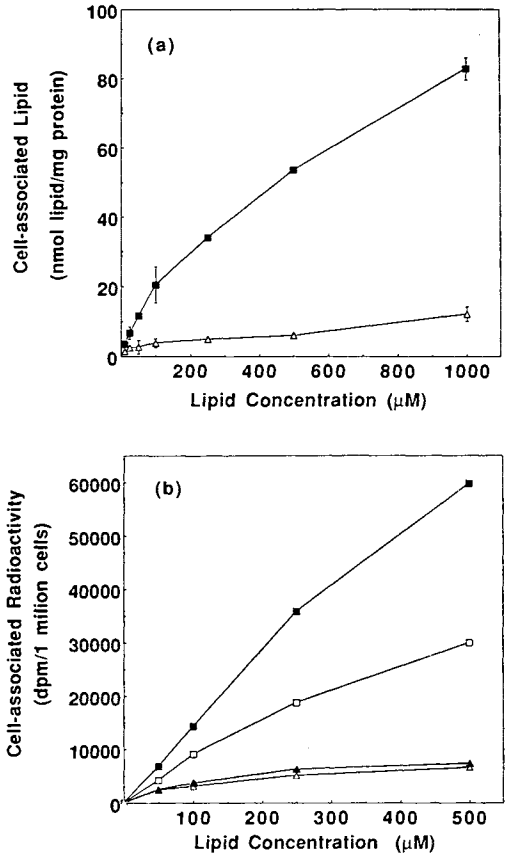

Fig. 1. (a) Concentration-dependent uptake of liposomes by P388D1 cells. Cells were incubated with ^{125}I-labeled liposomes of CHEMS/DOPC (2.5/7.5) (△) or CHEMS/DOPE (2.5/7.5) (■) for 1 hr at 37°C and nonattached liposomes were washed off before the measurement of cell-associated radioactivity. (b) Divalent ion dependent uptake of liposomes by RAW 264.7 cells. Cells were incubated with ^{125}I-labeled CHEMS/DOPC (4/6) (▲, △) or CHEMS/DOPE (4/6) (■, □) liposomes suspended in either DMEM (1.8 m*M* Ca^{2+}/0.8 m*M* Mg^{2+}) (▲, ■) or RPMI1640 (0.5 m*M* Ca^{2+}/0.4 m*M* Mg^{2+}) (△, □) for 1 hr at 37°C. Values are the means of duplicates in a single experiment and the bars represent the range of values from the means.

Table II. Percentage of Calcein Remaining in Liposomes[a]

Time (hr)	CHEMS/EPE (1/2)	CHEMS/EPC (1/2)	OA/EPC (3/10)	LysoPC/EPC (3/10)
0	97[b]	95	95	97
1	95	85	10	29
2	94	81	10	26
3	92	78	11	26
4	91	73	12	29
24	44	29	13	19

[a] Liposomes incubated at 37°C in the presence of serum.
[b] Values are the mean of duplicate measurements from a single experiment that agreed to within 15%.

Fluorescent Microscopy of Cytoplasmic Delivery of Fluorophores

Calcein is a very sensitive indicator for delivering liposome contents into the cytoplasm because it does not readily cross membranes even under mildly acidic conditions (7), and when a 50 m*M* self-quenched solution is diluted into a larger volume, the fluorescence intensity increases more than 20-fold. When encapsulated in control liposomes (CHEMS/DOPC) and incubated with cells, fluorescence is present in a punctate (vacuolar) pattern in the cells (Fig. 2a). If delivered by pH-sensitive liposomes, a diffuse calcein fluorescence is visible throughout the cells (Fig. 2b), indicating that calcein molecules distribute in the cytoplasm. Although the cells shown in Fig. 2b are incubated with liposomes for 1 hr, the diffuse cytoplasmic fluorescence with a lower intensity can be seen at as early as 15 min after addition of the pH-sensitive liposomes.

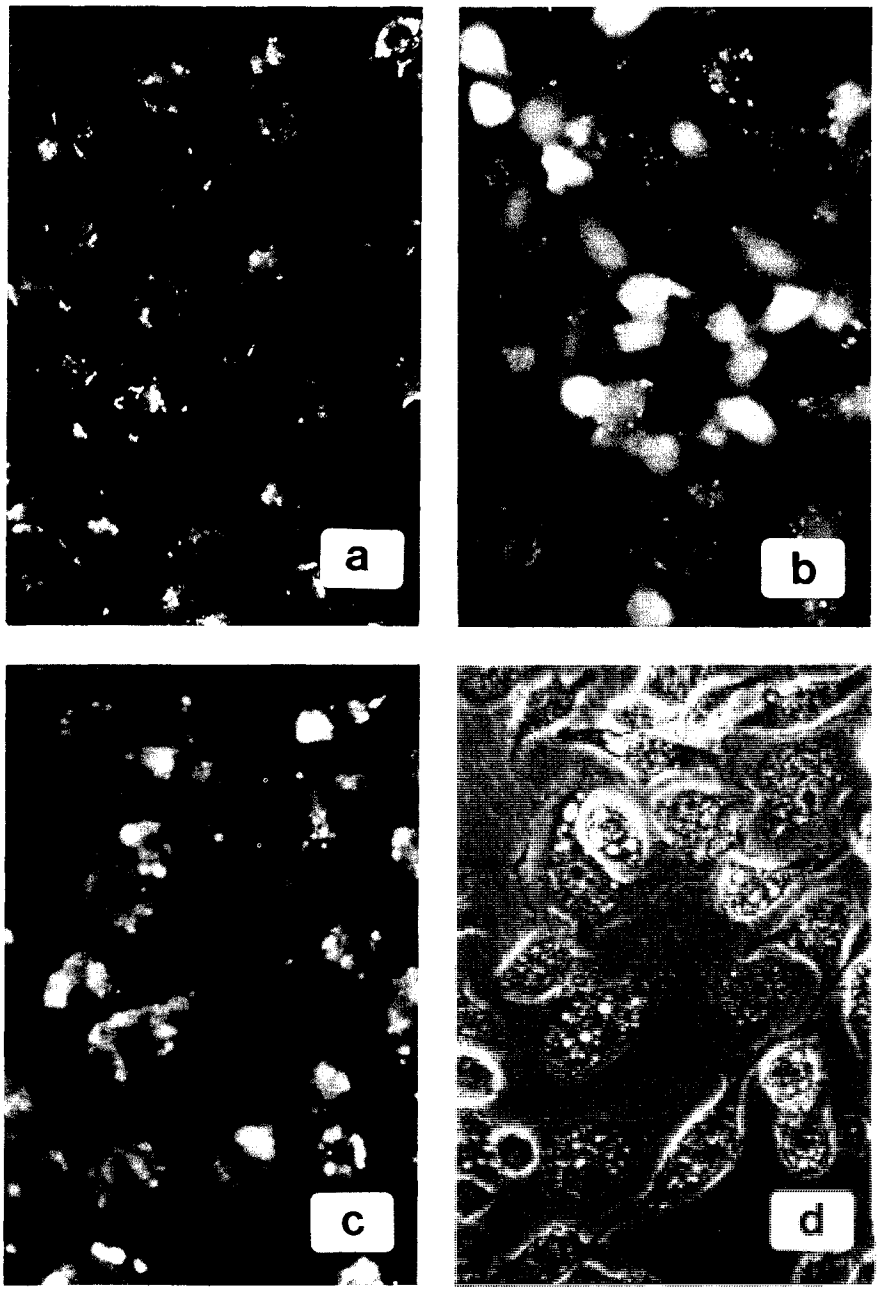

Fig. 2. Fluorescence of RAW264.7 cells after incubation with calcein-containing liposomes for 1 hr. (a) Punctate fluorescence resulting from incubation with 500 μ*M* CHEMS/DOPC (4/6) liposomes. (b) Diffuse (cytoplasmic) fluorescence obtained by incubation with 100 μ*M* CHEMS/DOPE (4/6) liposomes. (c) Vacuolar fluorescence from incubation with 100 μ*M* CHEMS/DOPE (4/6) vesicles in the presence of 20 m*M* NH_4Cl. (d) Phase-contrast image of the field shown in c. Bar indicates 10 μm.

In the presence of 20 m*M* NH_4Cl, a concentration that dissipates the pH gradients in the acidic granules (35), a punctate fluorescence is observed in cells incubated with the pH-sensitive liposomes (Fig. 2c). The brightly fluorescent vacuoles indicate that calcein is released and confined in these compartments. This supports the idea that transfer of calcein to the cytoplasm is a pH-dependent phenomenon.

Divalent cations, Ca^{2+}/Mg^{2+}, are also required for cytoplasmic delivery via pH-sensitive liposomes. When divalent cations were chelated by the addition of 1 m*M* EDTA in DMEM, cytoplasmic calcein fluorescence from pH-sensitive liposomes was not observed (data not shown). Only the punctate fluorescence which was similar to that of cells treated with NH_4Cl was obtained; even the fluorescence intensity observed under these two conditions, NH_4Cl and EDTA treatment, was similar. These findings indicate that both protons and divalent cations are involved in the destabilization/fusion of CHEMS/DOPE vesicles resulting in cytoplasmic delivery. All the phenomena describing the cytoplasmic delivery via CHEMS/DOPE vesicles and the factors altering the outcomes are similar in RAW 264.7 and P388D1 cells. No appreciable differences in their response to the pH-sensitive liposomes were observed.

When either P388D1 or RAW 264.7 cells were incubated with sulforhodamine 101 for 3 days, fluorescence was found to be concentrated in the perinuclear vacuoles (lysosomes) as previously described (36). Incubation of these cells with fresh medium containing empty CHEMS/DOPE or CHEMS/DOPC vesicles does not change the fluorescent distribution in the cell; in other words, fluorescence is observed only in the perinuclear vacuole, and not in the cytoplasm (data not shown). This result indicates that pH-sensitive liposomes are not likely to destabilize the lysosomal membranes and cause release of sulforhodamine 101 from these intracellular compartments into the cytoplasm. The possibility of fusion between pH-sensitive liposomes and the endosomal/lysosomal membrane or destabilization of endosomal membranes by the pH-sensitive liposomes cannot be excluded. Since the intracellular vacuoles are known to become disrupted due to the hypertonicity of a 20% glycerol solution (37), sulforhodamine in the perinuclear vacuoles could be released into the cytoplasm by exposing the cells to 20% glycerol for 5 min. This treatment of glycerol results in a bright cytoplasmic fluorescence in the control cells and the cells incubated with the pH-sensitive or non-pH-sensitive liposomes. The glycerol treatment also serves as a positive control demonstrating the rupture of the intracellular vesicles and redistribution of the dye into the cytoplasm. Whether the effect of hypertonic glycerol treatment is limited only to the disruption of lysosomal or involves other intracellular membranes is not clear, so that other mechanisms that induce redistribution of the dye from the lysosomes to the cytoplasm cannot be ruled out.

When large fluorescent molecules, such as FITC–dextran (MW 4200) and FITC–poly-GL (MW 69,000) are delivered in pH-sensitive liposomes, cytoplasmic fluorescence is observed in the cells, which can be differentiated from punctate fluorescence in the vacuoles. Only punctate fluorescence is observed when these two macromolecules are delivered in the non-pH-sensitive CHEMS/DOPC composition. However, due to the background fluorescence of vesicles containing the nonquenched fluorophore adhering to the cell surface and the substratum, photographs of cells (P388D1 and RAW 264.7) treated with the pH-sensitive liposomes do not conclusively document the phenomenon.

The efficiency of cytoplasmic delivery from one other pH-sensitive composition, oleic acid/DOPE, was also examined on the P388D1 and RAW 264.7 cells using calcein as a fluorescent marker. A very dim diffuse fluorescence, much less than that seen with the CHEMS/DOPE vesicles (Fig. 2b), is observed (data not shown).

In cells (P388D1) incubated with pH-sensitive liposomes containing HPTS/DPX, cytoplasmic fluorescence is observed when the blue-band filter (high-pH form of HPTS) is used (Fig. 3b). When the violet-band filter (both pH forms of HPTS) is used, a punctate and diffuse fluorescence pattern is observed in cells incubated with the pH-sensitive liposomes (Fig. 3a). A similar result is obtained when the RAW 264.7 cell is used (data not shown).

These results indicate that membrane-impermeant fluorophores can reach the cytoplasm when they are delivered by pH-sensitive liposomes but end up predominantly in the perinuclear vacuoles while delivered by non-pH-sensitive liposomes.

Protein Synthesis Inhibition by Liposomal DTA

Diphtheria toxin is a highly potent inhibitor of protein synthesis. Estimates as low as one molecule per cell, under appropriate conditions, can totally inhibit protein synthesis (38). The A chain of diphtheria toxin is unable to cross either the plasma membrane or the endosomal membrane without the assistance of the B chain of the toxin. Hence DTA is not inhibitory to cells. The inhibition of leucine incorporation by DTA delivered by liposomes is shown in Fig. 4. Neither nonencapsulated DTA (up to 10^{-6} *M*) plus empty vesicles, equivalent to the concentration of DTA-containing liposomes, nor DTA-containing control liposomes (up to 5×10^{-8} *M* DTA/10^{-3} *M* lipid) inhibit leucine incorporation into cellular protein. Inhibition is observed only when cells are incubated with DTA-containing pH-sensitive liposomes. A dose-dependent inhibition from DTA encapsulated in pH-sensitive liposomes is observed (Fig. 4a). The concentration of CHEMS/DOPE vesicles that caused a 50% inhibition (IC_{50}) is 40 μM which contain about 7.0×10^{-10} *M* (or 1.4×10^{-13} mol) of DTA. Ammonium chloride blocks the inhibition of protein synthesis elicited by DTA-containing pH-sensitive liposomes (data not shown). This indicates that a low-pH pathway is involved in the introduction of DTA from the pH-sensitive liposomes into the cytoplasm. This inhibition of protein synthesis experiment was not examined in RAW 264.7 cells, although the outcome is expected to be similar to that obtained from P388D1 cells due to the similar behaviors observed in other experiments.

Cell-associated DTA can be calculated based upon the amount of DTA encapsulated in liposomes (17.5×10^{-15} mol DTA/nmol CHEMS/DOPE lipid or 52.1×10^{-15} mol/nmol CHEMS/DOPC lipid) and the measured amount of vesicle uptake per cell (Fig. 1a). This assumes that all the cells take up liposomes, encapsulated molecules do not leak from the liposomes, and 1 million cells corresponds to 0.3 mg of cellular protein. When the dose–response curves are plotted on the basis of the amount of cell-associated DTA (Fig. 4b), the pH-sensitive composition is at least 100 times more ef-

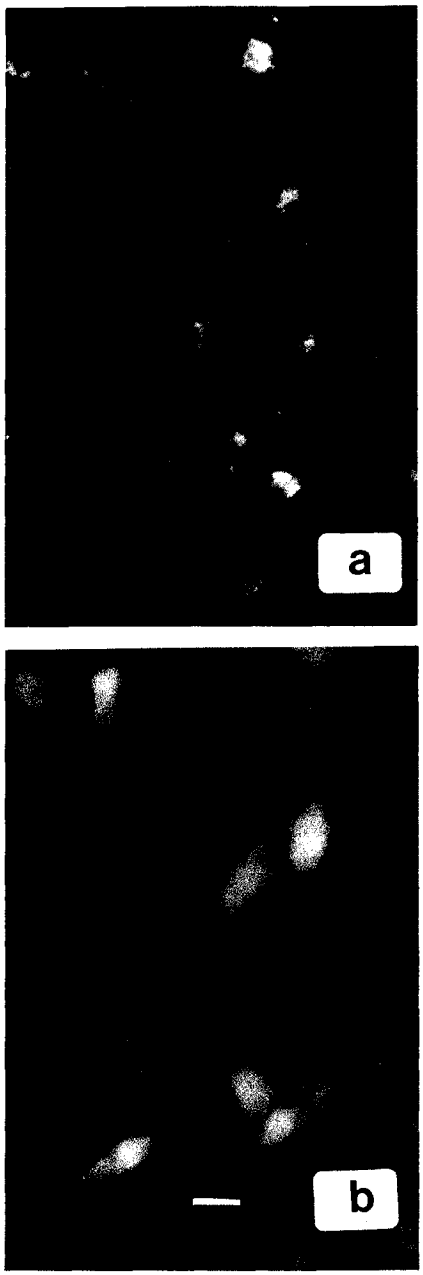

Fig. 3. Cellular location of liposomal HPTS in P388D1 cells. (a) Vacuolar fluorescence and diffuse fluorescence observed under violet-band filter set after incubation with 50 μ*M* CHEMS/DOPE (4/6) vesicles containing HPTS/DPX. (b) Cytoplasmic fluorescence viewed under blue-band filter set after incubation with 50 μ*M* corresponding pH-sensitive liposomes. Bar indicates 10 μm.

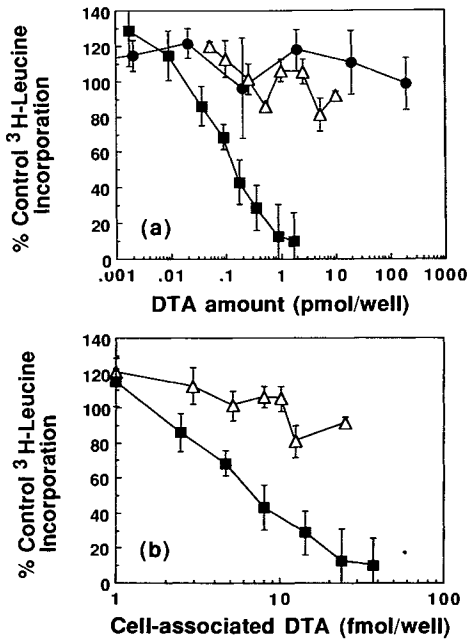

Fig. 4. Inhibition of ^{3}H-leucine incorporation by liposomal DTA in P388D1 cells. (a) Result expressed as amount of DTA in DTA-containing CHEMS/DOPC (2.5/7.5) vesicles (△), in DTA-containing CHEMS/DOPE (2.5/7.5) vesicles (■), or in a nonencapsulated form (●) incubated with 1×10^5 cells for 1 hr. (b) Result expressed as cell-associated DTA after 1 hr of incubation with DTA-containing CHEMS/DOPC (△) or DTA-containing CHEMS/DOPE vesicles (■). The cell-associated amount of DTA was calculated based on the result shown in Fig. 1a and the encapsulation ratio of DTA in the liposomes (moles DTA/moles lipid; see text). Values are the means of quadruplicate measurements in a single experiment and bars represent the standard deviations from the means.

ficient at delivering DTA to the cytoplasm than the non-pH-sensitive composition.

Acidification of HPTS

The fluorescence ratio from cell-associated HPTS/DPX as a function of time after incubation is shown in Fig. 5. Since the Fl 450/413 of cell-associated HPTS correlates with the pH of the environment HPTS is exposed to, this assay is used to estimate the pH of the cell-associated HPTS. In this experiment, the liposomes containing HPTS/DPX have a Fl 450/413 of 2.1 at pH 7.4, which is not significantly different from the value obtained using the nonencapsulated, unquenched HPTS solution at pH 7.4. At the zero-time point, shown in Fig. 5, the P388D1 cells have already been incubated with the HPTS/DPX-containing liposomes for 1 hr at 37°C. Vesicles not firmly attached to the cells are removed using three successive washes at 4°C. At the zero-time point, the assay indicates that the average HPTS molecule experiences a pH 6.6 environment. This is because a fraction of the liposomes has been internalized and they have delivered their contents into a low-pH compartment. As the incubation continues, the Fl 450/413 ratio progressively declines until the Fl 450/413 reaches 0.25 (corresponding to pH 6.1) after 4.5 hr (Fig. 5). The Fl 450/413 ratio remains constant at 0.25 for 24 hr, the last time point measured. There is no significant difference in the rate of acidification of intracellular HPTS whether the fluorophore is delivered in the pH-sensitive or non-pH-sensitive lipid vesicles. The similar result is obtained when RAW 264.7 cells were studied (data not shown). Only the Fl 450/413 ratio is slightly higher at 4.5 hr after washing. The variability of this assay is about 15%. Although fluorescence microscopy indicates cytoplasmic delivery from the pH-sensitive liposomes, the result with the spectrofluorimetric assay suggests that the fraction of HPTS delivered into cytoplasm via the pH-sensitive liposomes is less than 10% of the cell-associated contents.

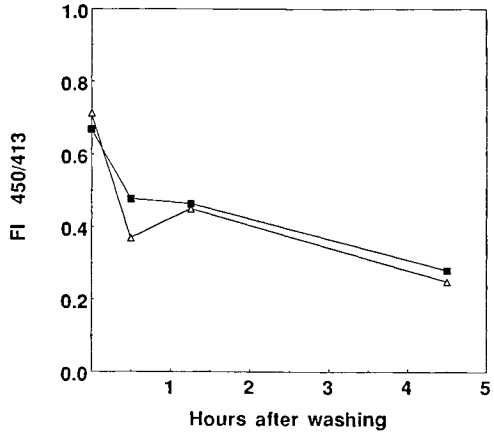

Fig. 5. Time course of acidification of liposomal HPTS in P388D1 cells. Cells were treated with HPTS/DPX-containing CHEMS/DOPC (4/6), 500 μ*M* (△), or CHEMS/DOPE (4/6), 50 μ*M* (■), vesicles for 1 hr. Nonattached vesicles were removed and fresh medium was added to cells at time 0. At the time indicated, cell-associated fluorescence observed at 510 nm when excited at wavelengths of 450 and 413 nm was measured as described under Materials and Methods. Values represent one measurement from a single experiment. The variation between experiments is within 15% of the value reported.

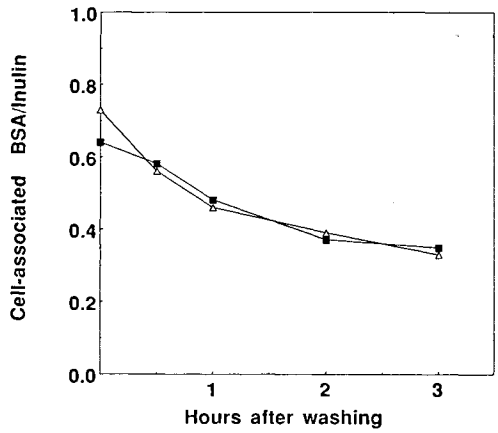

Fig. 6. Time course of degradation of liposomal BSA in P388D1 cells. Empty CHEMS/DOPC (4/6) liposomes (500 μ*M*) plus ^{125}I-BSA/^{3}H-inulin-encapsulated CHEMS/DOPE (4/6) vesicles (50 μ*M*) (■) and empty CHEMS/DOPE (4/6) vesicles (50 μ*M*) plus ^{125}I-BSA/^{3}H-inulin-containing CHEMS/DOPC (4/6) vesicles (500 μ*M*) (△) were incubated with cells for 2 hr. At time 0, nonattached liposomes were washed off and fresh medium was added for continued incubation. Cell-associated radioactivity was measured at the indicated times after washing. Values are the means of duplicates in a single experiment. The ranges of values are within 10% of the means.

Degradation of BSA

The degradation rate of BSA was investigated to estimate the fraction of BSA that escaped the lysosomotropic pathway. This assay is based on the fact that BSA degradation in the cytoplasm (half-life, 20–34 hr) (39,40) is much slower than BSA degradation in the lysosomes (half-life, <1 hr) (8). The degradation products of BSA are released into the extracellular medium, while the inulin is resistant to lysosomal enzymes and remains within the cells. Thus the cell-associated BSA/inulin ratio can be used as an indicator for the extent of degradation of liposomal BSA in the cells. This experiment was conducted so that a similar level of lipid became cell-associated with both the pH-sensitive liposomes and the non-pH-sensitive liposomes. The initial BSA/inulin ratio was 1.0. After a 2-hr exposure to liposomes, the cells were washed and the incubation continued in the absence of liposomes. Immediately after the wash the ratio was 0.73 for control liposomes and 0.65 for pH-sensitive liposomes because the BSA was being degraded inside the cells during the incubation. This ratio progressively declines, regardless of the liposome composition, to 0.34 by 3 hr (Fig. 6). The BSA/inulin ratio in duplicate determinations varied 10% from the mean value. Thus cytoplasmic delivery of less than 10% of the contents would be undetectable by this method. The similarity in the BSA degradation rate between the two compositions indicates that the cytoplasmic delivery from the pH-sensitive composition is less than 10% of the cell-associated contents. A similar conclusion is reached when BSA-containing CHEMS/DOPE and CHEMS/DOPC liposomes were incubated with RAW cells (data not shown).

DISCUSSION

pH-sensitive liposomes were originally developed to release their contents in the pH environment (pH 6.6–7.2) found in the vicinity of certain tumors (41). Since the pH change from the plasma to the tumor is rather modest, investigators turned their attention to the endosome. Liposomes are internalized into cells via the endosome and the pH in this prelysosomal compartment is reduced from 7.4 to between 5.3 and 6.5 within minutes after the endosome is formed. The pH-sensitive liposomes are designed to mimic enveloped viruses which have membrane fusion proteins that undergo a conformational change when the pH is reduced to about 6.0. The change in protein conformation induces fusion between the viral and the endosomal membrane and the viral genetic material is delivered into the cytoplasm. Lipid mixtures composed of PE and a pH-titratable fatty acid or lipid derivative have been developed to achieve this goal (42,43). Biophysical studies have demonstrated that such vesicles either fuse or leak their contents at low pH. Ellens and colleagues have demonstrated that CHEMS/DOPE vesicles, triggered by H^+, become destabilized at pH <5.5 and fuse at pH <5.0 (16,33).

In this work, we demonstrate that CHEMS/DOPE vesicles deliver various fluorophores (calcein, FITC–dextran, FITC–poly-GL) and biologically active DTA into the cytoplasm. The fact that both low and high molecular weight molecules are transferred into the cytoplasm implies that disruption of the intracellular membranes or fusion between the liposomal membrane and the intracellular compartment membranes is the most likely mechanism involved in the cytoplasmic delivery. The destabilization/fusion events are most likely to occur in the endosomes, since these vacuoles are the first compartment where liposomes encounter a low pH. Cytoplasmic fluorescence is not observed in the presence of agents that dissipate the pH gradient in intracellular vacuoles; a pH-sensitive event is necessary for the cytoplasmic delivery from the CHEMS/DOPE vesicles. It is also interesting that calcium/magnesium is required for cytoplasmic delivery since biophysical studies have indicated a re-

quirement of millimolar levels of these divalent cations for fusion to occur in model systems (33).

The inhibition of protein synthesis by DTA-containing CHEMS/DOPE vesicles but not by nonencapsulated DTA, empty liposomes, or DTA-containing CHEMS/DOPC liposomes further confirms that DTA is introduced into the cytoplasm by pH-sensitive liposomes. There is a greater than five order of magnitude difference between the cytotoxicity of CHEMS/DOPE DTA and that of nonencapsulated DTA. Moreover, the CHEMS/DOPE composition is at least 100-fold more efficient in delivering cytotoxic DTA than the CHEMS/DOPC composition. A 50% inhibition was obtained by incubating 7×10^{-10} *M* DTA, encapsulated in 40 μM pH-sensitive liposomes, with cells for 1 hr (Fig. 4).

The calculated amount of DTA which becomes cell associated after 1 hr of incubation with CHEMS/DOPE vesicles is 30,000 molecules per cell, if we assume that no leakage occurs during the incubation. The actual cell-associated DTA is probably lower than the amount calculated due to the leakage of contents induced by cell contact (44). To estimate the fraction of the 30,000 cell-associated DTA molecules that reach the cytoplasm in an active form, we can use data from studies on the efficiency of translocation of intact diphtheria toxin into the cytoplasm of susceptible cells (45). In this work the reciprocal half-time for inhibition of protein synthesis in a cell-free system is a linear function of DTX concentration, with a slope of 1.2 nM^{-1} min^{-1}. If we assume the cytoplasmic volume to be 2 pl and the half-time of inhibition of protein synthesis by 40 μM DTA-containing DOPE/CHEMS liposomes to be 360 min (assay was carried out with a pulse of ^{3}H-leucine for 6 hr after removal of nonattached liposomes), the amount of DTA required to elicit this degree of inhibition is 3 molecules per cell. Thus the cytoplasmic delivery efficiency of the CHEMS/DOPE system is at least 0.01% of the cell-associated contents.

The correlation between diphtheria toxin effects on protein synthesis reported in the cell-free system (45) and DTA effects in the P388D1 cells can only be inferred; thus we emphasize that this estimate is a lower bound on the efficiency of cytoplasmic delivery by the CHEMS/PE system. It is interesting, however, that Moynihan and Pappenheimer (45) conclude that only 0.4% of the diphtheria toxin that becomes cell associated reaches the cytoplasm. Colombatti and co-workers have shown that the cytotoxicity of diphtheria toxin is about 1000 times greater than antibody-linked DTA in target cells (46). This indicates that the CHEMS/DOPE vesicles are about two orders of magnitude more efficient than antibody-linked DTA and within an order of magnitude as efficient as intact toxin at transporting DTA molecules into the cytoplasm.

The CHEMS/DOPE composition seems to be the most efficient liposomal composition reported to date to deliver DTA-inhibitory activity. McIntosh and Heath (47) showed that DTA-containing phosphatidylserine (PS) vesicles did not inhibit protein synthesis in lymphoma cells even after 21 hr of lag time. Ikuta and colleagues (48) demonstrated that DTA-containing liposomes (EPC/cholesterol/PS) could kill HIV-infected leukemia cells but not noninfected cells with continuous incubation for 3 days. The killing resulted from the facilitated transport of DTA through the altered plasma membrane of the infected cells rather than from cytoplasmic delivery by the liposomes. Using a similar lipid composition (DOPC/cholesterol/PS or DOPC/cholesterol/DOPE), Jansons and Panzner (49) reported that approximately 50% inhibition of protein synthesis in human lymphoblastoid cells was obtained by incubating DTA-containing (DTA = 1.2×10^{-8} *M*) vesicles with cells for 2.5 hr. They were not able to get greater than 70% inhibition with longer incubations or higher concentrations. Attaching antibodies to the surface of liposomes, Collins and Huang (13) showed that only the DTA-containing pH-sensitive immunoliposomes could inhibit protein synthesis ($IC_{50} = 1.4 \times 10^{-9}$ *M*) in target cells after 3 hr of incubation, while nontargeted pH-sensitive liposomes or non-pH-sensitive immunoliposomes were ineffective. The cell-associated DTA using the pH-sensitive immunoliposomes was not quantitated, so the efficiency of delivery is unknown. In the absence of targeting, we can obtain a 50% inhibition of protein synthesis at 7.0×10^{-10} *M* of DTA after a 1-hr incubation of CHEMS/DOPE-DTA with P388D1. However, the macrophage-like nature of P388D1 cells may also contribute to the higher apparent delivery efficiency of the CHEMS/DOPE vesicles when compared to results in the other studies.

When the efficiency of cytoplasmic delivery by CHEMS/DOPE was compared with another pH-sensitive composition, OA/DOPE, the CHEMS/DOPE vesicles delivered more calcein to the cytoplasm. Moreover, CHEMS/DOPE seems to be able to deliver macromolecules better than the OA/PE composition as well. Baldwin and coworkers (50) employed OA/PE vesicles to deliver pokeweed antiviral protein, a DTA-like protein synthesis inhibitor which cannot readily cross the cell membrane. They showed that the OA/PE-encapsulated compound was 2500-fold more potent than nonencapsulated molecules in inhibiting CV-1 cell growth after 8 hr of incubation, while CHEMS/DOPE-DTA is at least 10^5 times more potent than nonencapsulated DTA in our system. In addition, the greater stability of CHEMS/DOPE in the presence of serum makes CHEMS/DOPE a more suitable formulation for *in vivo* use.

Both HPTS acidification and BSA degradation experiments clearly show that pH-sensitive liposomes are taken up by cells by an endocytotic pathway. This pathway routes the majority of liposomes and their contents into an acidic environment and eventually into the lysosomes where iodoalbumin is degraded. This is the same as the fate of non-pH-sensitive liposomes. The sensitivity of these two assays to discern the fate of the encapsulated molecules is limited by experimental variation, which is always between 3 and 15%. Since there is no significant difference found in the rates of HPTS acidification and BSA degradation between pH-sensitive and control liposomes, the efficiency of the cytoplasmic delivery is less than the sensitivity of the assays.

In summary, we conclude that the CHEMS/DOPE composition can deliver macromolecules to the cytoplasm of cells. This process requires a low-pH environment and the presence of divalent cations to effect cytoplasmic delivery. Whether a true fusion of the liposomal membrane with the endosomal and/or lysosomal membranes occurs cannot yet be determined. However, cells whose lysosomes have been labeled with sulforhodamine 101 do not show cytoplasmic fluorescence when incubated with the pH-sensitive liposomes. This indicates that the pH-sensitive liposomes do not cause extensive leakage of the endosomal/lysosomal contents. It suggests that leakage from these intracellular com-

partments is probably not a significant delivery pathway as opposed to endosome/lysosome liposome fusion for the CHEMS/DOPE composition. The efficiency of cytoplasmic delivery in the endocytotically active cells studied here is greater than 0.01% and less than 10% of the cell-associated liposomal contents. This is superior to other liposome compositions reported to date but still leaves much room for improvement.

Incorporating ligands onto the surface of pH-sensitive vesicles as has been done with antibodies (12,13) may increase the efficiency of cytoplasmic delivery. This is because cellular uptake of liposomes is more efficient by receptor-mediated endocytosis than by nonspecific adsorptive endocytosis (51) and the receptor–ligand binding may bring the liposome membrane and the endosome membrane into close proximity. The latter can accelerate the fusion between the two membranes (52). An alternative approach is to incorporate viral fusion proteins (53) or synthetic peptides (54) into the liposome bilayer, where they may assume a fusogenic conformation in the acidic endosome and induce fusion between the liposomal and the endosomal membranes (55). At present, we are exploring both approaches to increase the efficiency of cytoplasmic delivery from pH-sensitive liposomes.

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