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Interactions of mammalian cells with lipid dispersions containing novel metabolizable cationic amphiphiles

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Several novel cationic amphiphiles, based on a hydrophobic cholesteryl or dioleoylglyceryl moiety, have been prepared whose hydrophobic and cationic portions are linked by ester bonds to facilitate efficient degradation in animal cells. Dispersions combining such cationic species with phosphatidylethanolamine (PE), certain structural analogues of PE or diacylglycerol can mediate efficient transfer of both nonexchangeable lipid probes and the DNA plasmid pSV2cat into cultured mammalian (CV-1 and 3T3) cells. The abilities of different types of cationic lipid dispersions to mediate transfection of mammalian cells with pSV2cat could not be directly correlated with their abilities to coalesce with other membranes, as assessed by their ability to intermix lipids efficiently with large unilamellar phosphatidylcholine/phosphatidylserine vesicles in the presence or absence of DNA. The cytotoxicities toward CV-1 cells of dispersions combining PE with most of the degradable cationic amphiphiles studied here compare favorably with those reported previously for similar dispersions containing other types of cationic amphiphiles. Fluorescent analogues of two of the diacylglycerol-based cationic amphiphiles examined in this study are shown to be readily degraded after incorporation into CV-1 cells from PE/cationic lipid dispersions.

Introduction

Liposomes combining phospholipids with various cationic amphiphiles have been shown to interact with artificial and biological membranes with uniquely high efficiencies, and to mediate the transfer of both membrane-bound and surface-adsorbed molecules into cultured animal and plant cells [1–8]. Most of the cationic amphiphiles used to date to prepare such liposomes have been species lacking readily cleavable bonds (e.g., ester linkages) which would permit their efficient degradation by recipient cells after incorporation into cellular membranes. While such biochemical stability may be advantageous in some cases, the long-term persistence of unnatural cationic lipid species in cellular membranes may constitute a significant disadvantage in other potential applications of cationic liposomes. Various important regulatory processes in animal cells have been shown to be perturbed by cationic amphiphiles such as long-chain alkylamines and related compounds [9–13], and the surface charge of a membrane has been shown to be a generally important factor in regulating the activities of a variety of membrane-associated proteins [14–18]. The introduction of significant amounts of a biochemically stable cationic amphiphile into cellular membranes may therefore have undesirable long-term consequences for cellular function.

In the light of the above considerations, we have prepared a series of novel cationic amphiphiles, based on cholesteryl and diacylglyceryl hydrophobic moieties, that are designed to be readily susceptible to metabolic degradation after incorporation into animal cells. In

Abbreviations: CAT, chloramphenicol acetyltransferase; ChoSC, cholesteryl hemisuccinate choline ester; ChoTB, cholesteryl (4′-trimethylammonio)butanoate; CPS-, [[*N*-[4-[7-(diethylamino)-4-methylcoumarin-3-yl]phenyl]carbamoyl]methyl]thio; CPS-DASC, 1-palmitoyl-2-(12-CPS-stearoyl)-3-succinyl-*sn*-glycerol choline ester; CPS-DATB, 1-palmitoyl-2-(12-CPS-stearoyl)-3-(4′-trimethylammonio) butanoyl-*sn*-glycerol; (12-CPS)-18 PC, 1-palmitoyl-2-(12-CPS-stearoyl)phosphatidylcholine; (12-DABS)-18 PC, 1-palmitoyl-2-(12-[[4-[[4-(dimethylamino)phenyl[azo]phenyl]sulfonyl]methylaminostearoyl)-phosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; DOSC, 1,2-dioleoyl-3-succinyl-*sn*-glycerol choline ester; DOTAP, 1,2-dioleoyloxy-3-(trimethylammonio)propane; DOTB, 1,2-dioleoyl-3-(4′-trimethylammonio)butanoyl-*sn*-glycerol; D-MEM, Dulbecco's modified Eagle medium (supplemented with glutamine and gentamycin); EDTA, ethylenediaminetetraacetic acid, trisodium salt; Hepes, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; POPS, 1-palmitoyl-2-oleoylphosphatidylserine.

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this study, we describe the abilities of cationic lipid dispersions containing these compounds to interact with model membranes (lipid vesicles) and to mediate the transfer of both vesicle-bound lipids and vesicle-associated DNA to cultured animal cells. Our findings indicate that several of these species exhibit the useful properties reported previously for other types of cationic amphiphiles [1,3,5–8] and can moreover be efficiently metabolized by animal cells.

Materials and Methods

Materials

Dioleoylphosphatidylcholine and 1-palmitoyl-2-oleoylphosphatidylcholine were obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). Dioleoylphosphatidylethanolamine and 1-palmitoyl-2-oleoylphosphatidylserine were prepared from the corresponding phosphatidylcholines (via the phosphatidic acid in the latter case) using procedures described previously [19]. Oleoyl and 4-bromobutanoyl chlorides were freshly prepared from the corresponding carboxylic acids by reaction with oxalyl chloride. 3-Dimethylamino-1,2-propanediol, oxalyl chloride, 4-bromobutanoic acid and choline chloride were obtained from Aldrich (Minneapolis, MN). 4-Pyrrolidinopyridine (Aldrich) was recrystallized from ethyl ether/hexane and stored at −20 °C under nitrogen. Anhydrous trimethylamine (Eastman) was dissolved in dry chloroform to give a 40% stock solution, which was used in the preparations described below. Phospholipase C (*Bacillus cereus*) was obtained from Sigma (St. Louis, MO.). The DNA plasmid pSV2cat [20] was prepared by standard methods [21].

Syntheses

1,2-Dioleoyloxy-3-(trimethylammonio)propane (DOTAP). A solution of 75 mg (0.65 mmol) of 3-dimethylamino-1,2-propanediol, 60 μl of dry pyridine and 0.45 gm (1.6 mmol) of oleoyl chloride in dry diethyl ether (7.5 ml) was stirred for 40 h in the dark at 25 °C. The mixture was quenched with methanol, concentrated under nitrogen, redissolved in hexane (7.5 ml) and washed three times with 0.1 M KOH in 1:1 methanol/water (7.5 ml) at 0 °C, then once with 0.1 M aqueous NaCl. The hexane phase was concentrated under N_2 and applied in 99:1 (v/v) hexane/acetic acid to a 2.2 × 12 cm column of Bio-Sil A, which was eluted successively with 20% diethyl ether in hexane (200 ml), chloroform (3 × 100 ml) and 1% methanol in chloroform (5 × 100 ml). Pure 1,2-dioleoyloxy-3-(dimethylamino)propane eluted at 1% methanol: yield 326 mg (78%).

The product from the above procedure was reacted overnight in the dark with 400 μl methyl iodide in 3 ml each of chloroform and dry DMSO at 25 °C. The reaction mixture was repeatedly mixed with cyclohexane and reconcentrated under N_2 to remove excess methyl iodide, then partitioned between chloroform and 1:1 methanol/1 M aqueous NaCl. The chloroform layer was washed five times with methanol/aqueous NaCl, then concentrated under N_2 and applied in 99:1 (v/v) chloroform/acetic acid to a column of Bio-Sil A. The column was eluted with an ascending gradient of methanol in 99:1 chloroform/acetic acid, eluting pure DOTAP (287 mg = 63%) at 7% methanol.

1,2-Dioleoyl-3-(4′-trimethylammonio)butanoyl-sn-glycerol (DOTB). Dioleoylphosphatidylcholine (150 mg) was converted to 1,2-dioleoylglycerol by digestion for 5 h at 25 °C with 25 units of phospholipase C in a two-phase mixture of diethyl ether (4.5 ml) and 1 mM aqueous $CaCl_2$, 50 mM Tris-HCl (pH 7.3) (4.5 ml). Hexane (4.5 ml) was added, and the organic layer was washed three times with 1% aqueous NaCl, dried over Na_2SO_4 and concentrated under N_2. The recovered dioleoylglycerol was reacted overnight at 4 °C with 150 μl of 4-bromobutanoyl chloride in 3 ml of 99:1 chloroform/trimethylamine. The chloroform solution was washed twice with 1:1 methanol/0.2 M aqueous $NaHCO_3$, then concentrated under nitrogen. The residue was reacted with 3 ml of 20% trimethylamine in dry chloroform for 16 h at 25 °C, then the mixture was concentrated under N_2 and the products partitioned between chloroform and 1:1 methanol/0.1 M HCOONa (pH 3.0). The recovered crude 1,2-dioleoyl-3-(4′-trimethylammonio)butanoyl-*sn*-glycerol was applied to a column of Bio-Sil A, which was eluted with an ascending gradient of methanol in chloroform. The pure compound eluted at 8% methanol: yield 78 mg (55%).

Cholesteryl (4′-trimethylammonio)butanoate (ChoTB). 150 mg of cholesterol was reacted with 4-bromobutanoyl chloride in 99:1 chloroform/trimethylamine, then with 20% trimethylamine in chloroform under exactly the same conditions as described above for the preparation of DOTB from dioleoylglycerol. The final product was purified on a column of Bio-Sil A, eluting with a gradient of methanol in chloroform to yield pure cholesteryl (4′-trimethylammonio)butanoate at 8% methanol, in a yield of 123 mg (47%).

1,2-Dioleoyl-3-succinyl-sn-glycerol choline ester (DOSC). 1,2-Dioleoyl-*sn*-glycerol, freshly prepared from 100 mg dioleoyl phosphatidylcholine as described above, was converted to 1,2-dioleoyl-3-succinyl-*sn*-glycerol as described previously [22]. This material was reacted for 6 h in the dark at 25 °C with 150 mg of choline chloride, 100 mg of dicyclohexylcarbodiimide and 15 mg of 4-pyrrolidinopyridine in 3 ml of 1:1 dry chloroform/DMSO. The products were partitioned between chloroform and 1:1 methanol/1 M aqueous NaCl, and the chloroform phase was washed five times with fresh upper phase, then concentrated under N_2. Chromatography on Bio-Sil A, eluting with a gradient of methanol in chloroform, yielded pure 1,2-dioleoyl-3-succinyl-*sn*-

glycerol choline ester (48.3 mg, 45% yield) at 12% methanol.

Cholesterol hemisuccinate choline ester (ChoSC). 200 mg of cholesterol was reacted overnight with 150 mg succinic anhydride and 20 mg 4-pyrrolidinopyridine in 3 ml dry chloroform at 25° C. The products were partitioned between 2 : 1 hexane/chloroform and 1 : 1 methanol/water, and the organic layer was washed three times with methanol/water, then dried under N_2. The crude cholesterol hemisuccinate was converted to the choline ester as described above for the preparation of DOSC from dioleoylsuccinylglycerol, and the products were purified on a column of Bio-Sil A, eluting with a gradient of methanol in chloroform. Pure cholesterol hemisuccinate choline ester was eluted at 12% methanol: yield 127 mg (44%).

CPS-DATB and CPS-DASC were prepared starting from (12-CPS)-18 PC, using the procedures described above for the corresponding preparations of DOTB and DOSC from dioleoyl PC.

Methods

Except where otherwise indicated, lipid dispersions containing cationic lipid analogues were prepared by first bath-sonicating dried lipid samples in distilled water to clarity (2–5 min), then adding an equal concentration of 308 mM NaCl, 40 mM Hepes (pH 7.4), and sonicating further for 2 min. Lipid/DNA mixtures for transfections were prepared by incubating lipid dispersions with DNA for 5 min, typically at a total lipid concentration of 0.5–2 mg/ml, before addition to cell monolayers. Reverse-phase evaporation vesicles were prepared as described previously [23] and filtered through 0.1 μm pore-size Nucleopore filters. Lipid mixing between cationic lipid dispersions and large unilamellar PC/PS vesicles was assayed by a resonance energy transfer-based procedure as described previously [24], incubating cationic lipid dispersions (2 μM), colabeled with 1 mol% (12-CPS)-18 PC and 0.35 mol% (12-DABS)-18 PC, with a 9-fold excess (18 μM) of unlabeled PC/PS vesicles.

CV-1 cell monolayers for cytotoxicity and transfection assays were grown in D-MEM containing 5% fetal bovine serum. For assays of lipid cytotoxicity monolayers of CV-1 cells, grown in 6-well multiwell dishes to 25–35% or to 85% confluency, as indicated, were washed twice with 154 mM NaCl, 20 mM Hepes (pH 7.2), then incubated with varying concentrations of liposomes in the same medium for 90 min at 37° C. The medium was then drained off the cell monolayers and replaced, without washing, with D-MEM containing 5% serum. The cells were cultured for a further 36 h at 37° C, then the monolayers were washed twice with 154 mM NaCl, 40 mM Tris (pH 7.4) and solubilized with 154 mM NaCl, 40 mM Tris, 1 mM EDTA (pH 7.4) containing 1% sodium dodecyl sulfate. Protein was assayed using the procedure described by Markwell et al. [25].

For transfection of cells using cationic lipid dispersions, cell monolayers were washed twice with HEPES-buffered saline, then incubated with lipid/DNA mixtures in the latter medium (total volume 3 ml per 100-mm culture dish) at 37° C, for 90 min except where otherwise indicated. Following this incubation period, the incubation medium was either replaced or supplemented with 12 ml of D-MEM containing 5% serum, and the cells were cultured for a further 36 h before harvesting. Chloramphenicol acetyltransferase activity in plasmid pSV2cat-transfected cells was assayed as described previously [20], using chloramphenicol and acetyl-CoA concentrations of 3.5 μM and 2.5 mM, respectively. Conversion of D-*threo*-[*dichloroacetyl*-^{14}C]-chloramphenicol (Amersham) to acetylated products was assessed by autoradiography of thin-layer chromatograms and scintillation counting of spots cut from the chromatoplates. Cell transfections using DEAE-dextran were carried out as described previously [26].

For fluorescence microscopy, CV-1 cell monolayers grown on cover slips were viewed using a Zeiss epifluorescence microscope (model IM-35) with 40 × Planapo lens; samples were photographed using Kodak T-Max film (ASA 400).

Results

In Fig. 1 are shown the structures of the cationic lipid analogues that were examined in detail in this study. All of the cationic amphiphiles shown could be used to prepare aqueous dispersions, alone or in com-

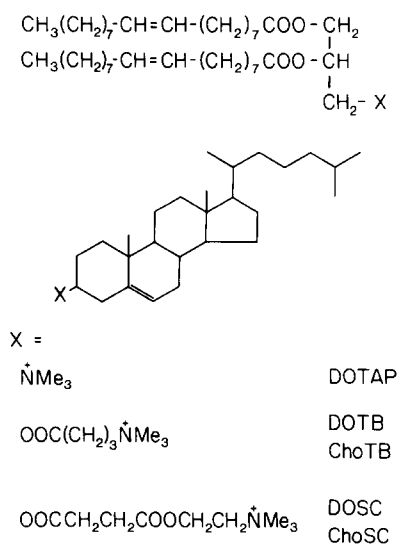

Fig. 1. Structures of the cationic lipid analogues examined in this study. DOTAP, DOTB and DOSC are based on a 1,2-(dioleoyloxy)-propane structure (racemic for DOTAP, derived from *sn*-glycerol for DOTB and DOSC), while ChoTB and ChoSC carry instead a cholesterol-derived hydrophobic moiety.

bination with phospholipids, which in most cases were stable at ionic strengths ranging up to at least physiological values. Several related compounds, including notably the betaine and (3′-trimethylammonio)propanoyl derivatives of cholesterol and 1,2-dioleoylglycerol, were also prepared in preliminary studies. However, these latter species were found to be markedly inferior to the species shown in Figure 1 in their ease of preparation and/or their chemical stability in aqueous dispersions, and they were not studied further.

Lipid mixing assays

We have shown previously (2,4) that large unilamellar vesicles prepared from DOTAP and phosphatidylethanolamine can rapidly intermix lipid components with choline phospholipid-rich natural or artificial membranes, which are comparatively refractory to such interactions with most other types of lipid vesicles. In the experiments illustrated in Fig. 2, we compared the abilities of different DOPE/cationic lipid dispersions (combining PE with cationic lipid in a 3 : 1 molar ratio) to intermix lipids with large unilamellar POPC/POPS vesicles. All of the types of DOPE/cationic lipid dispersions examined, when freshly prepared in a medium of

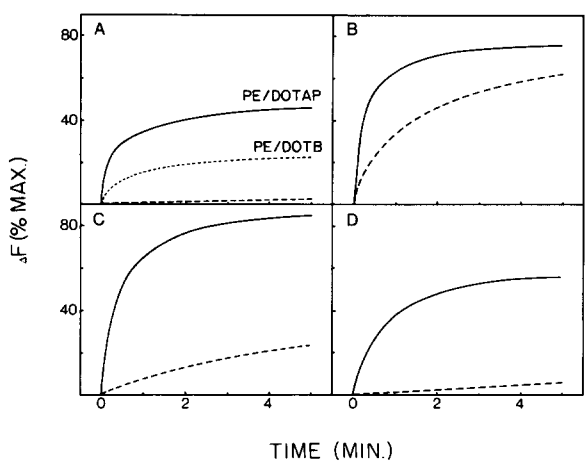

Fig. 2. Time courses of lipid mixing, monitored by the CPS-PC/DABS-PC fluorescence assay as described in the text, when various fluorescence-labeled DOPE/cationic lipid dispersions were mixed at zero time with unlabeled POPC/POPS vesicles. Cationic lipid mixtures, labeled with (12-CPS)-18 PC and (12-DABS)-18 PC, were dispersed by bath sonication in distilled water, then further sonicated for 2 min after addition of an equal volume of 308 mM NaCl, 40 mM Hepes (pH 7.4) and incubated for 5 min at room temperature in the presence or absence of plasmid pSV2cat (1 μg per 10 μg lipid). The dispersions (diluted to 2 μM lipid) were then mixed at 37 °C with a ninefold excess of 80:20 (molar proportions) POPC/POPS large unilamellar vesicles while recording the sample fluorescence. Panel (A). Solid curve: 3:1 DOPE/DOTAP, without DNA; upper dashed curve: 3:1 DOPE/DOTB, without DNA; lower dashed curve: 3:1 DOPE/DOTAP, with DNA. No lipid mixing was detected on this time scale using DOPE/DOTB dispersions preincubated with DNA. Panels (B)–(D). 3 : 1 DOPE/DOSC, DOPE/ChoTB and DOPE/ChoSC, respectively, with (dashed curves) or without (solid curves) DNA.

physiological ionic strength, showed rapid mixing of lipids with the POPC/POPS vesicles. However, the extent of this rapid lipid mixing varied with the nature of the cationic lipid component, decreasing in the order ChoTB > DOSC > ChoSC > DOTAP > DOTB. Interestingly, essentially the same sequence was found to describe the stabilities of these dispersions (i.e., their resistance to aggregation and precipitation) during prolonged incubations in media of physiological ionic strength (not shown).

Lipid-mixing experiments similar to those described above were also carried out using DOPE/cationic lipid dispersions that were preincubated with the DNA plasmid pSV2cat. As shown in Fig. 2, preincubation of the lipid dispersions for 5 min with the plasmid, at a DNA/lipid weight ratio of 1 : 10, strongly diminished their ability to undergo subsequent lipid mixing with POPC/POPS vesicles, to the extent that lipid mixing was virtually abolished for DOPE/DOTB and DOPE/DOTAP vesicles incubated with the plasmid. As little as 2% by weight of DNA was sufficient to produce a marked inhibition in the efficiency of lipid mixing between the cationic lipid dispersions and POPC/POPS vesicles (not shown). Results essentially equivalent to those shown in Fig. 2 were observed when the cationic lipid dispersions were incubated for 30 min rather than 5 min with the plasmid before the addition of unlabeled POPC/POPS vesicles.

The results of the lipid-mixing assays described above were mirrored in fluorescence-microscopic observations of the interactions of PE/cationic lipid dispersions with cultured CV-1 cells. In the experiment illustrated in Fig. 3, cell monolayers were incubated with PE/DOTB dispersions, labeled with (12-CPS)-18 PC (at a 65 : 25 : 10 molar ratio of DOPE/DOTB/(12-CPS)-18 PC), that were preincubated in the absence or presence of DNA. Cells incubated with the lipid dispersion alone in Hepes-buffered saline showed substantial incorporation of the labeled phosphatidylcholine into cellular membranes over a time scale of tens of minutes (Fig. 3A). By contrast, when the lipid dispersion was mixed with one-tenth by weight of DNA before addition to the cells, comparatively little transfer of the labeled lipid to the cells was observed (Fig. 3C). Large, highly fluorescent aggregates (of micron or larger dimensions) were observed adhering to the cover slip before washing when the cells were incubated with lipid/DNA mixtures but not when the cells were incubated with lipid alone. Results similar to those shown in Figure 3 were also observed using labeled DOPE/ChoTB or DOPE/DOTAP dispersions (not shown).

Cytotoxicity assays

For determinations of the cytotoxicity of various preparations of cationic liposomes toward CV-1 cells, cell monolayers were incubated for 90 min with lipid or

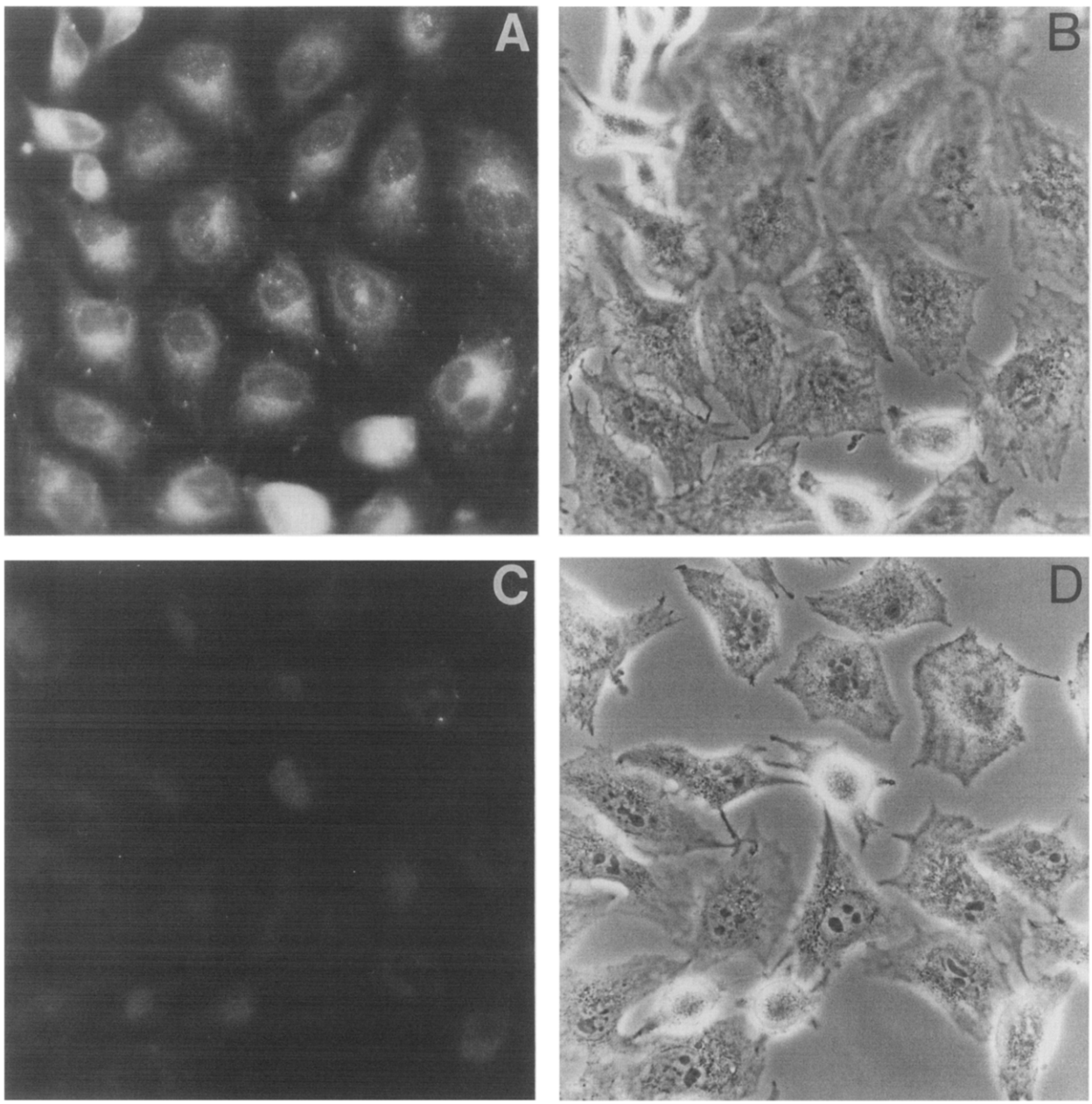

Fig. 3. Fluorescence (A, C) and phase-contrast (B, D) micrographs of CV-1 cell monolayers that were incubated for 45 min at 37 °C with a bath-sonicated 65:10:25 DOPE/(12-CPS)-18 PC/DOTB dispersion (15 μg/ml), then post-incubated for 45 min in lipid-free Hepes-buffered saline. (A, B). Cells were incubated with lipid alone. (C, D). Cells were incubated with lipid plus DNA (1 μg DNA per 10 μg lipid). Identical photographic conditions were used for panels (A) and (C).

lipid/DNA dispersions in Hepes-buffered saline, then serum-containing medium was added, and the cells were cultured for a further 36 h. The cell yield was then determined by protein assay as described in Materials and Methods. The results described below were obtained using dispersions combining DOPE with various cationic lipid analogues in a 3:1 molar ratio, since such dispersions delivered lipid probes and DNA to CV-1 cells at least as efficiently as did dispersions containing higher proportions of the cationic lipid component.

Near-confluent cell monolayers were found to be comparatively resistant to the cytotoxic effects of mixtures of DOPE and the novel cationic lipids examined here, in most cases showing little evidence of cytotoxicity up to at least 80–100 μg/ml lipid, in agreement with previous findings using other types of cationic lipid dispersions [3]. Subconfluent monolayers were appreciably more sensitive to incubation with cationic lipid/DNA complexes; cell yields were decreased by roughly one-half when monolayers at 25–35% con-

fluency were treated with < 10 μg/ml DOPE/ChoSC, 10 μg/ml of DOPE/DOSC, 30 μg/ml of DOPE/DOTAP, 100 μg/ml of DOPE/DOTB or > 200 μg/ml of DOPE/ChoTB dispersions containing pSV2cat (at 10:1 (w/w) lipid/DNA). Cationic lipid dispersions without DNA were somewhat more cytotoxic toward subconfluent cell monolayers (typically by 2–3-fold) than were lipid/DNA dispersions, possibly reflecting the greater efficiency of DNA-free dispersions in transfering their lipid components to cells (see above). Significantly lower cytotoxicity was observed when cells were incubated with the cationic lipid dispersions for up to several hours in Hanks' balanced salt solution or serum-free medium in place of Hepes-buffered saline.

Transfection of mammalian cells with pSV2cat bound to cationic liposomes

In preliminary experiments, optimal conditions were established for transfection of CV-1 cells with the plasmid pSV2cat using DOPE/DOTB or DOPE/DOTAP dispersions as carriers. For transfection of the cells with a fixed amount of plasmid (usually 2 or 5 μg per 100-mm culture dish), optimal expression of chloramphenicol acetyltransferase (CAT) activity was obtained using a 3:1 molar ratio of DOPE to cationic lipid and a lipid/DNA ratio of 10:1 (w/w). Essentially equivalent efficiencies of transfection were observed when the lipid/DNA mixtures were incubated together for times ranging from 2 to 30 min before application to the cell monolayers.

Using lipid/DNA mixtures prepared as described above, optimal transfection efficiencies were observed when CV-1 cells were incubated with the lipid and plasmid in Hepes-buffered saline (rather than D-MEM) for 90 to 180 min. At the end of this incubation period, the incubation medium could be either supplemented with four volumes of serum-containing medium or decanted and replaced with serum-containing medium, giving essentially equivalent final levels of CAT activity in either case. The level of CAT expression was significantly decreased if the cell monolayers were washed immediately after incubation with the lipid/DNA mixtures. Even gentle washing at this stage may remove a fraction of the cells, some of which typically become rounded, and possibly weakened in their attachment to the substrate, during incubation with the cationic lipid dispersions (see Fig. 3).

In Table I we compare the levels of CAT activity measured when dispersions combining dioleoyl PE with several different cationic lipid analogues, or the cationic polymer DEAE-dextran, were used as carriers to transfect CV-1 or 3T3 cells with the plasmid pSV2cat. Using the transfection conditions described above, the transfection efficiency varies with the structure of the cationic lipid analogue in the order DOTB > DOTAP > ChoTB > DOSC > ChoSC for CV-1 cells, and in the order DOTAP > DOTB > ChoTB for 3T3 cells. Dispersions containing DOTB, DOTAP and ChoTB varied somewhat in their relative efficiencies of transfection depending on the experimental conditions. For transfection of CV-1 cells in a D-MEM rather than a Hepes/NaCl medium, for example, the relative transfection efficiencies observed using DOTAP-, DOTB- and ChoTB-containing dispersions varied in the ratio 3.2:1.0:0.43. All of the cationic lipid dispersions examined, except those containing ChoSC, mediate much more efficient introduction of functional pSV2cat into either CV-1 or 3T3 cells than does DEAE-dextran (Table I).

TABLE I

Relative efficiencies of transfection of CV-1 and 3T3 cells with plasmid pSV2cat complexed to DOPE/cationic lipid dispersions or DEAE-dextran

Monolayers of CV-1 or 3T3 cells grown to 25–35% confluency were incubated with pSV2cat (5 μg per 100-mm culture dish) using as carrier either the indicated cationic lipid/DOPE dispersions (50 μg/dish) or DEAE-dextran, under the conditions described in Materials and Methods. After incubation for 90 min, the incubation medium was decanted and replaced with D-MEM containing 5% serum, and the cells were cultured for a further 36 h before determination of expressed CAT activity as described in the text. n.d., not determined.

Plasmid carrier	Relative efficiency [a] (DOTB/DOPE = 1.00) for	
	CV-1 cells	3T3 cells
DOTB/DOPE (1:3)	1.00	1.00
DOTAP/DOPE (1:3)	0.27	5.81
ChoTB/DOPE (1:3)	0.088	0.50
DOSC/DOPE (1:3)	0.029	n.d.
ChoSC/DOPE (1:3)	0.0029	n.d.
DEAE-dextran [b]	0.0045	0.048

[a] Activities shown are normalized to the level of CAT activity measured in the same experiments for cells transfected using DOPE/DOTB (3:1) dispersions as carrier (typically 5–8 nmol chloramphenicol acetylated per h per mg cell-extract protein using CV-1 cells, and 0.6–1 nmol chloramphenicol acetylated per h per mg cell-extract protein for 3T3 cells).

[b] Similar relative efficiencies of cellular transfection by DEAE-dextran vs. DOTB/DOPE dispersions were observed in experiments using cell monolayers at 85% confluency (not shown).

Efficient transfection of CV-1 cells could also be achieved using cationic lipids in combination with certain neutral lipids other than phosphatidylethanolamine. As shown in Table II, dispersions combining DOTB with either *N*-methyl DOPE or dioleoylphosphatidyl 2-amino-1-butanol mediate transfection of CV-1 cells by pSV2cat with efficiencies comparable to those observed using DOTB/DOPE dispersions. Vesicles combining DOTB with dioleoylglycerol in place of DOPE also mediate significant, though less efficient incorporation of lipid-associated pSV2cat into CV-1 cells, with cytotoxic effects no greater than those observed using DOPE/DOTB dispersions. A similar pat-

TABLE II

Relative efficiencies of transfection of CV-1 cells with plasmid pSV2cat complexed to different neutral lipid/DOTB or -ChoTB dispersions

Monolayers of CV-1 cells were transfected with pSV2cat (5 μg per 100-mm culture dish), using as carriers dispersions of the indicated mixtures of cationic and neutral lipids in a 1:3 molar ratio (50 μg/dish), and were subsequently assayed for CAT activity as described in Materials and Methods. Activities shown are normalized to the level of CAT activity measured in the same experiments for cells transfected using dispersions combining DOPE with the indicated cationic species (see Table I footnote a).

Neutral lipid component	Relative transfection yield (DOPE = 1.00) using codispersion with	
	DOTB	ChoTB
DOPE	1.00	1.00
Dioleoylphosphatidyl 2-amino-1-butanol	1.86	0.11
N-Methyl DOPE	2.34	0.26
1,2-Dioleoylglycerol	0.15	0.037

tern of results is obtained using vesicles combining the above neutral lipids with ChoTB in place of DOTB (Table II).

In parallel experiments, we examined the mixing of lipids between POPC/POPS large unilamellar vesicles and sonicated dispersions combining DOTB with the different neutral lipids noted above. The results of these experiments are summarized in Table III. In the absence of DNA, all of these types of cationic lipid dispersions exhibit substantial lipid mixing with PC/PS vesicles, with rates that vary considerably with changes in the neutral lipid component. In the presence of DNA (at a 1:10 weight ratio of DNA to lipid), the rate of lipid mixing is drastically reduced in all cases. The lipid-mixing rates given in Table III for different lipid dispersions, in the absence or presence of DNA, show no apparent correlation with the relative efficiencies with which such dispersions mediate transfection of CV-1 cells with pSV2cat (Tables I and II).

TABLE III

Lipid mixing between bath-sonicated cationic lipid dispersions and large unilamellar PC/PS vesicles in presence and absence of DNA

Dispersions of the indicated cationic lipid mixtures, labeled with (12-CPS)-18 PC and (12-DABS)-18 PC, were prepared as described in the legend to Fig. 2. Subsequent lipid mixing between these vesicles (2 μM) and a 9-fold excess of 80:20 POPC/POPS large unilamellar vesicles at 37°C was monitored as described in Materials and Methods.

Lipid dispersion	Lipid mixing rate (% max./min.)	
	−DNA	+DNA
DOTB/DOPE (1:3)	70	< 0.1
DOTB/dioleoylphosphatidyl 2-amino-1-butanol (1:3)	50	0.4
DOTB/*N*-methyl DOPE (1:3)	181	3.8
DOTB/1,2-dioleoylglycerol (1:3)	811	< 0.1
ChoTB/DOPE (1:3)	355	12.7
ChoTB/dioleoylphosphatidyl 2-amino-1-butanol (1:3)	219	10.8
ChoTB/*N*-methyl DOPE (1:3)	294	6.4
ChoTB/1,2-dioleoylglycerol (1:3)	511	14.2

Metabolism of cationic lipid analogues

In a final series of experiments, we examined the ability of fluorescent analogues of DOTB and DOSC to undergo metabolic conversion to other lipid species after introduction into CV-1 cells. The fluorescent probes used for these experiments, designated CPS-DATB and CPS-DASC, were derived from a diacylglycerol moiety carrying a palmitoyl chain at the 1-position and a coumarin-labeled stearoyl chain at the 2-position. Strong fluorescence labeling of cellular membranes, with little discernible residual adsorption of unincorporated lipid aggregates on cell surfaces, was observed by fluorescence microscopy when CV-1 cell monolayers were incubated with CPS-DASC- or CPS-DATB-containing dispersions for 1 h at 37°C in Hepes-buffered saline, then incubated for a further 1 h

TABLE IV

Catabolism of fluorescent analogues of DOTB and DOSC incorporated into CV-1 cells from DOPE/cationic lipid dispersions

CV-1 cell monolayers were incubated with lipid dispersions containing the fluorescent cationic species for 1 h in Hepes-buffered saline, then incubated successively for 1 h in lipid-free saline and for 2 h or 4 h in D-MEM without serum.

Fluorescent analogue	Total incubation time (h)	% migrating after incubation [a] as		
		unmodified compound [b]	lower-R_F derivative [b]	neutral lipids [b]
CPS-DATB	4	34.4	39.1	26.5
	6	28.0	41.5	30.5
CPS-DASC	4	34.6	12.6	52.8
	6	22.4	14.3	63.2

[a] After the indicated incubations the cells were harvested, and the lipids were extracted and analyzed by TLC as described in the text. The percentages given represent the relative amounts of the three major fluorescent products identified in the lipid extracts; smaller amounts of several other fluorescent products (see text) were not quantitated.

[b] The following R_F values were measured for these species in the solvent system 50:15:10:10:5 (by vol.) $CHCl_3$/acetone/CH_3OH/CH_3COOH/H_2O: 0.51, 0.30 and > 0.90, respectively, for CPS-DATB, its lower-R_F derivative and the fraction designated 'neutral lipids'; and 0.47, 0.29 and > 0.90, respectively, for CPS-DASC, its lower-R_F derivative and the 'neutral lipid' fraction. For reference, (12-CPS)-18 PC migrated with an R_F of 0.30 in this system, although it migrated somewhat differently from the lower-R_F derivatives of CPS-DATB and CPS-DASC in other solvent systems.

in the same medium after removal of unadsorbed cationic lipid.

In Table IV are summarized the results of thin-layer chromatographic analyses of the lipids extracted from CV-1 cells that were incubated as just described with DOPE/cationic lipid dispersions containing CPS-DASC or CPS-DATB, then postincubated for 2 or 4 h in serum-free D-MEM. Three major fluorescent spots were observed by thin-layer chromatography in each case, corresponding to unconverted CPS-DATB or CPS-DASC, neutral lipids (migrating near the solvent front in the chromatographic system employed), and a third, slower-moving compound that may represent the products of cleavage of the palmitoyl chain from the 1-position of the parent compounds. Considerably smaller amounts of several other fluorescent compounds, which migrated just ahead of the major unlabeled phospholipid spots, were also identified in these samples. As indicated in Table IV, very substantial degradation of both CPS-DASC and CPS-DATB is observed even at relatively short times after these cationic lipid probes are added to CV-1 cells in the form of PE/cationic lipid dispersions.

Discussion

The novel cationic amphiphiles examined in this study differ from those described in most previous work [1,3,5–7] in that the hydrophobic and the cationic portions of the molecules are linked through readily cleavable ester bonds, which should allow efficient intracellular metabolism of the cationic species after their incorporation into animal cells. In agreement with this suggestion, fluorescent analogues of DOTB and DOSC were shown to be substantially metabolized within a few hours after introduction into CV-1 cells. It is possible that the metabolic conversion of these compounds is in fact even more efficient than our experimental results would suggest, since lipid incorporation into cell membranes from adsorbed lipid aggregates is demonstrably a gradual process (as observed by fluorescence microscopy) and may least partly limit the rate of metabolism of these cationic lipids under our experimental conditions. The cytotoxicities of most of the cationic species examined in this study toward confluent cell monolayers compare favorably with those reported previously for other cationic amphiphiles [3,6,28–31].

As has been demonstrated previously for PE/DOTAP vesicles [2,4], dispersions combining DOPE with the various cationic lipid species studied here intermix lipids efficiently with PC/PS liposomes and mediate efficient transfer of nonexchangeable fluorescent lipids into the membranes of cultured cells. The addition of even small amounts of DNA to these cationic lipid dispersions (at 2–10% by weight, or approx. 0.17–0.85 mol DNA phosphate per mol cationic lipid) sharply reduces their ability to intermix lipids with negatively charged lipid vesicles and cellular membranes. A similar inhibition of lipid mixing could be produced by adding polyvalent anions (phosphate or EDTA) to the cationic lipid dispersions, or even in some cases by incubating the dispersions for prolonged periods with physiological concentrations of monovalent cations alone (results not shown). These effects probably reflect the growth of the cationic lipid aggregates by extensive aggregation and/or fusion, to a point where lipid mixing with other membranes becomes very inefficient.

Lipid dispersions containing several of the cationic amphiphiles examined here (particularly DOTB, DOTAP and ChoTB) mediate transient transfection of CV-1 and 3T3 cells with the plasmid pSV2cat with efficiencies much greater (by ten- to two hundred-fold) than those observed using DEAE-dextran. A comparable efficiency of transfection (relative to DEAE-dextran) has been reported when CV-1 cells are treated with pSV2cat complexed to liposomes combining DOPE with the cationic lipid analogue DOTMA [3]. Vesicles containing 1 : 1 DOPE/DOTAP have in fact been found to transfect LTA (mouse fibroblast) cells with the plasmids pSV0cat and pSV2cat with efficiencies 2–4-fold greater than those measured using a commercially available preparation of 1 : 1 DOPE/DOTMA under identical conditions (Raymond, M. and Gros, P., personal communication).

While the ability of cationic liposomes to mediate introduction of nucleic acids into eukaryotic cells is well-documented, the precise mechanism of this process remains unclear. The observations presented in this study strongly suggest, in agreement with some previous proposals [6,32], that the mechanism of transfer of DNA to animal cells from cationic liposomes may not entail a simple fusion of liposomal and cellular membranes. This conclusion is suggested by several lines of evidence. First, DNA is observed virtually to abolish the ability of DOPE/DOTAP and DOPE/DOTB dispersions to intermix lipids with lipid vesicles, yet such dispersions are nonetheless highly effective in mediating the uptake of DNA by CV-1 and 3T3 cells. Second, we find no direct correlation between the abilities of different types of cationic lipid dispersions to intermix lipids with POPC/POPS model membranes, either in the absence or presence of DNA, and the abilities of such dispersions to mediate transfection of CV-1 cells with pSV2cat. This is true both when we compare lipid dispersions combining DOPE with different cationic lipids and when we compare lipid dispersions combining DOTB or ChoTB with different neutral lipids. While such results do not preclude the possibility that some transfer of DNA from cationic liposomes to the cytoplasm of animal cells may occur by a fusion-related

mechanism, they suggest that such a mechanism may be at best inefficient under the conditions used to transfect cells with DNA/cationic lipid complexes. It is conceivable that DNA is transfered to the cytoplasm from cationic liposomes primarily by other mechanisms, e.g., through transient destabilization of endocytic vesicles containing DNA/cationic lipid complexes, or even possibly through mediated uptake of DNA by a process similar to that recently suggested for the uptake of intact oligonucleotides by animal cells [33,34].

Further study will clearly be required to define fully the mechanism(s) by which cationic lipid dispersions interact with animal cells and transfer liposome-associated molecules to various intracellular compartments. A better understanding of the nature of these processes, and of how their efficiencies depend on the composition and physical properties of the 'carrier' lipid dispersion, may allow significant further improvements in the utility of cationic lipid dispersions for delivery of polyanionic or lipophilic molecules (and possibly other types of bioactive molecules as well) to animal cells. The availability of readily degradable cationic amphiphiles, such as those examined in this study, offers the potential to extend the use of cationic lipid carrier systems to include a variety of potential applications in which the persistent incorporation of unphysiological cationic amphiphiles into cellular membranes is undesirable.

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