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Synergistic Effect between Components of Mixtures of Cationic Amphipaths in Transfection of Primary Endothelial Cells

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Abstract: To date, the primary approach to improving the transfection properties of cationic lipids has been the synthesis of new kinds of cationic amphipaths. Recently, however, it was found that combining two cationic lipid derivatives having the same head group but tails of different chain lengths can provide another, and often superior, approach to higher transfection efficiency. For example, the combination of medium-chain and long-chain homologues of *O*-ethylphosphatidylcholine transfected DNA into primary human umbilical artery endothelial cells (HUAECs) more than 30-fold more efficiently than did either compound separately. Here it is reported that this synergism of mixtures is not limited to *O*-ethylphosphatidylcholine homologues, but is also exhibited by other common cationic amphipathic transfection reagents; for example, combining DC-Chol (3β-[*N*′,*N*′-dimethylaminoethane)-carbamol] cholesterol), dimethylditetradecylammonium bromide, or DMTAP (1,2-dimyristoyl-3-trimethylammonium-propane) with EDOPC increased transfection significantly both in the absence and in the presence of serum. Furthermore, combining a poorer transfection agent—dimethyldioctadecylammonium bromide—with dimethylditetradecylammonium bromide increased transfection by about an order of magnitude with a maximum at an intermediate composition. Lack of synergy occurred with some mixtures, such as DMTAP and DOTAP (1,2-dioleoyl-3-trimethylammonium-propane), in which case transfection activity was a linear function of composition both in the absence and presence of serum. Although the mechanism of enhanced transfection by mixtures is not fully understood, the existence of a number of optimal mixtures with diverse cationic compounds indicates that attention to mixture formulations can lead to greatly improved transfection by cationic amphipathic carriers.

Keywords: EDOPC; DC-Chol; DMTAP; DOTAP; ditetradecyldimethylammonium bromide; dioctadecyldimethylammonium bromide

Introduction

Cationic lipids have been widely used for the delivery of plasmid and antisense DNA and siRNA into eukaryotic cells.[1−5] Compared to viral vectors, cationic lipids have several advantages, such as relatively low immune responses, a very high DNA-carrying capacity, and ease of production. Despite the promise of cationic lipids as gene vectors, however, their transfection efficiency remains low, and much research activity has been aimed at increasing efficiency. To date, the primary approach to improving the transfection properties of cationic lipids has been synthesis of new kinds

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of cationic amphipaths, although inclusion of noncationic helper lipids, such as phosphatidylethanolamine or cholesterol, in established cationic lipids has also been pursued to a limited extent. Hundreds of cationic lipids have been developed so far, and enormous attention has been given to altering the head group of these compounds.[6] In our previous study,[7] however, we showed that an alternative strategy can be very effective, namely, use of a mixture of cationic homologues with the same head group but different chain lengths. In particular, EDLPC (1,2-dilauroyl-*sn*-glycero-3-ethylphosphocholine) dramatically enhanced the transfection by EDOPC (1,2-dioleoyl-*sn*-glycero-3-ethylphosphocholine) of HUAECs (human umbilical artery endothelial cells).

To extend that research, we investigated whether cationic lipids other than *O*-ethylphosphatidylcholine derivatives can also have synergistic effects in mixtures. Here we report that other commonly used cationic lipids, such as DC-Chol (3β-[*N*′,*N*′-dimethylaminoethane)-carbamoyl] cholesterol), dimethylditetradecylammonium bromide, and DMTAP (1,2-dimyristoyl-3-trimethylammonium-propane), when combined with EDOPC, enhanced dramatically the transfection of HUAECs relative to that of either component independently. In addition, similarly to the combination of EDLPC and EDOPC, the combination of ditetradecyl (14-carbon chain) and dioctadecyl (18-carbon chain, DDAB) homologues of dimethylammonium bromide also transfected DNA into HUAECs more efficiently than either compound separately. Nevertheless, mixtures did not invariably yield optimal transfection activity. For example, when EDOPC was combined with DDAB or DOTAP (1,2-dioleoyl-3-trimethylammonium-propane), transfection did not improve significantly. Likewise, in mixtures of DMTAP and DOTAP, transfection by DMTAP increased monotonically with DOTAP content. Possible mechanistic insights are provided by the observations that increased transfection correlated positively with fusion between transfection lipids and anionic liposomes. Not surprisingly, DNA uptake by the nucleus of treated cells also correlated positively with transfection activity.

Experimental Methods

Materials. EDOPC triflate salt was synthesized as described.[8] (EDOPC chloride, which has similar transfection activity, is available commercially from Avanti Polar-Lipids, Inc. (Alabaster, AL).) DC-Chol, DDAB, DMTAP, DOTAP, NBD-PE (NBD-phosphatidylethanolamine), Rh-PE (lissamine rhodamine B-phosphatidylethanolamine), DOPG (1,2-dioleoyl-*sn*-glycero-3-[phosphor-*rac*-(1-glycerol)]), and egg PC were obtained from Avanti. Dimethylditetradecylammonium bromide was from Sigma (St. Louis, MO). CMV-β-galactosidase plasmid was purchased from Clontech Laboratories Inc. (Palo Alto, CA) and propagated and purified by Bayou Biolabs (Harahan, LA). A fluorescein-labeled 12-base double-stranded oligodeoxyribonucleotide (the "Dickerson" dodecamer) was purchased from IDT Inc. (Coralville, IA). HBSS (HEPES buffered saline solution) and Opti-MEM were obtained from Cambrex (Walkersville, MD) and Invitrogen Corporation (Carlsbad, CA), respectively.

Cell Culture. Human umbilical artery endothelial cells (HUAECs) were obtained from Cambrex (Walkersville, MD) and maintained in EGM-2 MV containing 5% fetal bovine serum (FBS) (Cambrex) at 37 °C with 5% CO_2. At confluence, the cells were passaged using 0.25 mg/mL Trypsin/EDTA (Cambrex) and were used at passages 5−10 for these experiments.

Transfection. The cells were seeded in 96-well plates at 24 h before transfection at densities appropriate to give about 80% confluence at the time of transfection. Cationic lipids were hydrated in HBSS and diluted in Opti-MEM, then pipetted into an equal volume of plasmid DNA solution (also diluted in Opti-MEM) at a 3:1 weight ratio or 1.03:1 molar ratio as indicated at final DNA concentration of 20 μg/mL under vortexing. The resultant DNA−lipid complexes were incubated at room temperature for 15 min and then added to cells that were either in medium lacking serum or in medium containing serum. At 2 h after their addition, the lipoplexes were removed and fresh medium containing serum was added. Cells were assayed for β-galactosidase activity 24 h after transfection with a microplate fluorometric assay,[9] or X-gal staining was done to determine the number of transfected cells histochemically according to the procedure provided by Invitrogen Life Technologies (Carlsbad, CA).

Membrane Fusion Assay. Membrane fusion of fluorescently labeled, cationic lipoplexes with anionic liposomes was measured using a FRET procedure.[10] The lipoplex lipids were labeled with 0.5 mol % each of NBD-PE and Rh-PE and hydrated at 1 mg/mL in PBS. Lipoplexes were then

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prepared as described above. Two hundred microliters of the resulting lipoplex dispersion was treated with a 3-fold molar excess of unlabeled egg PC liposomes containing 20% DOPG. The experiments were done at 37 °C in a spectrofluorometer with excitation and emission wavelengths of 320 and 535 nm, respectively. Calculation was as follows: % membrane fusion = $(Fn - F_0)/(F_{100} - F_0) \times 100\%$, where Fn is the fluorescence after the addition of anionic liposomes, F_0 is the initial fluorescence of lipoplexes, and F_{100} is the fluorescence when anionic lipid was mixed directly with cationic lipids in chloroform and then lipoplexes were prepared as above.

Fluorescence Microscopy. Lipoplexes were prepared as described above with the indicated cationic lipids and a fluorescein-labeled, 12-base, ds oligonucleotide and incubated with cells for 2 h in the presence of serum. The lipoplexes were then removed, and the distribution of oligonucleotide was imaged under a fluorescence microscope (Nikon DIAPHOT-TMD).

Results and Discussion

The structures of the cationic amphipaths used in the experiments, all commonly used as transfection reagents, are shown in Figure 1. They are the phospholipid derivative EDOPC, the cholesterol derivative DC-Chol, the ditetradecyl (14-carbon chain) and the dioctadecyl (18-carbon chain) homologues of dialkyldimethylammonium bromide, and the dimyristoyl (14-carbon chain) and the dioleoyl (18-carbon chain) homologues of 1,2-diacyl-3-trimethylammonium-propane.

We first describe the variation of transfection activity as a function of the composition of mixtures of the cationic amphipaths listed above. Since it is not at all surprising that, if one transfection agent is better than another by itself, then adding the former to the latter one should increase activity, we focus here on how transfection increases upon adding the poorer to the better transfection agent; that is, the increase in transfection activity is expressed as relative to the better transfection agent (obviously comparison of the mixture with the poorer transfection agent would give a much greater increase). Exceptions to this procedure were EDOPC/DOTAP and DMTAP/DOTAP, for which transfection activities are described relative to EDOPC or DMTAP, although DOTAP has higher transfection activity in both cases (details are given below). Figure 2 shows that combining EDOPC with DC-Chol enhances by 4.5- and 6.7-fold the extent of transfection of HUAECs in the absence or presence of serum, respectively, compared to DC-Chol alone. The ratio of EDOPC to DC-Chol is important, and different ratios are optimal, depending upon whether serum is present or absent, i.e., optimal transfection in the absence of serum was obtained at a lipid weight ratio of 90/10, whereas in the presence of serum, optimal activity was at 60/40.

Figure 3 depicts the transfection of HUAECs by combinations of EDOPC with dialkyldimethylammonium compounds, namely, dimethylditetradecylammonium bromide and dimethyldioctadecylammonium bromide. Similar to the

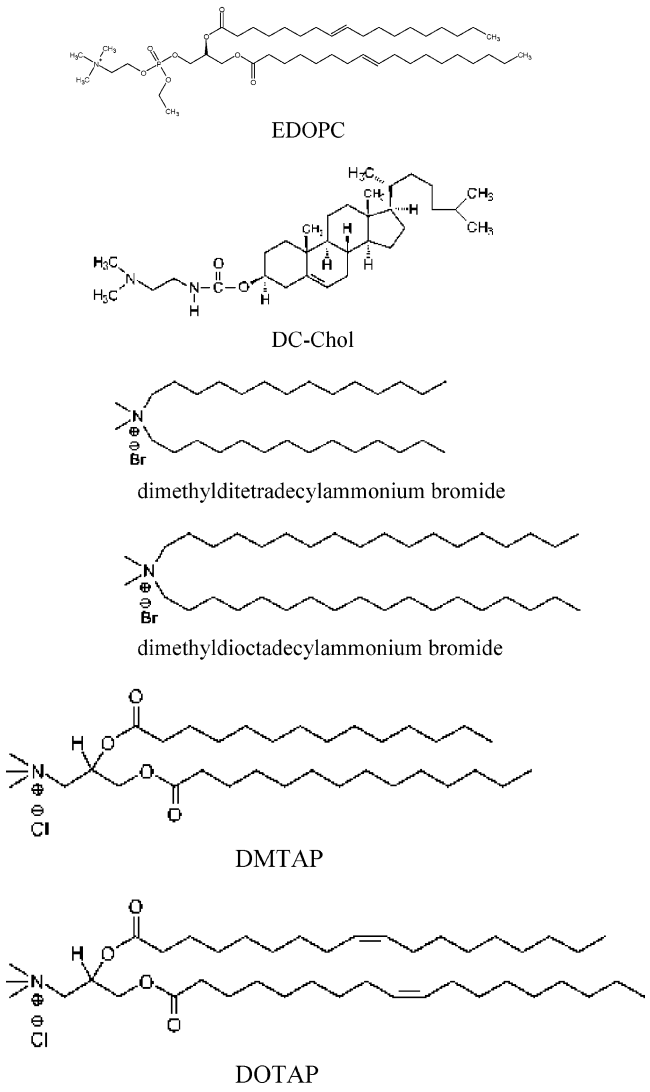

Figure 1. Structures of the cationic lipids used in this investigation.

combination of EDOPC/DC-Chol, the mixture EDOPC/dimethylditetradecylammonium bromide exhibited a pronounced maximum at intermediate compositions, depending upon serum concentration. Specifically, transfection was increased by 12.5- and 3.3-fold in the absence and presence of serum, respectively, compared to that of dimethylditetradecylammonium bromide alone. A difference from the results of Figure 2 is that transfection both in the presence and in the absence of serum was maximal at the same composition, namely, when the ratio of EDOPC to dimethylditetradecylammonium bromide was 30/70 (w/w). In the case of the mixture of dimethyldioctadecylammonium bromide/dimethylditetradecylammonium bromide, optimal transfection in the absence of serum was obtained at a weight ratio of 70/30, and transfection was enhanced 8.8-fold compared to dimethylditetradecylammonium bromide alone. Optimal transfection in the presence of serum was obtained at a weight ratio of 40/60, and transfection was enhanced 2.3-fold compared to dimethylditetradecylammonium bromide alone. It is noteworthy that, in the case of the EDOPC/

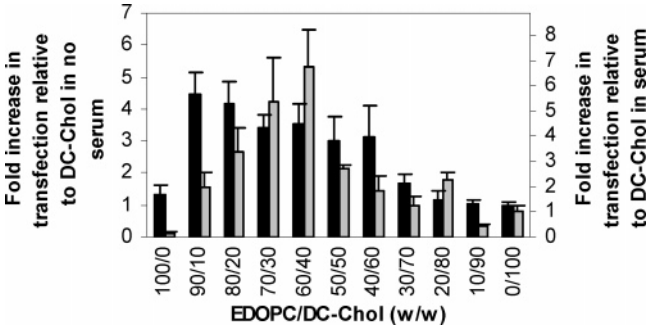

Figure 2. Transfection of HUAECs by mixtures of EDOPC with DC-Chol at a weight ratio of total lipid to DNA of 3:1. The figure shows increased transgene expression relative to that of DC-Chol in the presence (gray bars) or absence of serum (black bars). Data represent the mean ± SD of quadruplicate wells in one typical experiment. The β-Gal expression of HUAECs with EDOPC lipoplexes in the absence of serum was ~30 microunits per well.

dimethyldioctadecylammonium bromide mixture, a large jump in activity occurred with the addition of a small portion of the second component, specifically, 10% dimethyldioctadecylammonium bromide in EDOPC (absence of serum) and 10% EDOPC in dimethyldioctadecylammonium bromide (presence of serum). Transfection activity remained relatively constant for all intermediate compositions.

The next combinations to be described involve EDOPC combined with the dialkyltrimethylammonium propane compounds, DOTAP and DMTAP, as well as the combination of the two TAP compounds with each other. Figure 4 shows that the transfection−composition relationship of EDOPC/DMTAP was bell-shaped: like that of EDOPC/EDLPC, transfection in the absence or presence of serum was maximal at a ratio of EDOPC/DMTAP of 80/20 or 60/40, at which compositions the transfection activity had increased by 11- or 31-fold, respectively. Synergism was not exhibited by the EDOPC/DOTAP mixture, although DOTAP itself had a good transfection activity. Instead, transfection by DMTAP/DOTAP followed a different pattern, in which activity increased monotonically with DOTAP content.

As indicated, the data above were expressed as the fold increase relative to the better transfection agent. The extent to which the transfection agents are more efficient depends upon the particular combination, however, so it is difficult to directly compare the transfection efficiency of different combinations from different figures. To address this problem, Table 1 summarizes the transfection efficiency of the optimal combinations expressed as fold increase relative to the single compound, EDOPC.

Among all the lipid compositions tested, the formulations with the highest transfection activity in serum, EDOPC/dimethylditetradecylammonium bromide (30/70), EDOPC/DC-Chol (60/40), and EDOPC/DMTAP (60/40), were chosen for determination of transfection efficiency (percentage of cells expressing detectable galactosidase) by X-gal staining. The percentages of X-gal positive cells were 17% for EDOPC/dimethylditetradecylammonium bromide (30/70)

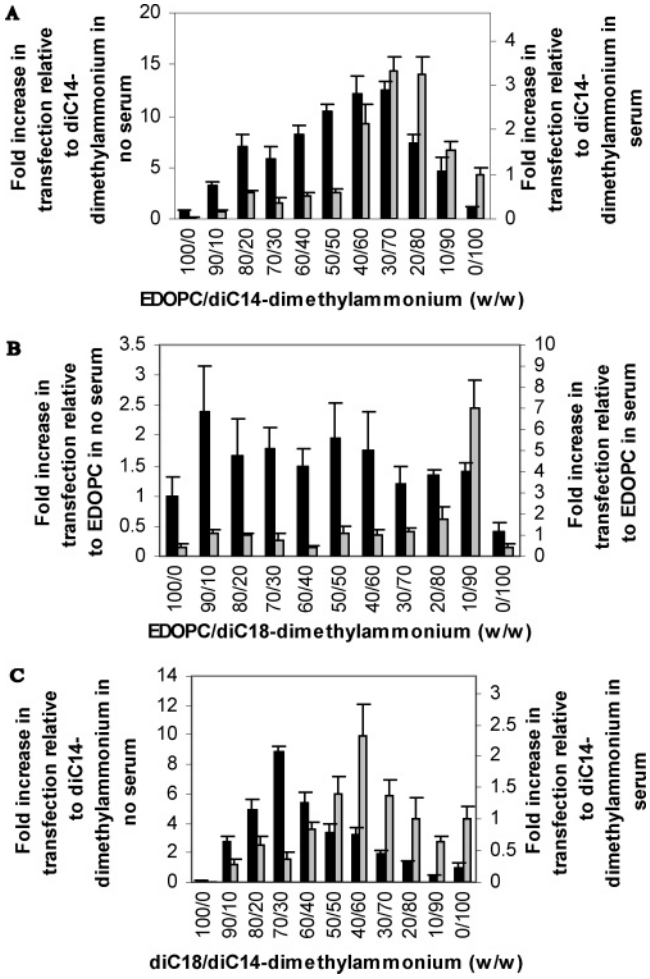

Figure 3. Transfection of HUAECs by mixtures containing dimethyldialkylammonium bromides at a constant total lipid/DNA weight ratio of 3/1. (A) EDOPC and dimethylditetradecylammonium bromide. (B) EDOPC and dimethyldioctadecylammonium bromide. (C) Dimethylditetradecylammonium bromide and dimethyldioctadecylammonium bromide. The figure shows the increase in transgene expression relative to that of dimethylditetradecylammonium bromide in A and C and relative to EDOPC in B, all in the presence (gray bars) and absence of serum (black bars). Data represent the mean ± SD of quadruplicate wells in one typical experiment.

and 8% for both EDOPC/DC-Chol (60/40) and EDOPC/DMTAP (60/40). The transfection efficiency of pure EDOPC for these cells was less than 1%.

Phosphatidylethanolamine and cholesterol have been widely used as helper lipids to improve the transfection properties of various cationic lipids. For EDOPC, at least in the case of HUAECs, we did not find any significant effect of including DOPE (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine) in the lipid formulation; nor did inclusion of DOPE in DOTAP formulations increase the transfection of HUAECs (unpublished data).

In the experiments described above, the weight ratio of lipids to DNA was held constant at 3:1. This ratio is optimal for EDOPC on HUAECs, but because there is some

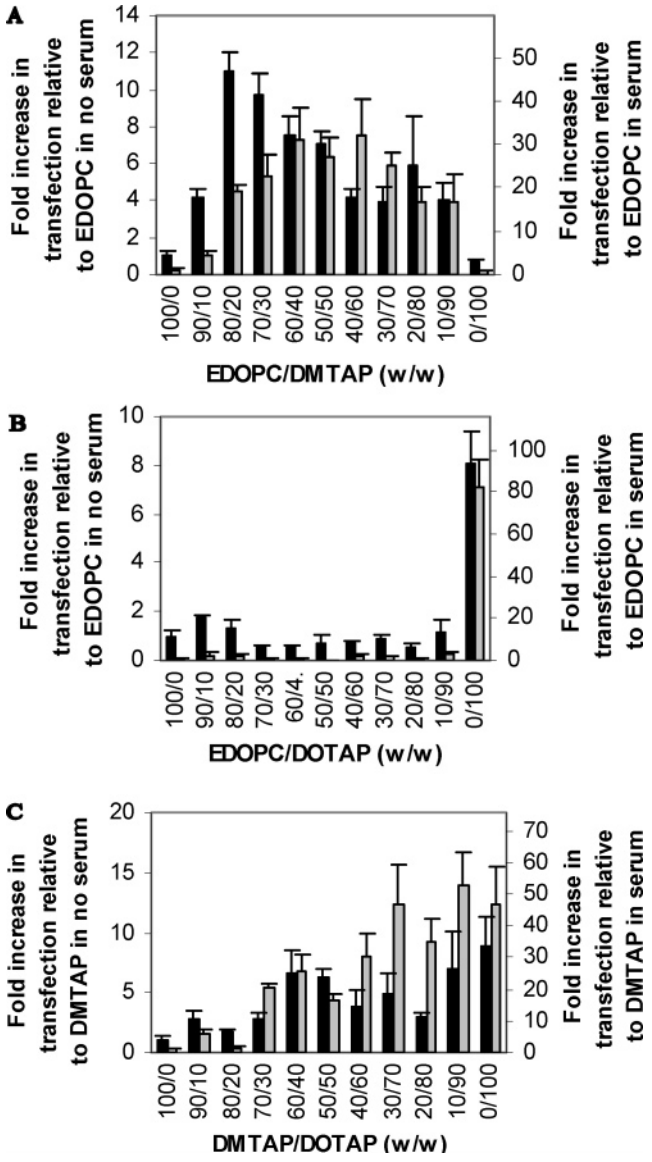

Figure 4. Transfection of HUAECs by mixtures containing diacylTAP compounds at a constant total lipid/DNA weight ratio of 3/1. (A) EDOPC and DMTAP. (B) EDOPC and DOTAP. (C) DMTAP and DOTAP. The figure shows transgene expression relative to that of EDOPC in A and B and relative to DMTAP in C, all in the presence (gray bars) or absence of serum (black bars). Data represent the mean ± SD of quadruplicate wells in one typical experiment.

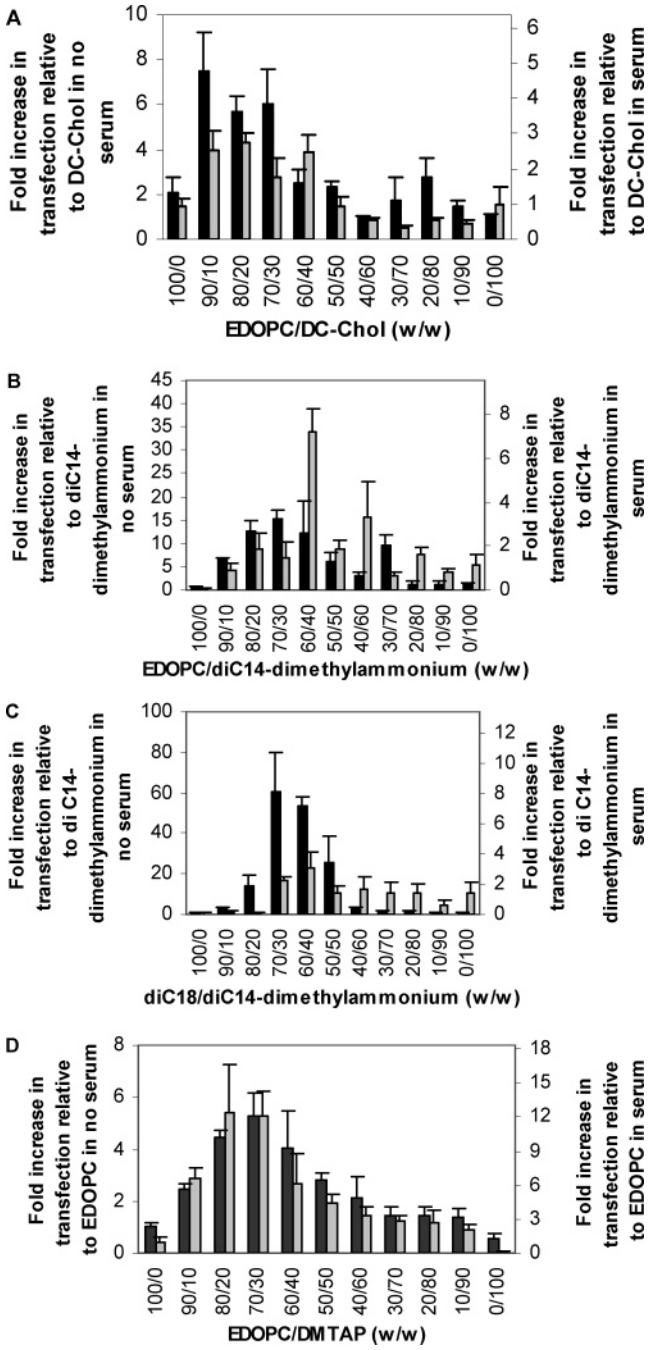

Figure 5. Transfection of HUAECs by mixtures of EDOPC/DC-Chol (A), EDOPC/diC14-dimethylammonium (B), diC18-/diC14-dimethylammonium (C), and EDOPC/DMTAP (D). The procedure was similar to that of Figures 2, 3, and 4 except that the molar ratio rather than the weight ratio of lipids to DNA was held constant. Gray and black bars denote transfection in the presence and absence of serum, separately. Data represent the mean ± SD of quadruplicate wells in one typical experiment.

difference between the MW of EDOPC and that of the other compounds, in the case of mixtures, the molar ratio varied somewhat as a function of the composition of lipids (as shown in Table 2). In order to assess whether transfection activity could have been qualitatively altered by the change of +/− charge ratio, transfection activity as a function of lipid composition was measured at a constant lipid/DNA molar ratio. For these determinations, those mixtures showing a bell-shaped transfection−composition relationship were chosen, namely, EDOPC/DC-Chol, EDOPC/diC14-dimethylammonium, diC18-/diC14-dimethylammonium and EDOPC/DMTAP. Again, the increase in transfection activity is expressed relative to the better transfection agent. As shown in Figure 5, similar relationships were found in these experiments as were found in the experiments in which the lipid/DNA weight ratio was held constant, although the composition of maximal transfection efficacy was slightly shifted.

Table 1. Summary of the Transfection Efficiency of the Optimized Combinations

combinations	serum	fold increase relative to EDOPC
EDOPC/DC-Chol (90/10)	−	3.1
EDOPC/DC-Chol (60/40)	+	39.8
EDOPC/diC14-dimethylammonium (30/70)	−	19.3
EDOPC/diC14-dimethylammonium (30/70)	+	117.2
EDOPC/DMTAP (80/20)	−	11.0
EDOPC/DMTAP (60/40)	+	31.3

In some cases, another parameter that changes as a function of composition of the mixtures is the anionic counterion. For example, EDOPC is the triflate salt, DC-Chol, DMTAP, and DOTAP are chloride salts, while diC14- and diC18-dimethylammonium are bromide salts. The influence on transfection activity of the counterion has been examined previously, and, indeed, it was demonstrated[11] that the counterion does have an influence on transfection, with activity decreasing in the order triflate ∼ bromide > chloride. Even though these effects were demonstrable, they were nevertheless much smaller than those described here, with activity decreasing only 50% as a consequence of replacing triflate with chloride analogue. In addition to the fact that the known counterion effects are much smaller than the mixture synergism effect described here, the change in counterion as a function of composition is linear and cannot therefore account for the bell-shaped activity−composition curve that characterizes mixture synergism. It is thus reasonable to conclude that the synergism described here for lipid mixture cannot be attributed to differences in the anionic counterion.

For efficient transfection, it is essential that the plasmid DNA be released from the cationic lipids and become accessible to the transcription apparatus.[4,12] Once taken up by cells through endocytosis, cationic lipids from lipoplexes must fuse with endosome/lysosome membranes, and subsequently, perhaps also, other cellular membranes, so that the DNA can become sufficiently free to enter the nucleus and be transcribed there.[13−16] We thus determined whether formulations with better transfection activity fused more readily with anionic lipid bilayers than did formulations with lesser activity. For this investigation, those formulations with the highest transfection activity in serum, EDOPC/DC-Chol (60/40) (∼40-fold increase compared to EDOPC in serum), EDOPC/dimethylditetradecylammonium bromide (30/70) (∼117-fold increase compared to EDOPC in serum), and EDOPC/DMTAP (60/40) (∼31-fold increase compared to EDOPC in serum), were chosen for comparison in terms of fusion activity with EDOPC. The lipoplexes were prepared at a weight ratio of total lipid to DNA of 3:1. The fusion partner of these cationic compositions in the fusion assay was anionic liposomes containing 20% DOPG in egg PC. Figure 6 shows that both the rate and the extent of fusion of those formulations with the highest transfection activity were much higher than that of pure EDOPC.

DNA must be released from a lipid aggregate and enter the nucleus to be expressed. Although the experiments described above show that formulations with higher transfection activity also had higher membrane fusion activity, membrane fusion does not necessarily ensure that the DNA is released from the lipoplexes, because DNA could presumably unbind from the lipid surface but remain entrapped in the lipid matrix generated by the mixing of the anionic and cationic lipids. Indeed, the absence of a necessary correlation between the extent of lipid mixing of cationic lipoplexes with anionic cellular membranes and the transfection activity of those lipoplexes has been observed by other investigators.[17−21] So, to assess release of DNA, we examined the intracellular distribution of fluorescent oligonucleotide in cells treated with different lipoplex formulations. We chose an oligonucleotide for these experiments because oligonucleotides are not only more readily released from lipoplexes but also more rapidly taken up by the nucleus. Figure 7 reveals that the oligonucleotide of EDOPC lipoplexes remained in the

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Table 2. Molecular Ratio of Lipid to DNA at a Weight Ratio of 3:1 for Different Compounds

	EDOPC	DC-Chol	diC14-dimethyl-ammonium	diC18-dimethyl-ammonium	DMTAP	DOTAP
molar ratio of lipids to DNA	1.03	1.84	1.91	1.57	1.68	1.42

cytoplasm, largely in small compartments, whereas the oligonucleotide from formulations with better transfection activity, such as EDOPC/DC-Chol (60/40), EDOPC/dimethylditetradecylammonium bromide (30/70), and EDOPC/DMTAP (60/40), more frequently escaped from the lipoplexes and entered nuclei. Two hundred cells were counted, and we found that the percentage of cells with distinct fluorescence in their nuclei was 0% for EDOPC, 20% for EDOPC/DC-Chol (60/40), 8% for EDOPC/dimethylditetradecylammonium bromide (30/70), and 15% for EDOPC/DMTAP (60/40).

A marked increase in the transfection activity of EDOPC in HUAECs was first observed in mixtures with EDLPC, a homologue with the same head group but a shorter chain length than EDOPC. As shown here, however, other common cationic lipids that, like EDLPC, have shorter chains and/or a smaller hydrophobic mass, such as DC-Chol, dimethylditetradecylammonium (14-carbon chain) bromide, and DMTAP (14-carbon chain), also synergistically increased the transfection activity of EDOPC. It appears that it is not the mixture *per se* that is important, but the mixture with a less hydrophobic lipid, because the mixtures of EDOPC with longer chain length analogues, such as DDAB (18-carbon chain) and DOTAP (18-carbon chain), did not exhibit significantly enhanced transfection activity. Moreover, mixing EDMPC with a longer chain lipid EdiC22:1PC had no beneficial effect (unpublished data), indicating that it is the shorter chain, not the difference in chains that makes the difference. These findings indicate that a synergistic effect of components in mixtures is not limited to *O*-ethylphosphatidylcholine derivatives, and that the presence of the second cationic lipid influences the properties of the lipoplex in a way that apparently involves an optimal combination of hydrophobic volumes of the lipid. A similar mixture effect was seen with the ditetradecyl (14-carbon chain) and dioctadecyl (18-carbon chain) homologues of dimethylammonium bromide, where the combination transfected DNA more efficiently than either compound separately and intermediate compositions exhibited maximal transfection activity. Thus, hydrophobicity is clearly very important; however, it should be emphasized that it not clear whether it is the average amount of hydrophobicity or its distribution within the biliayer (a heterogeneous distribution is possible in a mixture of two molecules with dissimilar hydrophobic moieties) that is critical. Furthermore, the transfection activity of mixtures of dimyristoyl (14-carbon chain) and dioleoyl (18-carbon chain) homologues of trimethylammonium propane, which increased monotonically with DOTAP content, indicates that, besides the hydrophobic moiety, the hydrophobic−hydrophilic balance of cationic lipids is also critical for transfection activity.

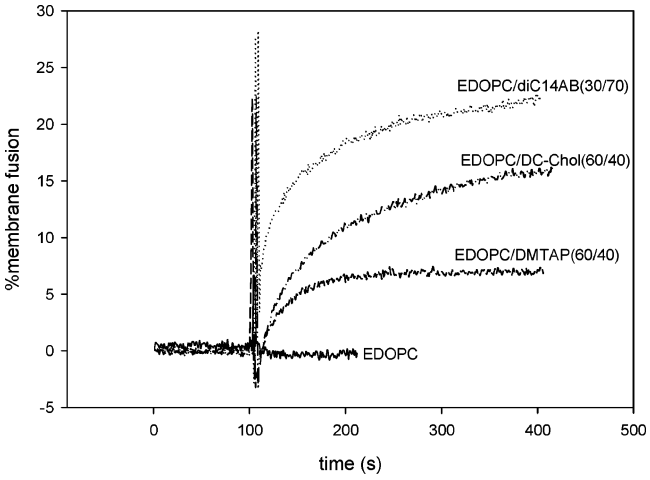

Figure 6. Fusion of fluorescence-labeled, cationic lipoplexes with anionic liposomes. The cationic lipids were labeled with 0.5 mol % each of NBD-PE and Rh-PE and were treated with a 3-fold molar excess of unlabeled egg PC liposomes containing 20% DOPG. Fusion of 100% corresponds to complete mixing of the anionic and cationic lipids.

Experiments on fusion of cationic lipids with anionic lipids and intracellular distribution of the fluorescent oligonucleotide in cells treated with different cationic lipids showed that the formulations with higher transfection activity had greater fusion capability and led to more DNA uptake by cell nuclei. They do not, however, explain why the inclusion of certain other cationic lipids in lipoplexes containing EDOPC should significantly change their membrane fusion ability. One general consideration is that including two cationic lipids instead of one increases the degrees of freedom in lipoplex−membrane interactions and could allow a larger range of membrane curvature, which is likely to be important in membrane fusion. Also, some of our studies[22,23] have shown that the way in which lipoplexes evolve structurally after contacting and exchanging lipid with cellular membranes is a critical factor for the DNA unbinding from lipoplex surfaces and for overall lipoplex performance. Specifically, we found a relationship between the transfection efficiency of a given cationic lipid and the mesomorphic phase generated upon mixing that lipid with anionic membrane lipids; formulations that are effective DNA carriers form phases of high negative interfacial curvature (inverted hexagonal, bilayer cubic, and inverted micellar cubic) when mixed with anionic lipids, whereas less effective formulations form phases of lower curvature. It is clear that mixtures of cationic lipids with dissimilar hydrophobic tails should allow

(22) Tarahovsky, Y. S.; Koynova, R.; MacDonald, R. C. DNA release from lipoplexes by anionic lipids: Correlation with lipid mesomorphism, interfacial curvature, and membrane fusion. *Biophys. J.* **2004**, *87*, 1054−1064.

(23) Koynova, R.; Wang, L.; Tarahovsky, Y.; MacDonald, R. C. Lipid phase control of DNA delivery. *Bioconjugate Chem.* **2005**, *16*, 1335−1339.

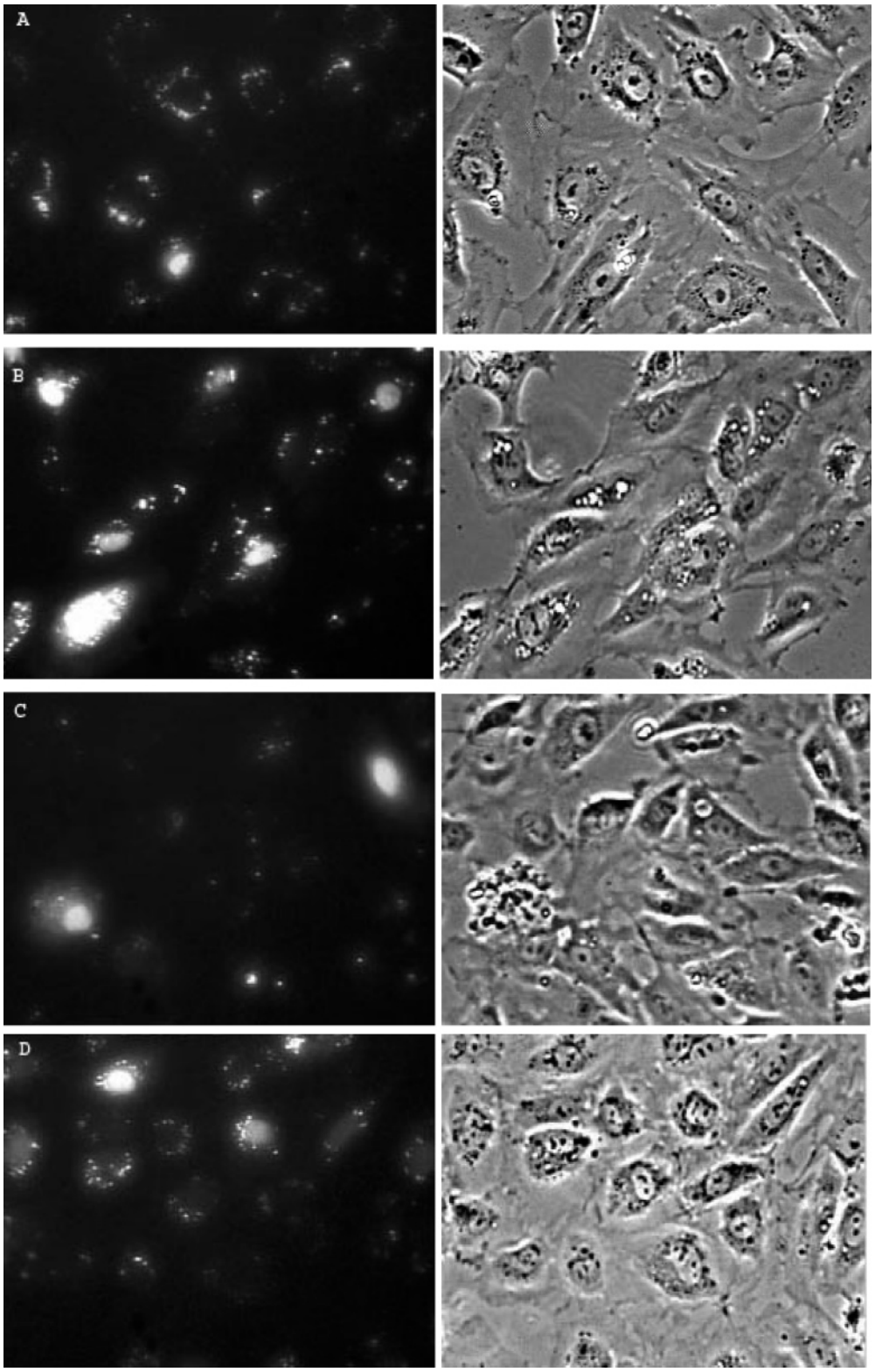

Figure 7. Distribution of fluorescent oligonucleotide in cells treated with various lipoplex formulations. Lipoplexes were prepared by combining the indicated cationic lipids with a fluorescein-12-base oligonucleotide and incubated with cells for 2 h in the presence of serum. The lipoplexes were then removed by washing, and the distribution of oligonucleotide was determined by imaging 3 h later under a fluorescence microscope. (A) EDOPC. (B) EDOPC/DC-Chol (60/40). (C) EDOPC/dimethylditetradecylammonium bromide (30/70). (D) EDOPC/DMTAP (60/40). The left column is the fluorescence image, and that on the right is phase contrast bright field.

increased possibilities for curvature, so if the curvature of the phase from which DNA escapes is critical, then the proper choice of lipid mixtures could offer numerous new possibilities for optimizing lipoplex-based DNA delivery. Further studies are under way to test this hypothesis.

Another incompletely understood aspect of the mixture effect is that its magnitude depends upon the cell line; mixtures containing EDOPC have a significantly larger synergistic effect on HUAECs than on the other cell lines we have tested, namely, MLE-12 (mouse lung epithelial cells) and A549 (human lung carcinoma epithelial cells). For those cells, EDOPC is an effective transfection agent in the absence of other cationic compounds. If lipoplex−cell membrane fusion is rate limiting or if phase changes occur upon mixing of lipoplex lipids with cell membrane lipids, then the differences in transfection of different cell types could be attributable, at least in part, to variations in amounts and types of lipids from one cell type to another.

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