Optimization of Lipid Composition in Cationic Emulsion as *In Vitro* and *In Vivo* Transfection Agents

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Purpose. To enhance *in vitro* and *in vivo* transfection activity by optimizing lipid composition of cationic lipid emulsions.

Methods. Various emulsion formulations having different cationic lipids as emulsifiers, and additional helper lipids as co-emulsifiers, were prepared. The stability of the emulsion and its complex with DNA was investigated by measuring the particle size change in phosphate buffer saline (PBS) over a period of 20 days. The activity of the emulsions in transfecting pCMV-beta into COS-1 cells in the presence or absence of 80% serum was evaluated. We also evaluated *in vivo* transfection activity using intravenously administered pCMV-Luc+ as a reporter gene.

Results. Among the cationic emulsifiers, 1,2-dioleoyl-*sn*-glycero-3-trimethylammonium-propane (DOTAP) formed the most stable and efficient emulsion gene carrier. Addition of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) increased *in vitro* transfection activity, but slightly compromised the stability of the emulsion. The loss was compensated for by including small amounts of Tween 80 in the emulsion. The *in vitro* and *in vivo* transfection activities were also increased by adding Tween 80. Even though *in vitro* transfection activity of liposomes was high in the absence of serum, the transfection activity of emulsions was far greater than that of liposomes in the presence of serum and for *in vivo* applications.

Conclusions. By including DOPE as an endosomolytic agent and Tween 80 as a stabilization agent, the cationic emulsion becomes a more potent gene carrier for *in vitro* and *in vivo* applications, especially in the presence of serum.

KEY WORDS: Gene transfer; DOTAP; DOPE; nonionic surfactant; poly(ethylene glycol).

INTRODUCTION

Gene transfer into adult animals resulting in generalized or tissue-specific expression could allow precise *in vivo* manipulations of biological processes to cure diseases and to induce immune responses against pathogens (1,2). Various nonviral gene delivery systems have been prepared to accomplish gene therapy and a DNA vaccine. Cationic liposomes have been widely used in gene transfer both *in vitro* and *in vivo.* To date, many cationic lipids have been synthesized in the hope of finding one with a high transfection activity and a low cytotoxicity (3–5).

Since Felgner introduced the first liposomal transfection agent (3), many scientists have sought to formulate a better and more efficient liposomal transfection agent (4). One of the most important features of a successful liposomal gene carrier is the cationic lipid itself (5). Some have proven to be good agents in the absence of serum for *in vitro* transfection, while at the same time failing for *in vivo* applications. Although the manner in which cationic lipids change transfection activity has not been studied systematically, attempts have been made to understand the underlying mechanism (6,7).

A cationic lipid formulation usually requires additional components to enhance its *in vitro* and *in vivo* transfection activity (8). 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) is one such example, especially for *in vitro* applications (4,5,8). Due to its well-known fusogenic properties, DOPE has been used to facilitate endosomal escape (9). Recently, nonionic surfactants, such as Tween 80, have been used as additional additives in cationic liposomes, presumably to enhance its physical stability (8,10). It has been reported that the stability-enhanced liposomes have a better transfection activity, especially under *in vivo* conditions (6,11).

In our previous work, we have shown that the oil-in-water (o/w) cationic emulsion system is physically stable and can successfully facilitate the transfer of genes in the presence of up to 90% (v/v) serum in COS-1 and CV-1 cells (12). This stable emulsion delivered genes *in vivo* more efficiently to endothelial cells in the mouse nasal cavity than commercialized liposomes (13).

In this article, we describe a process for selecting lipid components of defined composition that produces high *in vitro* and *in vivo* transfection activity. We do so by adopting the principles used to develop liposomal transfection agents. Liposomes and emulsions are similar in many respects. While lipids form bilayer leaflets that separate inner and outer aqueous phases in liposomes, they are used as emulsifiers and distribute mainly at the interface between the inner oil and the outer aqueous phases in o/w emulsions. Various emulsion formulations with different cationic lipids as emulsifiers, and additional helper lipids as co-emulsifiers, were prepared as gene carriers. Throughout this article, we compare the transfection activity of emulsions and liposomes made with an identical lipid composition excepting the oil component, squalene. We will refer to the latter as 'counterpart liposome' in this paper. The two carriers show a profound difference in transfection activity.

MATERIALS AND METHODS

Materials

Squalene, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Tween 80, 7 lauryl ether (Brij 7), and pluronic F68 (F 68) were purchased from Sigma Chemical Company (St. Louis, MO). 1,2-dioleoyl-*sn*-glycero-3-trimethylammonium-propane (DOTAP), 1,2-dimyristoyl-*sn*-glycero-3-trimethylammonium-propane (DMTAP), 1,2-dipalmitoyl-*sn*-glycero-3-trimethylammonium-propane (DPTAP), 1,2-distearoyl-*sn*-glycero-3-trimethylammonium-propane (DSTAP), dimethyldioctadecylammonium bromide (DDAB), 1,2-dioleoyl-*sn*-glycero-3-ethylphosphocholine (DOEPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-

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ethanolamine-N-[polyethylene glycol) 2000] ($PEG_{2000}PE$) were purchased from Avanti Polar Lipids, (Alabaster, AL) and were used without further purification. Dulbecco's modified Eagle's medium (DMEM) (Cat. No. 1600-034) and fetal bovine serum (FBS) were purchased from Gibco BRL/Life Technologies (New York, NY). All other chemicals and reagents were of tissue culture grade.

Plasmid DNA

The pCMV-beta encoding *Escherichia coli* (*E. coli*) *lac*Z (β-galactosidase) gene expression plasmid driven by the human cytomegalovirus immediate-early promoter was purchased from Clontech Laboratories (Palo Alto, CA). The plasmid pCMV-Luc+ consists of a cytosolic form of *Phontinus pyralis* luciferase cDNA, which was obtained from the plasmid pGL3 (Promega, Madison, WI) using Xba I and HindIII and was subcloned into the plasmid pcDNA3.1 (Invitrogen) in our laboratory. The plasmids were amplified in the *E. coli* DH5-α strain and purified by using a Qiagen mega-kit (Qiagen Inc., Chatsworth, CA) according to the manufacturer's instructions. DNA purity was determined by agarose gel electrophoresis and by measuring optical density. DNA having $OD_{260}/OD_{280} \geq 1.8$ was used.

Preparation of Lipid Carriers

The emulsions contained 100 μl/ml squalene and various compositions of lipid emulsifiers (Table 1). Emulsions were prepared as described previously (12). Briefly, lipid emulsifiers were weighed, dispersed in water, and sonicated until clear in an ice/water bath by using a probe type sonicator (High intensity ultrasonic processor, 600W model, Sonics and Materials, Danbury, CT). The lipid dispersion was added to the oil and the mixture was sonicated further in an ice/water bath for ca. 4 min to form the emulsion. To prepare liposome carriers, the lipid dispersions were sonicated for another 4 min after the solution became clear. They were stored at 4°C until used. The average particle size and the surface potential of the lipid carriers (liposome or emulsions) or the DNA/carrier complexes were measured by using photon correlation spectroscopy (Malvern Zetasizer, Malvern Instruments Ltd., England) as described previously (12). The emulsion and liposomes were diluted 300 and 3 times, respectively, with water or phosphate buffered saline (PBS) for the measurement. Short-term stability of the emulsions was monitored by measuring the time-dependent absorbance changes at 600 nm in PBS for 20 days (14).

Cell Culture

A simian kidney cell line (CV-1), its derivative (COS-1), and a mouse embryo fibroblast cell (NIH3T3) were cultured in DMEM supplemented with 10% FBS at 37°C in humidified 5% carbon dioxide incubator. A human large cell lung carcinoma, H1299, was also cultured in RPMI1640 with 10% FBS under the same growth condition as above. Cells were seeded at 2×10^4 cells per well on to 96-well plates 12 hours before transfection. Cells were *ca.* 70–80% confluent at the time of transfection.

In Vitro Gene Transfer

In this paper, the following terms will be used to describe the carrier-to-DNA ratios: C/D, the weight ratio between cationic lipid in lipid formulation and DNA in the complex; C/D_{min}, the minimum C/D ratio to form a carrier/DNA complex without showing free DNA on agarose gel; and C/D_{opt}, the C/D ratio that shows the highest transfection activity.

For a single well, 500 ng of pCMV-beta were mixed with an appropriate amount of carrier ($C/D = 1 \sim 28$) to form a complex in 40 μl of serum-free DMEM. After washing the COS-1 cells with serum-free DMEM, 160 μl of serum-free DMEM were added. To test the effect of serum, 160 μl of FBS was added instead of 160 μl of serum-free DMEM. After one hour of incubation, the cells were washed with serum-free DMEM to remove the remaining carrier/DNA complexes. The cells were fed again with DMEM containing 10% (v/v)

Table 1. Size, Polydispersity, and Zeta Potential of Emulsion Having Different Lipid Compositions

Emulsifier composition				
Type/Identity	Concentration (mg/ml)	Size (nm)	Polydispersity	Zeta Potential (mV)
DOEPC	28.0	149.6	0.189	65.2 ± 3.4
DDAB	21.6	153.5	0.201	79.7 ± 6.4
DMTAP	20.3	131.2	0.161	57.8 ± 4.8
DPTAP	22.2	139.8	0.138	46.5 ± 3.2
DSTAP	24.1	179.3	0.168	57.4 ± 1.3
DOTAP	24.0	143.5	0.154	64.9 ± 5.5
DOTAP/DOPE	21.8/2.2	156.6	0.168	62.3 ± 3.1
DOTAP/DOPE	20.0/4.0	164.5	0.156	62.5 ± 2.4
DOTAP/DOPE	18.0/6.0	304.1	0.321	61.2 ± 2.7
DOTAP/DOPE	12.0/12.0	508.1	0.528	55.8 ± 3.4
DOTAP/DOPE/Brij 7	20.0/4.0/1.7	174.8	0.203	55.9 ± 2.5
DOTAP/DOPE/F 68	20.0/4.0/28.4	232.2	0.254	53.4 ± 3.2
DOTAP/DOPE/$PEG_{2000}PE$	20.0/4.0/9.4	180.2	0.201	57.4 ± 3.8
DOTAP/DOPE/Tween 80	20.0/4.0/2.3	174.7	0.188	60.2 ± 2.1
DOTAP/DOPE/Tween 80	20.0/4.0/4.5	156.4	0.145	58.5 ± 2.6
DOTAP/DOPE/Tween 80	20.0/4.0/9.0	141.5	0.186	61.3 ± 3.0
DOTAP/DOPE/Tween 80	20.0/4.0/22.5	98.4	0.284	59.4 ± 2.4

FBS and cultured for 24 hours after transfection. The transfected cells were assayed for β-galactosidase activity using a photometric assay as described (5).

In Vivo Gene Transfer and Luciferase Assay

To prepare carrier/DNA complexes, DNA solution containing 10 μg of pCMV-Luc+ and the carrier, whose amount corresponds to C/D_{opt}, each diluted with 100 μl of PBS, respectively, were mixed by inversion. The complex solution was incubated at room temperature for 20 min and was injected into female Balb/c mice weighing approximately 20–25 g (6–8 weeks old) via the tail vein. The mice were sacrificed after 24 h, and organs such as heart, lung, liver, kidney, and spleen were removed and homogenized by using a high-speed homogenizer (T-25-Ultra-Turrax, Janke & Kunkel GmbH & Co KG, Germany) in lysis buffer (0.1 M Tris-HCl, 2 mM EDTA, 0.1% Triton X-100, pH 7.8, 5 μl/mg for each collected organ). After two freeze/thaw cycles, the homogenized organ lysates were centrifuged at 4°C for 10 min at 12,000 rpm in an Eppendorf centrifuge. A portion of the supernatants was assayed for protein concentration by using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). Luciferase activity in the lysates was quantified by using a Promega kit (Madison, WI, USA) with a luminometer (Turner Designs Luminometer Model TD-20/20, Promega).

RESULTS AND DISCUSSION

Cationic Lipid Changes *In Vitro* Transfection Activity of Emulsions

We have selected three commercially available cationic lipids—DOTAP, DOEPC, and DDAB—as emulsifiers. The concentration of emulsifier was set to 35 mM, and squalene at 10% (v/v) was used as an oil phase in the emulsions. Counterpart liposomes made with these three cationic lipids were also prepared for comparison. Five hundred nanograms of pCMV-beta were mixed with the carrier to form a complex at C/D = 4. The complexes were applied to COS-1 cells with or without 80% serum for *in vitro* transfection.

Among emulsions of the 3 cationic lipids, the DDAB-containing emulsion showed the lowest transfection activity while the DOTAP-containing emulsion (DOTAP emulsion hereafter) had the highest activity. In the presence of 80% serum, all of the three emulsions retained *ca:* 50–70% of their transfection activities compared to the serum-free condition (Fig. 1A). The situation was dramatically different in liposomes (Fig. 1B). Even though liposomes generally exhibited higher transfection activity than emulsions in the absence of serum, the transfection activity of liposomes was reduced to a barely detectable level by adding 80% serum. The transfection activity of emulsions far surpassed that of liposomes in the presence of serum. This result agrees well with that in our previous papers (12).

It has been widely acknowledged that the acyl chain length and the degree of unsaturation affect transfection activities of liposomes (5). To test whether the same principle holds in case of emulsions, we have prepared emulsions using a series of monovalent cationic lipids, 1,2,-diacyl-*sn*-glycero-3-trimethylammonium-propanes as cationic emulsifiers. DMTAP (with 14 carbons in each acyl chain with zero unsaturation; denoted c14:0), DPTAP (c16:0), and DSTAP (c18:0) at 35 mM were used as emulsifiers. Squalene at 10% (v/v) was used as the oil phase. Counterpart liposomes were prepared for comparison.

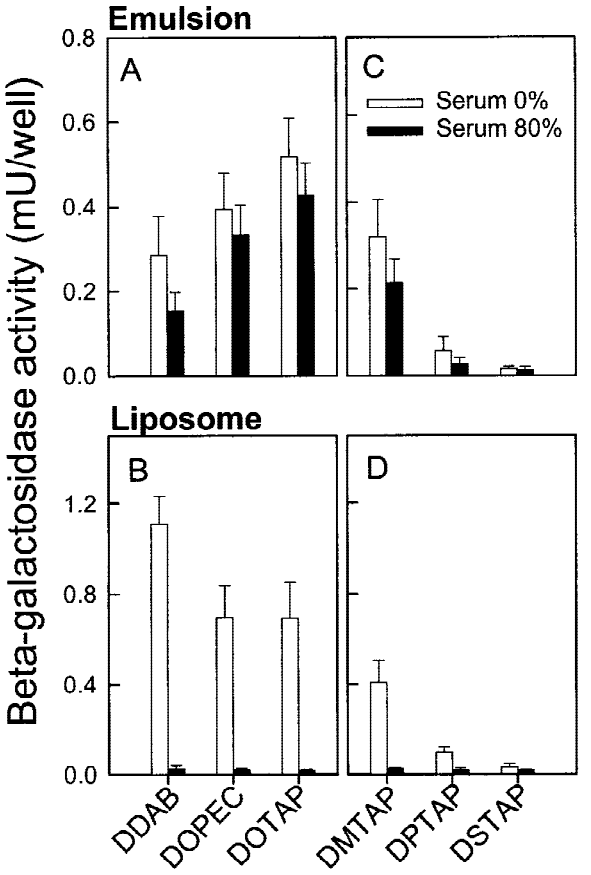

Fig. 1. Transfection activity of (A and C) emulsions and (B and D) liposomes comprising different cationic lipids in COS-1 cells. Six commercially available cationic lipids, DOTAP, DOPEC, and DDAB (A and B) and DMTAP, DPTAP, and DSTAP (C and D) were selected to prepare emulsions and liposomes at 35 mM. For emulsion, squalene at 10 %(v/v) was used as an oil phase. Fifty micrograms of pCMV-beta was complexed with the carrier at C/D = 4. Open and filled bars indicate transfection activity without or with 80% serum, respectively.

In the absence of serum, the transfection activity of emulsions decreased as the acyl chain length increased (Fig. 1C). The DMTAP emulsion had the most potent transfection activity among the cationic lipids having saturated acyl chains. Transfection activities of all three emulsions made with saturated lipids, however, did not exceed that of the DOTAP (c18:1) emulsion. Transfection activity of the DOTAP emulsion, for instance, was 22 times greater than that of its saturated analog, DSTAP emulsion, in the absence of serum. Similar observations were made in liposomes in the absence of serum (Fig. 1D). It is well-known that unsaturation and a decrease in acyl chain length reduce bilayer stiffness and increase the fusogenic properties of liposomes (5). In the case of emulsions, the shorter chain lipid has a higher hydrophile-lipophile balance value (15), and therefore, is a better emulsifier that forms a more stable emulsion. Among the emulsions made with the saturated series of cationic lipids, DMTAP formed the smallest in size, followed by DPTAP,

and then DSTAP (Table 1). Thus, emulsion stability may be related closely to its transfection activity (10,12).

In the presence of 80% serum, all of the emulsions had a high gene expression level (Fig. 1C). Liposomes, however, had negligible gene expression levels to an undetectable level (Fig. 1D). Of the emulsions examined, the highest activity was achieved with DOTAP in the presence or absence of serum.

Addition of DOPE Increases *In vitro* Transfection Activity of Emulsions

It has been shown for liposomal gene carriers that the transfection activity of cationic lipids is often enhanced by DOPE (5,8). The ratio between cationic lipid and DOPE for maximal transfection activity varies, depending on the cationic lipid (5). To investigate whether DOPE can enhance *in vitro* transfection activity in cationic emulsions, we prepared emulsions containing different weight ratios of DOTAP to DOPE (Fig. 2). Counterpart liposomes were also made for comparison. In all lipid formulations, the concentration of total lipids (DOTAP + DOPE) was 24 mg/ml. As the DOTAP:DOPE ratio changed from 1:0 to 5:1, the transfection activities of emulsions increased. The transfection activity at its maximum was 1.7 times greater than that without DOPE in the emulsion (Fig. 2A). Above 5:1, however, the activity declines. When the DOTAP:DOPE ratio was 1:1 in the emulsion, the transfection activity was greatly attenuated by serum, compared to emulsions having a lower DOPE content. Interestingly, in case of liposomes, the maximum transfection activity was also observed at a DOTAP:DOPE ratio of 5:1 (Fig. 2B), which is considerably lower in DOPE content than the commercialized DOTAP carriers (5,8). Not surprisingly, the liposomes lost most of their activity uniformly in the presence of serum.

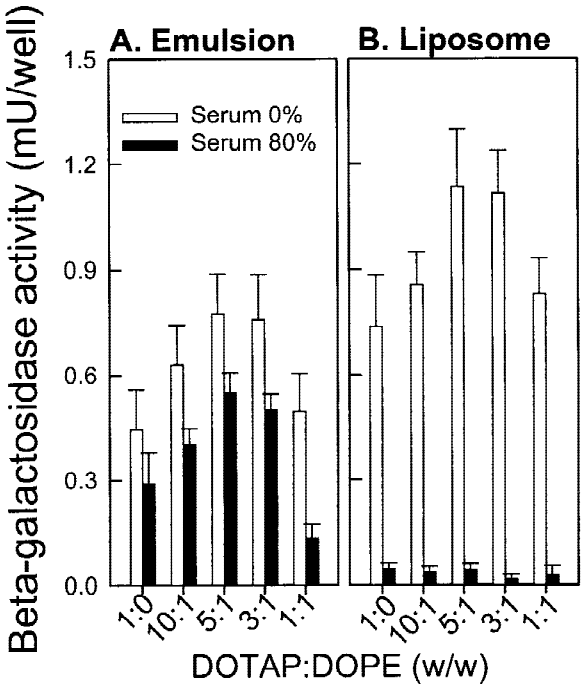

Fig. 2. Changes in *in vitro* transfection activity of (A) emulsions and (B) liposomes in COS-1 cells. The emulsions containing different weight ratios of DOTAP to DOPE were prepared. Counterpart liposomes were also made for comparison. In all lipid formulations, the concentration of total lipids (DOTAP + DOPE) was 24 mg/ml.

It seems that DOPE plays a similar role in emulsions as it does in conventional cationic liposomes (5,8). If the emulsion/DNA complex enters cells via endocytosis, it has to escape to the cytosol by fusing with the endosomal membrane. In this case, DOPE would help the complex to interact and to fuse with the endosomal membrane, resulting in an increased transfection activity. There is, however, another factor that controls the transfection activity of emulsions. Emulsions become unstable and have a bigger particle size at high DOPE contents because it is not a good emulsifier (Table 1). Therefore, upon forming a complex with DNA, the emulsion produces large aggregates at high DOPE contents (see below). These large aggregates are ineffective in transfecting cells (10,12–14). Therefore, the effect of DOPE on the transfection activity is probably due to a balance between enhanced fusogenic ability and decreased physical stability of the emulsion/DNA complex.

Nonionic Surfactants Alter *In Vitro* Transfection Activity of Emulsions

While increasing the transfection activity in COS-1 cells, the emulsion stability was somewhat compromised upon adding DOPE. Our next task, therefore, was to increase emulsion stability while maintaining or even further increasing transfection activity. It has been reported that the inclusion of nonionic surfactants containing PEG chains in the headgroup increases the stability of o/w emulsions (6,10). Our previous results also showed that non-ionic surfactants prevent effectively the formation of large DNA/emulsion complexes and protect against serum inactivation (12). We, therefore, examined whether the nonionic surfactants containing (PEG) could stabilize the DOTAP/DOPE emulsion and enhance its transfection activity. To this end, the DOTAP/DOPE (20/4 mg/ml) emulsions with four different types of nonionic surfactants at 10 mole % of total emulsifiers were prepared. Tween 80, Brij 7, F68, and $PEG_{2000}PE$ have ca. 7, 20, 45, and 150 covalently bonded PEG units per molecule, respectively. Counterpart liposomes were also prepared for comparison.

It has been reported that the PEG chains in the liposome interfere with liposome/DNA interaction by providing steric hindrance (6,12,13). In such cases, the length and the concentration of PEG chains are expected to influence the degree of emulsion–DNA interaction (10,12). Plasmid DNA (1 μg) and emulsions were mixed at various C/D values, and the mixture was incubated for 15 min at room temperature to evaluate C/D_{min} by agarose gel assay. In DOTAP/DOPE containing emulsion or liposome, the C/D_{min} was 4. The C/D_{min} values for Brij 7, Tween 80, $PEG_{2000}PE$, and F68 were 6, 8, 14, and 16, respectively, for emulsions; and 6, 6, 12, and 14, respectively, for liposomes. The bigger C/D_{min} value indicates that a larger amount of emulsion or liposome is required to form complexes with DNA, due to the steric hindrance exhibited by the larger PEG chains, as expected.

We also carried out *in vitro* transfection experiments with these carriers. For each liposome or emulsion, the transfection activity was evaluated at various C/D ratios to find C/D_{opt}. For an emulsion or liposome made with DOTAP/DOPE (20/4 mg/ml), C/D_{opt} was 4. This ratio increased upon incorporating PEG lipids into emulsions and liposomes (Fig. 3).

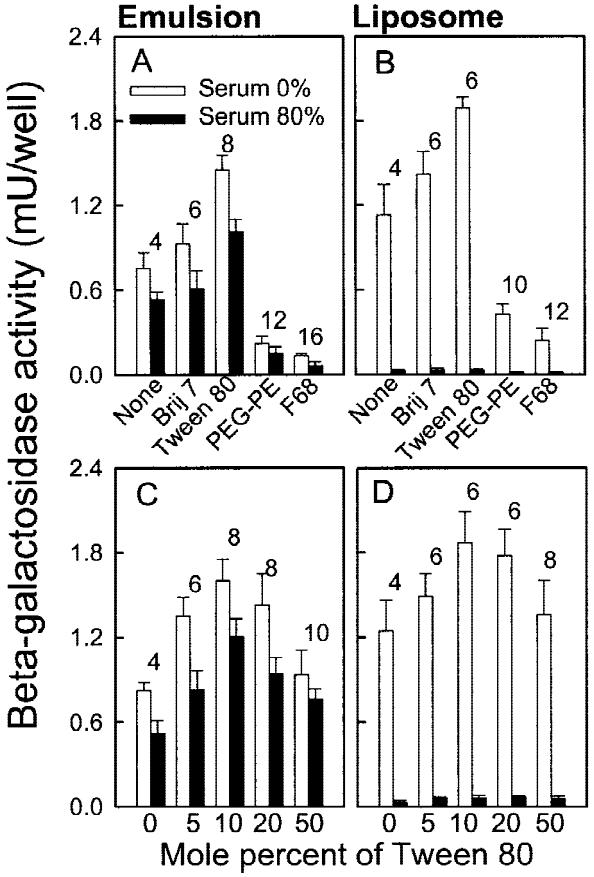

Fig. 3. *In vitro* transfection activity of (A and C) emulsions and (B and D) liposome comprising different PEG lipids (A and B) or as a function of Tween 80 content (C and D) at C/D_{opt}. Emulsions (A) and liposomes (B) were made with DOTAP/DOPE at 20/4 mg/ml and Tween 80, Brij 7, F68, and $PEG_{2000}PE$ (Table 1). The amount of PEG lipids was 10 mol% of total lipids. Emulsions (C) and liposomes (D) contained DOTAP/DOPE at 20/4 mg/ml and different concentrations of Tween 80. The numbers on bars represent C/D_{opt} values for the carrier/DNA complex.

Emulsions containing Tween 80 and Brij 7 at C/D_{opt} showed higher transfection activity than those without PEG-lipids (Fig. 3A). In particular, the emulsions containing Tween 80 produced the highest transfection activity. In contrast, the transfection activity of liposome and emulsion was reduced to ca. 29% and 17% by adding $PEG_{2000}PE$ and F68, respectively, when compared to that without PEG lipids. In addition to preventing the formation of large insoluble emulsion/DNA complexes, these surfactants are reported to impede access of the complex to cells and subsequent escape from the endosome (6,10,12). This is probably the reason why emulsions containing PEG lipids that have longer PEG units show very low transfection activity. In counterpart liposomes, we observed similar results in the absence of serum. However, the activity declined to a basal level by adding serum for all liposomal agents as expected. (Fig. 3B).

Emulsions containing different mole percentage of Tween 80 were prepared while fixing the DOTAP/DOPE ratio at 20:4 mg/ml to determine the optimum Tween 80 content. Before the transfection experiments, C/D_{min} (data not shown) and C/D_{opt} (Fig. 3C) were determined. Both values increased as the Tween 80 content in the emulsion increased, probably due to the lowered emulsion/DNA interaction by PEG chains. *In vitro* transfection experiments were performed with emulsions having different Tween 80 contents (Fig. 3C). The maximum transfection activity was observed at 10 mole % Tween 80 with or without serum. Above 10 mole %, the activity decreased gradually. As mentioned consistently in this paper, the transfection activity in the presence of serum was maintained significantly for all emulsion formulations containing PEG lipids. On the other hand, for the counterpart liposomes, the transfection activity was completely lost in the presence of 80% serum (Fig. 3D).

To investigate whether the inclusion of Tween 80 in the emulsion could increase transfection activity in cells of different origins, we performed *in vitro* transfection with 4 different cell lines using DOTAP/DOPE (20/4 mg/ml) and DOTAP/DOPE/Tween 80 (20/4/4.5 mg/ml) emulsions (Fig. 4). The inclusion of Tween 80 significantly increased the transfection activity for all emulsions and liposomes, although the magnitude of the effect varied among the four cell lines. Also, serum diminished transfection activity in case of liposomes but only slightly in emulsions, which is consistent with our other data.

Even though it has been hypothesized that Tween 80 may have a similar fusogenic property to DOPE (10), it seems highly probable that Tween 80 elicits its effect by stabilizing the emulsion. It has been shown that PEG lipids enhanced physical stability of emulsions (9,10,12). Also, stealth liposomes, which are stabilized by PEG lipids, are well known (6). To investigate this issue further, the stability of DOTAP, DOTAP/DOPE, and DOTAP/DOPE/Tween 80 emulsions was monitored by measuring the time-dependent absorbance change at 600 nm in PBS (Fig. 5A). Initially, turbid emulsion solutions scattered light in all three samples. In the case of DOTAP and DOTAP/DOPE/Tween 80 emulsions, the suspensions remained turbid for 20 days. The DOTAP/DOPE emulsion, by comparison, produced unstable larger particles as time progressed. These particles floated, resulting in a more transparent solution with lower absorbance. This result shows that the DOTAP/DOPE emulsion is unstable and that it could be rendered stable by including Tween 80.

One of the major drawbacks of using cationic liposomes

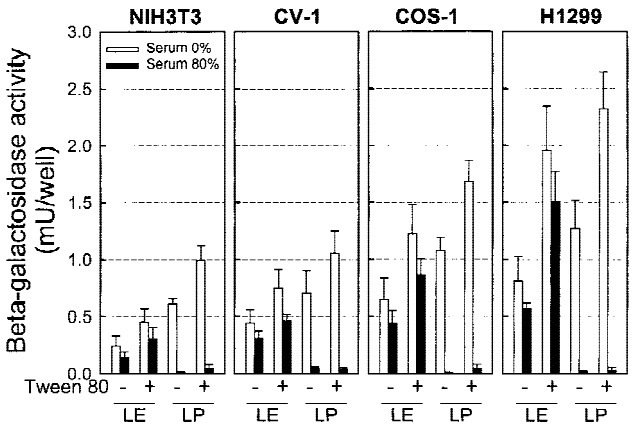

Fig. 4. *In vitro* expression level in emulsions (C/D = 8) and liposomes (C/D = 6) using NIH3T3, CV-1, COS-1, and H1299 cells. The liposome or emulsion formulation contained either DOTAP/DOPE (20/4 mg/ml) or DOTAP/DOPE/Tween 80 (20/4/4.5 mg/ml). LE and LP represent lipid emulsion and liposome, respectively.

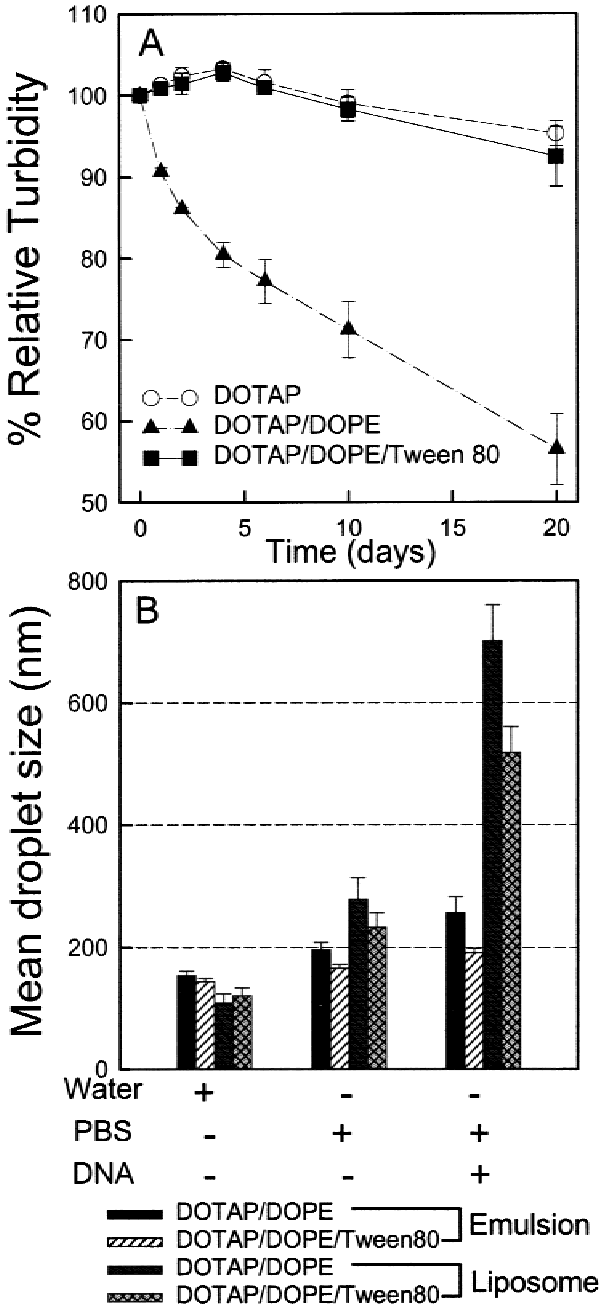

Fig. 5. (A) Changes in relative turbidity of the emulsions containing different co-emulsifiers in PBS as a function of time, and (B) particle sizes of the carrier in water or PBS and carrier/DNA complex in PBS. The stability of emulsions composed of DOTAP, DOTAP/DOPE (20/4 mg/ml), and DOTAP/DOPE/Tween 80 (20/4/4.5 mg/ml) was monitored by measuring time-dependent changes in the absorbance at 600 nm in PBS (A).

is that they form large aggregates upon mixing with DNA. For this reason, it has been difficult to obtain small and homogeneous cationic lipid/DNA complexes. The particle size of the complexes is a critical factor for systemic administration. In this regard, the size of emulsions and complexes in PBS or in water was investigated (Fig. 5B). The size of DOTAP/DOPE/Tween 80 emulsion/DNA complex increased slightly compared to the emulsion size in PBS. In contrast, in DOTAP/DOPE emulsion, the complex size was *ca.* 1.5 times bigger than the emulsion particle. Even though the size of liposomes was smaller in water than that of emulsions, the former was bigger in PBS and became even bigger (up to *ca.* 7 times) upon complexation with DNA. Therefore, the best formulation for systemic administration would be DOTAP/DOPE/Tween 80 emulsion, since it forms a small and stable complex.

In Vivo Transfection Via Intravenous Administration

To evaluate *in vivo* transfection activities of emulsions having DOTAP/DOPE and DOTAP/DOPE/Tween 80, 10 μg of pCMV-Luc+ was complexed with emulsions at C/D_{opt} from Fig. 3 in PBS and administered intravenously into BALB/c mice via the tail vein. Counterpart liposomes were used for comparison. Twenty-four hours after injection of the complex, luciferase activity in different organs was analyzed. No signs of toxicity were noticed under our experimental condition. We determined the levels of gene expression in various tissues from lung, heart, spleen, liver, and kidney (Fig. 6). In all lipid formulations, the expression level in lung lysates was 10~1000-fold higher than that in other tissue lysates, consistent with the results obtained using liposome/DNA complexes (6,14). In lung lysates, emulsions showed at least 2–4 times higher luciferase activities than counterpart liposomes. Among all of the lipid formulations, the DOTAP/DOPE/Tween 80 emulsion yielded the most potent transfection activities in lung lysates, as well as in the other tissue lysates.

In this study, we have used identical cationic lipids and co-lipids to formulate emulsions and liposomes. Even though the surface of the emulsion and liposome particles could dif-

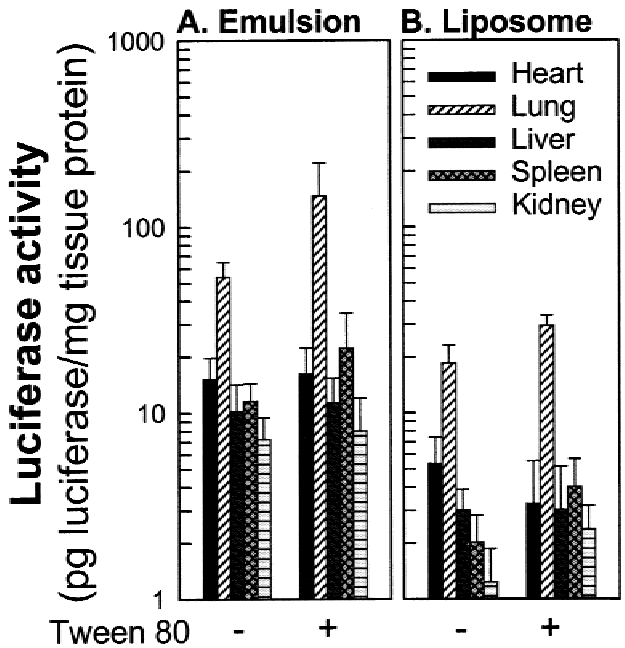

Fig. 6. *In vivo* transfection activity of (A) emulsion and (B) liposomes containing DOTAP/DOPE (20/4 mg/ml) or DOTAP/DOPE/Tween 80 (20/4/4.5 by weight). Each complex contained 10 μg pCMV-Luc+ in 100 μl of PBS. The complex was prepared at C/D_{opt} obtained from *in vitro* experiments (Fig. 3). Amount of luciferase was expressed as picograms per milligram of total protein extract. Background of luciferase activity in each organ was measured from the organs of untreated mice and was negligible.

fer due to the added squalene, the most important physical difference between the two carriers is probably physical stability. Also, the stability of the emulsion and its DNA complex is apparently related to *in vitro* and *in vivo* transfection activity. As with liposomes, DOPE and PEG lipids further enhanced the transfection activity of emulsions. Also, the transfection activity of the emulsion was far greater than the liposome preparation in the presence of serum and for *in vivo* applications. Considering that there are numerous cationic lipids synthesized up to now, it would be an interesting project to formulate and evaluate emulsion using those lipids for *in vivo* applications.

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