A Cationic Lipid Emulsion/DNA Complex as a Physically Stable and Serum-Resistant Gene Delivery System

Sun Woo Yi,[1,2] Tae Young Yune,[1] Tae Woo Kim,[1] Hesson Chung,[1] Young Wook Choi,[2] Ick Chan Kwon,[1] Eunhee Bae Lee,[1] and Seo Young Jeong[1,3]

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Purpose. To develop a non-viral gene delivery system in the form of an oil-in-water (o/w) lipid emulsion.

Method. Cationic lipid emulsions were formulated with soybean oil, 1,2-dioleoyl-*sn*-glycero-3-trimethylammonium-propane (DOTAP) as a cationic emulsifier and other co-emulsifiers. The physical characteristics of the lipid emulsion and the emulsion/DNA complex were determined. The *in vitro* transfection efficiency of the emulsion/DNA complex was determined in the presence of up to 90% serum.

Results. The average droplet size and zeta potential of emulsions were *ca.* 180 nm and *ca.* +50 mV, respectively. Among the emulsions, a stable formulation was selected to form a complex with a plasmid DNA encoding chloramphenicol acetyltransferase. By increasing the ratio of emulsion to DNA, zeta-potential of the emulsion/DNA complex increased monotonously from negative to positive without any changes in the complex size. The complex was stable against DNase I digestion and an anionic poly-L-aspartic acid (PLAA). The complex delivered DNA into the cells successfully, and the transfection efficiency was not affected by complex formation time from 20 min to 2 h. More importantly, the cationic lipid emulsion facilitated the transfer of DNA in the presence of up to 90% serum.

Conclusions. The cationic lipid emulsion/DNA complex has physical stability and serum resistant properties for gene transfer.

KEY WORDS: gene transfer; lipid emulsions; poly(ethylene glycol); transfection; cationic lipids.

INTRODUCTION

Cationic lipids have been used widely in transferring genes for *in vitro* and *in vivo* applications (1–4). Cationic lipids have been formulated as liposomes to deliver genes into cells (5). Transfection using cationic liposomes is convenient and highly efficient when compared with other non-viral gene carriers (1,6). Since liposomal vectors do not have the potentially deleterious side effects of viral vectors including host inflammatory and immune responses, much effort has been devoted to finding cationic lipids or lipid formulations that increase the transfection efficiency (1,2,5).

There are, however, some limitations in most of the cationic liposome formulations. Up to now, most of cationic liposomes have been shown to form aggregates and flocculates upon mixing with DNA (7,8). For this reason, it has been difficult to obtain homogeneous cationic lipid/DNA complexes, also known as lipoplexes (3). For the transfection experiments, it has been essential to use freshly prepared complex (8). In addition, the reproducibility in the transfection efficiency has been a problem since metastable preparations of liposome/DNA complexes have been used in most published studies (9–11). Recent publications have demonstrated increasing need to develop and to characterize physically stable liposome/DNA complexes (12,13). To correlate the structural parameters of liposome/DNA complexes to their transfection efficiency *in vivo*, it is critical to develop reproducible, stable and well-defined formulations (9,13). It is known that the transfection efficiency of cationic liposomes is compromised by serum components (10,11). For instance, many formulations leading to efficient *in vitro* gene transfer in the absence of serum are not effective in the presence of as low as 5–10% (v/v) serum (4,10–12).

Emulsions and liposomes are two of the most widely studied colloidal delivery systems in the pharmaceutical field (14–16). The oil-in-water (o/w) emulsion is made of oil dispersed in an aqueous phase with a suitable emulsifying agent such as phospholipids or non-ionic surfactants (14). We have formulated cationic lipid emulsions as an alternative gene delivery system. Our emulsion systems contained soybean oil as a core-oil and DOTAP as a cationic lipid. Cholesterol, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), and polymeric lipids were also included in the formulations. Castor oil emulsions have been introduced as alternative gene transfer vectors to overcome problems associated with liposomes (17). Our experiments, however, differ significantly from the castor oil system in several regards. While *in vitro* transfection using castor oil emulsion was performed in the presence of 20% (v/v) serum, we have performed the transfection experiments in the presence of 90% (v/v) serum. Also, our formulations contain 10% (w/v) soybean oil and 1.2 ~ 1.6% (w/v) emulsifier while the castor oil emulsion includes only 0.025% (w/v) oil and *ca.* 0.075% (w/v) emulsifier (17). Our formulations are similar to the lipid emulsion used for clinical nutrition (Intralipid®, Vitrum A.G., Stockholm, Sweden) (16). Compared to our formulation, the castor oil emulsions were very dilute and had excess emulsifier.

The physical stability of the carrier and its complex with DNA has been regarded as one of the important factors in its use (12,13). In addition, the stability of lipid emulsion is a critical factor for patient safety because larger particles may cause oil embolism when administered systemically (18). In this regard, the most stable cationic lipid emulsion formulation was selected through an emulsion stability test. The physicochemical properties of the cationic lipid emulsion/DNA complex were investigated and were compared with Lipofectamine, a commercially available cationic liposome reagent. The resulting emulsion/DNA complex was physically stable and facilitated transfer of a plasmid DNA encoding chloramphenicol acetyltransferase *in vitro*. More importantly, the cationic lipid

[1] Biomedical Research Center, Korea Institute of Science and Technology, 39-1 Hwawolkokdong, Sungbuk-ku, Seoul 136-791, Korea.
[2] Department of Pharmaceutics, College of Pharmacy, Chung Ang University, Seoul, Korea.
[3] To whom correspondence should be addressed. (e-mail: syjeong@kist.re.kr)

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emulsion facilitated the transfer of the plasmid DNA successfully in the presence of up to 90% (v/v) serum in COS-1 and CV-1 cells.

MATERIALS AND METHODS

Materials

Soybean oil, glycerol, poly-L-aspartic acid (PLAA, Molecular weight 1.1×10^4) and cholesterol were purchased from Sigma Chemical Company (St. Louis, MO). DOPE, DOTAP, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine-N-[poly (ethylene glycol) 2000] ($PEG_{2000}PE$) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine-N-[poly (ethylene glycol) 5000] ($PEG_{5000}PE$) were obtained from Avanti Polar Lipids (Alabaster, AL). Lipofectamine, cell culture medium and fetal bovine serum (FBS) were purchased from Gibco BRL/Life Technologies (New York, NY). Chloramphenicol acetyltransferase was obtained from Promega (Madison, WI), D-threo-[dichloroacetyl-1-^{14}C] chloramphenicol (CAT assay grade) was acquired from Amersham Life Science (UK), and acetyl-coenzyme A was from Boehringer Mannheim GmbH (Germany). All other chemicals and reagents were of tissue culture grade.

Preparation of Lipid Emulsions

The oil-in-water emulsions with various components and different compositions were prepared as potential emulsion vectors. The first step in preparing the emulsion was to make separately the oil and aqueous phases (14). The oil phase consisted of soybean oil, DOTAP, and DOPE. PEG_{2000} PE, PEG_{5000} PE, or cholesterol was also added to the oil phase. The aqueous phase contained 2.25% (w/v) glycerol for isotonicity. Each phase was heated to 70°C and stirred to solubilize the components completely. The aqueous phase was transferred to the oil phase to mix the two solutions roughly. The cationic lipid emulsion was prepared at 8,000 rpm for 10 minutes at room temperature by mixing the two solutions using a high-speed homogenizer (T-25-Ultra-Turrax, S25-18G, IKA Werke, Janke & Kunkel GmbH & Co KG, Germany). The mixture was passed 10 times through a Microfluidizer® M110S (Microfluidics Co., Newton, MA) with an inlet air pressure of 80 psi. The lipid emulsion so produced was subsequently sterilized by filtration through a 0.22 μm filter device (sterile and nonpyrogenic PVDF filter media, Whatman Inc., Clifton, NJ) as described by Lidgate *et al.* (19). The emulsion was kept at 4°C until further use.

Size and Zeta-Potential of Lipid Emulsions and the Emulsion/DNA Complex

The average droplet size and the zeta-potential were determined by the quasielastic laser light scattering with a Malvern Zetasizer® (Malvern Instruments Limited, England, 20, 21). Stability of lipid emulsions was assessed by measuring size changes with time after a 300-fold dilution in PBS. The zeta-potential of the emulsion was measured after a 300-fold dilution in water. The mixtures containing 5 μg of plasmid DNA and varying amounts of DOTAP in the cationic lipid emulsion to DNA ratios (nmol: μg) were used to measure the size and the zeta-potential of the emulsion/DNA complex. We note that the amount of emulsion was quantified in this report as the number of moles of DOTAP in the formulation (4). The size and zeta-potential of the emulsion/DNA complex were measured after dilution with RPMI 1640 and with water, respectively.

Purification of Plasmid DNA and Cell Culture

A plasmid DNA containing the chloramphenicol acetyl transferase (CAT) gene driven by the Simian virus 40 promoter (pSV-CAT) was purchased from Promega (Madison, WI). The plasmid was amplified in *Escherichia coli* HB101 and purified using Qiagen mega-kit (Qiagen Inc., Chatsworth, CA) according to the manufacturer's instruction. COS-1 and CV-1 cells were cultured in RPMI 1640 supplemented with 10% (v/v) FBS and in DMEM supplemented with 10% (v/v) FBS, respectively.

Agarose Gel Electrophoresis

The complex formation between plasmid DNA and the cationic lipid emulsion was detected by gel electrophoresis as described elsewhere (11). The pSV-CAT was diluted to a concentration of 5 μg per 100 μl in RPMI 1640 medium. The lipid emulsion diluted in an equal volume of the same vehicle was added to the DNA solution and incubated for one hour at room temperature. Ten microliters of Lipofectamine also diluted in 100 μl RPMI 1640 was added to the DNA solution. The DNA/liposome mixture was incubated to form a complex for 20 min at room temperature according to the manufacturer's protocol. Twenty microliters of the samples and 2 μl of the loading buffer were loaded into 1% (w/v) agarose gel including 0.5 μg/ml ethidium bromide (EtBr). The gel was electrophoresed at 5 V/cm in TAE buffer and visualized under UV light.

Complex Stability Against DNase I Digestion

Two microliters of either the cationic lipid emulsion or Lipofectamine were mixed with 1 μg of DNA and allowed to form complexes for 1 hour at room temperature. Plasmid DNA or the cationic lipid emulsion/DNA complex containing the same amount of DNA was exposed to DNase I for 30 minutes at 37°C to investigate the stability of the complex against nuclease digestion (4). The DNA was retrieved by phenol/chloroform extraction followed by ethanol precipitation and visualized on agarose gel containing ethidium bromide.

Stability of the Emulsion/DNA Complex Against Poly-L-Aspartic Acid

The stability of the lipid emulsion/DNA complex or Lipofectamine/DNA complex against an exchange reaction by an anionic polymer was investigated (11). Different concentrations of poly-L-aspartic acid (PLAA) were added to the complexes to vary the charge ratio of PLAA/plasmid DNA from 0 to 800. The reaction mixture was electrophoresed to determine whether DNA dissociated from the complex.

In Vitro Transfection Assay

Cells were seeded at 5×10^5 cells in a 60 mm-diameter dish one day before the transfection experiments. After washing

the cells with serum-free media, 3.8 ml of media was added to each well in the case of the transfection performed without serum. To test the effect of serum on the transfetion efficiency, the media contained appropriate amounts of FBS to vary total serum concentration from 0% (v/v) to 90% (v/v) of the final volume. Two hundred microliters of the carrier/DNA mixture were prepared and added to the cells. After 4 h incubation, the cells were washed with serum-free media to remove the remaining carrier/DNA complexes. The cells were fed again with RPMI 1640 or DMEM containing 10% (v/v) FBS, cultured for 48 hours after transfection, harvested with 0.25 M Tris-HCl (pH 7.8) and disrupted by freezing and thawing (22). CAT activity of the cell lysates was determined either by TLC autoradiography or by reading the radioactivity on an image plate (BAS-MP 2040P, Fuji photo film co., Ltd. Tokyo, Japan) with a Fuji BAS2000 imaging analyzer (Fuji). The CAT activity is expressed as the relative activity based on the percent conversion of acetylated chloramphenicols (ACh) in total chloramphenicols (Ch). Protein concentration in the lysates was measured by means of the Bio-Rad® protein assay (Bio-Rad Laboratories, CA), and lysates corresponding to equal amounts of protein were analyzed (23).

Statistical Analysis

Statistical analysis was performed using Student's paired *t* test for comparison. A probability of less than 0.05 was considered to be significant statistically.

RESULTS

Preparation of Oil-In-Water Lipid Emulsions and Stability of the Lipid Emulsions

Six different cationic emulsion formulations were prepared as potential non-viral vectors. The components and the composition of the emulsions are presented in Table I. All of the formulations consisted of 10% (w/v) soybean oil. Two of the emulsions contained DOTAP, DOPE and PEG_{2000}PE as emulsifiers. In one of the formulations, PEG_{5000}PE was used instead of PEG_{2000}PE. Cholesterol substituted for PEG_{2000}PE in the last formulation. When diluted 300-times in water, the average droplet size and the zeta-potential of the emulsions were *ca.* 180–350 nm and *ca.* + 50 mV, respectively, and did not change for 24 h (data not shown). Stability of the lipid emulsions was tested in PBS by measuring the changes in droplet size with time. Since PBS provides counter-ions for the cationic emulsion, only formulations that are stable in the absence of electrostatic repulsion can maintain the droplet size (20). The average droplet size of emulsion formulations B through F increased by up to 500% in 2 h in PBS (Table I). In formulation A, however, particle size did not change beyond the statistical error range for 2 h ($p > 0.05$) indicating that this formulation was stable in PBS. Physical stability is a prerequisite for emulsions as parenteral drug delivery systems (14–16,18,21). We have, therefore, decided to restrict further transfection experiments to formulation A.

Table I. The Composition of Lipid Emulsions Used in this Study which Incorporate Various Emulsifiers and Soybean Oil

	Oil phase, % (w/v)				Size (nm) after dilution in PBS	
Formulations[a]	DOTAP	PEG_{2000} PE	PEG_{5000} PE	Cholesterol	0 h	2 h
A	0.8	0.4	—	—	181	187
B	0.8	—	—	—	253	553
C	1.2	—	—	—	250	619
D	0.8	0.04	—	—	222	432
E	0.8	—	0.04	—	265	313
F	0.8	—	—	0.08	344	1515

[a] All formulations contain 10% (w/v) soybean oil and 0.4% (w/v) DOPE in oil phase. The aqueous phase contained 2.25% (w/v) glycerol and comprised of water quantity sufficient to 100%.

Emulsion/DNA Complexes

The physical characteristics of the emulsion/DNA complex were investigated as a function of the cationic lipid emulsion-to-DNA ratio. Different amounts of the lipid emulsion were mixed with 5 μg of DNA. The ratio of the cationic lipid emulsion to DNA varied from 1.4 to 69 (nmole of DOTAP per 1 μg of DNA). The zeta-potential increased monotonously as the DOTAP-to-DNA ratio increased (Fig. 1A). The size of the emulsion/DNA complexes, however, decreased slightly from *ca.* 180 nm to *ca.* 175 nm in the range investigated (Fig. 1B). It has been reported that liposome/DNA complexes are physically unstable and undergo a change in overall structure when the net charge of the liposome/DNA complex approaches neutrality (7,8,13). Unlike the liposomes, when the zeta-potential of the emulsion/DNA complex approached 0 at the DOTAP-to-DNA ratio (nmol: μg) of *ca.* 8, changes in the size of the complex were minimal with the average size ranging from 175 to 180 nm. Regardless of the emulsion/DNA ratio in the complex, size did not change for up to 48 hours (169 ± 18 nm).

Without the size changes, one might question whether indeed the emulsion/DNA complex had been made. To verify complex formation, gel electrophoresis was performed (4). When the lipid emulsion was mixed with the DNA solution (containing 5 μg of pSV-CAT), free DNA disappeared when the ratio of DOTAP to DNA (nmol:μg) was above 20 (Fig. 1C). Free DNA that did not form a complex with the lipid emulsion was clearly visible at the position of migration of naked DNA at complex ratios (nmol:μg) of 2.2, 6.8, and 14. Lipofectamine formed a complex with DNA without a trace of free DNA at the Lipofectamine/DNA ratio recommended by the manufacturer (Fig. 1C). At low emulsion concentrations, we observed pale fluorescent band sometimes in the well indicating that the emulsion/DNA complex remained in the well and was stained by EtBr. In most samples, although the complex is detected in the agarose gel well by eyes, the EtBr fluorescence was not detected under UV light. It means that the cationic emulsion can also condense the plasmid DNA so strong that EtBr could not intercalate base of DNA.

Stability of the Emulsion/DNA Complex Against DNase I Digestion and Exchange with Poly-L-Aspartic Acid (PLAA)

The electrophoresis experiments suggest that DNA in the complex might be highly condensed. Accordingly, we examined

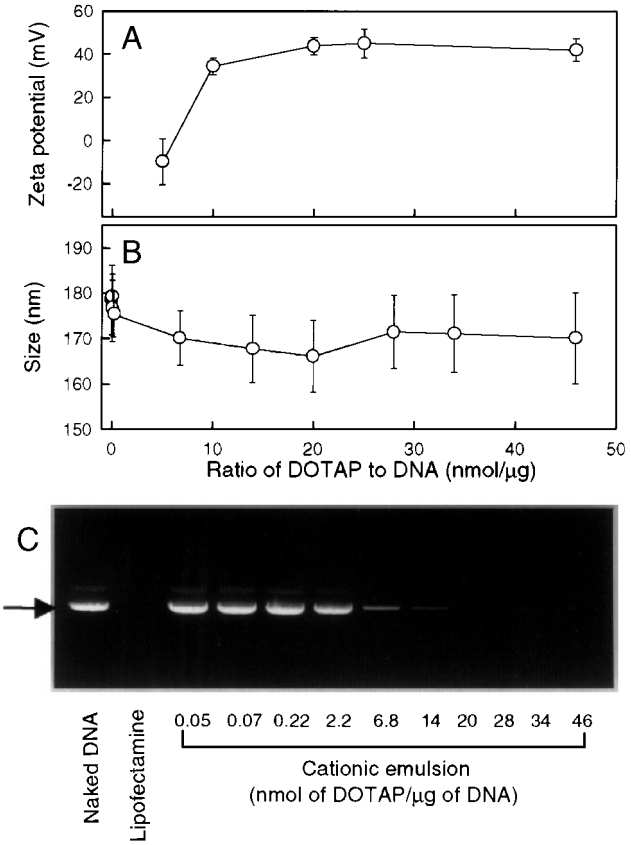

Fig. 1. (A) Zeta potential and (B) particle size of the emulsion/DNA complex as a function of the DOTAP-to-DNA ratio at 22 ± 2°C. One hundred micro-liters of the lipid emulsion diluted in RPMI 1640 was mixed with 100 μl of the DNA solution diluted in the same vehicle as the emulsion. The mixtures containing 5 μg of pSV-CAT at various DOTAP-to-DNA ratios were incubated for one hour at room temperature and then diluted with 1.8 ml of RPMI 1640 for the size measurement and with water for the zeta-potential measurement. Data points and error bars represent the mean value (n = 3) and the standard deviation, respectively. (C) Gel electrophoresis of the complex formed between the lipid emulsion and DNA as the DOTAP-to-DNA ratio varied from 2.2 to 46. The unbound free DNA in the agarose gel (position indicated by the arrow) was visualized under UV light. Lipofectamine/DNA complex and naked DNA were loaded for comparison and as a negative control, respectively.

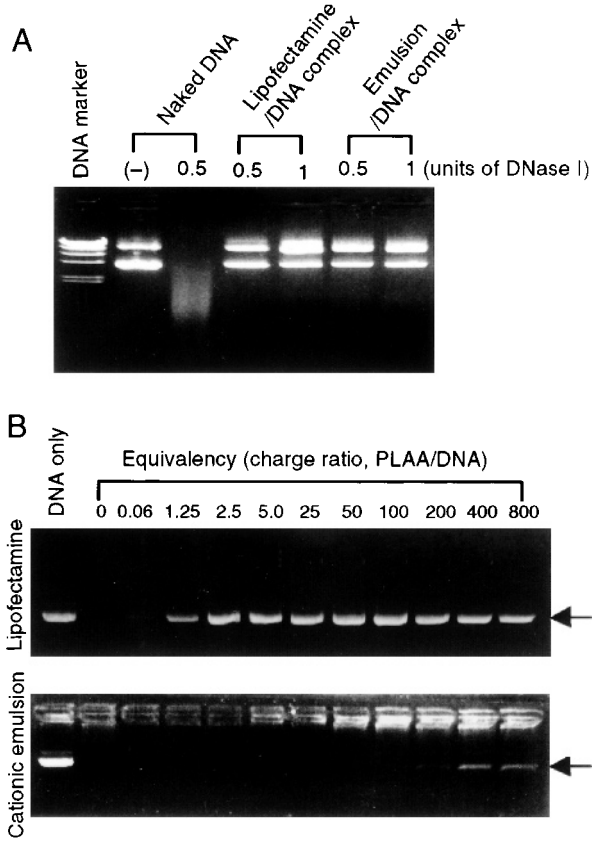

Fig. 2. Stability of the emulsion/DNA complex at DOTAP-to-DNA ratio of 24. A) Complex stability against DNase I digestion. Naked DNA with or without 0.5 unit of DNase I was loaded. The emulsion/DNA and Lipofectamine/DNA complexes were treated with 0.5 or 1 units of DNase I. The undigested DNA was visualized on an agarose-EtBr gel under UV light. B) Stability of the emulsion/DNA complex against an exchange reaction by poly-L-aspartic acid (PLAA). The emulsion/DNA and Lipofectamine/DNA complexes were exposed to various amounts of PLAA. Dissociated free DNA, at the position of the arrows, was visualized on the agarose-EtBr gel under UV light.

whether DNA in the complex could be protected from enzymatic digestion by DNase I. The sensitivity of DNA in the complex to DNase I digestion has been used to test the stability of DNA in the complex (4,24). The study was performed at a DOTAP-to-DNA ratio (nmol:μg) of 24 where free DNA did not exist. The pSV-CAT alone or its complex with the cationic lipid emulsion or with Lipofectamine was mixed with DNase I, the undigested DNA was retrieved and visualized as described under MATERIALS AND METHODS (Fig. 2A). Naked DNA was completely digested by 0.5 units of DNase I. In the case of the emulsion/DNA and Lipofectamine/DNA complexes, however, some of DNA molecules were nicked by 1 units of DNase I without severe smearing of digested DNA. The result suggests that DNA in the emulsion/DNA complex as well as the Lipofectamine/DNA complex was protected from a nuclease digestion and thus likely to survive in serum that contains DNase I.

In serum, there are many anionic materials that would compete with and substitute for DNA in the complex. Such a substitution has been assumed to be a major destabilization mechanism for the complex (8,11,24). Therefore, we investigated the stability of the emulsion/DNA complex against poly-L-aspartic acid (PLAA) as a model polyanion (24). The lipid emulsion/DNA complex or Lipofectamine/DNA complex was mixed with different concentrations of PLAA at a charge ratio of PLAA/plasmid DNA as high as 800. As shown in Fig. 2B, DNA dissociated from the Lipofectamine/DNA complex above a PLAA/DNA charge ratio of 1.25. In distinct contrast, the emulsion/DNA complex was stable up to a PLAA/DNA charge ratio of 200. Even at charge ratio of 800, only a trace of free DNA was detected. This result indicates that the interaction between the emulsion and DNA in the complex is much stronger than that between Lipofectamine and DNA. Therefore, the emulsion/DNA complex is more likely to maintain its physical

integrity in the presence of anionic competitors. This result inspired us to proceed with the *in vitro* transfection experiments.

Expression of CAT Gene in COS-1 Cells

To establish the transfection efficiency as a function of the ratio between the cationic lipid emulsion and DNA, COS-1 cells were transfected with the emulsion/DNA complex containing 5 μg of pSV-CAT. Twenty microliters of Lipofectamine were used in place of the lipid emulsion for comparison. Naked DNA and the lipid emulsion alone were used as negative controls. The CAT assay was performed using the CAT enzyme as a positive control. As shown in Fig. 3A, CAT activity was observed above a DOTAP-to-DNA ratio (nmol:μg) of 2.2. The ratio of acetylated chloramphenicols-to-total chloramphenicols was used as a measure of transfection efficiency (22). Transfection efficiency increased by increasing the emulsion-to-DNA ratio.

One of the inconveniences associated with handling liposome/DNA complexes is the need to perform the experiments rapidly. As reported in the literature, transfection efficiency is maximal within 1 h of complex formation (10,12,13). A complex that retains biological functions for a longer time post-preparation would offer a considerable advantage. We have evaluated how incubation time affects transfection efficiency for complexes DNA and either the cationic lipid emulsion or Lipofectamine. Transfection efficiency using Lipofectamine/DNA complex was clearly dependent on incubation time with a maximum of 20 min as described by the manufacture (data not shown). The emulsion/DNA complex, however, offered a transfection efficiency that was essentially independent of incubation time up to 120 min (Fig. 3B).

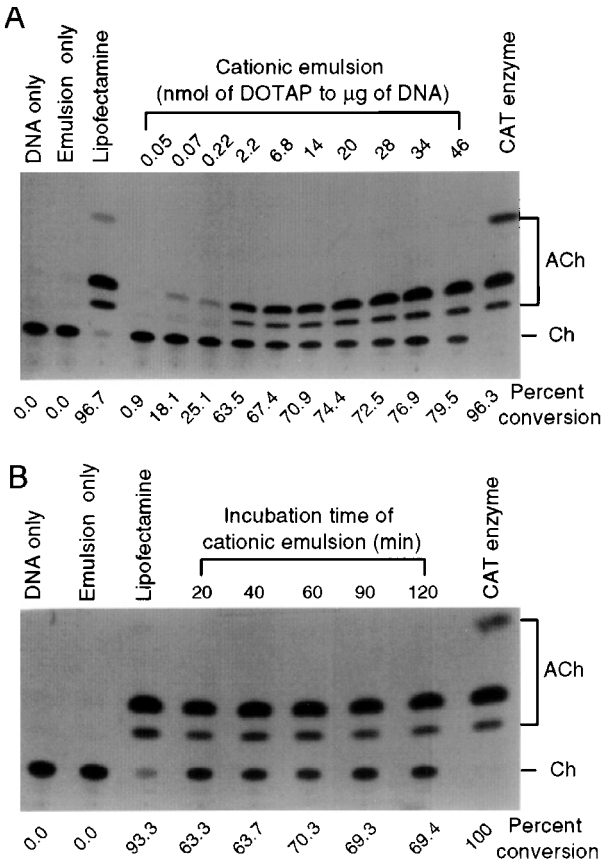

Fig. 3. (A) Transfection efficiency of the emulsion/DNA complex containing 5 g of pSV-CAT at various DOTAP-to-DNA ratios using COS-1 cells. Lipofectamine/DNA was used for comparison. Carrier/DNA complex incubation time was 20 min. Naked DNA and the lipid emulsion without DNA were used as negative controls. (B) Transfection efficiency as a function of the emulsion/DNA complex formation time from 20 min to 2 h at DOTAP-to-DNA ratio of 24. The CAT activity is expressed at the bottom as relative activity based on the percent conversion of acetylated chloramphenicols (ACh) in total chloramphenicols (Ch).

Transfection Efficiency in the Presence of Serum

It has been reported that the transfection efficiency of liposome/DNA complexes is drastically reduced in the presence of serum (4,10,11). *In vitro* transfection in the presence of serum would be a necessary if not a sufficient requirement for *in vivo* use (10,12,17). Accordingly we have quantified the *in vitro* transfection for two different cell lines, COS-1 and CV-1, in the presence of up to 90% serum. The lipid emulsion at a DOTAP-to-DNA ratio (nmol:μg) of 24 was allowed to form a complex with 5 μg of DNA. Lipofectamine (20 μg)/DNA complex was formed in the same manner. Cells were exposed to the carrier/DNA complexes for 4 h in cell culture media containing various concentrations of FBS. Afterwards, the medium was changed with fresh growth media. The transfected cells were harvested and assayed for CAT activity 48 h after transfection.

In the presence of serum, the transfection efficiency of the Lipofectamine/DNA complex decreased sharply even with 10% (v/v) of serum in COS-1 and CV-1 cells (Fig. 4) in agreement with the literature (10). In contrast, the transfection efficiency of the emulsion expressed as percent conversion was 99.3% and 63.2% in COS-1 and 37.1% and 22.4% in CV-1 in the absence and presence (90%) of serum, respectively (Fig. 4). Unlike the Lipofectamine/DNA complex, serum attenuated slightly the transfection efficiency of the emulsion/DNA complex in these two cell lines.

DISCUSSION

In developing non-viral gene carriers, those that are efficient *in vitro* often fail to show the same efficiency when applied *in vivo* (4,11). One of the reasons for poor efficacy *in vivo* is the sensitivity to serum; gene transfer mediated by cationic lipids is adversely affected by serum (10,12,17). With a view to developing an efficient gene carrier *in vivo*, we have performed transfection experiments in the presence of serum *in vitro* to mimic the *in vivo* situation (10,17). Most of the *in vitro* experiments in the literature, however, have been performed at low serum concentrations (up to *ca.* 20% (v/v)) (4,10,12,17). In the current study, we have performed *in vitro* transfection studies with the positively charged emulsion/DNA complex in the presence of up to 90% (v/v) serum in COS-1 and CV-1 cells (Fig. 4). These studies revealed a major difference between the cationic lipid emulsion and Lipofectamine. In agreement with work published by other laboratories, Lipofectamine, like most liposomal carriers, loses its ability to transfer DNA in the presence of ≥ 10% (v/v) serum (4,5,10–12). For the emulsion/DNA complex, however, more than 60% of transfection efficiency was retained in the presence of up to 90% (v/v) serum.

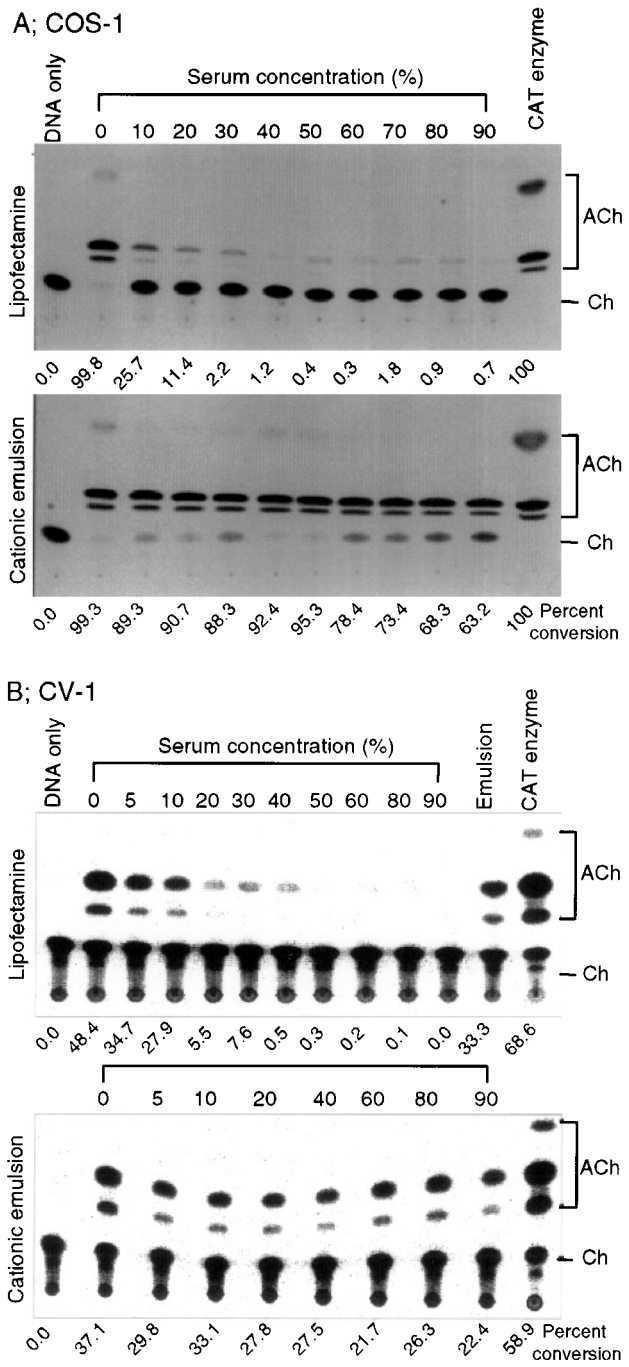

Fig. 4. Transfection efficiency as a function of serum concentration in (A) COS-1 cells and in (B) CV-1 cells. Cells were exposed to the carrier/DNA complexes for 4 h in cell culture medium containing various concentrations of FBS as described under MATERIALS AND METHODS. Transfection result was visualized on a TLC autoradiogram (COS-1) or by Fujix BAS2000 imaging analyzer (CV-1). The CAT activity is expressed at the bottom as relative activity based on the percent conversion of acetylated chloramphenicols (ACh) in total chloramphenicols (Ch).

In COS-1 cells, even though the ratio of the acetylated chloramphenicol decreased slightly with 90% (v/v) serum, considerable CAT activity was still observed. Similar phenomena were also observed in CV-1 cells. When cells were exposed to serum-free media during the transfection experiments, cell death to the extent of 40% was evident for the two carriers studied. The cytotoxicity of the emulsion/DNA complex as well as Lipofectamine/DNA complex diminished markedly by having as low as 10% (v/v) serum (data not shown). The cells incubated under serum-free conditions, with or without liposome/DNA complexes, showed significant levels of cell death due to a lack of growth factors provided by the serum that are required for cell survival (10). Therefore, the fact that transfection is efficient in the presence of serum is a big advantage when transfection in serum-containing medium is beneficial, as in *ex vivo* applications. One of the reasons for the serum-resistant properties of the cationic lipid emulsion may be the stability of the emulsion/DNA complex. The cationic emulsion/DNA complex was shown to be considerably more stable than Lipofectamine/DNA complex against the anionic exchange reaction by PLAA (Fig. 2B).

The stability of our emulsion formulation in serum may come from the stability provided by the polyethylene glycol moiety of $PEG_{2000}PE$. It is well known that incorporation of non-ionic surfactants with PEG moiety (PEG-lipid) in emulsions as well as in liposomes increases the physical stability of the formulation (9,17). It has been shown that PEG-lipids create a steric barrier and to prevent the DNA-induced aggregation of lipid particles (9,17). In our emulsion systems, Formulation A (Table 1), which contains 0.4% (w/v) $PEG_{2000}PE$, is physically more stable than other formulations that have a smaller amount of or no PEG-lipid. We have not investigated how the physical stability of the emulsion and emulsion/DNA complex is related to their serum stability in this study. Further studies are needed to understand the origin of the serum stability of this emulsion carrier.

Along with the serum stability of the emulsion/DNA complex, our newly formulated complex has another advantage. Its transfection efficiency was not affected by time up to 2 h post complex formation (Fig. 3B). This means that the experimenter has a wider time window to work within before using the complex. Indeed, it is possible that the complex is stable for considerably longer than 2 h. This was not examined in the current study.

We conclude from these results that the cationic lipid emulsion described in this study can be used to facilitate gene transfer into cells. The cationic lipid emulsion gene delivery system offers several advantages over existing liposome formulations. In particular, this cationic lipid emulsion formed a relatively small and physically stable emulsion/DNA complex capable of delivering a plasmid DNA in the presence of 90% (v/v) serum. The latter serum stability feature suggests that our emulsion system will be useful in *in vivo* as well as in *in vitro* transfection applications, especially where serum-containing medium is essential for cell culturing as in the case in primary cell cultures.

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