The Role of Organ Vascularization and Lipoplex-Serum Initial Contact in Intravenous Murine Lipofection

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Following intravenous administration of cationic lipid-DNA complexes (lipoplexes) into mice, transfection (lipofection) occurs predominantly in the lungs. This was attributed to high entrapment of lipoplexes in the extended lung vascular tree. To determine whether lipofection in other organs could be enhanced by increasing the degree of vascularization, we used a transgenic mouse model with tissue-specific angiogenesis in liver. Tail vein injection of N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTAP)/cholesterol lipoplexes resulted in increased lipoplex entrapment in hypervascularized liver but did not boost luciferase expression, suggesting that lipoplex delivery is not a sufficient condition for efficient organ lipofection. Because the intravenously injected lipoplexes migrated within seconds to lungs, we checked whether the effects of immediate contact with serum correlate with lung lipofection efficiency of different DOTAP-based formulations. Under conditions mimicking the injection environment, the lipoplex-serum interaction was strongly dependent on helper lipid and ionic strength: lipoplexes prepared in 150 mM NaCl or lipoplexes with high (>33 mol%) cholesterol were found to aggregate immediately. This aggregation process was irreversible and was inversely correlated with the percentage of lung cells that took up lipoplexes and with the efficiency of lipofection. No other structural changes in serum were observed for cholesterol-based lipoplexes. Dioleoyl phosphatidylethanolamine-based lipoplexes were found to give low expression, apparently because of an immediate loss of integrity in serum, without lipid-DNA dissociation. Our study suggests that efficient in vivo lipofection is the result of cross-talk between lipoplex composition, interaction with serum, hemodynamics, and target tissue “susceptibility” to transfection.

Cationic lipids, which are extensively employed as in vitro transfection agents, are promising agents for in vivo delivery of nucleic acids for a variety of applications from functional proteomics (1) to therapeutics (2). Obviously, the applications of cationic lipid-DNA complexes (lipoplexes) as in vivo delivery vehicles are useful only when the lipoplex-mediated transfection (lipofection) is high enough to modify the function of the protein/gene of interest.

The most challenging route of in vivo administration of lipoplexes is the intravenous (i.v.) route. Following mouse tail vein injection, most of the transgene expression occurs in the lungs, producing more than 80% of the total expressed protein in the animal (3–5). Despite significant efforts to optimize lipoplex performance following i.v. administration (4, 6), the “built-in” biological factors determining the efficiency of lung lipofection remain unclear.

It has been shown that within minutes after injection, 60–80% of the lipoplexes are entrapped in the lungs (4, 7, 8). This “first-pass” entrapment was ascribed to the highly extended lung capillary bed and was proposed to explain the predominant expression in the lungs (8, 9). Another factor thought to affect lung lipofection is the interaction of lipoplexes with serum. Incubation in pure serum or in serum-containing media can decrease the lipoplex delivery and alter intracellular processing (10, 11), lower or reverse the zeta (ζ)-potential (12), and even cause lipoplex disintegration upon prolonged exposure (6, 7, 12). However, the importance of the above mentioned serum effects for lung transgene expression following i.v. administration is in question because: (a) the injected lipoplexes are rapidly cleared from the circulation (4, 7) and internalized in lungs (13), thus ruling out long-term exposure to serum; (b) the pre-incubation of DOTAP/cholesterol lipoplexes with serum for as long as 30 min does not significantly impair lipofection in the lungs (8, 14).

In this work, we focused on formulations based on DOTAP, the most commonly used cationic lipid for various in vivo applications. Our goal was to clarify the mechanisms underlying murine lipofection, especially the role of organ vascularization and lipoplex-serum initial contact. To do this, we first checked whether the extent of vascularization is critical for the process of lipofection in the liver. We injected DOTAP/cholesterol lipoplexes into transgenic mice with VEGF-induced, liver-specific angiogenesis. Our results show that hypervascularization in the liver does not increase level of lipofection, despite increased entrapment of the lipoplexes. The fact that enlarging the vascular bed and increasing vascular permeability in liver are not sufficient to promote efficient lipofection leads to the conclusion

1 The abbreviations used are: i.v., intravenous(ly); DOTAP, N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride; VEGF, vascular endothelial growth factor; CF, carboxyfluorescein; CFPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(carboxyfluorescein); LRPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl); DoPE, 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine; HPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(7-hydroxycoumarin); EYFP, enhanced yellow fluorescent protein; UHV, unsized (non-extruded) heterogeneous vesicles; LUV, large unilamellar vesicles; cryo-TEM, cryogenic transmission electron microscopy; FRET, fluorescence resonance energy transfer; CMV, cytomegalovirus; FACS, fluorescence-activated cell sorter.

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that lungs might be an organ that is just more susceptible than liver to this process. Next, we checked that the interaction of immediate lipoplex-serum interaction to the process of lung lipofection. We found that the first contact between injected lipoplexes and serum affects lipoplex structure, the mode of delivery, and lipofection efficiency in the lungs.

**MATERIALS AND METHODS**

**Lipids and Fluorescent Probes—**DOTAP, carboxyfluorescein-PE (CFPE), and lissamine rhodamine-PE (LRPE) were obtained from Avanti Polar Lipids (Alabaster, AL). DOPE was purchased from Lipoid (Ludwigshafen, Germany). Cholesterol and spermidine base were purchased from Sigma. The pH-sensitive probe 7-hydroxycoumarin-PE (HCPE) was prepared in our laboratory.

DNA—pCMV-EYFP<sub>mito</sub>, a plasmid coding for enhanced yellow fluorescent protein (EYFP) carrying mitochondrial localization signal was purchased from Clontech (Palo Alto, CA). pCMV-Luc coding for the luciferase gene was constructed by insertion of a 875-bp CMV promoter-enhancer fragment into pGL3-enhancer (Promega, Madison, WI). pCMV-p53, based on pcDNA3 was provided by Dr. Mats O. Oren, Weizmann Institute of Science, Rehovot, Israel. All plasmids were propagated in Escherichia coli and purified in sterile endotoxin-free form using the Qiagen EndoFree Plasmid Mega kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol and were analyzed for purity and topology as described previously (15).

**Liposome Preparation—**For all preparations we used water purified with the WaterPro PS HPLC/UV Hybridrt Filter model (LABCONCO, Kansas City, MO), which delivers Type I 18.2-megohm/cm sterile (pyrogen-free to 0.06 endotoxin unit/ml) water. Unsize (non-extracted) heterogeneous vesicles (UHV) were prepared by mixing lipids in tert-butyl alcohol (Baker, Deventer, Holland) in a sterile isopropanol tube. The lipids were freeze-dried overnight and rehydrated in sterile water with gentle vortexing to make a 20 mM DOTAP concentration. Extruded large unilamellar vesicles (LUV) were prepared from UHV by stepwise extrusion through polycarbonate filters of 400- and 30-nm pore size, as described elsewhere (16). For fluorescent labeling, a fluorescent lipid was added at concentrations of 0.25–0.5 mol % to the lipids before the freeze-drying step.

**Preparation of Lipoplexes—**The lipoplexes were prepared under aseptic conditions. Briefly, volumes of DNA and cationic liposomes were made equal with sterile pure water, and DNA was added dropwise to the cationic liposomes with gentle agitation. The lipoplexes were prepared in 50% of the final volume, allowing for 9% NaCl or 50% DMSO in the remaining 10% of the volume to be added just before injection, to obtain an isotonic concentration of 5% dextrose or 0.9% NaCl in the remaining 10% of the volume to be added just before injection, to obtain an isotonic concentration of 5% dextrose or 0.9% NaCl in the formulations. When lipoplexes were prepared in different final volumes (200 or 800 μl), the volumes of DNA and lipid were correspondingly adjusted before the last mixing step, but the total dose of the components per animal was kept constant. When spermidine was used for pre-condensation of plasmid, it was added to plasmid solution at an amine/phosphate charge ratio of 0.5 10 min before adding the plasmid to the cationic liposomes. The lipoplexes were allowed to form for at least 15 min before injection.

**Transgene Expression and Function—**The protocols for the experiments were approved by the Ethics Committee of the Hebrew University’s Authority for Animal Facilities. For p53 efficacy study, B16-F10.9 melanoma cells (250,000 cells/mouse) were injected into the tail veins of 30 g (Harlan Laboratories, Rehovot, Israel) re-

**Fluorescence Resonance Energy Transfer (FRET) —**The plasmid DNA for FRET experiments was labeled covalently with carboxyfluorescein (CF). Such labeling only minimally affects plasmid topology and physical properties. Liposomes were labeled with LRPE. First, the emission spectra (excitation 490 nm, emission 510–600 nm) of DNA-CF or LRPE-labeled liposomes were measured separately on the spectrofluorimeter. In the next step, cationic liposomes with or without LRPE were added to DNA-CF at a charge ratio of 5 (+/-), and the spectra were recorded again. Finally, the spectra were recorded after the addition of various amounts of mouse serum to the lipoplexes.

**Fluorescent Lipid Quantification—**For CFPE or LRPE quantification, the liposomes were mixed with mouse serum at different ratios, and then immediately a drop of the lipoplex dispersion was placed on a slide, covered with a cover glass, and viewed in transmitted mode using Nomarsky contrast. For estimating size of aggregates, 3-μm latex beads (Sigma) were added to the formulations.

**Lipoplex-Serum Interaction—**For comparison of serum protein association with different lipoplexes, the latter were added to the mouse serum at a ratio of 1:2 (v/v). After a 5-min incubation, the mixtures (total 100 μl) were centrifuged at 14,000 rpm for 10 min. At the end of the procedure, 98% of the lipoplexes were in the pellet according to TLC analysis of fluorescent phospholipid or DOTAP in the supernatant (20). The lipids from the pellet were extracted with 200 μl of isopropanol, and the pellet was redissolved in 100 μl of 20% sodium dodecyl sulfate. The protein was quantified by a modified Lowry method (21).

**Cryogenic Transmission Electron Microscopy (Cryo-TEM) —**The cryo-TEM work was performed at the Hannah and George Krumholz Laboratory for Advanced Microscopy at the Technion, Haifa, Israel. Lipid dispersions and lipoplexes were prepared in exactly the same concentrations and conditions as throughout our study. For preparation of lipoplex-serum mixtures, lipoplexes were incubated in serum at a lipoplex:sерum ratio of 1:2 for ~5 min. The cryo-specimens were prepared, imaged, and processed as described (23).

**Physical Parameters of the Lipoplexes Before and After Incubation with Serum—**The mass-weighted size distribution of the liposomes and lipoplexes in buffer was determined at 25 °C using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). Viscosity of lipoplex dispersions was determined at 25 °C with a capillary microviscometer (Cannon Instrument, State College, PA).

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**Monitoring Membrane Surface Electrical Potential (V<sub>SM</sub>)—**The surface potential of both HCPE-labeled DOTAP/DOPS (1/1) and DOTAP/chol (1/1) liposomes was calculated from 515 nm excitation curves of the 7-hydroxyxocoumarin moiety of HCPE by recording excitation of HCPE at 405 nm and 380 nm, using emission at 450 nm, as described (16). The change of surface potential after the formation of lipoplexes and addition of serum was calculated from the change in degree of dissociation of the 7-hydroxyxocoumarin moiety of HCPE. Details and calculations are explained elsewhere (16, 24).
RESULTS

We started our work using LUV DOTAP/cholesterol (1:1)-based lipoplexes (with pre-condensation of the plasmid with spermidine at an amine/phosphate charge ratio of 0.5) because these have been shown to exhibit high transfection efficiency after i.v. administration (17). Throughout the study, to decrease the number of variables and to avoid toxicity inherent in the dose of cationic lipid and DNA (2), we kept the plasmid dose constant at 40 μg/mouse (121 nmol DNA phosphate/mouse) and the DOTAP-to-DNA +/− charge ratio constant at 5 (605 nmol of DOTAP/mouse).

Demonstration of in Vivo Protein Function by Means of Lipofection

We used a p53-coding plasmid to demonstrate that the gene phenotype and function of p53, which is a pro-apoptotic protein, indeed can be characterized in the lungs by lipoplex-mediated transfection. For this purpose, we injected the spermidine-based formulation, as described above, into mice with advanced lung metastases. Such treatment led to a significant decrease in the number of metastases (27.0 ± 1.4) compared with untreated animals (120.5 ± 13.4). The control group, in which the empty cassette pcDNA3 was used, also showed some regression of disease (78.5 ± 0.7), probably because of nonspecific, bacterial plasmid-dependent mechanisms (2).

Study of Mechanisms of Lipofection

Role of Organ Vascularization in Lipofection

Tail vein injection of pCMV-EYFP<sub>mito</sub>-based lipoplexes into normal mice produced visible EYFP expression predominantly in the lungs (Fig. 1A) and to a much smaller extent in the heart (Fig. 1B). The EYFP<sub>mito</sub> gene expression in lungs was confirmed by Northern blot (Fig. 1A, inset) and quantified by FACS (1.4 ± 0.3% EYFP-positive cells). On the other hand, in the liver no detectable EYFP expression was observed at 6–24 h post-injection (Fig. 1C).

To determine whether transgene expression in the liver could be boosted by means of increased vascularization, we injected LUV DOTAP/cholesterol (1:1)-based lipoplexes, containing either EYFP- or luciferase-expressing plasmid, into mice with VEGF-overexpressing livers (19). Such transgenic animals are characterized by extensive neovascularization and high vascular permeability, associated with greatly enlarged organ weight (up to 2.5 times). The results show that following tail vein injection, there was an increased liver accumulation of fluorescently labeled lipoplexes (Fig. 1D). Thus, as soon as 6 h post-injection, the normal livers contained on average 0.616 nmol of LRPE/organ, whereas VEGF-overexpressing livers contained on average 2.033 nmol of LRPE/organ. Despite more than a 3-fold boost in lipoplex entrapment, no visible EYFP expression was detected at 6–12 h post-injection. To increase the sensitivity of detection, we also measured luciferase expression levels. The expression was very low, 600 fg/mg tissue protein (about 100 times less than the corresponding values in the lungs) and did not differ from the normal transfected liver tissue.

Effect of Formulation Parameters on Transgene Expression in Lungs

We varied the following formulation parameters.

**Presence and Nature of Helper Lipid**—Table I shows the transfection efficiencies of the formulations prepared with different mole ratios of cholesterol or with DOPE instead of cholesterol. The optimal cholesterol mole fraction in the lipoplexes is ~33%. Further increasing cholesterol content decreases transfection. Formulations based on DOTAP alone also showed inferior transfection efficiency (not shown). Replacing cholesterol with DOPE drastically decreases transfection (Table I).

**Lipoplex Size**—To determine the effect of lipoplex size in the “pre-injection state,” we kept lipid composition (DOTAP/cholesterol (1:1) constant to avoid independent effects. We prepared lipoplexes of different sizes by: 1) starting from the ~500-nm UHV or from the 120-nm LUV; and 2) preparing lipoplexes from UHV in a final volume of 200 μl (“high concentration”) or 800 μl (“low concentration”). According to Table I, the high concentration UHV-derived lipoplexes (Formulation 2) were ~600 nm in size, whereas the corresponding LUV-derived lipoplexes (Formulation 6) were ~160 nm. The low concentration UHV-based lipoplexes (Formulation 5) were of intermediate size, ~380 nm. Lung transgene expression was highest for the high concentration UHV-based formulation and lowest for the LUV-based formulation (Table I).

**High Versus Low Ionic Strength Medium for Preparation of Lipoplexes**—The effects of media of various ionic strengths on the size and transgene expression efficiency of UHV DOTAP/cholesterol (1:1)-based lipoplexes were investigated. The presence of electrolyte resulted in partial aggregation of lipoplexes, although the aggregates could be redispersed by pipetting. Transgene expression in lungs (Table I) with 150 mM NaCl-based lipoplexes (Formulation 4) was 10-fold less efficacious than with 5% dextrose-based lipoplexes (Formulation 2). This effect of lipoplex medium on transfection was not a consequence of viscosity differences between the formulations, given that those differences are small (Table II) and the viscosity values are lower than that of mouse serum (1.5–2 centipoise).
Lipoplex-Serum Interaction in Lipofection

The formulations are abbreviated as in the following example: 200 dext UHV DOTAP/cholesterol (1:1)/pDNA, i.e. the lipoplexes were prepared in 200 μl of 5% dextrose from UHV of DOTAP/cholesterol (mol ratio DOTAP/cholesterol = 1:1) complexed with the plasmid. The sizes were measured by non-invasive back-scattering particle size analyzer and, in case of aggregation, estimated by light microscopy (as described under "Materials and Methods") from (+) aggregates sized well below 3 μm) to (+ + + (+) aggregates sized well above 3 μm). NM, not measured. The dynamic light scattering measurements give the unimodal population, which represents >90% by mass; sometimes there was "dust" (very large aggregates) in the sample, which we did not include in this table. The explanations of "low serum" and "high serum" are found under "Results." Both luciferase expression and percent of fluorescent-lipid-positive cells in lungs were determined at 12 h post-injection (n = 3–5). Notice the correlation between the level of aggregation in low serum, lipoplex delivery to lungs, and lipofection in Formulations 1–5.

| Formulation* | Lung luciferase expression | Initial lipoplex size | Size under "low serum" conditions | Aggregation by microscopy, "low serum" | Lipoplex-positive cells in lungs |
|--------------|----------------------------|-----------------------|----------------------------------|-------------------------------------|-------------------------------|
|              | pg/mg of tissue protein     | nm                    | nm                               | %                                   |                               |
| 200 dext UHV DOTAP/cholesterol (2:1)/pLuc (1) | 85 ± 3.3                  | 604                   | 1194 (2.0)                       | +                                   | 11.2                          |
| 200 dext UHV DOTAP/cholesterol (1:1)/pLuc (2) | 57 ± 2.7                  | 620                   | 1096 (1.8)                       | + + +                              | 9.3                           |
| 200 dext UHV DOTAP/cholesterol (1:2)/pLuc (3) | 6.6 ± 1.2                 | 1366                  | 5000 (3.7)                       | + + +                              | 3.4                           |
| 200 NaCl UHV DOTAP/cholesterol (1:1)/pLuc (4) | 5.5 ± 2.3                 | 830                   | 6850 (8.3)                       | + + +                              | 3.1                           |
| 800 dext UHV DOTAP/cholesterol (1:1)/pLuc (5) | 79.5                      | 520                   | 472 (1.0)                        | NM                                  | 10.2                          |
| 200 dext LUV DOTAP/cholesterol (1:1)/pLuc (6) | 11.3 ± 10.4               | 142                   | 310 (2.2)                        | NM                                  | 4.4                           |
| 200 dext UHV DOTAP/DOPE (1:1)/pLuc (7) | 1.82 ± 0.2                | 142                   | 1190 (2.8)                       | + + +                              | 8.4                           |

* Formulation numbers are boldface and enclosed in parentheses.

# Effect of Formulation Parameters on Lipoplex Delivery in Lung Tissue

We tracked the delivery and distribution of fluorescently labeled lipoplexes in the lung tissue at different times post-injection. For all UHV-based formulations, the greater part of the lipid dose (about 70%) reached the lung within the first 10 s post-injection, indicating a considerable first-pass effect. In the case of LUV-based lipoplexes, however, only 30% of the fluorescent was recovered in the lungs by this time. The reason for this might be that the smaller, LUV-based, lipoplexes are poorly entrapped in the lung vasculature. With time, the amount of fluorescent in lungs steadily decreased (40% after 30 min and 2% after 12 h for all UHV-based formulations; 15% after 30 min and 0.5% after 12 h for LUV-based lipoplexes). On the other hand, there was a significant dependence of UHV DOTAP/cholesterol-based lipoplex lung distribution on formulation parameters as early as 10 s post-injection. Thus, according to histological tissue examination, lipoplexes administered in 150 mM NaCl produced a very heterogeneous fluorescence distribution (Fig. 2A), i.e. fluorescence was associated with "islands" of cells, whereas other areas were fluorescence-free. Increasing the mole ratio of cholesterol above (1:1), even when lipoplexes were injected in low ionic strength medium, also led to the appearance of the same heterogeneous fluorescence pattern (not shown). On the other hand, lipoplexes administered in 5% dextrose produced a homogeneous distribution of fluorescence throughout the lung tissue with most of the endothelial cells being fluorescent (Fig. 2B).

Twelve hours post-injection, despite decrease in total fluorescence in the lung tissue, the above described differences in the distribution remained (not shown). As quantified by FACS for the formulations that initially distributed heterogeneously (Table I, Formulations 3 and 4), the percent of lung cells that contained lipoplexes was roughly three times less than for the formulations that produced homogeneous lung distribution (Formulations 1, 2, and 5).

The fluorescent phospholipids used for lipoplex labeling when extracted from the lungs 0.5–12 h post-injection demonstrated emission spectra and TLC separation patterns identical to those of the reference lipids (not shown), proving that they remained intact in the tissue and were not metabolized.

### Table II

| Formulation | Viscosity | Ψp potential before addition of serum | Ψp potential in serum | Bound serum proteins | Residual cholesterol after incubation in serum |
|-------------|----------|-------------------------------------|----------------------|----------------------|-----------------------------------------------|
| 200 dext UHV DOTAP/cholesterol (1:1)/pLuc (2) | 1.07     | 168.2                               | 170.8                | 1.31                 | 95.6                                          |
| 200 dext UHV DOTAP/cholesterol (1:2)/pLuc (3) | NM       | NM                                  | NM                   | 1.05                 | 93.8                                          |
| 200 NaCl UHV DOTAP/cholesterol (1:1)/pLuc (4) | NM       | NM                                  | NM                   | 1.14                 | NM                                            |
| 200 dext UHV DOTAP/DOPE (1:1)/pLuc (7) | 1.19     | 157.3                               | 122.0                | NM                   | NM                                            |

Abbreviations and formulation numbers (boldface, in parentheses) are as in Table I. The parameters were recorded before or after a short (30 s–5 min) incubation of preformed formulations in "low serum/lipoplex ratio" (except for serum-protein binding, which was measured in "high serum/lipoplex ratio"). For surface potential (Ψp) measurements, serum was added under stirring to the cuvette with the HCPE-labeled lipoplexes. NM, not measured.

## Effect of Formulation Parameters on Different Aspects of Serum-Lipoplex Interaction

Encouraged by the correlation between lung delivery and transgene expression efficiency of DOTAP/cholesterol-based formulations, we attempted to explain the observed differences in delivery and efficiency on the basis of short (<5 min) lipoplex-serum contact. Such interaction should be relevant to delivery to lungs in view of the fact that the major part of the injected dose as well as the differences in the lipoplex distribution were found by us as early as 10 s following i.v. injection. Thus, any physical modification of lipoplexes during their initial contact with serum may affect lung lipoplex delivery and transgene expression.

### Lipoplex Aggregation—We studied changes of lipoplex size in different serum conditions: (a) lipoplexes were added to serum at a volume ratio of 2:1, equivalent to 88 nmol of DOTAP/mg of protein (hereafter "low serum" conditions); (b) lipoplexes were added to serum at a volume ratio of 1:2, equivalent to 22 nmol
DOTAP/mg protein (hereafter “high serum” conditions). The rationale for low serum conditions is to mimic the situation during injection, when a large amount of lipoplexes interacts initially with a small amount of serum (see “Discussion”). Because size measurement by dynamic light scattering is accurate only for spherical particles ≥1 μm (25), we also estimated the size of aggregates using light microscopy (Table I and Fig. 2, insets). According to our data, the pattern of lipoplex aggregation under low serum conditions closely correlated with lipoplex distribution in lungs. Thus, the UHV DOTAP/cholesterol (1:1), 150 mM NaCl-based formulation in conditions of low serum immediately formed aggregates sized well above 3 μm (Fig. 2A, inset). Conversely, the same lipoplexes, but based on 5% dextrose, only modestly aggregated under the same conditions (Table I and Fig. 2B, inset). Increasing the cholesterol mole fraction in the lipoplexes above 50% (Formulation 3 in Table I) also caused strong aggregation of the lipoplexes under low serum conditions even when lipoplexes were prepared in 5% dextrose (Table I). It is of interest that lipoplexes of any composition and in any medium did not aggregate when placed under high serum conditions (Table I).

To check the reversibility of aggregation upon transition from low serum to high serum, which imitates the gradual dilution of the injected lipoplexes in the overwhelming amount of serum, we performed the mixing of lipoplexes with serum in two steps. First, we mixed 40 μl of UHV DOTAP/cholesterol (1:1), 150 mM NaCl-based lipoplexes with 10 μl of mouse serum and then immediately added an additional 70 μl of serum. The aggregation produced in the first step was not reversed by further dilution in serum, suggesting that what occurs at the site of injection is virtually irreversible.

Lipoplex Chemical Composition—We measured the quantity of serum proteins that binds to DOTAP/cholesterol (1:1) lipoplexes following a 5-min incubation in serum and did not find large differences among formulations (Table II). To assess whether cholesterol desorbs from lipoplexes to serum components, we measured the amount of cholesterol in different formulations before and after a 5-min incubation in serum. There was no decrease in cholesterol at the DOTAP/cholesterol mole ratios checked (Table II).

Lipoplex z-Potential—We measured z-potential, which is a widely accepted parameter for physical characterization of particle surface charge, before and after incubation of lipoplexes in serum. z-Potential of cationic liposomes and of lipoplexes at a +/− charge ratio of 5 measured in low ionic strength medium (10 mM NaCl) was positive (+72.1 mV). In 150 mM NaCl, as expected, the initial z-potential of lipoplexes was less positive (+54.5 mV) than in 10 mM NaCl. Following the addition of a small (1:5 v/v) amount of serum, the z-potential reversed to slightly negative (−13.8 mV), indicating a certain loss of colloidal stability of lipoplexes.

Lipoplex Microstructure—The aggregation of the lipoplexes in serum did not correlate with transfection efficiency for all of the formulations (see Table I). We considered it possible that the nanostructural differences among formulations could also be responsible for their variable efficiencies. First we characterized the studied formulations in the “pre-injection” state, i.e., before their contact with serum. According to cryo-TEM, the non-extruded heterogeneous vesicles, UHV (previously referred to as multilamellar vesicles, (26, 27)), were found to have only one or two bilayers (Fig. 3A, inset) and, apart from size, were similar to the extruded LUV (not shown). This was a rather surprising finding. Moreover, the lipoplexes prepared from LUV and UHV, apart from their sizes, were structurally very close to each other (a typical lipoplex is shown in Fig. 3A).

Similarly, formulations based on cholesterol or DOPE as helper lipids also showed much similarity (not shown).

In the next step, we studied the lipoplexes after a short (<5 min) incubation in serum. According to Fig. 3, the finer details of the systems are difficult to discern due to the high concentration of electron-dense materials (serum proteins), but some important features could be observed. Five percent dextrose, UHV DOTAP/cholesterol (1:1)-based lipoplexes in serum showed some indications of accelerated vesicle fusion, but, in general, the lipoplexes preserved their shape and morphology after the addition of serum (Fig. 3B). The serum proteins did not penetrate into the vesicles but appeared to be associated only with the outer membranes. When 5% dextrose UHV DOTAP/DOPE (1:1)-based lipoplexes were incubated in serum, however, many small vesicles and membrane fragments appeared (Fig. 3C), suggesting significant lipoplex disintegration.

Lipoplex Electrical Surface Potential, Ψo—To find out whether serum protein association with lipoplexes is accompanied by neutralization of lipoplex electrical surface potential, we used the electrical surface potential-sensing probe, HCPE. This non-exchangeable and non-transferable probe, having all
the advantages of 4-heptadecyl-7-hydroxycoumarin (16, 24), is positioned in the lipid assembly slightly above the plane of the DOTAP quaternary amine. HCPE is extremely useful for sensing micro-environmental changes in surface pH, which are expected to occur in the presence of serum, where acidic serum proteins bind to cationic membranes. When DOPE- and cholesterol-based lipoplexes were compared, the former, but not the latter, showed a considerable decrease in surface potential 30 s after the addition of serum (Table II). This neutralization of the DOTAP/DOPE bilayers by acidic serum proteins could indicate increased bilayer accessibility to serum. In contrast, DOTAP/cholesterol lipoplexes showed a low degree of Ψ2 neutralization after incubation with serum.

Liposomal Bilayer-DNA Association—DOPE is expected to weaken the association of DOTAP with plasmid DNA due to internal salt bridges between the phosphate group of DOPE and the quaternary amine of DOTAP (16, 24), rendering the lipoplex more accessible to nucleic acid stains (28) and acidic serum proteins (29). It has been suggested that serum displaces DNA from lipoplexes based on DOPE (8, 28). To find out whether disintegration of DOTAP/DOPE lipoplexes in serum is accompanied by lipid-DNA dissociation, we studied lipoplex integrity with FRET. We used carboxyfluorescein-labeled plasmid and LRPE-labeled cationic liposome as the donor-acceptor pair in resonance energy transfer. There is significant FRET in both DOTAP/cholesterol (1/1) and DOTAP/DOPE (1/1) lipoplexes (Fig. 4). The efficiency of FRET was calculated as described elsewhere (30) and was found to be ~50% for both formulations. After the addition of excess of serum to lipoplexes of DOTAP/cholesterol or DOTAP/DOPE, there was no evidence of lipid-DNA dissociation (no increase in fluorescence intensity at 520 nm and no decrease at 580 nm) in either type of lipoplexes. However, the emission at 520 nm showed a significant decrease, which could be ascribed to changes in the probe environment upon the addition of serum.

DISCUSSION

Efficient transgene expression in murine lungs following i.v. injection of cationic lipoplexes is a composite of several factors, some of which have been studied in this work.

Role of Organ Vascularization—We show that in the first seconds post-injection, lungs accumulate 60–70% of the dose. However, as early as 1 h post-injection, this amount drops to less than 40%. Despite the fact that within 1 h other organs receive a significant proportion of transfection-competent lipoplexes (8, 13), the expression in other organs is only a small fraction of that in the lungs. Interestingly, LUV-based lipoplexes, which are poorly entrapped in the lung vasculature (this study), still produce predominant, albeit decreased (in comparison with UHV-based lipoplexes), expression in the lungs (3). In view of this finding, it appears that high lipoplex delivery to an organ is a necessary, but not the only, precondition for efficient lipofection. This conclusion is supported by our results showing that artificially increasing the liver vascular bed and vascular permeability increased lipoplex entrapment but did not increase lipofection in this organ (Fig. 1D). The mechanism of such limitation is not known but appears to involve the susceptibility of a particular organ to transfection with lipoplexes. One of the reasons for liver being less "lipofectable" than lung could be due to the destruction of lipoplexes...
by Kupffer macrophages. However, it should be kept in mind that liver can be successfully transfected by alternative mechanisms, e.g. by high volume, naked DNA injection (11) or by hepatocyte-targeted lipoplexes (31).

**Role of Initial Contact between the Injected Lipoplexes and Serum—**During the injection of lipoplexes, they mix with about 50–100 μl of serum, presuming cardiac flow through the tail and abdominal veins to be ~300 μl of serum per 3 s (32). This situation is modeled by our experimental conditions of a low serum/lipoplex ratio, i.e. 50–100 μl serum/200 μl of lipoplex dispersion. At this lipoplex/serum ratio the cationic membrane surfaces are only partially associated with anionic serum proteins, which therefore may act as bridges between lipoplexes, thereby inducing lipoplex aggregation (Fig. 2, insets).

The extent of aggregation is significantly increased by the presence of high cholesterol (>33 mol %) in the lipoplexes. The mechanisms of this phenomenon are worth discussing here. For a phospholipid/cholesterol mixture to maintain a bilayer structure, the additive average packing parameter, \( P \), should be within the range of 0.74 to 1.0 (33, 34). At \( P \geq 1.0 \) (>40 mol % cholesterol (34)), the oversaturation with cholesterol leads to lateral separation of cholesterol-rich domains with subsequent exclusion of cholesterol microcrystals (35), which were observed by us previously in DOTAP/cholesterol (1:1 or 1:2) lipoplexes. Cholesterol in lipoplexes also induces dehydration and defects in the cationic lipid bilayer (36). In serum, the presence in lipoplexes of dehydrated domains engenders hydrophobic interactions, thereby enhancing aggregation.

The strong aggregation of lipoplexes prepared in 150 mM NaCl at conditions of low serum could be explained by countercations present in both the formulation and in serum (8.58 milliseimens/cm in serum (37)) that cause additional shielding of the remaining electric charges of the lipoplexes. The ‘preventive’ effect of dextrose on aggregation cannot be ascribed to viscosity but rather to the local low ionic strength at the site of injection. It should be stressed here that NaCl, even at 150 mM concentration, does not disrupt or weaken the DNA-DOTAP interaction, as demonstrated by the absence of free DNA in the supernatant after ultracentrifugation of DOTAP/cholesterol lipoplexes, and by ethidium bromide displacement studies.\(^5\)

A possible mechanism that we offer to explain the loss of lipoplex transfection efficiency following immediate aggregation at the injection site is quite straightforward. A dramatic decrease in the total number of particles (Fig. 2A, inset) means that there are insufficient lipoplexes to associate with the many lung endothelial cells (Fig. 2A). In addition, large aggregates that succeed in binding to cell membranes could be unable to enter the cells because of their size or could be physically dislodged by erythrocytes. It is worth stressing that once the aggregation at a low serum/lipoplex ratio has occurred, it is irreversible, even if lipoplexes are subsequently diluted in a large excess of serum. Thus, the structural changes of lipoplexes at site of injection conditions determine, to a large extent, the transfection efficiency.

It has been suggested that modest aggregation aids in retention of the particles in capillaries and in anchorage on the cell surface (8, 9). We show that the optimal size of lipoplex aggregates lies within the range of 400 to 1200 nm (Table I). Above this range, aggregation lowers transfection for the reasons described. Below this range, the LUV-based lipoplexes, despite their gross structural similarity to the UHV-based lipoplexes, cannot be efficiently entrapped in the lung vasculature (8, 38). In cell culture, the transfection with LUV-derived lipoplexes is inferior to that of UHV-derived lipoplexes for reasons that are not completely understood (39). Whatever the reason for such inferior efficiency in cell culture, it could be speculated that the difference between these two types of lipoplexes in vivo involves the additional factor of hemodynamics, which places a constraint on the minimum size required for lipoplex vascular entrapment and retention on the target cells.

**Role of Serum-mediated Changes of Lipoplex Structure and Electrostatics—**DOTAP/cholesterol lipoplexes retain their lipofection activity in serum, despite extensive protein binding to their external surface and reversal of \( \zeta \)-potential to a negative value. We think that the explanation could be the fact that the electrical surface potential, \( \Psi_o \), is only slightly affected by serum in DOTAP/cholesterol lipoplexes. In contrast, \( \zeta \)-potential fails to correlate with efficiency of lipofection (12, 40) because it is a measure of particle charge at the plane of shear, distant from the bilayer surface. The \( \Psi_o \) stability in serum agrees well with cryo-TEM images of DOTAP/cholesterol lipoplexes, showing that many liposomal surfaces are not available for protein binding (Fig. 3B). Presumably, the residual positive charge is important for cellular entry and processing of lipoplexes (2).

DOTAP/DOPE lipoplexes, in contrast, show an instability of \( \Psi_o \) which correlates with the binding of serum proteins to all liposomal surfaces (Fig. 3C) soon after exposure to serum. Because DNA does not dissociate from DOTAP in either type of lipoplex in the presence of serum, liposomal disintegration and surface potential neutralization are the main explanations for the inferior DOTAP/DOPE efficiency. The bilayer instability of DOPE-containing lipoplexes (because DOPE is a hexagonal-phase-prefering lipid) was proposed to explain superior DOTAP/DOPE efficiency in cell culture transfections (41). In the case of intravenous injection, this instability of DOPE-based lipoplexes in serum stems from the same cause and is definitely a disadvantage.

**Conclusion—**An understanding of the interplay between lipoplex composition, its interaction with serum, hemodynamics, and target tissue properties could explain the biodistribution and efficiency of transfection by intravenously administered lipoplexes.

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**References**

1. Shoshani, T., Faerman, A., Mett, I., Zelin, E., Tenne, T., Gordin, S., Moshel, Y., Elbaz, S., Budanov, A., Chajut, A., Kalinski, H., Kamar, I., Ronen, A., Mor, O., Keshet, E., Leshkovitz, D., Einiat, P., Skaliter, R., and Feinstein, E. (2002) Mol. Cell. Biol. 22, 2283–2293
2. Dass, C. R. (2002) Int. J. Pharm. 241, 1–25
3. Liu, F., Qi, H., Huang, L., and Liu, D. (1997) Gene Ther. 4, 517–523
4. Mahato, R. I., Anwer, K., Tagliaferri, F., Meaney, C., Leonard, P., Wadwia, M. S., Logan, M., French, M., and Rolland, A. (1998) Hum. Gene Ther. 9, 2983–2999
5. Song, Y. K., Liu, F., Chu, S., and Liu, D. (1997) Hum. Gene Ther. 8, 1585–1594
6. Li, S., and Huang, L. (1997) Gene Ther. 4, 891–900
7. Niven, R., Pearlman, R., Wedeking, T., Mackeigan, J., Noker, P., Simpson-Herren, L., and Smith, J. G. (1998) J. Pharm. Sci. 87, 1292–1299
8. Li, S., Tseng, W. C., Stolz, D. B., Wu, S. P., Watkins, S. C., and Huang, L. (1999) Gene Ther. 6, 585–594
9. Song, Y. K., Liu, F., and Liu, D. (1998) Gene Ther. 5, 1531–1537
10. Kscisz, V., Cicinna, C., Lacroix, P., Byk, G., Scherman, D., and Wils, P. (1998) Biochim. Biophys. Acta 1368, 276–288
11. Yang, J., Chen, S., Huang, L., Michalopoulos, G. K., and Liu, Y. (2001) Hepatology 33, 848–859
12. Zelphati, O., Uyechi, L. S., Barron, L. G., and Szoka, F. C., Jr. (1998) Biochim. Biophys. Acta 1390, 119–133
13. Barron, L. G., Gagne, L., and Szoka, F. C., Jr. (1999) Hum. Gene Ther. 10, 1683–1694
14. Sakurai, F., Nishioha, T., Saito, H., Baba, T., Okuda, A., Matsumoto, O., Taga, T., Yamashita, F., Takakura, Y., and Hashida, M. (2001) Gene Ther. 8, 677–686

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4. D. Simberg, S. Weisman, Y. Talmon, and Y. Barenholz, unpublished observation.
5. S. Even-Chen and Y. Barenholz, in preparation.
References

15. Even-Chen, S., and Barenholz, Y. (2000) Biochim. Biophys. Acta 1509, 176–188
16. Zuidam, N. J., and Barenholz, Y. (1997) Biochim. Biophys. Acta 1329, 211–222
17. Hung, K., Zheng, W., Baker, A., and Papahadjopoulos, D. (1997) FEBS Lett. 400, 233–237
18. Lavnikova, N., Prokhorova, S., Helyar, L., and Laskin, D. L. (1993) Am. J. Respir. Cell Mol. Biol. 8, 384–392
19. Dor, Y., Djonov, V., Abramovitch, R., Itin, A., Fishman, G. I., Carmeliet, P., Goelman, G., and Keshet, E. (2002) EMBO J. 21, 1939–1947
20. Simberg, D., Hirsch-Lerner, D., Nissim, R., and Barenholz, Y. (2000) J. Liposome Res. 10, 1–13
21. Peterson, G. L. (1983) Methods Enzymol. 91, 95–119
22. Shmeeda, H., Petkova, D., and Barenholz, Y. (1995) Am. J. Physiol. 268, H1759–H1766
23. Simberg, D., Danino, D., Talmon, Y., Minsky, A., Ferrari, M. E., Wheeler, C. J., and Barenholz, Y. (2001) J. Biol. Chem. 276, 47453–47459
24. Zuidam, N. J., and Barenholz, Y. (1998) Biochim. Biophys. Acta 1368, 115–128
25. Ostrowsky, N. (1993) Chem. Phys. Lipids 64, 45–56
26. Zuidam, N. J., Hirsch-Lerner, D., Margulies, S., and Barenholz, Y. (1999) Biochim. Biophys. Acta 1419, 207–220
27. Felgner, J. H., Kumar, R., Srudhar, C. N., Wheeler, C. J., Tsai, Y. J., Border, R., Ramsey, P., Martin, M., and Felgner, P. L. (1994) J. Biol. Chem. 269, 2550–2561
28. Zuhorn, I. S., Oberle, V., Visser, W. H., Engberts, J. B., Bakowsky, U., Polushkin, E., and Hoekstra, D. (2002) Biophys. J. 83, 2096–2108
29. Li, S., Rizzo, M. A., Bhattacharya, S., and Huang, L. (1998) Gene Ther. 5, 930–937
30. Lakowicz, J. R. (1999) in Principles of Fluorescence Spectroscopy (Lakowicz, J. R., ed) 2nd Ed., pp. 367–390, Kluwer Academic/Plenum Publishers, New York
31. Hwang, S. H., Hayashi, K., Takayama, K., and Maitani, Y. (2001) Gene Ther. 8, 1276–1280
32. Davies, B., and Morris, T. (1993) Pharm. Res. 10, 1093–1095
33. Israelachvili, J. N., Marcelja, S., and Horn, R. G. (1980) Q. Rev. Biophys. 13, 121–209
34. Kumar, V. V. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 444–448
35. Lagane, B., Mazeres, S., Le Grimellec, C., Cezanne, L., and Lopez, A. (2002) Biophys. Chem. 95, 7–22
36. Hirsch-Lerner, D., and Barenholz, Y. (1999) Biochim. Biophys. Acta 1461, 47–57
37. Elyahu, H., Servel, N., Domb, A. J., and Barenholz, Y. (2002) Gene Ther. 9, 850–854
38. Liu, F., and Huang, L. (2002) J. Controlled Release 78, 259–266
39. Audouy, S., and Hoekstra, D. (2001) Mol. Membr. Biol. 18, 129–143
40. Smith, J. G., Wedeking, T., Vernachio, J. H., Way, H., and Niven, R. W. (1998) Pharm. Res. 15, 1356–1363
41. Hui, S. W., Langman, M., Zhao, Y. L., Ross, P., Hurley, E., and Chan, K. (1996) Biophys. J. 71, 589–599