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Evaluation and Optimization of Different Cationic Liposome Formulations for *in Vivo* Gene Transfer

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Five commonly used cationic liposome formulations were tested for their ability to deliver DNA to established subcutaneous human tumor xenografts in SCID mice. Liposomes were complexed with a mammalian expression plasmid containing the bacterial β-galactosidase gene and delivered to tumors by direct injection. The optimal lipid to DNA ratios *in vivo* were markedly different than those observed *in vitro* for each liposome formulation. Tumor size at the time of inoculation also effected transfection efficiency significantly. Of the five liposome formulations tested, DC-Cholesterol was found to be superior to all others *in vivo.* Even under optimal conditions however, the efficiency of *in vivo* transfection was low in our system ($\sim$0.3%). Implications of these results for *in vivo* gene therapy of tumors are discussed.

Cationic liposomes represent an attractive alternative to viral gene delivery systems for various applications of gene therapy. Their simplicity of use, lack of toxicity, biological inertness, non-immunogenic properties and the ability to deliver very large pieces of DNA into tissues has resulted in their use in a number of clinical trials for gene therapy of cancer (reviewed in 1). Although preliminary studies with murine tumors produced encouraging results (2), comparatively little is known about the effectiveness of cationic liposome mediated gene delivery in the treatment of human cancer (3). Numerous lipid formulations have been synthesized and used for DNA delivery *in vivo* (reviewed in 1), however a systematic evaluation of the efficiency of *in vivo* gene transfer into human tissues by various liposome formulations has not been reported. Such information is essential to optimize protocols for the *in vivo* delivery of DNA to human tumors.

We evaluated the *in vivo* gene transfer efficiency of five commonly used liposome formulations including Lipofectin, Lipofectamine, Cellfectin, DC-Cholesterol and DMRIE-DOPE into human tumor xenografts established in SCID mice. Several parameters including the liposome to DNA ratios and the effect of tumor size at the time of injection were evaluated and the *in vivo* transfection efficiency for each lipid was determined.

MATERIALS AND METHODS

Mice. C.B.-17 *scid/scid* mice were obtained from our breeding colony. All mice were maintained in microisolation cages (Lab Products Inc., Federalsburg, MA) under pathogen-free conditions. Animals of both sexes were used in the studies at 8–12 weeks of age.

Liposomes and plasmid DNA. Construction and characterization of the bicistronic mammalian expression vector pCMVβIL-2 containing the bacterial β-galactosidase gene and the human interleukin-2 cDNA was described elsewhere (6). Lipofectin, Lipofectamine and Cellfectin were purchased from Gibco-BRL Inc. (Gaithersburg, MD). DC-Cholesterol was kindly provided by Dr. Leaf Huang (University of Pittsburgh, Pittsburgh, Pennsylvania). DMRIE-DOPE was a gift from Vical, Inc. (San Diego, CA).

Liposome mediated transfection of cells in vitro. A human lung squamous cell carcinoma cell line (2E9) which was established from a primary tumor in our laboratory was used for the transfection studies. Cells were plated at a density of 10^4 per well in 3 mls of DMEM-F12 containing 10% Fetal Calf Serum in Falcon multi-well tissue culture plates (35mm wells). After overnight incubation, the spent medium was removed, cells were rinsed 3 times with sterile PBS and 1 ml of serum-free DMEM medium containing the DNA: liposome complexes at various lipid to DNA ratios (nanomoles lipid to μg DNA, at a DNA concentration of 1 μg/ml) was added to the wells. After a 5 hour incubation in the incubator at 37°C

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under 5% CO_2, transfection medium was replaced with 3 mls of complete medium and the cells were incubated for another 19 hours. Cells were then fixed and stained for β-galactosidase activity as described (7). Transfection efficiency was evaluated by counting the number of blue-staining vs. clear cells using an inverted microscope. At least 200 cells were counted in each well.

Transfection of human tumor xenografts in vivo. Mice were inoculated with 1×10^6 2E9 cells in 100 μl of sterile DMEM subcutaneously in the ventral caudal midline area. Tumors were allowed to grow for 2–3 weeks and 10 μg of plasmid DNA, complexed with liposomes at various ratios (nanomoles of lipid to μg of DNA), was injected directly into the tumors in 50 μl of DMEM. For analysis of transfection efficiency, tumors were removed 16–20 hours post-injection and single cell suspensions were prepared by enzymatic digestion as described (8). The tumor cell suspension (in 3 ml of DMEM) was loaded onto a 100% Ficoll-paque cushion (4 mls, 15 ml Falcon tube) and centrifuged at 1000g for 15 minutes. Viable cells free of debris, erythrocytes and dead tumor cells were collected from the interphase, washed twice with DMEM and were stained for β-galactosidase activity. Percent transfection was determined by microscopic analysis of the cells. A minimum of 10^4 cells were screened for each sample.

RESULTS AND DISCUSSION

Determination of optimal in vitro and in vivo lipid to DNA ratios. The ratio of lipid to DNA molecules in liposome:DNA complexes is one of the critical variables in the optimization of *in vitro* transfections. Since the microenvironment that liposome:DNA complexes encounter *in vivo* is very different from that experienced *in vitro* (*i.e.* presence of serum proteins and extracellular matrix), the optimal ratios found *in vitro* will most likely not be the same *in vivo.* The changing conditions could also effect the activity of individual lipids to various degrees depending on their structure and charge. We therefore decided to test and compare the activities of each formulation both *in vitro* and *in vivo.* As expected each lipid had a different lipid to DNA optimum *in vitro* (between 6 to 1 and 10 to 1, nanomoles of lipid to μg of DNA) and the optimal transfection efficiencies varied between 12 to 55% with DMRIE-DOPE being the most effective formulation for *in vitro* gene delivery **(Table 1).** When the liposomes were evaluated *in vivo* however, both the optimal ratios and overall transfection efficiencies changed drastically **(Figure 1).** The lipid to DNA optima were lower (between 1 to 1 and 4 to 1) and the efficiency of transfection for individual formulations varied between 0.01–0.3%. The highest efficiency was obtained with DC-Chol which was 7 to 8-fold more effective than Lipofectin and 20 to 25-fold more effective than Cellfectin, Lipofectamine and DMRIE-DOPE **(Figure 1).** These results clearly establish that *in vitro* systems for evaluating liposome mediated gene delivery should not be used for optimization of *in vivo* gene delivery protocols.

Effect of tumor size on transfection efficiency. In the next set of experiments, we evaluated the effect of tumor size on the efficiency of transfection since results from therapy studies had indicated that tumor size at the time of DNA delivery might influence this parameter (unpublished observations). Mice were inoculated with various amounts of the human squamous cell lung

TABLE 1
Summary of the *in Vitro* Transfection Studies

Liposome Formulation	Optimal Lipid to DNA Ratio (nanomoles:μg)	Maximum Transfection Efficiency (%)
Lipofectin	8:1	12
DC-Cholesterol	10:1	24
Lipofectamine	6:1	35
Cellfectin	8:1	19
DMRIE/DOPE	8:1	55

Various lipid formulations were complexed with pCMVβIL-2 plasmid DNA at ratios of 2, 4, 6, 8, 10 and 12 nanomoles of lipid to 1 μg of DNA and were used to transform 2E9 cells in 6-well culture dishes as described in the materials and methods section. The percentages were determined by counting a minimum of 200 cells per well.

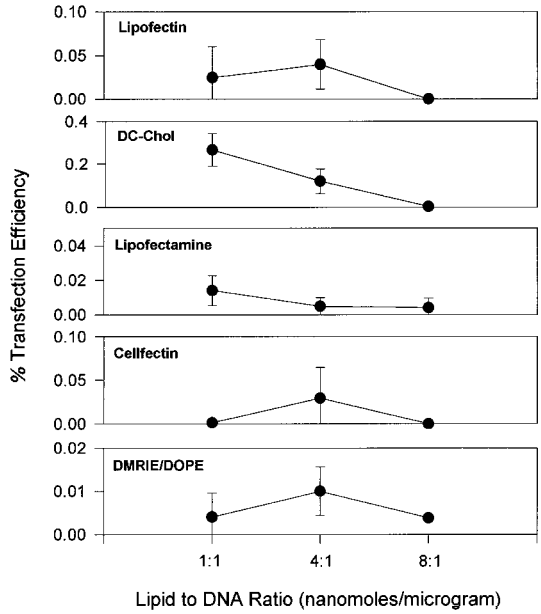

FIG. 1. Optimization of lipid to DNA ratios *in vivo.* Each lipid formulation was complexed with 10 μg of plasmid DNA at a given liposome (nanomoles) to DNA (μg) ratio in a total of 50 μl DMEM and was injected directly into the tumors. Efficiency of transfection was evaluated by analysis of single-cell suspensions prepared from tumors as described in the materials and methods. Each point is an average of 2 or 3 mice with the error bars corresponding to standard error.

carcinoma cell line 2E9 and tumors, upon reaching various sizes, were injected with 10μg of pCMVβIL-2 complexed with 10 nanomoles of DC-Cholesterol in 50μl of DMEM. Analysis of the transfection efficiency in these tumors revealed a very interesting pattern **(Figure 2).** A clear effect of tumor size on the efficiency of DNA delivery, which appeared to be nearly exponential, could be seen. A 4 to 5-fold reduction in transfection efficiency accompanied an increase from 20 mg to 100 mg in tumor size. In addition to more obvious explanations such as the decreasing amount of lipid:DNA complexes per cell with increasing tumor size, these results can also be explained by the changing physiological conditions within a tumor as it grows. For example, the 2E9 xenografts become highly vascularized and start developing largely necrotic centers once they reach 100 to 150 mg in size. It is possible that the efficiency of liposome:DNA complexes decrease drastically in the necrotic environment that typically contains large quantities of serum proteins which might disrupt the lipid:DNA complexes and inhibit their uptake by the cells. Poor efficiencies obtained *in vitro* in the presence of serum proteins would appear to support this notion.

Comparison of the transfection efficiencies of liposome formulations in vivo. The initial results obtained from the optimization studies **(Figure 1)** could not be used for direct comparison of the transfection efficiency of different formulations since tumor sizes, although constant within each group, varied between groups. In light of the results obtained from the tumor size studies, a more accurate comparison of each lipid would have to involve tumors of similar size. We therefore tested each formulation, using the optimal *in vivo* lipid to DNA ratios established for each, in mice carrying tumors of similar sizes. The results from such an experiment, where tumor sizes were between 18 to 30 mg, are shown in **Figure 3.** These results establish that DC-Cholesterol is at least 3 to 4-fold more effective than all other formulations. The order of efficiency has changed slightly (Lipofectamine and Cellfectin changed places) but the difference is not statistically significant. The naked DNA control sustains the notion that complexing of DNA with cationic liposomes in fact improves transfection.

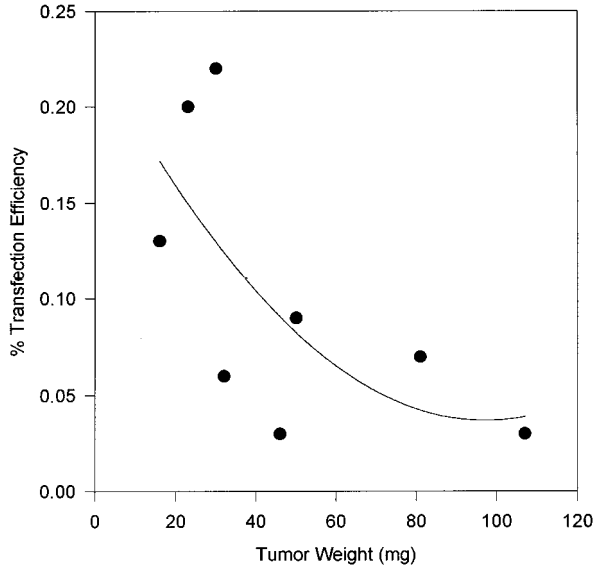

FIG. 2. Effect of tumor size on transfection efficiency. DC-Cholesterol (10 nanomoles) and plasmid DNA (10 μg) were complexed in 50 μl DMEM and were injected directly into tumors of various sizes. Tumors were removed 16–20 hours post-injection, weighed and processed to determine the efficiency of transfection. Sigmaplot version 2.0 was used to obtain the best fit curve with 2 degrees of freedom.

This is the first report, to our knowledge, where the *in vivo* tumor transfection efficiencies of different cationic liposome formulations were evaluated in a systematic manner. Here we show that both lipid to DNA ratio and tumor size are critical parameters that have to be considered prior to *in vivo* gene therapy of tumors. We also establish that, *in vivo*, DC-Cholesterol is superior to Lipofectin, Lipofectamine, Cellfectin and DMRIE-DOPE in the delivery of DNA to human lung tumor xenografts. Whether the conditions established here are applicable to other tumors remains

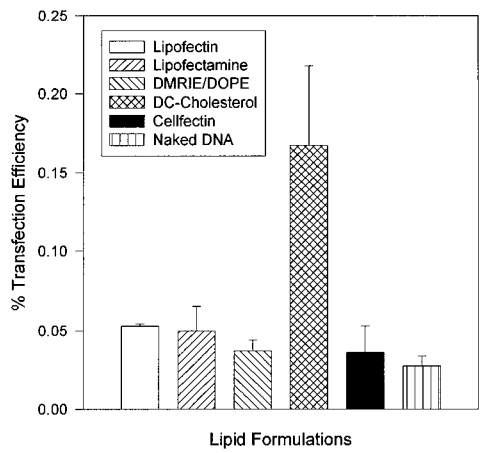

FIG. 3. *In vivo* transfection efficiencies of liposome formulations at optimal lipid to DNA ratios. Each liposome formulation was complexed with 10 μg of plasmid DNA at the optimal lipid to DNA ratio (4:1 for lipofectin, Cellfectin and DMRIE-DOPE, 1:1 for DC-Chol and Lipofectamine) and were delivered to tumors of similar size (18–30 mg) by direct injection. Naked plasmid DNA (10 μg) was injected in control experiments. Each bar is an average of 3 mice with the error bars corresponding to standard deviation.

to be shown since it is known that efficiency of transfection can vary significantly from one cell type to another.

Previous studies have analyzed transfection efficiency either by reporter enzyme activity (CAT, β-galactosidase or luciferase) in tissue extracts or by analysis of stained tissue sections which in general are poor predictors of efficiency on a per cell basis. In contrast, we have analyzed single-cell suspensions prepared by enzymatic disaggregation of tumors which should be more accurate. The overall transfection efficiencies obtained in this study, even under optimal conditions, did not exceed 0.3%. This result confirms the principal and well-known disadvantage of cationic liposome mediated gene delivery, *i.e.* low efficiency of transfection *in vivo.* Optimization of other parameters such as the quantity of DNA delivered per injection and the frequency of injections might lead to improved transfection efficiency. On the other hand, aggregation and precipitation of DNA:lipid complexes at high DNA concentrations and the dependence of transfection efficiency on tumor size (multiple injections would have to be done within a short period of time before tumors get too large) set limitations for these parameters.

Whether delivery of genes to tumors at the efficiencies reported here will have significant therapeutic consequences in human patients is not yet clear. In separate studies, we showed that a significant anti-tumor response could be provoked after cytokine gene transfection of human tumor xenografts in SCID mice at low transfection efficiencies (0.05–0.25%) but complete regression did not occur in all animals (6). Since the anti-tumor response by the host immune system has been shown to be strongly influenced by a local and sustained production of several different cytokines (reviewed in 9), further studies involving novel cationic liposome formulations and/or delivery protocols leading to improved transfection efficiencies are critical to achieving significant therapeutic effects with cytokine gene therapy of human cancer.

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