Gene Transfer *In Vivo* with DNA–Liposome Complexes: Safety and Acute Toxicity in Mice

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ABSTRACT

DNA can be introduced into a variety of cell types after formation of liposomal complexes with cationic lipids. In this report, conditions have been established to optimize the production of DNA–liposome complexes that efficiently transfect cells. The safety and toxicity of this method of gene delivery have been assessed after *in vivo* administration, either by intravenous or direct intratumor injection. Nine to eleven days after intravenous injection, DNA was found primarily in heart and lung tissue by PCR analysis. No abnormalities were evident from histologic examination of tissue, examination of tissue-specific serum enzymes, routine biochemical parameters, or electrocardiographic monitoring. DNA–liposome complexes can therefore be used for the delivery of recombinant genes *in vivo* with minimal toxicity.

OVERVIEW SUMMARY

Retroviral-mediated gene transfer is the standard procedure used in human gene transfer/therapy clinical protocols. Stewart et al. have utilized a different approach: DNA–liposome complexes to transfer genes *in vivo*. Their clinical protocol was recently approved by the RAC (and is the first *in vivo* gene therapy protocol to be approved). This manuscript presents a portion of the safety data on which the approval was based.

INTRODUCTION

DESPITE SUBSTANTIAL PROGRESS in the understanding of eukaryotic gene expression, a major obstacle to the treatment of human disease has remained the inability to express recombinant genes at specific sites *in vivo*. Although it has been possible to express recombinant genes in a tissue-specific fashion in transgenic animal models using cell-specific promoters or en-

hancers, the application of this technology to human somatic cells has been difficult. The development of novel technologies has now allowed this problem to be addressed by several methods. One approach to site-specific gene expression *in vivo* utilizes catheter technology, together with either retroviral vectors or DNA–liposome complexes, which can be introduced directly into arterial segments or elsewhere *in vivo* (Nabel et al., 1989, 1990; Lim et al., 1991). Within transduced arterial segments, expression of these recombinant genes can cause potent changes in the biology of the vessel wall. In a recent study, for example, expression of a foreign histocompatibility antigen produced an intense focal vasculitis (Nabel et al., 1992a). Recently, we have adapted this technique to introduce foreign histocompatibility genes into tumor nodules to stimulate their rejection *in vivo* (Plautz et al., 1992). This approach to DNA–liposome-mediated gene delivery therefore has potential therapeutic applications to cardiovascular disease, malignancy, and other acquired or inherited diseases.

DNA–liposome complexes (Felgner et al., 1987; Wang and Huang, 1989; Pinnaduwage et al., 1989; Felgner and Rhodes, 1991; Gao and Huang, 1991) have several desirable features in terms of gene transfer. First, DNA is delivered through a nonviral vector and is thus incapable of propagation in animal cells;

therefore, issues of health and safety of the vector are minimized. Second, cationic lipids can be formulated which contain hydrolyzable bonds, allowing the generation of biodegradable products after they have fused with cell membranes and delivered recombinant genes to cells (Gao and Huang, 1991). Several questions still remain, however, regarding optimal conditions for transfection *in vitro* and *in vivo* and potential side effects and toxicity of *in vivo* treatment using such liposomes. We report here experiments in which we have determined optimal conditions for liposome-mediated transfection of cell lines *in vitro*. In addition, we have examined the toxicity of DNA–liposomes delivered into the systemic circulation and into tumors *in vivo*.

MATERIALS AND METHODS

Plasmids

A plasmid containing the *Escherichia coli lacZ* gene under the control of the Rous sarcoma virus long terminal repeat (LTR) (RSV-β-gal) (Norton and Coffin, 1985) was used for transfection of porcine primary endothelial and HeLa cells. In addition, a plasmid containing the *lacZ* gene under the control of preproendothelin-1 5′-flanking DNA (−1,410 to +83) (Wilson *et al.*, 1990) was used for transfection of endothelial cells. For *in vivo* toxicity analysis, the RSV-β-gal plasmid and a plasmid derived from the PLJ vector containing the cDNA encoding an H-2K^s mouse MHC class I gene (Plautz *et al.*, 1992) were used.

Liposome preparation

The DNA–liposome mixture was prepared with lipid concentrations of dioleoyl phosphatidylethanolamine (DOPE)/3β [N-(N′,N′-dimethylaminoethane)-carbamoyl] cholesterol (DC-chol) (2:3) molar ratio) between 2.5 and 25 μM added to 0.2 ml of serum-free media or Ringer's lactate solution containing plasmid DNA in polystyrene tubes. The liposomes were prepared as previously described (Gao and Huang, 1991). After mixing gently, the solution was allowed to stand at room temperature for 15–20 min. For transfection analysis, cells were grown in 60-mm tissue culture dishes at 75% confluency or greater. Cells were washed twice with serum-free media or lactated Ringer's solution and then placed in 0.5 ml of the same media. The DNA–liposome solution (0.2 ml) was then added slowly to the cells, with gentle mixing, with a final volume of 0.7 ml. This resulted in DNA concentrations between 0.7 and 7 μg/ml (~0.1–1 nM), and lipid concentrations of 7–70 μM.

Cell culture, transfection analysis, and toxicity *in vitro*

Primary porcine endothelial cells, derived from the Yucatan minipig (YPE cells), were incubated with medium 199 (M199) supplemented with 10% FBS, 2 mM L-glutamine, 50 units/ml penicillin, and 5 μg/ml streptomycin. HeLa cells were maintained in Dulbecco's modified Eagles medium (DMEM) supplemented with 5% fetal calf serum, 2 mM L-glutamine, 50 units/ml penicillin, and 5 μg/ml streptomycin. Transfections were performed as described above and allowed to proceed for 1–5 hr, after which the cells were placed in media supplemented

as described above. At 24–48 hr after transfection, the enzymatic activity of the *E. coli* β-galactosidase was used to identify transfected cells by staining with X-gal chromagen (Plautz *et al.*, 1991). At least 1,000 cells were counted after staining to determine percent positivity. Results are representative of at least two independent transfection experiments. There is negligible staining of porcine endothelial cells transfected with other plasmids, including CAT, PDGF, or acidic FGF expression vectors (data not shown). Toxicity of the procedures *in vitro* was assessed by cytopathic effect and trypan blue exclusion.

Animal studies

Adult female mice (strain BALB/c) were used for all *in vivo* experiments. Intravenous injections were carried out *via* the tail vein using a 21-gauge needle. For intravenous injections, the DNA–liposomes were prepared, as described for the *in vitro* transfection studies, in 0.2 ml of serum-free M199 or lactated-Ringer's solution. After 15–20 min of incubation of DNA and lipid, the mixture was diluted to 0.7 ml and 0.1–0.2 ml of this dilution was then injected immediately into the tail vein. Blood was collected from the tail vein immediately prior to intravenous injection of the DNA liposomes and 9–11 days later. At ~2–3 weeks following injection, liver, kidney, lung, heart, and brain were extracted for histologic and PCR DNA amplification analysis using primers and conditions described previously (Nabel *et al.*, 1992a). Briefly, tissue samples were collected and treated with a proteinase K solution: 100 mM NaCl, 100 mM EDTA, 50 mM Tris · HCl pH 8.0, 1% SDS, and 0.6 mg/ml Proteinase K. Proteinase K solution (600 μl) was added to each sample (~0.1 gram) and incubated at 55°C overnight, followed by phenol and chloroform extraction. The top aqueous layer was collected, precipitated in EtoH, and washed once with 70% EtoH. The DNA was dissolved in 10 mM Tris · HCl pH 8.0 and 1 mM EDTA at ~1 mg/ml. The polymerase chain reactions (PCR) were performed as described (Nabel *et al.*, 1992a; Plautz *et al.*, 1992) with a 2-min annealing and extension at 72°C and 1 min dissociation at 94°C. Genomic DNA from CT26 K^s cells was used as positive control (1 μg). The sensitivity of detection has been estimated to be between 1 copy per 10^3 to 10^5 genomes. Intratumor injection of CT26 cells (Fearon *et al.*, 1990) and analysis were also performed according to previous protocols (Plautz *et al.*, 1992). Serum samples were stored frozen at −20°C until measurement of tissue-specific enzymes and routine biochemical parameters.

Studies of cardiac toxicity

To evaluate the effects of intravenous DNA–liposome complexes on cardiac tissue, measurement of total creatine phosphokinase (CPK), CPK isoenzymes, and electrocardiography (EKG) were made on 15 mice prior to and following DNA–liposome injection. In 10 mice, total CPK values were determined prior to injection. Seven mice received H-2K^s DNA–liposome conjugates prepared as described above (0.1 cc), injected into the tail vein. CPK measurements were made on serum samples obtained 16 hr after injections. Three control mice were injected with 0.1 cc of saline, and total CPK was measured at 16 hr.

As an independent parameter to evaluate cardiac function, EKG measurements were performed in 5 mice prior to DNA–

liposome complex ($n = 3$) or saline ($n = 2$) injection, during injection, and 1 and 5 min following injection. The mice were anesthetized with ketamine, and surface lead electrodes were attached to the four limbs using alcohol. Surface lead I or II was monitored at a chart speed of 50 mm/sec. After the baseline EKG was obtained, continuous measurements were made during injection of 0.1 cc of H-2K^s DNA–liposome conjugate ($n = 3$) or saline ($n = 2$) and for 5 min following injections.

RESULTS

In vitro studies: Optimization of transfection conditions and examination of toxicity

Optimal conditions for transfection and toxicity of DNA–liposomes were first determined in vitro. To obtain maximal transfection without toxicity in vitro, we studied chemical parameters of the DNA–liposome complex and their effects on cells. In particular, we examined the ratio of DNA to cationic lipid, the absolute concentration of DNA or lipids, and the conditions for mixture of DNA and cationic lipids. The cationic lipid preparation was a formulation of two compounds, which include DOPE and DC-Chol (Gao and Huang, 1991). Transfection efficiencies of this formulation were equal to or greater than those of Lipofectin (BRL) in several cells lines in vitro (data not shown).

Endothelial cells, which are typically difficult to transfect, and HeLa cells, which can be transfected easily using a variety of techniques, were examined by transfection in vitro. To determine the optimal conditions for transfection of endothelial cells, the lipid formulation was initially used at different concentrations while the DNA concentration was held constant. Maximal transfection efficiency was seen using 0.7 μg/ml DNA (~0.1 nM) and 21 μM of DOPE/DC-Chol lipid (Fig. 1), with a sharp decline in the number of transfected cells with higher or lower lipid concentrations. Next, the DNA concentration was altered as the lipid concentration remained constant. This analysis revealed that the number of cells transfected varied with DNA concentration, in that the number of transfected cells decreased significantly with increments of DNA concentration as low as 0.05 nM (Fig. 2).

These results indicate that the ratio of DNA to lipid is critical for maximum transfection efficiency, but that, in addition, the absolute concentration of each component is important in determining the efficiency of transfection. An increase in DNA and lipid concentration beyond the optimal concentration of 0.7 μg/ml DNA (~0.1 nM) and 21 μM of DOPE/DC-Chol reduced the number of viable cells and did not increase the transfection efficiency. Lipid concentrations greater than 35 μM reduced the number of viable cells by 50% compared to untransfected control cells, whereas the optimal concentration of 0.7 μg/ml DNA (~0.1 nM) and 21 μM of lipid had no effect on cell viability after 5 hr of incubation.

To compare the optimal concentrations for transfection in a different cell type, transfections were performed on HeLa cells (Fig. 3). In this case, a slightly different optimal ratio of DNA and lipid were observed. Peak transfection efficiencies were obtained at the same lipid concentration as endothelial cells (21 μg/ml) but varied less with small differences in DNA concentrations (Fig. 4). DNA concentrations of 1.4–4.2 μg/ml were

equally effective. Again, when the ratio of DNA to lipid was maintained but the concentration of each was decreased threefold, very few cells were transfected, illustrating that both the ratio of DNA to lipid and the absolute concentration of each component are critical in maximizing the number of transfected cells.

In addition, we have investigated the effect of the degree of cell confluence on transfection efficiency. No toxicity was
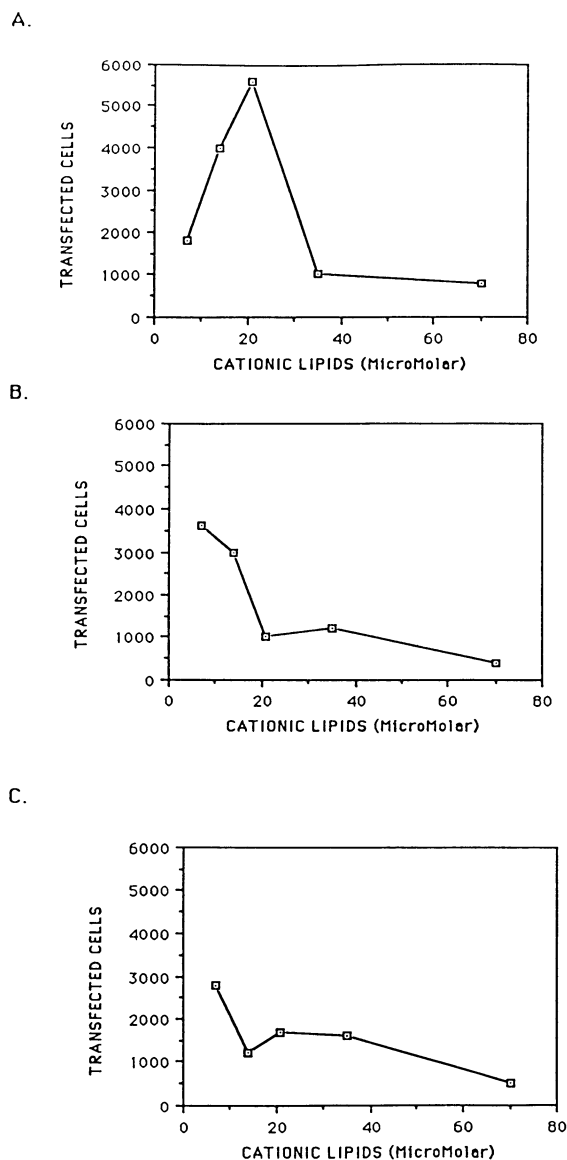
FIG. 1. Effect of lipid concentration on transfection efficiency in porcine endothelial cells. Porcine endothelial cells (Nabel et al., 1989) were transfected with DOPE/DC-Chol lipids at the indicated concentrations using a constant amount of DNA in each group. The DNA concentrations were tested at fixed concentrations of 0.7 μg/ml (0.13 nM) (A), 1.1 μg/ml (0.19 nM) (B), or 1.4 μg/ml (0.26 nM (C). A preproendothelin β-galactosidase reporter plasmid DNA was used (Methods and Materials), and 24–48 hr after transfection cells were fixed with 1.25% glutaraldehyde and stained with X-gal to determine the number of positive cells among 1–2 × 10^5 treated cells (Nabel et al., 1989).

A.

B.

C.

D.
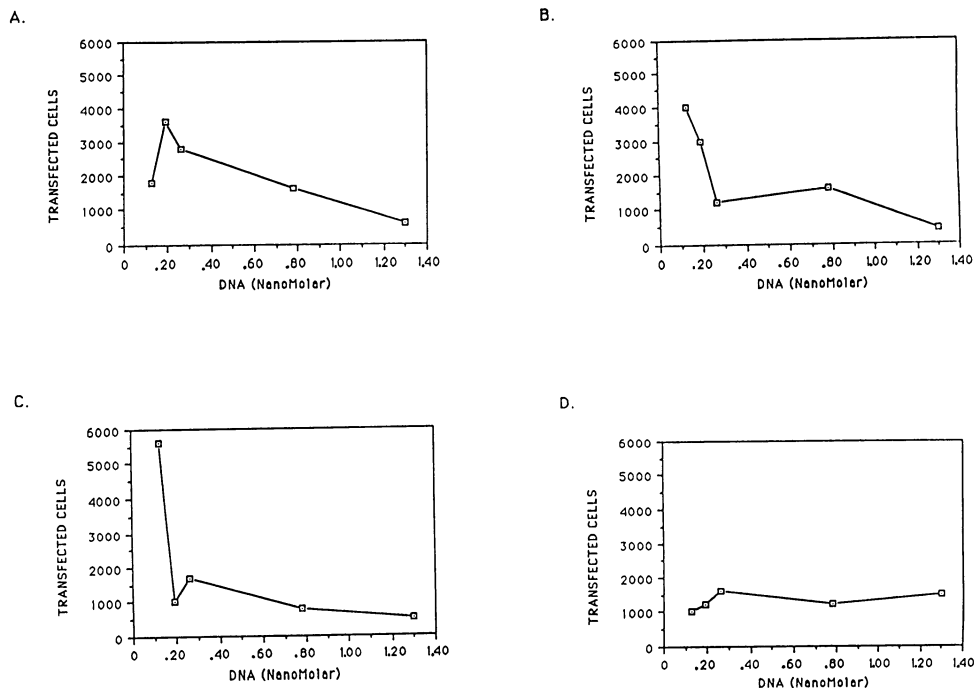
FIG. 2. Effect of DNA concentration on transfection efficiency in endothelial cells. Primary endothelial cells were transfected with 7 μM (A), 14 μM (B), 21 μM (C), or 35 μM DOPE/DC-Chol (D). The DNA concentration ranged from 0.13–1.3 nM (0.7–70 μg/ml) for each concentration of lipid. Transfections were performed and analyzed as described in Fig. 1 legend.

demonstrated on HeLa cells grown to 80% confluence or greater using up to 35 μM of lipid. When cells were transfected at a lower saturation density, however, cell viability was reduced dramatically with as little as 7 μM of lipid compared to the untransfected control cells. These results demonstrate that the optimal conditions for transfection and toxicity may differ somewhat, depending on the cell line and the degree of confluence of the cells transfected.

Another variable in the preparation of liposomes was the composition of the solution used to generate complexes of the cationic lipids with DNA. Among several media solutions analyzed, no substantial difference was noted in subsequent transfection efficiency or toxicity with M199, McCoy's, OptiMEM, or RPMI media. A significant improvement in transfection efficiency was observed, however, when standard Ringer's lactate was used to generate DNA liposomes. After incubation with cells for 1.5 hr, the efficiency of transfection increased threefold compared to the serum-free medium (Fig. 5), although prolonged incubation (>2 hr) resulted in a loss of cell viability in some cell types.

Animal studies: toxicity and distribution of DNA–liposome complexes after intravenous or intratumor administration

Since DNA–liposome complexes have shown promise as a gene delivery system, the question of their toxicity and distribution after systemic administration remained. To address these questions, we injected DNA–lipid complexes into the tail vein of BALB/c mice. These liposomes contained RSV-β-gal or a

PLJ-derived plasmid which contains the MHC class I cDNA, H-2K^s, complexed with DOPE/DC-Chol cationic lipid, and concentrations of DNA and cationic lipids were used that were optimal for transfection of porcine endothelial cells *in vitro* (see above). In one group, DNA–lipid complexes (0.1–0.2 ml) were injected intravenously. A second group of animals, which had been inoculated subcutaneously with a malignant colon adenocarcinoma CT26 ~2 weeks previously (Fearon *et al.*, 1990), received injection into the resulting tumor. Mice were sacrificed and organs were removed 2–3 weeks post-injection for analysis by PCR to detect plasmid DNA. In addition, tissue samples were obtained for histological analysis. Pre- and post-injection serum samples were also analyzed in nontumor-bearing animals to determine if blood proteins and/or biochemical parameters were affected.

PCR analysis of DNA from heart, lung, brain, liver, and kidney revealed that the intravenously injected DNA localized primarily to the lung and heart tissue (4 of the 6 mice) after 2–3 weeks. In one case, DNA localized only to the heart, with no evidence of recombinant DNA in other organs (Table 1A). The distribution of DNA has also been analyzed by *in situ* autoradiography of ^{35}S labeled DNA–liposome complexes. This analysis revealed the greatest uptake transiently, measured 5 hr after the injection, with highest levels in the liver. There was a rapid decrement within 24 hr, with most organs containing fewer than 1/10^5 cells with detectable radiolabel (Table 1B). Similar patterns were seen with intratumor injections, with consistent expression in the tumor and occasional expression in heart, kidney, lung or spleen.

Histological analysis of tissues from animals receiving intra-

venous injections of DNA–liposomes revealed no major abnormalities in tissues that contained the injected plasmid DNA or in other tissue. Autopsies were performed on 5 mice that received intravenous injections. Representative hemotoxylin and eosin-stained histologic sections were examined by light microscopy from formalin-fixed, paraffin-embedded tissue. These organs included brain, heart, lung, liver, spleen, kidney, and ovaries. Most findings were interpreted as incidental agonal findings, similar to those seen frequently in human postmortem exams. These findings included rare ischemic central nervous system neurons and small foci of contraction band necrosis in the heart. None of the ischemic changes seen in the heart were accompanied by an inflammatory response, suggesting that the events were extremely recent. The pulmonary parenchyma was variably atelectatic. In 4 mice, there were atelectatic foci in which the interstitial septae appeared slightly expanded by neutrophils. These changes, as well as the cardiac and neuronal findings described above, were extremely subtle, are observed in untreated, control animals, and probably of no significance.

In addition, there was no evidence of more subtle organ toxicity from analysis of serum enzymes or biochemical param-

eters. Blood samples from injected animals were compared to control samples obtained from the same animals prior to injection. Samples obtained 9–11 days after intravenous injection showed no statistical differences in liver function tests, glucose, amylase, BUN, and creatinine in 18 mice analyzed after injection of 0.1 ml or 0.2 ml of DNA (1 μg/ml)/lipid (21 μM) complex (Table 2).

Cardiac toxicity studies

Because plasmid DNA was detected by PCR in the heart, we wished to examine whether this organ showed signs of toxicity from this treatment. Although no pathologic changes were evi-
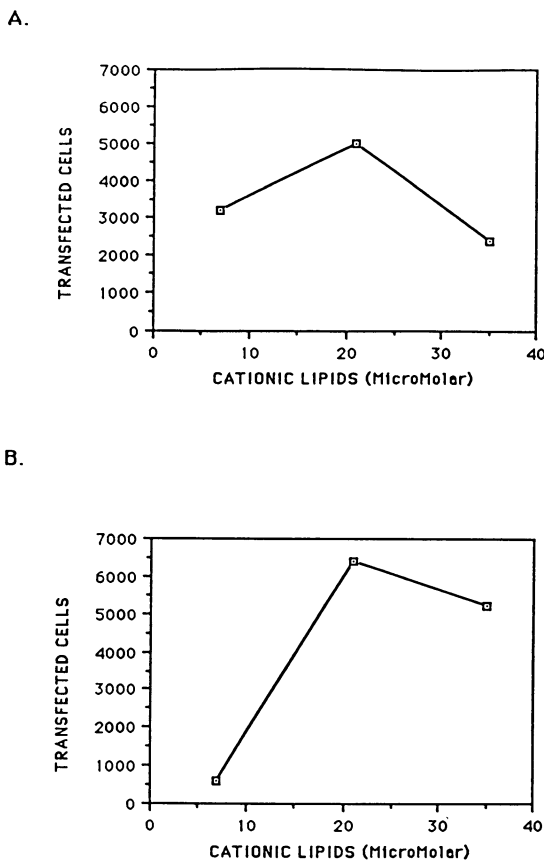
FIG. 3. Effect of lipid concentration on transfection efficiency in HeLa cells. HeLa cells were transfected with 1.4 μg/ml (0.40 nM) (A) or 4.2 μg/ml (1.20 nM) (B) of RSV-β-gal DNA. The concentration of DOPE/DC-Chol ranged from 7–35 μM. Transfected cells were characterized as described (see Fig. 1 legend), and results are representative of at least three independent transfections.
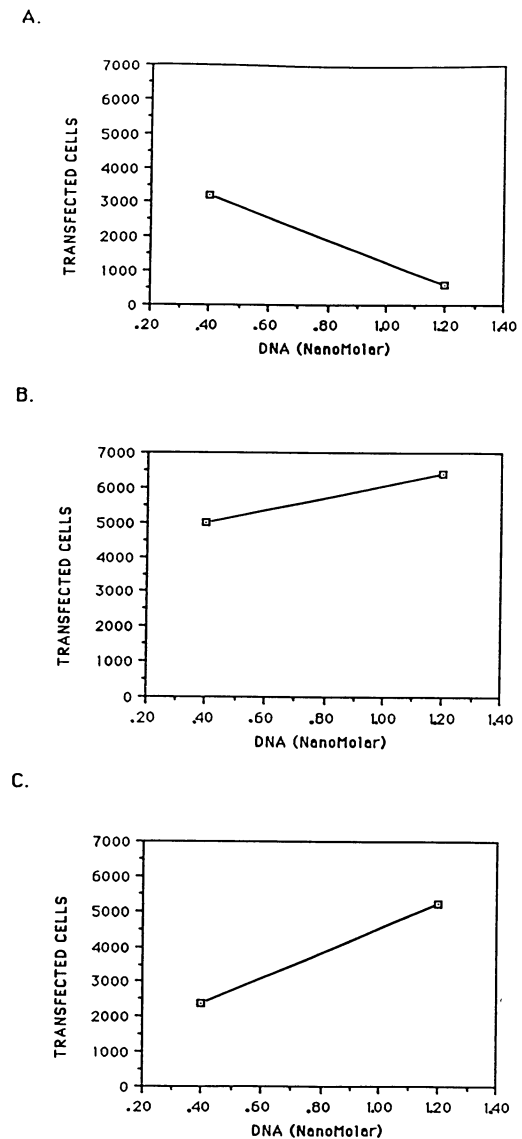
FIG. 4. Effect of DNA concentration on transfection efficiency in HeLa cells. HeLa cells were transfected with 7 μM (A), 21 μM (B), or 35 μM DOPE/DC-Chol (C). The DNA concentration ranged from 0.40 to 1.2 nM (1.4–4.2 μg/ml) for each concentration of liposome. Cells were stained as described (see Fig. 1 legend).
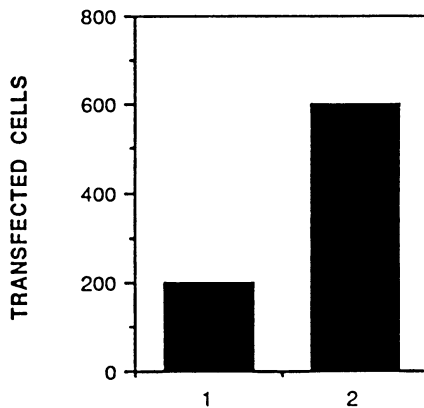
FIG. 5. Improved transfection efficiency in endothelial cells by formulation of DNA–liposome complex with Ringer's lactate solution. Cells were incubated with DNA–liposomes reconstituted in serum-free McCoy's medium (lane 1) or Ringer's lactate solution (lane 2) for 1.5 hr and analyzed after 36 hr. Transfection efficiencies of other complex media, including OptiMEM, DME, and M199, are similar to McCoy's media. Percent transfected cells were analyzed as described in Fig. 1 legend. Standard deviation was less than 10%.

dent at 10 days (see above), these studies did not address the potential for acute toxic effects. To evaluate the effects of DNA–liposome intravenous infusions upon cardiac function, total CPK measurements were measured in 10 mice prior to and following DNA–liposome or saline infusion. In the group injected with DNA–liposomes, no significant differences in CPK

TABLE 2. EVALUATION OF SELECTED SERUM ENZYMES AND CHEMISTRIES BEFORE AND AFTER INTRAVENOUS INJECTION WITH DNA–LIPOSOMES

Test	Pre-injection		Post-injection	
Albumin g/dl	2.9 ± 0.3	(9)	3.0 ± 0.3	(9)
Alk Phos IU/liter	196 ± 121	(14)	148 ± 74	(16)
Amylase units/liter	2363 ± 1861	(9)	1415 ± 394	(8)
Bilirubin mg/dl	0.2 ± 0.1	(9)	0.2 ± 0.1	(9)
BUN mg/dl	22 ± 7	(18)	25 ± 8	(17)
Creatinine mg/dl	1.1 ± 0.4	(9)	1.0 ± 0.1	(9)
Glucose mg/dl	156 ± 46	(18)	143 ± 24	(17)
Globulin g/dl	2.5 ± 0.5	(9)	2.9 ± 0.3	(9)
Phosphorous mg/dl	9.6 ± 2.1	(9)	8.0 ± 2.0	(8)
Protein, total g/dl	5.4 ± 0.6	(8)	5.9 ± .5	(9)
SGOT IU/liter	91 ± 29	(17)	125 ± 43	(17)
SGPT IU/liter	43 ± 18	(15)	50 ± 14.5	(17)

Blood samples were obtained from BALB/c female mice prior to intravenous injection and after 9–11 days. Serum enzyme and chemistry values were analyzed (Roche Biomedical Laboratory). Mean values, standard deviations, and the number of subjects tested (in parentheses) are shown.

levels were observed pre- or post-injection (Table 3). Similarly, the control group (3 animals) showed no statistically significant changes (Table 3).

As an independent evaluation of cardiac toxicity, electrocardiographic measurements were obtained on 5 additional mice to evaluate the cardiac rhythm and potential acute ischemic changes. The resting heart rate in the control and experimental groups was ~350 beats per minute. Following the infusion of

TABLE 1. LOCALIZATION OF RECOMBINANT GENES BY PCR AFTER INTRODUCTION OF *IN VIVO*

A.

Site of injection	Vehicle	Liver	Kidney	Brain	Heart	Lung	Tumor	Spleen
I.V.	DOPE/DC-Chol	0/6	1/6	0/6	4/6	4/6	ND	ND
Tumor	DOPE/DC-Chol	0/5	1/5	0/5	2/5	1/5	5/5	1/5
Tumor	Lipofection	0/6	1/6	1/6	2/6	0/6	3/6	0/6
Tumor	Retroviral vector	0/4	0/4	0/4	0/4	0/4	1/4	1/4

B.

Organ	Frequency of positive cells	
	5 hr	24 hr
Brain	$<10^{-5}$	$<10^{-5}$
Heart	$<10^{-5}$	$<10^{-5}$
Lung	ND	2×10^{-4}
Liver	$\sim10^{-2}$	2×10^{-4}
Spleen	$\sim5 \times 10^{-3}$	5×10^{-4}
Kidney	$<10^{-5}$	$<10^{-5}$

A. Mice were injected with 0.1–0.2 ml of the indicated vehicle, [liposome (DOPE/DC-Chol or Lipofectin) or H-2K^S encoding retroviral vector (Plautz *et al.*, 1992] (~5 × 10^5 G-418-resistant colony-forming units/ml), and DNA extracted from the indicated tissues for analysis by PCR. Mice received injections by tail vein (Row 1) or into CT26 tumors (Fearon *et al.*, 1990) previously inoculated into the hind flank. Tumor diameters at the time of injection were 1–2 cm. The number of animals that tested positive relative to the total number analyzed is indicated. ND, Not determined.

B. Mice were injected with 0.1 ml of DNA (2 μg)/liposome (12 n*M*) complexes (using DOPE/DC-Chol) by tail vein with nick translated ^{35}S-labeled DNA (3.5 × 10^7 cpm/μg). Tissues were removed at the indicated times after injection in separate animals and processed for counting by liquid scintillation counting and autoradiography by standard methods (Simmons *et al.*, 1989).

TABLE 3. MEASUREMENTS OF TOTAL CPK (UNITS/LITER)
BEFORE AND AFTER INTRAVENOUS INJECTION
WITH DNA–LIPOSOMES

	Control	H-$2K^S$ liposome
Pre-injection	933 ± 294	1,373 ± 360
Post-injection (16 hr)	1,126 ± 555	1,408 ± 575
	$p \leq 0.79$	$p \leq 0.96$

Serum samples were obtained from 10 BALB/c female mice prior to intravenous injection and 16 hr following injection of saline (control, $n = 3$) or H-2K^S liposome complexes ($n = 7$). Serum was analyzed by Roche Biomedical Laboratory (Burlington, NC).

saline or DNA–liposomes, normal sinus rhythm was maintained at 350 beats per minute without any arrhythmia (Fig. 6). There was no evidence of cardiac ischemia in either group, as determined by a lack of change in height of R waves, development of Q waves, or ST segment elevation.

DISCUSSION

The ability to deliver recombinant DNA to specific tissues *in vivo* is an essential step in the development of effective gene therapy in somatic cells. Site-specific expression can be achieved by direct gene transfer *in vivo* using cationic lipids mechanically directed to the arterial wall using catheter techniques (Nabel *et al.*, 1990; Lim *et al.*, 1991). Cationic lipids, however, must prove safe and effective before they can be applied widely to the treatment of human disease. In these studies, the efficacy and toxicity of a novel cationic lipid mixture has been evaluated *in vivo* in a mouse model. One of these lipid components, DC-Chol, is attractive for *in vivo* use since it specifically incorporates a degradable carbamoyl linkage that

generates cholesterol and a substituted ethylenediamine upon hydrolysis. Biodegradable liposomes containing this lipid also provide an efficient vehicle for transfection of DNA into cells.

We have found three parameters to be critical in achieving high rates of *in vitro* transfection using DOPE/DC-Chol liposomes: (i) the DNA/lipid ratio, (ii) the absolute concentration of the DNA and lipid, and (iii) the solution used to form the DNA–lipid complex. Conditions necessary to achieve maximum transfectability were compared for endothelial cells, which are typically difficult to transfect, and HeLa cells, which are easy to transfect by conventional methods. Although there was a difference in both the ratio and absolute concentration of DNA/lipid that was optimal for transfection of these cell lines, efficiency of transfection was dependent on both the ratio and absolute concentration of each component. In addition, lactated Ringer's solution was found to be more efficient than serum-free media for formation of DNA–lipid complexes. It is possible that the media exerts this effect by acting directly upon cells to affect their ability to take up or express DNA. Alternatively, serum-free media may contain factors that can bind to DNA–liposomes and prevent them from adhering to cells. Another important parameter in determining the activity of liposomes is the particle diameter. This diameter, as well as the absolute lipid concentration, can vary slightly between preparations; therefore, for each preparation of DOPE/DC-Chol, conditions to achieve the greatest number of transfected cells with minimal toxicity must be determined. Since such lipid mixtures are stable for ≥2 months, however, large quantities can be easily prepared and standardized for future use.

Using such biodegradable liposomes, we have not observed any acute adverse reactions in mice that have received intravenous or intratumor injections. There was no mortality from intravenous injections when optimal concentrations of DNA and lipid or *in vitro* transfection were used. In addition, no chronic toxicity was observed by histological or serological

TREATMENT
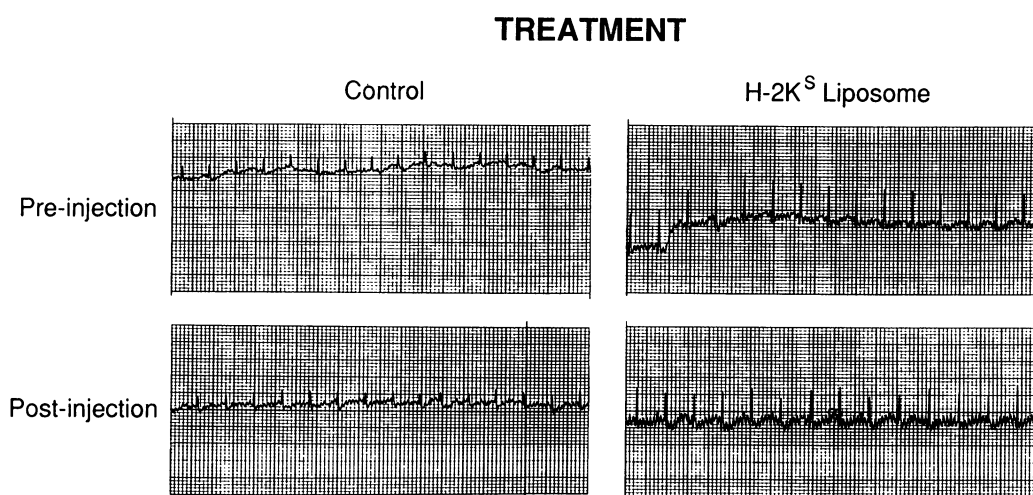
FIG. 6. Effect of DNA–liposome infusions on cardiac function measured by electrocardiography. Five mice underwent electrocardiographic (ECG) monitoring before and after intravenous infusions of saline (control, $n = 2$) or H-2K^s liposomes ($n = 3$). Female BALB/c mice were anesthetized by metathane inhalation. Surface electrodes were attached to the four limbs, and surface lead I or II was continuously recorded at 50 mm/sec paper speed. Representative tracings are shown for each group pre-injection and 5 min post-injection.

analysis. A previous report has shown that intravenous injection of pSV2CAT complexed with Lipofectin resulted in expression of the CAT gene only in the lungs (Brigham et al., 1989). We have found by PCR analysis that DNA localized most frequently in heart and lungs after intravenous injection with DOPE/DC-Chol. Whether these complexes localize to these tissues because they represent the first organ encountered in the circulation or because there is a specific affinity for these tissues is unknown. In either case, uptake does not appear to induce organ toxicity.

Despite the finding of H-2K^s DNA in cardiac tissue by PCR analysis, further studies of cardiac function during intravenous infusion of DNA–liposomes into mice revealed no effect on cardiac performance. There was no change in CPK levels after DNA–liposome infusion, suggesting that no acute myocardial injury was induced by introduction of these complexes. Similarly, no change in EKG rate, rhythm, or QRS morphology was observed following H-2K^s liposome infusion, indicating that they had no arrhythmogenic or ischemic effects on cardiac tissue. Therefore, the intravenous infusion of DNA–liposome conjugates appears safe with regard to cardiac function.

Another concern regarding the introduction of DNA into a given individual is the potential for insertional mutagenesis. In general, this problem is no greater than would be expected in retroviral gene transfer protocols (Cornetta et al., 1990, 1991; Rosenberg et al., 1990a,b; Temin, 1990). The major concern of insertional mutagenesis is the possibility that secondary malignancy may occur. Since transcriptional regulatory elements similar to those used in the retroviral vectors were used in the plasmids described in these studies, the risk of activation of a growth factor or inactivation of a recessive oncogene should be comparable to retroviral vectors. As with murine retroviruses, it is unlikely that the disruption of single gene products will lead to malignancy. Even in cases of primary transfections with dominant oncogenes, transfection of at least two gene products is required to transform primary cells in tissue cultures (Land et al., 1983), and the steps leading to malignant transformation are complex, with many steps proposed in some malignancies. Because DNA introduced by liposomal transfection is generally in episomal form (Plautz and Nabel, unpublished observations), it is likely that the risks would be less than those found in retroviral transformation.

The final concern regarding the introduction of DNA for therapeutic use is the potential to introduce new genetic material into germ cells. In a related study, this concern has now been addressed and appears to be minimal in animal studies (Nabel et al., 1992b). Taken together, these findings suggest that DNA complexed to cationic lipids can be used to deliver recombinant genes to mice in vivo without the risk of major toxicity. Therefore, this method of encapsidating DNA in liposomes could be used to deliver recombinant genes to specific sites in vivo and may eventually be applicable to the treatment of both inherited and acquired human disease.

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