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Pharmacodynamic Approach To Study the Gene Transfer Process Employing Non-viral Vectors

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ABSTRACT. In the present work we set out to apply pharmacodynamic concepts derived from dose–response curves (Potency and Efficacy) to characterize the gene transfer efficiency of a vector:DNA complex. We employed two widely used vectors, the cationic lipid DOTAP (*N*,*N*,*N*-trimethyl 1-2-3-bis (1-oxo-9-octadecenyl)oxy-(Z,Z)-1-propanaminium methyl sulfate) and the cationic polymer PEI (polyethylenimine, 800 kDa) to transfect several constructions of the green fluorescent protein cDNA. The analysis of dose–response curves indicated that in all cases the goodness-of-fit was > 0.99. Potency is a measure that provides information on gene activity per amount of DNA. Efficacy is a measure of maximum gene expression achievable using a specific vector:DNA complex, and depends on both the intrinsic efficacy of the gene (evaluated using different vectors to transfer the same gene construct) and on vector efficacy in DNA delivery (evaluated using a single vector to deliver different gene constructs). The results suggest that Potency and Efficacy are objective parameters for describing and comparing the goodness of vectors, as well as the intrinsic efficacy of a given gene construct. Furthermore, they are useful tools that may contribute to a better understanding of the mechanistic gene transfer process of each vector. BIOCHEM PHARMACOL **60**;12:1845–1853, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. gene transfer; non-viral vectors; Potency; Efficacy; dose–response; pharmacodynamic

Gene therapy offers good perspectives for the treatment of both inherited and acquired diseases, though several questions remain to be answered before genes can be used as pharmaceutical products in clinical practice. One of the difficulties of successful gene therapy is the poor efficiency of naked DNA delivery. To improve gene transfer efficiency, two main types of systems have been used, involving viral and non-viral vectors [1–5]. Although the former are more efficient, the latter offer increased safety as an advantage; neither satisfies the requirements of an ideal vector, however [6]. In any case, no consensus exists regarding the criteria employed to describe the goodness of gene transfer systems. Since the aim of gene therapy is to use nucleic acids as medicines, it seems reasonable to attempt to study genes as drug-like entities. The pharmacokinetic properties of nucleic acids transported both with and without vectors were addressed by a number of early studies. However, to our knowledge, no previous research has been carried out to analyze genedynamic parameters in the form of dose–response curves. Unlike viral vectors, non-viral vectors can be used to transfer nucleic acid sequences of essentially unlimited size and can easily be formulated as medicines. A wide variety of non-viral vectors have been reviewed for gene delivery [7–13]. They can be classified into two main groups, based on the formation of a DNA complex with lipids (lipoplexes) or polymers (polyplexes), respectively [14]. Among the existing gene delivery systems, the cationic liposomes represent one of the most promising for use in gene therapy, though the polycation polymer PEI† has also been found to be an inexpensive and useful reagent for gene delivery [15]. The present work studies the efficiency of DOTAP and PEI as lipid and polymer cationic-based non-viral vectors for the *in vitro* cell delivery of different constructs of the gene reporter green fluorescent protein on the basis of a dose–response curve. The parameters of Potency and Efficacy of the DNA complexes were obtained from the EC_{50} and E_{max}, respectively, in the manner of a pharmacological drug dose–response curve. This approach affords objective parameters and thus objective criteria for evaluating the advantages of each transfer system.

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† *Abbreviations:* DOTAP, *N*,*N*,*N*-trimethyl 1-2-3-bis (1-oxo-9-octa-decenyl)oxy-(Z,Z)-1-propanaminium methyl sulfate, $C_{43}H_{83}NO_8S\ CH_3SO_4^-$; PEI, polyethylenimine, $[CH_2CH_2NH]_x$; PEI-800, polyethylenimine average MW = 800 kDa; EGFP, green fluorescent protein; pCMV, citomegalovirus promoter; RFU, relative fluorescence units; PCR, polymerase chain reaction; *Neo*^{r}, neomycin resistance gene; G418, geneticin; and E_{max}, maximal effect.

MATERIALS AND METHODS

Cells, DNA Constructs, and Vectors

Murine melanoma B16 and lung carcinoma 3LL as well as human HeLa and 293 cell lines used in this study were cultured as recommended by the American Type Cell Collection. pEGFP-N1 (4.7 Kb), an EGFP expression plasmid vector driven by pCMV, was obtained from Clontech Laboratories. pNBT (10.6 Kb), provided by T. Blankenstein, expresses tumor necrosis factor (TNF) α under the control of the β-actin promoter. p3C-EGFP (6.45 Kb) was constructed by cloning EGFP into the *Hind*III site of pcDNA3 (Invitrogen). p3c-EGFP (6.07 Kb) was obtained by excising the enhancer region of pCMV situated between the *Sna*BI and *Mlu*I restriction sites of p3C-EGFP. mEGFP (or minimalistic EGFP; 1.6 Kb) was provided by Dr. Witig (MOLOGEN), and is a lineal DNA construction with capped ends that encompasses pCMV-EGFP-polyA from p3C-EGFP.

Cationic lipid DOTAP was obtained from Boehringer Mannheim and cationic polymer PEI 800 from Fluka (Sigma). As described by Boussif *et al.* [15], a 10-mM working stock solution was prepared, neutralized with HCl, and filtered through 0.22 μM (Millipore).

Plasmid Extraction and PCR Amplification

DNA was extracted from cells based on standard procedures [16] and was quantified using the DNA-binding dye PicoGreen (Molecular Probes) and salmon sperm DNA to perform as standard curve as previously quantified by spectrophotometry. Samples (2 μg) were amplified in a final volume of 20 μL containing 2.5 mM $MgCl_2$, 2 μL of 10× Taq polymerase buffer (Pharmacia), 0.2 mM deoxynucleotide triphosphates (dNTPs), 0.5 units of Taq polymerase (Pharmacia), and 0.8 μM of each primer. The PCR consisted of a single 3-min step at 94°, followed by 25 cycles of a three-step program (45 sec at 94°, 1 min at 60°, 1 min at 72°), and a final extension (5 min at 72°). The primer pair used amplifies part of *Neo*^{r} and the sequences are 5′-gcttgggtggagaggct-3′ (forward) and 5′-cgaacagttcg gctggcgcg-3′ (reverse).

Densitometry of PCR Products

PCR products were quantified based on band intensities of experimental lanes compared with a standard curve of plasmid DNA. After electrophoresis of the PCR products in agarose gels and ethidium bromide staining, images were obtained and stored as a "tiff" file using Image Store 5000 (UVP) software; image analysis was in turn performed with Gel Works 1.0 (UVP).

Quantitative PCR

The plasmid content of transfected cells was measured via quantitative PCR using an ABI PRISM 7700 Sequence Detector (PE Applied Biosystems). All quantitative PCR assays were performed with SYBR Green PCR master mix (PE Applied Biosystems) following the recommendations of the manufacturer. In order to determine the amount of genomic DNA, we used primers that amplify the ribosomal *18S* gene: 5′-aacggctaccacatccaagg-3′ (forward) and 5′-ttccaattacagggcctcga-3′ (reverse). To quantify the plasmid content, we used primers that amplify *Neo*^{r}. 5′-gggcacaaca gacaatcgg-3′ (forward) and 5′-agttcattcagggcaccg-3′ (reverse). Standard curves were performed using B16 genomic DNA and pEGFP-N1 plasmid DNA from a maxiprep, both previously quantified by spectrophotometry.

Transfection Procedure and G418 Selection

Tumor cell lines were seeded at 40,000 cells per well in 24-well dishes (Costar) 18 hr before transfection or cultured to 60–70% confluence in 75-cm^3 flasks (Costar). Lipoplexes were obtained following the instructions of the manufacturer and polyplexes were obtained as previously described [15] by mixing the specified vector:DNA weight ratios (for DOTAP from 2:1 to 10:1; for PEI from 0.1:1 to 1:1), using the DNA concentration indicated in the figures. Transfection mixture was added to cells in a serum-free medium and incubated for 30 min and then medium (5-fold transfecting volume) containing 10% fetal bovine serum (FBS) was added. Each transfection experiment was done in duplicate and at least 5 times. The cell culture damage of the vector was evaluated as loss of confluence with respect to a non-transfected flask. When indicated, transfected cells were grown in the presence of 2 mg/mL of G418 (Calbiochem). The number of colonies was calculated by counting groups of three or more cells that resist G418 in the medium after 11–13 days (under those conditions untransfected cells died in 10–12 days). The ability of the selected cells to grow was confirmed for one month.

Fluorescence Analysis

EGFP expression in the 24-well dishes was analyzed 48 hr after transfection, measuring fluorescence with a Cytofluor 2350 (Fluorescence Measurement System) from Millipore. The background fluorescence was evaluated measuring the fluorescence of non-transfected cells and cells transfected with a plasmid not containing EGFP. RFU were calculated by subtracting background fluorescence from the total fluorescence measured in each well.

To measure the percentage of transfected cells, the fluorescence of individual cells was quantified using an Axiovert 100 fluorescence microscope equipped with an MPM 200 photometer unit from Zeiss. Background fluorescence was calculated as the highest fluorescence measured in a sample of non-transfected cells. Positive cells were those with a higher than background fluorescence. At least 100 cells from each transfection were analyzed.

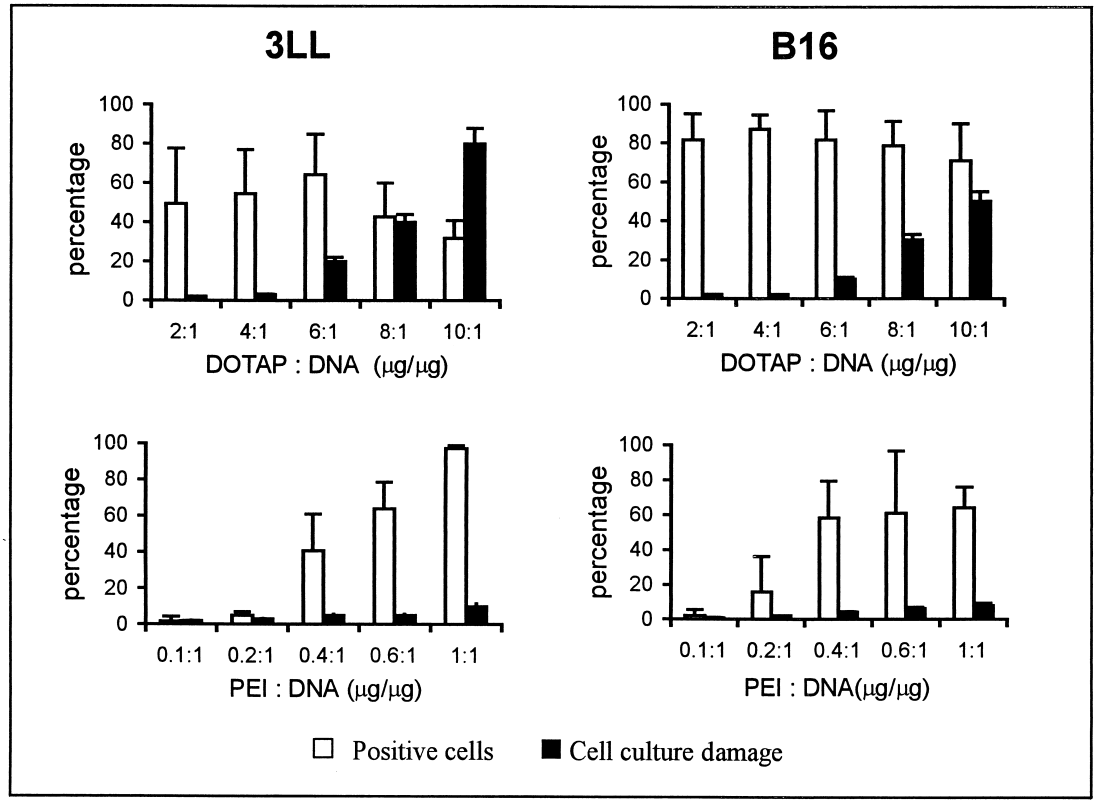

FIG. 1. Transfection efficiency and cell culture damage of DOTAP and PEI-800. B16 and 3LL cells were transfected with pEGFP-N1 and DOTAP or PEI-800 using the weight ratios indicated. Fluorescence of individual cells was measured 72 hr after transfection using a fluorescence microscope Axiovert 100 with an MPM 200 photometer (Zeiss). Background fluorescence was considered as the highest fluorescence measured in cells transfected with a control plasmid (a plasmid without EGFP). We considered positive cells those with higher fluorescence than background. Bars represent means ± SEM of 100 cells from at least five different experiments. Cell culture damage is expressed in percentage of loss of confluence 72 hr after transfection.

RESULTS

Effect of Vector:DNA Ratio on Cell Transfection

B16 and 3LL cells were transfected with pEGFP-N1 using PEI-800 or DOTAP as vectors in several vector:DNA weight ratios. To determine the optimal ratio, we considered two parameters: the percentage of transfected cells and cell culture damage (Fig. 1). pEGFP-N1, a green fluorescent protein expression plasmid, is driven by cytomegalovirus promoter. The latter drives high levels of transgene expression up to 48–72 hr following transfection, and then appears to be shut down; we therefore always analyzed fluorescence during this period of time.

None of the DOTAP:pEGFP-N1 weight ratios analyzed showed significant differences in the percentage of transfected cells, though some cell culture damage was observed when transfection was carried out with weight ratios of 6:1 or higher. On the other hand, PEI-800 showed a very low transfection efficiency with a 0.2:1 weight ratio (or lower), though the efficiency clearly increased at higher ratios. Some cell culture damage was observed with 1:1. Based on these data, we considered 4:1 to be the optimal weight ratio for DOTAP, versus 0.6:1 for PEI-800. These weight ratios also were optimal for other cell types, such as HeLa and 293 (data not shown).

Despite the absence of relevant RFU differences (data not shown), we observed that the standard deviation of cells transfected with DOTAP tended to be higher than that of cells transfected with PEI-800. Accordingly, when cells were analyzed as a function of relative fluorescence, differences indeed existed between these vectors (Fig. 2). PEI-800 fluorescence usually concentrates in the lower RFU ranges, which explains why its standard deviation is usually lower than in the case of DOTAP.

Dose–Response Curve Analysis

In order to compare the pharmacodynamic properties of different vector:DNA complexes, we analyzed cells transfected with DOTAP or PEI-800 and different DNA constructions of EGFP, using optimal weight ratios of the complexes and increasing concentrations of DNA. RFU was measured to trace dose–response curves and to calculate Potency and Efficacy. Potency derives from efficacy concentration 50 and consequently can be expressed in molar units (−log [mol/L]) or weight units (−log [g/L]). Potency depends on how DNA complexes interact with target cells.

Efficacy is an expression of E_{max}; consequently, it is a

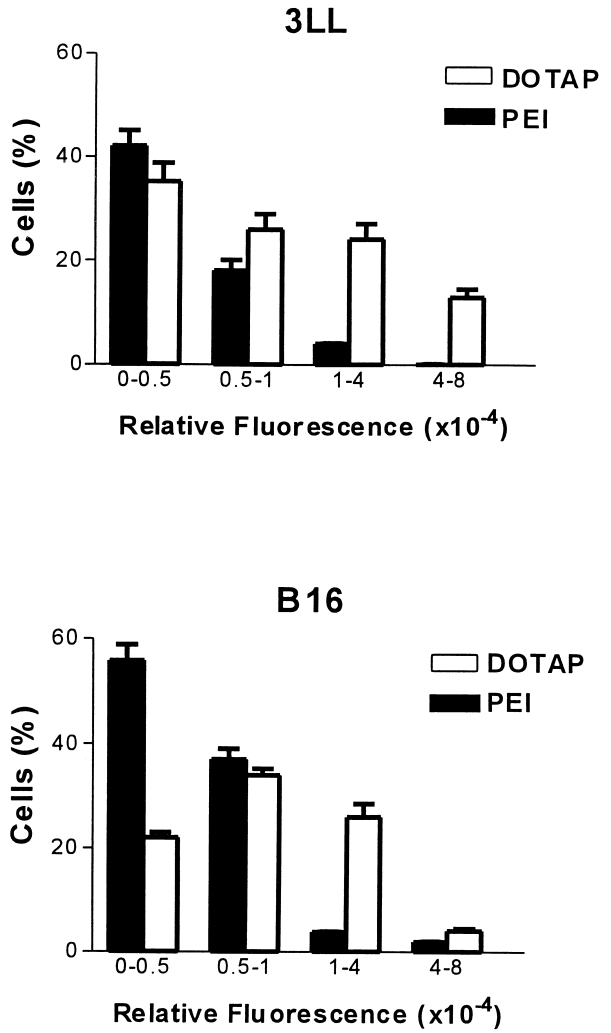

FIG. 2. Fluorescence pattern of B16 and 3LL cells transfected with pEGFP-N1 and DOTAP or PEI-800 using 4:1 and 0.6:1 vector:DNA weight ratios, respectively. Cells were analyzed 72 hr after transfection as in Fig. 1. The number of positive cells was plotted as a function of fluorescence intensity. Bars represent means ± SEM of 100 cells from at least five different experiments. Results are representative of other weight ratios.

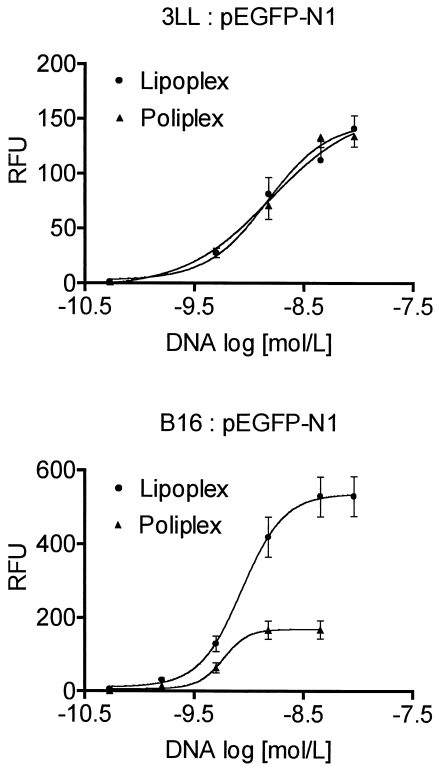

FIG. 3. Dose–response curves of B16 and 3LL cells transfected with pEGFP-N1 using DOTAP or PEI-800. Cells were transfected in 24-well dishes with optimal weight ratios (1:4 for DOTAP and 1:0.6 for PEI) and growing concentrations of plasmid:vector complexes. The total fluorescence of wells was measured 48 hr after transfection using Cytofluor 2350 (Millipore). Relative fluorescence was calculated by subtracting background fluorescence (fluorescence from cells transfected with a control plasmid) from total fluorescence. Fluorescence was plotted as a function of logarithm of molar concentration. Graphs were traced with the polynomial curve using GraphPad Prism 2.01. Each value represents the mean ± SEM (indicated with error bars) of duplicated transfections from at least five different experiments.

measure of the maximum gene expression achievable using a specific vector:DNA complex and can be expressed in either absolute or relative values. Two different types of Efficacy can be considered: Vector Efficacy and Intrinsic Gene Efficacy. In order to calculate the Vector Efficacy, a single gene construct must be transfected using different vectors. In our case, the pEGFP-N1 plasmid was transfected with DOTAP or PEI-800 (Fig. 3, Table 1). The results show that there were no relevant differences in Potency and Efficacy when the targets were 3LL and 293 cells. When B16 and HeLa cells were transfected, DOTAP reached a higher (3- to 4-fold) Vector Efficacy than PEI-800; its Potency on B16 cells was slightly lower, however. These results indicate that pharmacodynamic characteristics are dependent upon both vehicle and cell type, and suggest that Vector Efficacy is defined not only by DNA transduction capacity but also by the ability to influence mechanisms involved in plasmid expression.

In order to determine Intrinsic Gene Efficacy, different DNA constructions must be transfected using a single vector. In our study, three different DNA constructions were compared: two plasmids containing pCMV-EGFP either with or without the 5′ enhancer region of the CMV promoter (p3C-EGFP and p3c-EGFP, respectively), and the minimalistic *EGFP* gene (derived from p3C-EGFP). We used DOTAP to perform a dose–response curve on B16 cells (Fig. 4, Table 2). As was expected, the DNA constructs of the *EGFP* gene containing the 5′ enhancer region of the CMV promoter were found to drive to an 11- to 12-fold higher Intrinsic Gene Efficacy than the plasmid without it (relative efficacy 0.17). Interestingly, differences in Potency are observed when concentration is expressed in molar units. Since they describe the number of plasmid molecules (and therefore the number of *EGFP* gene copies), they are able to compare transcription efficiency from

TABLE 1. Pharmacodynamic parameters deduced from dose–response curve of Fig. 3

Curve parameters	B16		3LL		HeLa		293	
	Lipoplex	Poliplex	Lipoplex	Poliplex	Lipoplex	Poliplex	Lipoplex	Poliplex
Goodness of fit (r^2)	0.999	0.997	0.991	0.990	0.990	0.995	0.997	0.970
Hill slope	2.11	3.40	1.02	1.41	3.13	6.72	6.25	1.89
E_{max} (RFU)	537.0 ± 53.8	169.7 ± 26.2	156.4 ± 0.6	146.4 ± 9.5	155.70 ± 66.14	46.58 ± 13.82	86.19 ± 4.14	77.90 ± 15.58
Efficacy (relative)	1	0.31	0.29	0.27	0.29	0.09	0.16	0.14
EC_{50} (mol/L) ($\times 10^{10}$)	8.60 ± 0.34	5.84 ± 0.49	15.60 ± 3.97	14.70 ± 1.88	18.58 ± 0.10	19.51 ± 3.15	19.02 ± 0.11	19.61 ± 2.32
Affinity ($1/EC_{50}$) ($\times 10^{-9}$)	1.16 ± 0.04	1.71 ± 0.01	0.64 ± 0.21	0.68 ± 0.09	0.53 ± 0.02	0.51 ± 0.00	0.52 ± 0.00	0.50 ± 0.01
Potency ($-\log EC_{50}$)	9.06 ± 0.02	9.23 ± 0.01	8.80 ± 0.13	8.83 ± 0.06	8.73 ± 0.01	8.71 ± 0.64	8.72 ± 0.01	8.70 ± 0.01
EC_{50} (g/L) ($\times 10^3$)	2.79 ± 0.11	1.87 ± 0.0	5.91 ± 2.05	5.25 ± 0.72	6.23 ± 0.08	6.35 ± 1.07	6.21 ± 0.0	7.43 ± 0.0
Affinity ($1/EC_{50}$) ($\times 10^{-2}$)	3.58 ± 0.15	5.34 ± 0.06	1.69 ± 0.90	1.90 ± 0.03	1.60 ± 0.02	1.57 ± 0.19	1.61 ± 0.01	1.34 ± 0.02
Potency ($-\log EC_{50}$)	2.55 ± 0.02	2.73 ± 0.01	2.23 ± 0.19	2.28 ± 0.06	2.20 ± 0.01	2.19 ± 0.06	2.20 ± 0.01	2.12 ± 0.01

Maximal effect is the highest response that can be achieved with the system used (complexes/cells). Vector Efficacy is a relative value of maximal response where the highest response is assigned a value of 1. Efficacy dose 50 is the dose required to arrive at half the maximal effect. Affinity and Potency derive from Efficacy Concentration 50 and are calculated as $1/EC_{50}$ and $-\log EC_{50}$, respectively. Values are expressed as mean ± SEM.

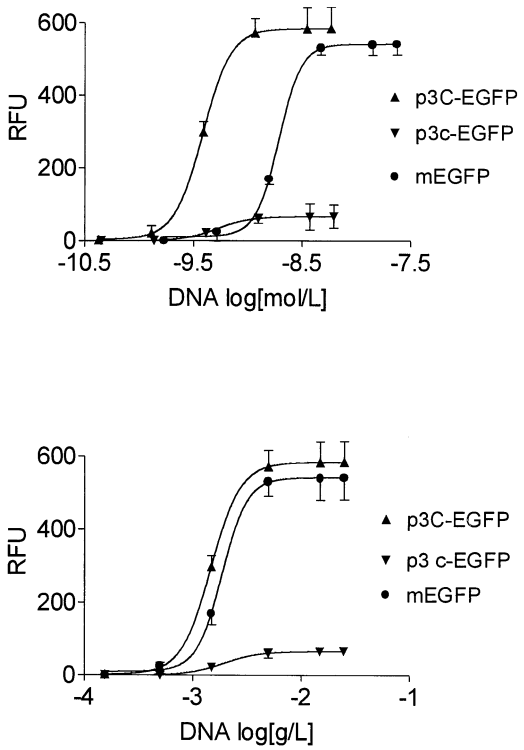

FIG. 4. Dose–response curve of B16 cells transfected with p3C-EGFP, p3c-EGFP, or mEGFP (minimalistic EGFP) using DOTAP. Cells were transfected and analyzed as in Fig. 3. Fluorescence was plotted as a function of logarithm of DNA concentration expressed in mol/L (upper panel) or in g/L (lower panel). Graphs were traced with the polynomial curve using GraphPad Prism 2.01. Each value represents the mean ± SEM (indicated with error bars) of duplicated transfections from at least five different experiments.

different plasmid constructions independent of their size. In contrast, Potency is found to be similar when the DNA concentration is expressed in g/L, because the latter describes the direct relationship between the weight of DNA (with its negative phosphate charge) and the weight of vector (with its positive charge).

Plasmid Contents of Transfected Cells

To investigate the way in which Vector Efficacy is correlated to different amounts of transfected DNA, we analyzed

TABLE 2. Pharmacodynamic parameters deduced from dose–response curve of Fig. 4

Curve parameters	B16:Lipoplexes		
	p3C-EGFP	p3c-EGFP	mEGFP
Goodness of fit (r^2)	0.999	0.999	0.999
Hill slope	3.31	2.84	4.14
E_{max} (RFU)	584.7 ± 28.6	65.6 ± 3.9	541.2 ± 30.1
Efficacy (relative)	1	0.17	0.92
EC_{50} (mol/L) ($\times 10^9$)	0.39 ± 0.02*†	0.54 ± 0.03†	1.93 ± 0.15
Affinity ($\times 10^{-9}$)	2.58 ± 0.01	1.87 ± 0.07	0.52 ± 0.04
Potency	9.41 ± 0.03	9.27 ± 0.02	8.71 ± 0.03
EC_{50} (g/L) ($\times 10^3$)	1.48 ± 0.15*	1.97 ± 0.11	1.88 ± 0.16
Affinity ($\times 10^{-2}$)	6.75 ± 0.19	5.07 ± 0.32	5.31 ± 0.21
Potency	2.82 ± 0.01	2.70 ± 0.02	2.72 ± 0.01

EC_{50} and derived parameters (Affinity and Potency) are expressed in both mol/L and g/L. Values are expressed as means ± SEM. Statistical evaluation was performed by the unpaired Student's *t*-test.

*$P < 0.05$ when compared with p3c-EGFP.

†$P < 0.05$ when compared with minimalistic EGFP.

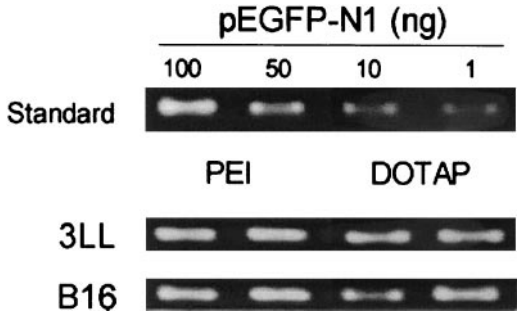

FIG. 5. Amount of *Neo*^{r} after transfection of B16 and 3LL cells with pEGFP-N1 using DOTAP or PEI-800. Cells were transfected in 24-well dishes using a dose of 15 μg/mL and the optimal weight ratios. Fluorescence was determined as in Fig. 3. Cells were then lysated and total DNA was extracted following standard procedures. Equal amounts of total DNA were analyzed to estimate *Neo*^{r} content by PCR and electrophoresis in agarose gels. The standard was performed by PCR by amplifying 100, 50, 10, and 1 ng of pEGFP-N1 plasmid previously quantified by spectrophotometry.

the plasmid contents of 3LL or B16 cells transfected with DOTAP or PEI-800 and a plasmid concentration of 15 μg/mL to achieve the maximal effect. Two days after transfection, relative fluorescence was measured and total DNA was extracted from the cells, following standard procedures. The amount of DNA extracted in each experiment was quantified using PicoGreen (Molecular Probes). PCR was performed on the total DNA extracted and the PCR products were then run on an agarose gel. After electrophoresis in agarose gels and ethidium bromide staining (Fig. 5), the plasmid contents were determined by PCR and densitometry of the products (data not shown). The results suggested that cells transfected with PEI-800 had higher amounts of plasmid than DOTAP. To confirm this observation, we performed quantitative PCR of the samples. Standard curves of genomic and plasmid DNA were plotted (Fig. 6) to calculate the plasmid/genome DNA ratio in each sample. The results indicate that the amount of plasmid delivered in B16 cells by PEI was higher than for DOTAP (Table 3), though EGFP expression was higher with DOTAP than with PEI. The observed Efficacy could not be explained by differences in plasmid transduction efficiency alone, thus suggesting that PEI-800 could limit the plasmid expression of transferred DNA.

G418 Selection

When comparing DOTAP versus PEI gene delivery, we observed that gene transfer mediated by the PEI:DNA complex resulted in a lower gene expression and higher amounts of intracellular DNA plasmid, thus suggesting that PEI could limit the nuclear availability of exogenous DNA. Lower nuclear availability of the plasmid would affect transgene expression as well as other nuclear operations such as integration on the target cell genome. We studied the integration ability of an exogenous DNA delivered with DOTAP and PEI vectors. It is well known that the plasmid efficiency of integration is low. In order to study this property, we used pNBT because in our hands it has relatively high integration efficiency. After transfection, cells were cultured in the presence of G418 to select those that had integrated the plasmid, and the number of colonies was counted (Table 4). There were significant differences between DOTAP and PEI-800. When using DOTAP, means of 80 and 33 colonies were selected to transfect B16 and 3LL cells, respectively, versus a mean of only 5 and none when using PEI to transfect the same cell lines. These results support the idea that the nuclear availability of plasmids transfected with DOTAP is higher than with PEI-800.

DISCUSSION

An improvement in the gene transfer efficiency of nonviral vectors is a key requirement for gene therapy to become a clinical reality. Whereas relatively high gene transfer efficiencies have been described by several authors using different vectors, the criteria for objective evaluation of vector efficiency [17] have generally been poor. Early pharmacokinetic concepts have successfully been incorporated to describe the kinetic process of a gene, based on objective parameters. Herein, we propose the introduction of the pharmacodynamic concepts of Efficacy and Potency to study genedynamic processes. For this study, we have used the parameters derived from the dose–response curves of E_{max} and EC_{50}. The green fluorescent protein gene was used to perform the pharmacological dose–response curve on mouse and human tumor cell lines, and the gene transfer

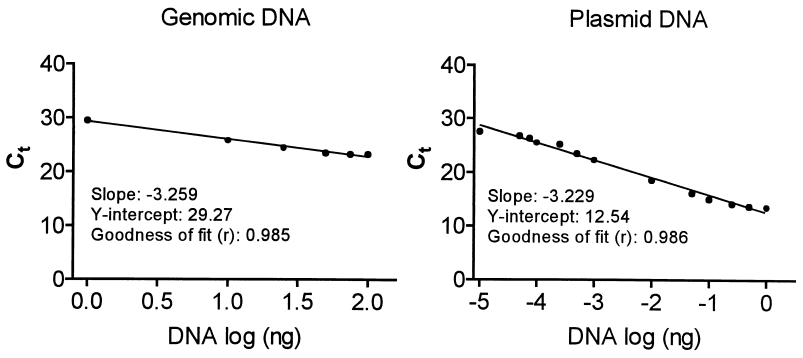

FIG. 6. Quantitative PCR standard curves were performed by amplifying 1, 10, 25, 50, 75, and 100 ng of B16 genomic DNA and 1×10^{-5}, 5×10^{-5}, 7.5×10^{-5}, 1×10^{-4}, 2.5×10^{-4}, 5×10^{-4}, 1×10^{-3}, 0.01, 0.05, 0.1, 0.25, 0.5, and 1 ng of pEGFP-N1 plasmid DNA from a maxiprep. Both DNA were previously quantified by spectrophotometry C_+, threshold cycle.

TABLE 3. Quantification of plasmid DNA from cells transfected with 15 μg/mL of plasmid

	B16:pEGFP-N1		3LL:pEGFP-N1	
	DOTAP	PEI	DOTAP	PEI
Genomic DNA (ng/μL)	561.0 ± 7.0	716.0 ± 41.0	257.6 ± 3.5	357.9 ± 21.0
Plasmid DNA (ng/μL)	1.4 ± 0.1	5.3 ± 0.1	$0.36 \times 10^{-4} \pm 0.01 \times 10^{-4}$	$0.49 \times 10^{-4} \pm 0.01 \times 10^{-4}$
Plasmid/genomic (ng/μg)	2.5*	7.4*	1.4×10^{-4}	1.4×10^{-4}

Quantitative PCR was performed using an ABI PRISM 7700 Sequence Detector. The amount of genomic DNA and plasmid was determined using the standard curves represented in Fig. 6 and the proper dilutions of the samples. Genomic and plasmid DNA are expressed as means ± SEM of duplicated transfections from at least 5 different experiments.
*This method discriminates samples with differences of 2-fold concentration or higher.

procedure was performed employing DOTAP (cationic lipid) and PEI (cationic polymer).

Cationic liposomes and polymers can complex to the negatively charged plasmid via electrostatic interactions with the phosphates on DNA. To determine the optimal cationic vector:DNA ratio for transfection [18], we used a fixed amount of pEGFP-N1 plasmid encoding the green fluorescent protein plus variable amounts of DOTAP lipid or PEI polymer. Transfection was performed *in vitro* on mouse 3LL and B16 cancer cell lines. Forty-eight hours later, the gene expression and cell culture damage were evaluated. Based on the ability of each vector complex to transfer the gene to a higher number of cells with the lowest toxicity, we consider the optimal ratio of vector:DNA to be 4:1 for DOTAP and 0.6:1 for PEI. The optimized preparations of the vector:DNA complex were then employed to study gene transfer efficiency on the basis of a dose–response curve. Tumor cells were distributed in 24-well plates, and increasing amounts of pEGFP-N1 plasmid were administered. The fluorescence per well was measured 24, 48, and 72 hr after transfection by means of a microtiter plate reader, followed by analysis of the data. Interestingly, when the relative fluorescence was plotted against the logarithm of the pEGFP-N1 plasmid concentration, a high goodness-of-fit ($r > 0.99$) was observed in all cases. This suggests that the curve parameters EC_{50}, E_{max}, and slope could contribute to define the dynamic process of gene transfer mediated by a vector:DNA complex. EC_{50} is the concentration of the plasmid that produces 50% of the maximum response. This value provides information on the Potency ($-\log EC_{50}$) of the system. In classical pharmacodynamic studies, EC_{50} is a measure of drug affinity for its receptor; however, in this study the interpretation of EC_{50} is more complex, since several barriers (plasma membrane, endosome, nuclear envelope) must be crossed before gene expression occurs. Therefore, in the present study potency is only a descriptive parameter of the amount of DNA:vector that yields half the maximal response. Potency can be expressed as a function of either the number of plasmid molecules (mol/L) or the weight (g/L) of DNA. When expressed as mol/L, we for example found potency of the largest plasmid to be greater than for the minimalistic plasmid, thus providing information on gene activity per number of plasmid copies of administered gene. Interestingly, when expressed as g/L, the potency values of all plasmids (employing the same vector) were found to be quite similar, suggesting that potency is directly dependent upon the amount of administered DOTAP or PEI. However, this is not such a useful way to analyze gene potency. On comparing plasmids (such as p3C-EGFP and p3c-EGFP) of similar molecular weight and potency (g/L), we observed significant differences in their relative fluorescence values, suggesting that E_{max} (the maximum relative fluorescence achieved from the dose–response curve) could be a more reliable parameter for comparing plasmids, and that not only potency but also E_{max} should be taken into account when determining the goodness of a DNA:vector system. E_{max} would constitute a parameter for evaluating the expression of the gene fraction that has been delivered into the cell, reached the nucleus, and is transcriptionally available. E_{max} can offer information on Vector Efficacy and Intrinsic Gene Efficacy, depending on the experimental procedure used in the study. Thus, when the same gene construct is delivered using different vectors, E_{max} gives

TABLE 4. G418 colony selection

Tumor line	Vectors	Incidence of positive selection (%)	Mean ± SE	Median	No. colonies/assay (distribution)
B16	DNA	0/12 (0)	0.0 ± 0.0	0	0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0
	DOTAP/DNA	12/12 (100)	80.2 ± 16.9	80	6, 8, 10, 64, 70, 70, 90, 91, 99, 120, 120, 215
	PEI/DNA	4/12 (25)	4.8 ± 2.4	0	0, 0, 0, 0, 0, 0, 0, 0, 4, 11, 18, 25
3LL	DNA	0/16 (0)	0.0 ± 0.0	0	0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0
	DOTAP/DNA	9/16 (56)	32.8 ± 13.1	6.5	0, 0, 0, 0, 0, 0, 0, 0, 2, 5, 8, 50, 58, 60, 65, 80, 197
	PEI/DNA	1/16 (6)	0.5 ± 0.5	0	0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 8

G418 colony selection after transfection with DOTAP or PEI-800. B16 and 3LL cells transfected with pNBT and DOTAP or PEI were grown in the presence of G418 (2 mg/mL). The number of colonies was determined 11–13 days after transfection when the confluence was minimal. Their ability to grow was confirmed for a month.

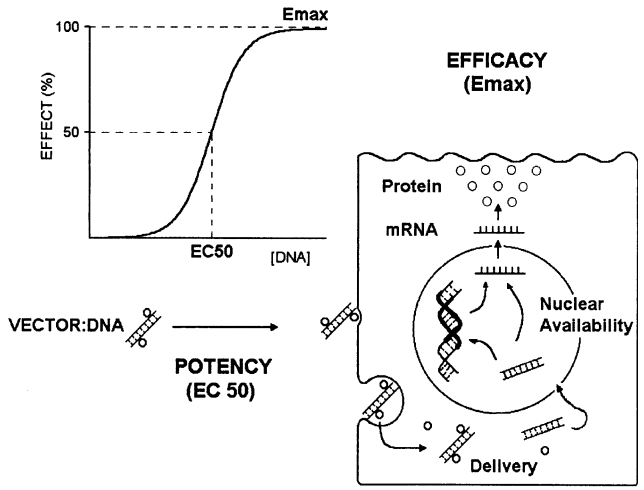

FIG. 7. Dynamic process of gene transfer.

information on Vector Efficacy. In contrast, when the same vector is used to deliver different constructions of the same gene (e.g. a gene driven by different promoters), E_{max} provides information on Intrinsic Gene Efficacy. In both cases, Efficacy must be considered a relative value that allows us to compare the efficacy of several vectors or gene constructs with respect to a reference regarded as the unit value. In our study, we found the Vector Efficacy of the DOTAP:DNA complex to be three times greater than that of the PEI:DNA complex which was employed to transfect B16 cells, though similar Efficacy values were recorded with the 3LL tumor cell line. We also found differences when employing DNA constructs of EGFP driven by different promoters, indicating that this model is useful for determining the Intrinsic Gene Efficacy. In this context, Efficacy is a useful descriptive parameter of the efficiency of gene transcription, though the information on how this is achieved must be procured by other procedures. The complete gene transfer process (Fig. 7) involves several consecutive steps: vector:DNA complex interaction with the target cell, gene delivery into the cell, and nuclear gene availability for expression and/or integration. Unlike viral vectors, the vector:DNA complex can be used to transfer expression cassettes of essentially unlimited size with a series of distinctive properties: they cannot replicate or recombine to form an infectious agent and have a low integration frequency. However, the final efficiency of gene transfer by vector:DNA is very limited, and depends on the success of each vector in passing through each of the steps of the gene transfer process. Therefore, the pharmacodynamic study of gene transfer may contribute to identify those steps in which a given vector either improves or limits the efficiency of the gene transfer process. Because gene transfer efficacy depends on the amount of DNA delivered and on the nuclear availability achieved by the exogenous gene, we performed transfection experiments to study the relative amount of plasmid delivered and its integration ability, using both DOTAP- and PEI-based vectors. The plasmid contents were displayed by PCR of the neomycin resistance gene. Agarose gel band densitometry indicated that the largest amounts of PCR products were obtained when cells were transfected with PEI:DNA complex. This observation is in agreement with the idea that PEI is a type of cationic polymer that requires no additional agents to achieve high transfection activity and that prevention of lysosomal degradation of DNA could contribute to increase intact DNA delivery into cells [15, 19]. There is agreement that cationic liposome:DNA [20–23] and PEI:DNA complexes [15, 24] enter the cell via an endocytic pathway; consequently, the buffering capacity of PEI in the physiological pH range could improve the efficiency of gene delivery. On the other hand, the greater amounts of plasmid obtained with PEI:DNA complex are in contrast with our own observations from the dose–response curve, where PEI:DNA complex showed the same or less efficacy than DOTAP:DNA complex in mediating gene expression. These data suggest that PEI can increase gene delivery into the cell, but also limits DNA availability for mediating its functional activity. Although the precise mechanisms of DNA entry into the nucleus and DNA release from the complex inform gene availability, they remain largely unknown. It has been described that plasmid DNA must dissociate from the cationic liposome complex prior to entry into the nucleus [23], since cationic lipid:DNA complex microinjected directly into the nucleus exhibits a low transfection efficiency. A mechanism of DNA delivery and release from cationic liposome complex has been proposed [25, 26] on the basis of anionic lipid exchange with the cytoplasmic surface of the cell membrane. DNA delivery from PEI complex is less well known, and although other mechanisms could be involved, we believe that the strong interaction between cationic polymer and DNA could lead to limited nuclear DNA availability. Since DNA integration is a process in which nuclear DNA availability must be good, we studied the integration ability of pNBT plasmid in 3LL and B16 cells transfected with either DOTAP or PEI:DNA complex as a relative measure of plasmid nuclear availability. Our results showed the number of G418-resistant colonies after cell transfection to differ depending on the vector used for transfection. Thus, the DOTAP:DNA complex was more efficient than the PEI:DNA complex in both tumor cell lines. These results support the idea that the vector used for DNA delivery can limit nuclear DNA availability for achieving DNA integration and gene expression, as suggested by the pharmacodynamic study. Thus, on comparing both vectors, PEI is seen to be the type of vector that offers advantages in the DNA delivery step, while DOTAP offers increased DNA nuclear availability.

In conclusion, the pharmacodynamic study of vector:DNA complexes is an appropriate procedure for describing the goodness of a vector and gene construct. The objective parameters of Potency and Efficacy derived from dose–response curves contribute to provide information on the goodness of the system in the first and last step of the gene transfer process, respectively. In addition, however, we

expect these parameters to contribute to reinforced understanding of the mechanistic gene transfer process of each vector.

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