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Cationic liposome-mediated expression of HIV-regulated luciferase and diphtheria toxin a genes in HeLa cells infected with or expressing HIV

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Abstract

HIV-regulated expression of the diphtheria toxin A fragment gene (HIV-DT-A) is a potential gene therapy approach to AIDS. Since cationic liposomes are safe and non-immunogenic for *in vivo* gene delivery, we examined whether LipofectAMINE or DMRIE reagent could mediate the transfection of HIV-DT-A (pTHA43) or the HIV-regulated luciferase gene (pLUCA43) into HIV-infected or uninfected HeLa cells. pLUCA43 was expressed at a 10³-fold higher level in HeLa/LAV cells than in uninfected HeLa cells, while the extent of expression of RSV-regulated luciferase was the same in both cell lines. Co-transfection of HeLa cells with pTHA43 and the proviral HIV clone, HXBΔBgl, resulted in complete inhibition of virus production. In contrast, the delivery of HIV-DT-A to chronically infected HeLa/LAV or HeLa/IIIB cells, or to HeLa CD4⁺ cells before infection, did not have a specific effect on virus production, since treatment of cells with control plasmids also reduced virus production. This reduction could be ascribed to cytotoxicity of the reagents. The efficiency of transfection, as measured by the percentage of cells expressing β-gal, was ~5%. Thus, cationic liposome-mediated transfection was too inefficient to inhibit virus production when the DT-A was delivered by cationic liposomes to chronically- or *de novo*-infected cells. However, when both the virus and DT-A genes were delivered into the same cells by cationic liposomes, DT-A was very effective at inhibiting virus production. Our results indicate that the successful use of cationic liposomes for gene therapy will require the improvement of their transfection efficiency.

Keywords: HIV; Diphtheria toxin A fragment; Liposome, cationic; Transfection

1. Introduction

The genetic approach to HIV infection is based on the notion that transfer of therapeutic genes into target cells will render them resistant to HIV replication. HIV gene therapy strategies have been based on RNA-targeted antivirals (antisense DNA and RNA, ribozymes, RNA decoys), intracellular proteins that compete or interfere with viral functions, and inducible toxins that kill host cells subsequent to infec-

Abbreviations: DME-HG, Dulbecco's modified Eagle's medium, high glucose; FBS, heat-inactivated fetal bovine serum; AAV, adeno-associated virus; RSV, Rous sarcoma virus; LFA, LipofectAMINETM reagent.

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tion by the virus. In each case, the therapeutic benefit will be determined by the antiviral gene chosen, intracellular expression and stability, delivery system, target cells and tissues, and localization of the gene product [1].

Expression of a gene encoding the diphtheria toxin A fragment (DT-A), controlled by tissue-specific regulatory elements, has been used previously to kill selected cell populations [2,3]. DT is composed of two chains: the B chain (342 amino acids), which mediates adsorption of the toxin to the cell surface, and the enzymatically active A chain (193 amino acids), which is a lethal inhibitor of protein synthesis when expressed in eukaryotic cells. The B chain is required for the endocytotic uptake and translocation into the cytoplasm of the active A chain, which inactivates elongation factor 2 (EF-2) required for translocation of polypeptidyl-tRNA from the acceptor to the donor site on the eukaryotic ribosome. The inactivation involves transfer of the ADP-ribosyl group of NAD to a specific site on EF-2. Binding to the cytosolic NAD protects DT-A against proteolysis and permits its activity for long periods within cells. EF-2 is the only protein ADP-ribosylated by DT-A, and this specificity depends on the presence in EF-2 of a unique modified histidine residue, diphtamide [4].

Due to the potent toxicity of DT-A, HIV-regulated expression of the DT-A gene can be an effective strategy to eliminate HIV-infected cells. The DT-A coding sequence has been linked to *cis*-acting control elements unique to HIV, resulting in DT-A gene expression that is highly dependent on *trans*-activation by the HIV regulatory proteins Tat and Rev [5–7]. Tat and Rev are expressed early in HIV-infected cells, upregulate the expression of HIV, and are essential for efficient viral replication. Harrison et al. [5,6] proposed that expression of a lethal DT-A gene responsive to Tat and Rev should allow for selective killing of HIV-infected cells.

Previous studies have shown that stable expression of the HIV-regulated gene for diphtheria toxin A fragment (HIV-DT-A), after transfection by electroporation or by retroviruses, confers resistance against HIV infection [6–9]. Selected HeLa cell clones containing HIV-DT-A sequences were transfected by electroporation with an HIV proviral clone, HXBΔBgl, and tested for HIV production. These

clones produce less HIV, by p24 assay, than either parental HeLa cells or cells expressing an HIV-DT-A frameshift mutant [6]. Similarly, selected H9 cell clones expressing the HIV-DT-A gene produce less p24 antigen when transfected with an HIV proviral clone or infected with laboratory or clinical HIV-1 strains [7,8]. Significant protection against HIV, dependent both on the stock of HIV-1 used and on the dose, was also observed in selected promonocytic U937 cells after transduction with HIV-DT-A [9]. These results suggest that virus-induced expression of the DT-A gene caused the death of infected cells.

In the studies described above, the cells stably expressing HIV-DT-A were subsequently challenged with HIV, either by transfection with the proviral clone or by direct infection with the virus. If expression of the HIV-DT-A gene can specifically destroy HIV-infected cells, the strategy could also be applicable to cells which are already infected. Since even one molecule of DT-A may be sufficient to kill a cell, even transient expression of HIV-DT-A in an already chronically or *de novo* HIV-infected cell could cause the selective suicide of the cell.

Two of the major problems of gene therapy are the effective delivery of the therapeutic gene into target cells, and the safety of the vector carrying the gene. Cationic liposomes provide a simple means of introducing DNA and other polynucleotides into eukaryotic cells, a process generally referred to as ‘lipofection’. Small unilamellar liposomes composed of cationic lipids and the membrane fusion-promoting lipid, phosphatidylethanolamine, spontaneously complex with negatively charged polynucleotides. The mechanism of interaction between cationic liposomes and DNA is poorly understood. Several models of the liposome-DNA complexes, situating the DNA either outside or inside the liposome, have been proposed [10]. Recently, electron microscopy has been used to study the relationship between the structure of the liposome-DNA complex and its transfection efficiency [11–14]. The morphology of the complexes was found to be dependent on incubation time and the lipid:DNA ratio. Interestingly, Zabner et al. [14] have reported that even under optimal conditions for transfection, DMRIE-DNA complexes constitute a very heterogeneous population.

Plasmid-liposome complexes have several advantages as gene transfer vectors: (i) they can be used to

transfer expression cassettes of essentially unlimited size; (ii) they cannot replicate or recombine to form an infectious agent, (iii) they are non-infectious and appear to be non-immunogenic in vivo and (iv) they are simple to manufacture and use, and therefore cost-effective [15–17]. Cationic liposomes have been utilized for gene delivery in vivo [18–22], and a direct gene transfer protocol using a liposome-DNA complex has been approved for injection into solid tumors in patients [23]. Several clinical trials utilizing liposome-mediated transfection are in progress. Thus, it will be of interest to establish conditions under which cationic liposomes can be used for eventual in vivo transfection of genes with therapeutic activity against HIV. Our approach contrasts with ex vivo gene therapy where transfected cells can be selected in the laboratory before injection into patients.

As a first step toward the utilization of cationic liposomes for HIV gene therapy in vivo we investigated (i) whether cationic liposomes could be used to express HIV-regulated luciferase and DT-A genes in HeLa cells, (ii) whether transient expression of the DT-A gene could reduce virus production in chronically infected HeLa/LAV and HeLa/IIIB cells and (iii) whether this expression could protect HeLa CD4⁺ cells against de novo HIV infection. Our results are the first demonstration that HIV-regulated luciferase could be expressed in chronically HIV-infected cells and that co-transfection of HIV-DT-A with an HIV proviral clone could prevent virus production. Some of our results have been presented earlier in preliminary form [24].

2. Materials and methods

2.1. Materials

LipofectAMINE™ reagent [25] containing the polycationic lipid 2,3-dioleoyloxy-*N*-[2-(spermine-carboxamido)ethyl]-*N,N*-dimethyl-1-propanaminium trifluoroacetate (DOSPA) and the neutral lipid, dioleoylphosphatidylethanolamine (DOPE) (3:1, w/w), and Lipofectin Reagent [15] containing the monocationic lipid *N*-[1-(2,3 dioleoyloxy)-propyl]-*N,N,N*-trimethylammonium chloride (DOTMA) and DOPE (1:1, w/w) were obtained from Gibco BRL Life

Technologies (Gaithersburg, MD). DMRIE reagent [26,27] (a 1:1 (w/w) mixture of 1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethylammonium bromide (DMRIE) and DOPE) was synthesized by VI-CAL (San Diego, CA). Alamar Blue dye was purchased from Alamar Biosciences Inc. (Sacramento, CA). The penicillin, streptomycin and L-glutamine solutions were obtained from Irvine Scientific (Santa Ana, CA). The β -Gal Staining Kit was obtained from Invitrogen Corporation (San Diego, CA).

2.2. Cells and virus

HeLa cells were obtained from the American Type Culture Collection. HeLa/LAV and HeLa CD4⁺ were obtained through the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH, Bethesda, MD) from Drs. Joerg Berg and Matthias Wable [28] and Dr. Richard Axel [29], respectively. HeLa and HeLa/LAV cells were maintained at 37°C, under 5% CO₂ in DME-HG medium (Irvine Scientific) supplemented with 10% (v/v) FBS (Sigma; St. Louis, MO), penicillin (100 U/ml), streptomycin (100 μ g/ml) and L-glutamine (4 mM). HeLa CD4⁺ cells were grown in RPMI 1640 medium with 10% FBS, penicillin (100 U/ml), streptomycin (100 μ g/ml), L-glutamine (2 mM) and 0.6 mg/ml Geneticin (G418) sulfate (Gibco). The cells were passaged 1:20 once a week. FACS analysis indicated that over 90% of the cells were CD4-positive.

The supernatant of chronically infected H9/HTLV-III_B cells was used as a source of the HIV-1 strain, HIV-1_{III_B}. The culture supernatants were harvested at times of peak p24 production and stored at –80°C. The p24 concentration of the stock was 1.4 μ g/ml as determined by ELISA [30]. The tissue culture infectious dose, 50% endpoint (TCID₅₀), was determined as described by Johnson and Byington [31]. The infectious titer per 1 μ g p24 was 7.1×10^3 TCID₅₀.

The presence of cell surface CD4 was examined by direct immunofluorescence assay, using fluorescein isothiocyanate (FITC)-conjugated Leu3 antibody (Becton-Dickinson), with FITC-conjugated mouse IgG as a control. The fluorescence distribution was analyzed with a fluorescence-activated cell sorter (FACScan; Becton-Dickinson, Mountain View, CA).

2.3. Plasmids

The plasmids used for transient transfection assays, pTHA43 (containing Tat + Rev-regulated diphtheria toxin A gene; HIV-DT-A), pTHA44 (a DT-A frameshift mutant of pTHA43) and pLUCA43 (containing the HIV LTR and *env* sequences including HIV *cis*-acting repressive sequences and the Rev responsive element (RRE) in the 3' untranslated region) have been described previously [5]. The plasmid pRSVLUC encodes the luciferase reporter gene under control of the Rous sarcoma virus promoter [32]. The HIV proviral clone, HXBΔBgl, kindly provided by Dr. E. Terwilliger, was derived from HXB2 [33] by deletion of 500 base pairs between two Bgl II sites in the *env* region [6,7]. The plasmid pCMV.SPORT-βgal containing the *E. coli* gene encoding β-galactosidase (β-gal) under control of the cytomegalovirus (CMV) promoter was obtained from GIBCO.

2.4. Transfection of cells

HeLa and HeLa/LAV cells were seeded at 2×10^5 cells/well in 2 ml of DME-HG medium in 24-well plates the day before transfection. Cells were ~60–70% confluent at the time of transfection. LipofectAMINE™ reagent (LFA) was provided at 0.5 mM (corresponding to 2 mg/ml) and DMRIE/DOPE was prepared to 0.747 mM in Milli-Q purified sterile water (Millipore; Bedford, MA) (corresponding to 1 mg/ml). For all experiments, the indicated amounts of lipid and DNA were complexed for 20–25 min at room temperature in Opti-MEM medium without serum (GIBCO). During lipid/DNA complex formation, cells were washed twice with 1 ml Opti-MEM medium, and 0.2 or 0.3 ml of Opti-MEM medium was placed on each well. The lipid/DNA mixture was then added gently in a volume of 0.2 ml per well. The transfection solution remained on the cells for 4 h at 37°C in a 5% CO₂ incubator and was then replaced with 2 ml of DME-HG medium. Cells were further incubated for indicated periods of time before the luciferase or Alamar Blue assays and p24 determination. Cells treated with either cationic lipids, DNA or medium alone served as controls. Treatment with DNA alone was not toxic in any of the experiments reported in this study. Treatment with the

lipids alone served as a control for the cytotoxic effect of the transfection procedure.

Transfection efficiency was measured by β-gal staining. Briefly, transfected cells were washed with PBS, fixed in the fixative solution containing 2% formaldehyde and 0.2% glutaraldehyde, and stained with an X-gal containing solution. The cells were checked under a phase contrast microscope for the development of blue color.

2.5. HIV infection of transfected HeLa CD4⁺ cells

The virus and infected cells were handled in a BL-3 facility. Twenty-four hours after transfection, HeLa CD4⁺ cells were exposed to HIV-1_{IIIB} for 2 h at 37°C, washed twice to remove unbound virus and cultured in RPMI medium (2 ml per well). Control cells were treated similarly but not exposed to virus. The infection was monitored by determining viral p24 in culture supernatants by an antigen capture ELISA assay [30], using a Molecular Devices (Menlo Park, CA) V_{max} microplate reader.

2.6. Cell viability assay

Cell morphology was evaluated by inverted phase contrast microscopy at 25× magnification. The number of viable cells used for experiments was determined by Trypan Blue exclusion. Cell viability after transfection was quantified by a modified Alamar Blue assay [34]. Briefly, 1.0 ml of 10% (v/v) Alamar Blue dye in the appropriate medium was added to each well. After incubation for various times (described under figure legends) at 37°C, 200 μl of the supernatant was collected from each well and transferred to 96-well plates. The absorbance at 570 nm and 600 nm was measured with a microplate reader. Cell viability (as a percentage of mock-treated control cells) was calculated according to the formula $(A_{570} - A_{600})$ of test cells $\times 100 / (A_{570} - A_{600})$ of control cells. After removal of the Alamar Blue/medium mixture, fresh growth medium was added, and cells were returned to the incubator. Thus, the Alamar Blue assay allows determination of viability over the culture period without the detachment of adherent cells. A good correlation was obtained between the Alamar Blue assay and Trypan Blue staining [35].

2.7. Luciferase assay

Luciferase activity was assayed using a kit obtained from Promega (Madison, WI) and a scintillation counter (1209 RACKBETA, Pharmacia LKB Nuclear, Gaithersburg, MD) according to the manufacturer's instructions to increase sensitivity. A standard curve of cpm vs. enzyme concentration was produced using purified luciferase.

3. Results

3.1. Trans-activation of the HIV-regulated luciferase gene in HeLa/LAV cells

In preliminary experiments the transfection activity of LFA, Lipofectin or DMRIE was optimized by using different amounts of the HIV-regulated luciferase gene, pLUCA43 [5]. Chronically infected HeLa/LAV cells were transfected with 0.5, 1 or 2 μg of the plasmid DNA complexed with 5 μl of LFA, Lipofectin or DMRIE. These lipid concentrations correspond to 6.25 μM LFA, 8.5 μM Lipofectin and 9.4 μM DMRIE. Luciferase activity was measured after 1 and 2 days. The highest level of expression was observed with 2 μg pLUCA43 complexed with LFA (a lipid:DNA (w/w) ratio of 5:1 and a charge ratio (+/-) of 6.25). A substantial level of expression was also observed with 2 μg pLUCA43 and DMRIE (a lipid:DNA (w/w) ratio of 2.5:1 and a charge ratio (+/-) of 0.8). Transfection with Lipofectin resulted in low expression of luciferase. In further experiments 2 μg plasmid DNA was complexed with 5 μl LFA or 8 μl DMRIE (a lipid:DNA (w/w) ratio of 4:1 and a charge ratio (+/-) of 1.0).

We first examined whether the HIV-regulated luciferase gene could be expressed in an HIV-dependent manner following transfection by cationic liposomes. The plasmids pLUCA43 (containing Tat + Rev-responsive luciferase) and pRSVLUC were transfected into uninfected or chronically infected HeLa cells. The HIV-regulated luciferase gene was expressed at a 10^3 -fold higher level in chronically infected HeLa/LAV cells compared to uninfected HeLa cells, while the level of expression of RSV-regulated luciferase was the same in both cell lines (Fig.

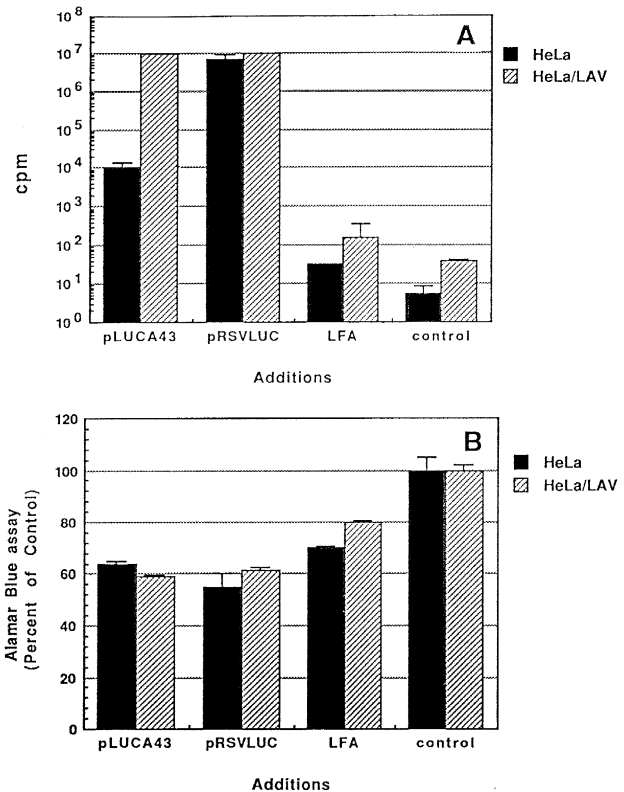


Fig. 1. HIV-regulated but not RSV-regulated luciferase gene expression was increased in chronically infected HeLa/LAV cells. HeLa and HeLa/LAV cells were transfected with 2 μg pLUCA43 or pRSVLUC complexed with 6.25 μM LFA (5 μl /400 μl Opti-MEM medium), at a lipid:DNA (w/w) ratio of 5:1 (charge ratio (+/-) = 6.25), as described in Section 2. (A) Cells were lysed 2 days post-transfection and luciferase activity was expressed as cpm. Data represent the mean \pm standard deviation obtained from duplicate wells. (B) Cell viability was measured 2 days post-transfection, by incubation with Alamar Blue for 2 h at 37°C, and was expressed as percent of the control. Data represent the mean \pm standard deviation obtained from duplicate wells from one experiment and are representative of three separate experiments.

1A). Transfection with either plasmid reduced cell viability by about 40%, while treatment with LFA alone reduced the viability of HeLa and HeLa/LAV cells by 30% and 20%, respectively (Fig. 1B).

3.2. Inhibition of p24 production by the HIV-regulated DT-A gene after co-transfection with an HIV proviral plasmid

To demonstrate that the HIV-regulated DT-A gene could be expressed in cells that also express the HIV

genome, following transfection by cationic liposomes, the plasmid HXB Δ Bgl was employed in a transient assay. If the DT-A gene is expressed and an active DT-A is produced, then it is expected that transfected cells are substantially impaired in their ability to produce HIV due to the high cellular lethality of DT-A. HeLa cells were transfected with the HIV proviral clone, HXB Δ Bgl, together with the HIV-regulated DT-A plasmid (pTHA43) complexed with DMRIE. The DT-A frameshift mutant plasmid (pTHA44) and the pRSVLUC plasmid were used as controls. HIV-1 expression and replication, as measured by p24 production on days 2 and 4, was completely inhibited in cells co-transfected with HXB Δ Bgl and pTHA43 plasmids. The amounts of p24 antigen determined in the culture supernatants of HeLa cells co-transfected with HXB Δ Bgl and pTHA44 or pRSVLUC were similar (Table 1). Co-transfection with HXB Δ Bgl and any of the three plasmids as well as treatment with DMRIE alone reduced cell viability by about 20–30% on day 4 post-transfection (data not shown). The results indicated that HeLa cells could efficiently express HIV-regulated DT-A after transfection with cationic liposomes. The subpopulation of cells transfected with HXB Δ Bgl and pTHA43 did not produce any detectable viral p24, while cells transfected with HXB Δ Bgl together with either of the control plasmids, pTHA44 or pRSVLUC, produced significant amounts of p24.

3.3. Delivery of HIV-DT-A to cells chronically infected with HIV

If the HIV-regulated DT-A gene can be expressed following transfection by cationic liposomes and is upregulated in infected cells by HIV-encoded Tat and Rev regulatory proteins, we hypothesized that HIV-infected cells may be targeted for destruction by DT-A. Chronically infected HeLa/LAV cells and uninfected HeLa cells were transfected with the HIV-regulated DT-A plasmid (pTHA43), the DT-A frameshift mutant plasmid (pTHA44) or pRSVLUC plasmid complexed with LipofectAMINETM (LFA). Transfection with any of the three plasmids inhibited virus production in HeLa/LAV cell culture supernatants by \sim 50% (Fig. 2A) and reduced cell viability by \sim 30–40%. Treatment with LFA alone reduced the viability of HeLa/LAV cells by \sim 15% (Fig. 2B). Thus, the observed inhibition of viral production could be attributed primarily to non-specific toxicity. Under the same experimental conditions, the viability of uninfected HeLa cells was reduced by \sim 30%, after transfection with any of the three plasmids. The viability was reduced by 20% following treatment with LFA alone (data not shown).

Inhibition of p24 production was also observed when HeLa/LAV cells were transfected with pTHA43, pTHA44 or pRSVLUC plasmids complexed with DMRIE, although the overall toxicity was lower by approximately 20% compared to that of

Table 1
p24 production (ng/ml) in HeLa cells co-transfected with HXB Δ Bgl and pTHA43, pTHA44 or pRSVLUC plasmids^a

Plasmids	Quantity of DNA (μ g)	p24 (ng/ml)	
		Day 2	Day 4
HXB Δ Bgl + pTHA43	0.5/0.5	0	0
	1.0/1.0	0	0
HXB Δ Bgl + pTHA44	0.5/0.5	0.25 \pm 0.10	1.34 \pm 0.15
	1.0/1.0	0.57 \pm 0.07	3.62 \pm 0.42
HXB Δ Bgl + pRSVLUC	0.5/0.5	0.17 \pm 0.03	0.75 \pm 0.17
	1.0/1.0	0.70 \pm 0.12	3.50 \pm 0.23

^a p24 production (ng/ml) is shown on days 2 and 4 following co-transfection of HeLa cells with either 0.5 μ g or 1.0 μ g each of HXB Δ Bgl and pTHA43, pTHA44 or pRSVLUC plasmids complexed with DMRIE/DOPE (8 μ l/500 μ l of Opti-MEM medium), at a lipid:DNA (w/w) ratio of 8:1 or 4:1. The charge ratio (+/-) for the 8:1 and 4:1 complex was 2.0 and 1.0, respectively. Day 1 was omitted because all samples were negative for p24 antigen. The transfection procedure was performed as described in Section 2. Data represent the mean \pm standard deviation obtained from triplicate wells. Results are from one experiment and are representative of two separate experiments.

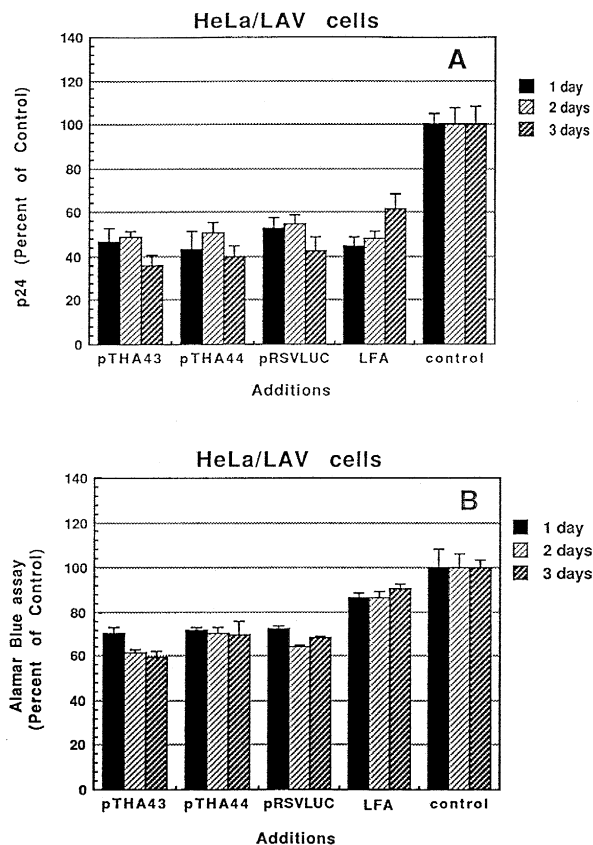


Fig. 2. Transient transfection of HeLa/LAV cells with HIV-DT-A did not have a specific effect on virus production. HeLa/LAV cells were transfected with 2 μ g of pTHA43, pTHA44 or pRSVLUC complexed with 6.25 μ M LFA (5 μ l/400 μ l Opti-MEM medium), at a lipid:DNA (w/w) ratio of 5:1 (the charge ratio (+/-) = 6.25), as described in Section 2. (A) The production of p24 antigen within a 24 h period was determined in culture supernatants on days 1, 2 and 3 post-transfection and was expressed as percent of control (100%: 68.8 \pm 3.6, 162.5 \pm 11.7 and 203.8 \pm 16.9 ng p24/ml on days 1, 2 and 3, respectively). Data represent the mean \pm standard deviation obtained from triplicate wells (n = 6). (B) Cell viability was measured on days 1, 2 and 3 post-transfection (incubation with Alamar Blue for 1 h 30 min at 37°C), and was expressed as percent of the control. Data represent the mean \pm SD obtained from triplicate wells. Results are from one experiment and are representative of three separate experiments.

LFA. Under the same experimental conditions, transfection of uninfected HeLa cells with any of the three plasmids complexed with DMRIE was not cytotoxic. In addition, DMRIE alone had no detectable toxic effects on either HeLa/LAV or HeLa cells (data not shown).

One likely explanation for the lack of inhibition of p24 production in HeLa/LAV cells by pTHA43 plasmid is that the efficiency of transfection was too low to affect significantly the level of p24 antigen in culture supernatants of cells normally producing high quantities of virus. Even if HIV-regulated DT-A expression could cause the death of a small portion of

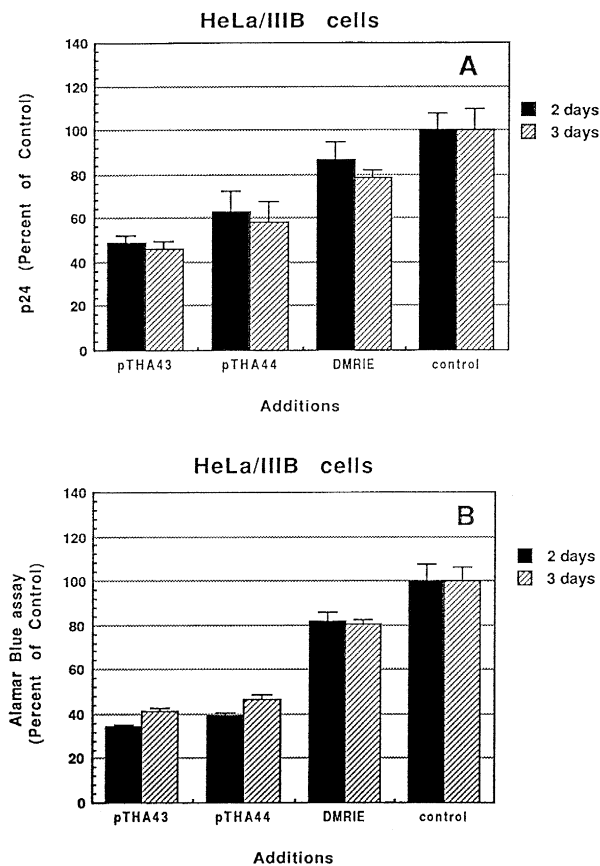


Fig. 3. Transient transfection of HeLa/IIIB cells with HIV-DT-A did not have a specific effect on virus production. HeLa/IIIB cells were transfected with 2 μ g of pTHA43 or pTHA44 complexed with 12 μ M DMRIE (8 μ l/500 μ l Opti-MEM medium), at a lipid:DNA (w/w) ratio of 4:1 (the charge ratio (+/-) = 1.0), as described in Section 2. (A) p24 production within a 24 h period was determined in culture supernatants on days 2 and 3 post-transfection, and was expressed as percent of control (100%: 1.2 \pm 0.1 and 1.74 \pm 0.16 ng p24/ml on days 2 and 3, respectively). Data represent the mean \pm standard deviation obtained from duplicate wells (n = 4). (B) Cell viability was measured on days 2 and 3 post-transfection (incubation with Alamar Blue for 2 h at 37°C), and was expressed as percent of the control. Data represent the mean \pm standard deviation obtained from duplicate wells. Values shown are from one experiment.

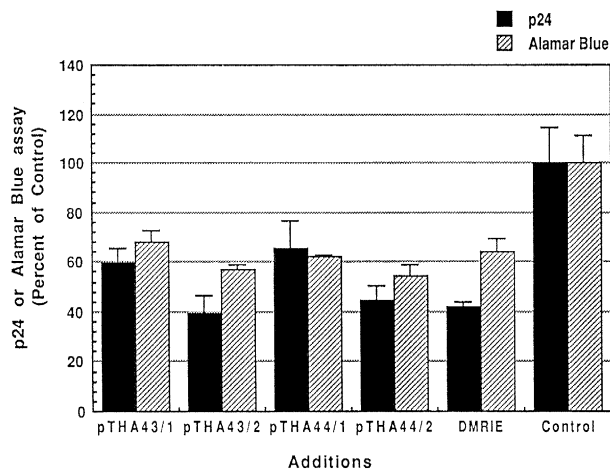


Fig. 4. Virus production after de novo infection of HeLa CD4⁺ cells transfected with HIV-DT-A. HeLa CD4⁺ cells were transfected with 1 or 2 μ g of pTHA43 or pTHA44 complexed with 12 μ M DMRIE (8 μ l/500 μ l Opti-MEM medium), at a lipid:DNA (w/w) ratio of 8:1 (the charge ratio (+/-) = 2.0) or 4:1 (the charge ratio (\pm) = 1.0). The cells were infected 24 h later with HIV-1_{IIIB} at 5×10^3 TCID₅₀ per well, as described in Section 2. One and 2 days post-infection, the culture medium was replaced with fresh medium, and 5 days post-infection the culture supernatants were analyzed for viral p24. The p24 antigen production within the previous 72 h period was expressed as percent of the control (100%: 0.21 ± 0.05 ng p24/ml). Cell viability was measured on day 5 post-infection (incubation with Alamar Blue for 2 h 40 min at 37°C) and was expressed as percent of the control. Data represent the mean \pm SD obtained from duplicate wells or quadruplicate control wells. Values shown are from one experiment and are representative of two separate experiments.

HeLa/LAV cells, without causing non-specific toxicity, the high level of virus production by cells not expressing the DT-A gene did not allow for the detection of any reduction in p24 levels. We therefore examined the effect of transfection on virus produc-

tion by a cell line producing much lower levels of virus.

HeLa/IIIB cells developed in our laboratory after infection of HeLa CD4⁺ cells with HIV-1_{IIIB}, produce 50 to 100-fold lower levels of p24 antigen than HeLa/LAV cells. Transfection of HeLa/IIIB cells with the pTHA43 plasmid complexed with DMRIE did not reduce viral p24 production in a specific manner. The production of p24 antigen in the culture supernatant was inhibited by $\sim 50\%$ and 60% after transfection with pTHA43 and pTHA44 plasmids, respectively (Fig. 3A). Transfection with either plasmid reduced the viability of HeLa/IIIB cells by $\sim 60\%$, while treatment with DMRIE alone reduced viability by $\sim 20\%$ (Fig. 3B).

3.4. Transfection of HIV-DT-A into HeLa CD4⁺ cells before infection

In contrast to data shown in Table 1, the data presented above (Figs. 2 and 3) show that expression of pTHA43, delivered using cationic liposomes, did not specifically reduce p24 production in chronically infected HeLa cells. Therefore, we next examined if p24 production could be reduced during de novo infection of previously transfected cells. HeLa CD4⁺ cells were transfected with pTHA43 or pTHA44 plasmid complexed with DMRIE (as before), and on the next day the cells were infected with HIV-1_{IIIB}. The production of p24 antigen was inhibited by $\sim 40\%$ and 60% after transfection with either of the two plasmids, at 1 μ g or 2 μ g, respectively (Fig. 4). Transfection with either plasmid or treatment with DMRIE alone reduced the viability of infected HeLa CD4⁺ cells by $\sim 40\%$ (Fig. 4).

Table 2

p24 production, luciferase activity, and β -gal activity in HeLa cells co-transfected with HXB Δ Bgl and pCMV.SPORT- β gal or pRSVLUC plasmids, and pCMV.SPORT- β gal and pRSVLUC plasmids^a

	HXB Δ Bgl + pCMV.SPORT- β gal	HXB Δ Bgl + pRSVLUC	pCMV.SPORT- β gal + pRSVLUC
p24 (ng/ml)	0.25 ± 0.01	0.29 ± 0.04	0
Luciferase (cpm)	0	$6.8 \times 10^4 \pm 1.9 \times 10^4$	ND
β -gal positive cells (%)	3.6	0	6.5

^a p24 production, luciferase activity and transfection efficiency are shown 2 days after co-transfection of HeLa cells with 1.0 μ g each of HXB Δ Bgl and pCMV.SPORT- β gal or pRSVLUC plasmids, or pCMV.SPORT- β gal and pRSVLUC plasmids, complexed with 12 μ M DMRIE/DOPE (8 μ l/500 μ l of Opti-MEM medium), at a lipid:DNA (w/w) ratio of 4:1 (charge ratio (+/-) = 1.0). The transfection procedure was performed as described in Section 2. Data represent the mean \pm standard deviation obtained from duplicate wells. Values shown are from one experiment and are representative of two separate experiments. ND: not determined.

3.5. Efficiency of transfection

HeLa cells were co-transfected with the HIV proviral clone, HXB Δ Bgl, together with the pCMV.SPORT- β gal plasmid or the pRSVLUC plasmid complexed with DMRIE. As a control for co-expression of β -galactosidase, the pCMV.SPORT- β gal plasmid was also co-transfected with the pRSVLUC plasmid. HIV-1 expression and replication (as measured by p24 production), luciferase activity and the efficiency of transfection were determined after 2 days. The amounts of p24 antigen determined in the culture supernatants of HeLa cells co-transfected with HXB Δ Bgl and pCMV.SPORT- β gal or pRSVLUC were similar (Table 2). The efficiency of transfection was determined by scoring the percentage of cells positive for β -galactosidase staining. Co-transfection with pCMV.SPORT- β gal and HXB Δ Bgl or pRSVLUC, yielded 3.6% or 6.5% of cells expressing β -gal, respectively (Table 2). Additional experiments utilizing pCMV.SPORT- β gal and other control plasmids also indicated that the efficiency of transfection was similarly low (data not shown).

4. Discussion

In this study, we investigated whether HIV-regulated luciferase and DT-A genes are expressed in HIV-infected or HIV-expressing HeLa cells following transfection by cationic liposomes, and whether transient expression of the DT-A gene could affect p24 production in chronically or acutely infected HeLa cells. Our results demonstrate that (i) expression of the HIV-regulated luciferase gene is specifically *trans*-activated in HeLa/LAV cells; (ii) co-transfection of HeLa cells with HIV-DT-A and the HIV proviral clone completely inhibits virus production; (iii) expression of HIV-DT-A in chronically infected HeLa/LAV or HeLa/IIIB cell cultures does not specifically reduce p24 production; (iv) transient expression of HIV-DT-A in HeLa CD4⁺ cell cultures does not protect against *de novo* HIV infection; (v) transfection by cationic liposomes reduces the viability of the cell culture; (vi) the observed reductions in p24 levels can be ascribed primarily to non-specific toxicity due to the transfection procedure; and (vii) the results in (iii) and (iv) can be explained by the low efficiency of lipofection in HeLa cells.

The introduction of a gene encoding a lethal product, e.g. DT-A, linked with regulatory elements that respond specifically to viral *trans*-activating proteins, could mediate the killing of HIV-infected cells at an early stage in the viral infectious cycle. For a highly toxic molecule such as DT-A, expression must be restricted to HIV-infected cells, as even low-level expression of the toxin in uninfected cells would be detrimental. Since cationic liposomes appear to be a relatively safe and non-immunogenic alternative to virus-mediated gene delivery for gene therapy *in vivo* [18–22], we examined whether cationic liposomes could be used to deliver HIV-DT-A into HIV-infected cells. In contrast to retroviral or adeno-associated virus (AAV)-based gene deliveries, liposome-mediated transfection (like other non-viral systems) is expected to result in a short transient expression of the heterologous gene due to the episomal nature of the transfected plasmid [36]. However, even low transient expression of an inducible suicide DT-A gene in an HIV-infected cell would be expected to result in cell death.

HeLa cells were selected in our study because they are relatively easy to transfect and are frequently used in studies of transient and stable transfections. Moreover, chronically HIV-infected HeLa cells are available, and HeLa cells expressing the CD4 molecule are infectable with HIV. In addition, HeLa cell clones stably expressing HIV-DT-A sequences produce less HIV, by p24 assay, than parental HeLa cells, after transfection with an HIV proviral clone using electroporation [6]. In our initial experiments we attempted to determine the level of luciferase expression under HIV control in HIV-infected monocytic and T lymphocytic cell lines. The level of expression in these cell lines was very low, and in some cases, difficult to reproduce.

Whether actual HIV infection could activate HIV-regulated luciferase has not been demonstrated previously. The expression of luciferase from the HIV-regulated luciferase gene (pLUCA43) was induced by the HIV proviral clone, HXB Δ Bgl, in HeLa cells transiently co-transfected by electroporation with pLUCA43 and HXB Δ Bgl [6]. DT-A expression from the HIV-DT-A (pTHA43) plasmid was assayed indirectly in transiently or stably transfected HeLa cells [5,6]. HeLa cells were transiently co-transfected by electroporation with pRSVLUC, and pTHA43 with or

without pH3tat + pH3art plasmids (expression plasmids for Tat and Rev, respectively). In the presence of both Tat- and Rev-encoding plasmids, the expression of luciferase was inhibited by 70%. The authors suggested that *trans*-activation of DT-A expression from pTHA43 by Tat + Rev results in inhibition of luciferase (presumably at the translational level) [5]. The decreased expression of a luciferase reporter gene was also observed in HeLa cell clones containing integrated HIV-DT-A sequences after co-transfection by electroporation with pRSVLUC and Tat- and Rev-encoding plasmids [6].

Since liposomes are approved for clinical trials [23], we examined the potential of using this delivery system for eventual gene therapy *in vivo*. In this study we wanted to establish whether the HIV-regulated luciferase gene (pLUCA43) or HIV-DT-A could be activated by an actual HIV infection, e.g. in chronically HIV-infected cells. We demonstrated for the first time that (i) the HIV-regulated luciferase was expressed at a significantly higher level in HIV-infected cells than in uninfected HeLa cells, and (ii) HIV-DT-A genes were expressed specifically in HIV-expressing HeLa cells following lipofection.

The first question raised by our results is why expression of HIV-DT-A did not confer protection against HIV infection. In order to be transcribed, transfected genes must be delivered into the cell, and ultimately to the nucleus in functional form. Our results indicate that, although transfection with cationic liposomes results in expression of HIV-regulated genes in HeLa cells, the percentage of transfected cells is too low to inhibit virus production in HIV-infected cells or to protect against *de novo* HIV infection. The low expression can be explained by the inefficient entry of the lipid-DNA complex into the cell, inefficient entry of the DNA into the nucleus or degradation of the DNA.

The precise mechanism(s) by which lipid-DNA complexes interact with the cell membrane are unknown. It has been suggested that the majority of DNA complexed to cationic liposomes is taken up through endocytosis, followed by its release from an early endosomal compartment (possibly mediated by the destabilization of the endosome membrane by the cationic lipids and DOPE) [37–40]. Since fusion of the endocytotic vesicles with lysosomes would lead to degradation of the DNA, the DNA must enter the

cytoplasm before this fusion event for successful transfection to occur. Recently, Xu and Szoka [41] have suggested that the endocytosed lipid-DNA complex destabilizes the endosomal membrane and induces flip-flop of anionic lipids located predominantly on the cytoplasmic face of the membrane. The diffusion of these lipids into the lipid-DNA complex was proposed to result in the displacement of the DNA from the complex and release of the DNA into the cytoplasm. It has also been proposed that liposome-mediated transfection involves translocation of DNA through pores across the membranes rather than delivery via fusion or endocytosis [42]. Our previous studies have shown that DOTMA liposomes can fuse with T lymphocytic cells [43]. More recent studies indicate that DOTAP liposomes and their complexes with DNA can undergo extensive fusion with the plasma membrane of monocytic cells (P. Pires, S. Simões, N. Düzgüneş and M.C. Pedroso de Lima, unpublished data). Very little is known about the mechanism by which DNA is transferred into the nucleus, except that only a small percentage of the cytoplasmically delivered DNA reaches the nucleoplasm [44–46]. Therefore, further studies are needed to understand the mechanism(s) by which DNA complexed to cationic liposomes is delivered into the nucleus and how these events are related to the efficiency of transfection.

The low efficiency of transfection obtained in our study suggests that additional methods need to be employed for the efficient delivery of the DT-A gene to HIV-infected cells, either by significantly improving transfection procedures, e.g. by using transferrin-cationic liposome complexes [47], or by appropriately altering the transfection vectors. It has been shown recently that high efficiency of lipofection and long-term expression of heterologous genes can be achieved by using plasmids containing the inverted terminal repeats (ITRs) from AAV [48,49]. The authors of these studies speculate that the presence of the ITRs facilitates the nuclear accumulation of the transfected plasmid. Episomal self-replicating plasmids contain a viral origin of DNA replication and a virally encoded early gene that *trans*-activates the viral DNA origin of replication, allowing for episomal replication in the transfected host cell [50]. It has been shown recently, using a liposome delivery system, that a construct containing an episomal DNA

vector of the human papovavirus BKV and a luciferase reporter gene yields widespread and long-lasting expression of luciferase in mice [51]. The use of some of these altered vectors may enhance the efficacy of HIV-DT-A gene expression and may confer protection against HIV infection. Targeting cationic liposome-DNA complexes to HIV-infected cells by covalently coupling anti-gp120 or anti-gp41 antibodies or soluble CD4 [52] to liposomes may be an alternative strategy to enhance gene delivery and expression. The possible immunogenicity of such targeting constructs [53,54] may be a complicating factor in their use in vivo. Nevertheless, strategies to reduce the immunogenicity of targeting ligands, such as the use of human monoclonal Fab fragments or synthetic peptides [55] may be developed. In a different targeting strategy, efficient transfer of the pCMV-luciferase gene to HepG2 hepatoma cells was obtained with lipopolyamine-condensed DNA particles presenting galactose ligands that recognize the asialoglycoprotein receptor on these cells [56].

The next question raised by our findings is the relatively high toxicity of the lipofection procedure to HeLa cells. In our study, uninfected CD4⁻ or CD4⁺ HeLa cells and infected HeLa/LAV or HeLa/IIIB cells were exposed to lipid-DNA complexes, or lipids alone, in Opti-MEM medium without serum, for 4 h at 37°C. Our results demonstrate that (i) cells differ in their sensitivity to the toxic effect of cationic liposomes, (ii) the formation of complexes between cationic liposomes and DNA increases the toxic effect of liposomes, and (iii) in HeLa/LAV cells, virus production is more sensitive to the toxic effects of liposome-DNA complexes or liposomes alone than is the overall viability of the cells as measured by a metabolic assay.

Both cationic liposomes alone and liposome-DNA complexes show various levels of growth inhibition and toxicity to cells in vitro, depending on the cell type and the confluency of the cell culture [15,26,35,57–62]. The molecular and cellular basis of this variability is not known. In contrast to the high toxicity observed in vitro, very low toxicity was reported following administration of various liposome-DNA complexes to animals [63–67].

Nevertheless, a proper comparison of toxicities in vitro and in vivo would have to involve the use of much higher concentrations of serum or plasma in

cell culture experiments. These conditions, however, are likely to inhibit the transfection activity and efficiency of liposome-DNA complexes. It is also likely that the interaction of the complexes with plasma components in vivo inhibits their toxic effects. It is interesting to note that under the same conditions cationic liposomes alone, either LFA or DMRIE, are less toxic than their complexes with DNA. These results confirm our previous observations [35] that the concentration of net positive charge per se is not a determinant of toxicity. Cytotoxicity is more likely to be associated with the structure of the cationic molecules and their complexes with DNA, their interactions with the cell membrane and their ability to be metabolized by the cell.

Although our studies have focused on HIV-controlled DT-A expression, we are aware of the potential limitations of toxin gene therapy in vivo. Nevertheless, the efficient and stable delivery of this gene therapy system by lipofection can be translated readily to other therapeutic strategies, such as those utilizing anti-HIV ribozyme genes [68–70].

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