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Delivery of an anti-HIV-1 ribozyme into HIV-infected cells via cationic liposomes

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Abstract

Cationic liposome-mediated intracellular delivery of a fluorescein-labeled chimeric DNA–RNA ribozyme targeted to the HIV-1 5′ LTR was investigated, using THP-1, THP-1/HIV-1_{IIIB} or HeLa/LAV cells. Different fluorescence patterns were observed when the cells were exposed to Lipofectamine, Lipofectin or DMRIE:DOPE (1:1) complexed to the ribozyme. With Lipofectamine intense cell-associated fluorescence was found. Incubation with Lipofectin resulted in less intense diffuse fluorescence, while with DMRIE an intense but sporadic fluorescence was observed. Differentiated THP-1/HIV-1_{IIIB} cells were more susceptible to killing by liposome–ribozyme complexes than THP-1 cells. Under non-cytotoxic conditions (a 4-h treatment) complexes of 5, 10 or 15 μM Lipofectin or DOTAP:DOPE (1:1) and ribozyme, at lipid:ribozyme ratios of 8:1 or 4:1, did not affect p24 production in THP-1/HIV-1_{IIIB} cells in spite of the intracellular accumulation of the ribozyme. A 24-h exposure of THP-1/HIV-1_{IIIB} cells to 5 μM Lipofectin or DOTAP:DOPE (1:1) complexed with either the functional or a modified control ribozyme reduced virus production by approximately 30%. Thus, the antiviral effect of the liposome-complexed ribozyme was not sequence-specific. In contrast, the free ribozyme at a relatively high concentration inhibited virus production by 30%, while the control ribozyme was ineffective, indicating a sequence-specific effect. Both Lipofectin and DOTAP complexed with ribozyme were toxic at 10 and 15 μM after a 24-h treatment. A 4-h treatment of HeLa/LAV cells with Lipofectin at 5, 10 or 15 μM was not toxic to the cells, but also did not inhibit p24 production. In contrast, treatment of HeLa $CD4^+$ cells immediately after infection with HIV-1_{IIIB} at the same lipid concentrations and lipid:ribozyme ratios was cytotoxic. Our results indicate that the delivery of functional ribozyme into cells by cationic liposomes is an inefficient process and needs extensive improvement before it can be used in ex vivo and in vivo applications.

Keywords: Human immunodeficiency virus (HIV); Ribozyme; Cationic liposome; THP-1 cell; HeLa $CD4^+$ cell

Abbreviations: DME-HG, Dulbecco's Modified Eagle's Medium, high glucose; FBS, heat-inactivated fetal bovine serum; DOPE, dioleoylphosphatidylethanolamine; DMRIE, a 1:1 (w/w) mixture of 1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide (DMRIE) and DOPE; DOTAP, a 1:1 (w/w) mixture of *N*-(1-(2,3-dioleoyloxy)propyl-*N*,*N*,*N*-trimethylammonium propane) (DOTAP) and DOPE; LF, Lipofectin reagent; LFA, LipofectAMINE™ reagent; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate

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1. Introduction

Ribozymes (Rz) are RNA molecules capable of catalytically cleaving specific phosphodiester bonds in complementary RNA molecules [1–4]. When delivered intracellularly, they can interfere with both pre-integration and post-integration events of the HIV replication cycle, by cleaving incoming viral RNA and transcribed mRNAs [5]. Delivery of Rz to cells has been attempted by (i) endogenous expression of the Rz-encoding gene or (ii) exogenous delivery of chemically-synthesized (pre-formed) Rz targeted to highly conserved regions of the virus [6].

Chang et al. [7] have demonstrated that an anti-HIV-1 *gag* Rz can be expressed under the control of the constitutive human β-actin promoter in HeLa $CD4^+$ cells. The stable expression of this anti-*gag* Rz in HeLa $CD4^+$ cells reduces *gag* transcripts and p24 levels upon infection with HIV-1 [1]. Since then, many of the genetic elements of the retroviral genome have been targeted including functional proteins, leader sequences and regulatory protein sequences [2,8]. A hairpin Rz designed to cleave HIV-1 RNA in the 5′ leader sequence suppresses virus expression in HeLa cells cotransfected with proviral DNAs [9,10]. Human T-cell lines, primary T cells and $CD34^+$ hematopoietic stem/progenitor cells transduced with retroviral vectors containing Rz were shown to be resistant to challenge with diverse strains of HIV-1, including uncloned clinical isolates [11–16].

A number of problems need to be addressed before pre-formed Rz can be used successfully for therapy: (i) stabilization of the Rz against serum and cellular nucleases without compromising its catalytic activity; (ii) efficient delivery to the target cells; (iii) intracellular localization of an active Rz; (iv) co-localization of the Rz with its mRNA target inside cells. In contrast to endogenous expression, exogenous delivery permits the use of chemically modified Rz (MRz) which are more resistant to degradation by nucleases, while maintaining cleavage capability [17,18]. Chimeric DNA–RNA hammerhead Rz, with DNA in helices I and III, have an increased resistance to nucleases and a six-fold greater catalytic activity than an analogous all-RNA Rz [19].

Several cationic liposome formulations have been developed to facilitate delivery of DNA [20–23], mRNA [24] and antisense oligonucleotides into eukaryotic cells [25–30]. In the present study we evaluated the intracellular delivery and anti-HIV effect of a fluorescein-labeled chimeric DNA–RNA Rz targeted to the HIV-1 5′ LTR, using four cationic liposomal preparations. Lipofectamine, Lipofectin, and liposomes composed of either DMRIE:DOPE [(a 1:1 (w/w) mixture of 1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethylammonium bromide (DMRIE) and DOPE):(dioleoylphosphatidylethanolamine)] (1:1, w/w) or DOTAP:DOPE [*N*-(1-(2,3-dioleoyloxy)propyl-*N*,*N*,*N*-trimethylammonium propane)] (1:1, w/w), were complexed with the Rz and added to cultures of differentiated monocytic THP-1 cells and chronically infected THP-1/HIV-1_{IIIB} cells, as well as chronically and de novo infected HeLa $CD4^+$ cells. Differentiated THP-1 and THP-1/HIV-1_{IIIB} cells were used in our study because they represent a convenient model of monocyte-derived macrophages [31] and chronically infected macrophages [32,33], respectively. Because HeLa cells are often used as a model system for cationic liposome-mediated DNA delivery, chronically infected HeLa/LAV cells and de novo infected HeLa $CD4^+$ cells were also included in our study. Some of our results have been presented earlier in preliminary form [34,35].

2. Materials and methods

2.1. Materials

LipofectAMINE™ reagent and Lipofectin reagent were obtained from GIBCO BRL Life Technologies, (Gaithersburg, MD). LipofectAMINE™ reagent [36] contains the polycationic lipid 2,3-dioleyloxy-*N*[2(sperminecarboxamido) ethyl]-*N*,*N*-dimethyl-1-propanaminium trifluoroacetate (DOSPA) and the neutral lipid, DOPE (3:1, w/w). Lipofectin reagent [20] contains the monocationic lipid *N*-[1-(2,3 dioleyloxy)-propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) and DOPE (1:1, w/w). DMRIE reagent [37] was synthesized by VICAL (San Diego, CA). Small unilamellar liposomes composed of DOTAP and DOPE (1:1, w/w) were prepared in our laboratory [38]. Alamar Blue dye was purchased from Alamar Biosciences (Sacramento, CA). DOTAP and DOPE were obtained from Avanti Polar Lipids (Alabaster, AL). Penicillin, streptomycin and L-

glutamine were obtained from Irvine Scientific (Santa Ana, CA). PBS was obtained from Zymed Laboratories (South San Francisco, CA).

A 38-mer, 5′ fluorescein-labeled, chimeric DNA–RNA hammerhead Rz with two phosphorothioate linkages at the 3′ terminal (mol. wt. 12,519.2), was targeted to the HIV-1 5′ LTR. The catalytic activity of the Rz was first tested in a cell-free system. A control Rz with a scrambled sequence in the binding arms was also designed and synthesized (MRz). The sequences of these Rz are as follows:

Rz: 5′-ACACAACAcugaugaGTCCGTGAG-GACgaaaCGGGC∗A∗C-3′

MRz: 5′-CAAACAACcugaugaGTCCGTGAG-GACgaaaACCGG∗G∗C-3′

where the '∗' indicates a phosphorothioate internucleotide linkage, capital letters represent deoxyribonucleotides and lowercase letters represent ribonucleotides.

The mixed oligodeoxyribo- and ribonucleotides were synthesized on an automated synthesizer (Applied Biosystems 394 RNA/DNA) as described previously [19].

2.2. Cells and virus

THP-1 cells were obtained from the American Type Culture Collection (TIB-202). A chronically HIV-infected cell line (designated THP-1/HIV-1_{IIIB}) was developed in our laboratory by infecting THP-1 cells with HIV-1_{IIIB} at a low multiplicity of infection [31], and the clone designated as THP-$1/_{IIIB30}$ was used in further experiments [39]. Cells were maintained at 37°C, under 5% CO_2 in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated FBS (fetal bovine serum) (Sigma; St. Louis, MO) (RPMI/10), penicillin (100 units/ml), streptomycin (100 μg/ml) and L-glutamine (2 mM). The cells were passaged 1:6 once a week.

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 Wabl [40] and Dr. Richard Axel [41], respectively. HeLa/LAV cells were maintained at 37°C, under 5% CO_2 in DME-HG (Dulbecco's Modified Eagle's Medium, high glucose) (Irvine Scientific) supplemented with 10% FBS (DME/10), penicillin (100 units/ml), streptomycin (100 μg/ml) and L-glutamine (4 mM). HeLa $CD4^+$ cells were grown in RPMI 1640 medium with 10% FBS, penicillin (100 units/ml), streptomycin (100 μg/ml), L-glutamine (2 mM) and 0.6 mg/ml Geneticin (G418) sulfate (GIBCO). The cells were passaged 1:20 once a week. Flow cytometric analysis indicated that over 90% of cells were CD4-positive.

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. Treatment with liposome–Rz complexes

Uninfected monocytic THP-1 cells and chronically infected THP-1/HIV-1_{IIIB} cells (1 ml at 1×10^6 cells/ml) were plated in 48-well plates, treated with 160 nM phorbol 12-myristate 13-acetate (PMA) [37] for 24 h at 37°C, and exposed to liposome–Rz complexes 5 or 6 days post-differentiation. HeLa/LAV cells were plated in 24-well plates (2×10^5 cells/2 ml) and treated after 48 h, at ~60% confluency, in the absence or presence of 8% FBS.

LipofectAMINE™ reagent (LFA) was provided at 0.5 mM (corresponding to 2 mg/ml) and Lipofectin reagent (LF) was provided at 0.68 mM (corresponding to 1 mg/ml). DMRIE/DOPE (referred to as DMRIE in the rest of the paper) was prepared to 0.747 mM in sterile water, corresponding to 1 mg/ml. DOTAP/DOPE (referred to as DOTAP) was prepared at 0.71 mM (corresponding to 1 mg/ml). Lyophilized fluorescent Rz was stored at 4°C, dissolved in 10 mM Tris, 1 mM EDTA, 0.1 M NaCl buffer, pH 8.0, and then diluted in the appropriate medium without serum or antibiotics.

For all experiments, the indicated amounts of lipid and Rz were complexed for 30 min at room temperature in RPMI or DME-HG medium without serum or antibiotics. Meanwhile the cells were washed twice with 1 ml medium, and 0.4 ml of medium was placed on each well. The lipid–Rz mixture was then added gently in a volume of 0.1 ml per well. The mixture remained on the cells for 4 h or 24 h at 37°C in a 5% CO_2 incubator. Cells treated with either medium or

Rz alone served as controls. Following treatment, cells were washed with PBS and viewed by phase contrast and fluorescence microscopy, using a Nikon Diaphot microscope (Nikon Instrument Group; Melville, NY). Cell morphology was evaluated by inverted phase contrast microscopy at 250 × or 400 × magnification. Photographs of fluorescence and phase-contrast fields were made on Fuji color print film at ISO (ASA) 1600 or on Kodak black and white print film at ASA 400. The treated cells were fed every 2–3 days with fresh medium (RPMI/10 for THP-1 and HeLa $CD4^+$ cells, or DME/10 for HeLa/LAV cells) and incubated for the indicated periods of time before the Alamar Blue assays and monitoring of the p24 level in supernatants. p24 values represent the amount of virus produced (in ng/ml) between the time of medium change and the time of p24 determination. This time period is indicated in the figure legends. Results were compared to medium-treated controls. Statistical significance was evaluated by the unpaired Student's *t*-test, using StatView software (BrainPower, Calabasas, CA). p values of ≤ 0.05 were taken to indicate a significant difference.

2.4. HIV infection of HeLa $CD4^+$ cells

The virus and infected cells were handled in a BL-3 facility. HeLa $CD4^+$ cells were plated in 12-well plates at 2×10^5 cells/well, and on the next day, at ~50% confluency, they were exposed to HIV-1_{IIIB} for 2 h at 37°C, at 5×10^3 $TCID_{50}$ per well. Following infection, the cells were washed to remove unbound virus and were treated with liposome–Rz complexes or medium alone. 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 [42], using a Molecular Devices (Menlo Park, CA) V_{max} microplate reader.

The supernatant of chronically infected H9/HTLV-IIIB cells was used as a source of the HIV-1 strain, HIV-1_{IIIB}. 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 μg/ml as determined by ELISA. The tissue culture infectious dose, 50% endpoint ($TCID_{50}$), was determined as described by Johnson and Byington [43]. The infectious titer per 1 μg p24 was 7.1×10^3 $TCID_{50}$.

2.5. Cell viability assay

The number of viable cells used for experiments was determined by Trypan Blue exclusion. Cell viability after treatment with free or liposome-complexed Rz was quantified by a modified Alamar Blue assay [44]. 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 [39].

3. Results

3.1. Delivery of liposome–Rz complexes to uninfected, differentiated THP-1 cells

Differentiated THP-1 cells were exposed to the liposome–Rz complexes for 4 or 24 h in RPMI medium with 8% FBS. The cells were exposed to Lipofectamine at 3 or 8 μM, and to Lipofectin and DMRIE at 15 or 40 μM, complexed with Rz, at a lipid:Rz (w/w) ratio of 1.3:1. Under these conditions, the charge ratio (+/−) for Lipofectamine was 1.7, while for Lipofectin and DMRIE this ratio was about 0.3. Thus, a net positive charge was not absolutely required for the complex to be taken up. Different fluorescence patterns were observed with the three liposomal preparations. With Lipofectamine, intense cell-associated fluorescence was found. Treatment with Lipofectin resulted in less intense diffuse fluorescence, while with DMRIE, an intense but sporadic fluorescence was observed (Figs. 1 and 2). The Rz alone, at concentrations of 12 to 40

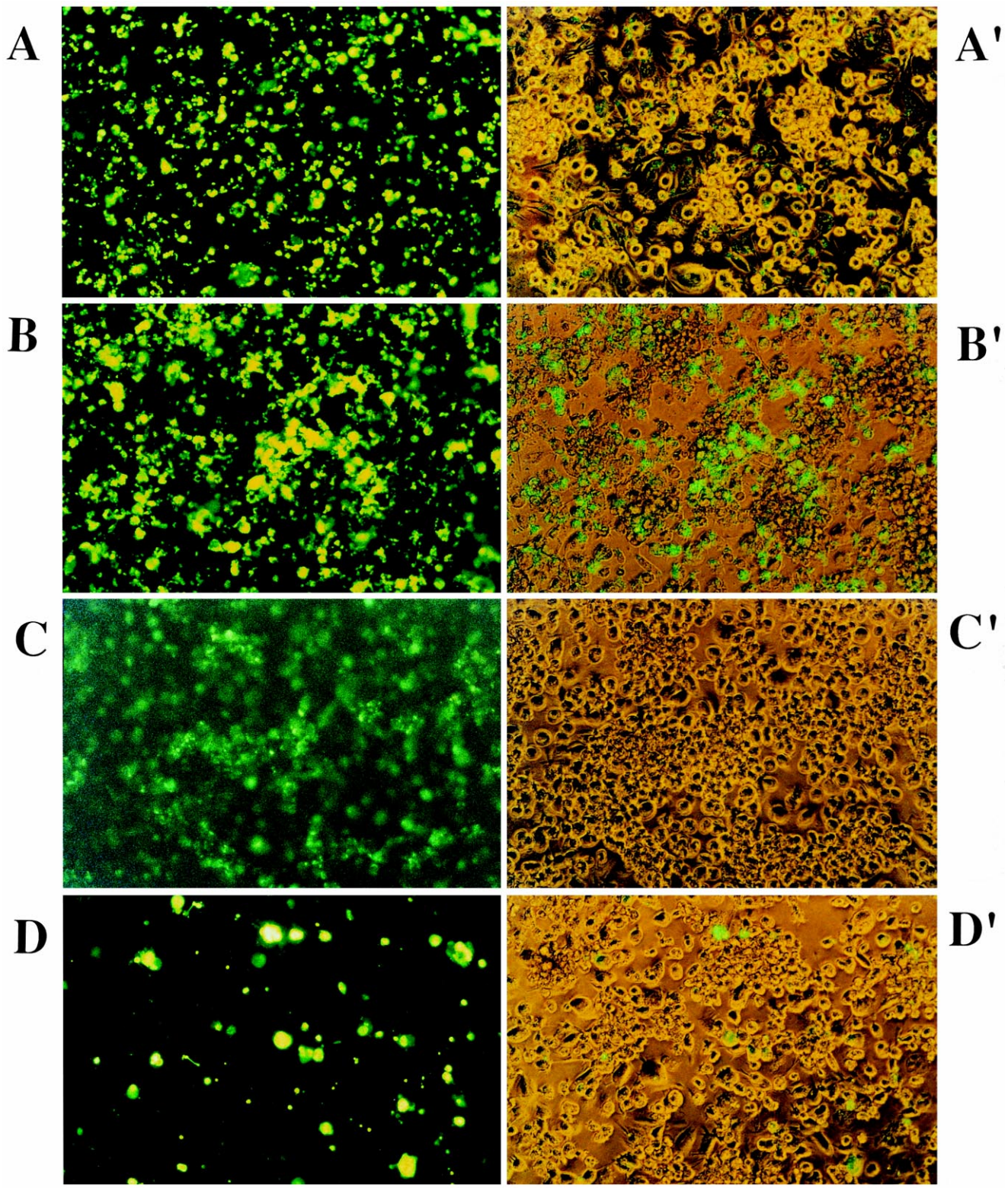

Fig. 1. Fluorescence (A–D) and phase contrast (A′–D′) micrographs of differentiated uninfected THP-1 cells treated for 4 h with LFA at 3 μM (A) or 8 μM (B), and LF (C) or DMRIE (D) at 40 μM, complexed with Rz, at a lipid:Rz (w/w) ratio of 1.3:1.

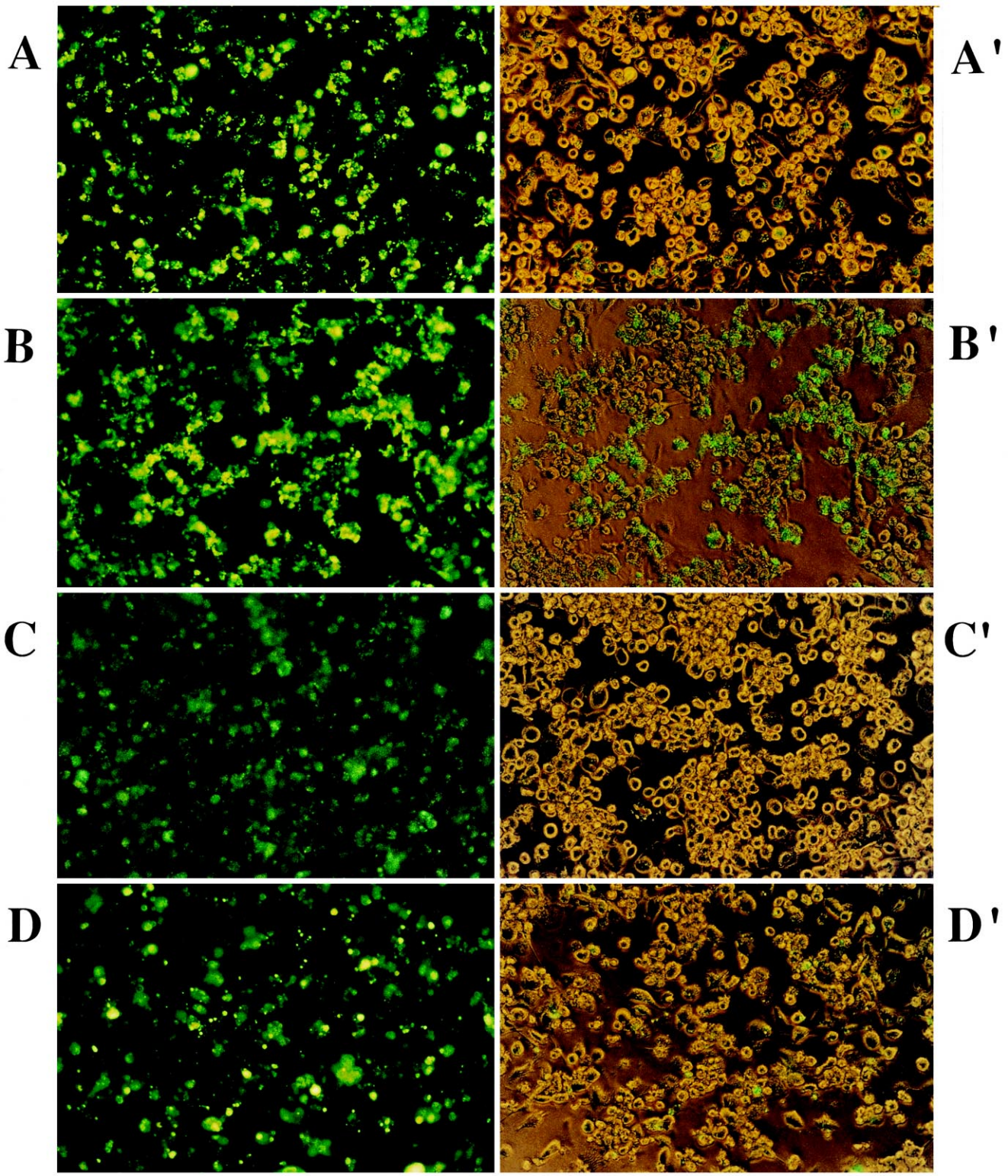

Fig. 2. Fluorescence (A–D) and phase contrast (A′–D′) micrographs of differentiated uninfected THP-1 cells treated for 24 h with LFA at 3 μM (A) or 8 μM (B), and LF (C) or DMRIE (D) at 40 μM, complexed with Rz, at a lipid:Rz (w/w) ratio of 1.3:1.

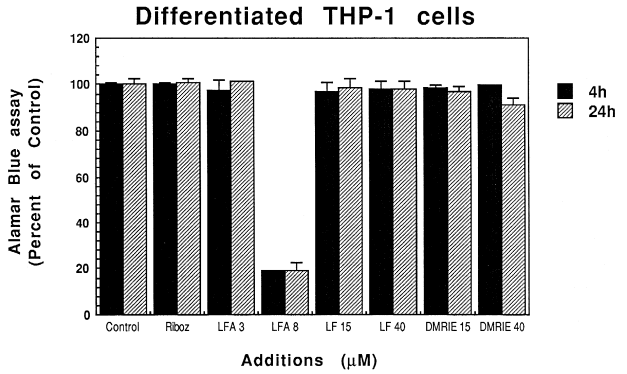

Fig. 3. Effect of LFA, LF or DMRIE complexed with Rz on the viability of differentiated, uninfected THP-1 cells. PMA-treated THP-1 cells, 5 days post-differentiation, were exposed to the liposome–Rz complexes (LFA: Lipofectamine, LF: Lipofectin) or the Rz alone (40 μg/ml = 3.2 μM) for 4 or 24 h (see Section 2 for details). Cell viability was measured on day 13 for the 4-h treatment, and on day 12 for the 24-h treatment, by incubating the cells with Alamar Blue overnight at 37°C. The viability is expressed as percent of the control. Data represent the mean ± standard deviation obtained from duplicate wells.

μg/ml (0.96 to 3.2 μM), showed no significant cellular accumulation (data not shown). The cells were further incubated and fed every 2–3 days with RPMI/10 medium before cell viability was quantified on days 12 or 13 post-treatment. The only cytotoxic treatment was that with 8 μM Lipofectamine which caused disintegration of the cells (Fig. 3).

3.2. Delivery of liposome–Rz complexes to differentiated THP-1 / HIV-1$_{IIIB}$ cells

Differentiated THP-1/HIV-1$_{IIIB}$ cells were exposed to Lipofectamine at 3 μM, and to Lipofectin or DMRIE at 15 μM, complexed with Rz, at a lipid:Rz (w/w) ratio of 4:1 or 1.3:1. Micrographs were taken following a 4-h treatment in RPMI medium with 8% FBS. The fluorescence patterns obtained with the three liposomal preparations were similar to that described for differentiated THP-1 cells (data not shown). The cells were further incubated and fed every 2 days with fresh RPMI/10 medium; viral p24 production was measured on days 2, 4, 6 and 8. Only 3 μM Lipofectamine was cytotoxic at the ratio of 1.3:1, causing disintegration of the cells and inhibition of p24 production (Fig. 4B). At the ratio of 4:1, the cationic liposome–Rz complexes were not toxic and did not inhibit p24 production (Fig. 4A).

Because transfection with Lipofectin resulted in diffuse cytoplasmic fluorescence further experiments were performed with this lipid as well as with its analog DOTAP which has acyl instead of alkyl chains. Differentiated THP-1/HIV-1$_{IIIB}$ cells were treated with Lipofectin or DOTAP at 5, 10 or 15 μM, complexed with Rz, at a lipid:Rz (w/w) ratio of 8:1 or 4:1, or Rz alone at 5.5 μg/ml (0.44 μM). The

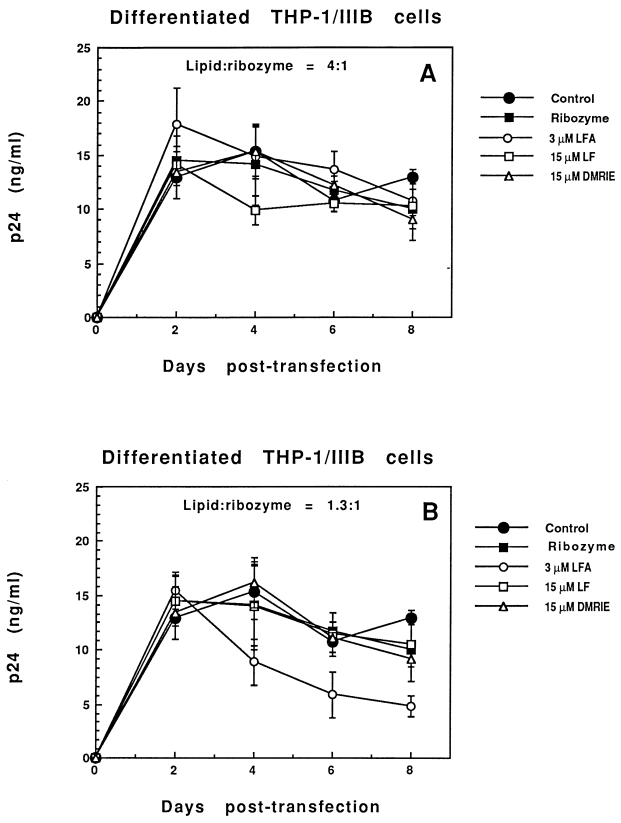

Fig. 4. The effect of Rz, complexed with LFA (3 μM), LF or DMRIE (both 15 μM), on p24 production in differentiated THP-1/HIV-1$_{IIIB}$ cells. Differentiated THP-1/HIV-1$_{IIIB}$ cells in 48-well plates, 6 days post-differentiation, were exposed to the liposome–Rz complexes (LFA: Lipofectamine, LF: Lipofectin), at lipid/Rz ratios of 4:1 (w/w) (A) or 1.3:1 (B), or the Rz alone at 15 μg/ml (1.2 μM), for 4 h (see Section 2 for details). Viral p24 antigen production within the previous 48-h period was determined on days 2, 4, 6 and 8. Data represent the mean ± standard deviation of p24 determinations in duplicate, in supernatants of duplicate wells (n = 4).

charge ratio (+/−) for the 8:1 complex was 1.8 while for the 4:1 complex this ratio was 0.9. Following a 4 or 24-h treatment in RPMI medium with 8% FBS, micrographs were taken. After a 4-h treatment, p24 production was determined on days 3, 5, 7, 9 and 14, and cell viability was measured on day 14. After a 24-h treatment, p24 production was determined on days 2, 4 and 6, and cell viability was measured on day 6. A concentration-dependent, cell-associated diffuse fluorescence was observed after a 4-h treatment. However, neither Lipofectin- nor DOTAP–Rz complexes reduced cell viability or affected p24 production after a 4-h treatment (data not shown).

A 24-h exposure to 5 μM Lipofectin or DOTAP complexed with Rz, at a lipid:Rz (w/w) ratio of 8:1 or 4:1, reduced the p24 levels at day 2 (Fig. 6). Fluorescence patterns observed with DOTAP are shown in Fig. 5. At Lipofectin:Rz ratios of 8:1 or 4:1,

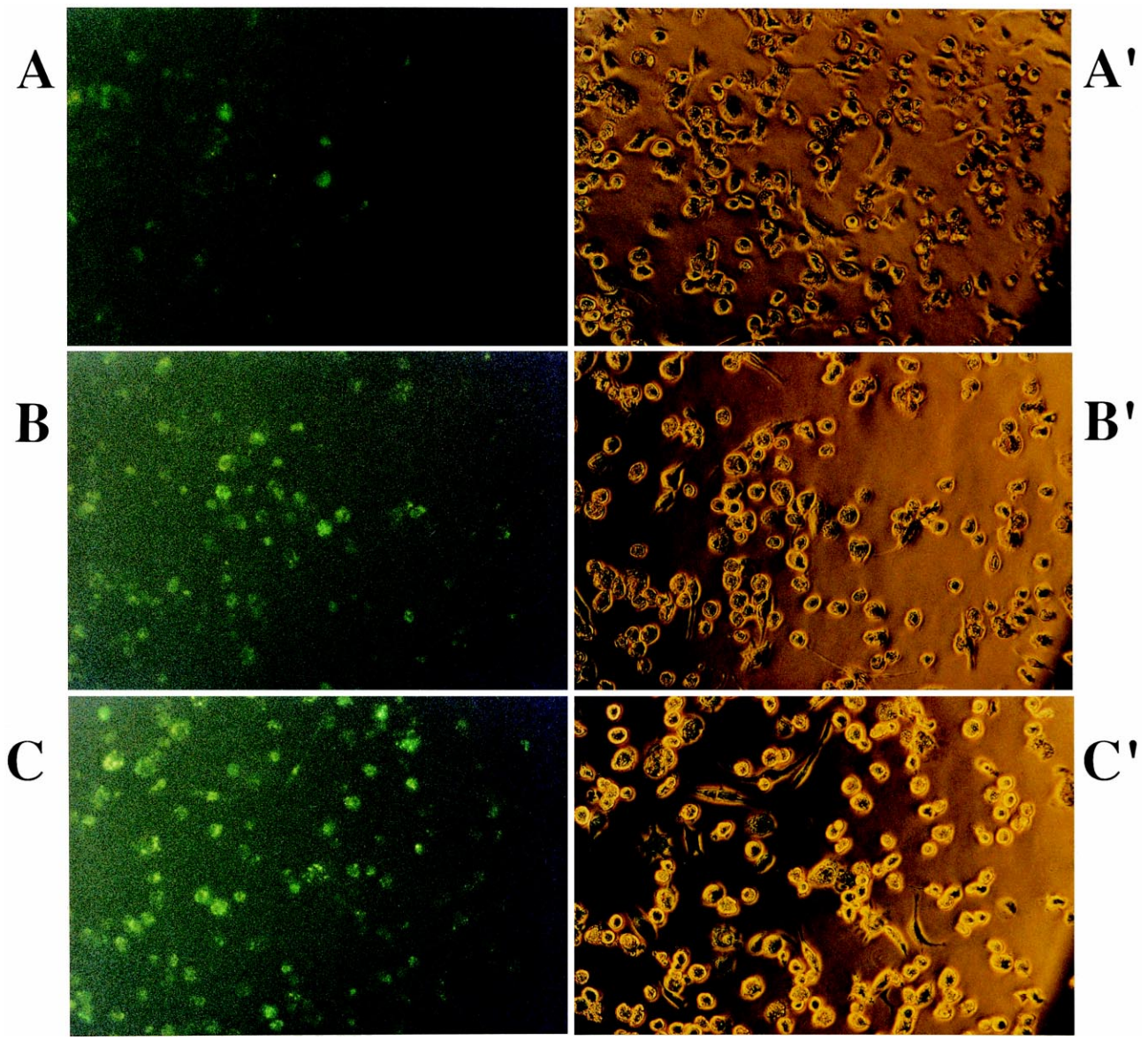

Fig. 5. Fluorescence (A–C) and phase contrast (A′–C′) micrographs of differentiated THP-1/HIV-1_{IIIB} cells treated with free Rz (5.5 μg/ml = 0.44 μM) (A), and 5 μM DOTAP at a lipid:Rz (w/w) ratio of 8:1 (B) or 4:1 (C) for 24 h.

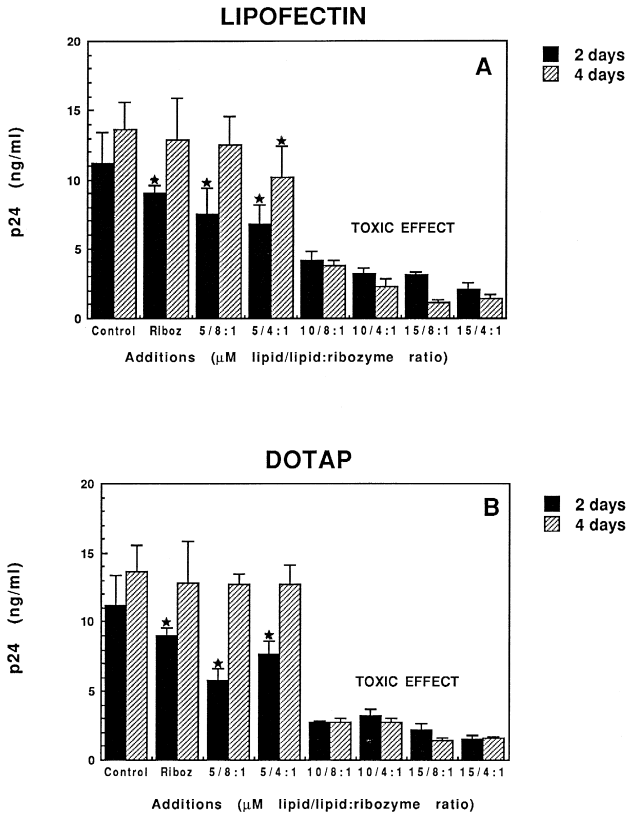

Fig. 6. Effect of LF (A) or DOTAP (B) complexed with Rz on p24 production in differentiated THP-1/HIV-1_{IIIB} cells. PMA-treated THP-1/HIV-1_{IIIB} cells, 6 days post-differentiation, were exposed to the liposome–Rz complexes, at lipid:Rz ratios (w/w) of 8:1 or 4:1, or the Rz alone at 5.5 μg/ml (0.44 μM), for 24 h (see Section 2 for details). The p24 antigen production within the previous 48-h period was measured on days 2 and 4. Data represent the mean ± standard deviation of p24 determinations in duplicate, in supernatants of duplicate wells ($n = 4$) except in controls which were measured in five wells. Asterisks (*) indicate that the values were significantly different from the untreated controls. LF and DOTAP at 10 and 15 μM were cytotoxic.

the p24 levels were reduced by 32.5% or 39.4%, respectively. These values were significantly lower than the untreated controls ($0.005 < p \leq 0.01$, or $p \leq 0.005$, respectively). With the DOTAP:Rz complex at the ratio of 8:1 or 4:1, the p24 levels were reduced by 48.6% or 31.2%, respectively. These values were significantly lower than the untreated controls ($p \leq 0.005$, or $0.005 < p \leq 0.01$, respectively). At this lipid concentration (5 μM), the lipid:Rz ratio of 8:1 corresponds to 0.93 μg Rz/ml (0.074 μM) and the 4:1 ratio corresponds to 1.9 μg Rz/ml (0.15 μM). When cells were treated for 24 h with free Rz, at 5.5 μg/ml (0.44 μM), the p24 level was reduced by 23.7% compared to untreated controls ($0.01 < p \leq 0.025$). On day 4 and 6 the p24 values were significantly lower only with the Lipofectin:Rz complex at the ratio of 4:1 (Fig. 6A) (day 6; data not shown). The p24 levels were reduced by 25.4% ($0.005 < p \leq 0.01$) and 19.1% ($0.025 < p \leq 0.05$) on day 4 and 6, respectively. Thus, on day 2 virus production was reduced to a greater extent by the liposome-complexed Rz than by a three to six-fold higher concentration of free Rz. This concentration of free Rz corresponded to the Rz added with lipid:Rz complex at 15 μM lipid at the ratio 4:1. Therefore, liposome-mediated delivery of the Rz enhanced its anti-HIV effect, as determined by reduction of p24 levels on day 2. However, treatment with the Lipofectin:Rz (8:1) and DOTAP:Rz (8:1 and 4:1) complexes could not prevent virus production from increasing over time. On days 4 and 6, the level of p24 antigen produced within the previous 48-h period was similar in the treated and control wells. Under the same conditions, both liposomal preparations at 10 and 15 μM were highly cytotoxic, causing substantial disintegration of the cells (data not shown) and a decrease in p24 levels (Fig. 6).

To determine whether the modest inhibitory effect observed at 5 μM lipid concentration was sequence-specific, differentiated THP-1/HIV-1_{IIIB} cells were exposed to 5 μM Lipofectin or DOTAP complexed with the functional Rz, or with control MRz (Table 1). When cells were treated for 24-h with free Rz at 5.5 μg/ml (0.44 μM), the p24 level was reduced by 30.7% compared to untreated controls ($p \leq 0.005$). Under the same conditions MRz was not inhibitory. However, both Rz and MRz were equally inhibitory when they were complexed with cationic liposomes. For example, Lipofectin complexed with Rz or MRz at a ratio of 8:1 reduced the p24 levels by 30% ($p \leq 0.005$). Under the same conditions DOTAP complexed with Rz or MRz reduced the p24 levels by 34.5% ($p \leq 0.0005$) or 28.5% ($p \leq 0.005$), respectively.

Interestingly, the charge ratios (+/−) for the 8:1 and 4:1 complexes are 1.8 and 0.9, respectively. Thus, it appears that the functional delivery of Rz in the 4:1 complex is not dependent on the presence of a net positive charge on the complex. However, it is

Table 1
Effect of LF and DOTAP complexed with anti-LTR or MRz on p24 production in differentiated THP-1/HIV-1_{IIIB} cells[a]

Treatment	p24 (ng/ml)				
	Free Rz	LF:Rz		DOTAP:Rz	
		8:1	4:1	8:1	4:1
None	3.68 ± 0.43[b]	–	–	–	–
Anti-LTR Rz	2.55 ± 0.14	2.58 ± 0.23	3.15 ± 0.23	2.41 ± 0.05	2.21 ± 0.23
MRz	3.81 ± 0.36	2.54 ± 0.09	2.69 ± 0.27	2.63 ± 0.11	3.11 ± 0.17

[a]PMA-treated THP-1/HIV-1_{IIIB} cells, 6 days post-differentiation, were exposed to the liposome–Rz complexes, at lipid:Rz ratios (w/w) of 8:1 or 4:1 (0.93 and 1.9 μg Rz/ml (0.074 and 0.15 μM), respectively), or the Rz alone at 5.5 μg/ml (0.44 μM), for 24 h. The p24 production within the previous 48-h period was measured on day 2. Data represent the mean $\pm$ standard deviation of p24 determinations in duplicate, in supernatants of duplicate wells ($n = 4$), except in controls which were measured in three wells.
[b]Untreated controls.

possible that positively charged regions of the complexes mediate their attachment to the cells.

3.3. Delivery of liposome–Rz complexes to chronically infected HeLa / LAV cells

Chronically infected HeLa/LAV cells were exposed to Lipofectin at 5, 10 and 15 μM, complexed with Rz, at a lipid:Rz (w/w) ratio of 8:1 or 4:1 in DME medium with 8% FBS. Micrographs were taken following a 4-h treatment at 37°C. The cells were further incubated and fed every day with fresh DME/10 medium; p24 production was measured on days 1, 2 and 3. Although a concentration-dependent, cell-associated diffuse fluorescence was observed, the treatment did not affect p24 production, nor did it cause cytotoxicity (data not shown).

3.4. Delivery of liposome–Rz complexes to de novo-infected HeLa CD4$^+$ cells

One explanation for the lack of inhibition of p24 production in HeLa/LAV cells by liposome–Rz complexes is that the efficiency of delivery was too low to affect significantly the level of p24 antigen in culture supernatants of cells producing high quantities of virus. Therefore, we next examined if p24 production could be reduced by Lipofectin–Rz complexes in de novo infected HeLa CD4$^+$ cells, producing much lower levels of virus.

HeLa CD4$^+$ cells were infected with HIV-1_{IIIB} at 5×10^3 $TCID_{50}$ per well, as described in Section 2. Immediately after infection the cells were exposed to Lipofectin at 5, 10 and 15 μM, complexed with Rz, at a lipid:Rz (w/w) ratio of 4:1 for 4 h in RPMI medium with 8% FBS. On 1 and 2 days post-treatment, the culture medium was replaced with fresh medium, and 3 days later the culture supernatants were analyzed for viral p24. Under these conditions treatment of de novo infected HeLa CD4$^+$ cells with the Lipofectin–Rz complexes was cytotoxic while the Rz alone was not toxic (Fig. 7). A similar cytotoxic effect was observed when uninfected HeLa CD4$^+$

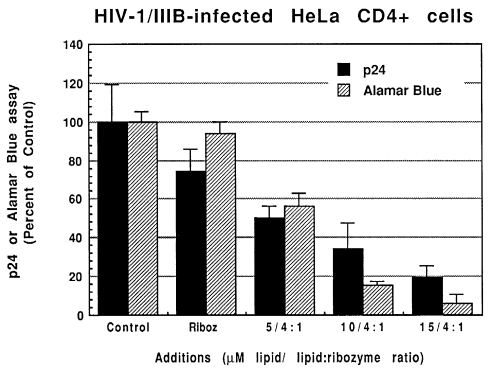

Fig. 7. Effect of LF complexed with Rz on p24 production in de novo infected HeLa CD4$^+$ cells. The cells were exposed to the LF–Rz complexes at a lipid:Rz ratio (w/w) of 4:1, or to the Rz alone at 5.5 μg/ml (0.44 μM), for 4 h (see Section 2 for details). The p24 antigen production within the previous 72-h period was measured on day 5 and was expressed as percent of the control (100% = 0.56 ± 0.11 ng p24/ml). Cell viability was measured on day 5 post-infection (incubation with Alamar Blue for 90 min at 37°C) and was expressed as percent of the control. Data represent the mean $\pm$ standard deviation obtained from duplicate wells or quadruplicate control wells.

cells were incubated with the Lipofectin–Rz complexes (data not shown).

4. Discussion

Our results demonstrate that (i) treatment with liposome–Rz complexes results in cell-associated fluorescence in a concentration-dependent manner; (ii) different cellular fluorescence patterns are observed with the four cationic liposomal preparations (Lipofectamine, Lipofectin, DMRIE and DOTAP); (iii) treatment of THP-1/HIV-1_{IIIB} cells with both functionally active or MRz complexed with 5 μM Lipofectin or DOTAP for a 24-h period causes a reduction in HIV production; (iv) differentiated chronically infected THP-1/HIV-1_{IIIB} cells are more susceptible to killing by liposome–Rz complexes than uninfected THP-1 cells; (v) CD4-expressing HeLa cells are more susceptible to killing by liposome–Rz complexes than chronically infected HeLa/LAV cells.

Compared to the wide variety of methods used to deliver DNA, progress in introducing RNA molecules into cells has been slow, probably because of the rapid degradation of RNA by both exo- and endoribonucleases [24]. Only a few studies have reported results obtained with exogenous delivery of Rz. In one study polybrene-treated H9 cells were incubated with 1 or 100 μM anti-HIV-1 *gag* Rz and subsequently infected with HIV-1 [45]. The formation of syncytia was assessed 7 days after infection. The Rz inhibited the formation of syncytia by 9% and 67%, at 1 and 100 μM, respectively, when compared to untreated controls. These results suggest that the exogenous administration of anti-HIV-1 Rz may be effective in blocking viral infection in H9 cells. However, the pre-formed Rz RNAs were short-lived, and 5 h after addition no intact Rz RNAs could be detected inside the cells. In a subsequent study, the anti-HIV-1 *gag* Rz (142 nucleotides in length) was encapsulated in liposomes composed of 50 mol% dipalmitoylphosphatidylglycerol. This procedure enhanced the uptake and protected the RNA molecules from degradation over a 48-h period in H9 cells [2]. Similarly, encapsulation of an all-RNA Rz in liposomes containing dimyristoylphosphatidylglycerol (DMPG), phosphatidylethanolamine and cholesterol, or cardiolipin, phosphatidylcholine and cholesterol increased its resistance to serum ribonucleases for at least 24 h and enhanced the accumulation of Rz in HepG2 cells to up to 1.5% of the added dose after 24 h [30].

Cationic liposomes as an exogenous delivery system for Rz have likewise been described in only a few studies. In one study chimeric DNA–RNA Rz complexed with Lipofectin were shown to be more stable in H9 cells over a 48-h period than their all-RNA counterparts [19]. Lipofectin was also used to deliver hammerhead Rz targeted to human urokinase receptor mRNA, into human osteosarcoma cells [46]. While free Rz were degraded immediately in medium, Rz complexed with Lipofectin were stable for up to 22 h and were efficiently transported into the cytoplasm. Another cationic liposome formulation, Transfectam (lipopolyamine), was used to deliver a chimeric hammerhead Rz directed against *bcr-abl* mRNA, to a cell line (EM-2) derived from a patient with chronic myelogenous leukemia [47]. The Rz decreased the level of *bcr-abl* mRNA and inhibited expression of the *bcr-abl* gene product, as well as cell growth. A multi-unit Rz that targets *bcr-abl* mRNA was delivered into murine myoloblasts transformed with the *bcr-abl* gene (32D cells), using either Lipofectin or folic acid–polylysine [48]. Both delivery systems protected Rz from degradation in serum-free-medium up to 24 h and enhanced Rz uptake; however, the folate receptor-mediated uptake was up to 10-fold more efficient than that mediated by Lipofectin. Another cationic lipid, DOTAP, was used to deliver hammerhead Rz targeted to the MDR-1 mRNA, into human pleural mesothelioma cells [49]. A 42-mer chimeric DNA–RNA hammerhead Rz targeted to a leukocyte-type 12-lipoxygenase mRNA, was delivered into porcine aortic vascular smooth muscle cells after complexation with Transfectam [50]. The Rz almost completely inhibited specific mRNA expression at 4 μM, but appeared to be ineffective at 0.5 μM. We should note that the use of Rz concentrations in the range 0.5–4 μM in our experiments would have been toxic to the cells.

Our results indicate that cationic liposome–Rz complexes are readily taken up by differentiated THP-1/HIV-1_{IIIB} cells. However, in spite of the intracellular accumulation of the Rz, its effect on virus production is confined to a very limited set of non-cytotoxic conditions. The lack of specific inhibi-

tion by the liposome-complexed functional Rz vs. the MRz may be explained by a non-specific oligonucleotide effect. In contrast, the free MRz did not inhibit p24 production when compared with the free functional Rz (Table 1). Since the functionally active chimeric Rz have been effective against cellular targets [19], the lack of major efficacy against HIV is most likely due to the inability of the Rz to enter the cytoplasm in sufficient quantities to inhibit virus production. It is also not clear what fraction of the Rz is free to interact with substrate, and what fraction is bound to cellular proteins or remain in the endocytotic pathway. It is also possible that cationic lipids augment the production of nucleases which degrade the Rz. Our results suggest that cationic liposome–Rz complexes are sequestered in intracellular membrane-bound compartments, such as endosomes, similar to the fate of cationic liposome–DNA complexes [51]. Such sequestering, together with the low efficiency of cytoplasmic delivery could diminish the potential effectiveness of the Rz, especially in chronically infected cells producing high quantities of virus. Although we have utilized four cationic liposome formulations and two Rz:lipid ratios in our studies, it is possible that a more extensive matrix of Rz and lipid concentrations may enhance the cytoplasmic delivery of exogeneous Rz.

It will be of interest to investigate whether endosome disrupting peptides, such as those used to enhance the expression of transferrin/polylysine-complexed DNA [52], or transferrin–cationic liposome complexes [53] will facilitate the delivery of Rz into the cytoplasm. We have shown that peptides that interact with and destabilize membranes at mildly acidic pH enhance gene delivery by cationic liposomes [54]. Likewise, the intracellular delivery of Rz in pH-sensitive liposomes, which are thought to destabilize the endosome membrane at the mildly acidic pH of the endosome lumen [55,56], may result in enhanced cytoplasmic delivery. Preliminary experiments in our laboratory have shown that the Rz used in this study is effective in inhibiting HIV production by macrophages when delivered in pH-sensitive liposomes (N. Düzgüneş, E. Pretzer and S. Simões, unpublished data). The recent development of sterically stabilized pH-sensitive liposomes with a prolonged circulation half-life [57] may render feasible the in vivo delivery of Rz to target cells and tissues.

Differentiated THP-1/HIV-1_{IIIB} cells were more sensitive to cytotoxic effects of liposome–Rz complexes than differentiated THP-1 cells. Interestingly, the formation of complexes with Rz significantly changes the toxic effect of liposomes alone. For example, a 4-h treatment of differentiated THP-1 cells with the Lipofectamine–Rz complex at 8 μM lipid caused a decrease in cell viability (Fig. 3), while 8 μM Lipofectamine alone was not toxic even after a 24-h treatment [39]. HeLa $CD4^+$ cells were more susceptible to cytotoxic effects of liposome–Rz complexes than uninfected THP-1 cells, chronically infected THP-1/HIV-1_{IIIB} cells or HeLa/LAV cells. These data are consistent with previous observations that both the efficiency of cationic lipid-mediated transfection and the toxicity of lipids or lipid–DNA complexes, vary for different cell types [20,24,26,51,58,59].

In summary our results indicate that the effective delivery of functional Rz into cells by cationic liposomes is an inefficient process and needs extensive improvement before the technique can be used successfully in ex vivo and in vivo applications. Viral targets such as HIV, especially in chronically infected cells, may not be as well suited for exogenous Rz delivery as are intracellular targets present in smaller molar amounts. It is possible that delivery of gene constructs for continuous endogenous expression of the Rz is a more promising strategy.

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