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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Human immunodeficiency virus type-1 (HIV-1) infection increases the sensitivity of macrophages and THP-1 cells to cytotoxicity by cationic liposomes

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Abstract

Cationic liposomes may be valuable for the delivery of anti-sense oligonucleotides, ribozymes, and therapeutic genes into human immunodeficiency virus type 1 (HIV-1)-infected and uninfected cells. We evaluated the toxicity of three cationic liposomal preparations, Lipofectamine, Lipofectin, and 1,2-dimyristoylpropyl-3-dimethyl-hydroxyethyl ammonium bromide (DMRIE) reagent, to HIV-infected and uninfected cells. Monocyte/macrophages were infected with HIV-1BaL and treated with liposomes in medium containing 20% fetal bovine serum (FBS) for 4 h or 24 h at 37°C. Uninfected monocytic THP-1 cells and chronically infected THP-1/HIV-1Mbl cells were treated with phorbol 12-myristate 13-acetate (PMA) and exposed to liposomes in the presence of 10% FBS. Toxicity was evaluated by the Alamar Blue assay and viral p24 production. The toxic effect of cationic liposomes was very limited with uninfected cells, although concentrations of liposomes that were not toxic within a few days of treatment could cause toxicity at later times. In HIV-1BaL-infected macrophages, Lipofectamine (up to 8 μM) and Lipofectin (up to 40 μM) were not toxic after a 4-h treatment, while DMRIE reagent at 40 μM was toxic. While a 4-h treatment of THP-1/HIV-1Mbl cells with the cationic liposomes was not toxic, even up to 14 days post-treatment, all three cationic liposomes were toxic to cells at the highest concentration tested after a 24-h treatment. Similar results were obtained with the Alamar Blue assay, Trypan Blue exclusion and a method that enumerates nuclei. Infected cells with relatively high overall viability could be impaired in their ability to produce virions, indicating that virus production appears to be more sensitive to treatment with the cationic liposomes than cell viability. Our results indicate that HIV-infected cells are more susceptible than uninfected cells to killing by cationic liposomes. The molecular basis of this differential effect is unknown; it is proposed that alterations in cellular membranes during virus budding cause enhanced interactions between cationic liposomes and cellular membranes.

Keywords: HIV-1; Cationic liposome; Cytotoxicity; Macrophage; (Human)

1. Introduction

Cationic liposomes provide a simple and efficient means of introducing DNA and other polynucleotides into eukaryotic cells. Small unilamellar liposomes composed of cationic lipids spontaneously complex with negatively charged compounds. In most cases transfection becomes efficient only when liposomes contain the membrane fusion-promoting lipid, dioleoylphosphatidylethanolamine (DOPE), and the nucleic acid-lipid complexes have a net positive charge [1,2]. Cationic liposomes have been used to facilitate delivery of DNA [3–7], mRNA [8], antisense oligonucleotides [9] and proteins [10,11] into living cells. Unlike viral vectors, liposomes are noninfectious and appear to be non-immunogenic in vivo. They have been utilized for gene delivery in vivo [1,12–15], and a direct gene transfer protocol using a liposome-DNA complex has been approved for injection into solid tumors in patients [16].

Although many studies have been carried out to improve liposome-mediated transfection (lipofection), these techniques suffer from variable transfection efficiency. The composition of the cationic liposomes, the lipid-DNA ratio, overall lipid concentration, the cell type and the density of the cell culture are critical for efficient transfection [2,3,17]. Since the first report of transfection by the cationic
lipid reagent. Lipofectin [3], several different kinds of cationic lipids have been developed, including quaternary ammonium detergents, cationic derivatives of cholesterol and diacyl glycerol, and lipid derivatives of polyamines [1,18].

Cationic liposomes and DNA-liposome complexes may enter the cells by membrane fusion or spontaneous endocytosis, but the precise mechanism of their interaction with the cell membrane has not been elucidated. Liposomes composed of N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium (DOTMA), the cationic component of Lipofectin, were found to undergo lipid mixing with lymphocytic A3.01 and Raji cells in serum-free medium. However, lipid mixing was inhibited by the presence of serum, although the liposomes could still bind to the cell membrane [19]. Cationic liposomes can undergo fusion with negatively charged liposomes and erythrocyte membranes [4,20,21]. The diffuse pattern of fluorescence in cell membranes following incubation with DNA-complexed DOTMA/DOPE liposomes has been interpreted as reflecting the fusion of the lipid DNA complex with the plasma membrane of the cells, resulting in delivery of the exogenous DNA into the cytoplasm [3,4]. On the other hand, the fusion activity of certain cationic liposomes (measured by their ability to fuse with negatively charged liposomes) can be abolished by preincubation with DNA; nevertheless, these lipids can mediate efficient uptake of DNA by cultured cells [22]. In addition, the fusion activities of different cationic liposomes, in the absence or presence of DNA, do not correlate with their transfection activities [22]. It is possible that the majority of DNA-liposome complexes are taken up through adsorption-mediated endocytosis. Although a recent study has indicated that the fusion of cationic liposomes with cultured Hep G-2 cells in phosphate-buffered saline occurs following endocytosis [23], it is not known whether DNA-liposome complexes can also undergo fusion after endocytosis, and what the effect of serum is on this process. 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. The endosome membrane may be destabilized by the cationic lipid, resulting in the release of the intact DNA-liposome complex into the cytoplasm [22–26]. Very little is known about the mechanism by which DNA localizes in the nucleus, except that only a small percentage of the cytoplasmically delivered DNA reaches the nucleoplasm [4,27]. It is possible that the cationic liposome-DNA complex enters the nucleus as an intact complex [28]. Lipofectin complexes of antisense oligonucleotides also localize in the nucleus, as well as in discrete structures in the cytoplasm [29].

Cells of monocyte/macrophage lineage can be easily infected by HIV and are thought to play an important role in the pathogenesis and progression of HIV-related disease [30–32]. Large numbers of infected macrophages have been found in lymph nodes, liver, spleen, lungs, skin, brain, and nearly all tissues and organs from infected subjects [31–33], and chronically infected macrophages are considered to be a reservoir for HIV in infected individuals. Inhibition of virus production in such cells would require agents that are effective in inhibiting HIV gene expression or maturation, or in cleaving viral RNA sequences. These agents may have limited access to the cytoplasm, and cationic liposomes may be useful for the delivery of antisense oligodeoxynucleotides, ribozymes or therapeutic genes into chronically infected macrophages. As a first step towards the use of such liposomes in macrophages, we investigated the toxicity of three cationic liposomal preparations, Lipofectamine, Lipofectin and liposomes composed of DMRIE and DOPE (1:1), to human monocyte-derived macrophages and a differentiated monocytes cell line, THP-1. Since the cationic liposomes would eventually be used to deliver antiviral agents to HIV-infected cells, we also investigated the toxicity of the liposomes to macrophages infected with the monocytotropic strain HIV-1_H1M, as well as to chronically infected THP-1/HIV-1_H1M cells. Our results indicate that cationic liposomes have a differential toxicity for HIV-infected cells under certain conditions. Some of our results have been presented earlier in preliminary form [35].

2. Materials and methods

2.1. Materials

Lipofectamine Reagent [36] containing the polycationic lipid 2,3-dioleloxy-3-[3(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate (DOSPA) and the neutral lipid, dioleoylphosphatidylethanolamine (DOPE) (3:1, w/w), and Lipofectin Reagent [3] containing the monocationic lipid N-[1-(2,3 dioleloxy)-propyl]-N,N,N-trimethylammonium chloride (DOTMA) and DOPE (1:1, w/w) were obtained from Gibco BRL Life Technologies, Inc. (Gaithersburg, MD). DMRIE reagent [37] (a 1:1 (w/w) mixture of 1,2-dimyristoylpropyl-3-dimethyl-hydroxyethylammonium bromide (DMRIE) and DOPE) was synthesized by VICAL, Inc. (San Diego, CA). Alamar Blue dye was purchased from Alamar Biosciences Inc. (Sacramento, CA). The enzyme-free, PBS-based, cell dissociation buffer was obtained from Gibco BRL Life Technologies, Inc. (Gaithersburg, MD). Triton X-100, Nonidet P-40 (NP-40) and naphtol blue black (Buffalo Black NBR), were obtained from Sigma (St. Louis, MO).

2.2. Cells and virus

Monocytes were obtained from HIV seronegative buffy coats by centrifugation on a Ficoll-Hypaque (Histopaque-1077; Sigma, St. Louis, MO) gradient and plastic adherence. Briefly, mononuclear cells separated by centrifuga-
tion were counted and plated in Dulbecco's Modified Eagle's Medium-high glucose (DME-HG) (Irvine Scientific, Santa Ana, CA) without serum, at 1.4 \( \times \) \( 10^4 \)/ml per well or 5.6 \( \times \) \( 10^3 \)/0.2 ml per well in 48-well or 96-well plates, respectively. It was assumed that approximately 5–10% of the cells plated would be recovered as macrophages. The cells were allowed to adhere overnight, after which the wells were washed and the medium was replaced with DME-HG supplemented with 20% (v/v) heat-inactivated fetal bovine serum (FBS) (Sigma, St. Louis, MO), 10% (v/v) human AB serum (Advanced Biotechnologies, Columbia, MD), penicillin (100 U/ml), streptomycin (100 \( \mu \)g/ml) and L-glutamine (4 mM). The cells were left undisturbed in this medium for 5–6 days, by which time differentiation occurred. Macrophages were then maintained at 37°C, under 5% CO\(_2\) in DME/20 (DME-HG + 20% heat-inactivated FBS) medium supplemented with penicillin (100 U/ml), streptomycin (100 \( \mu \)g/ml) and L-glutamine (4 mM). Every 2 days the medium was removed completely and replaced with fresh medium.

Infection of macrophages was performed 8 days post-isolation. The virus and infected cells were handled in a BL-3 facility. A monocytotropic strain of HIV-1, recovered from primary lung cultures and designated HIV-1Bal, [30], was used in these experiments. Cells were exposed to the virus for 2 h at 37°C; then the medium was removed and the cells were washed three times to remove unbound virus. After infection macrophages were cultured for an additional 12 days in fresh DME/20 medium, 1 ml/well or 0.25 ml/well in 48-well or 96-well plates, respectively, with medium changes 3 times/wk. The progress of infection was monitored by determining viral p24 in culture supernatants, by an antigen capture ELISA assay described previously [38], using a Molecular Devices (Menlo Park, CA) Vmax microplate reader.

Viral inocula were standardized by their p24 content, and cultures were infected at a concentration of 5 or 1.4 ng p24 per well in 48-well or 96-well plates, respectively. HIV-1Bal, obtained from Advanced Biotechnologies Inc. (Columbia, MD) was propagated in macrophages, harvested at times of peak p24 production and stored in 1 ml aliquots at \(-80^\circ\)C. The reverse transcriptase activity of the virus stock solution was 8.3 \( \times \) \( 10^3 \) cpm/ml, and the p24 concentration was 50 ng/ml.

THP-1 cells were obtained from the American Type Culture Collection (TIB-202). A chronically HIV-infected cell line (designated THP-1/HIV-1inf) was developed in our laboratory by infecting THP-1 cells with HIV-1inf at a low multiplicity of infection [39]. THP-1/HIV-1inf cells were cloned by limiting dilution in flat-bottom 96-well plates. After 50 and 71 days in culture, supernatants were screened for HIV production, and it was found that 29 of 30 clones obtained (96.7%) were positive for p24 production. Subsequently, samples of all clones were treated with 160 nM phorbol 12-myristate 13-acetate (PMA), for 24 h at 37°C, to check for differentiation. Fifteen of the clones adhered strongly after PMA treatment and produced virus. These clones were expanded and cryopreserved. The clone designated as THP-1/IIIb30 was used in further experiments. Cells were maintained at 37°C, under 5% CO\(_2\) in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated FBS (RPMI/10), penicillin (100 U/ml), streptomycin (100 \( \mu \)g/ml) and L-glutamine (2 mM). The cells were passaged 1:6 once a week.

2.3. Exposure of cells to cationic liposomes

HIV-1Bal-infected macrophages were treated with liposomes in the presence of 20% FBS, on day 12 post-infection. Uninfected macrophages were treated with liposomes on day 11 post-isolation. Uninfected monocytic THP-1 cells and chronically infected THP-1/HIV-1inf cells (1 ml at \( 1 \times 10^6 \) cells/ml) were plated in 48-well plates, treated with 160 nM PMA for 24 h at 37°C and exposed to liposomes in the presence of 10% FBS, 6 or 7 days post-differentiation. Infection of THP-1/HIV-1inf cells was monitored by the p24 level in supernatants. p24 values represent the amount of virus produced (in ng/ml) between the time of medium change and time of sampling for p24. This time period is indicated in the Figure legends for each experiment.

The cells were exposed to Lipofectamine at 3 or 8 \( \mu \)M, and to Lipofectin and DMRIE at 15 or 40 \( \mu \)M, for 4 h or 24 h at 37°C; the medium with liposomes was then removed and fresh medium was added. Lipofectamine, the formulation containing the lipid DOSPA (carrying a spermine headgroup) with a charge of +5 at neutral pH, was used at concentrations recommended by the manufacturer's protocol (Lipofectamine at 25 \( \mu \)l/ml is equivalent to 12.5 \( \mu \)M lipid). The monocationic liposomal preparations, Lipofectin and DMRIE, were used at concentrations of 15 \( \mu \)M, which is equivalent to the highest recommended concentration (Lipofectin and DMRIE at 20 \( \mu \)l/ml are equivalent to 13.7 \( \mu \)M and 15 \( \mu \)M lipid, respectively), and 40 \( \mu \)M to obtain a higher positive charge concentration, slightly less than that of 3 \( \mu \)M Lipofectamine. To achieve the number of positive charges equivalent to that for 8 \( \mu \)M Lipofectamine, both Lipofectin and DMRIE would have to be used at concentrations of 120 \( \mu \)M (~170 \( \mu \)l/ml), which is above the toxic level. Following treatment with liposomes, cells were cultured and fed every 2–3 days with fresh medium (DME/20 for macrophages and RPMI/10 for THP-1 cells). Control cells were treated similarly with the equivalent amount of appropriate medium but not exposed to liposomes. Liposome-related toxicity was evaluated by the Alamar Blue assay and p24 production.

2.4. Cell viability assays

Cell morphology before and after treatment with liposomes was evaluated by inverted phase contrast mi-
croscopy at 25 × magnification. The number of viable cells in suspension used for experiments was determined by Trypan Blue exclusion.

Both macrophages and differentiated THP-1 cells that do not multiply after PMA-treatment adhere to the plastic, and accurate counting of adherent cells is difficult. Direct counting is often unsatisfactory, since it is difficult to select truly representative microscopic fields. To overcome this problem we quantified cell viability after treatment with liposomes using a modified Alamar Blue assay [40].

The Alamar Blue assay was recently introduced by Alamar Biosciences (Sacramento, CA) as an indicator of cell viability allowing continuous monitoring of cell proliferation and/or cytotoxicity [40]. This colorimetric assay measures the oxido-reductive capacity of cells due to the production of metabolites, as a result of cell growth, and permits determination of viability over the culture period without the detachment of adherent cells. The non-toxic Alamar Blue dye can be washed off and the culture continued without termination of the experiment. This assay has been applied to determine the proliferation of lymphocytes [41], and the adhesion rate and viability of monocytes and macrophages [42]. Briefly, 1.0 ml of appropriate medium and 0.1 ml of Alamar Blue, or 0.2 ml of medium and 20 μl of Alamar Blue, was added to 48-well or 96-well culture plates, respectively. 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 using a Molecular Devices Vmax microplate reader. Cell viability (as a percentage of mock-treated control cells) was calculated according to the formula, \((A_{570} - A_{600}) \times 100 / (A_{570} - A_{600})_\text{control cells}\) of test cells. After removal of the Alamar Blue/medium mixture, fresh growth medium was added and the cells were returned to the incubator.

The results obtained by the Alamar Blue assay were correlated with two indirect methods: Trypan Blue exclusion and nuclear counting [43]. Adherent cells were detached, using the enzyme-free dissociation buffer, and counted by the Trypan Blue exclusion method. For nuclear staining, adherent cells were washed with PBS and treated with 0.1 ml of the counting solution, 1% (w/v) Triton X-100 in 0.1 M citric acid with 0.05% (w/v) naphtol blue black, pH 2.2. After 30 min incubation at room temperature the solution was mixed and the suspension of fixed stained nuclei was counted in a hemocytometer.

3. Results

3.1. Effect of liposomes on uninfected macrophages

Uninfected macrophages were treated with the cationic liposomes for 24 h, and cell viability was quantified by the Alamar Blue assay on days 7, 9, 11 and 15 after treatment. Lipofectamine and Lipofectin were not toxic at the concentration range tested. The toxic effect of DMRIE was very limited, and a decrease in cell viability by ~ 20% could be observed after 15 days (Fig. 1). A 4 h treatment of uninfected macrophages with the cationic liposomes was not toxic, even up to 15 days post-treatment (data not shown).

3.2. Effect of liposomes on chronically infected macrophages

Chronic HIV-1 infection is usually established in infected macrophages 10–12 days after virus challenge, as determined by stabilization of both the number of HIV antigen-expressing cells detected by immunofluorescence and proviral DNA determined by the polymerase chain reaction [44]. We treated HIV-1BaL-infected macrophages with the cationic liposomes on day 12 post-infection, when

![Fig. 1. Effect of cationic liposomes on viability of uninfected macrophages. Macrophages in 96-well plates were exposed to the cationic liposomes (LipA: Lipofectamine. Lipof: Lipofectin) for 24 h, 11 days post-isolation, as described in Section 2. Cell viability was measured by the Alamar Blue assay on days 7, 9, 11 and 15 (incubation for 2.5–4 h at 37°C) and was expressed as percent of the control. Data represent the mean ± SD obtained from duplicate wells.](image-url)
chronic infection was already established. Infection of macrophages reduced cell viability by about 50% (data not shown). We also determined p24 production in chronically infected macrophages before treatment with the cationic liposomes. Only wells exhibiting similar cell viability and p24 production were exposed to the cationic liposomes; this procedure allowed us to avoid inter-experiment variability caused by differences both in initial infection and its progression.

HIV-1 BaL-infected macrophages were treated with the cationic liposomes for 4 h; cell viability was quantified on days 6 and 13, and p24 production was measured on days 7 and 14 after treatment. After a 24 h treatment, cell viability and p24 production were determined on days 6 and 14 after treatment. Neither Lipofectamine nor Lipofectin was toxic after a 4 h treatment, while a decrease in cell viability by ~ 50% could be observed with DMRIE at 40 μM (Fig. 2A). A 24 h exposure to Lipofectin at 15 or 40 μM reduced cell viability by ~ 20% and 30%, respectively (Fig. 2B). At the same concentrations, DMRIE caused greater cytotoxicity, reducing cell viability by ~ 40% and 70% at 15 and 40 μM, respectively. Lipofectamine was not toxic after a 24 h treatment. Similar results were obtained when the effects of cationic liposomes were determined by measuring p24 levels in the harvested supernatant (Fig. 3). However, there were some differences between the viability assay and p24 production. For example, while no detectable cytotoxicity was observed at 15 μM DMRIE after a 4 h treatment (Fig. 2A), the production
of p24 antigen in the culture supernatant was inhibited by ~ 30% (Fig. 3A).

3.3. Effect of liposomes on differentiated uninfected THP-1 cells

PMA-treated THP-1 cells were exposed to the cationic liposomes for 24 h, and cell viability was quantified on days 6, 9, and 13 after treatment (Fig. 4). While Lipofectamine at 3 and 8 μM and Lipofectin at 15 μM were not toxic, 40 μM Lipofectin caused a reduction in cell viability by ~ 40% on days 6 and 9, and by 70% on day 13. Although the toxic effect of DMRIE was limited, a decrease in cell viability by 30–40% could be observed after 13 days at both concentrations (Fig. 4).

3.4. Effect of liposomes on differentiated THP-1/HIV-1 cells

Differentiated THP-1/HIV-1 cells were treated with the cationic liposomes for 4 h; cell viability was quantified on days 5 and 14, and p24 production was measured on days 5, 8 and 14 after treatment. A 4 h exposure to any of the three liposomes did not reduce cell viability and p24 production even up to 14 days post-treatment (data not shown). Following a 24 h treatment of these cells, cell viability was determined on day 1, 8 and 12, and p24 production was measured on days 3, 7 and 12 after the end of the treatment period. At the lower concentration, none of the three liposomes reduced cell viability after a 24 h treatment as measured by the Alamar Blue assay (Fig. 5A). However, Lipofectin and DMRIE at 15 μM inhibited viral p24 production measured on days 7 and 12 after treatment by ~ 40% (Fig. 5B), although they did not show an effect on day 3. All three cationic liposomes were toxic at the higher concentration after a 24 h treatment (Fig. 5A,B). Interestingly, concentrations of liposomes that were not toxic at early time points subsequent to treatment could cause significant toxicity at later times. For example, p24 production was not affected by 40 μM Lipofectin or DMRIE on day 3, while on days 7 and 12 it was reduced by ~ 80% (Fig. 5B).

3.5. Comparison of the Alamar Blue assay with the Trypan Blue exclusion and the nuclear counting methods

PMA-treated THP-1 and THP-1/HIV-1 cells were exposed to the cationic liposomes for 24 h. On day 9 after treatment, the Alamar Blue assay was performed, and cell numbers were determined by counting. DMRIE at 15 and 40 μM was not toxic for differentiated uninfected THP-1 cells, while a 24 h exposure to Lipofectin at 40 μM reduced cell viability by ~ 50% (Fig. 6A). Lipofectin at 40 μM and DMRIE at 15 and 40 μM reduced the viability of differentiated THP-1/HIV-1 cells by ~ 50%, 60%, and 90%, respectively (Fig. 6B). After removal of the Alamar Blue/medium mixture the cells were quantified by the Trypan Blue assay and the nuclear staining method. In the Trypan Blue assay only live cells were counted because the affected cells were disintegrating to such an extent that very few cells took up and retained the Trypan Blue. The results were consistent with phase contrast microscopic observations (described below) that the cytotoxic effect was caused by significant damage of the cells and not by mere changes in cell membrane permeability. The viability of control mock-treated cells was 91–96%; however, the absolute number of cells was only 10% of the initially plated 10⁶ cells/well. Thus, during the course of the experiment, approximately 90% of cells were detached and lost. The number of viable cells quantified in the

Fig. 4. Effect of cationic liposomes on viability of differentiated uninfected THP-1 cells. PMA-treated THP-1 cells in 48-well plates were exposed to the cationic liposomes (LipA: Lipofectamine. Lipof: Lipofectin) for 24 h, 7 days post-differentiation, as described in Section 2. Cell viability was measured on days 6, 9, and 13 (incubation overnight at 37°C) and was expressed as percent of the control. Data represent the mean ± SD obtained from duplicate wells or triplicate control wells.
Trypan Blue assay corresponded very well to the number of nuclei stained with the naphtol blue black dye. For example, the number of viable THP-1/HIV-1Hm cells and their nuclei, counted in duplicate control wells with the Trypan Blue or naphtol blue black methods, was $1.24 \pm 0.07 \cdot 10^5$ and $1.07 \pm 0.03 \cdot 10^5$, respectively.

When expressed as a percentage of controls, both the Trypan Blue exclusion and the nuclear counting methods gave results similar to that obtained with the Alamar Blue assay (Fig. 6A and B). Variation in the results was found only in the case of THP-1/HIV-1Hm cells treated with 40 \( \mu \)M DMRIE, where a very significant cytotoxic effect was observed. The trend in the toxic effect of the cationic liposomes was similar to that presented in Figs. 4 and 5A, although some inter-experiment variations were observed.
Fig. 7. Effect of cationic liposomes on the morphology of differentiated uninfected THP-1 cells. PMA-treated THP-1 cells in 48-well plates were exposed to the cationic liposomes for 24 h, 7 days post-differentiation, as described in Section 2. Phase contrast micrographs were taken 3 days after treatment (magnification: 400×). Frame a shows untreated cells. Frames b–f show cells treated with 8 μM Lipofectamine (b), 15 μM Lipofectin (c), 40 μM Lipofectin, 15 μM DMRIE (e), and 40 μM DMRIE (f).
3.6. Cell morphology

Cell morphology can be a sensitive indicator of the toxicity of certain compounds. PMA-treated THP-1 cells were exposed to the cationic liposomes for 24 h, and micrographs were taken 3 days after treatment in the same experiment whose results are shown in Fig. 4. Lipofectamine, at the concentrations used in our studies, had no significant effect on the morphology of differentiated THP-1 cells compared to untreated control cells (Fig. 7b). 40 µM Lipofectin was highly cytotoxic, causing disintegration of cells (Fig. 7d). Changes of cell morphology were also seen with 40 µM DMRIE (Fig. 7f).

4. Discussion

In this study, we investigated the toxicity of one polycationic (Lipofectamine) and two monocationic (Lipofectin and DMRIE) liposomal preparations to uninfected and HIV-infected cells. Our results demonstrate that (i) macrophages and PMA-treated THP-1 or THP-1/HIV-1R cells differ in their sensitivity to the toxic effect of cationic liposomes; (ii) HIV-infected cells are more susceptible to killing by cationic liposomes than uninfected cells; (iii) concentrations of liposomes that were not toxic at early time points subsequent to treatment could cause toxicity at later times; (iv) in infected cells, virus production is more sensitive to the effects of cationic liposomes than the overall viability of the cells as measured by a metabolic assay.

Chronically HIV-1-infected macrophages and differentiated THP-1/HIV-1R cells were selected in our study because of the importance of macrophages in the pathogenesis and progression of HIV infection [32,33]. The avid endocytosis of liposomes by mononuclear phagocytes [45–47] should promote cationic lipid-mediated transfection of therapeutic genes and delivery of anti-HIV anti-sense oligodeoxynucleotides or ribozymes via the endocytotic pathway.

Both cationic lipids alone and lipid-DNA complexes show various levels of growth inhibition and toxicity to cells, depending on the cell type and the conjuguity of the cell culture [3,8,22,48–50]. The molecular and cellular basis of this variability is not known. Generally, transfected cells are temporarily growth-arrested or grow more slowly than untransfected cells [51]. In our study, macrophages and differentiated THP-1 cells were exposed to liposomes in the presence of 20% or 10% FBS, respectively. Although earlier reports on lipofection emphasized the use of serum-free conditions, recent studies indicate that successful transfections with cationic liposomes can be achieved in the presence of serum, provided that the lipid-DNA complexes are prepared under serum-free conditions. The presence of serum can also diminish the growth inhibition and/or toxic effects associated with lipofection [52,53].

Two questions raised by our findings are (i) why differentiated THP-1 and THP-1/HIV-1R cells were more sensitive than macrophages to the toxic effect of cationic liposomes, and (ii) why THP-1 cells were more sensitive to Lipofectin (Fig. 4), while DMRIE was more toxic to macrophages (Fig. 2). The established human monocytoid cell lines have been used to study HIV-monocyte/macrophage interactions, the relationship between virus production and cell differentiation, and virus latency [54–56]. Compared to other monocytoid cell lines, differentiated THP-1 cells behave more like native monocyte-derived macrophages [57]. More than 80% of the PMA-treated THP-1 cells change morphologically and adhere to the substratum; 60 to 70% of these cells are able to phagocytose yeast particles and immunoglobulin G-coated erythrocytes [58]. PMA-treated THP-1/HIV-1R cells represent a convenient model of HIV-1 production in differentiated cells [39]. The monocytoid cells are a useful tool for studying specific aspects of virus-cell interactions; however, none of these cell lines properly represents monocyte/macrophages. The capacity of HIV-1 isolates to replicate in established monocytoid cell lines does not correlate with the tropism of the virus for primary mononuclear phagocytes [56,59]. HIV-1, unlike HIV-1R, does not replicate in any of the monocytoid cell lines in spite of high CD4 expression in some of the lines, and both the expression of cell surface markers and cytokine production vary significantly among these cell lines. For example, the monocytic marker CD14 is expressed in few THP-1 cells (4%), but in a majority of blood monocytes (97%) [59]. Thus, the different sensitivity of macrophages and THP-1 cells to cationic liposomes may be related to the differences in the intrinsic properties of the cells. The structural characteristics of the different lipids may also determine how they interact with the different cell surface components on these two cell types. Interestingly, the formation of complexes between cationic liposomes and RNA significantly changed the toxic effect of liposomes [60]. A 4-h treatment of differentiated THP-1 cells with the Lipofectamine/ribozyme complex at 8 µM lipid caused a decrease in cell viability by ~80% [60], while 8 µM Lipofectin alone was not toxic even after a 24-h treatment (Fig. 4). In contrast, a 24-h treatment with the Lipofectin/ribozyme complex at 40 µM lipid was not toxic, while with 40 µM Lipofectin alone cell viability was significantly reduced (Fig. 4). Incubation of endothelial cells for 4 h with 24 µM (or greater) Lipofectin in the presence of antisense oligonucleotides in serum-free medium (followed by overnight incubation in serum-containing medium without the cationic lipids) was toxic to these cells, as determined by the loss of adherent cells at the end of this incubation period [29].

Another question raised by our results is why chronically HIV-1-infected cells are killed more effectively by
cationic liposomes than are uninfected cells. The interaction of cationic liposomes with cells involves surface binding and internalization, and it is unclear which cellular component(s) are involved in these processes. The effects of HIV infection on monokine production and metabolic and immune functions of macrophages, both in vivo and in vitro, are not well defined [33,34]. It is possible that the continuous production (budding) of the virus and expression of viral envelope proteins can alter the susceptibility of the cell membrane in HIV-infected cells to interaction with cationic liposomes. Our results indicate that cells with relatively high overall viability could be impaired in their ability to produce virions. Thus, virus production appears to be more sensitive to treatment with the cationic liposomes than cell viability measured by the Alamar Blue assay. This observation suggests that cationic liposomes, by inserting into intracellular membranes, may affect the transport of viral components to the cell surface and virus budding. Cytoplasmic vesicles in epithelial cells in which antisense oligonucleotide/Lipofectin complexes accumulate appear to be larger than those in which the oligonucleotide alone accumulates [29]. One interpretation that has been offered for this observation is that the lipid promotes fusion of smaller cytoplasmic vesicles [29].

Our results indicate that cationic liposomes have prolonged effects on cells after the initial incubation period. Although cationic liposomes may appear to have no cytotoxicity within a few days after treatment, they may become toxic later. It is therefore important to monitor cytotoxicity for relatively long time periods. This delayed cytotoxicity may be related to the stability of the cationic lipids used in this study, and the inability of the cells to metabolize them. In this respect it will be of interest to compare the toxic effect of these lipids which contain ether bonded alkyl chains to liposomes containing \( (\text{N}1\text{-}(\text{2,3-dioleoyloxy})\text{propilyl-N,N,N-trimethylammonium methysulfate}) \) (DOTAP), which has ester bonded acyl chains [20,22].

It is interesting to note that Lipofectamine is less toxic than Lipofectin in the cellular systems tested, even at higher concentrations of the cationic lipid component and a higher number of charges per DOSPA molecule. For example, 8 \( \mu M \) Lipofectamine reagent corresponds to 24 \( \mu M \) DOSPA, equivalent to 120 \( \mu M \) in cations, while 15 \( \mu M \) Lipofectin corresponds to 15 \( \mu M \) DOTMA, equivalent to the same concentration of cations. This observation indicates that the concentration of positive charge per se is not a determinant of toxicity. Cytotoxicity is more likely to be linked to the structure of the cationic molecule as well as its ability to be metabolized by the cell.

Our observations point to the importance of evaluating lipid-dependent effects on virus replication when the therapeutic effects of antisense oligonucleotides, ribozymes or genes delivered by cationic liposomes are being investigated. Although this study has concentrated on the toxic effects of pure lipids, the toxicity of nucleic acid/cationic liposome complexes needs to be examined just as thoroughly when evaluating the sequence-dependent effects of therapeutic nucleic acids on virus production or cell proliferation.

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References