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Transgene expression kinetics after transfection with cationic phosphonolipids in hematopoietic non adherent cells

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Abstract

Cationic lipids are considered to be capable of efficiently and safely mediating DNA transfer into cells, although expression is transient. A new family of cationic lipids, called phosphonolipids, has been developed, with the relationship between the hydrophobic domain of the lipid molecules and the significant enhancement of transduction efficiency in a non-adherent cell line characterised in the present study. The kinetics of transfection efficiency were also investigated. Our results demonstrate that the peak of the transient expression of these reporter genes mediated by cationic lipids occurred within 3 to 14 days, depending on the aliphatic chain length of the complex used and on its formulation in the presence or absence of DOPE. Furthermore, the kinetics of transgene expression were found to differ in adherent and non-adherent cells. These results were obtained using three different techniques: CPRG, luminescence, and FACS-gal, and were in agreement with electron microscopy studies. We thus hypothesized that the plasma membrane composition of cells could affect the efficiency of transfection with cationic lipids. Our results suggest that phosphonolipids constitute a promising class of compounds for gene transfer protocols, and that galenic optimization should improve and modify the transfection efficiency of these DNA–lipid complexes. © 1998 Elsevier Science B.V.

Keywords: Gene transfer; Cationic lipid; Kinetics; Hematopoietic cell

1. Introduction

Over the past decade cationic lipids have been extensively used to deliver genes to a variety of different cell lines and tissues [1–7] and have shown promise for in vivo gene transfer as well as in gene therapy clinical trials [8–16]. Although a large number of cationic lipids have been developed [1,5,17–24], their mechanism of gene delivery and the relationship between the structure and function of these compounds is yet to be fully elucidated.

In general, cationic lipids are composed of three

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main structural features: a cationic polynucleotide binding domain, a hydrophobic domain and a negatively charged counter-ion. The transfection mechanism of the DNA–lipid complex across the cell plasma membrane is not yet fully understood, but it has been shown that only a small proportion of the complex is conveyed to the nucleus producing a short-term and transient expression that usually declines within a few days. To the present time, the kinetics of transgene expression have not been well studied or documented. They are, however, a critical parameter for the development of optimal vectors to transfect a target cell or tissue with a gene of interest.

In a previous study [25], we reported on the transfection of a reporter gene (βgal) using a new family of vectors called cationic phosphonolipids. A series of 24 new molecules were synthesized [26], with 7 of them displaying a high transfer efficiency in hematopoietic cell lines. The present work deals with investigations carried out on the kinetics of transgene expression in non-adherent cells transfected with a phosphonolipid–DNA complex or lipoplex [27]. To assess β-galactosidase activity we carried out three different assays for a 32-day period following transfection; these being a Chlorophenol Red β-D-galactopyranoside or CPRG assay, a luminescence assay, and flow cytometry. It is currently accepted that most cationic lipids must be formulated with a neutral phospholipid (e.g., di-oleylphosphatidylethanolamine (DOPE)) in order to provide optimal transfection activity [28,29]. In fact, DOPE stabilizes most types of cationic lipids in a lipid bilayer and enables the penetration of cationic lipids into the cell by increasing membrane fusion [28]. The neutral phospholipid requirement for transfection activity and its influence on the kinetics of transgene expression were also investigated in the present study.

We show here that the length of the lipid aliphatic side-chain plays a critical role in the transfection efficiency and we determine the kinetic rate of transgene expression. Moreover, we demonstrate a peak in expression with C14 aliphatic side-chain compounds three days after transfection. In contrast, expression with C18 compounds was maximum at day 14.

Finally, we show that the addition of DOPE to the formulation of these compounds drastically shifted the activity of the C14 complexes and resulted in peak expression 14 days after transfection.

2. Materials and methods

2.1. Cell-culture

2.1.1. K562 cells

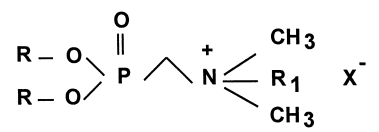
The human hematopoietic non adherent cell line K562 was obtained from American Type Culture Collection (no. ccl 243 ATCC, Rockville, MD, USA) and was maintained in RPMI-1640 medium (Gibco-BRL, Cergy Pontoise, France) supplemented with 10% fetal calf serum (FCS), 0.2 mM glutamine, 100 U/ml of penicillin, 100 U of streptomycin and 1% fungizone. Cells concentrations were maintained between 10^5/ml and 10^6/ml.

2.2. Plasmid

The plasmid used was pCMVLacZ, containing the *LacZ* gene encoding β-galactosidase under the control of the cytomegalovirus (CMV) promoter. It was grown in Epicurian coli (Stratagene), extracted and purified on QUIAGEN-500 columns (Coger). The structure and purity were checked after enzymatic digestion with *Eco*RI by electrophoresis in a 0.7% agarose gel followed by ethidium bromide staining to detect DNA. DNA concentration was determined by absorbance at 260 nm.

2.3. Cationic liposomes

A series of seven cationic phosphonolipids synthesized by our group was studied in this report. These original molecules were obtained by the Mannich reaction from fatty phosphites followed by quaternarization of the resulting aminophosphonates [26]. Their structures are presented in Fig. 1 and Table 1. Each of the cationic phosphonolipids was prepared alone



R = C14:0 $X^- = I^-$ R1 = CH_3 : GLB73

R = C18:1 $X^- = I^-$ R1 = CH_3 : GLB43

Fig. 1. Structure of cationic phosphonolipid molecules.

Table 1
Formula of the cationic phosphonolipids used

R	R1	X	Phosphonolipids
C12:0	CH_3	I^-	GLB 416
C14:0	CH_3	I^-	GLB 73
C14:0	CH_3	Cl^-	GLB 84
C16:0	CH_3	I^-	GLB 239
C18:0	CH_3	I^-	GLB 212
C18:1	CH_3	I^-	GLB 43
C18:1	CH_3	Cl^-	GLB 58

or in combination with the neutral lipid DOPE (Sigma, Saint Quentin Fallavier, France). The phosphonolipids were formulated by mixing chloroform solutions of the different lipids in glass vials, then chloroform was removed by rotary evaporation to produce dried lipids films. A total of 1 ml of sterile pyrogen-free DI water was added per mg of lipid and the vials were sealed and stored one night at +4°C. Small unilamellar vesicles (suv) were prepared by sonicating the compounds for 10 min in a sonicator (Prolabo, Paris, France) [30].

2.4. Transfection protocol

K562 non adherent cells were seeded 1 h before transfection onto a 24-well plate at 100,000 cells per well in 250 μl of RPMI medium. The cationic phosphonolipid–DNA complexes were prepared by adding the appropriate amounts of RPMI (serum free), pCMVβgal DNA (1 to 8 μg) and the liposome formulation (12.5 μl) into a 5-ml polystyrene tube to get a total volume of 200 μl. Then, the tube was incubated 30 min at room temperature. A total of 200 μl of the resultant transfection complex were added to each well, and the cells were incubated for 2.5 h at 37°C. Then, 1 ml of the appropriate growth medium supplemented with 10% of calf foetal serum per well was added and the cells were cultured until carrying out β-galactosidase activity detection assays (every 3 or 4 days during one month). In long term-cultures, the medium was changed every 3 or 4 days and the cells were passaged every week. At the end of the transfection period, the cells were removed, centrifuged and washed twice with PBS. The cell pellet obtained was divided into three parts: the first one was used for the CPRG analysis, the second one for the chemiluminescent β-galactosidase assay, and the last one for the flow cytometric FACS-gal assay. All the experiments were carried out 4 times.

2.5. CPRG (Chlorophenol red galactopyranoside) test

The cells were assayed for expression of β-galactosidase using the CPRG (Chlorophenol red galactopyranoside) spectrophotometric test as described previously [29]. Succinctly, the cells were lysed by adding 50 μl of lysis buffer (K_2HPO_4 0.1 M, triton 0.2%) per well. Fifty microliters of the cell extract were transferred to microtiter plate and 50 μl of phosphate buffered-saline containing 0.5% bovine serum albumin was added to each well. The β-galactosidase activity present in each well of the transfection plate was converted into an absolute level of β-galactosidase protein per well from the β-galactosidase standard curve. This standard curve was prepared by serially diluting 2-fold a β-galactosidase standard (obtained from Sigma) in PBS (phosphate-buffered saline) containing 0.5% BSA (bovine serum albumin) to give 250, 125, 62.5, 31.25, 15.62, 7.81, 39, 1.95 ng of β-galactosidase per well. A total of 150 μl of substrate, 1 mg/ml of ChloroPhenol red galactopyranoside in β-galactosidase buffer (60 mM sodium dibasic phosphate buffer, pH 8, 1 mM magnesium sulfate, 10 mM KCl, 50 mM β-mercaptoethanol) were added to each well, and the plate was read at 580 nm in a microtiter plate reader allowing the color to develop (10 min to 24 h depending on the level of expression obtained).

2.6. Luminescent detection of β-galactosidase

β-Galactosidase activity was measured with a luminescent β-galactosidase detection kit (Clontech).The cells were lysed in 50 μl of lysis buffer (Clontech), 25 μl of the supernatant were mixed to 50 μl of reaction buffer (Clontech) in a microtiter plate. The plate was incubated at room temperature for 90 min. β-Galactosidase activity in the supernatant was quantified with a luminometer (LMR Dynex) to integrate light emission over a 5-s reaction period. β-Galactosidase activity present in each well of the microtiter plate was converted into

an absolute level of β-galactosidase protein per well from the β-galactosidase standard curve.

2.7. Analysis of βgal expression in viable cells using a FACS-gal assay

To investigate gene expression in the cells, a FACS-gal assay [31] was also used. According to this flow cytometry method, enzyme activity is measured by using a fluorogenic substrate (fluorescein di-β-D-galactopyranoside) (FDG) hydrolyzed by β-galactosidase inside cells. For this assay 1.10^5 cells in a 35-mm tissue culture plate were transfected with an optimal phosphonolipid-to-DNA ratio. The FACS-gal assays were done basically as described elsewhere [32]. Briefly, aliquots of cells were suspended in 50 μl of growth medium and warmed at 37°C. FDG (2 mM in 98% distilled water) (Sigma) was warmed at 37°C and 50 μl were added to each 50 μl aliquot of cells. The cells and FDG were rapidly mixed and immediately incubated in a 37°C bath for 1 min. The cells were removed from the water bath, 1 ml or more of ice cold growth medium or phosphate-buffered saline (PBS) was added to the cells and incubated on ice until analyzed on a FACScan (Becton–Dickinson). With this fluorescent assay, the percentage of positive cells (LacZ +) was evaluated by comparison with a control transfected with the same quantity of lipid–DNA complex (in this control the DNA used was pBR322).

2.8. Electron microscopy

Cells were fixed with 1.25% phosphate buffered glutaraldehyde solution for 30 min at room temperature and centrifuged (2000 rpm, 10 min). After washing with the same buffer, cells were post-fixed in 1% osmium tetraoxyde for 30 min, then washed with 0.1 M phosphate buffer. Cells were kept at 4°C during night in 70% ethanol. They were dehydrated in ethanol 95% for 2×15 min and in 100% ethanol for 3×20 min. Cells were embedded in Epon-Araldite Resin (Epox 812 ml, Araldite 506 3 ml, DDSA 5 ml, NMA 4.5 ml and DMP 30 1.5%) Ultrathin sections (50 nm) were cut with a diamond knife and stained with an ultrastainer (Leica). They were examined with a Jeol JEM 100S microscope.

3. Results

3.1. Cationic lipids

With the aim of mimicking the phosphate linkage of natural phospholipids, a series of new cationic phosphonolipids was designed. The novel family of

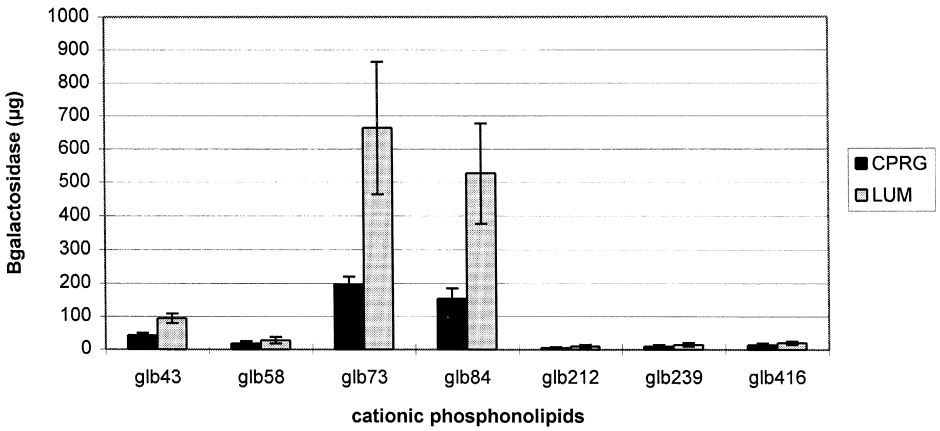
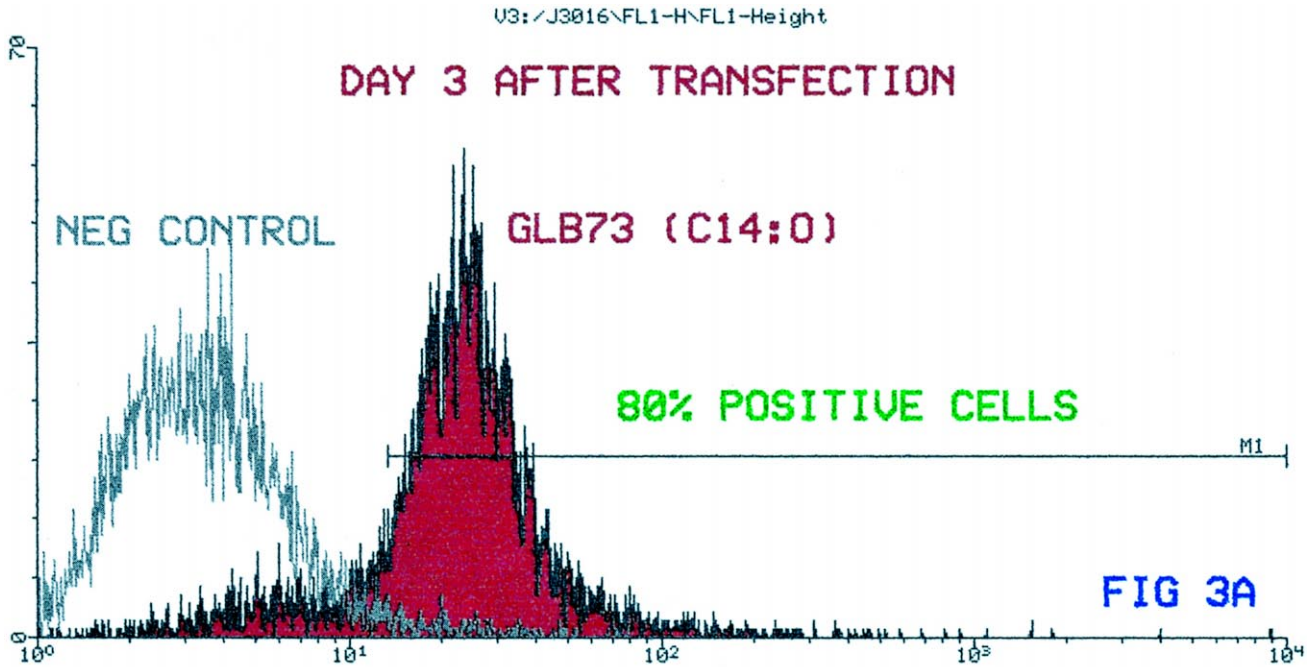


Fig. 2. Comparison of 7 cationic phosphonolipids efficiency in the K562-cell population transfection performed with pCMV βgal plasmid DNA (GLB43 (R = C18:1, R1 = CH_3, X = I), GLB58 (R = C18:1, R1 = CH_3, X = CI), GLB73 (R = C14:0, R1 = CH_3, X = I), GLB84 (R = C14:0, R1 = CH_3, X = CI), GLB212 (R = C18:0, R1 = CH_3, X = I), GLB239 (R = C16:0, R1 = CH_3, X = I), GLB416 (R = C12:0, R1 = CH_3, X = I)). Three days after transfection, the cells were assayed for β-galactosidase activity with CPRG (■) and luminescent (□) tests. Each point indicates the mean value of the β-galactosidase quantity derived from 4 transfections and the standard deviation from this mean.



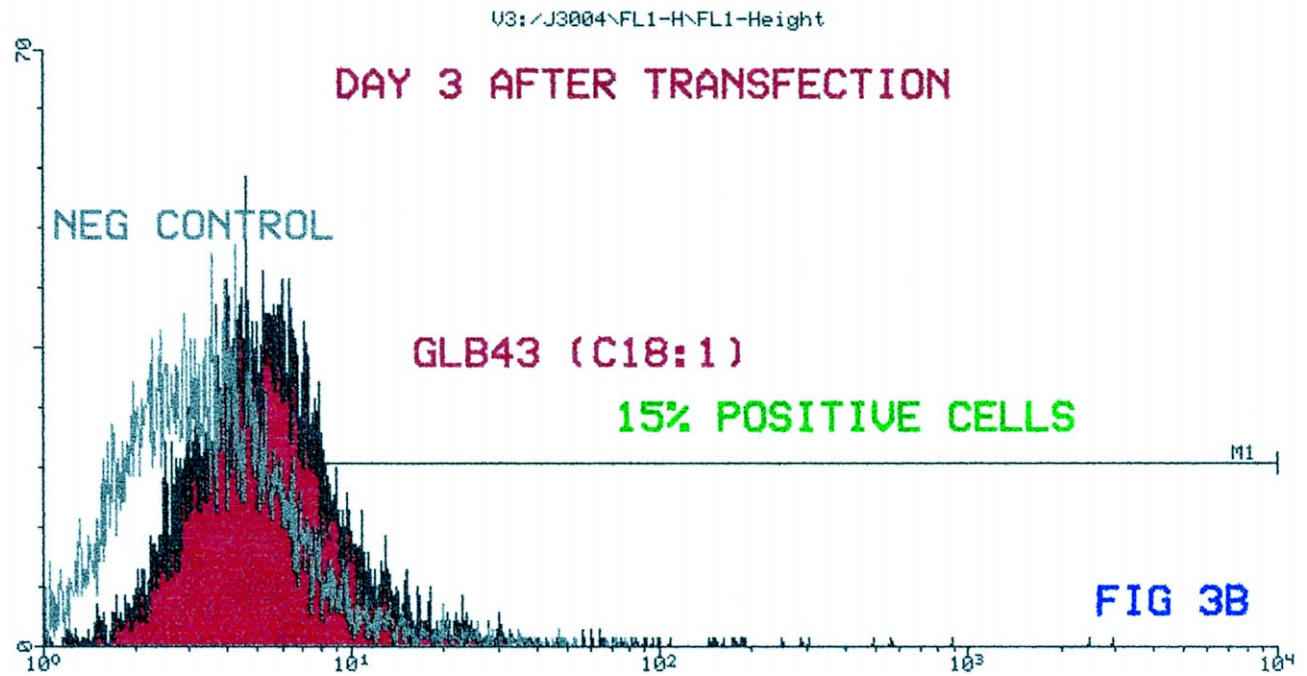


Fig. 3. Flow cytometry analysis of the positive cell percentage in a K562-cell population transfected with pCMV βgal plasmid DNA. Cells were assayed 3 days after lipofection. (A) Obtained with C14:0 compound (GLB 73) and (B) with C18:1 compound (GLB 43). Grey curve: negative control (K562 cells transfected with a pBR 322 plasmid DNA).

cationic lipids used was synthesized as previously described [26]. In order to understand the relationship between structure and activity on the one hand, and between structure and kinetics of reporter gene expression on the other, 7 phosphonolipids containing C12 to C18 acyl chains were synthesized (Table 1). Different formulations that included the neutral phospholipid DOPE were prepared so as to evaluate the role of DOPE in both the transfection activity of these molecules and in the kinetics of transgene expression.

3.2. Influence of aliphatic chain length on transfection efficiency (at day 3)

To assess the relationship between phosphonolipid hydrophobic domain structure and transfection activity, a panel of phosphonolipids differing only in the composition of the aliphatic side-chain was studied, i.e., GLB43 (C18:1), GLB58 (C18:1), GLB73 (C14:0), GLB84 (C14:0), GLB212 (C18:0), GLB239 (C16:0), GLB416(C12:0). The counter-ion used was Cl^- in GLB84 and GLB58, and I^- in all other compounds.

In order to compare transfection activities, K562 cells were assayed for β-galactosidase expression 72 h after transfection using the three different assays outlined in Section 2. The results obtained at day 3 after transfection using the CPRG test and the luminescence assay are presented in Fig. 2. Irrespective of the assay employed, di-myristil (C14) had the most efficient level of transfection, followed by di-oleyl (C18:1); with the three least efficient transfecting compounds being di-palmityl (C16:0), di-steryl (C18:0) and di-lauryl (C12:0), all of which had similar levels of efficiency. The decline in the transfection activity of C12:0 was correlated with a visible reduction in the number of cells per well resulting from a possible toxicity of the cationic lipid molecule. This was confirmed by the cytotoxicity assay (data not shown).

A comparison of the saturated compound di C18:0, with di C18:1 highlighted the beneficial effect of additional unsaturation. As previously reported, two phosphonolipid analogues with an iodide anion (GLB43, GLB73) resulted in higher transfection efficiencies than the same compounds with a chloride anion (GLB58, GLB84). It should be noted that the transfection activities measured with the luminescence assay were higher than those obtained with the less sensitive CPRG test, although the two sets of data were in good agreement. The transfection activities of these different compounds were also compared using a FACS-gal assay (Fig. 3). In this assay, the intensity of the fl1 (green fluorescence) was proportional to β-galactosidase expression. The fl1 fluorescence intensities measured 72 h after the transfection of K562 cells with GLB73 (C14:0) or GLB43 (C18:1) are displayed in Fig. 3a and b, respectively. Fig. 3a shows that 80% of the K562 cells were efficiently transfected with GLB73, whereas only 15% of the K562 cells could be transfected with GLB43 (Fig. 3b). The fl1 intensity was compared to the intensity measured in control cells transfected with the same

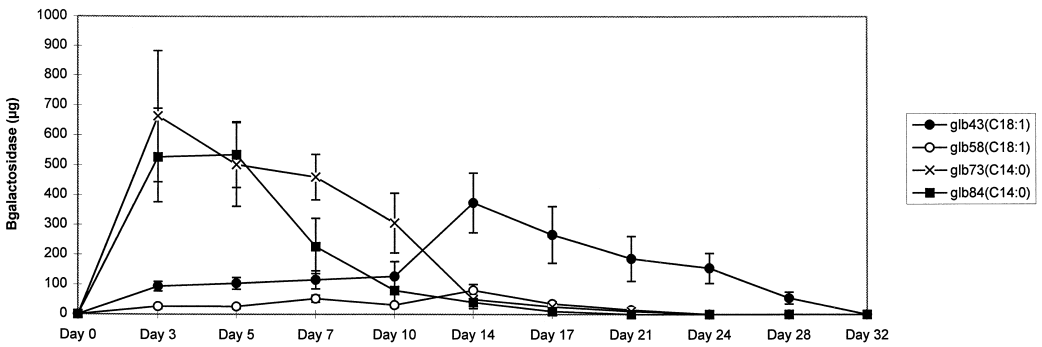


Fig. 4. Influence of aliphatic chain length on the transgene kinetic expression, transfections of the K562 cells were performed with various cationic phosphonolipids and pCMV βgal plasmid DNA. The cells were assayed for β-galactosidase activity within 3 and 32 days after transfection. Each data point indicates the mean value of the β-galactosidase quantity derived from four transfections and the standard deviation from this mean.

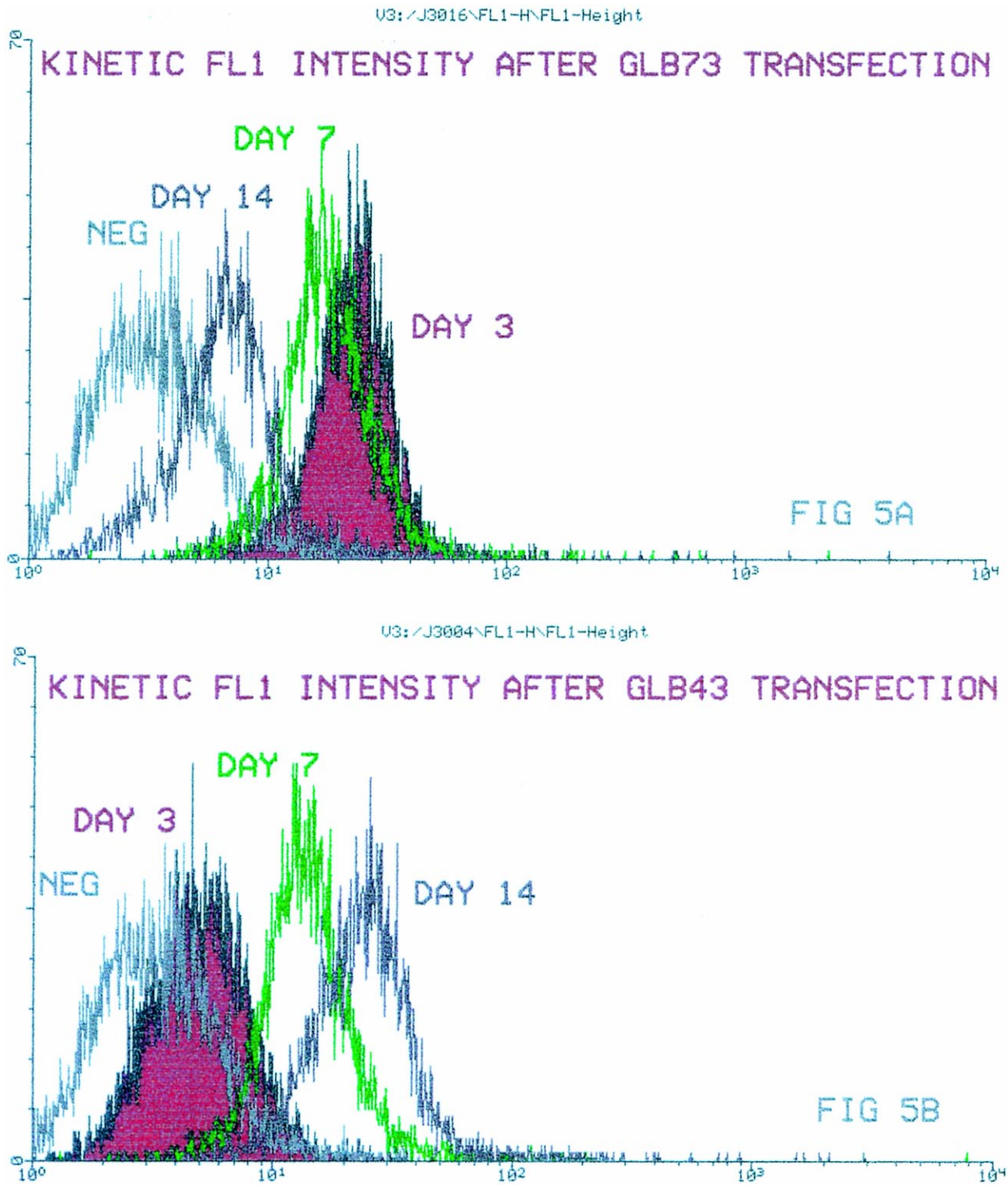


Fig. 5. Flow cytometry analysis of the transgene kinetic expression, after transfection using a C14:0 (GLB73) (A) or a C18:1 (GLB43) (B). K562 cells were transfected using a pCMV βgal plasmid DNA and assayed for β-galactosidase activity 3, 7 and 14 days after lipofection. Grey curve: negative control (K562 cells transfected with a pBR 322 plasmid DNA).

quantity of lipid–DNA complex with pBR322 as DNA. The results obtained with the FACS-gal assay were in agreement with those of the two other tests, with transfection using GLB73 (C14:0) greater than that obtained with GLB43 (C18:1).

3.3. Influence of aliphatic chain length on transgene expression kinetic

In most experiments described in the literature, transfected cells are usually assayed for reporter gene expression 24 to 48 h after transfection. In the present work, K562 cells were transfected with the 4 most efficient phosphonolipids (GLB43 (C18:1, I^-), GLB58 (C18:1, Cl^-), GLB73 (C14:0, I^-), GLB84 (C14:0, Cl^-), and assayed for β-galactosidase expression on different days (3, 5, 7, 10, 14, 17, 21, 24, 28, 32 days). The same experiment was carried out with GLB212, GLB239, GLB416, however the yield of β-galactosidase was very low, at less than 10 ng (data not shown).

Results describing the kinetics of the luminescence assay are presented in Fig. 4. They highlight the fact that the aliphatic chain length of the phosphonolipid used for the transfection alters transgene expression kinetics. Maximal transgene expression was observed between days 3 and 5 after transfection for the C14:0 compounds (GLB73, GLB84). β-Galactosidase levels began to decline on day 6 and were undetectable after 14 days. The two C14:0 compounds exhibited similar patterns of activity. For the C18:1 compounds (GLB43, GLB58), maximal transgene expression was only observed at day 14. When the cells were transfected using GLB43 it was possible to detect β-galactosidase activity up to day 28. The profiles obtained with the two C18:1 compounds were similar, although GLB58 provided a lower yield than GLB43. It was therefore concluded that, irrespective of the aliphatic chain length (C18 or C14), the counter-ion used (I^- or Cl^-) did not influence the kinetic expression of the transgene.

Similar results were obtained with the FACS-gal assay and the β-galactosidase activity detection test (Fig. 5). The fl1 fluorescence was maximum 3 days after transfection using GLB73 (Fig. 5a), whereas with GLB43 the maximum occurred after 14 days (Fig. 5b).

3.4. Relationship between phosphonolipids formulation and transfection efficiency (at day 3)

Most currently produced cationic lipid reagents contain a mixture of a cationic lipid and a neutral phospholipid (i.e., dioleylphosphatidylethanolamine (DOPE)). The transfection activity of the 7 cationic phosphonolipids formulated in the presence or absence of DOPE (in 2 ratios; 1:1, 2:1) was compared 72 h after transfection using the luminescent β-galactosidase detection test. The results obtained are

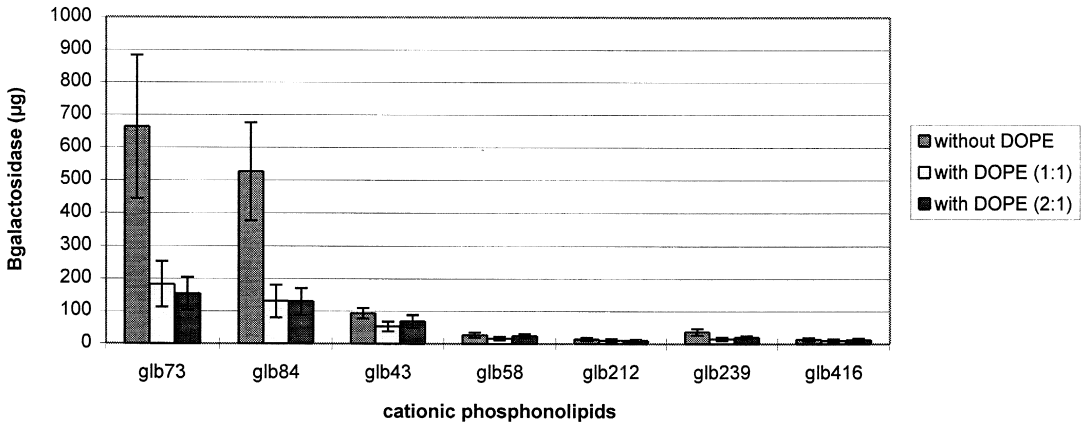
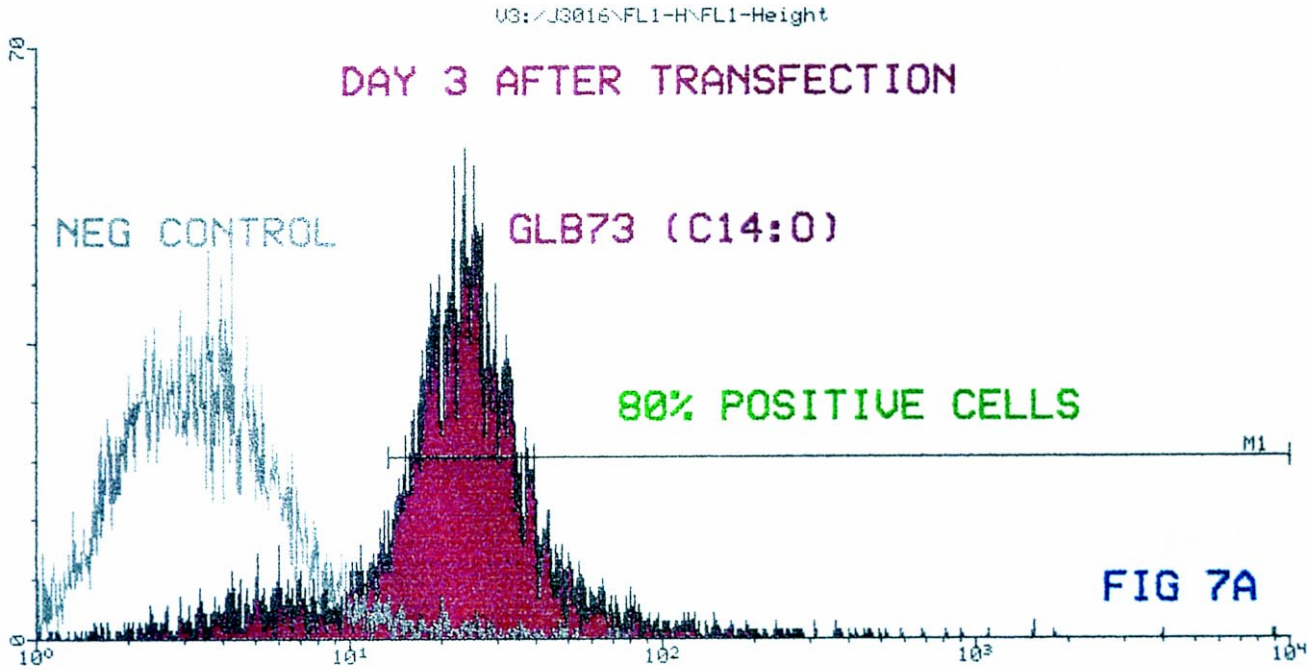


Fig. 6. Comparison of 6 different DOPE-containing cationic phosphonolipids reagents in transfection assay carried out on a K562-cell population with pCMV βgal plasmid DNA. Three days after transfection, β-galactosidase activity was determined with a luminescent assay. Each data point indicates the mean value of the β-galactosidase quantity derived from four transfections and the standard deviation from this mean.



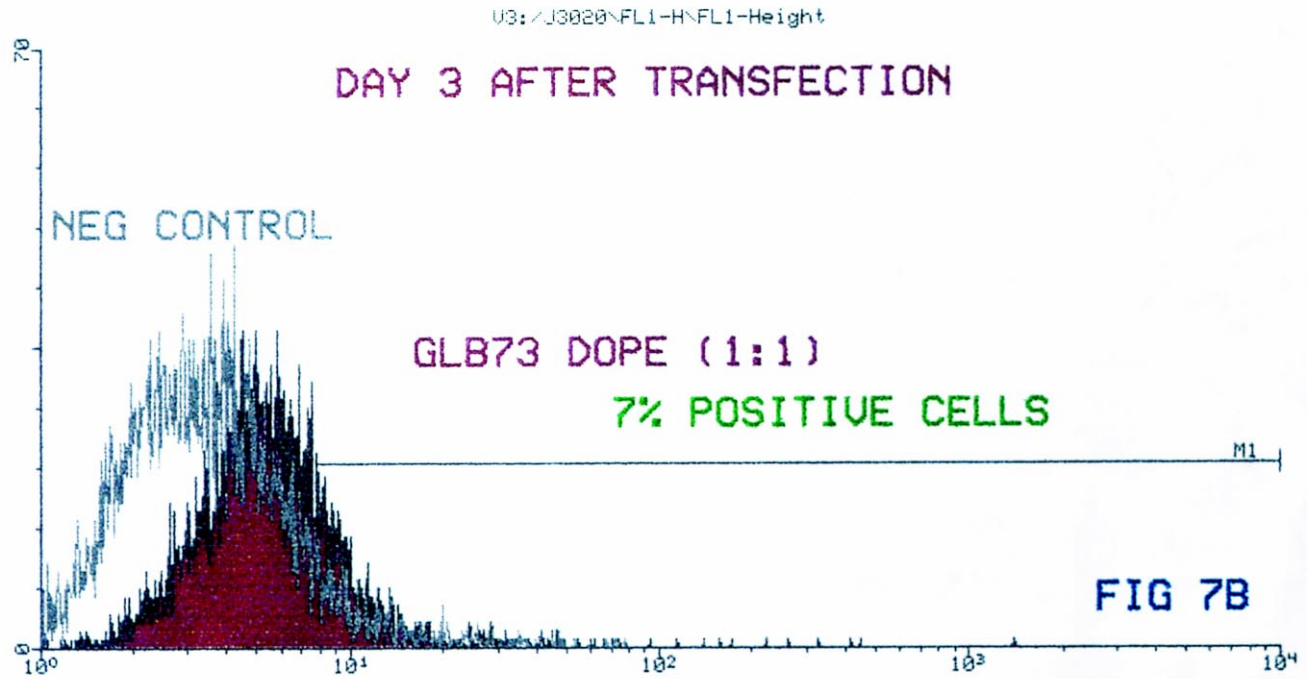


Fig. 7. Flow cytometry analysis of the positive cell percentage in a K562-cell population transfected with pCMV βgal plasmid DNA. The cells were assayed 3 days after lipofection. Curves obtained with C14:0 compound GLB73 formulated without DOPE (A) and with DOPE (1:1) (B). Grey curve: negative control (K562 cells transfected with a pBR 322 plasmid DNA).

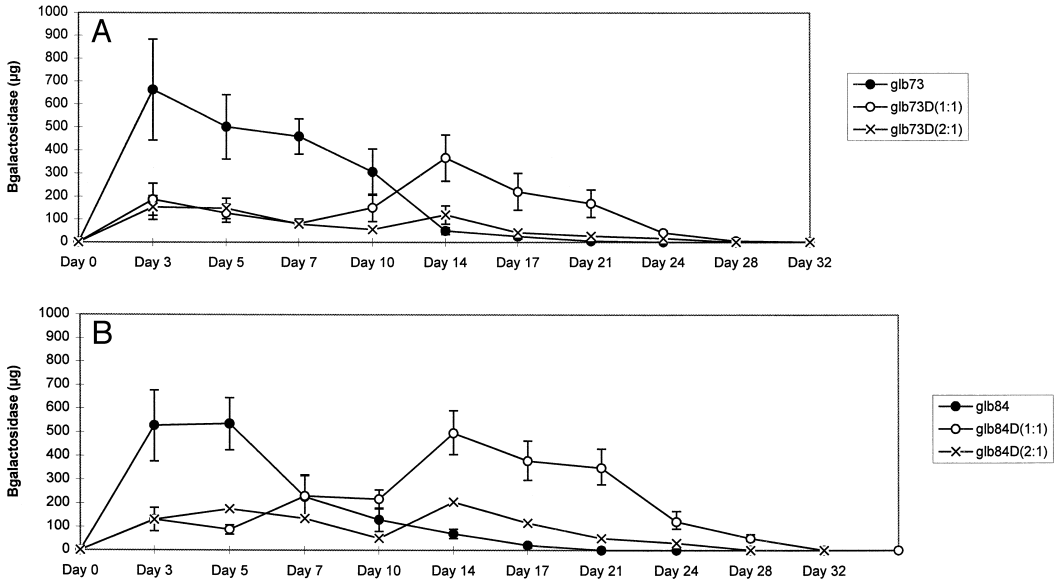


Fig. 8. Influence of DOPE on transgene kinetic expression after transfection. K562 cells were transfected with a pCMV βgal plasmid DNA and assayed for β-galactosidase activity within 3 and 32 days after lipofection with a luminescent assay. (A) was obtained with C14:0 compound, GLB 73, (B) with GLB 84. Both were formulated with and without DOPE. Each data point indicates the mean value of the β-galactosidase quantity derived from four transfections and the standard deviation from this mean.

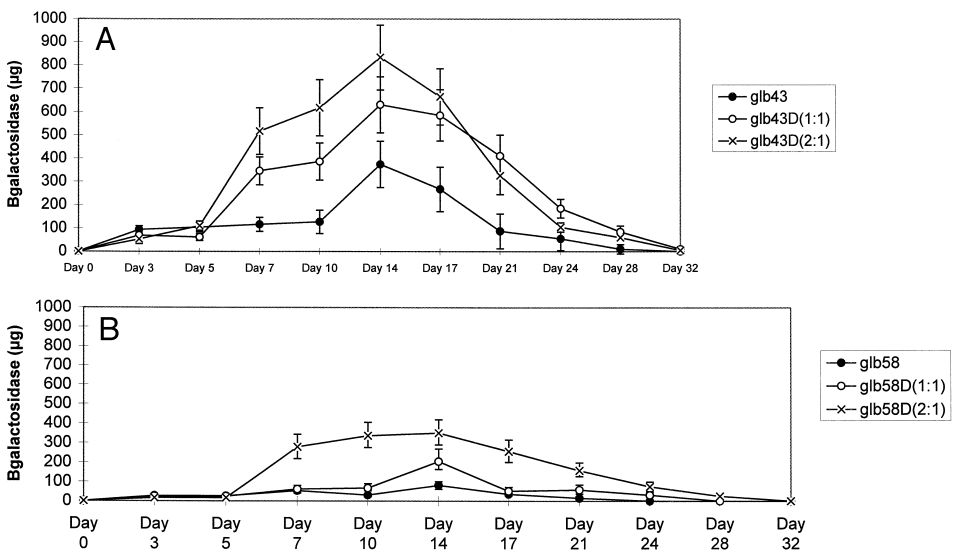


Fig. 9. Influence of DOPE on transgene kinetic expression after transfection of a K562-cell population with a pCMV βgal plasmid DNA. β-galactosidase activity was assayed within 3 and 32 days after lipofection with a luminescent assay. (A) Obtained with C18:1 compound GLB 43 and (B) with GLB 58. Both were formulated with and without DOPE. Each data point indicates the mean value of the β-galactosidase quantity derived from four transfections and the standard deviation from this mean.

summarized in Fig. 6. All of the C14:0 cationic phosphonolipids formulated with DOPE decreased transfection activity to similar levels displayed by that of the C18:1 compounds.

It has been previously reported [29] that a 2-5-fold activation in the adherent COS 7 cell line was obtained with cationic vesicles formulated with 50% DOPE; however no such result was observed in the

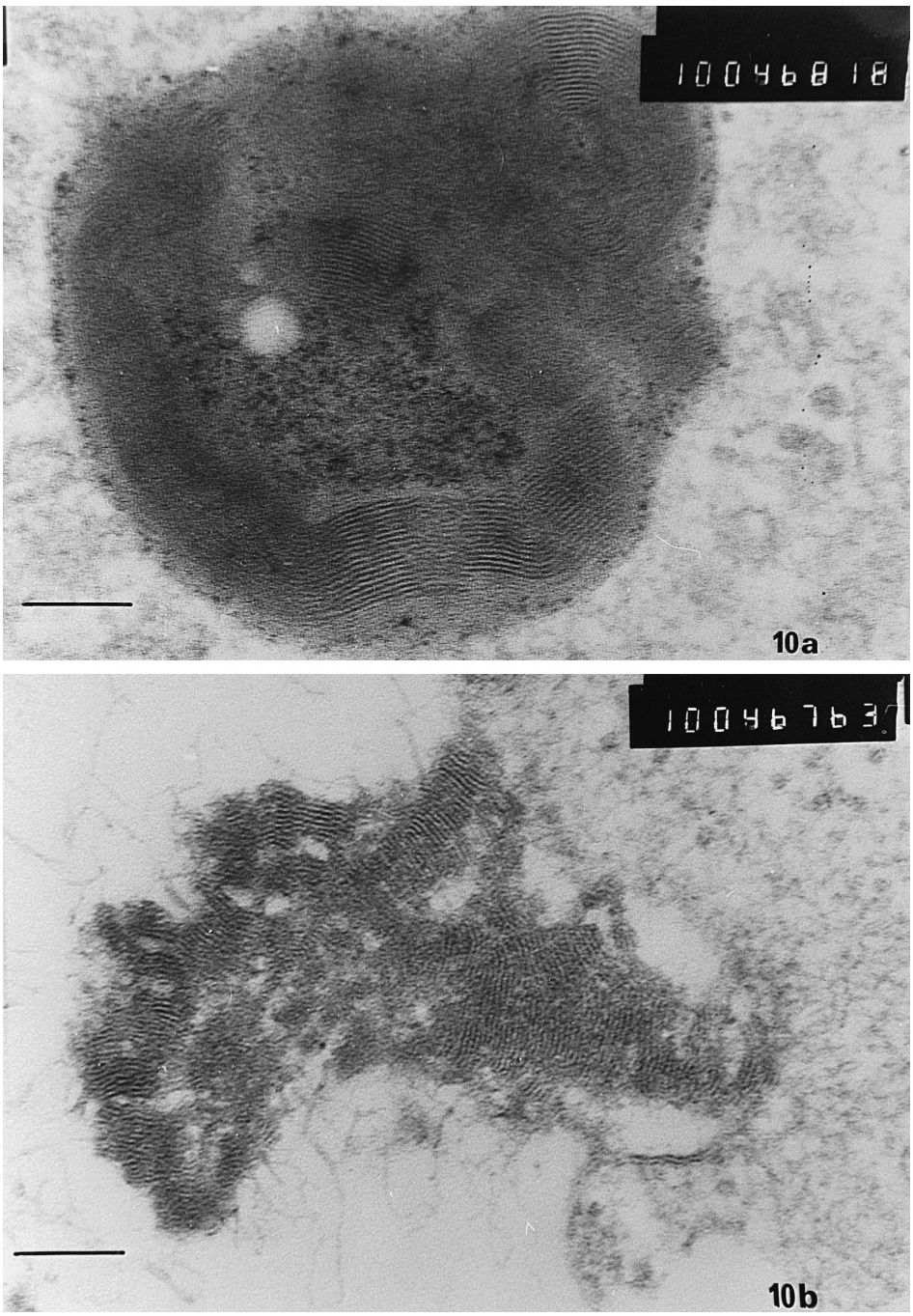


Fig. 10. Electronic photomicrographs of lipoplexes. (A) Lipoplex A formulated with DNA C18:1 compound and DOPE, concentric regular lamellar pattern, (B) Lipoplex B formulated with C14:0 compound, fingerprint-like pattern. Bar indicates 0.1 μm.

present study. The C12:0, C16:0 and the C18:0 cationic phosphonolipids formulated with DOPE were always less active than the C14:0 and the C18:1 analogues formulated in the presence or absence of DOPE. Therefore, regardless of the phophonolipid–DOPE ratio used, the transfection activity at day 3 was always higher when DOPE was not added to the formulation.

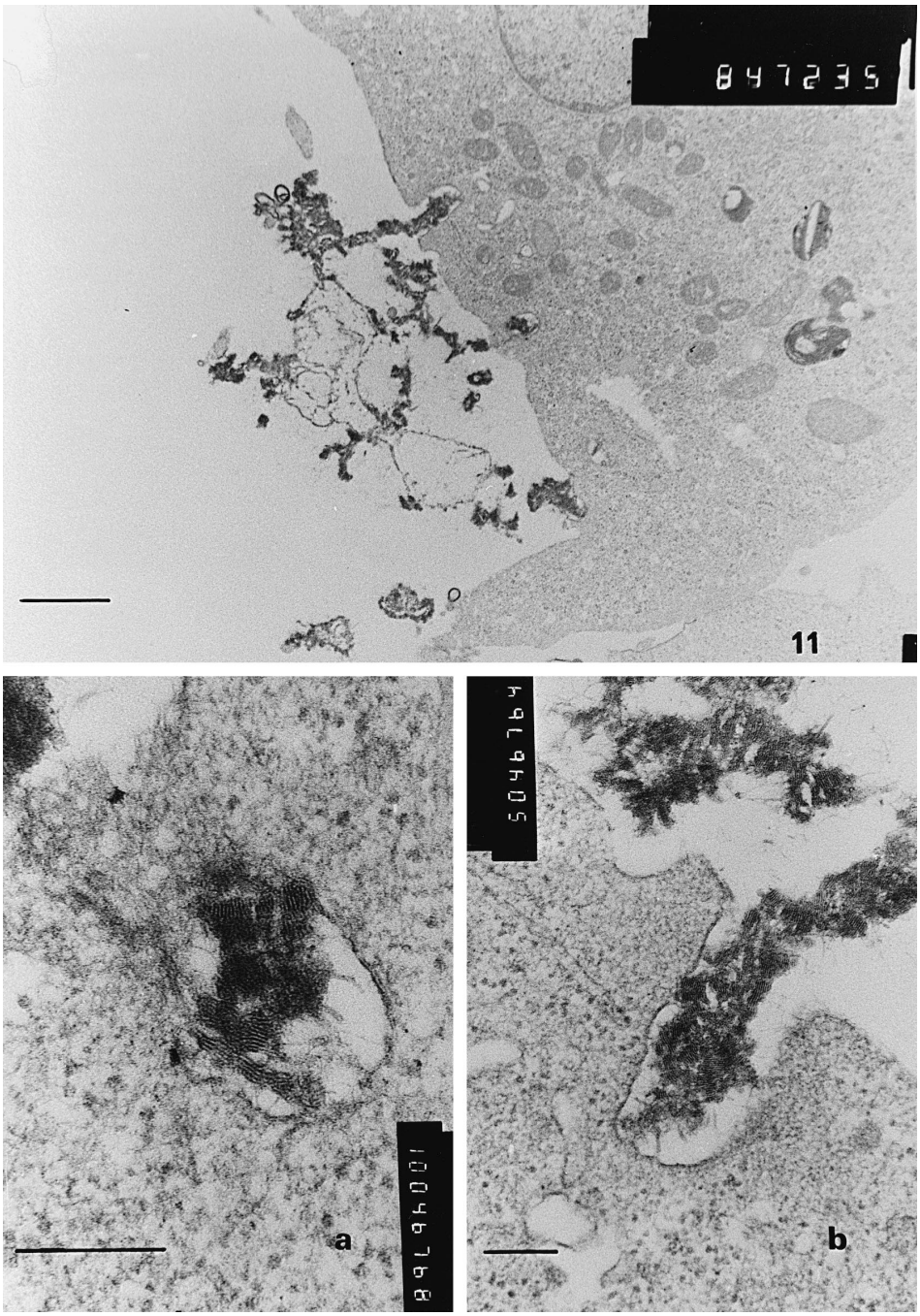


Fig. 11. Lipoplex B 2.5 h after transfection start: low magnification showing the lipoplex penetration. Bar indicates 1 μm. 11A: Detail: Lipoplex B begins to penetrate in a fold of the cell membrane. Bar indicates 0.2 μm. 11B: Detail: Lipoplex B in the cytoplasm of K562 cell inside a vacuole. Bar indicates 0.2 μm.

FACS-gal assay results 72 h after transfection of K562 cells with GLB73 formulated in the presence or absence of DOPE are presented in Fig. 7a and b. The addition of DOPE to the GLB73 formulation induced a decrease in the fl1 fluorescence intensity, with 80% of cells efficiently transfected using GLB73 compared to only 7% when the formulation contained DOPE.

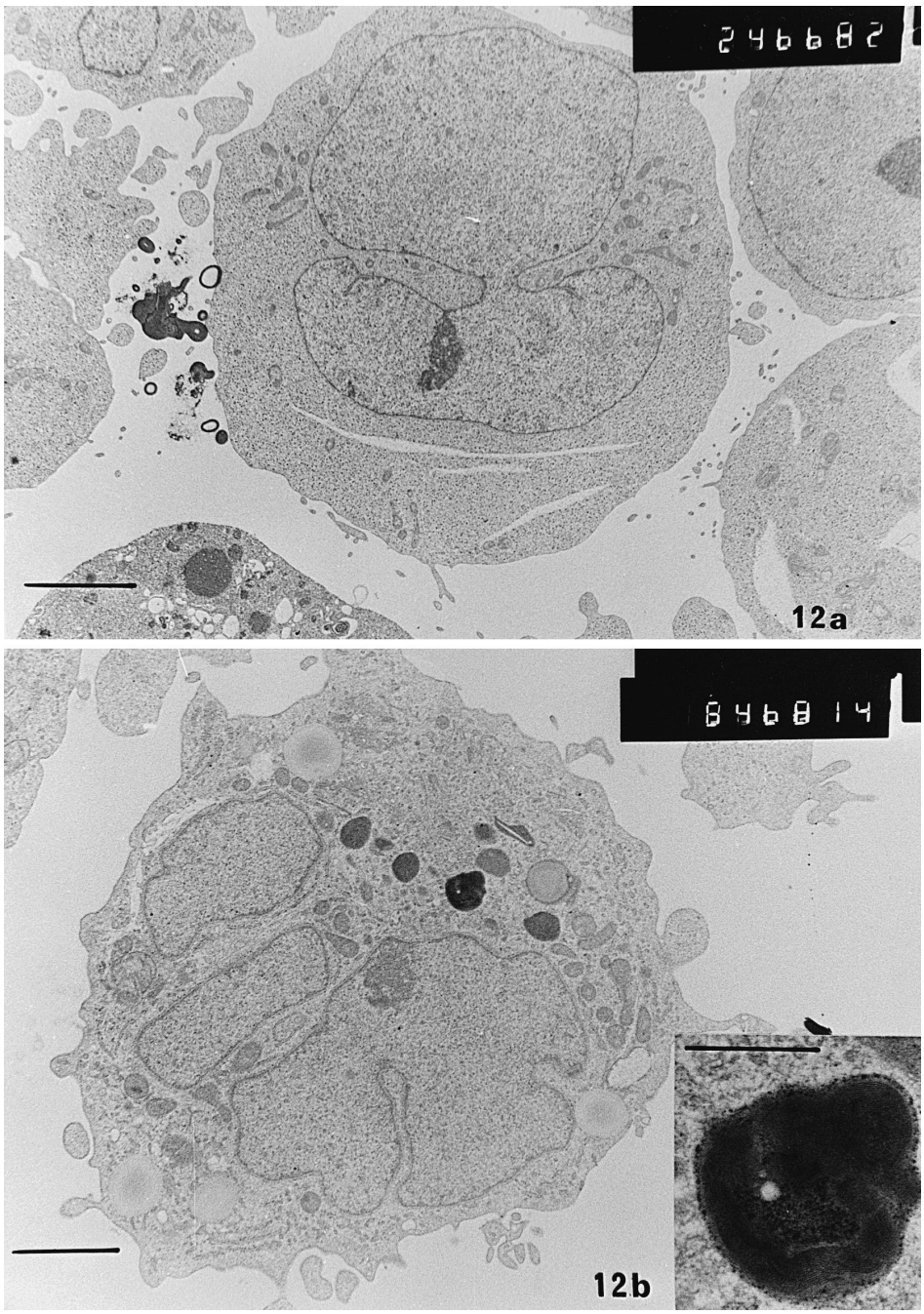


Fig. 12. (A) Lipoplex A (C18:1 DOPE DNA) 2.5 h after transfection starts, the lipoplex is outside the cell. Bar indicates 5 μm. (B) Lipoplex inside the cell, 4 days after transfection starts. Bar indicates 2 μm—insert: lipoplex at high magnification. Bar indicates 0.4 μm.

3.5. Influence of DOPE on transgene kinetic expression

3.5.1. C14:0 compounds

K562 cells were transfected with C14:0 phosphonolipids formulated in the presence or absence of DOPE. After transfection, the cells were assayed for β-galactosidase expression for up to 32 days. Two phosphonolipid–DOPE ratios were used (1:1 and 2:1), with the luminescence assay results for these experiments presented in Fig. 8a and b. When GLB73 was used without DOPE, β-galactosidase activity was maximum at day 3 (Fig. 8a). In the presence of DOPE in the cationic lipid formulation the maximum activity was delayed to day 14, irrespective of the ratio of DOPE used. Although the profile of the kinetic expression of the transgene was the same after transfection with GLB73 DOPE (1:1) and GLB73 DOPE (2:1), the prior had a higher transfection efficiency than the latter. In this experiment, a higher β-galactosidase activity after transfection with GLB73 in the absence of DOPE was observed at day 3. Similar results were observed with GLB84 (Fig. 8b), demonstrating once more that the counter-ion does not affect the kinetics of transgene expression.

3.5.2. C18:1 compounds

Similar experiments were carried out with the C18:1 phosphonolipids (Fig. 9a and b). Irrespective of the C18:1 compound and the formulation used (with or without DOPE), β-galactosidase activity was always maximum at day 14 after transfection. Fig. 9a and b show that the most appropriate phosphonolipid–DOPE ratio for optimal transfection activity was 2:1, regardless of the C18:1 phosphonolipid used. Moreover, the two C18:1 profiles were similar, although the β-galactosidase activity with GLB58 was lower than that with GLB43 in both the presence and absence of DOPE.

No influence of the counter-ion on the kinetics of transgene expression was observed and all results were confirmed with the CPRG test and FACS-gal assay.

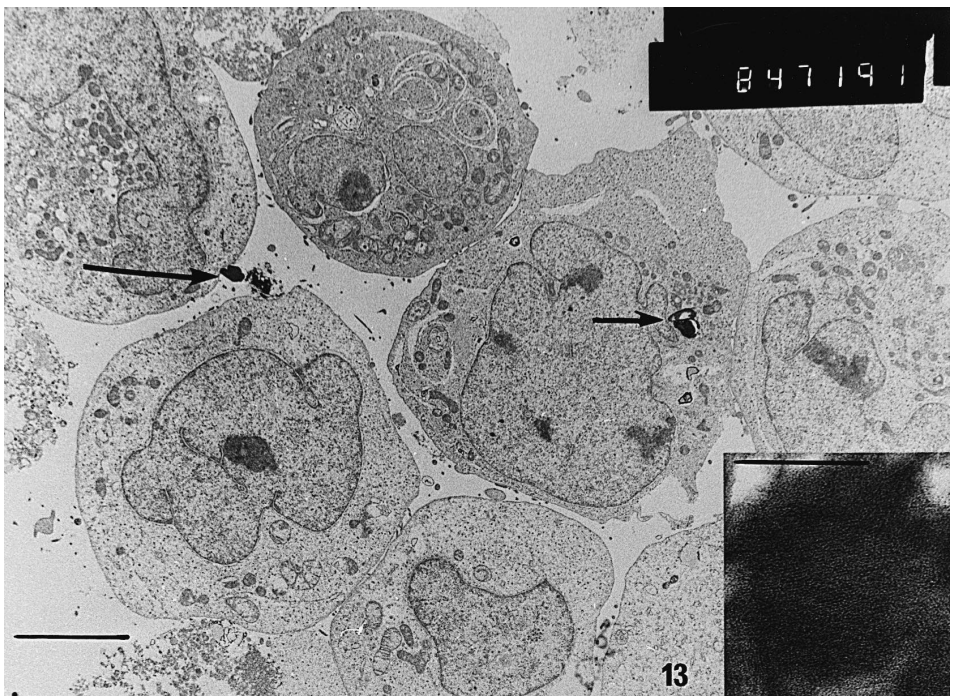


Fig. 13. Lipoplex A 17 days after transfection: lipoplex is observed outside the cell (long arrow) and inside the cell (short arrow). Bar indicates 5 μm. Insert: detail of lipoplex inside the cell (short arrow) at high magnification. Bar indicates 0.2 μm.

3.6. Electron microscopy study

Electron microscopy enabled us to follow the fate of the lipid–DNA complexes from 2.5 h to 17 days post-transfection. In a first set of experiments, the cells were transfected using two different lipoplexes, 2.5 h after the transfection the cells were fixed and electron microscopy study was carried out. Two lipid–DNA complexes were used, consisting of a C18:1 compound with DOPE (Lipid A) and a C14:0 compound (Lipid B). The two lipoplexes provided very different results concerning the shape. The first, DNA–lipid A, developed a highly ordered pattern as illustrated in Fig. 10a with regular concentric lamellae and a 'myeline figure' aspect. It possibly represents a strand of DNA surrounded by several bilayers of lipids. Lipoplex B (Fig. 10b) showed a bilayer structure but with a quite different fingerprint-like pattern with aggregated and fused particles. We noticed that 2.5 h after transfection, microscopy images showed that lipoplex B had bound to the plasma membrane and was incorporated into the cell by endocytosis (or phagocytosis). The mechanism is demonstrated in Fig. 11, highlighting the different steps that the process involves. Invaginations closely follow the particle boundaries. Fig. 12a shows that 2.5 h after transfection with lipoplex A (C18:1 DOPE), the lipoplex was still outside the cell, it could be observed within the cytoplasm only after 4 days following transfection (Fig. 12b). When the K562 cells were transfected with a C18:1 compound in the presence of DOPE, lipoplex structures were present inside and outside the cells for up to 17 days post-transfection (Fig. 13).

4. Discussion

The objective of this work was to examine the influence of (i) hydrophobic domain chemical structure and (ii) formulation (with or without DOPE) of cationic phosphonolipids on the kinetic expression of a transgene in a non-adherent hematopoietic cell line. The cationic phosphonolipids studied consisted of compounds with C18:1 to C12:0 symmetric hydrocarbon side-chains. All compounds were formulated in both the presence and absence of DOPE (1:1, w:w). The major findings of the study highlighted that:

-Cationic phosphonolipids can transfect the hematopoietic non-adherent cell line K562, with transgene expression being detected up to 28 days after transfection.

-Formulation of the phosphonolipids in the presence of DOPE did not enhance the transfection efficiency of cationic phosphonolipids in the non-adherent K562 cell line.

-The formulation and chemical structure of cationic phosphonolipids influence the kinetic expression of the transgene.

Many authors have stressed the fact that non-adherent cells are difficult to transfect with cationic vectors [33,34]. We report here a transfection efficiency with the K562 cell line equivalent to that observed with other adherent cell lines such as CFT1 and COS 7 [35] using cationic phosphonolipids. Optimal results were obtained for all cell lines with a C14:0 compound (i.e., GLB73). However, it was necessary to include DOPE in the formulation to obtain efficient transfection in the CFT1 and COS 7 adherent cell lines. In most publications, the lipid formulations tested have been prepared with DOPE, and the cells assayed for gene reporter activity 24 or 48 h after transfection. In the present study, under these transfection conditions (i.e., with DOPE) an efficient transfection of a lipid–DNA complex into non-adherent K562 cells was not obtained. Optimum results were obtained 72 h after the transfection of a C14:0 compound not formulated with DOPE. The most efficient transfection using a lipid formulation prepared with DOPE was observed 7 or 14 days after transfection. With a C18:1-DOPE compound, transgene expression was observed for up to 28 days. It could be of interest to know if the conclusion that the apparently poor transfection of non adherent cells by cationic lipids is a consequence of failure to look for transfection at long times is also valid for other types of cationic lipids. Under optimized conditions for transfection, our results were not consistent with a marked decrease in transfection efficiency between adherent and non-adherent cells described by some authors [33,34]. These observations led us to assume that cationic phosphonolipids do not induce differences in transfection efficiency between adherent and

non-adherent cell lines. On the other hand, they modify the kinetic expression of the transgene.

Several authors (see e.g., Refs. [28,29]) have reported that the inclusion of DOPE in the cationic lipid formulation increases transfection activity by enhancing mixing and fusion of liposomes and cell membranes. DOPE forms inverted hexagonal phase structures at ambient temperature [36,37], and may therefore facilitate the formation of similar structures when formulated with cytofectins. In this study, we compared the transfection efficiency of different cationic phosphonolipids formulated in the presence or absence of DOPE. Irrespective of the aliphatic chain length, the inclusion of DOPE in the cationic phosphonolipid formulation resulted in a decrease in transfection efficiency when the cells were assayed for β-galactosidase activity 3 days after transfection. DOPE is not always required to obtain transfection efficiency as has been illustrated by the high activity frequently observed with the DOTAP reagent [38].

The EM images obtained with a C14:0-DNA complex were similar to those described by Labat-Moleur et al. [34] who used Transfectam. The complexes appeared as aggregated and fused liposomes together with tubular spaghetti-like structures which may be considered as characteristic dark fingerprints. Sternberg et al. [39] reported improved transfection activity of the spaghetti-like structures. When DOPE and the C18:1 compound (i.e., GLB43) were used to prepare the lipoplex, the EM appearance was similar to that reported by Zabner et al. [40] who used DMRIE/DOPE and observed coiled bilayer-like structures with a myeline figure aspect. Zhou and Huang [41] also reported the same myeline figure aspect. These very different morphological features provided by the two types of lipoplex could explain variations observed in transfection activity 3 days after transfection.

Various reports have shown that chemical changes in the three principal structural motifs of cytofectins can modify transfection efficiency [38,42,29]. Such structure/activity studies have begun to establish general principles which can be applied to obtain optimum transfection efficiencies. While there has been no previous report on the importance of the chemical structure on the kinetic expression of transgenes, we have been able to establish one such relation. Using a C14:0 compound (i.e., GLB73) an elevated expression was observed at an earlier stage after transfection, but decreased rapidly such that no expression could be detected after 7 days. Adding DOPE to the C14:0 formulations (i.e., GLB73, GLB84) delayed the peak transgene expression such that it was observed only at day 14. Using C18:1 compounds (i.e., GLB43, GLB58) in the presence or absence of DOPE, maximal transgene expression was observed 14 days after transfection.

According to the hypothesis originally advanced by Akao et al. [43] stating that cytofectin transfection activity and transfection lipid bilayer fluidity are directly correlated, one of the primary requirements of an amphiphile for DNA transfection would be that the T_c (phase transition temperature between the gel and the crystalline phases) is lower than 37°C. Consequently, the transfection lipid is assumed to be under a fluid crystalline state at cell culture temperatures. Researchers supporting this hypothesis [29,43] have relied on the analysis of a limited number of cytofectin analogues and cell lines. At cell culture temperature, C14:0 compounds are supposed to be under lamellar phase (assumed $T_c > 37°$), and C18:1 compounds under inverted hexagonal phase (assumed $T_c < 37°$). The hexagonal inverted phase structures have been described as transfection enhancing structures [44]; DOPE is known to form inverted hexagonal structures at ambient temperature [36,37], such that it may facilitate the formation of similar structures when formulated with cytofectins. We postulated that the lipid composition of non-adherent cells could interfere with the uptake mechanism of the lipoplex. The same reasoning could be applied to the release of the lipoplex from the endosome because in our experiments DOPE did not enhance the release of the lipoplex from the endosome. EM studies showed lipoplex formulated with DOPE in endosomes for up to 17 days following transfection.

The results described here are not due to a particular characteristic of the cationic phosphonolipid because with adherent cells (CFT1, COS 7) [35] we confirmed all of the classical published hypotheses dealing with the DOPE-enhancing transfection effect. No differences were observed in the kinetics of transgene expression after transfection with C14:0 or C18:1 compounds.

It is important to highlight that experiments concerning adherent and non-adherent cells were per-

formed under the same conditions. Non-adherent cells have often been reported as being difficult to transfect, and hypotheses explaining the low transfection efficiency of cationic vectors on this cell type are limited. Labat-Moleur et al. [34] noted that lymphocytes and presumably other non-adherent cells are difficult to transfect with cationic vectors because DNA particles do not efficiently bind to the cell surface. This suggests that the particles bound to membrane components involved in Ca^{2+}-mediated cell anchoring to the extracellular matrix are present on adherent cells only. Our EM studies were not consistent with these observations: cationic phosphonolipid cell-binding and endocytosis images were observed 2.5 h after transfection. Examples in the literature comparing the lipid composition of erythrocyte (non-adherent cells) and liver cell (adherent cells) plasma membranes have shown variations in phospholipid percentages [45,46]. PE compounds represent 7% of the liver cell lipid membrane and 18% of the erythrocyte lipid membrane, and are known to be under inverted hexagonal phase at ambient temperature. The presence of DOPE or C18:1 compounds entails the formation of a structure which favours neither lipoplex penetration nor its release by endosomes. Additional studies focussed on the composition of plasma membranes of adherent and non-adherent cells would aid in the explanation of our results. Moreover, one may assume that other elements in the cell environment might affect the transfection process.

The results reported here are important, for they demonstrate that the short term activity of phosphonolipids depends on their formulation, and can thus be altered. Moreover, these findings highlight that some specific molecules can be used to obtain maximum expression activity two weeks after transfection. This could provide the basis for further research in this field.

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