The role of endosome destabilizing activity in the gene transfer process mediated by cationic lipids

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Abstract **We used a ^{32}P-labeled pCMV-CAT plasmid DNA to estimate the DNA uptake efficiency and unlabeled pCMV-CAT plasmid DNA to quantify the CAT activity after transfection of COS cells using each of the three following cationic compounds: [1] vectamidine (3-tetradecylamino-*N*-tert-butyl-*N'*-tetradecylpropionamidine, and previously described as diC14-amidine [1]), [2] lipofectin (a 1:1 mixture of *N*-(1-2,3-dioleyloxypropyl)-*N,N,N*-triethylammonium (DOTMA) and dioleylphosphatidylethanolamine (DOPE)), and [3] DMRIE-C (a 1:1 mixture of *N*-[1-(2,3-dimyristyloxy)propyl]-*N,N*-dimethyl-*N*-(2-hydroxyethyl) ammonium bromide (DMRIE) and cholesterol). Surprisingly, a high CAT activity was observed with vectamidine although the DNA uptake efficiency was lower as compared to lipofectin and DMRIE-C. Transmission electron microscopy (TEM) revealed endocytosis as the major pathway of DNA–cationic lipid complex entry into COS cells for the three cationic lipids. However, the endosomal membrane in contact with complexes containing vectamidine or DMRIE-C often exhibited a disrupted morphology. This disruption of endosomes was much less frequently observed with the DNA–lipofectin complexes. This comparison of the three compounds demonstrate that efficient transfection mediated by cationic lipids is not only correlated to their percentage of uptake but also to their ability to destabilize and escape from endosomes.**

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Key words: pCMV-CAT plasmid DNA; Cationic lipid; DNA uptake efficiency; CAT activity; COS cell; Vectamidine; Lipofectin; DMRIE-C; Electron microscopy

1. Introduction

Synthetic cationic lipids have been used extensively to facilitate delivery of DNA [1–6], mRNA [7,8], oligonucleotides [9] and proteins [10,11] into cultured cells. They have also been tested successfully for direct gene transfer into animals [12–14] and some of them are being evaluated in humans under clinical trials [15–17]. Their common structure is composed of an hydrophobic moiety (usually two hydrocarbon chains) and one positively charged headgroup. Most of them tend to form liposomes, much like phospholipids, when dispersed in an aqueous phase. Despite their wide use and potential applications as vectors for gene therapy, it is only recently that serious attempts have been made to better understand their mechanism of action. Successive and distinct steps are involved in cationic lipid-mediated gene delivery. Early steps, including the initial interaction of the cationic liposome with the DNA to form a complex [18–21] and internalization of the complexed DNA by the cell [22–27], are relatively well characterized. Release of DNA from the complex [28] and its accessibility to the transcription apparatus are still poorly understood. Endocytosis has been suggested as the main pathway of DNA–cationic lipid complex internalization by the cell [22–27] and the efficiency of this step has been proposed to be an important rate-limiting factor in transfection [24,25]. Recent studies have concluded that early escape of the complex from the endosomal/lysosomal compartment is crucial for efficient gene transfer and expression [23,26]. Indeed, entry of the complex into the lysosomal compartment has been suggested to cause massive DNA degradation and to prevent transfection. However, the relative importance of each step (efficiency of complex uptake by endocytosis and escape from endosomes) in determining the transfection yield is still a matter of debate.

We have designed a new cationic lipid (vectamidine) capable of transfecting DNA [1] and mRNA [8] into mammalian cells with high efficiency. In contrast to many other cationic compounds, the positive charge of vectamidine is associated with an amidine function rather than an amine. This feature may explain the novel properties of the DNA–vectamidine complex in transfection studies. Therefore, we compared vectamidine with two other commercial cationic lipid reagents (lipofectin and DMRIE-C), at the level of DNA uptake and transgene expression efficiency in the COS cell line. Interestingly, no correlation was found between these two parameters. Electron microscopy revealed that for the three different cationic compounds, DNA–cationic lipid complexes were located in endosomal structures. However, most endosomal membranes in contact with DNA vectamidine complexes (84% of cases) and several with DNA–DMRIE-C complexes (64% of cases), were discontinuous or absent. Endosomes containing DNA–lipofectin complexes exhibited disrupted structure much less frequently (21% of cases). More importantly, the frequency of the observed endosomal destabilization by the DNA–cationic lipid complex can be directly correlated to the efficiency of transfection mediated by the cationic lipid.

2. Materials and methods

2.1. Reagents

Vectamidine (diC14-amidine) was synthesized as described [1] and is available from Biotech. Tools (Brussels, Belgium). Vectamidine liposomes were prepared by the ethanol injection method [8]. [^{32}P]dCTP and the DNaseI/DNA polymerase mixture (cat. no. N 5000) were from Amersham. Lipofectin and DMRIE-C were purchased from Gibco BRL. DNA polymerase I was obtained from Boehringer Mannheim.

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2.2. Labeling of plasmid DNA with [^{32}P]dCTP

The plasmid DNA pCMV-CAT (containing the chloramphenicol acetyl transferase, *CAT*, bacterial gene under the control of the CMV immediate early promoter/enhancer) was kindly provided by Dr. R. Debs and was previously described [12]. The pCMV-CAT plasmid DNA was purified using a Quiagene kit according to the manufacturer's instructions. To label the pCMV-CAT plasmid DNA with ^{32}P, we used a modified nick translation technique: a 100 μl reaction mixture was prepared to contain 50 mM Tris-HCl, pH 7.5, 10 mM $MgSO_4$, 0.1 mM DTT, 50 μg/ml BSA, 0.1 mM dATP, 0.1 mM dGTP, 0.1 mM dTTP, 10 μg DNA, 2 μl (10 U/μl) DNA polymerase I, 2 μl of the DNA polymerase I/DNaseI mixture and 5 μl of 3000 Ci/mmol [^{32}P]dCTP. Under these conditions, the low DNaseI/DNA ratio and the increased DNA polymerase/DNaseI ratio allows incorporation of the labeled nucleotide into the plasmid DNA without significant alteration of plasmid integrity as assessed by agarose gel electrophoresis. Radiolabeled plasmid DNA was separated from non-incorporated [^{32}P]dCTP by chromatography using a G50 column (Pharmacia) according to manufacturer's instructions.

2.3. Cell culture

COS cells from the American Type Culture Collection (Rockville, MD) were grown in DMEM supplemented with 10% FBS, 1 mM pyruvate, 2 mM glutamine, 100 μg/ml kanamycin and non-essential amino acids in a 5% CO_2 atmosphere. Cells were passaged every 2 days.

2.4. Transfection and reporter gene assay procedures

Tranfection of cells was performed as described [1] with some modifications. Briefly, cells were plated in 6-well plates at 4×10^5 cells per well 20–24 h before transfection. To prepare transfection complexes, 1 μg of DNA and the desired amount of lipid (2 μg of vectamidine, 5 μg of lipofectin or 10 μg of DMRIE-C) were separately diluted in 50 μl of HBS at room temperature. They were mixed by pipetting up and down 3 times and incubated 15–20 min at room temperature. During this incubation time, cells were rinsed once and incubated in medium-free serum. The DNA–cationic lipid complexes were then diluted in 900 μl of medium-free serum and applied to cells for 4 h. At the end of the incubation, complexes were aspirated, cells trypsinized and transferred to 9-cm-diameter plates in 10 ml of serum-containing medium. About 48 h post-transfection, cells were washed 2 times with PBS and scraped in PBS with a rubber policeman. Cells were divided into two fractions and pelleted by centrifugation at 4°C. The first cell pellet was resuspended in 50 μl of 250 mM Tris-HCl, pH 7.8, and the cells were lyzed by three freeze-thawing cycles, heated for 5 min at 60°C and assayed for CAT activity as described [1]. Quantification of acetylated chloramphenicol was performed using the Phosphor Imager (Molecular Dynamics). The second cell pellet was lyzed in 50 μl of 0.5% Triton X-100 in PBS and assayed for protein concentration using the DC-protein assay (BioRad). CAT activity was expressed as pmoles of acetylated chloramphenicol/μg cell protein/h (CAT units).

2.5. DNA uptake measurement

In these experiments, ^{32}P-labeled plasmid DNA was used. DNA–lipid complexes were prepared and incubated with cells as described above. At the end of incubation with complexes, cells were washed 3 times with medium-free serum and once with the trypsin solution (Life Technology), then detached by trypsin treatment and pelleted by centrifugation followed by washing with PBS. This treatment allowed removal of complexes that are weakly attached to cell. Finally, cells were lyzed in 200 μl of buffer containing 10 mM Tris-HCl, pH 8, 150 mM NaCl, 10 mM EDTA, 0.5% SDS. The cell lysates were aspirated several times throughout a 0.4×12 mm syringe needle to disrupt viscosity then 50 μl of the lysate was mixed with 5 ml of Aquasol2 scintillation cocktail (Dupont) and the ^{32}P radioactivity was counted.

2.6. Electron microscopy

2.6.1. Vectamidine–DNA complex. Liposomes and DNA–liposome complexes were identified by transmission electron microscope (TEM) using a negative staining technique. Drops (50 μl) of freshly prepared samples were floated on collodion/carbon-coated 200 mesh nickel grids for 5 min. After washing for 30 s in PBS, the grids were incubated for 5–10 min with 2% phosphotungstic acid (pH 6.8). This solution was wicked off with filter paper and the grids were allowed to dry.

2.6.2. Interaction of DNA–cationic lipid complexes with cells. Transfected or untransfected COS cell cultures were scraped off the plates and centrifuged at $350\times g$ for 3 min to form pellets. Small fragments of the various pellets were fixed for 30 min at room temperature in 2.5% glutaraldehyde in 0.1 M Sorensen's buffer (pH 7.4). After washing in buffer, cells were post-fixed in 2% OsO_4 for 30 min at room temperature, dehydrated in ethanol (30%, 1×10 min; 70%, 3×10 min) and embedded in Epon 812 resin (70% ethanol/Epon, 1:1, 2×10 min; 70% ethanol/Epon, 1:2, 2×10 min; pure Epon, overnight). The polymerization was achieved at 60°C. Ultrathin sections (90 nm) were placed on 200 mesh nickel grids and stained with uranyl acetate and Reynold's lead citrate. The samples were examined in a Jeol $C\times100$ electron microscope at 60 kV.

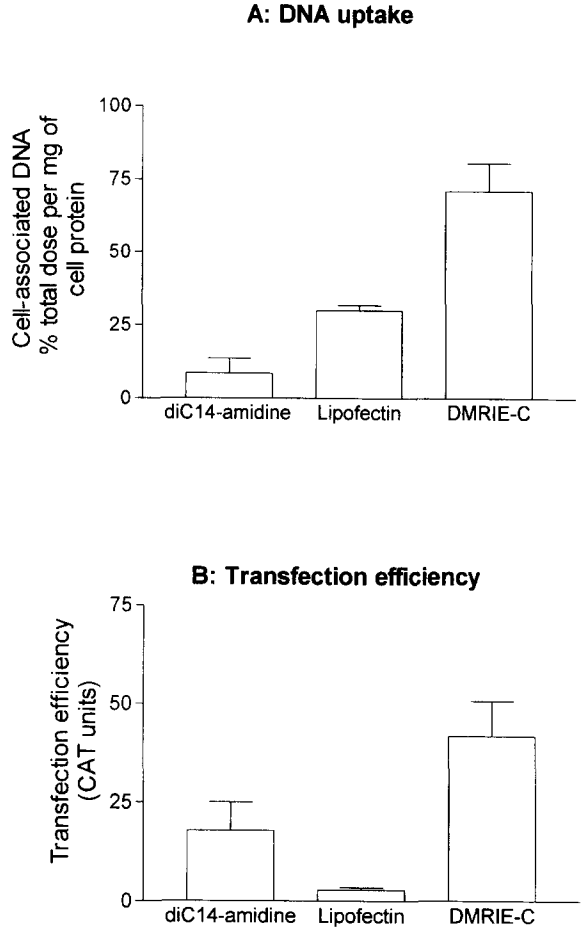

Fig. 1. Comparison of DNA uptake efficiency (% of cell-associated DNA) and transfection efficiency (CAT activity) in COS cells. Cells were plated in a 6-well multidishes at 4×10^5 cells per well (±24 h prior the time of transfection) then transfected for 4 h, using 1 μg of pCMV-CAT plasmid DNA and 2, 5 or 10 μg of Vectamidine, Lipofectin or DMRIE-C, respectively (for details, see Section 2). 48 h after transfection, cells were harvested and CAT activity quantitated (expressed as pmoles of acetylated chloramphenicol/μg cell protein/h = CAT units). B: Results are expressed as means ± SD from 2 different triplicate experiments. For DNA uptake measurements, ^{32}P-labelled pCMV-CAT plasmid DNA was used and cell-associated ^{32}P radioactivity measured at the end of the 4 h complexes incubation with cells (A). DNA uptake efficiency was expressed as the % of cell-associated DNA/mg of cell proteins. Results are expressed as means ± SD from 2 different triplicate experiments.

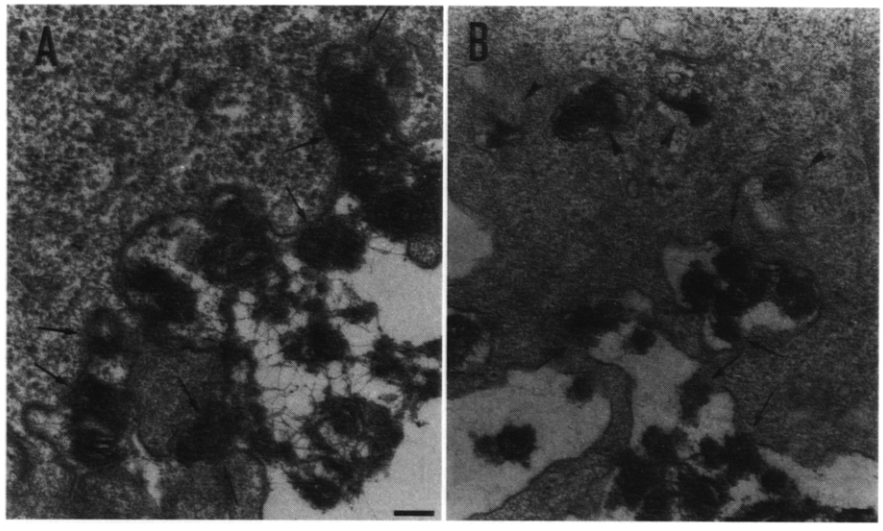

Fig. 2. Thin-section electron micrographs of COS cells transfected for 3 h with DNA–Vectamidine complexes. The complexes appear as dark aggregated ovoid structures formed by a pile of membranes. The complexes are bound to the plasma membranes (A) or/and included in endosomes (B). At the contact sites with the complexes, the plasma and endosomal membranes are frequently not visible (arrows). Most of complexes intruded into the cytoplasm are not or only partially surrounded by the endosomal membrane (arrowheads). Bars: 0.2 μm.

3. Results

3.1. Comparison between DNA uptake and transfection efficiency using vectamidine, lipofectin and DMRIE-C

In order to establish a correlation between the transfection efficiency and the uptake of DNA mediated by cationic lipids, COS cells were transfected with plasmid DNA containing the chloramphenicol acetyl transferase gene (*pCMV-CAT*) using vectamidine, lipofectin or DMRIE-C (at 2:1, 5:1 and 10:1; lipid to DNA weight ratios, respectively), and CAT activity was quantitated. The percentage of DNA comprised in the transfection complexes, under the lipid/DNA ratios used here, is close to 100% in all cases as evidenced by sucrose gradient analysis of the complexes (result not shown). In parallel, DNA uptake efficiency was measured using ^{32}P-labeled pCMV-CAT. Comparison of Figs. 1A and 1B clearly shows that there is no obvious correlation between the level of DNA uptake and transfection efficiency. This is evident for vectamidine, for which a weak uptake results in an unexpected high level of transfection. This result suggests that vectamidine may be more efficient in the steps following the DNA uptake step. Alternatively, vectamidine-mediated DNA delivery into the cells could proceed through a different pathway. The entry of the three DNA–cationic lipid complexes into cells was studied by electron microscopy.

3.2. Study of the pathway of DNA–cationic lipid complexes entry into cells by electron microscopy.

Ultrathin sections from transfected or untransfected cells were prepared and processed for TEM as described in Section 2. The complexes were identified by their higher electron density after staining. vectamidine–DNA complexes appeared as an aggregation of ovoid structures of multilamellar lipid membranes (Fig. 2). This observation on ultrathin section was confirmed by negative staining of vectamidine–DNA complexes adsorbed on a microscope grid (data not shown). The

Table 1

A Frequency of cell membranes destabilization and disruption at the complex attachment site

	Frequency of membrane disruption at the complex attachment site					
	Vectamidine		DMRIE-C		Lipofectin	
Plasma membrane	76%	$n=41$	28%	$n=32$	25%	$n=71$
Endosomes	81%	$n=32$	64%	$n=14$	21%	$n=24$

B Evaluation of intracellular complexes as compared to total cell-associated complexes

	Distribution of complexes in COS cells					
Liposomes included in complexes at the cell surface	523		2905		1087	
Liposomes included in complexes inside the cells	122	(18%)	897	(23%)	324	(23%)

A: COS cells were transfected with the DNA–cationic lipid complexes and ultrathin sections were prepared and observed by TEM as described in Section 2. For each lipid type, a number (n) of complexes, visible on ultrathin sections, either attached at the plasma membrane or included into endosomes, were randomly chosen. Among these complexes, those showing membrane destabilization and direct contact with the cytosol were counted and percentage of destabilization was obtained by dividing the obtained value by the total number (n) of complexes.

B: 20 different ultrathin sections from cells transfected with vectamidine, lipofectin or DMRIE-C were selected to contain complexes. The total number of liposomes included in complexes that are attached to the cell surface and liposomes included in complexes that are located inside the cell cytoplasm were counted. The percentage of intracellular complexes (indicated in parentheses) is expressed as the ratio of intracellular liposomes included in complexes versus total liposomes included in complexes (liposomes included in cytoplasmic plus cell surface attached complexes).

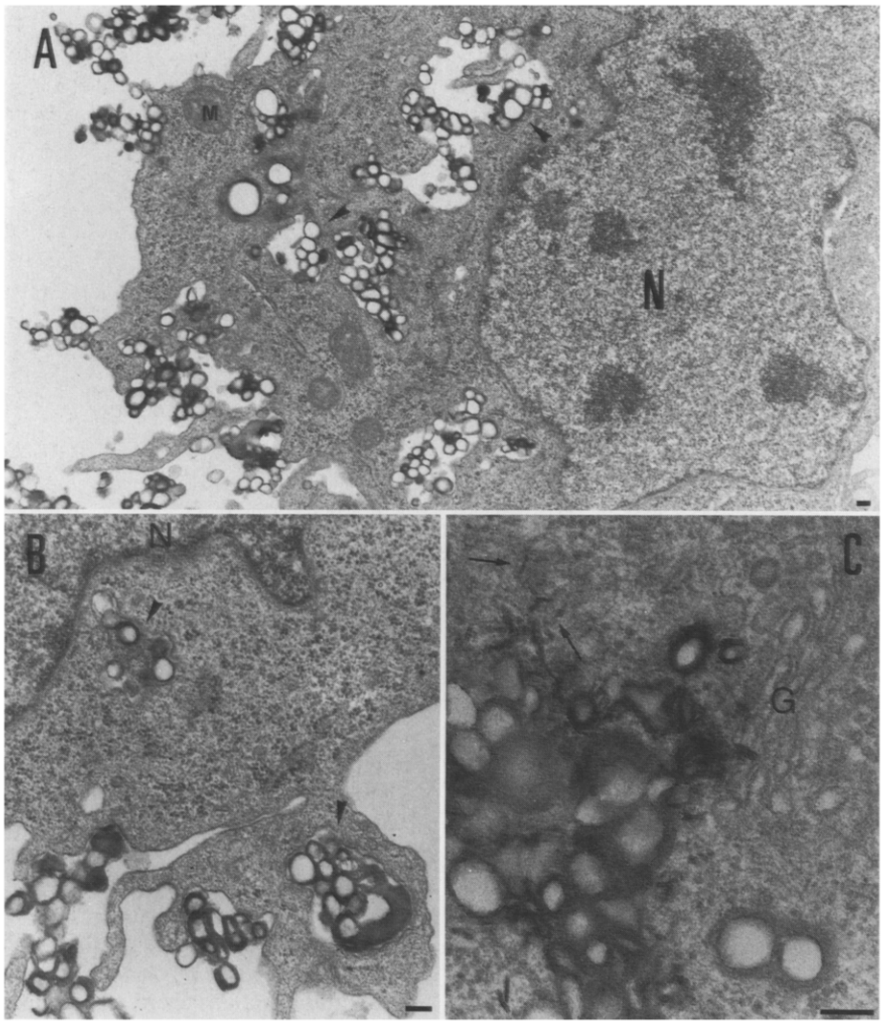

Fig. 3. Thin-section electron micrographs of COS cells transfected for 6 h with DNA–DMRIE-C complexes. The complexes look like aggregation of round plurilamellar hollow structures. A: General view of a COS cell showing many complexes bound to the cell surface or included in endosomal vesicles. B: Detail of a COS cell showing complexes in close contact with the plasma membrane or in endosomes. While the plasma membrane often seems continuous at the complex attachment site, discontinuities of endosomal membrane appear at the contact site with complexes (arrowheads). C: Detail of a COS cell showing a large and non-membrane-bound aggregate of complexes in the cytoplasm near the Golgi apparatus (G). Heavily stained membrane segments lie in the surrounding regions and seems derived from the complexes (arrows). N: nucleus, M: mitochondria. Bars: 0.2 μm.

size of individual liposomes entrapped in the aggregate (average: 240 ± 94 nm; $n = 50$) was in agreement with our previous dynamic laser light scattering results [8]. In some cells, these structures were bound to the plasma membranes (Fig. 2A) and/or included in endosomal vesicles (Fig. 2B). Occasionally, some structures were also seen associated with coated pits. The plasma or endosomal membrane was very often interrupted at contact sites with the DNA–vectamidine complexes (Fig. 2, arrows). Most of the complexes intruded into the cytoplasm were free or only partially surrounded by the endosomal membrane (Fig. 2B, arrowheads). In order to quantitate this observation, a number (n) of complexes were chosen randomly and those showing cell membrane destabilization and direct contact with cytoplasm were counted. Results are summarized in Table 1A. Plasma and endosomal membranes were found to be destabilized when in contact with vectamidine–DNA complexes in 76% ($n = 41$) and 81% ($n = 32$) of cases, respectively. However, a background should be subtracted from this values. Indeed, cell membranes were not always visible, even in regions devoid of complexes. This is most likely because of an artifact due to a vanishing contrast when the membrane is tilted with respect to the section plane.

The same TEM approach was applied to cells transfected with lipofectin or DMRIE-C. On ultrathin sections, DNA–DMRIE-C complexes appeared as an aggregation of round, plurilamellar and hollow structures (Fig. 3) whereas DNA–lipofectin complexes appeared as an aggregation of dark, ovoid plurilamellar and hairy particles (Fig. 4). The average size of individual DMRIE-C and lipofectin liposomes was smaller than that of DNA–vectamidine liposomes (198 ± 80 nm ($n = 50$) for DMRIE-C and 148 ± 53 nm ($n = 50$) for lipofectin as compared with 240 nm for vectamidine). Both DNA–DMRIE-C and DNA–lipofectin complexes were bound to the cell surface and were taken up by endocytosis. Interaction of the DNA–lipofectin complexes with the cells was frequently observed at coated pits.

In contrast to DNA–vectamidine complexes, which were detected in a small fraction of cells (only 2%, out of 160 randomly chosen ultrathin sections, contained complexes), DNA–lipofectin and DNA–DMRIE-C complexes were de-

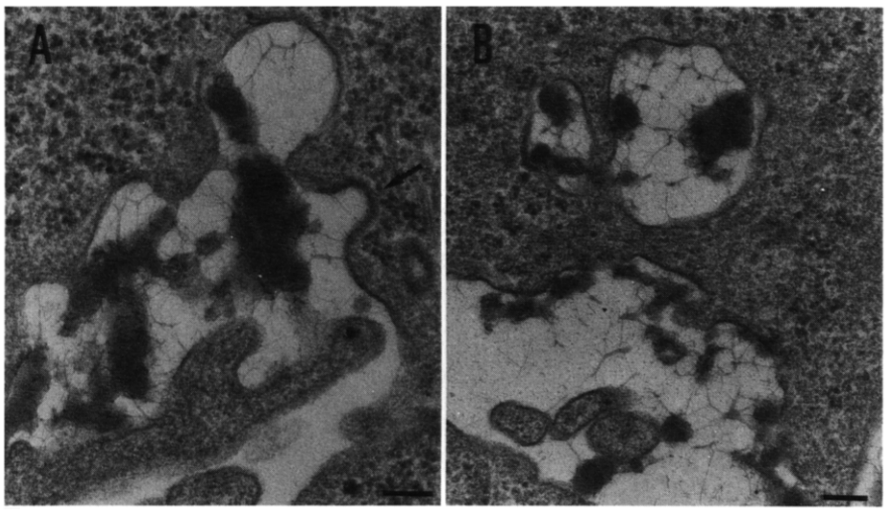

Fig. 4. Thin-section electron micrographs of COS cells transfected for 6 h with DNA–Lipofectin complexes. The complexes appear as aggregated, dark, ovoid, plurilamellar and hairy particles. Such particles are found at the cell surface (A) and in endosomal vesicles (B). Both plasma and endosomal membranes most often seems continuous at the complex attachment site. Arrow indicates a coated pit. N: nucleus. Bars: 0.2 μm.

tected in nearly all cells (96% and 94% for lipofectin and DMRIE-C, respectively, out of 122 randomly chosen ultrathin sections for each lipid, contained complexes). Moreover, the number of complexes per cell was significantly higher in the case of DMRIE-C and lipofectin as compared to vectamidine (Table 1B). These results are in agreement with the [^{32}P]DNA uptake measurement (Fig. 1A).

It was also interesting to note that with these two cationic lipids the plasma membrane often seemed continuous at the complex attachment site. Indeed, there was only 25–28% ($n = 103$) of situations where discontinuity appeared as compared with 76% for vectamidine. In the case of DMRIE-C, however, a 64% ($n = 14$) frequency of disruption at the complex attachment site in the endosome was observed. This disruption of endosomes was much less frequently observed (21%, $n = 24$) with the DNA–lipofectin complexes. Finally, these two cationic lipid–DNA complexes were also found in large membrane-bound vesicles near the Golgi apparatus and none were observed in the nucleus.

4. Discussion

The use of cationic lipids as DNA delivery systems for gene therapy is still limited by the relative inefficiency of the gene transfer process compared with viral vectors (i.e. adenovirus). Understanding the cellular pathways and limitations of the cationic lipid-mediated gene transfer could allow the design of more efficient cationic vectors. We compare here vectamidine with two reference compounds (lipofectin and DMRIE-C) regarding the DNA uptake efficiency and transgene expression efficiency of the corresponding DNA–lipid complexes. An interesting feature are the observed differences in complex morphology on ultrathin sections, when using different cationic lipids. At present, we do not know whether these differences in complex morphology affect their interaction with cells, but this aspect surely merits further investigations.

The first step in cationic lipid-mediated DNA transfection is the capture and uptake of the cationic lipid–DNA complexes by the cell. This process may be influenced by several parameters such as the charge and the size of the complexes [14,25]. It was suggested that large DNA–cationic liposome complexes are not efficiently taken up by the cell [25]. Electron microscopy showed that vectamidine tends to form large complexes with DNA. This could explain, at least in part, the lower DNA uptake observed with vectamidine in the cell line tested here. It is clear that our [^{32}P]DNA uptake measurement (Fig. 1A) did not allow discrimination between internalized DNA–lipid complexes and cell surface-bound complexes. However, Table 1B shows that the percentage of intracellular complexes relative to total cell-associated complexes is similar for each compound and is close to 20%.

The fact that DNA uptake efficiency did not necessarily lead to transgene expression efficiency (Fig. 1), clearly indicates that the uptake step is not the single limiting factor in transfection. Electron microscopy supported the idea that for all cationic lipids studied here, endocytosis is the main pathway of complex internalization by the cells. Moreover, the endosomal membranes were often discontinuous and absent in contact with DNA–vectamidine and DNA–DMRIE-C complexes. Endosomes containing DNA–lipofectin complexes less frequently exhibited this disrupted morphology. This suggests that vectamidine and DMRIE-C are more efficient in promoting escape of complexes from endosomes than was lipofectin and may explain the relative inefficiency of lipofectin, compared to vectamidine and DMRIE-C, in terms of transgene expression. Indeed, only complexes that escape from endosomes are expected to deliver their DNA to the cytoplasm and thus permit its entry into the nucleus and finally expression [23,26,28].

The plasma membrane was also often absent when in contact with DNA–vectamidine complexes and some complexes were found to be free in the cytoplasm. This suggests that, in the case of vectamidine, a direct translocation across the plasma membrane may be also involved in the complex entry into the cytoplasm. The frequency of such a mechanism is difficult to estimate relative to the endocytosis pathway with TEM [22,23]. Indeed, such an event is expected to be faster than endocytosis which would prevent its detection by electron microscopy.

Fusion between the cationic liposome of the complex and

the endosomal membrane has been proposed to explain the mechanism of endosomal escape and DOPE has been suggested to play a major role in the fusion process [23,24,26,29]. It is worth mentioning that both vectamidine and DMRIE-C contained a C14 hydrocarbon chain, but no DOPE, and were efficient in promoting complex escape from endosomes and transgene expression. This suggests that the interaction between cationic lipids containing a short saturated hydrocarbon chain and endosomal membranes may involve a different mechanism than the previously suggested DOPE-mediated fusion mechanism. Recent data support the idea that lipid fusion can occur even in the absence of DOPE [30].

In conclusion, this study reveals several important observations. Endocytosis is the major pathway of DNA–cationic lipid complex uptake by COS cells. Large DNA–cationic lipid complexes are not efficiently taken up by cells. However, the uptake efficiency is not the rate-limiting step of transfection in our case. The ability to destabilize and escape from endosomes/lysosomes, crucial for transfection, could be correlated with the presence of a short (C14) saturated hydrocarbon chain in the hydrophobic domain of the cationic lipid and to a lesser extent, to the presence of DOPE as a helper lipid. With vectamidine, a high transfection efficiency was observed despite the absence of DOPE and a low amount of cell-associated DNA.

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References

[1] Ruysschaert, J.M., El Ouahabi, A., Willeaume, V., Huez, G., Fuks, R., Vandenbranden, M. and Di Stefano, P. (1994) Biochem. Biophys. Res. Commun. 203, 1622–1628.
[2] Felgner, J.H., Kumar, R., Sridhar, C.N., Wheeler, C.J., Tsai, Y.J., Border, R., Ramsey, P., Martin, M. and Felgner, P.L. (1994) J. Biol. Chem. 269, 2550–2561.
[3] Gao, X. and Huang, L. (1995) Gene Ther. 2, 710–722.
[4] Felgner, P.L., Gadek, T.R., Holm, M., Roman, R., Chan, H.W., Wenz, M., Northrop, J.P., Ringold, G.M. and Danielsen, M. (1987) Proc. Natl. Acad. Sci. USA 84, 7413–7417.
[5] Leventis, R. and Silvius, J.R. (1990) Biochim. Biophys. Acta 1023, 124–132.
[6] Behr, J.P., Demeneix, B., Loeffler, J.P. and Perez-Mutul, J. (1989) Proc. Natl. Acad. Sci. USA 86, 6982–6986.
[7] Malone, R.W., Felgner, P.L. and Verma, I.M. (1989) Proc. Natl. Acad. Sci. USA 86, 6077–6081.
[8] El Ouahabi, A., Pector, V., Fuks, R., Vandenbranden, M. and Ruysschaert, J.M. (1996) FEBS Lett. 380, 108–112.
[9] Capaccioli, S., Di Pasquale, G., Mini, E., Mazzei, T. and Quattrone, A. (1993) Biochem. Biophys. Res. Commun. 197, 818–825.
[10] Debs, R.J., Freedman, L.P., Edmunds, S., Gaensler, K.L., Duzgunes, N. and Yamamoto, K.R. (1990) J. Biol. Chem. 265, 10189–10192.
[11] Walker, C. (1992) Proc. Natl. Acad. Sci. USA 89, 7915–7919.
[12] Zhu, N., Liggitt, D. and Debs, R. (1993) Science 261, 209–211.
[13] Hyde, S.C. et al. (1993) Nature 362, 250–255.
[14] Solodin, I., Brown, C.S., Bruno, M.S., Chow, C.Y., Jang, E.H., Debs, R.J. and Heath, T.D. (1995) Biochemistry 34, 13537–13544.
[15] Nabel, G.L. et al. (1993) Proc. Natl. Acad. Sci. USA 90, 11307–11311.
[16] Caplen, N.J. et al. (1995) Nature Med. 1, 39–46.
[17] Nabel, G.J., Chang, A.E., Nabel, E.G., Plautz, G.E., William, E., Fox, B.A., Felgner, P., Shu, S. and Cho, K. (1994) Hum. Gene Ther. 5, 57–77.
[18] Gershon, H., Ghirlando, R., Guttman, S.B. and Minsky, A. (1993) Biochemistry 32, 7143–7151.
[19] Sternberg, B., Sorgi, F.L. and Huang, L. (1994) FEBS Lett. 356, 361–366.
[20] Gustafsson, J., Arvidson, G., Karlsson, G. and Almgren, M. (1995) Biochim. Biophys. Acta 1235, 305–312.
[21] Mahato, R.I., Kawabata, K., Nomura, T., Takakura, Y. and Hashida, M.J. (1995) Pharmacol. Sci. 84, 1267–1271.
[22] Friend, D.S., Papahadjopoulos, D. and Debs, R.J. (1996) Biochim. Biophys. Acta 1278, 41–50.
[23] Zhou, X. and Huang, L. (1994) Biochim. Biophys. Acta 1189, 195–203.
[24] Worbel, I. and Collins, D. (1995) Biochim. Biophys. Acta 1235, 296–304.
[25] Hui, S.W., Langner, M., Zhao, Y.L., Ross, P., Hurley, E. and Chan, K. (1996) Biophys. J. 71, 590–599.
[26] Zabner, J., Fasbender, A.J., Moninger, T., Poellinger, K.A. and Welsh, M.J. (1995) J. Biol. Chem. 270, 18997–19007.
[27] Labat-Moleur, F., Steffan, A.-M., Brisson, C., Perron, H., Feugeas, O., Furstenberg, P., Oberling, F., Brambilla, E. and Bher, J.-P. (1996) Gene Ther. 3, 1010–1017.
[28] Xu, Y. and Szoka, F.C. (1996) Biochemistry 35, 5616–5623.
[29] Farhood, H., Serbina, N. and Huang, L. (1995) Biochim. Biophys. Acta 1235, 289–295.
[30] Martin, I. and Ruysschaert, J.M. (1997) FEBS Lett. 405, 351–355.