Pharmaceutical Research, Vol. 13, No. 9, 1996 Report

Intracellular Distribution and Mechanism of Delivery of Oligonucleotides Mediated by Cationic Lipids

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Received May 7, 1996; accepted June 17, 1996

Purpose. To study the parameters influencing the intracellular trafficking of oligonucleotides delivered by cationic 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) lipids and to elucidate the mechanism of uptake.
Methods. We have studied the intracellular localization of fluorescently labeled oligonucleotide (F-ODN) delivered by DOTAP using confocal microscopy and measured inhibition of luciferase synthesis. The delivery mechanism of ODN/DOTAP complexes was investigated using inhibitors of the endocytosis pathway.
Results. F-ODN delivered by DOTAP liposomes redistribute from punctate cytoplasmic regions into the nucleus. The nuclear uptake of F-ODN depends on: charge ratio (+/−), time of incubation, temperature and presence of serum. A positively charged complex is required for enhanced uptake. The association of neutral lipids with DOTAP reduced the optimum charge ratio without altering the delivery efficiency. DOTAP lipids increased >100 fold the antisense activity of a specific anti-luciferase ODN. Inhibitors of the endocytosis pathway show that the majority of F-ODN are introduced through an endocytic pathway mainly involving uncoated vesicles. Nuclear accumulation of oligonucleotides can be decreased by inhibitors of actin microfilaments, energy metabolism and proteins implicated in the fusion of endosomes. Nuclear uptake is independent of acidification of the endosomal vesicles and unaffected by inhibitors of microtubules.
Conclusions. Oligonucleotides are delivered by cationic lipids into the cytoplasm at an early stage of the endocytotic pathway which leads to a marked increase in antisense activity and oligonucleotide nuclear uptake.

KEY WORDS: oligonucleotides; cationic liposomes; intracellular distribution; gene therapy; drug delivery.

INTRODUCTION

The use of antisense oligonucleotides to down regulate specific gene products requires oligonucleotides to enter cells and hybridize to the target mRNA present in the cytoplasm and/or in the nucleus of cells (1). Nevertheless, the poor ability of oligonucleotides to cross the cell membrane greatly limit their potency. To bypass this problem, liposomes (anionic, cationic, fusogenic, pH-sensitive and immunoliposomes) have been successfully used to deliver and increase the cellular uptake of oligonucleotides into cells (2). Among these carriers, cationic lipids are reported to markedly prevent nuclease degradation, enhance the rate of oligonucleotide cellular uptake and modify their intracellular distribution (2, 3, 4). Antisense oligonucleotides complexed to cationic liposomes have been successfully used in various biological models that demonstrate the potential of these agents to deliver oligonucleotides (2). However, since earlier observation that cationic lipids alter intracellular trafficking of oligonucleotides (4) little has been published on the intracellular distribution of oligonucleotides and the parameters involved in it. These factors are nevertheless important for the optimal utilization of antisense oligonucleotides for cell biology experiments as well as for therapeutic applications.

Recently, Zabner and coworkers have described cellular mechanisms for gene delivery mediated by cationic lipids and identified movement of plasmid DNA from cytoplasm to the nucleus as limiting to successful gene transfer (5). From these results, it is clear that the behavior of plasmid DNA and oligonucleotides are different since oligonucleotides localize into the nucleus after microinjection (6) or after delivery by cationic lipids (4). Herein we investigated the uptake pathway and ODN intracellular distribution mediated by ODN/DOTAP lipids complexes.

MATERIALS AND METHODS

Cells and Reagents

CV-1 (monkey kidney fibroblast) and CV-1 luciferase expressing cell lines were cultivated as described (7). 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and dioleoylphosphatidylethanolamine (DOPE) were obtained from Avanti Polar Lipids Inc. (Alabaster, AL). Cholesterol, metabolic inhibitors and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) were obtained from Sigma. Monensin was purchased from Calbiochem.

Oligonucleotides

5′ end fluorescein-labeled phosphorothioate oligonucleotides were a generous gift of G. Zon of Lynx Therapeutics Inc, Hayward, CA. An antisense anti-rev 28-mer (5′-TCGTCGCTGTCTCCGCTTCTTCCTGCCA-3′) and a 25-mer complementary to the murine β actin messenger RNA (5′-TCTGGGTCATCTTTTCACGGTTGGC-3′) (8). The uniformly ^{35}S-radiolabeled 18-mer phosphorothioate oligonucleotide was a generous gift from Dr. M. Woodle of Genta Inc., San Diego, CA. To measure antisense inhibition, a phosphorothioate oligonucleotide targeting the coding region starting at nucleotide 341 of the luciferase gene (5′-TCATACTGTTGAGCA-3′) and a reversed sequence (5′-ACGAGTTGTCATACT-3′) as a control were used.

Cellular Uptake of ^{35}S-Oligonucleotides

CV-1 cells were seeded at 2.5×10^5 cells for 16–18h and then incubated with various concentrations of ^{35}S-labeled ODN (specific activity 150μCi/μmole) with or without DOTAP for 3h at 37°C in serum free medium. For the 24 hour incubations, 10% FBS was added after the initial 3h incubation in serum free medium. After incubation, culture medium was recovered and cells were washed with cold PBS. Radioactivity associated with the cells or with the culture medium pooled with the PBS washes were analyzed by liquid scintillation (ScintiVerse®, Fisher Scientific, NJ, USA) spectrometry.

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Preparation of Liposomes and Complexes with Oligonucleotides

Cationic liposomes (DOTAP; DOTAP:DOPE, 1:1 (molar ratio of components); DOTAP:cholesterol, 1:1 and DOTAP:DOPE:cholesterol, 1:1:1) were prepared as reported (7). For the preparation of ODN/lipid complexes, 0.25μg of F-ODN was added to different concentrations of cationic liposomes (charge ratio +/− range 0.625–20 fold). The mean diameter of DOTAP liposomes was 20–50nm. After addition of oligonucleotides the size increased, 90% of complexes have diameter of 120 ± 40nm and the remaining fraction have diameters >200nm as determined by light scattering (Zetasizer 4, Malvern Instruments Ltd., UK).

Antisense Activity of ODN Delivered by DOTAP Lipids

The luciferase-expressing CV-1 cells were seeded at 5×10^5 cells. After overnight incubation, cells were incubated with ODN alone or complexed to DOTAP in serum free medium for 4h at 37°C. Then, the medium was removed and replaced by 10% FBS containing medium. After 24h of incubation, the level of luciferase expression was measured using the luciferase assay kit (Promega, Madison, WI) on an Opticompt luminometer (GMG Instruments, Hamden, CT). Total protein per well was measured using the Bio-Rad protein assay. Cell viability was tested by a colorimetric MTT assay as described (9).

Intracellular Distribution Studies

F-ODN (0.2μg) alone or as complexes were incubated in DME-H21 medium without serum on CV-1 cells grown on coverslips. Cells were then incubated at 37°C for various times (30min. to 48h). In the time course study, the cells were incubated with the complexes for 3h in serum free medium, followed by incubation with medium containing 10% of serum for 24 or 48h points. Oligonucleotide concentration ranged from 140–160nM. The lipid concentration varied as a function of the charge ratio (2.4 and 76μM). After incubation, CV-1 cells were washed in PBS and coverslips were immediately mounted on hanging drop slides (Fisher Scientific) with PBS. Cells were immediately examined by confocal microscopy.

Effects of Endocytosis Inhibitors on ODN Delivery by DOTAP

Prior to incubation with F-ODN/DOTAP complexes, CV-1 cells were preincubated 30min. with either chloroquine (100μM), NH_4Cl (20mM), monensin (10μM), or bafilomycin A (200nM) at 37°C, or at 4°C with nocodazole (33μM). With azide/deoxyglucose (10mM/25mM), cytochalasin B (25μg/ml) or N-ethylmaleimide (40μM) cells were preincubated 15min. at 4°C. Then cells were washed once in serum free medium and incubated with F-ODN/cationic lipid complexes as above. During the three hours of incubation, inhibitors were present in serum free medium.

Confocal Laser Scanning Microscopy

A MRC-600 (Biorad) confocal laser scanning imaging system with Krypton/Argon mixed gas laser and equipped with an upright microscope (Nikon, NY) and a 60× oil immersion objective (Nikon, NY) was used. COMOS confocal software program was employed to control the confocal functions and to analyze images.

RESULTS

Intracellular Localization of F-ODN +/− DOTAP Lipids

F-ODN added in culture media (140nM) were poorly internalized and localized into punctuate cytoplasmic regions (Fig. 1B) probably corresponding to endocytic compartments (10). Whatever the fluorescent probe, fluorescein or rhodamine, the concentration used (up to 2.8μM) and time tested, (3, 24 or 48h), oligonucleotides were never detected in the nucleus of viable cells. In contrast, after their association with DOTAP lipids at a charge ratio +/− of 10, the cellular fluorescence increased and F-ODN were mainly localized in the nucleus (Fig. 1C). Some diffuse fluorescence can also be seen in the cytoplasm but never in nucleoli. Thus, the intracellular distribution of oligonucleotides is modified when delivered by DOTAP as also described with other cationic lipids (4).

The comparison of fluorescence intensity between Fig. 1B and C demonstrates that DOTAP have increased the cellular accumulation of oligonucleotides. The analysis of fluorescence intensity (pixels)/cells revealed that the mean of pixels was at least 10 times higher with F-ODN/DOTAP complexes than with F-ODN alone (data not shown). The nuclear fraction of fluorescent oligonucleotides delivered by DOTAP represented approximately 60% of the total cell associated.

Cellular Uptake of ^{35}S-Labeled ODN +/− DOTAP Lipids

To quantify the enhancement of the total cellular uptake we have used a uniformly labeled ^{35}S-oligonucleotide. After 3h of incubation with CV-1 cells, the uptake of ^{35}S-ODN free in solution was low and concentration dependent (Fig. 2), as reported (10). The total cell-associated radioactivity corresponded 0.5–2% of the dose added to the cells (1.5–4.5pmoles of oligonucleotides). The steady-state association of ^{35}S-ODN with CV-1 cells was reached after 3h (data not shown).

When ^{35}S-ODN were complexed to DOTAP, cellular uptake was increased by 3 to 20 fold as a function of the oligonucleotide concentration and +/− charge ratio of the complex (Fig. 2). The highest uptake was seen with complexes formulated with a 10 fold excess of positive charge. At the same ODN concentration used for confocal microscopy studies, the association of ^{35}S-ODN with cells was enhanced by a factor 8–10 with DOTAP at a charge ratio of 10 and by a factor 3–4 at charge ratio of 2.5.

Antisense Activity of ODN Delivered by DOTAP Lipids

We tested the antisense activity of an ODN targeting the luciferase gene. No inhibition of luciferase expression was detected up to 50μM of free antisense ODN (Fig. 3). When complexed to DOTAP lipids at 10/1 charge ratio, antisense ODN become active with an IC_{50} around 125nM (Fig. 3). Thus DOTAP increased the activity of a specific ODN by a factor >100.

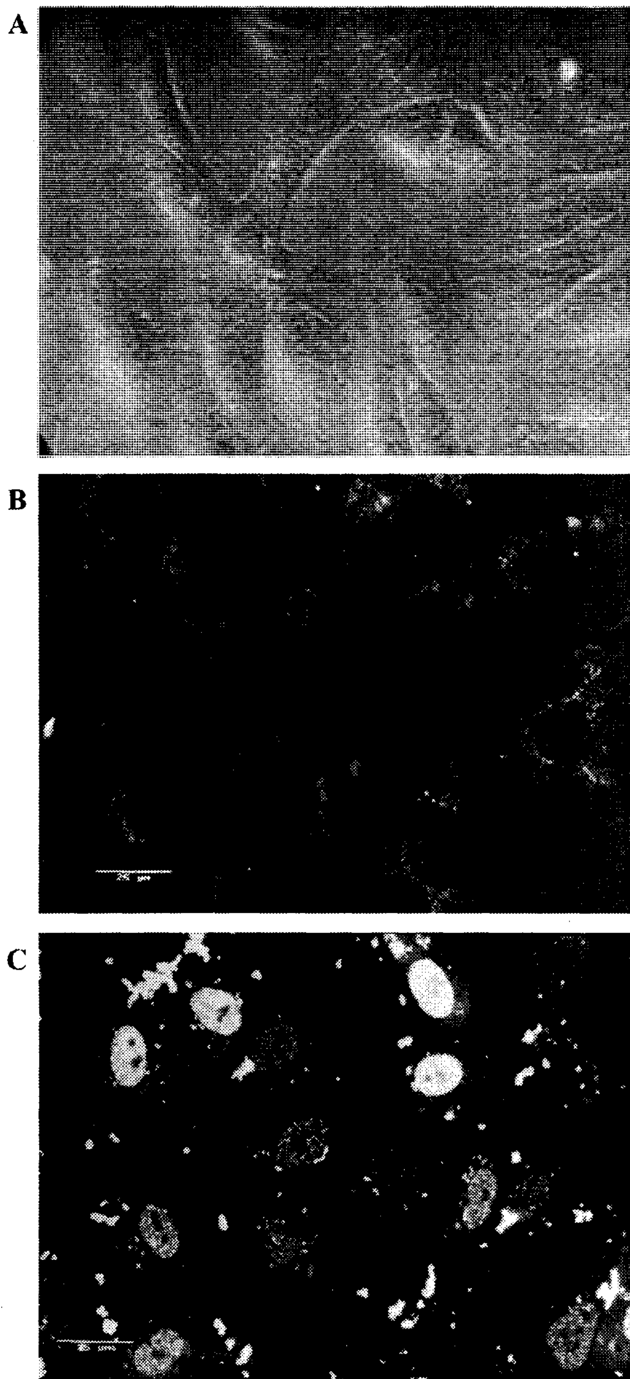

Fig. 1. Cellular uptake of F-ODN +/− cationic DOTAP lipids. F-ODN (140nM) alone or complexed to DOTAP (38μM) at a 10/1 charge ratio were incubated in serum free medium on CV-1 cells. After 3h at 37°C, cells were observed by confocal microscopy as described in materials and methods. **A**) Direct transmitted light of CV-1 cells; **B**) F-ODN incubated alone on CV-1 cells; **C**) F-ODN/DOTAP at 10/1 charge ratio (+/−).

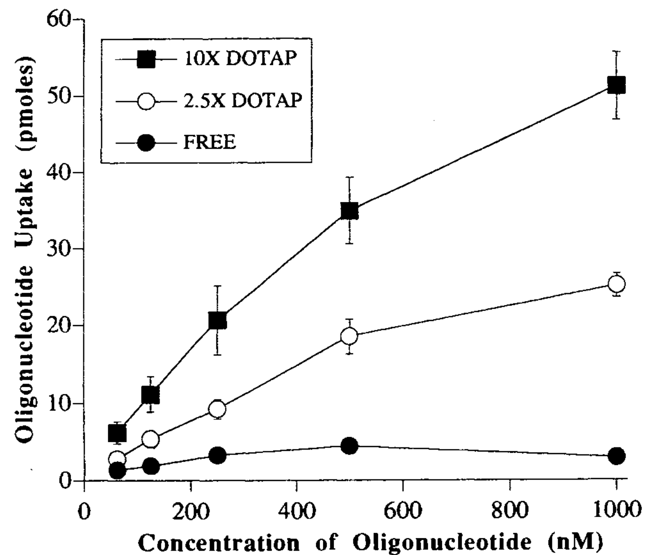

Fig. 2. Cellular uptake of ^{35}S-ODN +/− DOTAP. Various concentrations of ^{35}S-ODN +/− DOTAP were incubated on CV-1 cells 3h at 37°C in serum free medium. Then, the quantity of ^{35}S-ODN (pmoles) associated with cells was determined. The mean of two separate experiments is given. Where no error bars are visible the S. D. is smaller than the symbol.

Parameters Influencing the Oligonucleotide Nuclear Uptake Mediated by DOTAP

The charge ratio is critical for the nuclear delivery of ODN by DOTAP lipids since at 1/1 and lower ratios no fluorescent nuclei were seen (see Fig. 5). The maximum efficiency was obtained at 10/1 (+/−) where approximately 70 % of cells have F-ODN present in the nucleus. At higher charge ratios the efficiency of delivery decreased until the cytotoxic level of complexes was reached (data not shown). The cytotoxicity of the ODN, DOTAP liposomes and ODN/DOTAP complexes were monitored by an MTT assay. Toxicity was only seen when cationic lipids concentrations were ≥95μM. Phosphorothioate oligonucleotide were not cytotoxic up to 50μM.

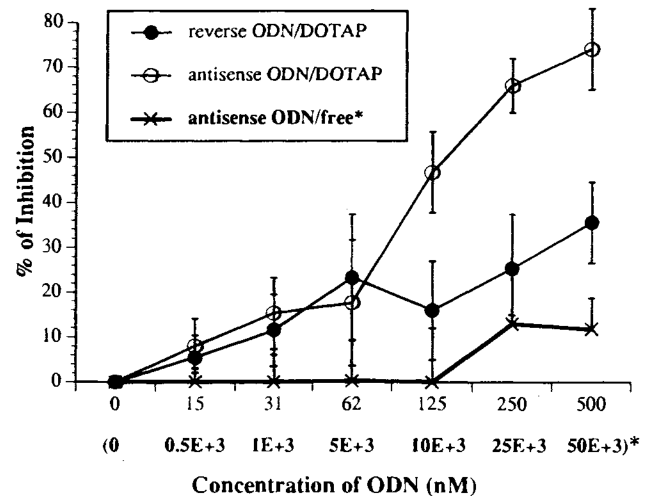

Fig. 3. Antisense activity of ODN +/− DOTAP. Antisense or reversed ODN +/− DOTAP at 10/1 charge ratio were tested to inhibit the luciferase synthesis in CV-1 luciferase expressing cells. After 24h the RLU/mg of protein was measured as described in materials and methods. The mean of two separate experiments is given.
*nM concentration of ODN used alone.

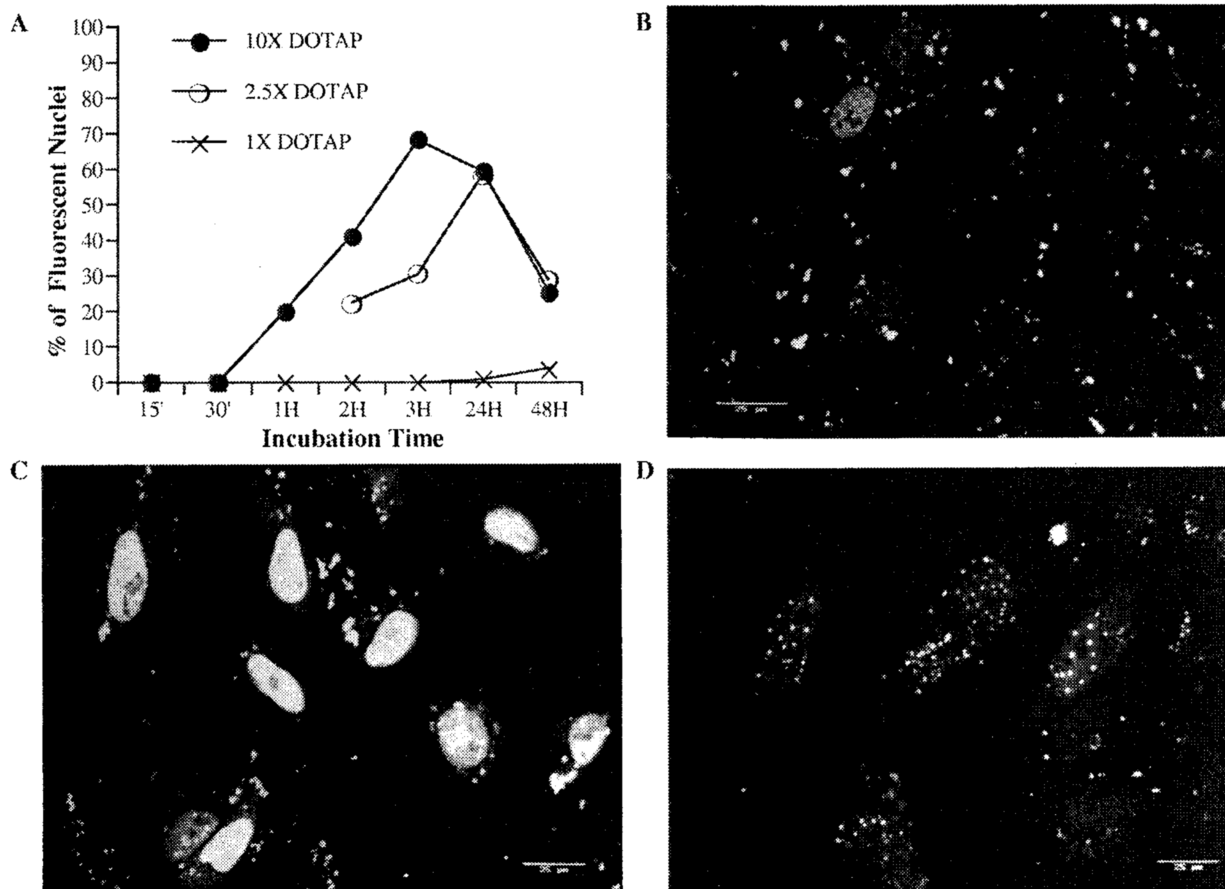

Fig. 4. Kinetics of nuclear uptake of F-ODN/DOTAP complexes. F-ODN (140nM) were associated with DOTAP at three different charge ratio (+/−) 10/1, 2.5/1 and 1/1. C. **A**) Percentage of cells with a fluorescent nucleus. The mean of two separate experiments is given and the S. D. did not exceed 10%; **B**) F-ODN/DOTAP at 2.5/1 charge ratio incubated for 3h at 37°C; **C**) F-ODN/DOTAP at 2.5/1 charge ratio after 24h; D) F-ODN/DOTAP at 2.5/1 charge ratio after 48h.

The intracellular oligonucleotide delivery by DOTAP lipids was temperature dependent. Fluorescent nuclei were seen only after incubation at temperatures ≥ 30°C and none were detected at 4, 10, 15 and 20°C with 10/1 (+/−) complexes.

The time course of nuclear uptake of F-ODN mediated by DOTAP lipids depended upon the charge ratio. At neutral ratio, no fluorescence was detected in the nuclei until 48h (Fig. 4A). The maximum percentage of fluorescent labeled nuclei was obtained with complexes at charge ratio 10/1 after 3h of incubation (Fig. 4A). With F-ODN/DOTAP at +/− charge ratio 2.5/1 some nuclei are labeled after 3h and the intensity of fluorescence is low (Fig 4B). After 24h the fluorescence intensity of nuclei is very bright and the number of nuclei equals the 10/1 ratio (Fig. 4C). We have quantified the cellular uptake of oligonucleotides at 24h after their addition to CV-1 cells. The total cell-associated radioactivity was enhanced by 80 and 120 fold for 2.5 and 10 +/− charge ratio, respectively at 24h than it was at 3h (data not shown). This high level of oligonucleotide cellular uptake was only observed when serum was added directly to serum-free medium. The enhancement of uptake upon addition of serum may be related to the alleviation of cell starvation. Addition of serum may promote a more metabolically active state conducive to the uptake of complexes attached to the cell surface. However when the complex was directly added to cells in serum delivery efficiency was greatly reduced. After 48h of incubation, the number of cells showing nuclear fluorescence diminished as well as the fluorescence intensity (Fig. 4D). In several nuclei, some very bright punctate loci can be seen probably due to a concentration of oligonucleotides in a particular region of nucleus (6,11).

Oligonucleotide Nuclear Uptake Mediated by DOTAP +/− "Helper" Lipids

The formulation of the neutral phospholipid, in particular a hexagonal phase competent lipid, DOPE, with most cationic lipids increases transfection of the cationic lipid-DNA complexes (7, 12, 13). Cholesterol has also been recently reported to have similar beneficial effects (14). The association of DOPE and/or cholesterol with DOTAP changed the optimum charge ratios: 10/1 for DOTAP, 5/1 for DOTAP:cholesterol, 2.5/1 for DOTAP:DOPE and 1.25/1 for DOTAP:DOPE:cholesterol but not the delivery efficiency (Fig. 5).

Effects of Endocytosis Inhibitors on Oligonucleotide Nuclear Uptake

Oligonucleotide delivery by cationic lipids has been suggested to be due to the fusion of cationic lipids with plasma

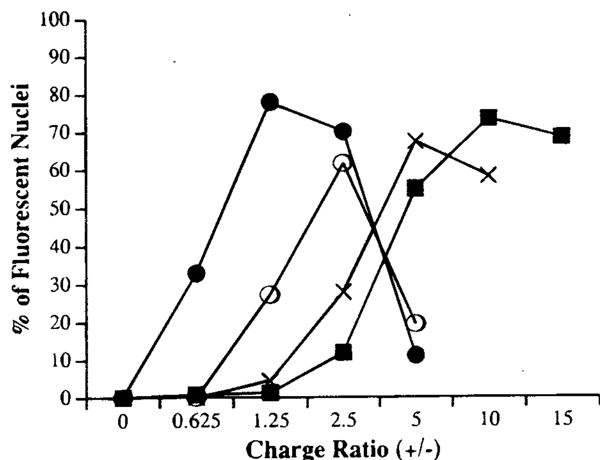

Fig. 5. Effects of "helper" lipids on delivery efficiency of DOTAP. CV-1 cells were incubated 3h in serum free medium with various complexes prepared at different charge ratios. Then, cells were treated as described in materials and methods and the number of fluorescent nucleus were reported as a function of the total cell number. The mean of two separate experiments is given and the S. D. did not exceed 10%. —●—, DOTAP:DOPE:CHOL; —○—, DOTAP:DOPE; —×—, DOTAP:CHOL; —■—, DOTAP.

membrane (4) whereas DNA has been proposed to enter via endocytosis (5, 7, 13). Thus, since the plasmid DNA and ODN delivery mediated by cationic lipids is different at least in term of intracellular distribution and of "helper" lipids association we examined the effects of inhibitors of endocytosis and intracellular trafficking.

Incubation of the cells with F-ODN/DOTAP complexes at 4°C completely blocked the intracellular delivery of F-ODN (Fig. 6) and no fluorescence was seen either in the cytoplasm or in the nucleus but F-ODN/DOTAP were still able to bind to cell membranes. Azide/deoxyglucose concentrations that can block ATP production in the cell (6), inhibited DOTAP-mediated delivery of oligonucleotides to the nucleus (Fig. 6).

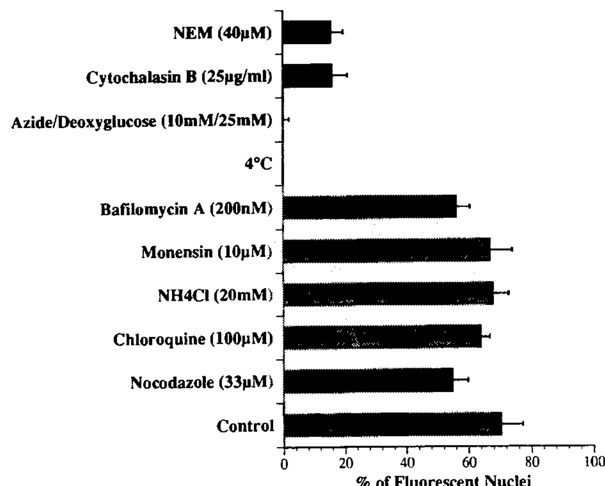

Fig. 6. Effects of endocytosis inhibitors on F-ODN nuclear uptake mediated by DOTAP. CV-1 cells were treated as described in materials and methods. Then, F-ODN/DOTAP complexes (10/1 charge ratio) were added to cells in serum free medium containing the inhibitors for 3h at 37°C. The number of cells having a fluorescent nucleus were determined as a function of the total number of cells. The mean and S. D. of two separate experiments are presented.

Cytochalasin B depolymerizes the microfilaments of actin and blocks the uncoated pit mediated endocytosis (15). The treatment with cytochalasin B greatly reduced the efficiency of cationic DOTAP lipids to mediate nuclear delivery of ODN, indeed the percentage of cells having a fluorescent nucleus fell as well as the fluorescence intensity (Fig. 6). These results suggest that the major delivery pathway of DOTAP lipids was by endocytosis. The role of endocytosis was examined further by using N-ethylmaleimide that is known to block the NSF protein activity required for fusion between endosomes (16). N-ethylmaleimide reduced the number and the intensity of fluorescent nucleus from DOTAP-mediated delivery of oligonucleotides as well as cytochalasin B (Fig. 6).

Chloroquine, NH_4Cl, and bafilomycin A which are known to increase the pH of endocytotic vesicles and thus prevent the acidification of the endocytic pathway (17) did not significantly modify the ODN nuclear uptake efficiency mediated by DOTAP (Fig. 6). Thus, cationic liposomes do not need acidification to release oligonucleotides into cells. Monensin, a monovalent carboxylic ionophore that blocks endocytosis and disrupts sodium and protons gradients across biological membranes (18), has no effect on the number of cells presenting a fluorescent nucleus, however, the intensity of fluorescence is decreased.

The microtubule depolymerizing-agent, nocodazole, which interferes with the later phase of endocytosis (19) has no significant effect on the nuclear delivery of oligonucleotides mediated by DOTAP (Fig. 6). Brefeldin A, which interferes with recycling between the endoplasmic reticulum and cis-golgi had also no effect (data not shown).

DISCUSSION

To understand the mechanism of how cationic liposomes enhance oligonucleotide potency a number of steps in the process must be characterized: these include complex formation, the mechanism of delivery and how and where the complex dissociates to release the oligonucleotide within the cell. Although previous work has shown increased potency and enhanced activity of ODN/cationic lipid complexes on cells (2–4) the mechanism was attributed to delivery via fusion of the complex with the plasma membrane (4). We show that addition of the ODN/DOTAP complex to cultured cells results in a dramatic increase in nuclear accumulation of the fluorescent oligonucleotide, as previously seen with another cationic lipid (4). Our studies indicate the concentration of oligonucleotide available to hybridize to the target either in the cytoplasm or in the nucleus is very much increased over oligonucleotide that is added without the carrier. The antisense activity of ODN is also increased by the use of DOTAP which suggests that nuclear localization of ODN correlates with an enhanced inhibitory activity. Most importantly our data support endocytosis as being the principal pathway for delivery of ODN via the cationic lipid complex which would make ODN delivery similar to DNA delivery (5, 7, 13).

The delivery of ODN by DOTAP lipids depends on several parameters including: charge ratio, time of incubation and neutral lipid composition. Modulation of the charge ratio and time of incubation can regulate the time course and extent of nuclear

accumulation. The inclusion of certain neutral lipids, especially DOPE or cholesterol, with cationic lipids has been demonstrated to increase the DNA delivery efficiency (12–14). We found the association of these molecules with DOTAP lipids did not effect the capacity of oligonucleotide delivery in contrast to gene delivery reports. However, the optimum charge ratio for nuclear uptake was reduced as the DOTAP was diluted with the neutral lipid. The reason for this change with the association of neutral lipids is not known but it could be related to the charge density of the liposome surface being more complementary with the charge density of the oligonucleotide and/or to the quantity of lipids required to form the complexes. However a positive charge is still required for effective delivery indicating that complexes bind to the cell membrane through electrostatic interactions.

To better characterize the uptake pathway of the complexes, we have used several inhibitors of endocytosis. These results show that complexes are endocytosed and suggest that internalization is mainly achieved by uncoated vesicles. The liberation of the oligonucleotide does not require acidification since lysosomotropic agents have no effect. Nocodazole had no effect on delivery which suggests release of ODN from the complex takes place in the early stages of endocytosis, probably at the level of the uncoated pits or early endosomes since nocodazole abolishes vesicle movement in CV-1 cells whereas cytochalasin B does not (20). This is supported by data of Wrobel and Collins that an early event in the endocytotic pathway plays a key role in destabilization of cationic liposomes (21). Interestingly, at 15–20°C where processing of material into endosomes is not affected no nuclear delivery of ODN was observed. This is probably due to the decreased ability of cationic liposomes to fuse with endosomes at this temperature (21). Indeed, we have recently proposed that destabilization of the endosomal membrane by the complex causes anionic lipids from the cytoplasmic face of the membrane to form an ion pair with the cationic lipid and release nucleic acids from the complex into the cytoplasm (22, O. Zelphati, unpublished data). Our results, taken together with the results from the groups cited above, point out the importance of an endocytic capability of the target cell in determining the delivery efficiency of oligonucleotides mediated by cationic agents.

The use of cationic liposomes is still greatly limited, most critically by the dramatic effect of serum on delivery efficiency (12). The serum effect although limiting for intravascular oligonucleotide delivery may not preclude the use of cationic lipid formulations for extravascular delivery such as to the lung or eye.

ACKNOWLEDGMENTS

This work was supported by NIH GM30163, GM 26691, DK46052 (F. C. S.), Glaxo Inc. and Association pour la Recherche contre le Cancer (O. Z.). We thank Tara Wyman for helpful discussions and Wayne Hendren and Chris Leamon for the luciferase expressing cell line and comments.

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