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Interaction of cationic liposomes and their DNA complexes with monocytic leukemia cells

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Abstract

Cationic liposomes complexed with DNA have been used extensively as non-viral vectors for the intracellular delivery of reporter or therapeutic genes in culture and in vivo. We examined the relationship between the characteristics of the lipoplexes, their mode of interaction with monocytic THP-1 cells and their ability to transfect these cells. We determined the size and ζ potential of cationic liposomes (composed of 1,2-dioleoyl-3-(trimethylammonium) propane (DOTAP) and its mixtures with neutral lipids), and lipoplexes at different (+/−) charge ratios. As the (+/−) charge ratio of the lipoplexes decreased to (1/1), a significant reduction in ζ potential and an increase in size was observed. The increase in size resulted from fusion between liposomes promoted by DNA, as demonstrated by a lipid mixing assay, and from aggregation of the complexes. Interaction of liposomes and lipoplexes with THP-1 cells was assessed by monitoring lipid mixing ('fusion') as well as binding and cell association. While no lipid mixing was observed with the 1/2 (+/−) lipid/DNA complexes, lipoplexes with higher (+/−) charge ratios underwent significant fusion in conjunction with extensive cell binding. Liposome binding to cells was dependent on the positive charge of the liposomes, and their fusion could be modulated by the co-lipid. DOTAP/phosphatidylethanolamine (1:1) liposomes fused with THP-1 cells, unlike DOTAP/phosphatidylcholine (1:1) liposomes, although both liposome types bound to the cells to a similar extent. The use of inhibitors of endocytosis indicated that fusion of the cationic liposomes with cells occurred mainly at the plasma membrane level. The presence of serum increased the size of the cationic liposomes, but not that of the lipoplexes. Low concentrations of serum (3%) completely inhibited the fusion of cationic liposomes with cells, while inhibiting binding by only 20%. Our results suggest that binding of cationic liposomes and lipoplexes to cells is governed primarily by electrostatic interactions, whereas their fusion is regulated by the lipid composition and sterically favorable interactions with cell surface molecules. In addition our results indicate no correlation between fusion of the lipoplexes with the plasma membrane and the levels of transfection.

Keywords: Cationic liposome; Lipoplex; Membrane fusion; Transfection; THP-1 cell

1. Introduction

Recent studies have shown that gene therapy con-

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stitutes a promising strategy for the treatment of cancer, infectious diseases and hereditary disorders [1–4]. Delivery of therapeutic genes into cells with high efficiency, i.e. with high levels of gene expression in a large percentage of treated cells, and in a stable manner, is a crucial issue in gene therapy [5,6]. Cationic liposomes have proven to be useful tools for delivery of genes and antisense oligonucleotides into cells in culture and in vivo [7–13]. For example, the delivery of the gene encoding the chloride transporter into the lungs of transgenic mice lacking this gene [14], and into cystic fibrosis patients [15] via cationic liposomes partially corrected this defect.

Cationic liposomes have several advantages as gene transfer vectors over viruses. Unlike viral vectors, cationic liposomes can be used to transfer DNA of essentially unlimited size. Since plasmid-liposome complexes ('lipoplexes' [16]) lack proteins, they are not likely to evoke immunogenic or inflammatory responses. Moreover, they cannot replicate or recombine to form an infectious agent and have low integration frequency [5,17]. Nevertheless, lipoplexes also present several deficiencies, such as the low efficiency of transfection and extensive interaction with negatively charged macromolecules in plasma and cell surfaces, which may be detrimental.

Despite the extensive use of cationic liposomes for gene delivery in vitro and in vivo [7,15,18–21] the mechanisms by which they deliver DNA into cells is not well understood. Initial studies suggested that they fused with the plasma membrane and that the fusion process mediated the entry of DNA into the cytoplasm [7,8,18]. These interpretations were based on the observation of diffuse fluorescence on the cell surface following the interaction of liposomes containing rhodamine-phosphatidylethanolamine (Rh-PE) in their membrane, and the correlation of this fluorescence with the ability of DOTMA/DOPE, but not DOTMA/DOPC, liposomes to mediate transfection. Supporting evidence was provided by the ability of cationic liposomes to fuse with erythrocyte membranes [22,23]. Questions remain as to whether the topology of the cationic liposome-DNA complexes allows the entry of the DNA through a fusion event, and whether this event takes place at the plasma membrane or in endocytotic vesicles or endosomes. Other studies have indicated that endocytosis of the lipoplexes is another possible mechanism of intracellular entry [24–26]. However, the problem of how the lipoplex induces the disruption of the endosome in order to gain access into the cytoplasm still needs to be resolved. Furthermore, electron microscopic observations are likely to miss rapid fusion events that take place at the plasma membrane, and to focus on lipoplexes in endocytotic vesicles that are readily detectable. Alternative methods, such as fluorescence dequenching, need to be employed to ascertain the rate and extent of fusion events at either the plasma membrane or the endosomal membrane, or both.

Recently it has been proposed that the interaction of cationic lipids with the endosome membrane induces flip-flop of negatively charged phosphatidylserine molecules in the cytoplasmic leaflet to the lumenal leaflet, and that this lipid displaces the DNA from its complex with the cationic lipid [27]. Although elegant and useful, this hypothesis does not account for the nature of the initial intermembrane interaction that then leads to the transbilayer movement of lipids. In addition to the above hypotheses on the mechanism of DNA entry, van der Woude et al. [28] have suggested the formation of a pore in the plasma membrane, based on the hemolytic activity of lipoplexes. In this case, whether the DNA permeates through the pore or whether the breakdown of the permeability barrier of the cell membrane is only a by-product of the fusion reaction must be resolved.

Similar to what has been described for divalent cation-induced fusion of anionic liposomes [29], multivalent anions, oligonucleotides or DNA trigger the fusion of cationic liposomes [23,30–32]. The presence of nucleic acids leads to the formation of clusters with surface charge and size that are different from the original cationic liposome preparation, depending on the lipid/DNA (+/−) charge ratio.

To gain insights into the questions raised above, we performed a systematic study of the interaction of cationic liposomes and lipoplexes with monocytic leukemia (THP-1) cells, in the presence or absence of serum in the culture medium. We quantitated lipid mixing between cationic liposomes induced by DNA at the different charge ratios. We examined the process of membrane lipid mixing between cationic liposomes and the cells, by monitoring the dequenching of a non-exchangeable fluorescent probe embedded in the liposome membrane. We assessed the effects

of the lipid/DNA (+/−) charge ratio, endocytosis inhibitors, and serum on membrane lipid mixing. We also established the levels of transfection under these conditions. Some of our results have been presented earlier in preliminary form [33,34].

2. Materials and methods

2.1. Materials

The cationic lipid 1,2-dioleoyl-3-(trimethylammonium) propane (DOTAP), egg phosphatidylcholine (PC), phosphatidylethanolamine (PE) produced by transphosphatidylation of PC, poly(ethylene glycol) (2000) distearoyl phosphatidylethanolamine) (PEG-DSPE) and L-α-phosphatidylethanolamine-*N*-(lissamine rhodamine B sulfonyl) (Rh-PE) were purchased from Avanti Polar Lipids (Alabaster, AL). NaN_3, NaF, antimycin A, ethidium bromide (EtBr), DNA from calf thymus, and *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES) were obtained from Sigma (St. Louis, MO). All the other chemicals and reagents used were of analytical grade.

2.2. Preparation of cationic liposomes and their complexes with DNA

Large unilamellar cationic liposomes (LUV) were prepared by multiple extrusion of multilamellar liposomes (MLV). Briefly, lipids dissolved in $CHCl_3$ were mixed at the desired molar ratio and dried in a rotatory evaporator. The dried lipid films were hydrated in HEPES-buffered saline (HBS; 150 mM NaCl, 10 mM HEPES, pH 7.4) at a final lipid concentration of 5 mM. The resulting MLV were extruded 5 times through two stacked polycarbonate filters of 100 nm pore diameter using a Lipex Biomembranes (Vancouver, BC) high pressure extrusion device. Phospholipid concentrations were determined by an assay for phosphate [35]. Liposomes were labeled by incorporating Rh-PE at a self-quenching concentration (5 mol% of total lipid). Liposome/DNA complexes (lipoplexes) were prepared by mixing appropriate amounts of DNA (also in HBS buffer) with the liposomes in order to obtain the desired (+/−) charge ratio.

2.3. Cells

THP-1 cells, a human monocytic leukemia cell line, were obtained from the UCSF Cell Culture Facility (San Francisco, CA) and maintained at 37°C under 5% CO_2, in RPMI 1640 medium containing 25 mM HEPES buffer and 2% sodium bicarbonate, supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Biochrom KG, Berlin) and antibiotics (100 μg/ml of streptomycin and 1 unit/ml of penicillin). For fusion, binding and cell association studies, the cells were harvested by centrifugation at $180\times g$ for 7 min at room temperature, washed twice in phenol red-free RPMI 1640 containing 25 mM HEPES buffer (pH 7.4), resuspended in the latter medium at a stock cell density of 20×10^6 cells/ml and kept on ice in polypropylene centrifuge tubes until use on the same day. Cell viability was determined by Trypan blue exclusion and was routinely above 95%.

2.4. Physico-chemical characterization of liposomes and their complexes with DNA (lipoplexes)

Liposomes and lipoplexes were characterized with respect to their size and ζ potential using a Malvern Autosizer II C (Malvern Instruments, UK) and a Coulter DELSA 440 (Coulter Corporation, Miami, FL), respectively. The DELSA 440 is a laser-based multi-angle particle electrophoresis analyzer that measures the electrophoretic mobility and ζ potential distribution simultaneously with the hydrodynamic size of particles in suspension. The ζ potential was automatically calculated from the electrophoretic mobility based on the Helmholtz-Smoluchowski formula [36]. Although adsorption of cationic liposomes onto the camera wall was noticeable, this appeared not to interfere with the reproducibility of the measurements.

Cationic liposomes and their complexes with DNA were diluted 10 times with HBS before analysis at 25°C. All the liposome/DNA complexes were prepared immediately before analysis. Samples of the prepared complexes were placed in the measuring cell, whose position was adjusted to cover a previously determined stationary layer, and an electric current of 3.0 mA was applied. Measurements were recorded and the ζ potential was calculated for each scattering angle (8.6°, 17.1°, 25.6° and 34.2°). Data

represent the mean ± standard deviation obtained for the different angles of two measurements. All cationic liposome/DNA complexes showed a unimodal distribution for ζ potential, while in size measurements a polymodal distribution was observed.

2.5. Ethidium bromide-DNA interaction

Plasmid DNA (0.138 mmol) was incubated with 0.2 mg/ml EtBr, in a final volume of 3 ml HBS. Following 2 min incubation, different amounts of the DOTAP liposome suspension were added in order to obtain the desired (+/−) lipid/DNA charge ratios. EtBr fluorescence was continuously monitored at 610 nm in a Spex fluorometer (SPEX Industries, Edison, NJ) (excitation wavelength: 500 nm; 1.0 mm excitation and 2.0 mm emission slits).

2.6. Fusion (lipid mixing) assay

Fusion (lipid mixing) between THP-1 cells and cationic liposomes was evaluated at 37°C by continuously monitoring the dequenching of Rh-PE incorporated in the liposome membrane at a self-quenched concentration. Fusion experiments were carried out using various concentrations of liposomes and THP-1 cells. Results are presented for a lipid concentration of 20 μM (liposomes) and 1×10^6 cells/ml (THP-1 cells), which were determined to be the optimal concentrations in terms of fluorescence increase (data not shown). The final incubation volume was 2 ml. The extent of fusion was measured following a 30 min incubation of the cationic liposomes and cells, and is given as a percentage of the maximal fluorescence. The fluorescence scale was calibrated, such that the initial fluorescence of the Rh-PE-labeled liposomes and cell suspension was set at 0% fluorescence. Maximal fluorescence (100%) was obtained upon addition of Triton X-100 at a final concentration of 0.5% (v/v). Fluorescence measurements were performed in the SPEX fluorometer. The fluorescence was read at an excitation wavelength of 568 nm and an emission wavelength of 586 nm using 0.5 and 1 mm slits in the excitation and emission channels, respectively. The sample chamber was equipped with a magnetic stirring device and the temperature was controlled with a thermostated circulating water bath.

To inhibit endocytosis at 37°C, THP-1 cells were pre-treated with 1 μg/ml of antimycin A, 10 mM NaF, 0.1% (w/v) sodium azide for 30 min at 37°C as described before [37,38]. Fusion was measured in the presence of these metabolic inhibitors. The effect of liposome composition on the extent of fusion was investigated by preparing DOTAP liposomes containing different amounts of PC, PE or PEG-DSPE. To evaluate the effect of DNA on fusion, complexes of DOTAP or DOTAP/PE with DNA were prepared at different (+/−) charge ratios, immediately before use. The effect of serum was studied following its addition to the reaction medium at various concentrations before addition the cationic liposomes to the cells.

2.7. Binding and cell association assays

The binding experiments were carried out at 4°C, while cell association studies were performed at 37°C. In both cases, a mixture containing 1×10^6 cells/ml and 20 μM of fluorescently labeled cationic liposomes (final volume 2 ml) in polypropylene tubes was continuously stirred using a magnetic stirrer on ice or in a water bath, respectively. After various incubation times, samples were centrifuged at $180\times g$ for 10 min at 4°C. The supernatant and the pellet were separated and kept at 4°C until use. The fluorescence of the supernatant and the pellet (resuspended in 2 ml of RPMI medium) was measured at 37°C, following detergent lysis. The effect of DNA and serum was evaluated in the same way as described above.

2.8. DNA-induced liposome-liposome fusion

DNA-induced fusion between cationic liposomes was monitored by the dequenching of the fluorescent probe Rh-PE incorporated into the membrane of one population of liposomes at a self-quenching concentration (5 mol%). Fusion was initiated by adding different amounts of DNA into a cuvette containing 4 μM of labeled and 16 μM of unlabeled cationic liposomes in 1.9 ml of HBS to obtain the desired (+/−) charge ratio. Fusion was monitored for 3 min at 37°C. Parallel experiments were performed in the presence of serum.

2.9. Cell viability

Cell viability was measured following incubation of cells with liposomes or liposome-DNA complexes for different periods of time. Membrane integrity was determined by lactate dehydrogenase (LDH) assay [39] or by Trypan blue exclusion. The reduction capacity of mitochondrial dehydrogenases was determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay [40].

2.10. Transfection activity

The transfection activity of lipid/DNA complexes was measured by luciferase expression from the plasmid pCMVluc (VR-1216 from Vical). THP-1 cells in suspension were rinsed twice with serum-free medium, resuspended in 0.3 ml of RPMI 1640 and transferred to 48 well plates (at a cell density of 0.5×10^6 cells/well) before lipid/DNA complexes were added. Lipid/DNA complexes were added gently to cells in a volume of 0.2 ml per well. After an incubation for 4 h (in 5% CO_2 at 37°C) the medium was replaced with RPMI 1640 containing 10% FBS, and the cells were further incubated for 24 h. The cells were then washed twice with phosphate-buffered saline (PBS) and 100 μl of lysis buffer (Promega, Madison, WI) were added to each well. The level of gene expression in the lysates was evaluated by measuring light production by luciferase using a scin-

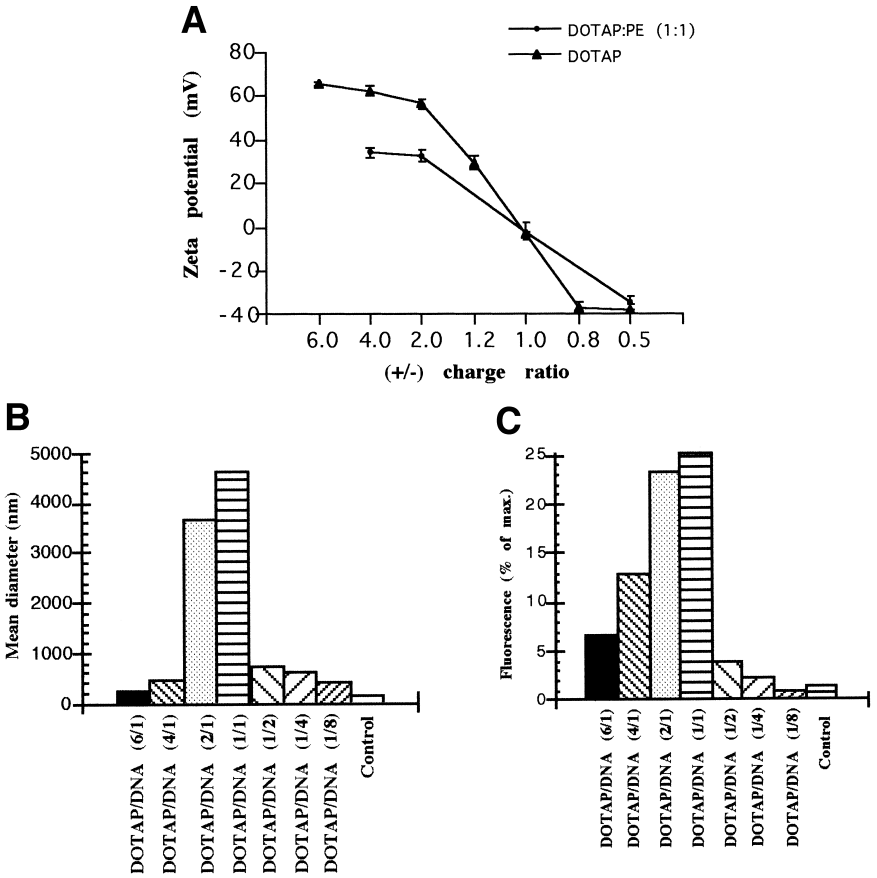


Fig. 1. The effect of DNA on the physico-chemical properties and on the fusion of DOTAP-containing liposomes. (A) DOTAP-containing liposomes and their complexes with DNA were characterized with respect to ζ potential as described in Section 2. Liposomes were prepared by extrusion of multilamellar vesicles in 10 mM HEPES, 150 mM NaCl, pH 7.4 as described in Section 2. Liposome-DNA complexes were prepared at different lipid/DNA (+/−) charge ratios immediately before analysis. Total lipid concentration was 4.1 mM. (B) The mean particle size was measured 5 min after adding DNA to liposome suspensions (20 μM) by light scattering analysis. (C) Fusion was evaluated by measuring the extent of Rh-PE fluorescence dequenching, at 37°C, upon addition of DNA to a mixture of fluorescently labeled (4 μM) and unlabeled (16 μM) DOTAP liposomes (3 min incubation).

tillation counter protocol (Promega). The protein content of the lysates was measured by the Dc Protein Assay reagent (Bio-Rad, Hercules, CA) using bovine serum albumin as the standard. The data were expressed as ng of luciferase (based on a standard curve for luciferase activity), per mg of total cell protein.

3. Results

3.1. Physico-chemical characterization of DOTAP-containing liposomes and their complexes with DNA

Significant changes occur in the physico-chemical features of cationic liposomes upon their complexation with negatively charged nucleic acids [31,41]. These changes are likely to alter the interaction of cationic liposomes with cell membranes. The ζ potential of the cationic liposomes and their complexes with DNA provides a measure of their net charge, while dynamic light scattering gives their mean particle size. Fig. 1 shows the values obtained for ζ potential and mean particle size of liposome/DNA complexes at different lipid/DNA (+/−) charge ratios, as well as the extent of fusion between liposomes. Complexation of DNA with DOTAP-containing liposomes leads to a decrease in the values of the ζ potential (Fig. 1A). The mean size of the complexes was considerably larger than that of the liposomes themselves, and varied from 260 nm for the 6/1 complex to above 4500 nm for the 1/1 (+/−) complex (Fig. 1B). The correlation between the extent of fusion between liposomes, measured as Rh-PE fluorescence dequenching upon addition of DNA, and the mean diameter of the resulting complexes are also illustrated in Fig. 1B,C. Similar to the charge ratio dependence of the mean diameter, the extent of Rh-PE fluorescence dequenching was maximal at the 1/1 lipid/DNA (+/−) charge ratio (Fig. 1C). It appears that when the resulting complexes exhibit an excess of positive or negative charge, electrostatic repulsive forces prevent extensive aggregation and/or fusion, thus leading to the formation of smaller complexes. In contrast, extensive aggregation and fusion are favored in the case of approximately neutral complexes where repulsive forces are minimal.

3.2. Ethidium bromide intercalation

The intercalation of ethidium bromide in between the base pairs of DNA results in a large enhancement of fluorescence. The addition of cationic liposomes to ethidium bromide-labeled DNA causes the condensation of the DNA, extrusion of the ethidium bromide, and a decrease in fluorescence [27,31]. Addition of DOTAP liposomes to DNA in HBS resulted in a decrease of EtBr fluorescence up to the 1/1 (+/−) charge ratio, as illustrated in Fig. 2. Subsequent addition of DOTAP liposomes up to a charge ratio of 2/1 (+/−) did not lead to any further decrease in the fluorescence.

3.3. Fusion of DOTAP-containing liposomes with THP-1 cells

The mode of interaction of lipoplexes with the plasma membrane of the target cell and the intracellular fate of the DNA component are not understood well. Two of the hypotheses that describe the mechanism of transfection mediated by cationic liposomes are that: (i) fusion with the plasma membrane results in cytoplasmic delivery of the genetic material [7], and (ii) lipoplexes are internalized by endocytosis [25,42]. These mechanisms are not mutually exclusive, and may be active to a greater or lesser extent in different cell types. We examined the

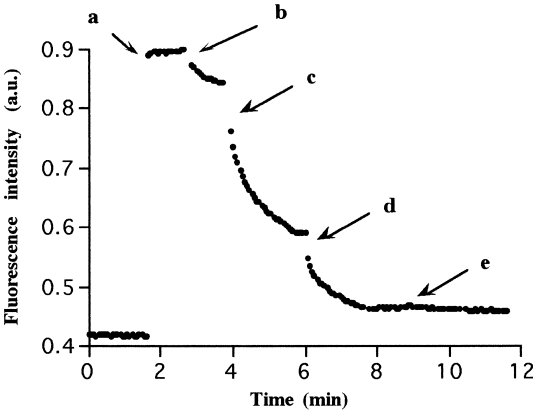


Fig. 2. Effect of addition of DOTAP-containing liposomes on the interaction of ethidium bromide with DNA. Following addition of DNA (a) and incubation with ethidium bromide, different amounts of DOTAP liposomes were added in order to obtain the desired (+/−) lipid/DNA charge ratios: 1/4 (b); 1/2 (c); 1/1 (d); 2/1 (e). The accessibility of EtBr to DNA was evaluated at 37°C as described in detail in Section 2.

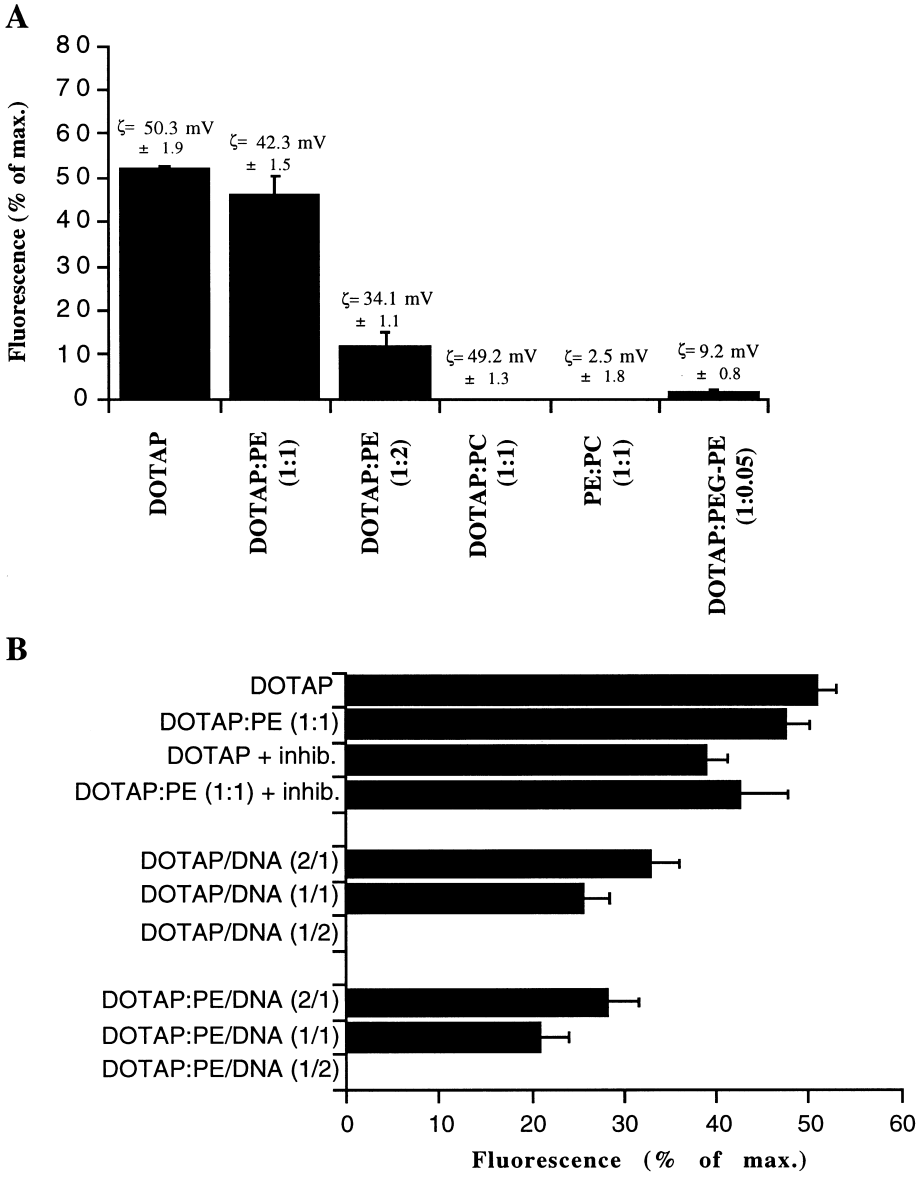


Fig. 3. (A) Fusion between liposomes and THP-1 cells. Fusion was monitored at 37°C by the dequenching of Rh-PE incorporated into the liposome membrane at 5 mol%. Liposomes were prepared as described in Section 2. The extent of fusion was measured after 30 min incubation of the liposomes with cells. Liposome concentration and cell density were 20 μM and 1×10^6 cells/ml, respectively. The ζ potential values are indicated in the figure for each liposome formulation. (B) Effect of inhibitors of endocytosis and of DNA on the fusion of DOTAP-containing liposomes with THP-1 cells. THP-1 cells were pre-treated with inhibitors of endocytosis for 30 min at 37°C and fusion was measured after 30 min incubation of the liposomes with treated or untreated (control) cells. Complexes of DOTAP or DOTAP/PE with DNA were prepared at different (+/−) charge ratios and the extent of fusion was measured following 30 min incubation with cells.

fusion of DOTAP liposomes with THP-1 cells, and the effect on this process of zwitterionic lipids known to have particular effects on membrane fusion in other experimental systems [29,43]. THP-1 cells were chosen for these studies since they can be used both in suspension and as adherent cells [44,45] and because of our previous experience with these cells in terms of liposome uptake and inhibition of endocytosis [38].

Pure DOTAP liposomes fused extensively with THP-1 cells, resulting in about 50% fluorescence dequenching within 30 min of addition of the liposomes

to the cells (Fig. 3A). When PE was included in the membrane of DOTAP liposomes at 50 mole%, the extent of fusion was not significantly affected. In contrast, the inclusion of 50 mole% PC completely inhibited fusion, as expected from the fusion inhibitory effect of PC in the divalent cation-induced fusion of net anionic liposomes [46] or the fusion of cationic liposomes with anionic liposomes [23]. In mixed DOTAP/PE liposomes, as the mol fraction of DOTAP was decreased at the expense of PE, the extent of fusion with THP-1 cells was reduced.

Previous studies had indicated that the inclusion of PEG-DSPE in phosphatidylserine-containing liposomes inhibited calcium-induced fusion of the liposomes [46,47]. When 5 mole% DSPE-PEG was included in DOTAP liposomes, fusion of the liposomes with THP-1 cells was also inhibited substantially (Fig. 3A).

3.4. Effect of inhibitors of endocytosis on liposome-cell interactions

To evaluate the relative contribution of endocytosis to the interaction of DOTAP-containing liposomes with THP-1 cells, we measured the extent of liposome-cell fusion in the presence of inhibitors of endocytosis. The results in Fig. 3B show that inhibition of endocytosis leads to only about 20% inhibition of fusion, suggesting that fusion of the cationic liposomes with THP-1 cells takes place primarily at the plasma membrane. Independent experiments utilizing fluorescence microscopy of THP-1 cells indicated that endocytosis was indeed inhibited under these conditions (unpublished data).

On the other hand, in order to exclude the possibility that internalization and accumulation of some cationic liposomes inside the endosomes could limit the maximal extent of fusion (50%), we measured the total cell association of DOTAP liposomes in the presence or in the absence of inhibitors of endocytosis. Experiments performed on total cell association, which involves the combination of cell binding, membrane fusion and endocytosis of the liposomes, show that the percentage of liposomes that have associated with the cells is similar to that observed for the extent of fusion (data not shown). Similar to what was described in Fig. 3B, the presence of inhibitors of endocytosis did not significantly affect the extent of liposome-cell association up to 90 min of incubation. This observation reinforces our previous finding that fusion of cationic liposomes with THP-1 cells occurs mainly at the level of the plasma membrane.

3.5. Binding studies

In order to obtain insights into the effect of DNA and liposome composition on the ability of cationic liposomes to fuse with THP-1 cells, we also examined the binding of the liposomes to the cells at 4°C, under which conditions no endocytosis is expected to occur. As illustrated in Fig. 4, pure DOTAP liposomes showed the greatest binding among the several formulations examined. The increase in the percentage of PE in the liposome membrane resulted in a decrease of liposome-cell binding, probably due to a reduction in the electrostatic interactions between positively charged liposomes and the negatively charged cell surface. However, no significant effect on binding was observed upon addition of PC to DOTAP liposomes in a (1/1) molar ratio. The different behavior in terms of extent of cell binding between DOTAP/PE and DOTAP/PC liposomes may be explained by the differences in their net charge (as illustrated by their ζ potential presented in Fig. 3A). In contrast, inclusion of 5% of PEG-DSPE drastically reduced the binding capacity of the resulting cationic liposomes. The binding of DOTAP or DOTAP/PE liposomes to THP-1 cells at 4°C showed saturation kinetics after a 90 min incubation, while lipoplexes reached saturation after 15 min (data not shown). Except for PC-containing liposomes a correlation between liposome-cell binding and fusion ability could be established. The absence of any lipid mixing between DOTAP/PC liposomes and the cell membrane (Fig. 3A), despite extensive binding of the liposomes to the cells, also supports our interpretation that the fluorescence dequenching is not due to non-specific probe transfer from bound liposomes to the cell membrane.

3.6. Effect of DNA complexation on cationic liposome-cell interactions

Complexation of DNA with DOTAP or DOTAP/PE liposomes resulted in the inhibition of fusion.

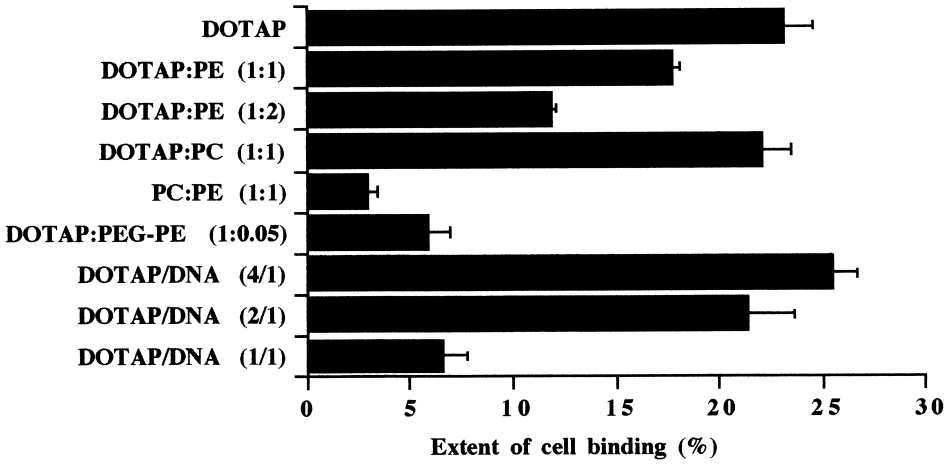


Fig. 4. Liposome-cell binding studies. The effect of liposome composition and of addition of DNA to DOTAP liposomes. Liposomes and their complexes with DNA were prepared as described in the legend to Fig. 1. Liposome-cell binding was measured after 15 min incubation.

Fusion was completely abolished when the lipid/DNA complexes exhibited a net negative charge (Fig. 3B). Complexation of DNA with DOTAP liposomes also led to a significant decrease in the binding of the resulting complexes to THP-1 cells, depending on the (+/−) charge ratio (Fig. 4).

3.7. Effect of serum on fusion and binding between DOTAP-containing liposomes and THP-1 cells

Transfection of certain cell types by some cationic liposomal compositions is sensitive to the presence of serum [7,48,49]. It is possible that negatively charged macromolecules present in serum affect the fusogenic properties of cationic liposomes and thus their ability to interact with cells. We therefore investigated the effect of serum on the physico-chemical properties of the cationic liposomes and of their complexes with DNA, as well as its effect on liposome-cell interactions.

When positively charged liposomes composed of DOTAP or DOTAP/PE (1/1) were added to medium containing 10% serum at 37°C, a substantial aggregation of the liposomes occurred as measured by turbidity (data not shown). Incubation of DOTAP liposomes with residual amounts of serum (1%) led to the formation of aggregates (approx. 800 nm in diameter) whose mean diameter further increased when the serum concentration was raised up to 3%. Further increase in serum concentration did not result in any significant change in the size of liposomes. Under the same experimental conditions, completely different results were observed when DOTAP liposomes were complexed with DNA at a 4/1 lipid/DNA (+/−) charge ratio, since serum had no effect on complex size (Fig. 5A).

The presence of serum drastically inhibited the fusion of DOTAP or DOTAP/PE liposomes with cells, even at very low concentrations, and fusion was abolished in the presence of 3% serum (Fig. 5B). To determine whether this inhibitory effect was due to inhibition of binding of liposomes to cells, we evaluated the effect of serum on binding (Fig. 5C). Curiously, the presence of 3% serum caused only a 20% reduction in binding, although this concentration of serum was sufficient to completely inhibit fusion. A strong inhibition of liposome-cell binding was only observed when a relatively high concentration of serum was present in the medium (up to 20%). The effect of increasing the percentage of serum on the extent of lipoplex-cell binding was less pronounced than its effect on fusion.

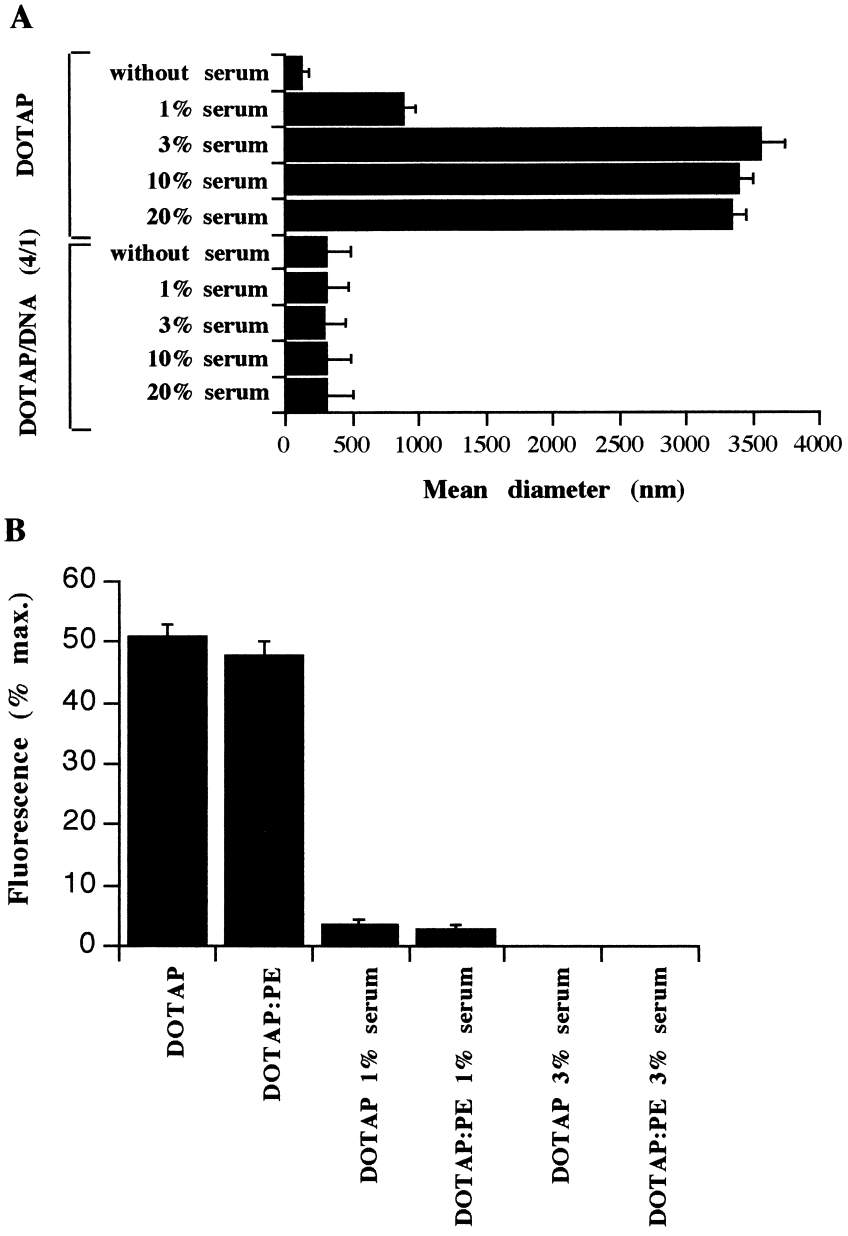
3.8. Transfection activity

Transfection activity was affected by the net charge of the complexes. When lipoplexes presented a net positive or neutral charge high levels of transfection were observed. However, when they were net negatively charged a reduction in the transfection levels was observed (Fig. 6). A significant decrease in the levels of luciferase gene expression was ob-

served upon incubation of the complexes with the cells in the presence of inhibitors of endocytosis, demonstrating that endocytosis represents the major route of intracellular gene delivery. This decrease cannot be attributed to any toxic effect of these compounds on the cells as evaluated by the Alamar blue assay (data not shown).

4. Discussion

The physico-chemical features of lipid-based gene delivery systems play an important role in their interaction with cellular membranes [50]. The mechanisms by which cationic liposomes deliver DNA into cells are not well understood, and are being investigated. In early studies on gene or oligonucleotide delivery via cationic liposomes, the proposed mechanism was that following binding, the lipid-DNA or lipid-oligonucleotide complexes fuse with the plasma membrane of a target cell [7,51]. More recent studies have shown that lipoplexes are first endocytosed and then destabilize the endosomal membrane, thereby micro-injecting the DNA into the cytoplasm [24–



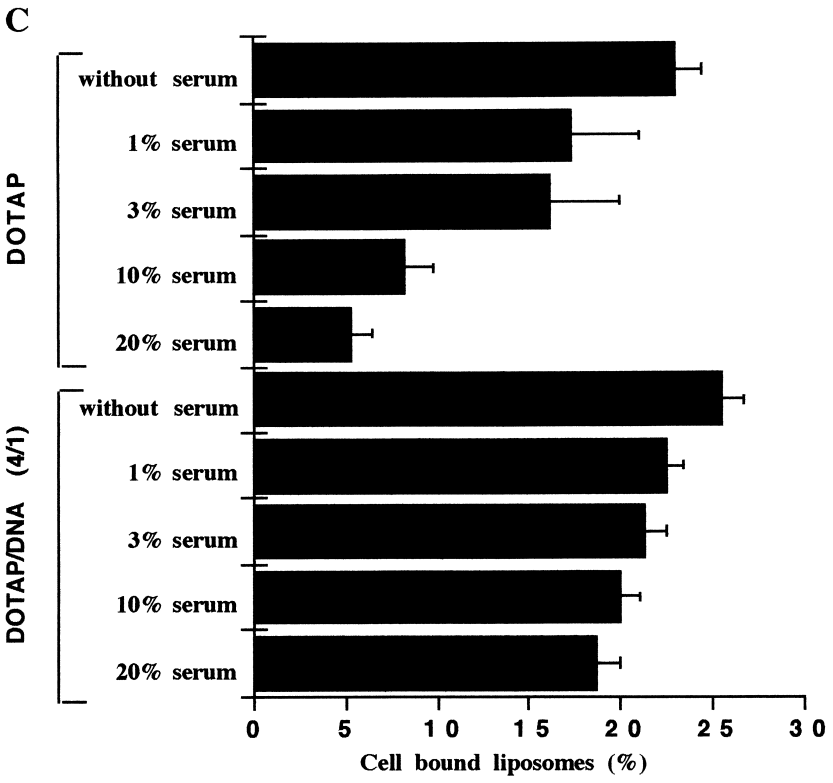


Fig. 5. The effect of serum on the mean diameter of DOTAP liposomes and of their complexes with DNA (A), on liposome-cell fusion (B) and on the extent of cell binding (C). Liposomes and liposome/DNA complexes were prepared as described in the legend to Fig. 1 in the absence or presence of 3% serum. Liposome- or complex-cell binding and fusion were evaluated in the absence or presence of different amounts of serum, as described in detail in Section 2.

26,52]. The characteristics of the lipoplexes resulting from the interaction of cationic lipids with DNA are still largely unknown. The current study focused on several types of interactions of lipoplexes with THP-1 suspension cells, namely cell binding and association, fusion with the plasma and endosomal membranes, and transfection. Our results emphasize the importance of the lipid-DNA electrostatic interactions involved in the formation and properties of the resulting complexes. A significant decrease of the ζ potential was observed when the lipid/DNA (+/−) charge ratio decreased from 2/1 to 1/1. As more DNA is complexed with cationic liposomes, the repulsion between the liposomes is reduced. Furthermore, the multianionic DNA mediates the close approach of the liposomes, leading to aggregation and fusion of the cationic liposomes. This results in an enhancement of the sizes of the complexes. Upon further addition of DNA, i.e. a decrease in the lipid/DNA (+/−) charge ratio, the complexes exhibit much smaller sizes, as a result of the repulsion between the excess negative charges. DNA-induced fusion between liposomes in complexes with lower or higher (+/−) charge ratios than 1/1 is strongly inhibited. Consequently, under these conditions the complexes maintain their small sizes for long periods of time. It is reasonable to assume that in addition to partially neutralizing the positive charge of DOTAP, DNA may form a bridge between the positively charged polar headgroups of two apposing liposomes. Dehydration of the membrane surface may occur during the formation of the complexes due to removal of bound water by DNA, as suggested by Rupert et al. [30] and Düzgünes et al. [23] for the case of didodecylammonium bromide (DDAB)/dipicolinic acid complexes and polyanion-induced fusion of dioleyloxypropyltrimethylammonium (DOTMA) liposomes, respectively. The neutralization of the positive charge results in a decrease of the effective size of the lipid polar headgroups, thus reducing the ability of the lipids to form lipid bilayers, and in a reduction in intervesicular electrostatic repulsion.

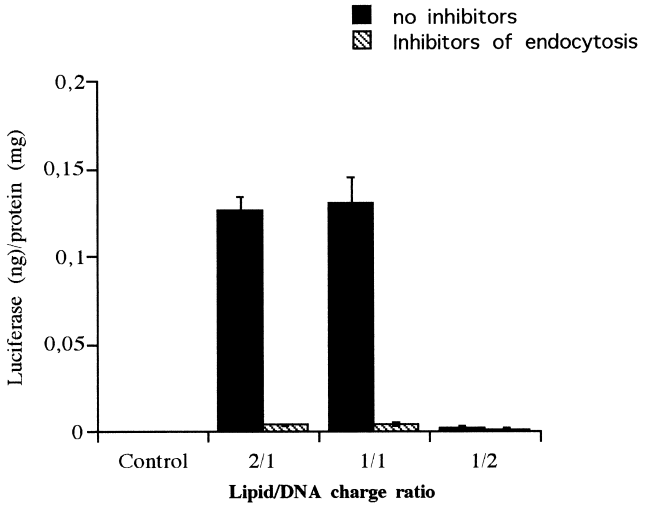


Fig. 6. Transfection of THP-1 cells in the presence or in the absence of endocytosis. Liposome/DNA complexes were prepared as described in the legend to Fig. 1. Transfection activity was measured by the expression of luciferase as described in Section 2.

This in turn would lead to more favorable conditions for the induction of fusion, since aggregation would be facilitated under these conditions [53].

Results obtained on the accessibility of EtBr to DNA present in complexes with different lipid/DNA (+/−) charge ratios show that, even in 1/1 complexes, DNA is completely condensed and covered by a lipid bilayer, and thus is most likely protected from nucleases. These observations, together with the effect of the addition of DNA on the ζ potential of the complexes, indicate that all the cationic lipids interact electrostatically with nucleic acids. This is only possible assuming that the final structure of the liposomes in the complexes is not spherical, but rather a planar lipid bilayer where the positive charges of both leaflets become available to interact with DNA.

It should be noted that electrostatic interactions between cationic liposomes or lipid-DNA complexes with cells represent a key element in their interaction with cells. Inclusion of PE in DOTAP-containing liposomes (maintaining the total lipid concentration at the expense of DOTAP) leads to a decrease in the extent of liposome-cell fusion. Despite the fusogenic properties of PE [25,45,46,54–57], the reduction of the net positive charge of the liposomes due to the presence of this lipid results in a lower extent of binding and, consequently, of fusion. Neutral liposomes composed of PC/PE (1/1), presenting a reduced binding ability, exhibit a very limited extent of fusion with cells. Similar results were observed upon inclusion of a low mole percentage of PEG-DSPE in DOTAP liposomes. The masking effect of PEG on the charge of the liposomes, as illustrated by the ζ potential measurements, drastically reduces their binding and fusion capacity. Nevertheless, besides the net charge of the liposomes, the nature of the co-lipid used also plays an important role in liposome-cell interactions. In fact, inclusion of PC instead of PE in DOTAP-containing liposomes leads to a decrease in the extent of liposome-cell fusion, this being completely abolished at 1/1 (DOTAP/PC) molar ratio. Since binding ability was not affected, we can conclude that this inhibitory effect was not due to unfavorable electrostatic interactions, but rather to a steric hindrance caused by the presence of the large and highly hydrated polar headgroup of PC [45,46].

Similar to the observations upon incorporation of PE in DOTAP liposomes, association of nucleic acids with DOTAP liposomes results in a significant reduction in binding and fusion with the cells, both being completely abolished when the complexes present a net negative charge (1/2 charge ratio).

Fusion of the cationic liposomes with THP-1 cells seems to occur mainly at the level of the cytoplasmic membrane, since only 20% inhibition of fusion was observed when cells were pre-incubated with inhibitors of endocytosis. Similar results were obtained in measurements of cell-associated liposomes at 37°C, where inhibitors of endocytosis decrease the cell association by only about 10%, indicating that liposomes are essentially associated with the plasma membrane. Furthermore, the similarity between the percentage of liposome-cell association and that of the extent of fusion suggests that the great majority of liposomes associated with the cells fuse with the plasma membrane and that fusion with the endosomal membrane, following endocytosis of the liposomes, plays a minor role in the overall extent of liposome fusion with cellular membranes at least for this type of cells.

Incubation of cationic liposomes with serum leads to an increase in their size, which is mainly due to aggregation promoted by serum components. The negatively charged serum proteins may form bridges

between cationic liposomes, as described for the effect of DNA. However, in contrast to what was observed for DNA, the increase in size of the liposomes is not due to fusion between them. It should be noted that aggregation was not observed when complexes prepared at high (+/−) charge ratios (4/1) were incubated with serum. Apparently in this case the binding of serum proteins was not sufficient to overcome the electrostatic repulsion between the complexes.

Fusion of cationic liposomes (DOTAP or DOTAP/PE) or of their DNA complexes with cells was completely abolished in the presence of 3% serum. This effect cannot be attributed to the lack of binding, since even 20% serum was not sufficient to completely inhibit liposome-cell binding. At low percentages of serum, its proteins may act as a physical barrier, preventing fusion but not binding. Moreover, the large sizes of the liposomes observed under these conditions may also limit their capacity to fuse with cells. On the other hand, at higher serum concentrations, the presence of large amounts of negatively charged proteins, besides constituting a physical barrier, also represents an electrostatic barrier that prevents both fusion and binding.

Overall, our findings indicate that addition of nucleic acids to cationic liposomes promotes a significant change in their physico-chemical properties as a result of aggregation and fusion. Consequently, liposome-cell interactions, particularly fusion, are drastically affected. Besides the unfavorable electrostatic interactions, due to the presence of DNA or negatively charged macromolecules present in serum, it appears that the resulting size change of the complexes also plays an important role in limiting lipid mixing.

Taking into account our observations that fusion of cationic liposomes with the plasma membrane is strongly inhibited upon addition of DNA, it seems that the only pathway of internalization of lipid-DNA complexes in transfected cells is through endocytosis. However, if fusion does not take place in the presence of DNA it remains to be clarified how DNA is released from the endosomes into the cytoplasm. One possibility would be fusion/destabilization between the liposomal and endosomal membranes promoted by the high radius of curvature of the latter membrane, as suggested by Xu and Szoka [27].

Our studies with THP-1 cells have shown a significant level of complex fusion with the plasma membrane and a low level of transfection. It should be noted that the limited efficiency of transfection cannot be attributed to loss in cell viability, since no toxic effects were observed upon incubation of the complexes with the cells. Inhibition of endocytosis reduced significantly the extent of complex fusion, the small level of fusion remaining being with the plasma membrane, whereas transfection was almost abolished. Hence, it appears that the main route of transfection in this system relies on the endocytosis of the complex.

This lack of correlation between fusion and transfection, also previously observed by Stegmann and Legendre [58] with adherent cells, may be partially attributed to the heterogeneity of the complexes formed upon addition of DNA to cationic liposomes. The evaluation of the conditions leading to high levels of transfection activity would be facilitated by the development of methodologies for the preparation of more homogeneous complexes. The fact that fusion of the complexes with the plasma membrane does not yield high levels of transfection, should not lead prematurely to discard this route. First, other cell types should be investigated, focusing on cells with low rates of endocytosis. Second, this system may be supplemented by means to direct the DNA to the nucleus following the fusion of the complex with the plasma membrane.

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