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Kinetic analysis of the initial steps involved in lipoplex–cell interactions: effect of various factors that influence transfection activity

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Abstract

We investigated the mode of interaction of lipoplexes (DOTAP:DOPE/DNA) with HeLa cells, focusing on the analysis of the initial steps involved in the process of gene delivery. We evaluated the effect of different factors, namely the stoichiometry of cationic lipids and DNA, the presence of serum in the cell culture medium, and the incorporation of the ligand transferrin into the lipoplexes, on the extent of binding, association and fusion (lipid mixing) of the lipoplexes with the cells. Parallel experiments were performed upon cell treatment with inhibitors of endocytosis. Our results indicate that a decrease of the net charge of the complexes (upon addition of DNA) generally leads to a decrease in the extent of binding, cell association and fusion, except for the neutral complexes. Association of transferrin to the lipoplexes resulted in a significant enhancement of the interaction processes referred to above, which correlates well with the promotion of transfection observed under the same conditions. Besides triggering internalization of the complexes, transferrin was also shown to mediate fusion with the endosomal membrane. The extent of fusion of this type of complexes was reduced upon their incubation with cells in the presence of serum, suggesting that serum components limit the transferrin fusogenic properties. Results were analyzed by using a theoretical model which allowed to estimate the kinetic parameters involved in lipoplex–cell interactions. The deduced fusion and endocytosis rate constants are discussed and compared with those obtained for other biological systems. From the kinetic studies we found a twofold enhancement of the fusion rate constant (*f*) for the ternary lipoplexes. We also concluded that HeLa cells yield a relatively low rate of endocytosis. Overall, our results estimate the relative contribution of fusion of lipoplexes with the plasma membrane, endocytosis and fusion with the endosomal membrane to their interactions with cells, this information being of crucial importance for the development of gene therapy strategies.

Keywords: Cationic liposome; Lipoplex; Endocytosis; Transferrin; Fusion rate constant; HeLa cell

Abbreviations: DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; DOPE, L-α-dioleoyl-phosphatidylethanolamine; DOTMA, *N*-(2,3-(dioleyloxy)propyl)-trimethylammonium; DC-Chol, 3β(*N*-(dimethylaminoethane)carbamoyl)cholesterol; DMRIE, *N*-(2,3-(dimyristyloxy)propyl)-dimethyl-*N*-(2-hydroxyethyl) ammonium bromide; FCS, fetal calf serum; Hepes, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; Rh-PE, *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine; Tf, transferrin

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1. Introduction

The possibility of delivering DNA efficiently to cells represents a crucial issue for the treatment of both genetic and acquired diseases. In order to improve gene delivery into cells both in vitro and in vivo, increasing attention has been focused on the development of methods and vectors capable of efficiently carrying the DNA into the target cells, as well as protecting it from degradation by nucleases. Although viral vectors are known for their high gene transfer efficiency and wide range of target cells, they have limitations that cannot be disregarded. Non-viral transfer vectors, such as molecular conjugates [1–3] or cationic liposomes [4], have a number of technological, therapeutic and toxicological advantages, thus providing a means to achieve a gene therapy strategy with a high degree of safety. However, inefficiency of transfection of these synthetic carriers [5] is a major problem confronting their use in gene therapy.

Cationic liposomes have been widely applied for the delivery of plasmid and antisense DNA into eukaryotic cells [4,6–16] and some clinical trials are currently in progress [17,18]. The mechanisms that enable cationic liposome/DNA complexes (lipoplexes [19]) to enter the cells, and their intracellular fate, are not completely characterized. Fusion of cationic liposomes with the plasma membrane was thought initially to be the mechanism of DNA delivery [4,13,20,21]. Although fusion between lipoplexes and the cellular membrane occurs [13], recent studies revealed that endocytosis is likely the main route for plasmid DNA delivery into the cells [7,13,22–27], since after transfection of cells the DNA was found mainly in endosome-like intracellular vesicles [13,25,26]. How the nucleic acids are released into the cytosol and transported into the nucleus [28], even if just a small fraction will not be degraded inside the lysosomal compartment [23,25], is still poorly understood. Because the lysosomal membrane is destabilized by cationic lipids [29], these may facilitate plasmid escape from lysosomes. It was suggested by van der Woude et al. [30] that incorporation through plasma membrane pores could also be an alternative mechanism for intracellular gene delivery.

As a result of its ability to form non-bilayer structures [31,32], as required for membrane fusion [33], DOPE is known to promote fusion [4,14]. In most experiments, the cationic liposomes are composed of an amphipathic mono- or polycationic carrier (DOTMA, DOTAP, DC-Chol or DMRIE) mixed with an equimolar amount of the 'helper' lipid DOPE. Depending on the type of cells and the cationic lipid used, DOPE may improve [13,15,34,35] or not [36,37] the expression of the transgene.

Transfection efficiency is determined by the limiting barriers involved in this process, such as the entry of lipoplexes into the cell, their escape from the endosome, dissociation of the plasmid from the lipid, translocation into the nucleus and finally transcription of the transgene. In an attempt to overcome some of the barriers mentioned to above and to achieve the successful use of lipoplexes as transfer vectors in gene therapy, researchers have employed human transferrin-associated lipoplexes (Tf-lipoplexes). The use of this ligand in combination with liposomes has proven to be effective in HeLa cells [38–40], where it could result in 100% transfection, although the DNA was only transiently transcribed. Recent work shows that transferrin facilitates the internalization of the ternary complexes through non-specific receptor-mediated endocytosis [40]. Whether it also facilitates fusion with the plasma membrane and/or the plasmid escape from the endosome is not clear.

The main reason for the limited knowledge of the cellular and molecular mechanisms of gene transfer is that in most cases transfection efficiency is dependent on the cell type used [9,30], and on liposome composition and properties [13,34], making it difficult to compare experimental results. This unpredictability, as well as the lack of agreement between cell culture and in vivo results, are some of the factors delaying the progress of improved methods in gene therapy.

In order to obtain more detailed and fundamental information about gene transfer mechanisms, we performed a series of studies focusing on the mode of interaction of cationic liposomes and of their DNA complexes (either plain or Tf-associated) with HeLa cells, and on different factors that affect lipofection such as lipoplex charge ratio, the inclusion of a 'helper' lipid, the presence of serum and the association of a ligand to the lipoplexes. We evaluated the extent of binding, association and fusion (lipid mixing) of lipo-

somes, lipoplexes and Tf-lipoplexes prepared at different lipid/DNA (+/−) charge ratios, in the presence or absence of inhibitors of endocytosis and in the presence or absence of serum. Additionally we performed a kinetic analysis of these processes. The cationic lipid was DOTAP, used successfully for in vitro and in vivo lipofection [8,41,42], with or without the 'helper' lipid DOPE. A human epithelial-like cell line (HeLa) was selected on the basis of its value for further studies, since it represents an important tool for gene transfer studies aimed at human gene therapy.

2. Materials and methods

2.1. Cells

HeLa cells, an epithelial-like adherent cell line derived from human tissue, were obtained from American Type Culture Collection, MD. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 and maintained in Dulbecco's Modified Eagle's Medium with High Glucose (DMEM-HG) (Sigma Chemical, St. Louis, MO) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Biochrom, Berlin, Germany), 1.168 g glutamine/l, 100 μg/ml of streptomycin and 100 units/ml of penicillin (Sigma). Cells were propagated by diluting the cell suspension 1/20 every 3–4 days. For use in lipid mixing experiments, HeLa cells were seeded in 35-mm culture dishes (Corning Costar Corp., Cambridge, MA) in a final volume of 2 ml, 3 days before experiments in order to allow growing to confluence (about 1.6×10^6 cells/dish). Cell viability was determined by Trypan blue exclusion.

2.2. Preparation of cationic liposomes and lipoplexes

The lipids DOTAP, DOPE and Rh-PE were purchased from Avanti Polar Lipids (Alabaster, AL). Liposomes containing DOTAP alone and DOTAP:DOPE at a 1:1 weight ratio were prepared by mixing the lipids and drying them from chloroform solution under vacuum using a rotatory evaporator. The dried lipid film was rehydrated with distilled water to a lipid concentration of 5 mg/ml and sonicated for 5 min. The resulting liposomes were then extruded 21 times through two stacked polycarbonate membranes (50 nm pore diameter), and diluted with distilled water to a final lipid concentration of 2 mg/ml. Phospholipid concentration was determined by a phosphate assay [43]. Liposomes were labeled by incorporating Rh-PE into the lipid mixture, at a self-quenching concentration (5 mol% of total lipid) and the suspension was stored at 4°C, under nitrogen, until use. The preparation of lipoplexes was carried out by gently mixing 5 μl of liposomes composed of DOTAP or DOTAP:DOPE and 100 μl of DNA solution containing a predetermined amount of DNA (deoxyribonucleic acid from calf thymus, purchased from Sigma, dissolved in distilled water) in order to obtain the desired lipid/DNA (+/−) charge ratio. The mixture was incubated for 15 min at room temperature before addition to the cells. The Tf-lipoplexes were obtained by gently mixing 5 μl of liposomes with 100 μl of human transferrin solution (Collaborative Biomedical Products, Bedford, MA) 15 min prior to the addition of 100 μl DNA solution, and the resulting mixture was further incubated for 15 min. The transferrin solution was prepared at 320 μg/ml in 20 mM Hepes buffer, pH 7.4 (Sigma) and 100 mM NaCl (Merck, Darmstadt, Germany). Lipoplexes were prepared immediately before experiments.

2.3. Fluorimetric measurements

2.3.1. Cell association and binding assays

Cell association experiments were performed at 37°C. HeLa cells were incubated (two times freshly washed with 1 ml serum-free DMEM-HG) with 5 μl of fluorescently labeled liposomes/lipoplexes, in a final volume of 1 ml (serum-free DMEM-HG). After different incubation times, the medium containing the non-associated liposomes/lipoplexes was collected and diluted to a final volume of 2 ml of serum-free DMEM-HG. Fluorescence was measured at 37°C following addition of Triton X-100 (Merck) at a final concentration of 0.5% (v/v). To assess the fluorescence associated with the cells, cells were rinsed and detached from the culture dishes with disposable scrapers (Corning Costar), and then suspended in 2 ml of medium. The fluorescence of the cell suspension was measured in the presence of Triton X-100 as described above.

Extent of cell association was determined according to the following equation:

$$\%\ \text{cell association} = \frac{F_{\text{cells}}}{F_{\text{non-associated}} + F_{\text{cells}}} \times 100 \quad (1)$$

where F_{cells} is the value of fluorescence associated with the cells and $F_{\text{non-associated}}$ is the value of fluorescence of non-associated liposomes/lipoplexes.

Binding studies were performed at 4°C (on ice). Cells (two times freshly washed with 1 ml serum-free DMEM-HG) were incubated with the liposomes/lipoplexes in a final volume of 1 ml (serum-free DMEM-HG). After different incubation times, the medium with the non-associated liposomes/lipoplexes was collected and diluted to a final volume of 2 ml of serum-free DMEM-HG. Cells were rinsed and detached from the culture dishes with disposable scrapers, and then suspended in 2 ml of medium. The experimental procedure for binding quantification was the same as that described for the cell association measurements. Extent of binding was determined according to Eq. 1.

2.3.2. Fusion (lipid mixing) assay

Lipid mixing between HeLa cells and cationic liposomes, plain lipoplexes or Tf-lipoplexes (ternary complexes) was evaluated by monitoring the increase of fluorescence of Rh-PE incorporated in the liposomal membrane at a self-quenching concentration.

Cells (two times freshly washed with 1 ml serum-free DMEM-HG) were incubated with the liposomes/lipoplexes in a final volume of 1 ml (serum-free DMEM-HG). After 1 h incubation on ice, the medium containing the non-associated liposomes/lipoplexes was removed. Cells were rinsed with 1 ml of culture medium and then incubated in 2 ml of the same medium at 37°C to promote fusion. After different incubation times, culture dishes were transferred to ice to stop fusion. Cells were detached as described previously and the extent of fusion was determined by measuring the fluorescence of cell suspension at 20°C. Values are given as a percentage of the maximal fluorescence which was obtained upon addition of Triton X-100 at a final concentration of 0.5% (v/v). The initial fluorescence of the bound Rh-PE-labeled liposomes/lipoplexes (and the scattering of the cells) was set as 0% fluorescence. This zero value was determined, for each experimental condition, after 60 min incubation of the liposomes/lipoplexes with the cells at 4°C; the unbound liposomes/lipoplexes were removed and the fluorescence of the cell suspension was measured at 20°C.

Extent of fusion was determined according to the following equation:

$$\%\ \text{fusion} = \frac{F_t - F_0}{F_{\max} - F_0} \times 100 \quad (2)$$

where F_t is value of fluorescence after t min, $F_{\max}$ is the value of fluorescence after addition of Triton-X100 and F_0 is the zero value.

The results of lipid mixing were expressed as a percentage of the maximal fluorescence (F_{cells}+ $F_{\text{bound liposomes/lipoplexes}}$, after addition of Triton X-100). Since different extents of binding were obtained for each charge ratio tested, fusion results were normalized taking into account the respective extent of binding.

Parallel experiments of cell association, binding and fusion were performed in the presence of drugs that interfere with the endocytotic pathway as well as in the presence of 10% serum (FCS). HeLa cells were pre-treated with the metabolic inhibitors of endocytosis, antimycin A (1 μg/ml), sodium fluoride (10 mM) and sodium azide (0.1%, w/v), for 30 min, at 37°C, prior to their incubation with the lipoplexes in the presence of the drugs.

All fluorescence measurements were performed in a SPEX Fluorolog 2 fluorometer (SPEX Industries, Edison, NJ). The fluorescence was read at excitation and emission wavelengths of 568 and 590 nm, respectively, using 0.5 mm excitation and 1 mm emission slits. For these experiments 4 ml volume and 10 mm path length disposable fluorimetric cuvettes (Hughes and Hughes, Tonedale, Wellington) were used. The sample chamber was equipped with a magnetic stirrer and the temperature was controlled with a thermostatic circulator.

2.3.3. Kinetic measurements

For kinetic studies, parallel experiments of binding, cell association and lipid mixing were performed by incubating lipoplexes (including both plain and ternary complexes) with cells, in the absence and in the presence of metabolic inhibitors of endocytosis, for different times, using different lipid concentra-

tions, while maintaining constant the lipid/DNA/transferrin mole ratio.

The binding studies were performed by incubating the lipoplexes with the cells at 4°C, for different times, as described previously. Cell association and fusion (lipid mixing) experiments were also carried out as described before, except that for some fusion experiments no pre-incubation at 4°C was performed. Therefore, the extent of fusion was determined as a percentage of the total amount of lipoplexes added to the cells.

2.3.3.1. Analysis of fusion kinetics. (i) Pre-binding at 4°C. By elevating the temperature the bound lipoplexes begin to fuse according to Nir et al. [44]

$$\frac{dL}{dt} = fL \tag{3}$$

$$L(t) = Lo.\exp(-ft) \tag{4}$$

$$\bar{F}(t) = Lo - L(t) = Lo(1-\exp(-ft)) \tag{5}$$

in which f is the fusion rate constant and $L(t)$, Lo and $F(t)$ are the molar concentrations of bound, total and fused lipoplexes, respectively. The fraction, F, of fused lipoplexes is given by

$$\bar{F} = F(t)/Lo = 1-\exp(-ft) \tag{6}$$

The rate constant, f, can be determined from Eq. 6, or, alternatively, Eq. 6 can simulate the experimental results.

We introduced some extension to these equations, in order to account for two types of effects:

1. As noted before [45], the fusion process might be initiated with some lag time.
2. As fusion progresses, the cellular plasma membrane becomes loaded with positively charged lipid molecules, which may result in reduced fusogenicity. Hence, it is instructive to characterize the fusion process at each time interval by the appropriate rate constant.

Consider a time interval between t_1 and t_2, such that

$$T = t_2 - t_1 \tag{7}$$

At the beginning of this time interval the concentration of unfused lipoplexes is L_1. Eqs. 3–5 still hold if t is replaced by T, i.e.,

$$F(T) = L_1 \bullet (1-\exp(-fT)) \tag{8}$$

in which

$$F(T) = F(t_2) - F(t_1) = F_2 - F_1 \tag{9}$$

where F_1 and F_2 denote the concentration of fused lipoplexes at times t_1 and t_2. From Eqs. 8 and 9, it follows that

$$1-(F_2-F_1)/L_1 = \exp(-fT) \tag{10}$$

By setting $L_1 = Lo - F_1$ and $\overline{F_1}/Lo$, Eq. 8 gives

$$(1-\overline{F_2})/(1-\overline{F_1} = \exp(-fT) \tag{11}$$

The fusion rate constant in Eq. 11 pertains to the time interval between t_1 and t_2. For $t_1 = 0$ Eq. 11 coincides with Eq. 6.

(ii) No pre-binding. Parallel experiments of fusion were performed without pre-binding at 4°C. Here the analysis followed the procedure [44], which also considers binding of liposomes to the cells and their dissociation. In principle a further extension in the program can also consider endocytosis and fusion of lipoplexes with endosomes.

3. Results

3.1. Effect of lipid/DNA charge ratio on the extent of lipoplex–cell interactions

In order to evaluate the influence of the charge ratio of the lipoplexes on their mode of interaction with cells, lipoplexes composed of DOTAP:DOPE and DNA were prepared at different lipid/DNA (+/−) charge ratios and incubated with HeLa cells for different periods of time, since incubation time has been shown to affect transfection activity [38].

As illustrated in Fig. 1, lipoplexes reach maximal cell association (which includes cell binding, membrane fusion and endocytosis) when prepared at a 1/1 (+/−) charge ratio. When the complexes were prepared at lipid/DNA charge ratios different from 1/1, no significant differences were obtained for their extent of cell association. While for these lipoplexes no significant variation in the extent of cell association is observed with incubation time, for the 1/1

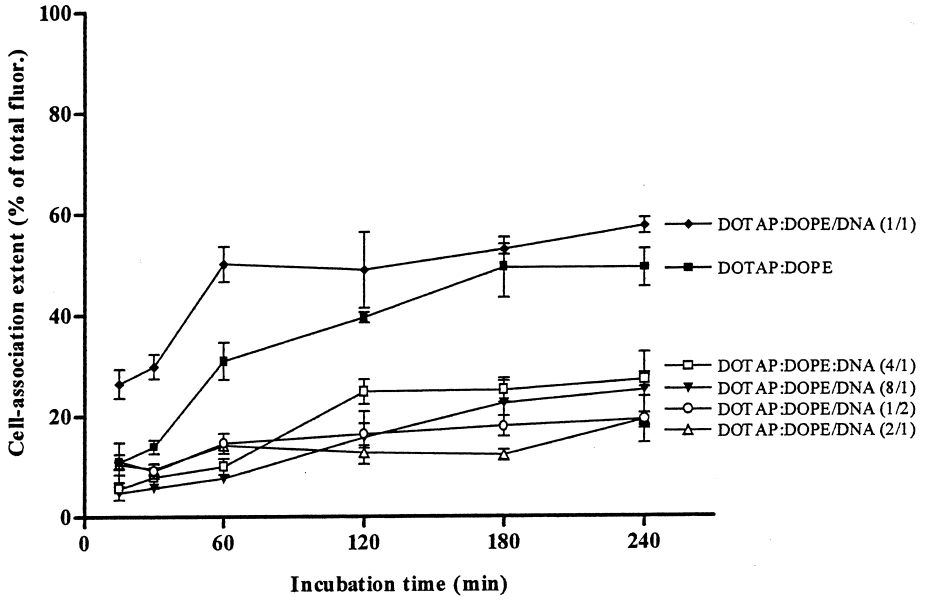

Fig. 1. Association of DOTAP:DOPE (1:1, w/w) liposomes or lipoplexes at various lipid/DNA (+/−) charge ratios with HeLa cells. The extent of cell association was measured at 37°C, after 15, 30, 60, 120, 180 and 240 min incubation at 37°C, as described in Section 2. For each culture dish, the cationic lipid concentration was approximately 7 μM and the cell density was approximately 1.6×10^6 cells/ml. The data are expressed as a percentage of the total fluorescence and represent the mean ± standard deviation from experiments carried out in triplicate.

lipoplexes a remarkable increase is observed for the initial times (1 h), after which a plateau is reached. As can also be observed in Fig. 1, complexation of DNA with liposomes generally leads to a decrease in the extent of their cell association, which can be attributed to the reduction of the positive net charge associated with the liposomes. However, the highest values of cell association observed for the 1/1 lipoplexes can be explained by their precipitation (sedimentation) over cells which occurs as a result of extensive aggregation favored by lack of repulsive forces (the complexes are approximately neutral).

No significant differences in the extent of binding (Fig. 2) or fusion (Fig. 3) are observed for the various lipoplexes, independently of the lipid/DNA charge ratio tested. It is possible that the 1/1 lipoplexes do not bind too strongly to the cells, such that they are washed away in the binding experiments, thus leading to low extents of binding. It is interesting to note that the 1/1 lipoplexes exhibit low values of binding and fusion as compared to liposomes. Since cell association encompasses binding, fusion with the plasma membrane, endocytosis and fusion with the endosomal membrane, and taking into con-

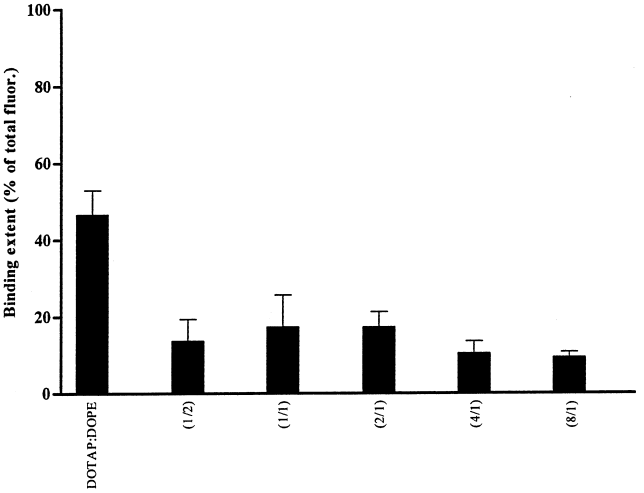

Fig. 2. Binding of DOTAP:DOPE (1:1, w/w) liposomes or lipoplexes at various lipid/DNA (+/−) charge ratios, to HeLa cells. The extent of binding was measured at 37°C, after 60 min incubation at 4°C, as described in Section 2. For each culture dish, the cationic lipid concentration was approximately 7 μM and the cell density was approximately 1.6×10^6 cells/ml. The data are expressed as a percentage of the total fluorescence and represent the mean ± standard deviation from experiments carried out in triplicate.

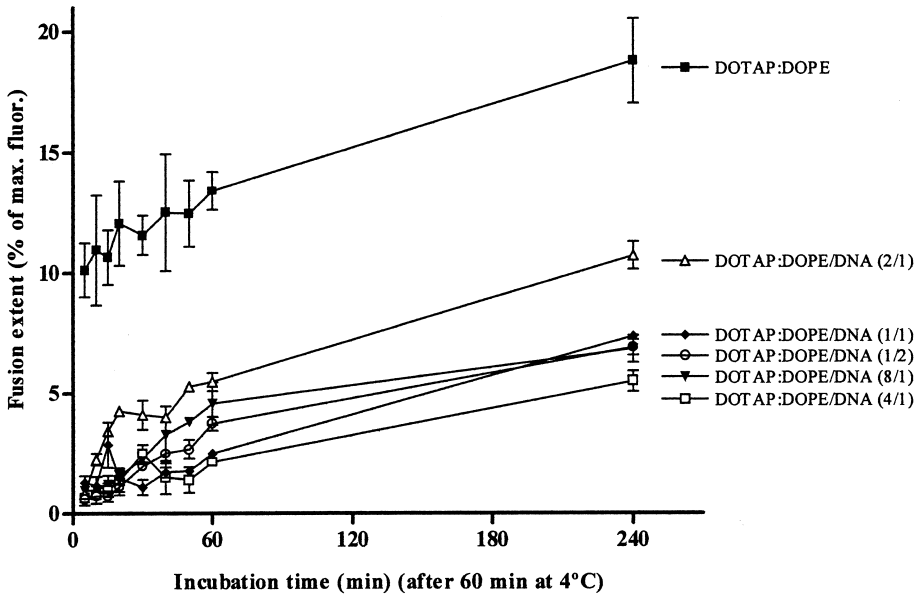

Fig. 3. Fusion (lipid mixing) between DOTAP:DOPE (1:1, w/w) liposomes or lipoplexes at various lipid/DNA (+/−) charge ratios and HeLa cells. Lipid mixing between HeLa cells and liposomes or lipoplexes was measured at 20°C following the dequenching of Rh-PE incorporated into the liposomal membrane at 5 mol%, after 5, 10, 15, 20, 30, 40, 50, 60 and 240 min incubation at 37°C, as described in Section 2. For each culture dish, the cationic lipid concentration was approximately 7 μM and the cell density was approximately 1.6×10^6 cells/ml. The data are expressed as a percentage of the maximal fluorescence and represent the mean ± standard deviation from experiments carried out in triplicate.

sideration the results reported above for the 1/1 lipoplexes, it can be suggested that endocytosis represents the major pathway for the internalization of these lipoplexes, which may also be extended for the lipoplexes prepared at other lipid/DNA charge ratios.

A similar pattern was observed when the complexes were prepared from pure DOTAP liposomes (data not shown), suggesting that the presence of the 'helper' lipid (DOPE) in the liposome composition does not affect the mode of interaction of the lipoplexes with HeLa cells.

3.2. *Effect of inhibitors of endocytosis and serum on lipoplex–cell interactions*

In an attempt to demonstrate that in fact endocytosis represents the major pathway for the internalization of the lipoplexes by HeLa cells, we carried out experiments to assess lipoplex–cell interactions, namely cell association, binding and fusion, in the presence of metabolic inhibitors of endocytosis. For that purpose, lipoplexes were incubated for 60 min with cells that have been pre-treated with these drugs. This condition was selected because our previous results (see Figs. 1 and 3) had shown that a great deal of lipoplex uptake and fusion occurred after that time. Under these conditions, a decrease in the extent of association of the various lipoplexes tested with HeLa cells is observed (Fig. 4A), this effect being particularly relevant for the 1/1 lipoplexes. However, lipid mixing experiments (Fig. 4B) performed with cells that had been pre-treated with these drugs show that no significant decrease in the extent of fusion is observed for the 1/1 lipoplexes, while a slight decrease is noted for the positively charged complexes (2/1 and 4/1). Therefore, these results provide further evidence that endocytosis is the main determinant to the extent of cell association for the 1/1 lipoplexes. On the other hand, these results are in close agreement with our previous findings that transfection is significantly inhibited when the endocytotic pathway is blocked [40]. Moreover, since lipid mixing is not significantly affected upon inhibition of endocytosis, whereas under the same conditions transfection is essentially abolished [40], it can also be concluded that no correlation between lipid mixing and transfection can be established,

which again is in agreement with previous findings [35,46].

In order to gain insights into the mode by which different factors affect the lipoplex biological activity, we investigated the effect of serum on the various steps involved in the interaction of lipoplexes with HeLa cells. This knowledge is of particular interest since transfection experiments should ideally be carried out in the presence of serum, in an attempt to mimic the conditions observed in vivo. Similarly to what has been described for the effect of inhibitors of endocytosis, a significant decrease in the extent of cell association is observed for the 1/1 lipoplexes when they are incubated with HeLa cells in the presence of 10% FCS, whereas no effect is noted for the other lipid/DNA charge ratios tested (Fig. 4A). On the other hand, no relevant effect was noted for the extent of fusion (Fig. 4B), as well as for the extent of binding (data not shown), independently of the lipoplex charge ratio tested.

3.3. Mode of interaction of transferrin-associated lipoplexes with HeLa cells

It was previously demonstrated that lipoplexes are internalized mainly by the endocytotic pathway [7,13,22–27]; the results from the present study with the 1/1 lipoplexes confirm these findings and reinforce the importance of the physico-chemical characteristics of the lipoplexes in this process. In an attempt to enhance transfection we explored this pathway, by promoting receptor-mediated endocytosis of the complexes. For that purpose, different ligands (e.g., transferrin) targeted to cell surface receptors that undergo endocytosis, were associated to lipoplexes [38–40]. Although these strategies were shown to be very promising, leading to a significant enhancement of transfection as compared to plain lipoplexes, studies on the mechanisms by which Tf-lipoplexes deliver DNA into cells showed that lipoplexes of this type interact and are internalized mainly by non-specific pathways [40]. Alerted by the results described in the previous sections for the plain lipoplexes, we extended these studies to investigate the mode of interaction of the ternary com-

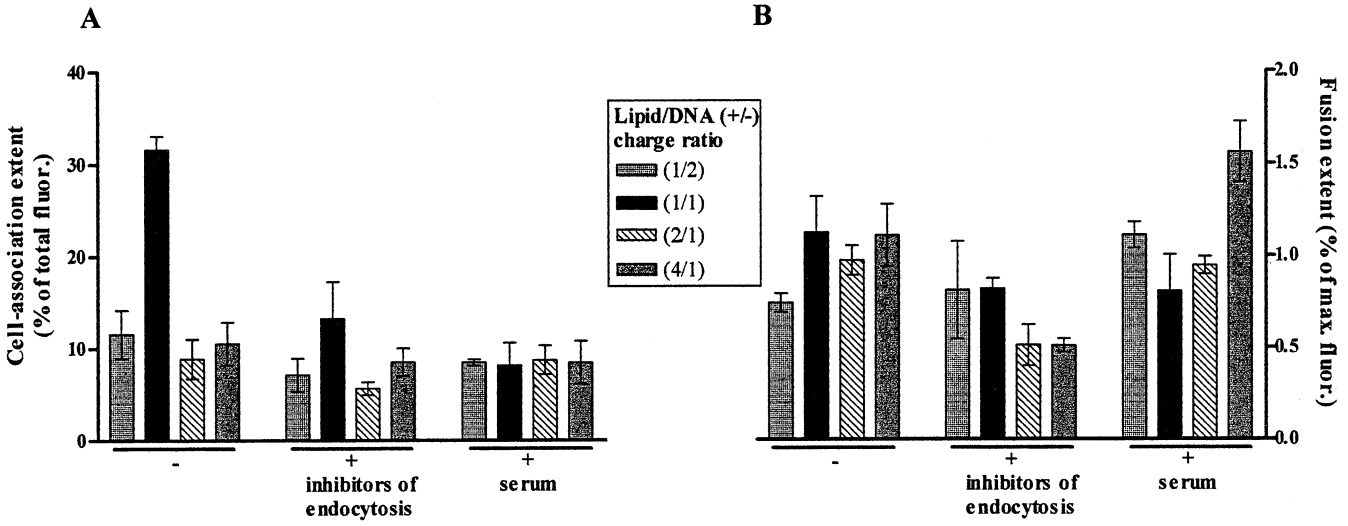

Fig. 4. Effect of inhibitors of endocytosis and serum on association and fusion (lipid mixing) of DOTAP:DOPE lipoplexes with HeLa cells. (A) The extent of cell association was measured at 37°C, after 60 min incubation at 37°C, as described in Section 2. The effect of inhibitors of endocytosis was investigated by treating HeLa cells for 30 min with 1 μg/ml of antimycin A, 10 mM sodium fluoride and 0.1% (w/v) sodium azide, at 37°C. The effect of serum was evaluated by incubating the cells with the lipoplexes in the presence of 10% FCS. Values are expressed as a percentage of the total fluorescence. (B) Fusion experiments were performed as described in the legend to Fig. 3. The effects of inhibitors of endocytosis and serum were evaluated as described above. Values are expressed as a percentage of the maximal fluorescence. Plain lipoplexes, were prepared at 1/2, 1/1, 2/1 and 4/1 lipid/DNA (+/−) charge ratios. For each culture dish, the cationic lipid concentration was approximately 7 μM and the cell density was approximately 1.6×10^6 cells/ml. The data represent the mean ± standard deviation obtained from experiments carried out in triplicate.

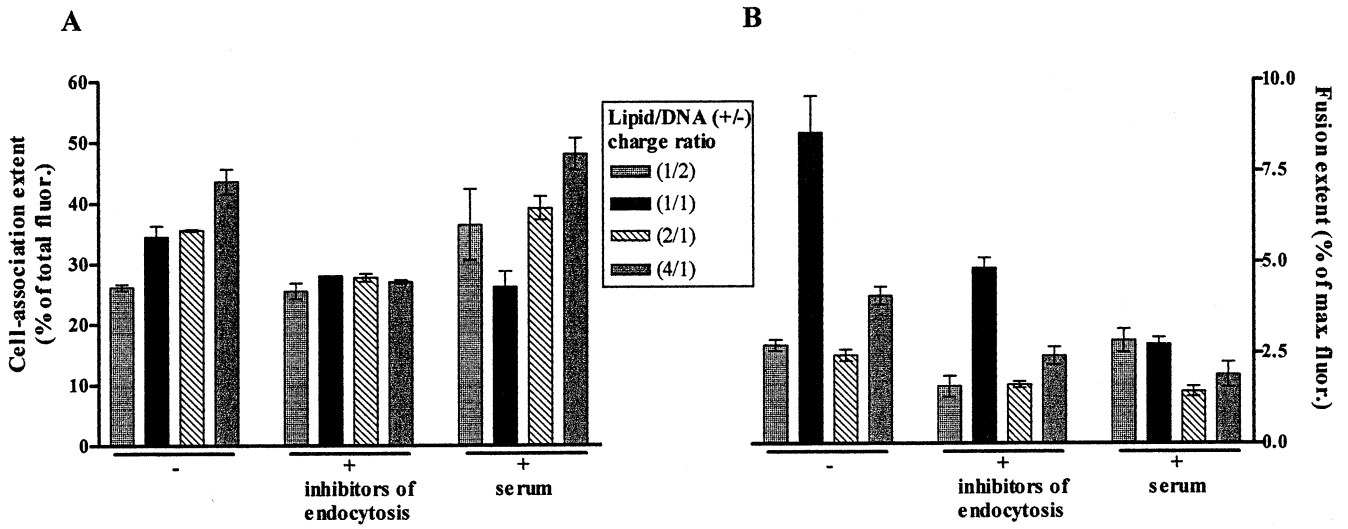

Fig. 5. Effect of inhibitors of endocytosis and serum on association and fusion (lipid mixing) of transferrin-associated DOTAP:DOPE lipoplexes with HeLa cells. (A) The extent of cell association was measured as described in the legend to Fig. 4A. (B) Fusion experiments were performed as described in the legend to Fig. 4B. Tf-lipoplexes were prepared at 1/2, 1/1, 2/1 and 4/1 lipid/DNA (+/−) charge ratios. The liposomes were associated with 32 μg of transferrin as described in Section 2. For each culture dish, the cationic lipid concentration was approximately 7 μM and the cell density was approximately 1.6×10^6 cells/ml. The data represent the mean ± standard deviation obtained from experiments carried out in triplicate.

plexes (containing transferrin) with cells, by quantifying cell association, binding and fusion in the absence or presence of inhibitors of endocytosis.

Association of transferrin to the lipoplexes results in a significant enhancement of the extent of cell association as compared to plain lipoplexes (compare Figs. 5A and 4A). The enhancement is observed for all the charge ratios tested, except for the 1/1 lipoplexes. An enhancement in binding was also observed upon association of transferrin to the lipoplexes (data not shown).

Upon cell treatment with inhibitors of endocytosis, a decrease in the extent of cell association of Tf-lipoplexes is observed for the neutral and positively charged complexes (Fig. 5A). This can be attributed to a reduction in their extent of internalization, since binding is not affected by the presence of these drugs (data not shown).

Similarly to what was described for cell association, the exposure of HeLa cells to the Tf-lipoplexes leads to an increase in their fusion extent for all the charge ratios tested (compare Fig. 5B with 4B). However, in contrast to what was described for the cell association studies, the enhancement observed for the extent of fusion of 1/1 lipoplexes induced by transferrin is much more significant than that observed for cell association. The extent of fusion of Tf-lipoplexes is significantly reduced upon treatment of the cells with inhibitors of endocytosis, suggesting that fusion of Tf-lipoplexes with HeLa cells occurs to a large extent following their internalization. This decrease in the extent of fusion can also justify the reduction in the cell association observed under the same conditions. Regarding the effect of serum on the interaction of ternary complexes with the cells, cell association remains unaltered (Fig. 5A) whereas fusion is significantly inhibited (Fig. 5B). Whether fusion is inhibited at the level of the plasma membrane, and/or inside the cell is a question that remains to be addressed.

3.4. Kinetic measurements

In this study we have established experimental and theoretical procedures for quantifying the binding, fusion and total cell association of both plain and ternary complexes. For that purpose different concentrations of lipoplexes, and different times for their incubation with cells, were tested both in the absence and in the presence of inhibitors of endocytosis. As shown in Fig. 6, the differences in cell association and fusion extents (as for the binding extent – data

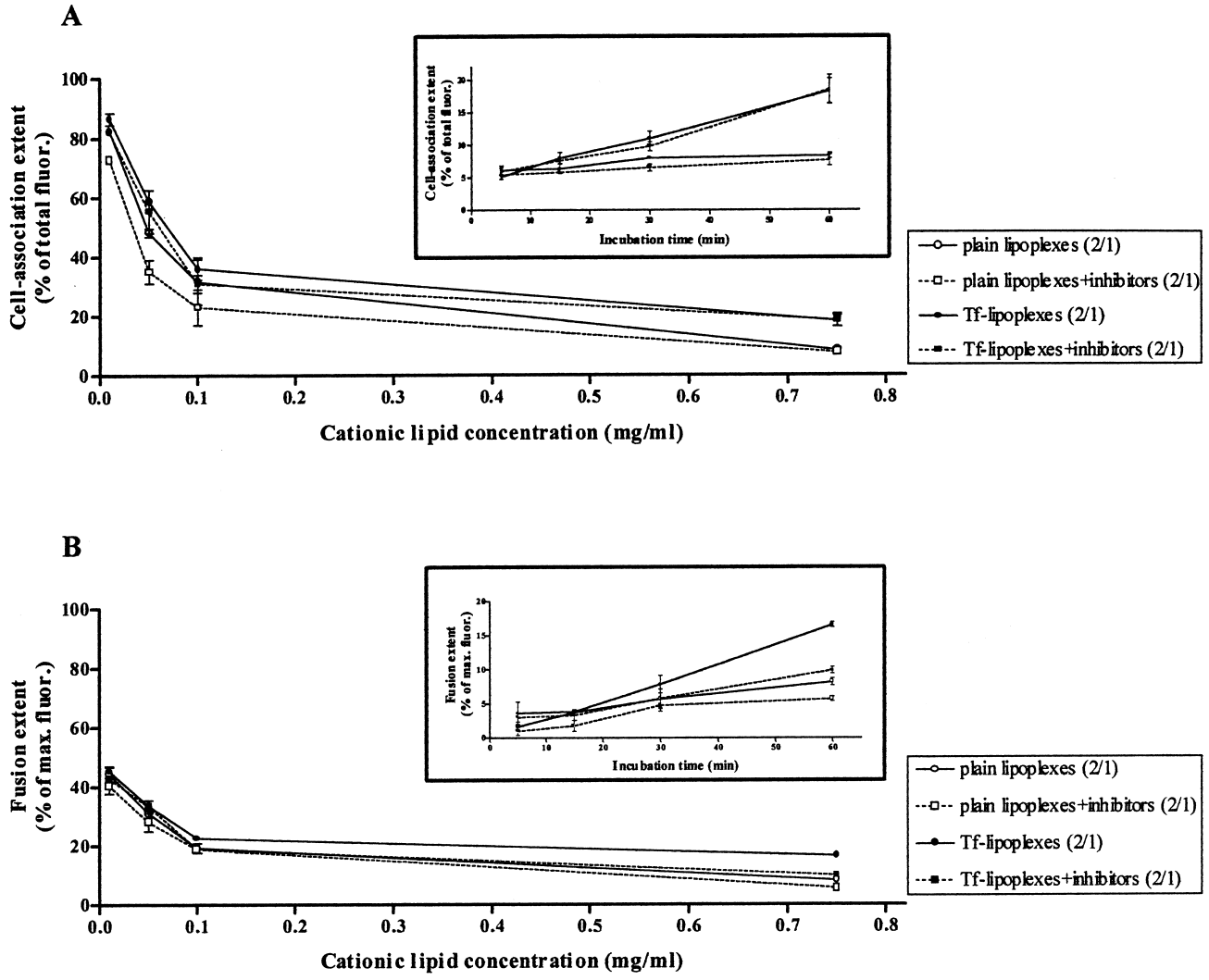

Fig. 6. Effect of lipid concentration on (A) association and (B) fusion (lipid mixing) of DOTAP:DOPE lipoplexes (plain and transferrin-associated) with HeLa cells. DOTAP:DOPE lipoplexes (2/1 lipid/DNA (+/−) charge ratio) were prepared at 0.75, 0.1, 0.05 and 0.01 mg DOTAP/ml. Tf-lipoplexes were prepared using a transferrin solution at an adequate concentration for each lipid concentration (24, 3.2, 1.6 and 0.32 μg transferrin/100 μl solution, respectively). Cell density was approximately 1.6×10^6 cells/ml. The extent of cell association was measured at 37°C, after 60 min incubation of the complexes with the cells at 37°C. Values are expressed as a percentage of the total fluorescence. Inset: extent of association of DOTAP:DOPE lipoplexes (2/1 lipid/DNA (+/−) charge ratio) with HeLa cells, after 5, 15, 30 and 60 min incubation. DOTAP:DOPE lipoplexes were prepared at 0.75 mg DOTAP/ml. The extent of fusion was measured at 20°C, after 60 min incubation at 37°C with the cells. Values are expressed as a percentage of the maximal fluorescence. Inset: extent of fusion (lipid mixing) between DOTAP:DOPE lipoplexes (2/1 lipid/DNA (+/−) charge ratio) and HeLa cells, after 5, 15, 30 and 60 min incubation. DOTAP:DOPE lipoplexes were prepared at 0.75 mg DOTAP/ml. Parallel experiments were performed in the presence of inhibitors of endocytosis as described in Section 2. The data represent the mean ± standard deviation obtained from experiments carried out in triplicate.

not shown) between plain and ternary complexes (prepared at 2/1 (+/−) charge ratio) are particularly evident for the highest lipid concentration (and consequently highest transferrin concentration). Regarding the effect of inhibitors of endocytosis, it should be noted that for the highest lipid concentration an inhibition of the fusion extent (Fig. 6B) was noted only for the Tf-lipoplexes, which again reinforces the idea that part of the fusion events with target membranes, mediated by this type of complexes, occurs following their internalization. On the other hand, no significant effect was observed on the extent of cell

association, independently of the type of complexes tested. It is also interesting to note that as lipid concentration decreases, the lipoplex–cell interactions, including binding, cell association and fusion, are enhanced, both in the absence and in the presence of inhibitors of endocytosis. This may be attributed to a larger number of available lipoplex binding sites at the cell surface. Moreover, at lower lipid concentrations no significant differences were found between transferrin-associated and plain lipoplexes for both cell association and fusion. This observation suggests that the role of transferrin in promoting binding and fusion of the lipoplexes to and with cells is only effective above a given concentration of this protein. Below this concentration lipoplex–cell interactions seem to be dictated by the properties of the lipoplexes. In fact, inspection of the fusion results shows that at the lowest concentration the extents of fusion for both plain and ternary complexes, after 1h incubation with the cells approaches 50%, whereas at the highest concentration the extents of fusion are 8.2% and 16.6% for the plain and the ternary complexes, respectively (experiments performed in the absence of inhibitors of endocytosis). This trend, as well as the slowing down of the kinetics of fusion with lipoplex concentration, is also observed for intermediate concentrations. As illustrated in the inserts on Fig. 6, for the highest lipid concentration there is an increase in cell association and fusion extents with time. Moreover, association of transferrin to the lipoplexes results in an enhancement of the extent of lipoplex–cell interactions, mainly for the longest incubation times. Still regarding the effect of incubation time on lipoplex–cell interactions, cell treatment with inhibitors of endocytosis results in a significant inhibition of the extent of fusion of Tf-lipoplexes. However, no clear effect was observed on the cell association extent. A similar pattern was observed for all the lipid concentrations tested.

3.5. Fusion rate constants

To further characterize the fusion processes involved in lipoplex–cell interactions, we applied a theoretical model to estimate the kinetic parameters. Table 1 summarizes the results in terms of the corresponding fusion rate constants. We will first focus on the results based on pre-incubation of lipoplexes with cells at 4°C. The kinetics of fusion curves corresponding to 0.05 mg/ml can be adequately simulat-

Table 1
Fusion rate constants (s^{-1}) deduced for different concentrations of lipoplexes (2/1 lipid/DNA (+/−) charge ratio) and time intervals.

System: plain (P) or ternary (T) lipoplexes	DOTAP concentration (mg/ml)	Time intervals (min) 0–t				
		5	15	30	60	30–60
P	0.01	$(1.2\pm0.1)\times10^{-3}$	$(1.1\pm0.2)\times10^{-3}$	$(6.5\pm1)\times10^{-4}$	$(4.8\pm1)\times10^{-4}$	3.0×10^{-4}
		$(3.4\pm0.3)\times10^{-3}$[a]	$(3.6\pm0.4)\times10^{-3}$[a]	$(5.3\pm1)\times10^{-4}$[a]		
T	0.01	$(2.3\pm0.2)\times10^{-3}$	$(8.5\pm0.2)\times10^{-4}$	$(7.0\pm1)\times10^{-4}$	$(5.6\pm1)\times10^{-4}$	4.0×10^{-4}
		$(6.6\pm1)\times10^{-3}$[a]	$(1.1\pm0.2)\times10^{-3}$[a]	$(4.6\pm1)\times10^{-4}$[a]		
P	0.05	$(2.8\pm0.4)\times10^{-4}$	$(3.3\pm0.5)\times10^{-4}$	$(2.8\pm0.5)\times10^{-4}$	$(2.1\pm0.5)\times10^{-4}$	1.4×10^{-4}
		$(2.0\pm0.5)\times10^{-3}$[a]	$(8.4\pm2)\times10^{-4}$[a]	$(1.2\pm0.4)\times10^{-3}$[a]		
T	0.05	$(3.5\pm1)\times10^{-4}$	$(6.0\pm2)\times10^{-4}$	$(3.6\pm1)\times10^{-4}$	$(3.7\pm1)\times10^{-4}$	3.8×10^{-4}
		$(2.0\pm0.5)\times10^{-3}$[a]	$(1.0\pm0.3)\times10^{-3}$[a]	$(1.0\pm0.3)\times10^{-3}$[a]		
P	0.1	$(2.8\pm0.5)\times10^{-4}$	$(1.2\pm0.3)\times10^{-4}$	$(6.7\pm1)\times10^{-5}$	$(5.5\pm1)\times10^{-5}$	4.4×10^{-5}
		$(2.5\pm1)\times10^{-3}$[a]	$(1.1\pm0.6)\times10^{-3}$[a]	$(1.2\pm0.6)\times10^{-3}$[a]		
T	0.1	$(3.3\pm0.5)\times10^{-4}$	$(1.4\pm0.4)\times10^{-4}$	$(7.8\pm1)\times10^{-5}$	$(5.3\pm1)\times10^{-5}$	2.8×10^{-5}
		$(3.8\pm1)\times10^{-3}$[a]	$(1.6\pm0.6)\times10^{-3}$[a]	$(1.1\pm0.5)\times10^{-3}$[a]		
P	0.5	$(1.9\pm0.2)\times10^{-4}$	$(7.6\pm2)\times10^{-5}$	$(5.4\pm1)\times10^{-5}$	$(3.9\pm1)\times10^{-5}$	2.3×10^{-5}
T	0.5	$(3.4\pm0.5)\times10^{-4}$	$(12.0\pm3)\times10^{-5}$	$(7.3\pm2)\times10^{-5}$	$(5.2\pm1)\times10^{-5}$	3.1×10^{-5}
P	1.0	$(8.4\pm2)\times10^{-5}$	$(6.1\pm1)\times10^{-5}$	$(3.6\pm1)\times10^{-5}$	$(2.6\pm1)\times10^{-5}$	1.6×10^{-5}
T	1.0	$(14.0\pm3)\times10^{-5}$	$(10.0\pm2)\times10^{-5}$	$(6.8\pm1)\times10^{-5}$	$(5.6\pm1)\times10^{-5}$	4.4×10^{-5}

[a]The values are lower bounds deduced from cases without prior pre-incubation of lipoplexes, but in the presence of inhibitors of endocytosis. In all other cases lipoplexes were pre-incubated with the cells at 4°C as described in Section 2.

ed with a single value of the fusion rate constant, whereas in all other cases the values of fusion rate constant decrease with time, i.e., with the extent of fusion. The corresponding fusion rate constants in the ternary system are about twofold larger than those obtained with the plain lipoplexes. From the fact that with 0.05 mg/ml no decrease is observed in the rate constant of fusion at later times, it can be concluded that inactivation of fusion does not exist in this biological system. In fact, if inactivation of fusion does exist, we expect it to occur for all lipoplex concentrations, although perhaps to varying degrees. The case of 0.05 mg/ml does not correspond to the smallest lipid concentration. Since no fusion inactivation is observed for this case we can rule this out for all other cases as well. We interpret the decrease in the fusion rate constant with time or with the extent of fusion for the larger lipoplex concentrations, i.e., 0.1 and 1.0 mg/ml, to reflect mostly a reduction in the fusogenicity of the plasma membranes upon excessive loading of positive charges. The last column in Table 1 emphasizes this point. In certain cases, the fusion rate constants have very similar values for equal amounts of positive charges added to the plasma membrane via fusion. For instance, the values of the fusion rate constants are very similar for 1 mg/ml at 5 min and 0.5 mg/ml at 15 min, corresponding to a ratio of 1:2 in the percents of fusion. The same similarity holds for these cases at the respective times 15 vs. 30 min and 30 vs. 60 min. The decrease in the fusion rate constants from 15 min to 60 min for the lowest lipoplex concentration might reflect the existence of a small number of sites per cell (< 20) where the tendency to fuse is larger.

Table 1 also gives lower bounds on fusion rate constants, which were deduced from experiments without pre-incubation at 4°C. In these cases, we considered fusion and cell association results in the presence of inhibitors of endocytosis. By using cell association results at a given time, e.g., 5 min, the deduced f value is underestimated, because the calculations considered the final values of cell association at that time interval. Table 1 illustrates that these values are larger than those based on pre-incubation of lipoplexes with cells at 4°C. It was noted before that pre-incubation of cells and virions on ice followed by exposure to a warm buffer resulted in a reduction in the deduced fusion rate constants [44,45,47].

4. Discussion

Since the role of membrane fusion in gene transfer has been questioned, we addressed studies to clarify the mechanisms by which cationic liposomes, currently the most used non-viral gene delivery systems, deliver genetic material into cells, an essential aspect to facilitate cell transfection. Although these mechanisms are not well characterized, there is clear evidence that lipoplexes are internalized through an endocytotic pathway [7,13,22–27], rather than through fusion with the plasma membrane as proposed in earlier studies [4,13,20,21].

In the present work we investigated the interactions of cationic liposomes and of their DNA complexes with HeLa cells, namely assessing the extent of binding, cell association and fusion. In addition we performed a kinetic analysis of these processes.

Our results indicate that in general complexation of DNA with DOTAP containing liposomes gives rise to a decrease in the liposome–cell interactions, which is in agreement with previous observations by Stegmann and Legendre [35]. The association between lipid and DNA, due to electrostatic interactions, leads to a reduction of the net positive charge of the liposomes and thus to less repulsion between them. This partial neutralization of the surface charges promotes aggregation and fusion of liposomes, which results in lipidic structures exhibiting a larger size [32]. The existence of such structural changes, together with the decrease in the net positive charge, may explain the observed decrease in the lipoplex–cell interactions. However, the analysis of the results obtained, in particular those for the 1/1 lipoplexes (see Figs. 1–3), shows that although their extents of binding and fusion are similar to those observed for the other lipid/DNA charge ratios tested, they exhibit the highest levels of cell association (even higher than for liposomes). This may be explained by the major contribution of their sedimentation over cells as compared to the electrostatic interactions of lipoplexes with the plasma membrane. These data also suggest that lipoplexes of this type are internalized mainly by endocytosis. A similar

interpretation can be made for the other lipoplexes. To confirm this possibility, we performed parallel experiments involving pre-treatment of cells with inhibitors of endocytosis. As illustrated in Fig. 4A, a decrease in the amount of cell-associated lipid was observed, this effect being particularly relevant for the 1/1 lipoplexes. However, this inhibitory effect was not notorious for the extent of fusion. Since cell association includes binding, fusion with the plasma membrane, endocytosis and fusion with the endosomal membrane, these results clearly show that the endocytotic pathway plays an important role in the internalization process of the lipoplexes. Since under these conditions transfection of HeLa cells was almost completely abolished [40], whereas, as reported here, lipid mixing was less affected, it may be concluded that no correlation between fusion and gene transfer can be established. This observation is in agreement with previous findings [35,46]. To further evaluate the role of the different parameters affecting lipoplex–cell interactions, we carried out experiments in the presence of 10% serum, since in some studies referred in the literature, serum has been reported to exhibit an inhibitory effect on transfection [48]. As referred above, the inhibitory effect of serum on cell association, for the 1/1 lipoplexes (Fig. 4A), is mainly attributed to a reduction in the extent of their endocytosis, since no significant effect was observed for the extents of binding and fusion. Under the same conditions transfection was slightly inhibited (H. Faneca, S. Simões, P. Pires, M.C. Pedroso de Lima, unpublished data), thus confirming the importance of endocytosis on transfection activity mediated by the lipoplexes. Curiously, an opposite picture emerged for the positively charged complexes tested, where no inhibitory effect of serum on cell association (i.e., endocytosis) was observed, whereas a significant reduction in transfection was noted. Overall, we may conclude that the interpretation of these results cannot be made in a simple and straightforward manner, since besides uptake of the lipoplexes, other stages also play a role in the intracellular gene delivery process, which in turn may be affected by the coating of lipoplexes with serum components. In this regard, it can be speculated that this process will contribute to the destabilization of the lipoplexes, thus facilitating the dissociation of DNA and its escape from the endosomes [49–51].

We extended our studies to investigate the interactions of Tf-lipoplexes with cells, since the presence of the ligand transferrin was shown to lead to a great enhancement of both transfection activity and efficiency in a large variety of cells [38–40]. The results presented here demonstrate that these ternary complexes have significantly higher binding, cell association and fusion extents as compared to plain lipoplexes. Our results demonstrate that cell association of Tf-lipoplexes is decreased by the presence of inhibitors of endocytosis. As mentioned before, besides promoting cell association, transferrin enhances the ability of lipoplexes to fuse with target membranes. However, our results indicate that the extent of fusion is significantly inhibited upon incubation of the cells with inhibitors of endocytosis. Together, these results suggest that fusion of the Tf-lipoplexes occurs mainly after endocytosis, thus most likely involving the endosomal membrane. This observation reinforces the idea that transferrin acquires fusogenic properties under acidic conditions, thus facilitating endosomal disruption and intracellular release of DNA [40]. This idea is also reinforced by the results from the kinetic studies where the effect of inhibitors of endocytosis is again only noted for the fusion extent of the Tf-associated lipoplexes and not of the plain lipoplexes. From the kinetic studies, and from the analysis of the obtained results by applying a theoretical model, we estimated the fusion rate constants (f) and found a twofold enhancement for the ternary lipoplexes when compared to the plain lipoplexes (Table 1). This increase in the fusion rate constants for the lipoplexes containing transferrin is not a dramatic effect though, and is probably rather non-specific. This effect results from an enhancement in their extent of binding thus being reflected in the extent of fusion. It is interesting to note that the largest fusion rate constants obtained, i.e., $(1–4)\times10^{-3}$ s^{-1} (Table 1) are about one order of magnitude below the values reported for virus-cell fusion at neutral pH [47,52]. We also found a reduction in the f value for the larger lipoplex concentrations, which may be due to the referred excessive loading of positive charges in the plasma membrane. Another interesting finding was that the fusion rate constants deduced from experiments without pre-incubation of lipoplexes with cells at 4°C are larger than those based on pre-incubation. We speculate that during the

binding of particles to the cells at 4°C, a certain fraction might be attached to sites of low fusogenicity on the cell surface and/or to sites that do not undergo endocytosis. Optimal fusion of these particles may require prolonged rearrangements, which may occur in the experiments performed at 37°C in the absence of pre-incubation.

From the kinetic studies we also observed that for DOTAP concentrations of 0.1 mg and below, the difference in cell association and fusion, with and without inhibitors of endocytosis, was minimal, both for plain and ternary lipoplexes. The conclusion is that HeLa cells yield a relatively low rate of endocytosis, which explains the low transfection efficiency obtained for HeLa cells as compared with other cells [39]. It can be expected that at relatively larger DOTAP concentrations, the ratio between the endocytosed and bound amounts would be larger, in particular at later times, meaning that the rate of endocytosis would be enhanced. For this purpose, parallel experiments using 2.0 mg DOTAP/ml were performed and the obtained results were analyzed as before. The results obtained from these experiments (not shown) indicate that the relative fractions of lipoplexes associated with the cells were 23% larger in the absence of inhibitors of endocytosis. However, due to the relatively slow rate of endocytosis and the decrease in the rate and extent of binding with excess loading of positive charges on the plasma membrane, a detailed analysis and simulation of the uptake of lipoplexes was not possible. Nevertheless, some estimates for the rate constants of endocytosis could be deduced by assuming a first order reaction. From the results of cell association for the 2 mg DOTAP/ml case, the values deduced for the rate constants of endocytosis are $3–5\times10^{-5}$ s^{-1} and $6–10\times10^{-5}$ s^{-1} for the plain and ternary lipoplexes, respectively. In this context, it should be noted that these values are more than an order of magnitude below the values reported for endocytosis of liposomes by macrophage like cells [53], or epidermal growth factor [54] by fetal rat lung cells, whereas Nunes-Correia et al. [55] reported 2.6×10^{-4} s^{-1} for endocytosis of influenza virus by MDCK cells in culture. Some interesting conclusions emerge from inspection of the fusion results. For the plain lipoplexes (2 mg/ml), the ratio between percents of fusion with or without inhibitors of endocytosis is similar to the ratio observed for cell association. However, for the ternary lipoplexes, the percent of fusion was 44% larger without inhibitors, whereas the corresponding percent of cell association was 23% larger. The same trend was also observed for 0.75 mg DOTAP/ml. These results imply that the presence of transferrin associated with lipoplexes enhances dramatically their rate of fusion with endosomes, which might be perhaps related to the enhancement of transfection efficiency by transferrin, well above the enhancement of cellular uptake induced by this protein [38,39].

Enhancement of transfection mediated by non-viral vectors, namely cationic liposomes, is of crucial importance for the establishment of the successful application of gene therapy protocols. The goal is to generate viable and safer alternatives to the use of viral vectors. It is well recognized that such an improvement will only be possible upon an extensive knowledge of the various steps involved in the transfection process. We and others tried to dissect this process aiming at identifying the involved mechanisms, as well as the key elements, thus allowing for the development of novel and fruitful strategies. Although fusion of lipoplexes with the plasma membrane has been demonstrated to occur, this was shown not to be an efficient pathway for the intracellular delivery of DNA [35,46]. In contrast, evidence has been reported suggesting the importance of the endocytotic pathway in the uptake of non-viral-vectors. By assessing the effect of different parameters usually involved in the transfection process, we were able to estimate the relative contribution of fusion with the plasma membrane, endocytosis and fusion with the endosomal membrane to the lipoplex–cell interactions. Moreover, the determination of the rate constants of endocytosis and fusion of different types of lipoplexes, by performing a detailed kinetic analysis of the referred processes, elucidated the effects of the different strategies explored in our laboratory at a fundamental level. Thus, the combination of such information with that resulting from transfection data [39,40,46] indicates that together with endocytosis, the destabilization of and fusion with the endosomal membrane constitute a crucial step in the transfection process. The present work emphasizes the importance of the information deduced from a combination of studies on binding, cell association, fusion and trans-

fection, in exploring approaches to enhance transfection.

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