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Anionic liposomes inhibit human immunodeficiency virus type 1 (HIV-1) infectivity in CD4^{+} A3.01 and H9 cells

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Mechanisms of gene transfer mediated by lipoplexes associated with targeting ligands or pH-sensitive peptides

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Association of a targeting ligand such as transferrin, or an endosome disrupting peptide such as GALA, with cationic liposome–DNA complexes ('lipoplexes') results in a significant enhancement of transfection of several cell types (Simões S et al, Gene Therapy 1998; 5: 955–964). Although these strategies can overcome some of the barriers to gene delivery by lipoplexes, the mechanisms by which they actually enhance tranfection is not known. In studies designed to establish the targeting specificity of transferrin, we found that apo-transferrin enhances transfection to the same extent as transferrin, indicating that internalization of the lipoplexes is mostly independent of transferrin receptors. These observations were reinforced by results obtained from competitive inhibition studies either by preincubating the cells with an excess of free ligand or with various 'receptor-blocking' lipoplexes. Transfection of cells in the presence of drugs that interfere with the endocytotic pathway provided additional insights into the mechanisms of gene delivery by transferrin- or GALA-lipoplexes. Our results indicate that transferrin-lipoplexes deliver transgenes by endocytosis primarily via a non-receptor-mediated mechanism, and that acidification of the endosomes is partially involved in this process.

Keywords: gene delivery; cationic liposomes; transferrin; transfection; endocytosis; GALA

Introduction

Non-viral gene delivery systems appear to be very promising systems for gene therapy since they obviate some of the safety limitations associated with viral vectors. Among such systems, cationic liposomes, first proposed by Felgner et al,[1] have been used extensively in vitro and in vivo.[2–7] Although a large amount of effort has been put into the development of these vectors, namely through the synthesis and evaluation of different lipid compositions,[8] as well as of new plasmids with more efficient promoters,[7] the results are still unsatisfactory. The heterogeneity of the complexes formed with the plasmid DNA, the lack of knowledge on the mechanisms by which these systems mediate gene delivery,[9,10] toxicity under certain conditions,[11,12] sensitivity to the presence of serum[1,13,14] and especially the lack of target cell specificity and the low levels of gene expression in vitro and in vivo compared with viral vectors raise some doubts regarding their potential as gene delivery systems in clinical applications.

In a previous article, we reported two different approaches for successfully enhancing transfection

mediated by cationic liposomes composed of 1,2-dioleoyl-3-(trimethylammonium) propane (DOTAP) and dioleoylphosphatidylethanol amine (DOPE).[15] The first approach was based on the promotion of cellular internalization of the cationic liposome–DNA complexes ('lipoplexes')[16] through receptor-mediated endocytosis. For that purpose iron-saturated human transferrin was associated with the lipoplexes at different (+/−) charge ratios. Transferrin is a useful ligand that binds to a cell-surface receptor expressed by most proliferating cells with particularly high expression on erythroblasts and tumor cells.[17]

The uptake of ferric transferrin (Tf) by cell-membrane receptors (TfR) and its intracellular cycle have been well established.[18,19] Complexes of diferric or monoferric Tf with the TfR are internalized via specialized regions of the cell plasma membrane known as coated pits through the endocytotic pathway. When the endosome lumen is acidified (pH 5.0–5.5) the iron ions are released from transferrin and move to their destination within the cell. Following removal of iron from Tf, the TfR with bound apotransferrin recycles to the cell surface, where the apo-Tf is released in response to the neutral pH of the extracellular medium, leaving the TfR free to bind additional ferric Tf molecules. Thus, the high affinity of ferric transferrin for the receptors at neutral pH in contrast to the high affinity of apotransferrin for the receptors at acidic pH appears to be essential for this cycle. Targeting to cellular transferrin receptors has been tried successfully before by linking the ligand to polycation conjugates[20,21] or by associating the protein with cationic liposomes.[22]

The second approach was based on the association of endosome-disrupting agents to the lipoplexes with the purpose of facilitating the cytoplasmic release of DNA from endosomes, thus preventing its lysosomal degradation and therefore enhancing transfection. Two different synthetic pH-sensitive peptides, 'GALA'[23,24] and the influenza virus hemagglutinin HA2 N-terminal peptide,[25] both low pH-activated membrane-active peptides, were used for that purpose.[15]

The ternary complexes resulting from the association of transferrin or fusigenic peptides with lipoplexes were significantly more efficient in mediating gene transfer than plain lipoplexes. This effect was observed in a variety of cell types, including epithelial and lymphoid cell lines, as well as in human blood monocyte-derived macrophages, and was particularly pronounced when the complexes presented a net negative charge.[15,26,27] Furthermore, the enhancement of gene delivery by transferrin or GALA was not affected significantly by the presence of serum and did not result in significant cytotoxicity.

Although based on rational strategies, the actual mechanisms by which these agents promote gene delivery when associated with lipoplexes remain to be elucidated. We therefore examined the different processes by which transgene expression is facilitated by the presence of a targeting ligand or a fusigenic peptide. By performing targeting specificity studies, we showed that transferrin promotes internalization of the lipoplexes, most likely via a non-specific process. These observations were reinforced by results obtained from competitive inhibition studies, either by preincubating the cells with an excess of free ligand or with various 'receptor-blocking' complexes. Transfection of cells in the presence of different drugs that interfere with the endocytotic pathway also provided insight into how ternary complexes enter cells and mediate gene delivery. Based on our observations and on recent findings, we discuss the potential mechanisms involved in the internalization of the ternary complexes, the dissociation of DNA from the complexes and its delivery into the cytoplasm.

Results

Targeting specificity studies

To demonstrate whether the internalization of Tf-lipoplexes was mediated by specific interaction with Tf receptors, we tested the effect of associating apo-Tf (which should have very low affinity for TfR at physiological pH) with DOTAP:DOPE DNA complexes on the levels of luciferase expression, using the same experimental conditions described for transferrin.

Figure 1a shows the levels of transfection obtained with COS-7 cells when complexes containing different lipid/DNA (+/−) charge ratios, prepared in the presence or absence of ferric Tf or apo-Tf, were incubated with the cells for 4 h at 37°C. As previously reported,[15] the association of ferric Tf resulted in a significant enhancement of transfection as compared with controls, ie plain lipoplexes prepared in the absence of Tf. Although this effect was observed for all the lipid/DNA (+/−) charge ratios tested, the highest levels of luciferase expression were achieved with ternary complexes with the 1/1 lipid/DNA charge ratio for both COS-7 and HeLa cells (Figure 1a and b).
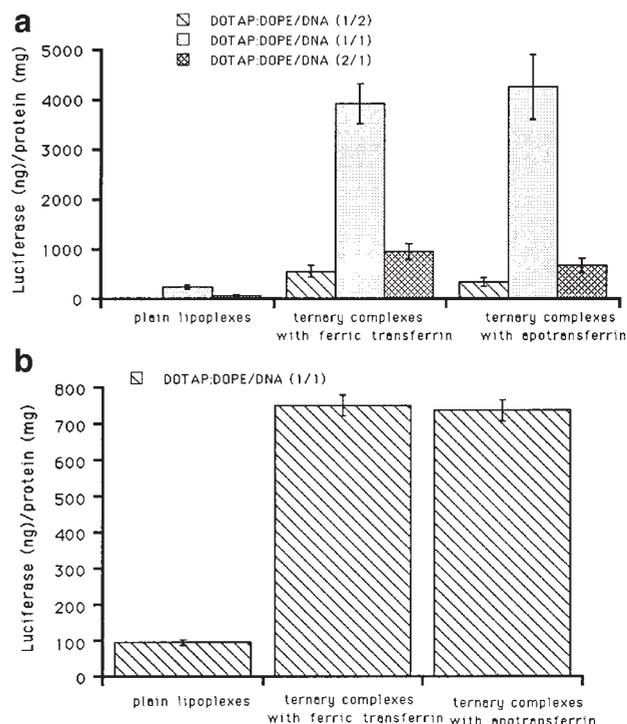
Figure 1 *Targeting specificity studies. Levels of transfection activity when complexes containing different lipid/DNA (+/−) charge ratios, prepared in the presence or absence of ferric transferrin or apotransferrin, were incubated with (a) COS-7 cells or with (b) HeLa cells. Cells were rinsed twice with serum-free medium and then covered with 0.3 ml of DME-HG before lipoplexes were added. The liposomes were complexed, in the absence or presence of 32 g of transferrin or of 32 g of apotransferrin, with 1 g of pCMVluc at the indicated theoretical lipid/DNA charge ratios, as described in detail in Materials and methods. After an incubation for 4 h, the medium was replaced with DME-HG containing 10% FBS and the cells were further incubated for 48 h. The level of gene expression was evaluated as described in Materials and methods. The data are expressed as nanograms of luciferase per milligram of total cell protein (mean ± standard deviation obtained from triplicate wells), and are representative of three independent experiments.*
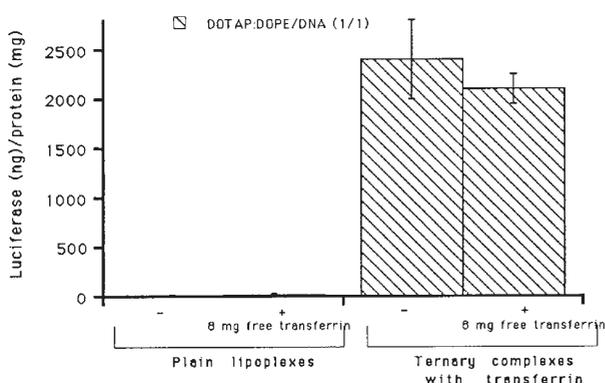
The results obtained with Tf and apo-Tf were strikingly similar (Figure 1). Although apo-Tf has a much lower affinity for its cell receptors at physiological pH than Tf, the association of this protein to the lipoplexes also resulted in a significant enhancement of the levels of gene expression for all the different lipid/DNA (+/−) charge ratios. As observed for transferrin, the condition leading to the highest levels of transfection was the 1/1 lipid/DNA charge ratio. This common capacity to enhance transfection of ferric- and apotransferrin was also demonstrated with HeLa cells, where only the best condition (1/1 lipid/DNA) was tested (Figure 1b). This enhancing effect of apo-Tf was not affected by the presence of serum (10%) during transfection (data not shown). It should be noted that the ability of apotransferrin-containing lipoplexes to enhance transfection cannot be attributed to the saturation of this protein due to sequestration of free iron from the cell culture medium, since no significant differences in the levels of transfection were observed when these complexes were incubated with cells in the presence of desferrioxamine (a chelating agent for free iron) (data not shown).

Competitive inhibition by various lipoplexes

In view of the unexpected results presented in the previous section regarding the specificity of transferrin to promote receptor-mediated endocytosis of the lipoplexes, we explored whether cellular receptors for Tf-lipoplexes could be blocked by competitive inhibitors.

Effect of excess free transferrin: Figure 2 illustrates the effect of pre-incubating COS-7 cells with 8 mg of free Tf (approximately 250 times the amount of protein that is associated with the lipid/DNA complexes) on the levels of luciferase gene expression. The cells were incubated with free Tf dissolved in 0.3 ml of cell culture medium for 30 min at 37°C before the addition of the control complexes (plain lipoplexes of DOTAP:DOPE/DNA) or the ternary complexes of Tf-DOTAP:DOPE/DNA. Surprisingly, the presence of this large excess of free Tf in the medium of transfection seemed to have no significant effect on the levels of transfection mediated by either plain lipoplexes or ternary complexes (both at a 1/1 (+/−) charge ratio). The relative levels of transfection obtained among the different lipid/DNA charge ratios was also maintained (data not shown).
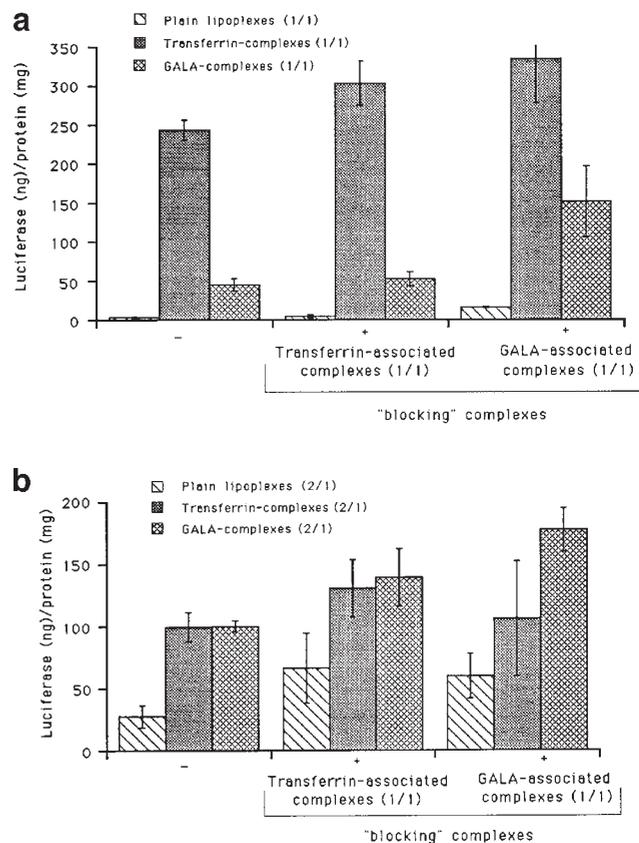
Effect of lipoplexes with different physico-chemical properties: It is possible that Tf associated with lipoplexes may interact differently with the cell membrane compared with free Tf. For example, it may bind to different, or possibly multiple, receptors or sites on the cell membrane. To examine this possibility we pre-incubated cells with different 'blocking' complexes (lipoplexes with the same lipid composition but containing the non-luciferase expressing plasmid, pCMV.SPORT- -gal) before the addition of the 'active' ternary complexes (lipoplexes containing the luciferase plasmid, pCMVluc). In previous studies we had measured the zeta potential ( ) of different (+/−) charge ratios and the effect of the association of Tf or pH-sensitive peptides (eg GALA) on the overall charge of these lipoplexes.[15,26] We had found that, for

some of the lipid/DNA charge ratios used, the association of transferrin or the GALA peptide led to the formation of net negatively charged complexes, which turned out to be more effective in mediating gene transfer than neutral or positively charged complexes.

In one group of experiments, 1/1 (+/−) Tf-lipoplexes (= −38.6 ± 5.2 mV) and 1/1 (+/−) GALA-lipoplexes (= −35.3 ± 3.6 mV) were preincubated with Hela cells for 1 h at 37°C before the medium was replaced with DME-HG and new complexes of the same composition and charge (but carrying the luciferase plasmid) were added and incubated for another hour. Transfection was evaluated 48 h later as described in Materials and methods (Figure 3a). Thus, we tested how the presence of negatively charged complexes would affect internalization of complexes of either the same or different composition, but exhibiting the same overall charge. In another group of experiments the 'blocking' complexes were the same as described above, but the 'active' complexes consisted of 2/1 (+/−) plain lipoplexes (= 42.8 ± 0.5 mV), 2/1 (+/−) Tf-associated complexes (= 25 ± 2 mV) and 2/1

Figure 2 Competitive inhibition studies. Effect of the incubation with the cells for 30 min of an excess of free transferrin before the addition of the plain or ternary lipoplexes at an optimized charge ratio (1/1 lipid/DNA) on the levels of transfection. COS-7 cells were pre-incubated with 8 mg transferrin (ie 250 × more protein than that associated with the lipoplexes). After an incubation for 1 h with the complexes, the medium containing an excess of transferrin was replaced with DME-HG enriched with 10% FBS and the cells were further incubated for 48 h. The level of gene expression was evaluated as described in Materials and methods. The data are expressed as nanograms of luciferase per milligram of total cell protein (mean ± standard deviation obtained from triplicate wells), and are representative of three independent experiments.

Figure 3 Competitive inhibition studies. Effect of the incubation with HeLa cells for 1 h at 37°C of 'blocking' complexes (containing a non-luciferase expressing plasmid, pCMV.SPORT- -gal) composed of transferrin- or GALA-lipoplexes at a 1/1 charge ratio (both net negatively charged). The medium was then replaced before the addition of (a) 'active' complexes (complexes carrying the luciferase plasmid, pCMVluc) of the same composition and charge ratio; or (b) complexes of the same composition but prepared at a 2/1 lipid/DNA charge ratio. Cells were further incubated for another hour at 37°C. Transfection experiments were performed as described in Materials and methods. The data, expressed as nanograms of luciferase per milligram of total cell protein, indicate the mean ± standard deviation obtained from triplicate wells, and are representative of two independent experiments.

(+/−) GALA-associated complexes (= 35.3 ± 5.2 mV) (Figure 3b). In this case, the effect of the negative charge and composition of the complexes on the entry of positively charged complexes with the same or different composition were thus evaluated.

As demonstrated in Figure 3a, no significant effect on gene expression was noted when neutral (plain lipoplexes 1/1) or net negatively charged 'active' complexes at a 1/1 lipid/DNA charge ratio (containing either Tf or GALA) were added, following the incubation of the cells with the net negatively charged 'blocking' complexes containing Tf. A slight potentiation of transfection was noted for all three types of complexes when the preincubation was carried out with the 1/1 (+/−) GALA-associated blocking complexes.

Similar results were achieved when either plain, Tf-associated (both positively charged) or GALA-associated complexes (negatively charged) at a 2/1 lipid/DNA charge ratio were added to the cells (Figure 3b). Again, the most significant change observed was the enhancement of the levels of gene expression observed when transfection was mediated by 2/1 GALA-associated complexes upon the pre-incubation of the cells with the 1/1 GALA complexes.

These results indicate that the different complexes, independently of their composition and charge, do not interfere with each other in any specific process of internalization or in their ability to mediate gene transfer. However, when GALA-associated 'blocking' complexes were pre-incubated with the cells, an enhancement of transfection was observed, independently of the composition and to some extent, of the overall charge of the 'active' complexes. In agreement with the data presented above, it appears that entry of such complexes into the cytoplasm is mediated by a process independent of specific receptors.

Effects of inhibitors of the endocytotic pathway
To define the mechanisms involved in the internalization and intracellular fate of the various lipoplexes further, HeLa cells were pre-treated before transfection with chemical agents that interfere with various aspects of the endocytotic pathway: (1) A mixture of antimycin A, NaF and NaN₃, which, by restricting metabolic activity of the cell, results in a strong inhibition of both receptor- and non-receptor-mediated endocytosis;[28–29] (2) cytochalasin B, a drug that is known to disrupt the microfilament network by inhibiting actin polymerization, thereby blocking phagocytosis and pinocytosis, but not receptor-mediated endocytosis;[30–33] (3) bafilomycin A₁, a specific inhibitor of the vacuolar ATPase proton pump present in the intracellular membrane compartments, thus preventing the acidification of the endocytotic pathway.[34–36]

Results presented in Figure 4a–c illustrate the effect of such drugs on the transfection activity of HeLa cells mediated by plain lipoplexes, and by ternary complexes containing either Tf or GALA. For all the cases, 1/1 and 2/1 (+/−) lipid/DNA charge ratios were used. Although varying with the type of drugs used and the composition and charge of the complexes tested, an inhibitory effect on transfection was evident for essentially all the conditions. In order to clarify this effect better and to facilitate the interpretation of the data, taking into account the physico-chemical features of the complexes, the percentage of inhibition of transfection activity was calculated as
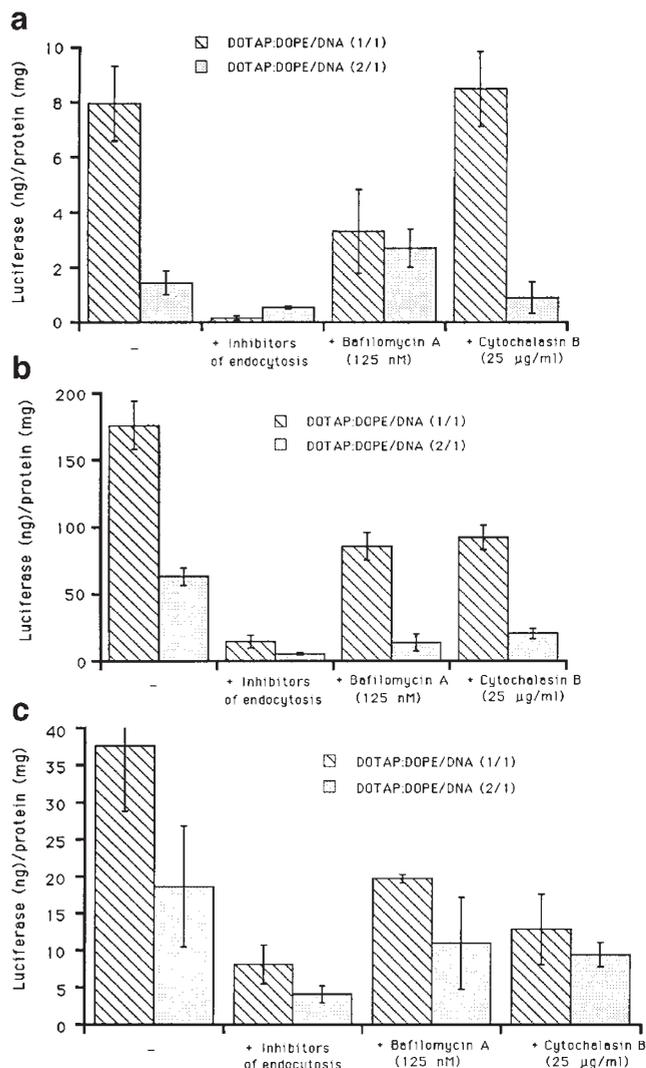
Figure 4 Effect of different drugs on transfection. HeLa cells were incubated for 30 min at 37°C, in the absence of serum, with either a mixture of antimycin A (1 g/ml), NaF (10 mM) and NaN₃ (0.1%), or with cytochalasin B (25 g/ml) or even with bafilomycin A₁ (125 nM). Cells were further incubated for 1 h at 37°C with (a) plain lipoplexes, (b) trasferrin-associated complexes and (c) GALA-associated complexes in the presence of the various drugs and then washed once with serum-free medium. Medium was then replaced with DME-HG containing 10% FBS, and the cells were further incubated for 48 h before evaluation of transfection. The data, expressed as nanograms of luciferase per milligram of total cell protein, indicate the mean ± standard deviation obtained from triplicate wells, and are representative of two independent experiments.

compared with that obtained with cells transfected in the absence of the drugs (Table 1). The following observations can be made from the data: (1) Transfection mediated by plain lipoplexes at a 1/1 (+/−) lipid/DNA charge ratio was strongly inhibited by the inhibitors of endocytosis (98% inhibition), this effect being less pronounced (62% inhibition) for the same type of complexes prepared at a 2/1 charge ratio. Moreover, inhibition of non-coated pit-mediated endocytosis by cytochalasin B did not lead to any noticeable effect on transfection activity of HeLa cells mediated by both of these complexes. On the other hand, the increase of endosomal pH due to the presence of bafilomycin A₁ resulted in a significant inhibition of transfection when neutral

Table 1 Inhibitory effect of different drugs (% of inhibition compared with controls) on transfection activity of Hela cells mediated by DOTAP/DOPE/DNA derived complexes

Drugs Formulation	Lipid/DNA charge ratio	Overall charge of the complexes	Size of the complexes (nm)	Antimycin A NaF NaN₃	Cytochalasin B	Bafilomycin A₁
Plain lipoplexes	(1/1)	neutral	900	98	no effect	58
	(2/1)	+	400	62	no effect	no effect
Transferrin-associated complexes	(1/1)	−	600	92	48	51
	(2/1)	+	600	92	67	78
GALA-associated complexes	(1/1)	−	−	79	66	48
	(2/1)	−	550	78	49	41

HeLa cells were pre-treated with chemical agents interfering with the endocytosis process before starting transfection, as described in detail in Materials and methods. Percentage of inhibition of transfection activity was estimated considering the mean values of luciferase expression observed for the different lipoplexes in the presence of the drugs, compared with mean values achieved with untreated cells. The overall charge and the size measurements of the complexes were evaluated by a Coulter DELSA 440 instrument.

complexes were used (about 60%) while no effect was observed for the 2/1 complexes. (2) The inhibitors strongly inhibited transfection by the transferrin-lipoplexes at both charge ratios. Cytochalasin B exhibited a clear inhibitory effect on transfection mediated by these complexes, the extent of inhibition being higher than 50% for the 2/1 (+/−) lipid/DNA complexes. Very similar effects to those described for cytochalasin B were also observed when bafilomycin A₁ was used. (3) As expected, luciferase gene transfer mediated by ternary complexes composed of DOTAP:DOPE liposomes, DNA and the fusigenic peptide GALA was also seriously reduced by the drug mixture composed of antimycin A, NaF and NaN₃ independently of the charge ratio of the complexes. Nevertheless, this effect was slightly less pronounced (about 80% inhibition) than that observed for the other types of complexes tested. Inhibition of phagocytosis and pinocytosis by cytochalasin B also resulted in a significant decrease in the levels of transfection. The extent of inhibition was similar for both charge ratios tested and comparable to that observed with transferrin-lipoplexes. Pretreatment of the cells with bafilomycin A₁ reduced the levels of transfection mediated by the GALA-lipoplexes. Nevertheless, the level of inhibition observed was below our expectations, especially considering the pH-sensitivity attributed to the GALA peptide.

Cell viability

As can be observed in Figure 5, the viability of HeLa cells was not affected upon transfection in the presence of the different drugs. Therefore, the inhibitory effects on the levels of gene expression observed under those conditions cannot be attributed to any cytotoxic effect of the drugs.

Discussion

In previous studies we have explored two different approaches to enhance gene delivery mediated by lipoplexes in an attempt to circumvent the limitations associated with the low levels of transfection usually achieved by these systems compared with viral vectors: (1) association of a targeting ligand (transferrin) to the complexes to promote their internalization, presumably by receptor-mediated endocytosis; and (2) association of a
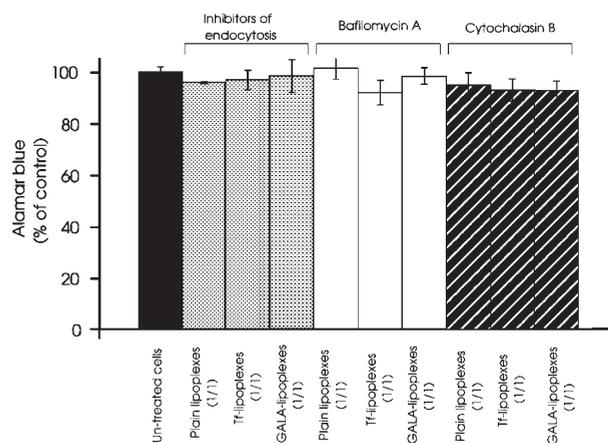
Figure 5 Effect of the inhibitors of the endocytotic pathway on the viability of HeLa cells. Cells in 48-well plates were exposed to either plain (1/1) (+/−) lipoplexes or with lipoplexes associated with the GALA peptide or transferrin under the experimental conditions described in the legend to Figure 4. Cell viability was measured by the Alamar Blue assay following 48 h of incubation and was expressed as the percentage of the untreated control cells. Data represent the mean ± standard deviation obtained from duplicate wells.

synthetic fusigenic peptide (GALA) to the complexes to promote endosomal destabilization and release of the genetic material into the cytoplasm. Nevertheless, the actual mechanisms by which the resulting complexes promoted gene transfer and enhance transfection were not studied.

Our results raise serious doubts as to whether the Tf-lipoplexes are internalized by specific receptor-mediated endocytosis. The striking similarity between the levels of enhancement obtained by complexation of apo-Tf (a protein that does not have any affinity for the TfR at physiological pH) and Tf (Figure 1a and b) suggests that internalization of these ternary complexes is being mediated by a non-specific process.

This lack of specificity was confirmed by some of the results obtained with competitive inhibition studies. The absence of any inhibition of transfection mediated by Tf-lipoplexes when TfRs were pre-saturated with an excess of free Tf (Figure 2), supports the hypothesis that these ternary complexes enter cells using a pathway that is

mostly independent of TfRs. Regarding the use of Tf, results of other authors show some controversy. Gene delivery mediated by Tf-coupled polyethyleneimine was shown to be specific for TfR, utilizing competitive inhibition experiments with free Tf.[37] On the other hand, the ability of Tf to potentiate transfection can be totally abolished by the use of certain cationic liposomes.[22] According to our results, it appears that the association of Tf with cationic liposomes and DNA due to electrostatic interactions, results in a loss of its targeting specificity, although it mediates a highly significant enhancement of transfection over plain lipoplexes. On the other hand, our results also indicate that an excess of positive charge on the ternary complexes results in a decrease of transfection activity.[15] It seems, therefore, that in a system composed of cationic liposomes, ligand and DNA, the observed effects can range from a lack of specificity to the total absence of enhancement of transfection, depending on the nature of the components and on the cationic lipid/DNA (+/−) charge ratio.

These observations led us to investigate whether the composition and the overall charge of the ternary complexes would have any effect on their pathway of internalization. Competitive inhibition studies showed that internalization of the various ternary complexes is not affected by the pre-addition of 'blocking' complexes, independently of their composition and charge (Figure 3a and b). This observation reinforces the possibility that the ternary complexes are internalized primarily by a receptor-independent pathway.

Inhibition of endocytosis in HeLa cells essentially abolished transfection activity by Tf-lipoplexes (independently of their overall charge), as well as the neutral plain lipolexes. Transfection by GALA-lipoplexes was also inhibited by about 80%. These results indicate that lipoplexes are internalized primarily via the endocytotic pathway, which is in agreement with observations reported by other authors.[9,10,31,38]

Pre-treatment of cells with cytochalasin B has been shown to result in inhibition of pinocytosis and phagocytosis but not of receptor-mediated endocytosis via clathrin-coated pits.[30,33] Since it is well known that internalization of Tf receptors occurs through a clathrin-coated process[18,39] it was surprising to observe the significant inhibitory effect of cytochalasin B on the levels of transfection mediated by Tf-associated lipoplexes. According to our data about 50% of the internalization for the ternary complexes at the optimal charge ratio (1/1 lipid/DNA) was not mediated by the normal route of endocytosis of transferrin. A possible explanation for these unexpected results may be the large size and heterogeneity of the complexes. The relatively large size of the ternary complexes (more than 500 nm) would hamper endocytosis through coated vesicles, but the presence of Tf could trigger their phagocytosis. In contrast, the smaller complexes within the heterogeneous mixture (200 nm) would follow the usual route of internalization of Tf through binding to its receptors on the cell surface. The effect of cytochalasin B was even more pronounced with the net positively charged 2/1 (+/−) ternary complexes, most likely due to a decrease in the targeting ligand specificity caused by the strong and dominant electrostatic interactions between this type of complex and the cell membrane.

This non-receptor-mediated internalization also seems to play an important role in the case of GALA-lipoplexes, since the inhibition of non-clathrin coated endocytosis resulted in a substantial decrease in the levels of transfection. Since these complexes also exhibit a large size, it is likely that the GALA peptide has the ability to trigger phagocytosis, although to a lesser extent than transferrin.

Surprisingly, the inhibition of phagocytosis did not affect transfection mediated by plain lipoplexes, independently of their charge. Although these complexes are not expected to bind to the cell surface via specific receptors, it is possible that they enter the cells through a non-specific clathrin-mediated process, especially those with a smaller size. Anionic liposomes are known to enter cells via coated pits.[40]

As demonstrated by Zabner *et al*,[9] escape of DNA from endosomes and its dissociation from the complexes are also crucial steps in the process of intracellular gene delivery, besides the entry of the lipoplexes into cells. In this context, the results obtained when cells were pre-treated with bafilomycin A_1 are of particular interest. The significant inhibition of transfection observed when GALA-lipoplexes were added to the cells in the presence of this drug, is consistent with the low pH-dependent membrane disruption activity of this peptide, presumably by inducing pore formation.[41–43]

It should be noted that the size of the pores (5 to 10 Å) caused by this peptide is too small to allow the escape of DNA from endosomes. It appears therefore that the enhancement of transfection promoted by GALA would result from a combination of other mechanisms. We can speculate that the structural changes of the peptide that occur at low pH would promote the deaggregation of the complexes. This would not only facilitate the dissociation of DNA from the complexes but also result in a larger exposure of the cationic lipids. This, in turn, together with the pore opening promoted by the peptide, may induce the flip-flop of the anionic lipids from the cytoplasmic leaflet of the endosomal membrane,[10] thus leading to an extensive membrane destabilization. Moreover, the fusigenic properties of the helper lipid under acidic conditions and consequently, its contribution to the escape of DNA from the endosomes should also be considered.[31,44–47]

The significant inhibition by bafilomycin A_1 of gene expression by Tf-lipoplexes suggests that the acidification of the endosome also plays an important role in intracellular gene transfer mediated by these complexes. Similar to what was proposed for the role of GALA, it is possible that structural changes induced by protonation of apo-Tf[48,49] would also result in deaggregation of the lipoplexes, thus triggering the cascade of events described above involving the cationic and helper lipids. In addition, we can speculate that upon acidification, Tf would acquire fusigenic properties that could also contribute to the destabilization of the endosome, analogous to membrane destabilization and fusion induced at low pH by insulin, clathrin or various colicins.[50–52] Thus, besides its major role in triggering both receptor- and non-receptor-mediated endocytosis, Tf may also be involved in overcoming the endosomal membrane barrier to gene transfer. This suggestion is in agreement with the observation that the association of Tf with lipofectin increased DNA entry by only two-fold, while transfection activity was enhanced by a much larger factor.[22]

Figure 6 illustrates a model describing the main steps
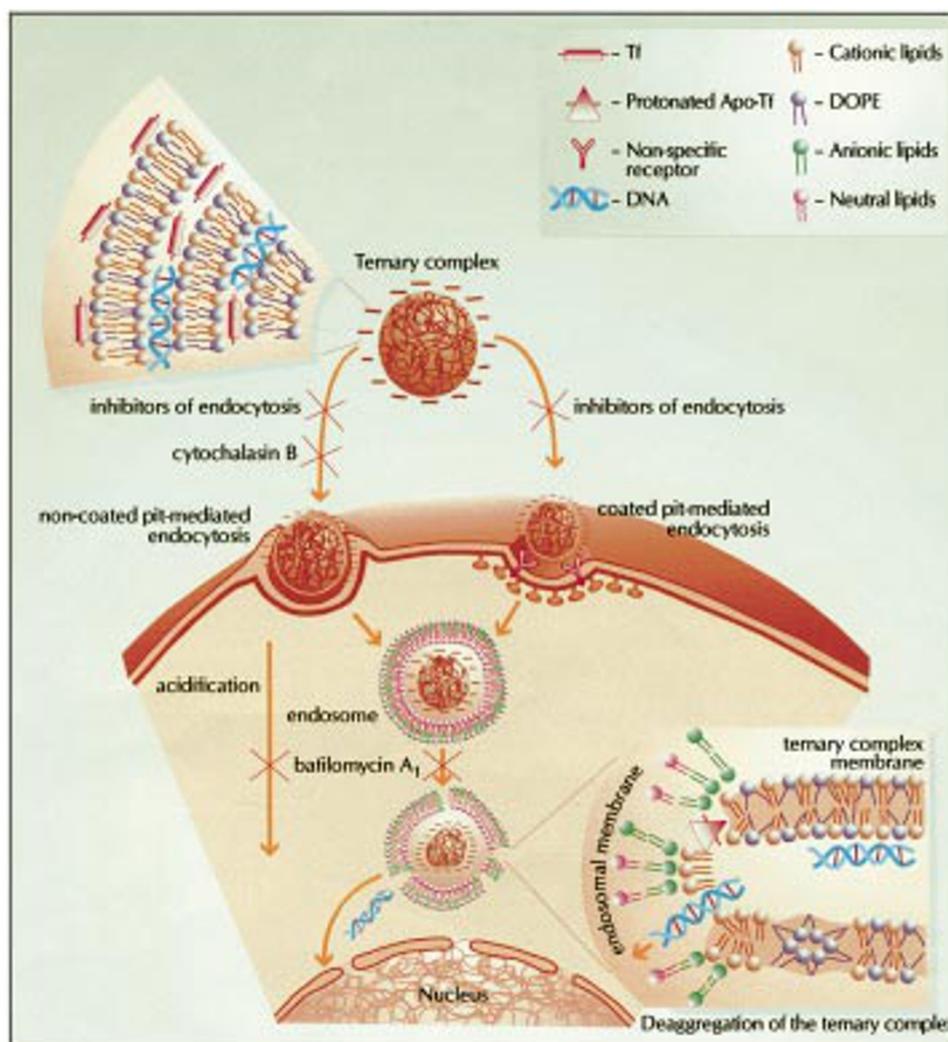
Figure 6 *Schematic model describing the main steps involved in the intracellular uptake and delivery of DNA mediated by transferrin (Tf)-lipoplexes. Due to electrostatic interactions, transferrin and DNA associate with cationic liposomes (DOTAP:DOPE) leading to the formation of negatively charged ternary complexes (considering a 1/1 lipid/DNA (+/−) charge ratio) by a still unknown mechanism. Presumably complexes are internalized either by receptor-mediated endocytosis via clathrin-coated pits or by phagocytosis via uncoated pits, depending on their size. Both of these endocytotic pathways can be inhibited by pre-treatment of the cells with inhibitors of endocytosis (mixture of antimycin A, NaF, NaN₃), whereas phagocytosis can be selectively inhibited by cytochalasin B. Efficient delivery of DNA into the cytoplasm (from where it can reach the nucleus) seems to be dependent on the acidification of the endosomal lumen, which can be partially inhibited by bafilomycin A_1. We speculate that this acidification process triggers a cascade of synergistic effects that would lead to dissociation of DNA from the ternary complexes and to destabilization of the endosomal membrane. Structural changes induced by protonation of apotransferrin would promote deaggregation of the complex. It is also possible that, under these conditions, apotransferrin becomes fusigenic, promoting destabilization of the endosomal membrane, therefore creating favorable conditions for flip-flop of anionic lipids from the cytoplasmic leaflet of the endosomal membrane. Electrostatic interactions between the anionic lipids present in the inner leaflet of the endosomal membrane and cationic lipids would not only promote deaggregation of the complexes but also facilitate DOPE to undergo a transition from a bilayer to an inverted hexagonal phase, thus acquiring fusigenic properties. All these events would lead to dissociation of DNA from the complexes and to its escape into the cytoplasm.*

involved in the intracellular uptake and delivery of DNA mediated by Tf-lipoplexes, as well as the effect of the different drugs on the endocytotic pathway followed by the complexes. This hypothetical model was constructed taking into account the above described observations, the recent findings in this field as well as other proposed mechanistic models related to lipoplex–cell interactions.[9,10,20,31,32,45,47] According to this model, the presence of Tf in the complexes promotes an enhancement of transfection by two different mechanisms: (1) by triggering internalization of the complexes through both non-coated pit (presumably phagocytosis) and coated pit-mediated endocytosis (after binding of the complexes to non-specific receptors); and (2) by promoting intracytoplasmic gene delivery in a pH-dependent manner. Similar mechanisms can also be proposed for the role of the GALA peptide in enhancing transfection mediated by lipoplexes.

Elucidation of the exact mechanism and relative contribution of endocytosis and fusion to transfection may aid

in the development of more effective means of gene delivery by cationic liposomes, especially for *in vivo* applications where transfection efficiency is of paramount importance.

Materials and methods

Materials

The cationic lipid 1,2-dioleoyl-3-(trimethylammonium) propane (DOTAP), and dioleoylphosphatidylethanolamine (DOPE), were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Iron-saturated, heat-inactivated human transferrin was obtained from Collaborative Biomedical Products (via Becton Dickinson, Bedford, MA, USA). The GALA peptide[23,24] and the peptide named HA-2, derived from the N-terminal sequence of the influenza virus hemagglutinin subunit HA2[25] were synthesized and purified by the UCSF Biomolecular Resource Center. GALA is a 30 amino acid, pH-sensitive, amphipathic peptide with the sequence WEAALAEA-LAEALAEHLAEALAEALEALAA. The pCMVluc plasmid (VR-1216) was kindly provided by Dr P Felgner (Vical, San Diego, CA, USA). The plasmid pCMV.SPORT-gal was obtained from Gibco BRL Life Technologies (Gaithersburg, MD, USA). Alamar Blue dye was purchased from AccuMed (Westlake, OH, USA). Apotransferrin, bafilomycin A_1, cytochalasin B, NaN_3, NaF, NaCl, 2-[N-morpholino] ethanesulfonic acid (MES) and N-[2-hydroxyethyl] piperazine-N -[2-ethanesulfonic acid] (HEPES) were obtained from Sigma (St Louis, MO, USA). Antimycin A was obtained from Calbiochem (La Jolla, CA, USA).

Liposome preparation

Cationic liposomes composed of DOTAP:DOPE (1:1 weight ratio) were prepared by first drying a film of lipid under argon and then in a vacuum oven at room temperature, and hydrating the lipid film with 1 ml deionized water at a final concentration of 5 mg/ml. The multilamellar vesicles obtained were then sonicated briefly under argon, extruded 21 times through polycarbonate filters of 50 nm pore diameter using a Liposofast device (Avestin, Toronto, Canada), diluted five times with deionized water and filter-sterilized utilizing Millex 0.22 m pore-diameter filters (Millipore, Keene, NH, USA).

Cells

HeLa cells (American Type Culture Collection, Rockville, MD, USA) were maintained at 37°C, under 5% CO_2, in Dulbecco's modified Eagles's medium high glucose (DME-HG) (Irvine Scientific, Santa Ana, CA, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Sigma, St Louis, MO, USA), penicillin (100 units/ml), streptomycin (100 g/ml) and L-glutamine (4 mM). For transfection, 0.2×10^6 HeLa cells were seeded in 1 ml of medium in 48-well culture plates and used at 80–90% confluence. COS-7 cells (UCSF Cell Culture Facility, San Francisco, CA, USA) were maintained under the same conditions described for HeLa cells. For transfection 0.3×10^5 cells were seeded in 1 ml of medium in 48-well culture plates and used at 40–60% confluence.

Preparation of the ternary complexes

Complexes were prepared by sequentially mixing 100 l of a solution of 100 mM NaCl, 20 mM Hepes, pH 7.4, with or without 32 g iron-saturated human transferrin[20] with 2.5, 5, 10 or 20 l liposomes and incubated at room temperature for 15 min. One hundred microliters of buffer containing 1 g of pCMVluc plasmid was then added and gently mixed, and the mixture was further incubated for 15 min at room temperature. Peptide complexes were prepared in a similar manner except that 0.6 g of GALA was used.[15]

Transfection activity

Cells were rinsed twice with serum-free medium and then covered with 0.3 ml of DME-HG 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 DME-HG containing 10% FBS, and the cells were further incubated for 48 h. The cells were then washed twice with phosphate-buffered saline (PBS), and 100 l of lysis buffer (Promega, Madison, WI, USA) were added to each well. The level of gene expression in the lysates was evaluated by measuring light production by luciferase using a scintillation counter protocol (Promega). The protein content of the lysates was measured by the Dc Protein Assay reagent (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as the standard. The data were expressed as nanograms of luciferase (based on a standard curve for luciferase activity), per milligram of total cell protein.

Zeta potential and hydrodynamic size measurements

Zeta potential measurements of the different lipid/DNA complexes, and ternary complexes with transferrin or peptides, were performed using a Coulter DELSA 440 instrument (Coulter Electronics, Miami, FL, USA). The DELSA 440 is a laser-based multiple angle particle electrophoresis analyzer that measures the electrophoretic mobility and zeta potential distribution simultaneously with the hydrodynamic size of particles in suspension. 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 zeta 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. The hydrodynamic size of the complexes was evaluated in the absence of any electric field.[53–55]

Targeting specificity studies

Parallel transfection experiments were performed using a protein as similar as possible to transferrin but to which the cell surface receptors for transferrin do not exhibit affinity. For that purpose, ternary complexes were prepared by associating 32 g of apotransferrin (iron-depleted transferrin) with the lipid–DNA complexes at different lipid/DNA (+/−) charge ratios. Complexes were added to COS-7 cells and transfection activity was evaluated as described above.

Competitive inhibition studies

To elucidate the cellular mechanisms of gene delivery by different lipoplexes, the following experiments were designed: (1) An excess of free transferrin (8 mg/0.3 ml of DME-HG medium) was added to COS-7 cells and

incubated for 30 min at 37°C before the addition of ternary complexes (cationic liposomes, pCMVluc plasmid, transferrin) which were incubated with the cells for 1 h always in the presence of an excess of transferrin. The medium was then replaced with DME-HG containing 10% FBS, and the cells were further incubated for 48 h before being harvested for luciferase activity measurements. (2) Lipoplexes with or without transferrin or the GALA peptide, and containing the plasmid pCMV.SPORT. -gal at different lipid/DNA charge ratios were incubated with HeLa cells in serum-free medium for 1 h at 37°C under the conditions described above. The medium was then replaced before the addition of similar complexes containing the luciferase plasmid (pCMVluc) which were incubated with the cells for another hour. Afterwards, the medium was replaced with DME-HG containing 10% FBS, and the cells were further incubated for 48 h before evaluation of transfection activity.

Effect of different drugs on transfection

Before the addition of lipoplexes, HeLa cells were incubated for 30 min at 37°C, in the absence of serum, with either (1) a mixture of antimycin A (1 g/ml), NaF (10 mM) and NaN$_3$ (0.1%) to inhibit endocytosis;[28,29] (2) cytochalasin B (25 g/ml) to inhibit pinocytosis and phagocytosis;[30–33] or (3) bafilomycin A$_1$ (125 nM) to prevent acidification of the endosomes.[34–36] Cells were further incubated for 1 h at 37°C with the different lipoplexes in the presence of the various drugs and then washed once with serum-free medium. The medium was then replaced with DME-HG containing 10% FBS, and the cells were further incubated for 48 h before evaluation of transfection. The viability of the cells transfected in the presence of these agents was evaluated and compared to that of untreated control cells.

Cell viability assay

Following transfection under the different experimental conditions, cell viability was quantified by a modified Alamar Blue assay.[11] The assay measures the redox capacity of cells due to the production of metabolites as a result of cell growth and allows determination of viability over the culture period without the detachment of adherent cells. Briefly, 1 ml of 10% (v/v) Alamar Blue dye in complete DME medium was added to each well 45 h following the initial transfection period (4 h). After 2.5–4 h of incubation at 37°C, 200 l of the supernatant were collected from each well and transferred to 96-well plates. The absorbance at 570 nm and 600 nm was measured with a microplate reader (Molecular Devices, Menlo Park, CA, USA). Cell viability (as a percentage of control cells) was calculated according to the formula $(A_{570} - A_{600})$ of treated cells $\times 100/(A_{570} - A_{600})$ of control cells.

Acknowledgements

References

1 Felgner PL *et al*. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc Natl Acad Sci USA* 1987; **84**: 7413–7417.

2 Hug P, Sleight RG. Liposomes for the transformation of eukaryotic cells. *Biochim Biophys Acta* 1991; **1097**: 1–17.

3 Philip R *et al*. *In vivo* gene delivery: efficient transfection of T lymphocytes in adult mice. *J Biol Chem* 1993; **268**: 16087–16090.

4 Singhal A, Huang L. Gene transfer in mammalian cells using liposomes as carriers. In: Wolf JA (ed). *Gene Therapeutics: Methods and Applications of Direct Gene Transfer*. Birkhäuser: Boston, 1994, pp 118–142.

5 Thierry AR *et al*. Systemic gene therapy: biodistribution and long-term expression of a transgene in mice. *Proc Natl Acad Sci USA* 1995; **92**: 9742–9746.

6 Felgner PL *et al*. Improved cationic lipid formulations for *in vivo* gene therapy. *Ann NY Acad Sci* 1995; **772**: 126–139.

7 Lasic DD, Templeton NS. Liposomes in gene therapy. *Adv Drug Deliv Rev* 1996; **20**: 221–266.

8 Felgner JH *et al*. Enhanced gene delivery and mechanism studies with a novel series of cationic lipid formulations. *J Biol Chem* 1994; **269**: 2550–2561.

9 Zabner J *et al*. Cellular and molecular barriers to gene transfer by a cationic lipid. *J Biol Chem* 1995; **270**: 18997–19007.

10 Xu Y, Szoka FC Jr. On the mechanism of DNA release from cationic liposome/DNA complexes used in cell transfection. *Biochemistry* 1996; **35**: 5616–5622.

11 Konopka K, Pretzer E, Felgner PL, Düzgüneş N. Human immunodeficiency virus type-1 (HIV-1) infection increases the sensitivity of macrophages and THP-1 cells to cytotoxicity by cationic liposomes. *Biochim Biophys Acta* 1996; **1312**: 186–196.

12 Konopka K, Harrison GS, Felgner PL, Düzgüneş N. Cationic liposome-mediated expression of HIV-regulated luciferase and diphtheria toxin A genes in HeLa cells infected with or expressing HIV. *Biochim Biophys Acta* 1997; **1356**: 185–197.

13 Ciccarone V, Hawley-Nelson P, Jessee J. Cationic liposome-mediated transfection: effect of serum on expression and efficiency. *Focus* 1993; **15**: 80–83.

14 Li S, Huang L. Lipidic supramolecular assemblies for gene transfer. *J Liposome Res* 1996; **6**: 589–608.

15 Simões S *et al*. Gene delivery by negatively charged ternary complexes of DNA, cationic liposomes and transferrin or fusigenic peptides. *Gene Therapy* 1998; **5**: 955–964.

16 Felgner PL *et al*. Nomenclature for synthetic gene delivery systems (editorial). *Hum Gene Ther* 1997; **8**: 511–512.

17 Wagner E, Curiel D, Cotten M. Delivery of drugs, proteins and genes into cells using transferrin as a ligand for receptor-mediated endocytosis. *Adv Drug Deliv Rev* 1994; **14**: 113–136.

18 Huebers HA, Finch CA. The physiology of transferrin and transferrin receptors. *Physiol Rev* 1987; **67**: 520–582.

19 Harford JB, Casey JL, Koeller M, Klausner RD. Structure, function and regulation of the transferrin receptor: insights from molecular biology. In: Steer CJ, Hanover JA (eds). *Intracellular Trafficking of Proteins*. Cambridge University Press: New York, 1991, pp 302–334.

20 Wagner E *et al*. Transferrin-polycation conjugates as carriers for DNA uptake into cells. *Proc Natl Acad Sci USA* 1990; **87**: 3410–3414.

21 Cotten M, Wagner E, Birnstiel ML. Receptor-mediated transport of DNA into eukaryotic cells. *Meth Enzymol* 1993; **217**: 618–644.

22 Cheng PW. Receptor ligand-facilitated gene transfer: enhancement of liposome-mediated gene transfer and expression by transferrin. *Hum Gene Ther* 1996; **7**: 275–282.

23 Subbarao NK *et al*. pH-dependent bilayer destabilization by an amphipathic peptide. *Biochemistry* 1987; **26**: 2964–2972.

24 Parente RA, Nir S, Szoka FC Jr. pH-dependent fusion of phosphatidylcholine small vesicles. *J Biol Chem* 1988; **263**: 4724–4730.

25 Wagner E *et al*. Influenza virus hemagglutinin HA-2 N-terminal fusogenic peptides augment gene transfer by transferrin-polylysine-DNA complexes: toward a synthetic virus-like gene-transfer vehicle. *Proc Natl Acad Sci USA* 1992; **89**: 7934–7938.

26 Simões S *et al*. Transfection of human macrophages by lipoplexes via the combined use of transferrin and pH-sensitive peptides. *J Leukocyte Biol* 1999; **65**: 270–279.

27 Simões S *et al*. Receptor ligand-mediated lipid-based gene delivery into T-lymphocytes. *Abstr. 12th Ann Mtg GTRV, Brussels*. 1997 (Abstr.).

28 Lee K-D, Nir S, Papahadjopoulos D. Quantitative analysis of liposome–cell interactions *in vitro*: rate constants of binding and endocytosis with suspension and adherent J774 cells and human monocytes. *Biochemistry* 1993; **32**: 889–899.

29 Slepushkin VA *et al*. Sterically stabilized pH-sensitive liposomes: intracellular delivery of aqueous contents and prolonged circulation *in vivo*. *J Biol Chem* 1997; **272**: 2382–2388.

30 Paccaud J-P, Siddle K, Carpentier J-L. Internalization of the human insulin receptor – the insulin independent pathway. *J Biol Chem* 1992; **267**: 13102–13106.

31 Zhou X, Huang L. DNA transfection mediated by cationic liposomes containing lipopolylysine: characterization and mechanism of action. *Biochim Biophys Acta* 1994; **1189**: 195–203.

32 Zelphati O, Szoka FC Jr. Intracellular distribution and mechanism of delivery of oligonucleotides mediated by cationic lipids. *Pharm Res* 1996; **13**: 1367–1372.

33 Matsui H, Johnson LG, Randell SH, Boucher RC. Loss of binding and entry of liposome–DNA complexes decreases transfection efficiency in differentiated airway epithelial cells. *J Biol Chem* 1997; **272**: 1117–1126.

34 Umata T, Moriyama Y, Futai M, Mekada E. The cytotoxic action of diptheria toxin and its degradation in intact Vero cells are inhibited by bafilomycin A1, a specific inhibitor of vacuolar-type H(+)-ATPase. *J Biol Chem* 1990; **265**: 21940–21945.

35 Pless DD, Wellner RB. *In vitro* fusion of endocytic vesicles: effects of reagents that alter endosomal pH. *J Cell Biochem* 1996; **62**: 27–39.

36 Cattani L *et al*. Bafilomycin A1 and intracellular multiplication of *Legionella pneumophila*. *Antimicrob Agents Chemother* 1997; **41**: 212–214.

37 Kircheis R *et al*. Coupling of cell-binding ligands to polyethyleneimine for targeted gene delivery. *Gene Therapy* 1997; **4**: 409–418.

38 Friend DS, Papahadjopoulos D, Debs RJ. Endocytosis and intracellular processing accompanying transfection mediated by cationic liposomes. *Biochim Biophys Acta* 1996; **1278**: 41–50.

39 Alberts B *et al*. *Molecular Biology of the Cell, 3rd edn*. Garland Publishing: New York, 1994.

40 Straubinger RM *et al*. Endocytosis of liposomes and intracellular fate of encapsulated molecules: strategies for enhanced cytoplasmic delivery. In: Gregoriadis G, Poste G, Senior J, Trouet A (eds). *Receptor-Mediated Targeting of Drugs*. Plenum Press: New York, 1985, pp 297–315.

41 Parente RA, Nir S, Szoka FC Jr. Mechanism of leakage of phospholipid vesicle contents induced by the peptide GALA. *Biochemistry* 1990; **29**: 8720–8728.

42 Fattal E, Nir S, Parente RA, Szoka FC Jr. Pore-forming peptides induce rapid phospholipid flip-flop in membranes. *Biochemistry* 1994; **33**: 6721–6731.

43 Mechtler K, Wagner E. Gene transfer mediated by influenza virus peptides: the role of peptide sequences. *New J Chem* 1997; **21**: 105–111.

44 Düzgüneş N *et al*. Proton-induced fusion of oleic acid/phosphatidylethanolamine liposomes. *Biochemistry* 1985; **24**: 3091–3098.

45 Farhood H, Serbina N, Huang L. The role of dioleoyl phosphatidylethanolamine in cationic liposome mediated gene transfer. *Biochim Biophys Acta* 1995; **1235**: 289–295.

46 Hui SW *et al*. The role of helper lipids in cationic liposome-mediated gene transfer. *Biophys J* 1996; **71**: 590–599.

47 Kichler A, Mechtler K, Behr J-P, Wagner E. Influence of membrane-active peptides on lipospermine/DNA complex mediated gene transfer. *Bioconj Chem* 1997; **8**: 213–221.

48 Aisen P. The transferrins. In: Jacobs A, Worwood M (eds). *Iron in Biochemistry and Medicine II*. Academic Press: London, 1980, pp 87–129.

49 Jarritt PH. Effect of iron on sedimetation-velocity and gel filtration behaviour of transferrin from several vertebrates. *Biochim Biophys Acta* 1976; **453**: 332–343.

50 Farias RN, Vinals AL, Morero RD. Insulin-mediated fusion of negatively charged phospholipid vesicles at low pH. *Biochem Biophys Res Commun* 1985; **128**: 68–74.

51 Maezawa S *et al*. Clathrin-induced fusion of phospholipid vesicles associated with its membrane binding and conformational change. *Biochemistry* 1989; **28**: 1422–1428.

52 Pattus F *et al*. pH-dependent membrane fusion is promoted by various colicins. *EMBO J* 1985; **4**: 2469–2474.

53 Woodle MC *et al*. Sterically stabilized liposomes. Reduction in electrophoretic mobility but not electrostatic surface potential. *Biophys J* 1992; **61**: 902–910.

54 Tomlinson E, Rolland AP. Controllable gene therapy. Pharmaceutics of non-viral gene delivery systems. *J Control Rel* 1996; **39**: 357–372.

55 Lourenço C, Teixeira M, Simões S, Gaspar R. Steric stabilization of nanoparticles: size and surface properties. *Int J Pharm* 1996; **138**: 1–12.