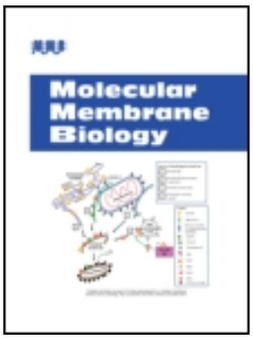

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Lipoplex size determines lipofection efficiency with or without serum

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Summary

In order to identify factors affecting cationic liposome-mediated gene transfer, the relationships were examined among cationic liposome/DNA complex (lipoplex)-cell interactions, lipoplex size and lipoplex-mediated transfection (lipofection) efficiency. It was found that lipofection efficiency was determined mainly by lipoplex size, but not by the extent of lipoplex-cell interactions including binding, uptake or fusion. In addition, it was found that serum affected mainly lipoplex size, but not lipoplex-cell interactions, which effect was the major reason behind the inhibitory effect of serum on lipofection efficiency. It was concluded that, in the presence or absence of serum, lipoplex size is a major factor determining lipofection efficiency. Moreover, in the presence or absence of serum, lipoplex size was found to affect lipofection efficiency by controlling the size of the intracellular vesicles containing lipoplexes after internalization, but not by affecting lipoplex-cell interactions. In addition, large lipoplex particles showed, in general, higher lipofection efficiency than small particles. These results imply that, by controlling lipoplex size, an efficient lipid delivery system may be achieved for *in vitro* and *in vivo* gene therapy.

Keywords: Cationic liposomes, DNA, lipofection, fusion, uptake.

Abbreviations: AFM, atomic force microscopy; Chol, cholesterol; DC-6-14, *O,O′*-ditetradecanoyl–*N*-(α-trimethylammonioacetyl)diethanolamine chloride; DMEM, Dulbecco's Modified Eagle Medium; DOPE, dioleoylphosphatidylethanolamine; FBS, fetal bovine serum; Lipofection, lipoplex-mediated transfection; Lipoplex, cationic liposome-DNA complex; NBD-PE, *N*-7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycerol-3-phosphoethanolamine; PBS, phosphate buffer saline; Rh-PE, Lissamine rhodamine phosphatidylethanolamine.

Introduction

Within a decade, gene therapy has gained rapid momentum as a new modality for the treatment of several diseases such as cancer, infection diseases and hereditary disorders (Morgan and Anderson 1993, Tolstoshev 1993, Crystal 1995, Taneja *et al*. 1997). However, clinical application of gene therapy depends on the development of suitable gene transfer vehicles (vectors) that acts with high efficiency, i.e. that lead to high levels of gene expression (Nabel *et al*. 1993, Lasic and Templeton 1996). Since their first introduction by Felgner *et al*. (1987), cationic liposomes have proven to be useful tools for delivery of genes to cells in culture and for gene therapy in pre-clinical and clinical trials (Zhu *et al*. 1993, Dean and McKay 1994, Lappalainen *et al*. 1994, Felgner *et al*. 1995, Liu *et al*. 1995, Thierry *et al*. 1995). For example, the delivery of the gene encoding the chloride transporter into the lungs of transgenic mice lacking this gene (Hyde *et al*. 1993) and into cystic fibrosis patients (Caplen *et al*. 1995) via cationic liposomes partially corrected this defect.

There are many advantages of cationic liposomes over viruses as gene transfer vectors. Unlike viral vectors, cationic liposomes can be used to transfer DNA of essentially unlimited size; they are simple, easily produced in large scale, have low immunogenicity and cannot form an infectious agent. There are, however, some drawbacks with lipid vectors, such as their lower efficiency than viral vectors in gene transfer, especially in the presence of serum or *in vivo* (Huang and Viroonchatapan 1999, Chesnoy and Huang 2000).

Many parameters are known to affect the efficiency of gene transfer by lipoplexes (lipofection). Among these parameters are the composition of liposomes, the cationic lipid to DNA ratio or (+/−) charge ratio, and the transfection protocol. Various efforts have been undertaken to understand these factors (Wheeler *et al*. 1996, Fasbender *et al*. 1997, Lee and Huang 1997, Escriou *et al*. 1998, Huang and Viroonchatapan 1999, Pires *et al*. 1999, Sakurai *et al*. 2000). However, until the importance of these factors is recognized and the mechanism(s) of their effects on lipofection is fully understood, optimization will remain largely a result of trial and error (Lee and Huang 1997, Huang and Viroonchatapan 1999, Chesnoy and Huang 2000).

This study used cationic lipid DC-6-14 complexed with lipid helpers cholesterol and DOPE at a molar ratio of 1/0.75/0.75, respectively, which showed high transfection efficiency *in vitro*, even in the presence of serum, and *in vivo* with low toxicity (Kikuchi *et al*. 1999). These liposomes were complexed with plasmid DNA pGL3-C encoding a luciferase reporter gene and were used to investigate the major factors affecting lipofection efficiency and the relationships among them. The effects of changing the cationic lipid/DNA (+/−) charge ratio on lipoplex-cell interactions were studied, namely, binding, uptake, fusion and intracellular distribution, and on lipoplex size and lipofection efficiency. Also, the relationship between each of these factors and lipofection efficiency, in the absence and presence of serum, was investigated.

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Results

Effect of (+/−) charge ratio on lipoplex-cell interactions

Significant changes occur in the biophysical features of cationic liposomes upon their complexation with negatively-charged DNA (Gershon *et al*. 1993, Sternberg *et al*. 1994, Pires *et al*. 1999). These changes are likely to alter the interaction of cationic liposomes with cell membranes.

The effect of changing the lipoplex (+/−) charge ratio on lipoplex-cell interactions was examined, namely lipoplex-cell binding, uptake and fusion. Lipoplex-cell binding and uptake would reflect the extent of lipoplex internalization into the cytoplasm (Wrobel and Collins 1995, Escriou *et al*. 1998, Huang and Viroonchatapan 1999, Pires *et al*. 1999, Ross and Hui 1999, Sakurai *et al*. 2000). Fusion between lipoplex and cell membrane and/or endosomal membranes would reflect the extent of DNA delivery into the cytoplasm (Felgner *et al*. 1987, Wrobel and Collins 1995, Xu and Szoka 1996, Huang and Viroonchatapan 1999, Pires *et al*. 1999, Ross and Hui 1999). As shown in figures1 (*a*) and (*b*), complexing liposomes with an increasing amount of DNA resulted in a gradual decrease in the rate of lipoplex-cell binding, uptake and fusion. This decrease seems to be due to the gradual decrease in the net positive charge of the cationic liposomes when complexed with an increasing amount of the negatively-charged DNA, which in turn results in a decrease in the electrostatic attractions between lipoplexes and cells (figure 1). These data demonstrate that lipoplex-cell interactions are dominated by the electrostatic attraction between them.

Effect of (+/−) charge ratio on lipoplex size

As shown in figure 2, free cationic liposomes had a mean diameter of 254 nm. Complexing of liposomes with an increasing amount of DNA, up to 1:1 (+/−) charge ratio resulted in a dramatic increase in the particle size; i.e. lipoplexes of 6:1, 3:1, 1.5:1 and 1:1 (+/−) charge ratios had mean diameters of 520, 1230, 1950 and 3818 nm, respectively. At the 1:1 (+/−) charge ratio, the very large particle mean size can be attributed to extensive and uncontrolled aggregation and fusion among lipid particles due to their neutralization at this charge ratio. This uncontrolled liposome–liposome fusion/aggregation resulted also in large differences in the measured mean diameter among different preparations and in high standard deviation at this (+/−) charge ratio (figure 2). However, complexing greater amounts of DNA with liposomes resulted, oppositely, in decreasing particle size. In this case at 0.75:1, 0.5:1, 0.25:1 and 0.125:1 (+/−) charge ratios, the particle mean diameters were 1350, 730, 324 and 243 nm, respectively (figure 1). This could be due to the increasing strength of repulsive forces among lipoplex particles resulting from the increased negative charge at these charge ratios, as was suggested before for another liposome system (Pires *et al*. 1999).

Effect of (+/−) charge ratio on lipofection efficiency

A431 cells were transfected with lipoplexes of different (+/−) charge ratios (see experimental procedures section). As illustrated in figure 3, lipofection efficiency was strongly affected by changing the (+/−) charge ratio of the lipoplexes. Lipoplex of 1.5:1 (+/−) charge ratio had the highest lipofection efficiency and increasing or decreasing this ratio resulted in a significant decrease in the lipofection efficiency. The decrease in lipofection efficiency was not due to cytotoxicity toward the treated cells, as lipoplexes of different charge ratios tested had no detectable cytotoxicity as measured by the trypan blue exclusion test (data not shown). These results indicate that both the decrease and increase in lipofection efficiency were independent of the decrease and increase in the charge ratio, as there was a maximum

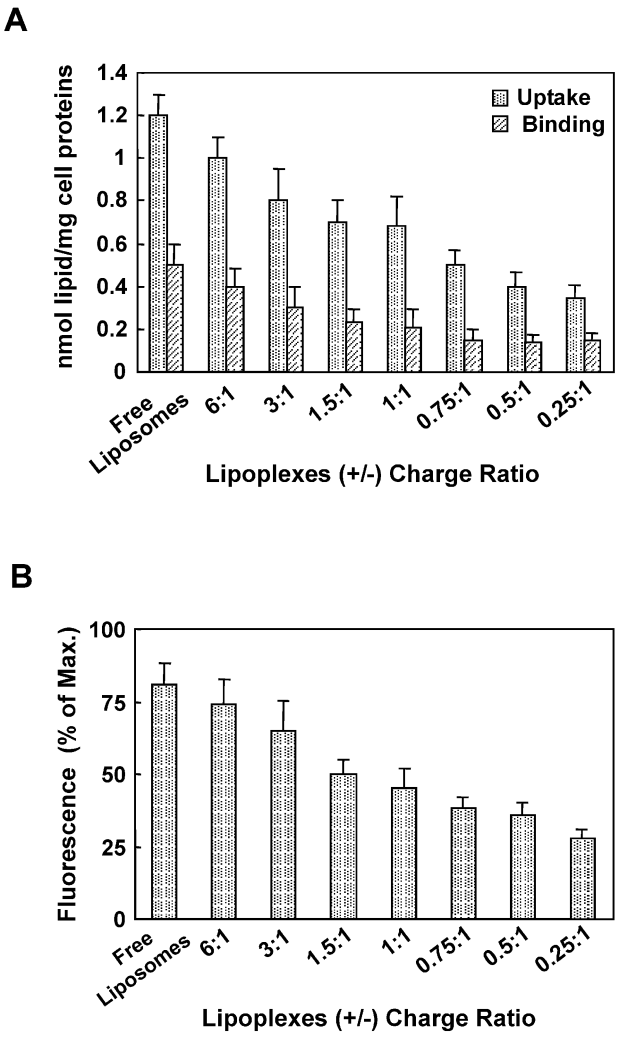

Figure 1. Effect of (+/−) charge ratio on lipoplex–cell interactions. (*a*) Changes in lipoplex–cell binding and uptake over different (+/−) charge ratios. A431 cells were incubated with liposomes or lipoplexes of the indicated (+/−) charge ratios for 15 min at 4°C for binding and for 1 h at 37°C for uptake. Liposomes corresponding to 10 nmol cationic lipids were added for all experiments and the amount of DNA was changed to obtain the indicated (+/−) charge ratio. (*b*) Changes in lipoplex–cell fusion over different (+/−) charge ratios. Liposomes were added as 5 nmol cationic lipids/ml for all experiments and the amount of DNA was adjusted to obtain the indicated (+/−) charge ratio. Lipoplexes were prepared immediately before analysis. Error bars represent standard deviations for at least three different experiments.

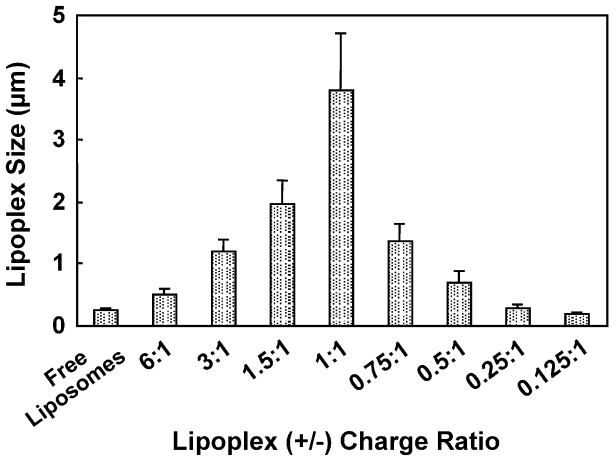

Figure 2. Effect of (+/−) charge ratio on lipoplex size. Cationic liposomes or lipoplexes of different (+/−) charge ratios were characterized with respect to mean particle size. All measurements were done in OptiMEM I medium. Lipoplexes were prepared at different lipid/DNA (+/−) charge ratios immediately before analysis. Error bars represent standard deviations for at least three different preparations.

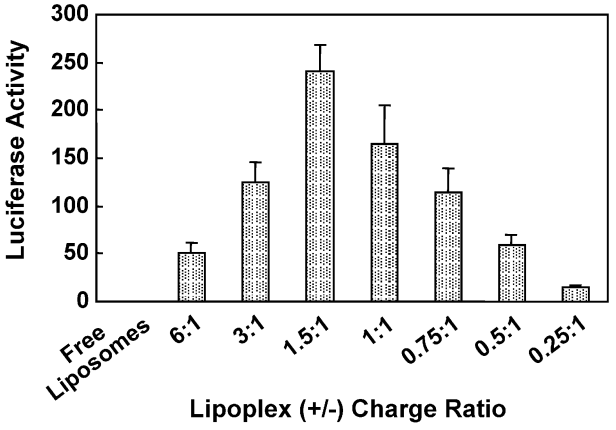

Figure 3. Effect of (+/−) charge ratio on lipofection efficiency. Plasmid DNA encoding firefly luciferase pGL3-C (1 μg) was complexed with different amounts of cationic liposomes to obtain the indicated charge ratios. Lipoplexes were prepared immediately before analysis, and luciferase activity was evaluated 24 h after lipofection. The values of luciferase activity are light counts per minute per μg of total cellular proteins. Error bars represent standard deviations for at least three different experiments.

efficiency at the 1.5:1 (+/−) charge ratio and an increase or decrease in this charge ratio resulted only in decreased lipofection efficiency (figure 3).

Lipoplex size determines lipofection efficiency

In view of all of the above results taken together, lipofection efficiency did not correlate with the change in the lipoplex charge ratio; also, it seems that the effect of changing the charge ratio of lipoplexes on the lipoplex interactions with cells, namely, binding, uptake and fusion (figure 1), cannot explain the effects of the lipoplex charge ratio on lipofection efficiency. Although lipoplex binding to and uptake by cells are essential for efficient internalization of lipoplexes into the cytoplasm (Escriou *et al*. 1998, Huang and Viroonchatapan 1999, Pires *et al*. 1999, Ross and Hui 1999, Sakurai *et al*. 2000), and lipoplex fusion directly with cytoplasmic membranes and/or with endosomal membranes are also essential for efficient delivery of DNA into the cytoplasm (Wrobel and Collins 1995, Xu and Szoka 1996, Huang and Viroonchatapan 1999, Pires *et al*. 1999, Ross and Hui 1999), lipofection efficiency changed independently of the change of all these parameters according to the results. Adding more DNA to liposomes resulted in only a gradual decrease in cell-lipoplex binding, uptake and fusion (figure 1), which would result in decreasing the amount of internalized lipoplexes in the cells and in the amount of DNA delivered into the cytoplasm; but in fact it resulted in increasing lipofection efficiency up to the 1.5:1 (+/−) charge ratio, and then a gradual decrease in lipofection efficiency as more DNA was added (figure 3). The effect of changing the lipoplex charge ratio on lipofection efficiency seemed to parallel only that on lipoplex size (compare figures 2 and 3). In order to check this possibility, changes were compared in lipoplex size and lipofection efficiency over different (+/−) charge ratios. As shown in figure 4, there was, in fact, a strong relationship between lipoplex size and lipofection efficiency at almost all studied charge ratios (R^2=0.9566). The only exception was lipoplex of 1:1 (+/−) charge ratio, as at this charge ratio the particle mean diameter was much larger than that at the 1.5:1 charge ratio but the lipofection efficiency was less (figure 3). As discussed above, at the 1:1 (+/−) charge ratio lipoplexes had significant heterogeneous particle sizes; and, therefore, some of their particles would be smaller than that of 1.5:1 (+/−) charge ratio, some larger, and some of the same particle size. Smaller particles would have less transfection efficiency as explained above; and larger particles could be too large to be uptaken by cells and therefore would be inefficient in transfecting the cells. Thus, lipoplexes of 1:1 (+/−) charge ratio would have overall less lipofection efficiency than those having the 1.5:1 (+/−) charge ratio, and this may explain why

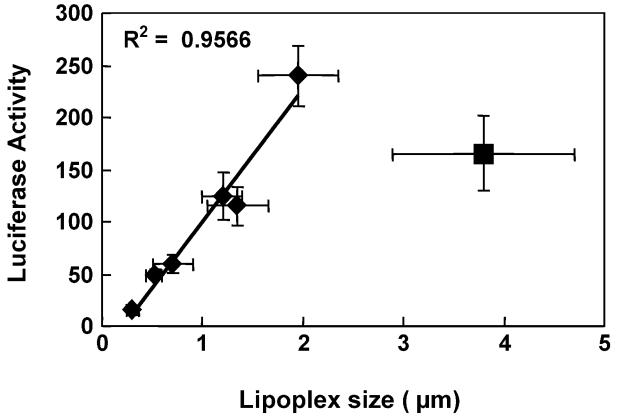

Figure 4. Lipoplex size determines lipofection efficiency. Lipoplex sizes were compared with lipofection efficiency over different (+/−) charge ratios. Error bars represent standard deviations of lipofection efficiency (Y error bars) and lipoplex sizes (X error bars).

this charge ratio was an exception from the lipoplex size–lipofection efficiency relationship.

It should be noted that these results were reproducible when two other cell lines, rat liver cancer cells (AH130) and human lung cancer cells (SCB-3), were used. Even though the extent of lipoplex-cell interactions and lipofection efficiency were different among the different cell lines, a strong relationship between lipoplex size and lipofection efficiency was found for all cell lines used (data not shown).

Effect of (+/−) charge ratio on intracellular distribution of fluorescently labelled lipoplexes

Fluorescently labelled liposomes or lipoplexes of different (+/−) charge ratios were incubated with cells for 1 h, and their intracellular distributions were imaged by using confocal microscopy, as explained in the experimental procedures section. As shown in figure 5(*a*), free liposomes became internalized efficiently into the cytoplasm and localized within small vesicles that appeared to be endosomes. Lipoplexes, as well, were internalized efficiently into the cytoplasm, but their intracellular distribution patterns were different for different lipoplex (+/−) charge ratios. As shown in figure 5(*b*), lipoplexes of 3:1 (+/−) charge ratio, which had a mean diameter of 1230 nm, were in intracellular vesicles much larger than those containing free liposomes, which had a mean diameter of 254 nm. Lipoplexes of 1.5:1 and 1:1 charge ratio, which had mean diameters of 1950 and 3818 nm, respectively, were within even larger intracellular vesicles that seemed to be phagosomes (figures 5(*c*) and (*d*)). Lipoplexes of 0.75:1, 0.5:1, 0.25 and 0.125:1 (+/−) charge ratios, which had decreasing particle size, were also within intracellular vesicles of decreasing size (figures 5(*e*–*h*)). Thus, lipoplexes with the 0.125:1 charge ratio, for example, were within small intracellular vesicles similar in size to those containing free liposomes (figure 5(*h*)). These intracellular distribution patterns were in good agreement with the original sizes of the internalized lipoplexes (figure 2). In addition, increasing the amount of DNA complexed with liposomes, which decreased lipoplex uptake by cells due to the decrease in the positive charge of the liposomes (figure 1(*a*)), resulted in a decrease in the overall internalized fluorescent particles in the cytoplasm. These results indicate that the size of intracellular vesicles containing lipoplexes, which correlates well with the original size of the lipoplexes, is crucial for the lipofection efficiency rather than the amount of lipoplexes internalized. Lipofection efficiency was higher when cells took up large lipoplex particles, leading to the formation of large intracellular vesicles. These large vesicles would be more easily disrupted, thus releasing DNA into the cytoplasm (Escriou *et al*. 1998).

Effect of serum concentrations on lipoplex size, lipoplex-cell interactions and lipofection efficiency

It is well known that serum has an inhibitory effect on lipofection efficiency (Escriou *et al*. 1998, Sakurai *et al*. 2000). The mechanism(s) of this inhibitory effect on lipofection efficiency has been explained as follows: negatively charged serum proteins interact with cationic liposomes, resulting in neutralization of the positive charge of lipoplexes and then minimization of the electrostatic interaction between the neutralized lipoplexes and cellular membrane and/or the release of DNA before the lipoplexes are being taken up by cells (Lee and Huang 1997, Pires *et al*. 1999). Others proposed that serum inhibitory effects on lipofection are due to the inhibitory effect of serum on the increase in liposomal size when liposomes are complexed with DNA (Escriou *et al*. 1998).

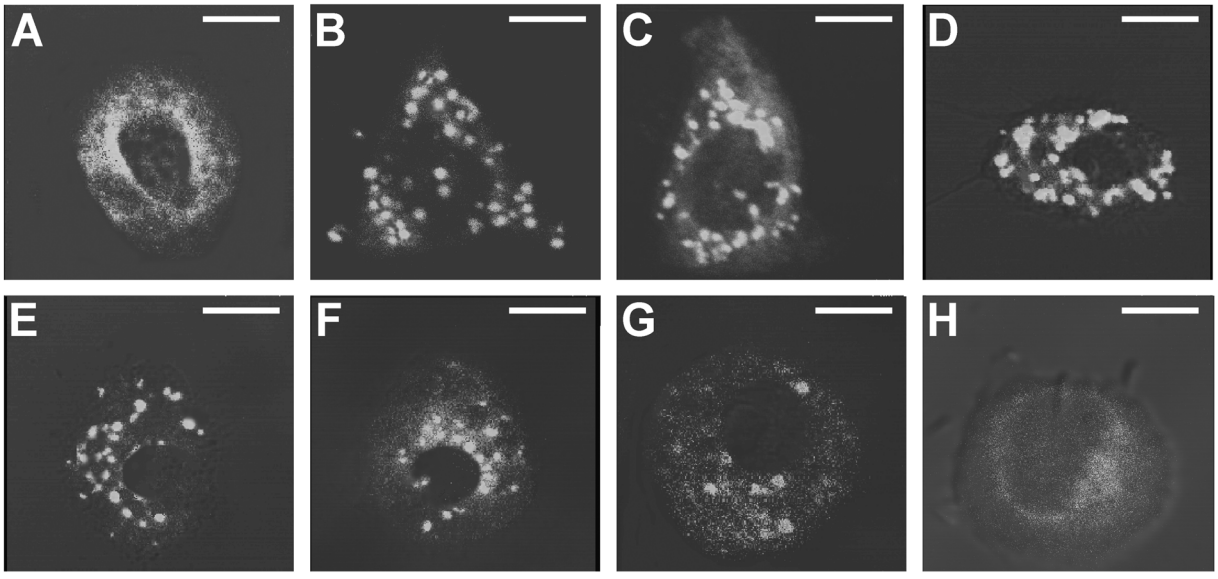

Figure 5. Confocal microscopic images of intracellular distribution of lipoplexes. Cells were transfected for 1 h with lipoplexes of different (+/−) charge ratios exactly as in the legend of figure 3. Then the cells were washed, fixed and imaged. Panels (*a*–*h*) represent free liposomes, 3:1, 1.5:1, 1:1, 0.75:1, 0.5:1, 0.25:1 and 0.125:1 (+/−) charge ratios, respectively. Bars indicate 10 μm. The panels shown are typical of the results of multiple evaluations.

To check these possibilities, the effect was investigated of increasing the percentage of serum in the incubation medium on lipoplex size, lipoplex-cell interactions (binding, uptake, and fusion) and lipofection efficiency. For all experiments, lipoplexes of 1.5:1 (+/−) charge ratio were used as they showed the highest lipofection efficiency (figure 3). As shown in figures 6(*a*) and (*b*), increasing the percentage of serum in the incubation medium resulted in a slight gradual decease in binding, uptake and fusion, compared with these interactions in the absence of serum. However, the increase in serum resulted in a significant decrease in lipoplex size (figure 7) and in lipofection efficiency (figure 8), which occurred in a similar pattern. As shown in figure 9, comparing the changes in lipoplex size and lipofection efficiency that resulted from an increase in the serum concentration in the incubation medium, a strong relationship was found between lipoplex size and lipofection efficiency in the presence of serum (R^2=0.9873).

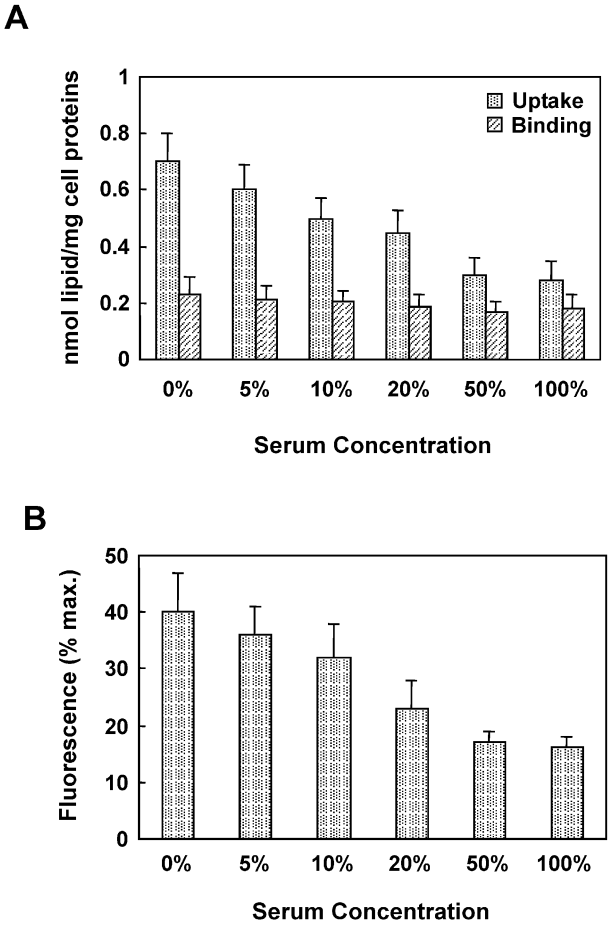

Figure 6. Effect of serum concentration on lipoplex–cell interactions. Changes in lipoplex–cell binding and uptake (*a*) and in lipoplex–cell fusion (*b*) with different serum concentrations in the incubation medium are shown. For all experiments, lipoplexes of 1.5:1 (+/−) charge ratio were used and were prepared immediately before analysis. Experimental conditions were the same as those for the 1.5:1 (+/−) charge ratio in the legend of figure 1(*a*) for binding and uptake, and in the legend of figure 1(*b*) for fusion experiments. Error bars represent standard deviations for at least three different experiments.

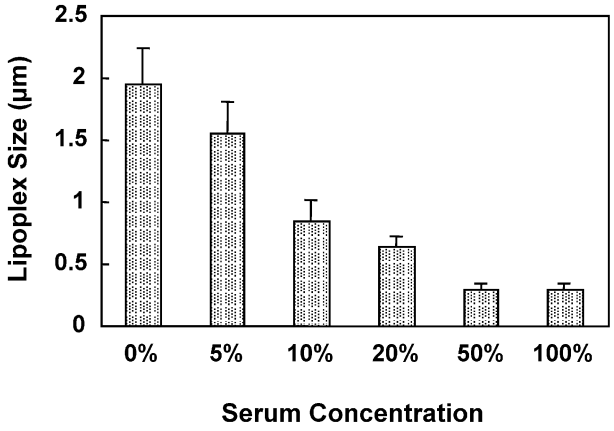

Figure 7. Effect of serum concentration on lipoplex size. Lipoplexes of 1.5:1 (+/−) charge ratio were characterized with respect to mean particle size in the presence of the indicated serum concentrations in the incubation medium (Opti-MEM I medium). Lipoplexes were prepared immediately before analysis. Error bars represent standard deviations for at least three different preparations for each serum concentrations.

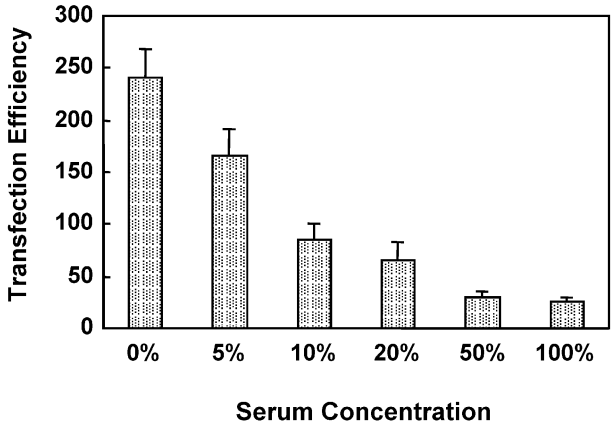

Figure 8. Effect of serum concentrations on lipofection efficiency. Experimental conditions were exactly the same as in the legend of figure 3. For all experiments, cells were transfected with lipoplexes of 1.5:1 charge ratio in the presence of the indicated serum concentrations in the incubation medium. Error bars represent standard deviations for at least three different experiments.

Similar results were found when AH130 and SCB-3 cell lines were examined. Although different extents of lipoplex–cell interactions and lipofection efficiency in the presence of serum were observed for different cell lines, there was a good correlation between lipoplex size and lipofection efficiency (data not shown).

Confocal microscopic images of the internalized lipoplexes in the cytoplasm in the presence of increasing concentrations of serum in the incubation medium showed also only a slight decrease in the overall number of internalized lipoplex particles in the cytoplasm, consistent with the results of the lipoplex–cell interaction experiments (figure 5). However, interestingly, these images showed a significant decrease in

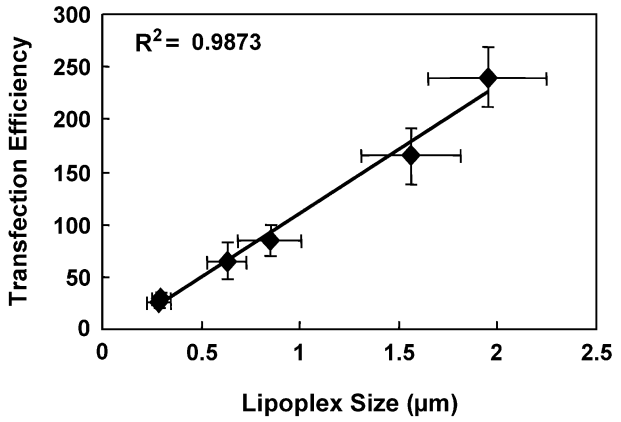

Figure 9. Lipoplex size determines lipofection efficiency in the presence of serum. Lipoplex size is compared with lipofection efficiency over different serum concentrations in the incubation medium. Error bars represent standard deviations of lipofection efficiency (Y error bars) and lipoplex sizes (X error bars).

the size of the intracellular vesicles containing lipoplexes when the serum concentration of the incubation medium was increased (data not shown).

Therefore, it was concluded that serum affects primarily lipoplex size, which in turn controls the size of the intracellular vesicles containing internalized lipoplexes and that this action plays a major role in the inhibitory effect of serum on lipofection efficiency.

Discussion

The biophysical characteristics of lipid-based gene delivery systems play an important role in their interaction with cells and finally in their gene delivery efficiency (Morgan and Anderson 1993, Lee and Huang 1997, Huang and Viroonchatapan 1999, Pires *et al*. 1999). This study investigated the effect of changing the (+/−) charge ratio of lipoplexes on lipoplex size, lipoplex–cell interactions (binding, uptake, fusion, and intracellular distribution) and lipofection efficiency. The results emphasize the importance of the lipid-DNA electrostatic interactions involved in the formation and the properties of the resulting complexes, which is in agreement with other reports for different liposome systems (Lee and Huang 1997, Escriou *et al*. 1998, Huang and Viroonchatapan 1999, Pires *et al*. 1999, Sakurai *et al*. 2000). Lipoplex size changed dramatically upon a change in (+/−) charge ratio and the maximum lipoplex size was found at the neutral charge ratio, 1:1 (+/−).

Lipoplex–cell interactions, i.e. binding, uptake and fusion, were found to be governed mainly by the electrostatic attraction between the positively charged cationic lipid and the negatively charged cell membranes, so charged due to the presence of negatively charged sialic acid residues. These results, also, are in agreement with other reports using different cationic liposomes and cells (Huang and Viroonchatapan 1999, Pires *et al*. 1999, Sakurai *et al*. 2000).

Lipofection efficiency changed significantly when the (+/−) charge ratio of the lipoplexes was changed. However, this change in efficiency, increase or decrease, was not correlated with the increase or decrease in the lipoplex (+/−) charge ratio. In addition, the change in lipofection efficiency over different charge ratios was not correlated with the changes in lipoplex–cell interactions such as binding, uptake and fusion. Thus, although lipoplex–cell electrostatic attractions appear to control lipoplex–cell interactions, they do not control lipofection efficiency. There was a strong correlation between lipofection efficiency and lipoplex size over a range of (+/−) charge ratios. Therefore, it was concluded that lipoplex size, rather than any of the lipoplex–cell interactions, has the determinant effect on lipofection efficiency. Another point to be noted is that, in general, large lipoplex particles were preferable for efficient lipofection, which is in agreement with other reports using other liposome systems (Wheeler *et al*. 1996, Fasbender *et al*. 1997, Escriou *et al*. 1998, Sakurai *et al*. 2000).

Confocal fluorescence imaging of the cellular distribution of the internalized lipoplexes of different (+/−) charge ratios showed a clear correlation between the sizes of intracellular vesicles containing the internalized lipoplexes and the original sizes of these particles. Interestingly, it was reported that large intracellular vesicles containing large lipoplex particles would be more easily disrupted, thus releasing DNA into the cytoplasm (Escriou *et al*. 1998). Therefore, it is reasonable to assume that lipoplex size controls the lipofection efficiency by controlling the size of the intracellular vesicles containing the lipoplexes and subsequently controlling the efficiency of DNA release from these vesicles into the cytoplasm, from which the DNA finally reaches the transcription apparatus.

The serum effects on lipoplex size, lipoplex–cell interactions, lipofection efficiency and intracellular distribution support the above conclusion. There was a clear correlation between lipofection efficiency and lipoplex sizes over different serum concentrations in the incubation medium. Therefore, it was concluded that the inhibitory effect of serum on lipofection efficiency was mainly caused by blocking the increase in lipoplex size after the liposomes were complexed with DNA, and not by inhibiting lipoplex–cell interactions. Confocal microscopic images of the intracellular distributions of lipoplexes in the presence of increasing concentrations of serum showed that the lipoplexes were within intracellular vesicles of decreasing size, which correlates with the decrease in lipoplex size under these conditions. This observation strongly supports the above hypothesis that lipoplex sizes control lipofection efficiency by changing the size of the intracellular vesicles.

It is of importance to mention that Ross and Hui (1999) reported that, for DOTAP-based liposomes, lipoplex size was a major determinant of lipofection efficiency; and they attributed this effect on efficiency to be due to the lipoplex size control of lipoplex–cell uptake and fusion. Although our results emphasize that lipoplex size is a major determinant of lipofection efficiency, in this system and as discussed above, lipoplex size did not control lipoplex–cell binding, uptake or fusion; rather lipoplex size appeared to control lipofection efficiency by controlling the size of the intracellular vesicles containing lipoplexes after internalization and that occurred in the presence or absence of serum.

It should be noted that large lipoplexes, such as 1.5:1 and 1:1 (+/−) ones, were found in the cytoplasm within large vesicles of several micrometres in diameter (figure 5). These sizes are much larger than those of endocytosis vesicles (several hundreds of nanometres), which are the suggested intracellular pathway for lipoplexes (Wrobel and Collins 1995). It was reported, however, that airway epithelial cells did take up large lipoplexes of ~2 μm in diameter through phagocytosis (Matsui *et al*. 1997), and so this could be the case for our large lipoplexes as well. The mechanism of internalization of such large particles into the cytoplasm is currently under investigation.

Interestingly, the cationic liposomes used in this study had substantial transfection efficiency even in the presence of 50–100% serum (figure 9). These results emphasize the results of the original study that introduced these liposomes (Kikuchi *et al*. 1999) in which they were effective *in vitro* in the presence of serum and *in vivo*. In addition, these liposomes had almost no toxicity at any charge ratio used, even at the 6:1 (+/−) one (figure 3), which indicates that these liposomes are a promising vehicle for lipid-based gene delivery.

All together, the results indicate that, in the presence or absence of serum, lipoplex size is the major determinant of the lipofection efficiency and that large particles are more efficient in transfection. Lipoplex size appears to determine lipofection efficiency by controlling the size of intracellular vesicles containing the lipoplexes, which in turn affects the efficiency of DNA release into the cytoplasm. In addition, serum inhibited lipofection efficiency mainly by reducing lipoplex sizes, emphasizing the importance of lipoplex size and the need for large lipoplex particles for efficient lipofection.

It is believed that these results are another significant step towards more detailed understanding of the factors affecting lipofection efficiency, as well as the relationships among these factors. In addition, this study indicates the need to develop lipoplexes resistant to the compacting effect of serum; which would be very useful for efficient *in vitro* lipofection, in which the presence of serum is usually preferable, and of course for efficient *in vivo* gene delivery. These studies are currently being carried out.

Experimental Procedures

Materials

The cationic lipid DC-6-14 was a generous gift from Daiichi Pharmaceutical Co. Ltd. (Tokyo, Japan). DOPE was kindly donated by the Nippon Oil and Fats Co., Ltd. (Tokyo, Japan). Cholesterol was purchased from Wako Pure Chemical (Osaka, Japan). The DNA expression plasmid pGL3-Control, containing a SV40 promoter and enhancer and Luciferase reporter gene was purchased from Promega (Madison, WI). Rh-PE and NBD-PE came from Avanti Polar Lipids (Alabster, AL). Opti-MEM I and DMEM were purchased from Gibco BRL (Rockville, MD).

Preparation of plasmid DNA

The above expression plasmid was amplified by using competent E. coli JM 109 (Nipon Gene, Japan) and purified by equilibrium centrifugation in CsCl-ethidium bromide gradients (Molecular Cloning/second edition, 1989).

Cells

A431, a human epidermoid carcinoma cell line, and SBC-3, a human lung cancer cell line, were maintained at 37°C under 5% CO_2 in DMEM medium containing 10% FBS. AH130, a rat liver cancer cell line, was maintained at 37°C under 5% CO_2 in RPMI 1640 medium containing 10% FBS.

Preparation of cationic liposomes and lipoplexes

Cationic lipid DC-6-14 with neutral lipid helpers, cholesterol and DOPE, at a molar ratio of 1:7.5:7.5, respectively, were dissolved in a chloroform-methanol mixture of 4:1 (v/v), and the solvent was removed in a rotary evaporator. The lipid mixture was evacuated in a desiccator for at least 2 h. The lipid mixture was then dispersed in 9% sucrose aqueous solution, and extruded 10 times at 70°C through a polycarbonate membrane (Nuclepore Co., Pleasanton, CA) with 0.1 μm pore size. For binding and uptake studies, 0.2 mol% NBD-PE was added to the lipid mixture. For the fusion study, 0.6 mol% of Rh-PE and NBD-PE each were added to the lipid mixture.

Lipoplexes were prepared by mixing the appropriate amount of DNA with the liposomes in order to obtain the desired (+/−) charge ratio.

Size determination

Mean particle sizes of liposomes and lipoplexes in Opti-MEM I medium were determined by a quasi-elastic light scattering method using a Nicomp 370 device (Santa Barbara, CA, USA). Where indicated, increasing serum concentrations in the medium were included. Sample size was analysed by multi-modal NICOMP vesicle analysis to determine the size of lipoplexes. Sizes presented represent multiple measurements of different samples made from different liposome preparations and on different days. Lipoplex sizes were reproducible and accurate over the measured size range. The range of particle sizes was within the accurate measurable size range of the Nicomp 370 according to the maker's guidelines.

Liposome- and lipoplex-cell binding and uptake

In order to evaluate lipoplex–cell binding and uptake, liposomes containing 2 mol% NBD-PE, or their complexes with DNA, were prepared. A431 cells were grown in 12-well plates to ~75% confluence. Liposomes or lipoplexes were incubated with cells, at 4°C for binding and at 37°C for uptake, for 1 h. The lipid concentration for all experiments was 10 nmol cationic lipid/ml incubation medium. The final volume of the incubation medium was set at 1 ml. The DNA concentration was changed, in each experiment, to obtain the indicated (+/−) charge ratio. Thereby, 6:1, 3:1, 1.5:1, 1:1, 0.75:1, 0.5:1 and 0.25:1 (+/−) charge ratios were prepared by mixing liposomes containing 10 nmol cationic lipid with 0.5, 1, 2, 2.5, 4, 6 and 12 μg plasmid DNA, respectively. After incubation, the cells were washed five times with ice-cold PBS and lysed by adding 400 μl of Cell Culture Lysis Reagent (CCLR) lysis buffer (Promega, Madison, WI) to each well. The cell lysates were inoculated into microtubes and centrifuged at a 15 000 rpm for 2 min. Aliquots of 100 μl of supernatants were added to each well of a 96-well plate and fluorescence was recorded at excitation and emission wavelengths of 568 and 586 nm, respectively, by a Wallac Multi Label Counter 1420 ARVO SX (Turku, Finland). The lipid equivalent (nmol) of measured fluorescence units was calculated from a calibration curve prepared by measuring the fluorescence of increasing known amounts of NBD-PE-labelled lipid as a standard under the same conditions (not shown). The protein contents of the lysates were measured with the Dc Protein assay reagent (Bio-Rad, Hercules, CA) with bovine serum albumin used as the standard. The data were expressed as nmol lipid, bound or uptaken, per mg of total

cellular proteins. For the study on the serum effect, different serum concentrations were included in the incubation medium as indicated in the figure legends.

Liposome- and lipoplex – cell fusion

Cationic liposomes containing 0.6 mol% Rh-PE and NBD-PE each, or lipoplexes at different (+/−) charge ratios were prepared. A431 cells (10^6) were suspended in ice-cold Opti-MEM I medium and kept on ice until used. Cells were added to a cuvette containing 2 ml of PBS only or with serum of different concentrations where indicated, at 37°C in the sample chamber of the fluorometer and allowed to equilibrate for 5 min. Then free cationic liposomes or lipoplexes of different (+/−) charge ratios were added to the cuvette to obtain a final concentration of 5 nmol cationic lipid/ml. The amount of DNA used to prepare lipoplexes in each experiment was adjusted to obtain the indicated (+/−) charge ratio, as explained above. The samples were stirred in a thermostated cuvette holder throughout the experiment. Fluorescence resulting from the dequenching of NBD-PE, due to lipid fusion, was recorded for 30 min at excitation and emission wavelengths of 465 and 530 nm, respectively. The initial fluorescence recorded upon liposome or lipoplex addition was set to zero. Fluorescence was monitored over a 30-min period. At the end of the incubation period, Triton X-100 was added to a final concentration of 1.0%; and the resulting fluorescence value was set to 100%. For the study on the serum effect, different serum concentrations were included within the incubation medium as indicated in the figure legends.

Lipofection activity

A431 cells were grown in 12-well plates to ~65% confluence. The cells were rinsed with PBS and then lipoplexes were added at a final volume of 400 μl in Opti-MEM I medium and a plasmid DNA dose of 1 μg for all experiments. Liposome concentrations were changed to prepare lipoplexes of the desired (+/−) charge ratios and were calculated as explained above. After an incubation of 1 h at 37°C under CO_2, the transfection medium was replaced with the normal growth medium DMEM containing 10% FBS and cells were further incubated for 23 h at 37°C under CO_2. The cells were then washed twice with PBS and lysed by adding 400 μl of CCLR lysis buffer (Promega, Madison, WI) to each well. The cell lysates were inoculated into microfuge tubes and were centrifuged at 15 000 rpm for 2 min. The level of gene expression was evaluated by measuring light production after mixing 10 μl of cell lysate with 50 μl of Luciferase Assay Reagent (Promega) by using a luminometer (Aloka, BLR-301, Tokyo, Japan). The protein contents of the lysates were measured with the Dc Protein assay reagent (Bio-Rad, Hercules, CA), with bovine serum albumin as the standard. The data were expressed as light counts per minute per μg of total cellular proteins. To assess serum effects, different serum concentrations were included in the incubation medium as indicated in the figure legends.

Confocal microscopic imaging of intracellular trafficking of lipoplexes

A431 cells, grown on coverslips within culture dishes, were transfected with liposomes or lipoplexes of different (+/−) charge ratios for 1 h. The conditions and lipoplexes and DNA concentrations were exactly the same as in the transfection experiments. The cells were then washed with PBS, fixed with 2% paraformaldehyde for 30 min, and washed twice with PBS. After having been dried, the coverslips were mounted on slide glasses with one drop of 2% n-propyl gallate. Drops of nail polish were applied to the edges of the coverslips for shielding purposes. Localization of NBD-PE-labelled lipoplexes was then examined by confocal microscopy (λ_{ex}=465 and λ_{em}=530 nm). For the study on the effect of serum, different serum concentrations were included in the incubation medium as indicated in the figure legends.

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