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Gene transfer mediated by fusion protein hemagglutinin reconstituted in cationic lipid vesicles

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Hemagglutinin, the membrane fusion protein of influenza virus, is known to mediate a low-pH-dependent fusion reaction between the viral envelope and the limiting membrane of the endosomal cell compartment following cellular uptake of the virus particles by receptor-mediated endocytosis. Here we exploited this activity of hemagglutinin to achieve efficient gene delivery to cultured cells. Hemagglutinin was reconstituted in the presence of the monocationic lipid dioleoyldimethylammonium chloride (DODAC) to permit plasmid binding to the virosome surface. Virosomes with 30 mol% DODAC exhibited a distinct binding capacity for plasmid without causing aggregation. The virosome fusion activity was not affected by the cationic lipid DODAC as demonstrated by low-pH-dependent lipid mixing with erythrocyte ghosts. Efficient cell transfection of BHK-21 cells was observed with virosomes containing 30 mol% DODAC and plasmid encoding for β-galactosidase (pCMV β-gal) associated to their surface. The transfection activity observed was dependent on the functional activity of hemagglutinin. Contrary to DNA/cationic lipid complexes the transfection was not dependent on the cationic lipid to DNA charge ratio. Importantly, transfection of BHK-21 cells with pCMV β-gal by DODAC-containing virosomes did not show any significant signs of cytotoxicity that is commonly observed with DNA/cationic lipid complexes. Together with the high levels of expression of the transgene this highlights the potential of DODAC-containing virosomes as a novel approach in nonviral gene transfer.

Keywords: *non-viral gene delivery; virosomes; hemagglutinin; intracellular delivery; carrier system; gene therapy*

Introduction

Human gene therapy requires vectors which are efficient, safe, simple to prepare and flexible. Although for gene transfer purposes recombinant viruses are attracting a great deal of attention, the current viral vectors do not meet all of these requirements. Therefore, the promising potential of nonviral techniques for gene transfer has been recognized.[1–4]

To date, several nonviral gene delivery systems are known. Since the introduction in 1987 of a lipid-based system with *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride, DOTMA,[5] cationic lipids have readily evolved into a widely used reagent for mediating *in vitro* transfection of cultured cells. At present a wide variety of cationic lipids are known, which are generally formulated as small unilamellar liposomes in a mixture with dioleoylphosphatidylethanolamine (DOPE). The positively charged headgroup of the cationic lipid facilitates binding of the negatively charged DNA to the liposomes resulting in formation of DNA/lipid complexes where the DNA is condensed. These complexes interact with cell membranes, possibly followed by endocytotic uptake and at some point part of the DNA escapes into the cytoplasm. Membrane destabilization has been suggested to play a role in the latter process.[6–8]

In another nonviral gene delivery system the DNA is condensed with poly-l-lysine conjugates containing both a ligand for cell attachment initiating receptor-mediated endocytosis of these particles and an endosomolytic agent facilitating the escape of the DNA from the endosomal cell compartment to the cell cytosol.[9,4,10] For the latter the membrane-destabilizing properties of the fusion peptide from hemagglutinin was employed.[11–13]

Hemagglutinin (HA), the membrane fusion protein of influenza virus mediating viral cell entry, is composed of two membrane subunits, HA1 and HA2.[14–16] The virus binds to cells through interaction of the HA1 subunits with sialylated lipids (gangliosides) or proteins in the membrane of target cells. This triggers uptake of intact virus particles by receptor-mediated endocytosis into the endosomal cell compartment. During maturation of the endosomes acidification occurs inducing a conformational change in the HA spike protein resulting in exposure of the HA2 N-terminal fusion peptides. By virtue of their hydrophobic nature, the exposed fusion peptides insert into the endosomal target membrane leading to fusion of the viral membrane with the endosomal membrane and release of the nucleocapsid into the cell cytosol. In cultured cell systems fusion of the viral membrane with the plasma membrane can also be induced by a temporary reduction of the pH in the external medium.

Previously, a procedure for the reconstitution of HA in

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so-called virosomes has been developed.[17,18] These virosomes show pH-induced fusion with target membranes. The application of influenza virosomes as a fusigenic carrier system for the delivery of membrane impermeable water-soluble compounds into target cells has been evaluated. The A chain of diphtheria toxin and gelonin, membrane-impermeable polypeptides encapsulated into virosomes were efficiently delivered to the cytosol of target cells.[19,20]

In the present study, we evaluated the potential of virosomes to act as a transmembrane carrier for plasmids. The data demonstrate that cationic virosomes can act as delivery vehicles for plasmid leading to expression of a reporter gene and that the delivery is dependent on the fusigenic activity of HA.

Results

Formation and characterization of cationic virosomes

For influenza virosomes to act as a transmembrane carrier for plasmid DNA, the plasmid needs to be either encapsulated in the interior of the virosome or attached to the virosome surface. Our approach here was to introduce a cationic lipid into the lipid bilayer of the virosomes to enable the binding of plasmid DNA to the surface.

Firstly, the effect of exogenous neutral and cationic lipids incorporated into the virosomal membrane on the pH-dependent fusion activity was evaluated. Virosomes were prepared with 30 mol% egg phosphatidylcholine (EPC) and in the presence of 10, 20, 30, 40 and 50 mol% of the monocationic lipid DODAC. For 10 and 20 mol% DODAC preparations 20 and 10 mol% EPC, respectively, was added resulting in a total of 30 mol% exogenous lipids. The mol% lipid added is in relation to the total phospholipids in the final virosome preparation. Viral lipids and the fusion protein HA dissolved in C12E8 detergent was added to the exogenous lipids previously dried to a lipid film. After complete solubilization of the lipids, the virosomes were formed by removal of the detergent with BioBeads. The lipid compositions in the different virosome preparations were analyzed by thin-layer chromatography. The data showed incorporation of DODAC and EPC in the expected ratios (data not shown). The size distribution of the different virosome preparations with and without cationic lipids were analyzed by quasi-elastic light scattering. Virosomes with EPC showed a size distribution similar to virosomes without additional lipids with a mean diameter of 170 nm. Addition of cationic lipids resulted in a broader size distribution and a larger mean diameter of 240 nm.

The pH-dependent fusion activity of the different virosome preparations was determined using a lipid mixing assay with erythrocyte ghosts as target membranes.[21] The virosome preparations were made with 10 mol% pyrene PC resulting in excimer fluorescence (fluorescence of dimers of a non-excited and excited pyrene molecule). Fusion of a virosome with a target membrane, eg erythrocyte ghosts, will result in a dilution of the fluorophore with loss of excimer fluorescence. Virosomes were mixed with erythrocyte ghosts at pH 7.4, 37°C. Following a binding period of 2 min the medium was acidified to pH 5.6 and the change in excimer fluorescence recorded. The fluorescence changes were then converted into percentage of fusion as outlined in Materials and methods.

Virosomes without any exogenously added lipids fused rapidly and extensively with erythrocyte ghosts (Figure 1a). The fluorescence changes recorded for virosomes with 10 to 30 mol% DODAC indicate slightly slower fusion kinetics than for DODAC-free virosomes, yet the final extent of fusion was apparently the same. The fusion activity for virosomes containing 30 mol% DODAC is shown in Figure 1a as a representative example. The fusion kinetics of virosomes with 40 or 50 mol% DODAC were significantly slower than observed for virosomes containing less DODAC, with fusion rates of approximately 4%/s and 3%/s for virosomes with 40 and 50 mol% DODAC, respectively, and 6.0%/s for virosomes lacking DODAC. None of the virosome preparations showed any change in pyrene excimer fluorescence during the initial 2-min incubation period with the erythrocyte ghosts at pH 7.4 and 37°C. These results indicate that fusion activity of virosomes containing 10, 20 or 30 mol% DODAC is similar to the one of virosomes without exogenous neutral or cationic lipids.

Exposure of virosomes to low pH in the absence of target membranes is known to irreversibly inhibit the fusion activity of HA.[17] The different virosome preparations with and without DODAC were incubated for 30 min at pH 4.5, 37°C. Following low pH incubation the virosome suspensions were neutralized and tested for fusion activity. None of the acid-treated virosomes, irrespective of the amount of DODAC present, showed fusion activity with erythrocyte ghosts. Figure 1b shows the fusion activity for virosomes with 30 mol% DODAC before and after acid pH incubation.

Cellular uptake of virosomes and fusion with the endosomal membrane was evaluated by fluorescence microscopy. Virosomes labeled with rhodamine-PE were taken up readily by the cells via endocytosis resulting in highly punctuated intracellular fluorescence in the BHK cells within 15–20 min. Over the following 30 to 60 min the fluorescence becomes diffuse over the cells indicative of fusion with cellular membranes (data not shown). This apparent fusion was inhibited when NH_4Cl (20 mm) was present in the cell medium. NH_4Cl is known to inhibit endosomal acidification. Under these conditions the fluorescence remained punctuated over the 60 min incubation period. The fusion activity of virosomes with BHK cells was also followed by fluorescence spectroscopy using pyrene-PC labeled virosomes (Figure 1c). The excimer fluorescence started to decrease after an initial lag phase of 10 to 15 min reaching a value corresponding to approximately 55% fusion within 1 h after addition of virosomes to the cells. The lag phase is attributed to the time required for binding followed by endocytosis and processing of the endosomes to sufficiently low pH to initiate fusion. The decrease in excimer fluorescence was completely inhibited when NH_4Cl (20 mm) was present in the medium. Fluorescence changes were converted into percentage fusion as outlined in Materials and methods.

DNA binding to cationic virosomes

For this binding study virosomes containing 30 mol% DODAC were chosen since preliminary transfection experiments indicated a high activity for this preparation. Virosomes with 30 mol% DODAC (20 nmol phospholipids) were incubated with 1–10 μg ^{3}H-labeled pCMV β-gal (8×10^3 dpm/μg) for 15 min at room tem-

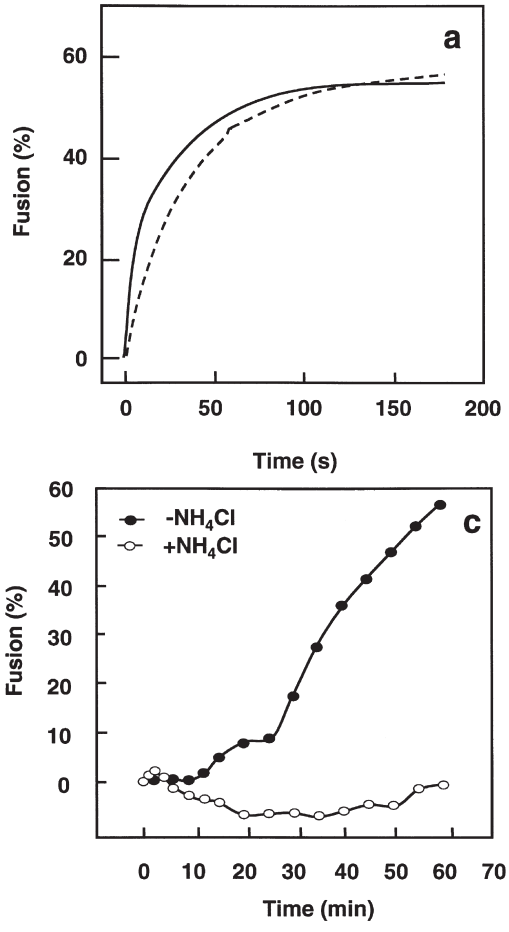

Figure 1 *Fusion of virosomes containing DODAC with erythrocyte target membranes and BHK cells. Low pH-dependent fusion of pyrPC-labeled virosomes with erythrocyte ghosts was determined at 37°C following acidification of the medium to pH 5.6. The decrease of pyrene excimer fluorescence was converted into extent of fusion as outlined in Materials and methods. (a) Fusion of virosomes lacking DODAC (solid curve) and fusion of virosomes containing 30 mol% DODAC (dashed curve); (b) virosomes inactivated by pre-exposure to an acidic medium. Solid curve, fusion of non-inactivated virosomes with 30 mol% DODAC; dashed curve, change of pyrene excimer fluorescence obtained with virosomes inactivated by a 30-min pre-incubation at pH 4.5 and 37°C in the absence of target membranes. (c) Fusion of virosomes with BHK cells. Virosomes (0.2 μm final) were incubated with 10^6 BHK cells in HBSS-BSA buffer in absence (●) and presence (○) of 50 mm NH_4Cl at 37°C. In the latter case the cells were pre-incubated with 50 mm NH_4Cl for 15 min before addition of virosomes. Fluorescence value for 100% fusion was obtained following solubilization of cells with detergent (octylglucoside, 50 mm final) and percentage fusion was calculated as outlined in Materials and methods.*

perature. Following incubation the virosome/DNA complexes were pelleted by centrifugation and the plasmid content in the pellet and in the supernatant was determined based on the radioactivity (Figure 2). When up to about 6.0 μg of DNA was added to the virosomes with 30 mol% DODAC virtually all DNA was recovered in the pellet together with the virosomes, without any significant amount of free DNA remaining in the supernatant. However, when DNA was added in excess of 6 μg there was no further increase of radioactivity (DNA) in the pellet but increasing amounts of free plasmid appeared in the supernatant.

Transfection of BHK-21 cells by cationic virosomes

The potential of cationic virosomes for intracellular delivery of plasmid DNA was tested with BHK-21 cells and pCMV β-gal as the reporter gene. Virosomes containing 0, 10, 20, 30, 40 or 50 mol% DODAC (30 nmol phospholipid) in HBS were mixed with 9 μg DNA. The volume was adjusted to 50 μl with HBS. After incubation for 30 min at room temperature aliquots of the mixture were added to BHK-21 cells in DMEM containing 5% FBS, previously seeded in 48-well plates (see Materials and methods). After 48 h incubation, the cells were analyzed for β-galactosidase activity using a staining procedure for whole cells and by a quantitative assay for cell lysate as outlined in Materials and methods. As an indicator for cell viability the level of cellular protein in the cell lysates was determined. The β-galactosidase

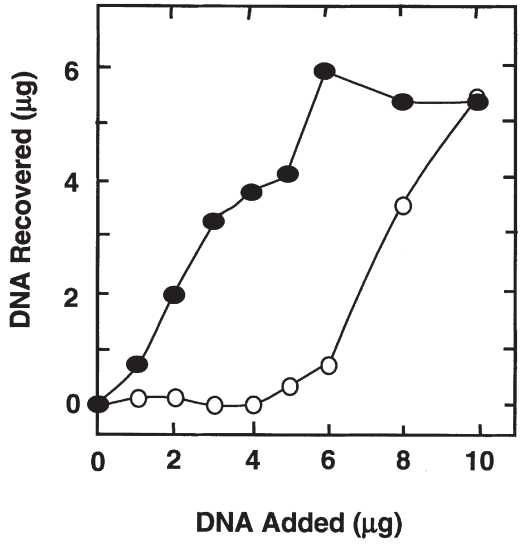

Figure 2 *DNA binding by DODAC-containing virosomes. Virosomes (20 nmol phospholipid) containing 30 mol% DODAC were mixed with different amounts of pCMV β-gal in 50 μl HBS. After a 15-min incubation period the mixtures were subjected to ultracentrifugation and the amounts of DNA recovered in pellet (●) and supernatant (○) were determined as outlined in Materials and methods. The data represent the mean of duplicate measurements.*

activity measured in cell lysates (units per mg protein) is plotted as a function of the DODAC content in the different virosome preparations (Figure 3). In cells treated with virosomes not containing DODAC and with virosomes containing 10 and 20 mol% DODAC only very low β-galactosidase activity was observed. However, in cells treated with virosomes with 30 mol% DODAC high β-galactosidase activity of approximately 15 U/mg protein was detected. A higher concentration of DODAC in the virosomes (40 and 50 mol%) resulted in a markedly lower gene expression than for 30 mol% DODAC. When plasmid was added without any virosomes, no detectable β-galactosidase activity was observed. The protein contents in the various cell lysates did not differ significantly following treatment with the different virosome preparations and was similar to the protein levels in lysates of cells treated with naked DNA only or not treated at all (Figure 3). This indicates that there was little or no cytotoxicity associated with transfection by the different virosome preparations.

The β-galactosidase activity of 10–15 U/mg protein detected in cells treated with virosomes was typically about twice the activity observed in cells treated with cationic lipid/plasmid complexes (4–6 U/mg protein). The lipid/plasmid complexes were formed by mixing DODAC-DOPE (1:1; mol:mol) LUVs with pCMV β-gal to give a charge ratio of 1.3 (+/−). The protein content in the cell lysate of cells treated with cationic lipid/plasmid complexes was approximately 50% lower than for non-treated cells. The reduced protein levels indicate toxic effects of the cationic lipid/DNA complexes on these cells. Note, highest β-galactosidase activity was detected in cells 24 h after treatment with cationic lipid/plasmid complexes and 48 h after transfection with virosomes.

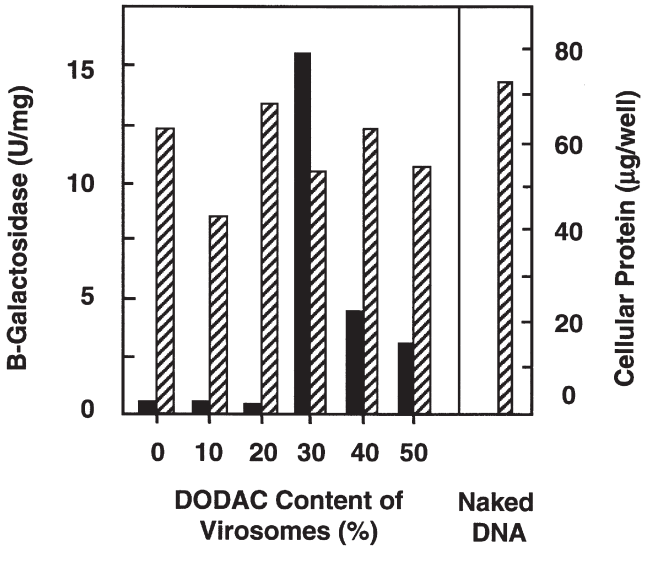

Figure 3 *Transfection of BHK-21 cells as a function of DODAC concentration in the virosomes. Plasmid DNA (6 μg pCMV β-gal) was mixed with virosomes (20 nmol total phospholipid) containing different amounts of DODAC in 50 μl HBS. BHK-21 cells were incubated with 2 μg per well of pCMV β-gal attached to virosomes with 0, 10, 20, 30, 40 and 50 mol% DODAC (mol% relative to total phospholipid) and naked plasmid. After 48 h incubation at 37°C the cells were lysed and the supernatants collected. Aliquots were assayed for β-galactosidase activity and the amount of protein as outlined in Materials and methods. Solid bars, β-galactosidase activity in U/mg protein; hatched bars, amounts of protein in cell lysate in μg per well.*

For the transfection with cationic lipid/DNA complexes the charge ratio is crucial for good transfection activity. However, in the case of cationic virosomes the charge ratio appeared to be less important. The transfection profile observed with 5 and 10 μg of DNA, respectively, added to 30 nmol of virosomes of different DODAC concentrations were similar. Again, the best transfection activity was obtained with virosomes containing 30 mol% DODAC as shown above for 9 μg (data not shown).

To visualize the transfection efficiency in terms of cell numbers, the cells were stained for β-galactosidase activity and photographed. The photographic image shown in Figure 4a is typical for cells treated with virosomes containing 30 mol% DODAC and pCMV β-gal, while Figure 4b shows cells treated with the same amount of free plasmid only. Incubation with virosomes results in a high number of blue cells (80–90%) while no appreciable positive staining is obtained with free plasmid only.

Dose-dependent transfection activity

The effect of DNA dose applied for transfection on β-galactosidase expression in BHK-21 cells was investigated. Virosomes containing 30 mol% DODAC (20 nmol phospholipid) were mixed with 6 μg of pCMV β-gal plasmid. After incubation, aliquots of the mixture with 0.5–5 μg DNA were added to the cells in 48-well plates. The β-galactosidase activity detected in the cell lysate is plotted as a function of the DNA dose added (Figure 5). The amount of β-galactosidase expression increased with increasing DNA dose from 0.5 to 2.5 μg plasmid. With higher dose than 2.5 μg the β-galactosidase expression leveled off at an activity corresponding to approximately 10 U β-galactosidase per mg protein.

Role of the hemagglutinin in transfection with cationic virosomes

Although these cationic virosomes fuse with erythrocyte ghosts and BHK cells in a low pH-dependent manner characteristic of HA-mediated fusion (Figure 1), more direct evidence is required to demonstrate that DNA delivery is indeed mediated by this mechanism. It was shown previously[19–21] that the intracellular fusion of virosomes with the endosomal membrane can be inhibited by reagents which interfere with endosomal acidification, eg ammonium chloride.[22] Furthermore, the fusion activity of HA can also be eliminated by a pre-incubation of virosomes at acidic pH in the absence of a target membrane.[23] Thus, the transfection activity of cationic virosomes was tested in the presence of NH_4Cl and with acid-inactivated virosomes.

For acid inactivation of HA, the virosomes were incubated for 30 min at pH 4.5 at room temperature. Subsequently, the suspension was neutralized and the virosomes (20 nmol phospholipid) were incubated with pCMV β-gal plasmid (6 μg DNA) and applied to cells. In a separate experiment endosomal acidification was prevented by adding NH_4Cl (20 mm) to the cell medium before the addition of virosomes and this concentration of NH_4Cl was maintained throughout the following 48 h incubation period. No significant amount of β-galactosidase was produced in cells treated with either acid inactivated virosomes or with virosomes in the presence of ammonium chloride (Figure 4c, d and Figure 6). The pro-

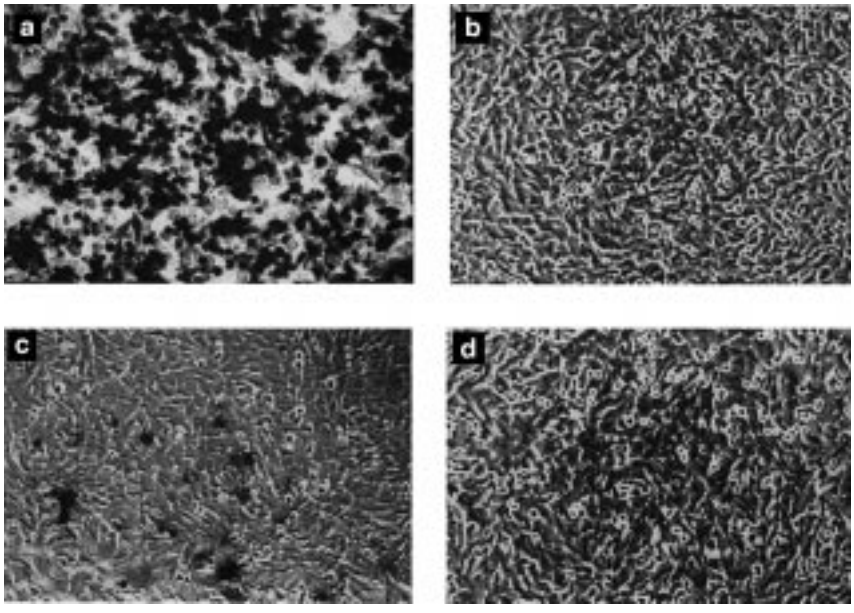

Figure 4 *Cytochemical staining of BHK-21 cells. After 48-h incubation of BHK-21 cells with 2 μg of pCMV β-gal attached to virosomes with 30 mol% DODAC (6 μg plasmid mixed with 20 nmol virosomes in 50 μl HBS), the cells were fixed and stained for β-galactosidase activity with X-gal, as outlined in Materials and methods. (a) Cells treated with plasmid attached to virosomes; (b) naked DNA; (c) cells treated with plasmid attached to virosomes in the presence of NH_4Cl; (d) plasmid attached to virosomes previously inactivated by a 30-min incubation at pH 4.5.*

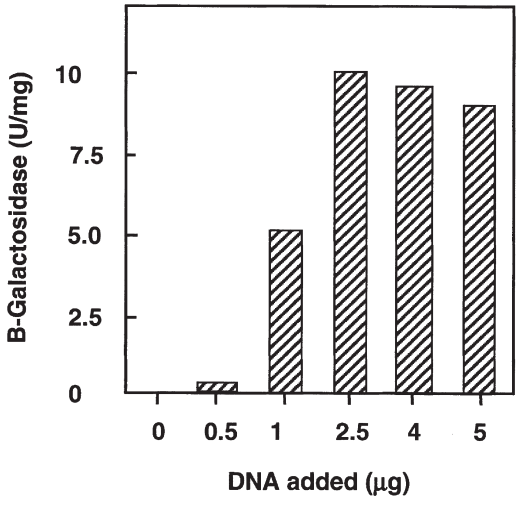

Figure 5 *Dose-dependent transfection of BHK-21 cells with plasmid attached to virosomes. pCMV β-gal was attached to virosomes containing 30 mol% DODAC as in Figure 4 (6.0 μg pCMV β-gal mixed with 20 nmol virosomes in 50 μl HBS). 0.5, 1, 2.5, 5 and 10 μg plasmid attached to virosomes were added to BHK-21 cells, four wells for each concentration. After 48-h incubation, the cells were lysed and the supernatants were analyzed for β-galactosidase activity and protein as outlined in Materials and methods.*

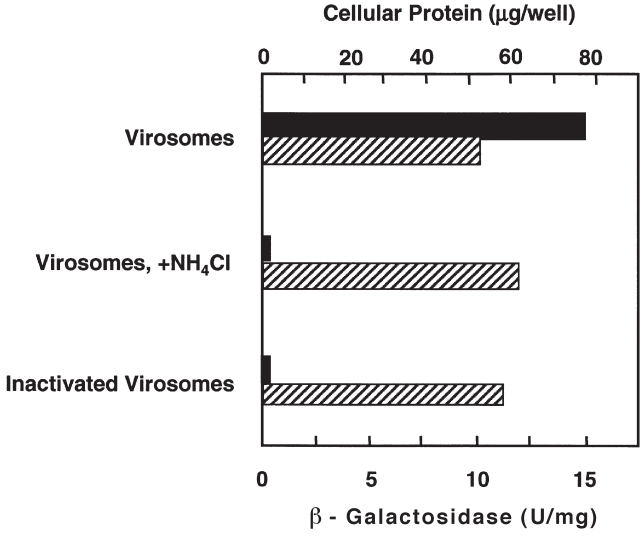

Figure 6 *HA-dependent transfection of BHK cells with DODAC-containing virosomes. Virosomes containing 30 mol% DODAC were mixed with pCMV β-gal as in Figure 5 and added to BHK-21 cells previously seeded in 48-well dishes (2 μg plasmid per well). After 48-h incubation, the cells were lysed and the supernatants were assayed for β-galactosidase activity and protein as outlined in Materials and methods. Solid bars, β-galactosidase activity (U/mg protein); hatched bars, amounts of protein in cell lysate (μg per well).*

tein levels measured in the different cell lysates were similar.

It is also possible to trigger HA-mediated fusion of virosomes with the cell plasma membrane.[20] Here, virosomes are first allowed to bind to the cell membrane by incubation at 4°C. The low temperature prevents the endocytosis of the virosomes. A brief acidification of the medium results in fusion of the bound virosomes with the plasma membrane. Virosomes with 30 mol% DODAC and associated plasmid were added to BHK-21 cells and incubated at 4°C for 60 min. Thereafter, the temperature was raised to 37°C and the medium acidified to pH 5.4. After 10 min the medium was exchanged for fresh medium (pH 7.4) containing 20 mm NH_4Cl and the cells were incubated for a further 48 h at 37°C. β-galactosidase

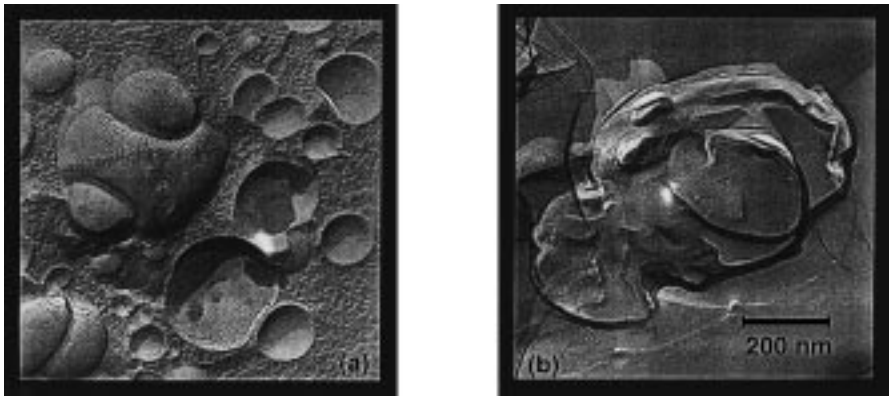

Figure 7 *Freeze-fracture electron micrograph of (a) virosomes with plasmid DNA attached to the surface; and (b) plasmid complexed to cationic LUVs. The plasmid/lipid complexes were prepared by mixing DODAC:DOPE (1:1 mol:mol) LUVs with plasmid DNA at a charge ratio of 3:1 (positive charge of DODAC to negative charge on plasmid).*

expression was also detected in cells treated with virosome/DNA in this manner, however, expression was approximately three- to four-fold lower compared with virosomes entered via endocytosis (data not shown).

Morphology of cationic virosomes

Freeze fracture electron micrographs of virosomes containing 30 mol% DODAC and complexed with plasmid show single vesicles with a diameter of 160 ± 60 nm without any aggregation (Figure 7a). In contrast, extensive aggregation is observed when DODAC/DOPE LUVs (1:1 molar ratio) were added to plasmid DNA to form the typical DNA/lipid complexes with a positive to negative charge ratio of 2 (Figure 7b).

Plasmid condensation by cationic virosomes

Transfection of cells with cationic liposomes as plasmid carriers is dependent on the degree of DNA condensation.[24] The extent of condensation can be monitored by determining intercalation of the fluorescent dye ethidiumbromide (EtBr).[24,25] The condensation of pCMV β-gal plasmid with virosomes containing 30 mol% DODAC was investigated. A fixed amount of DNA was incubated with increasing amounts of virosomes and subsequently the fluorescence measured following addition of EtBr. As comparison, the condensation of the plasmid with cationic liposomes (DODAC/DOPE in 1:1 molar ratio) was also determined. In Figure 8 the fluorescence of EtBr is plotted as a function of the lipid/DNA charge ratio. Maximal condensation of the plasmid cationic lipid vesicles required a slight charge excess of the cationic lipid as observed previously.[24] For a complete condensation of the plasmid with virosomes a three-fold excess of positive charge was necessary. Note, quantitative DNA binding and transfection of cells by DODAC-containing virosomes is already obtained with a positive-to-negative charge ratio of about 0.15 to 0.20 (see Discussion) and a higher ratio inhibits transfection activity rather than stimulates it. This indicates that transfection of cells with cationic virosomes requires the binding, but not necessarily the (complete) condensation of DNA.

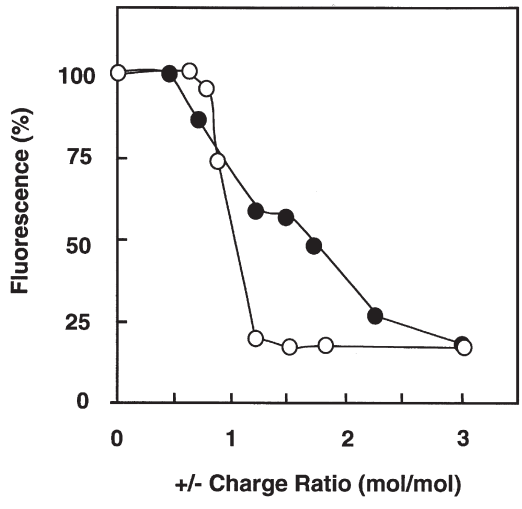

Figure 8 *Condensation of pCMV β-gal. Fixed amounts of DNA (6.25 nmol) were mixed with increasing amounts of virosomes containing 30 mol% DODAC or extruded liposomes, composed of DODAC and DOPE (molar ratio 1:1), in 500 μl HBS. After a 15-min incubation period at room temperature 40 μl 3 μm EtBr was added and the fluorescence was monitored as outlined in Materials and methods. Fluorescence of ethidium bromide intercalating in non-condensed DNA in DODAC-containing virosomes (●) and DODAC/DOPE liposomes (○).*

Discussion

A lipid-based nonviral gene delivery system was first introduced in 1987 consisting of a cationic lipid (DOTMA) and the fusigenic lipid DOPE in a 1:1 molar ratio. Pre-formed vesicles were mixed with a reporter gene plasmid to form DNA/lipid complexes.[5] The transfection activity observed *in vitro* was shown to be charge ratio-dependent and the highest activity was obtained with a slight charge excess of DOTMA relative to the negative charges of the DNA phosphate backbone. At the charge ratio typically employed condensed DNA structures are formed with the cationic liposomes as characterized with DNA-specific dyes and the loss of electrophoretic mobility.[24] The presence of DOPE greatly enhances the transfection efficiency and is thought to be the consequence of the non-bilayer forming properties of this lipid.[26]

Several drawbacks of this transfection system have to be noted. (1) The DNA/lipid complexes formed are not stable in the presence of serum,[27] limiting the application of this system *in vivo*. (2) The DNA/lipid complexes are very heterogeneous in size and have the tendency to

increase in size and precipitate during storage,[28] resulting in loss of activity. This represents a major problem for the pharmacologic formulation of this vector. (3) Cytotoxicity is often associated with lipofection. (4) Marked differences in the transfection efficiency of different cells lines have been observed *in vitro* and primary cells are poorly transfected, if at all. Since the introduction of DOTMA, numerous other cationic lipids and/or formulation methods have been developed. With all these cationic lipid/DNA complex systems, the transfection activity is affected by the cationic lipid to DNA charge ratio and the optimal charge ratio varies for different cationic lipids. However, these type of carriers still show relatively low transfection activity compared with viral vectors.

In the present study, the potential of the influenza virus fusion protein HA to promote uptake and intracellular delivery of plasmid DNA was investigated. Previous studies have shown that the membrane fusion mediated by reconstituted virosomes closely resembles that of the intact virus.[17,19,20] Cationic lipids were incorporated into virosomes to attach plasmid DNA to the lipid bilayer surface by electrostatic interaction. Exogenous lipids (up to 30 mol% EPC and/or DODAC) were successfully incorporated into the virosomal membrane without affecting the acidic pH-induced fusion properties with target membranes (Figure 1). DNA complexed to the surface does not affect the fusion activity significantly. The fusigenic property of the virosomes containing cationic lipids is solely HA-mediated exhibiting a similar pH dependence as compared with control virosomes. Furthermore, temporary exposure to acidic pH, known to inactivate HA, rendered the virosomes fusion inactive (Figure 1b, c).

Cationic virosomes (30 mol% DODAC) exhibit distinct DNA binding characteristics with a defined binding capacity of approximately 6 μg DNA per 20 nmol phospholipid (Figure 2). At saturation this corresponds to a DNA-to-DODAC ratio of approximately 1 μg/nmol. Since DODAC is a monocationic lipid and each DNA nucleotide ($M_r = 325$) carries one negatively charged phosphate group, this corresponds to a positive-to-negative charge ratio of approximately 0.4. This charge ratio relates to the total amount of DODAC present. However, the DODAC is expected to be distributed equally between the inner and outer membrane leaflets, therefore, the actual positive-to-negative charge ratio of these virosome/DNA complexes is more likely around 0.2.

The transfection activity of virosomes is clearly dependent on the relative DODAC density in the membrane. The highest activity is obtained with virosomes containing 30 mol% DODAC (Figure 3). The low transfection activity of virosomes with 10 or 20 mol% DODAC is presumably due to insufficient binding of DNA to the virosomes and the transfection observed is the result of random co-uptake of plasmid and virosomes with the medium during the endocytosis. This interpretation is supported by the observation that a mixture of pCMV β-gal with a three times higher amount of virosomes containing 10 mol% DODAC, did not result in notable transfection, while the absolute amount of DODAC present was the same as in the virosomes containing 30 mol% DODAC.

The diminished transfection activity at concentrations higher than 30 mol% DODAC can be rationalized in part by the reduced fusion activity observed with these virosomes. However, more relevant might be the aggregate formation when plasmid is added to virosomes with >30 mol% DODAC. The formation of larger aggregates will most likely prevent endosomal uptake and thereby reduce transfection activity. There is little if any aggregation observed with virosomes containing 30 mol% DODAC and distinct vesicles are observed by freeze fracture electron microscopy.

Virosomes containing 30 mol% DODAC were always most efficient in transfection independent of the DODAC-to-DNA charge ratio and the absolute amount of DODAC present. As outlined above, the positive-to-negative charge ratio for virosomes with 30 mol% DODAC and saturated with DNA is approximately 0.2. This is in marked contrast to the charge ratio required for optimal transfection *in vitro* by lipofection, which is characteristically greater than 1. A charge ratio greater than one for lipofection is necessary to compact fully the plasmid DNA and this is believed to be a prerequisite for cell entry and optimal transfection.[24] The charge ratio of 0.2 with virosomes implicates that in this case the plasmid DNA does not need to be compacted for transfection. Indeed, the degree of DNA condensation as investigated using the DNA binding dye ethidium bromide indicates that cationic virosomes do not compact the DNA at the charge ratio of 0.2 (Figure 8). Complete shielding or condensation of DNA by cationic virosomes requires a positive-to-negative charge ratio of approximately 3:1. Thus, it appears that only DNA binding and not DNA condensation is important for cationic virosome-mediated transfection. A similar finding was reported previously for transfection mediated by ternary complexes consisting of DNA, anionic liposomes and (cationic) histone H1.[29] In this system efficient transfection occurred under conditions where the plasmid DNA was sensitive to DNase I degradation and EtBr intercalation.

It is interesting to note that, in contrast to lipofection, serum did not interfere with cellular uptake of virosomes and concomitant transfection mediated by DODAC-containing virosomes and could be present throughout the experiment. Furthermore, transfection with virosomes is apparently not accompanied by cytotoxic effects, since the protein levels in cells treated with virosomes was similar to non-treated cells. In contrast, cells transfected with cationic lipid/DNA complexes typically show reduced protein levels. It might be possible that optimal charge density, stability in serum and cytotoxicity are interrelated. Certainly, a major advantage of cationic virosomes over DNA/lipid complexes is the much lower requirement in cationic lipid for similar or even higher efficiency of transgene expression. This in an important point, since dissociation of the cationic lipids/DNA complexes has to occur after cell entry in order for DNA to translocate into the nucleus and for subsequent transcription to occur.[25,30] Indeed, it was shown that after microinjection of DNA/lipid complexes into the cytoplasm only a tiny fraction of DNA is expressed. It is tempting to speculate that the less tightly bound DNA in the virosome complexes leads to more efficient DNA translocation and subsequent nuclear transcription.

Plasmid delivery with cationic virosomes and concomitant expression of reporter gene is dependent on the activity of the fusion protein HA based on the following observations. (1) Transfection is inhibited when the

fusion protein is inactivated by a temporary exposure of the virosomes to an acidic medium (Figures 4d and 6) before complex formation with plasmid. The transient exposure of virosomes to an acidic medium inhibits the pH-dependent fusion with erythrocyte ghosts and cells (Figure 1b). (2) Fusion of virosomes with cells is inhibited when endosomal acidification is prevented with NH_4Cl in the cell medium (Figure 1c). Similarly, the transfection activity is completely abolished with NH_4Cl (Figures 4c and 6). (3) Transfection can also be obtained when fusion of the virosomes with the plasma membrane is triggered by a brief acidification of the medium following the binding of virosomes to the cell membrane. This transfection activity is observed even in the presence of NH_4Cl. Therefore, the inhibition of transfection observed in (2) cannot be the result of inhibited gene expression by ammonium chloride.

The endosomal membrane has been recognized as one of the major barriers for gene delivery and efficient transfection. Therefore, nonviral vector systems with features that facilitate the escape of plasmid from the endosomal compartment to the cytosol are gaining importance. Of particular interest are carriers that promote membrane disruption or fusion at the acidic pH characteristic of endosomes. The mechanism of plasmid entry into the cytoplasm by the virosome system described here is not clear since the plasmid is not encapsulated in the aqueous interior of virosomes which is released into the cytoplasm upon fusion. However, HA-mediated fusion has been shown to be quite a leaky process causing the release of at least 10 000 MW dextran molecules from target liposomes.[31] This feature might result in plasmid to escape from the endosomes into the cytoplasm during fusion. Alternatively, cytoplasmic entry could also occur as a result of destabilization of the endosomal membrane following penetration of HA fusion peptides. The latter has previously been shown to facilitate DNA delivery by poly-l-lysine based molecular conjugates.[11–13]

A fusigenic liposome with a viral fusion protein for gene delivery was recently constructed by fusion of UV-inactivated Sendai virus with DNA/cationic lipid vesicles.[32] However, the application of an inactivated virus raises safety concerns regarding application for gene therapy. The advantage of the system presented here is its truly artificial construction.

The carrier system presented in this paper uses the strategy of enveloped viruses for cell entry and intracellular delivery of plasmid. The fusion protein HA reconstituted in virosomes with cationic lipids is shown to be a key component in this carrier system for intracellular delivery of a plasmid. It might be possible to improve the efficiency of the virosomes as plasmid carriers further by entrapping the plasmid inside. Further studies are required to investigate the potential of virosomes as carriers for gene therapy.

Materials and methods

Cationic lipid (DODAC)

The monocationic lipid dioleoyldimethylammonium chloride (DODAC) was provided by Steven Ansell of Inex Pharmaceuticals (Vancouver, BC, Canada).

Preparation of plasmid DNA

Throughout this study a 7164 bp plasmid containing the *E. coli lac*Z reporter gene encoding for β-galactosidase, driven by the cytomegalovirus early promoter and enhancer was used (pCMV β-gal; Clontech, Palo Alto, CA, USA). The plasmid was expanded in *E. coli* DH5α following standard techniques. Cells were lysed by alkaline lysis[33] and the plasmid was subsequently purified by anion exchange chromatography according to the manufacturer (Qiagen, Chatsworth, CA, USA). Radiolabeled plasmid was generated by growing *E. coli* DH5α containing pCMV β-gal in the presence of ^{3}H-thymidine-5′-triphosphate (DuPont NEN, Boston, MA, USA). The labeled plasmid was purified as described above. The activity was approximately 1.5×10^5 dpm/μg.

Preparation of virosomes

Influenza virus A, strain MRC-11 (H3N2 subtype) was grown and purified as described elsewhere.[21] Virosomes were prepared from the virus by a procedure described previously.[17,18] Virus (the equivalent of about 1.5 μmol membrane phospholipid) was sedimented at 4°C by centrifugation. The pelleted virus was resuspended and solubilized by the addition of 0.35 ml of 0.20 m octaethyleneglycol monododecyl ether (C12E8; Fluka Chemie, Buchs, Switzerland) in Hepes-buffered saline (HBS: 5.0 mm Hepes, 0.15 m NaCl, pH 7.4). The suspension was flushed through a 25-gauge needle several times to ensure complete dispersion of the virus. After a 30-min incubation the viral nucleocapsid was removed by centrifugation at 4°C.

For the preparation of cationic virosomes, DODAC was dissolved in chloroform and the required amount dried under a stream of nitrogen gas followed by an additional 2 h under vacuum. The dry lipid film was rehydrated and dissolved in 70 μl of 0.20 m C12E8 in HBS. After 30 min the supernatant containing solubilized viral lipids and protein in C12E8 was added and after an additional 30-min incubation diluted with HBS to give a final concentration of 0.10 m C12E8. The clear suspension was transferred to a vial containing 0.25 g of wet BioBeads SM2 (BioRad, Hercules, CA, USA) and gently shaken at room temperature to remove the C12E8. After 30-min incubation, another 0.25 g of wet BioBeads SM2 was added, followed by a subsequent 2 h incubation at room temperature with gentle shaking. For membrane fusion measurements virosomes were labeled with 1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphocholine (pyrPC; Molecular Probes, Eugene, OR, USA) by adding the viral C12E8-containing supernatant to 0.15 μmol pyrPC either dried on its own or dried together with an appropriate amount of DODAC. DODAC was added as mol fractions relative to the amount of viral phospholipid used (1.5 μmol).

After the removal of C12E8, virosomes were purified on a discontinuous sucrose density gradient, composed of 0.40 ml 40% (w/v) sucrose in HBS and 1.0 ml 10% (w/v) sucrose in HBS by centrifugation for 90 min at 150 000 *g* and 4°C. The virosomes were collected from the interface of the sucrose layers. The phospholipid contents of the virosome preparations were determined by phosphate analysis[34] after degradation of the lipids with perchloric acid.[35]

Determination of fusion activity

The fusion activity of virosomes was evaluated by monitoring low pH-dependent dilution of pyrPC into erythrocyte ghost target membranes.[21] Right-side-out erythrocyte ghosts were prepared from hypotonically lysed red blood cells.[36] Erythrocyte ghost phospholipid contents were determined by phosphate analysis[34] after extraction[37] and digestion of the lipids.[35] Ghosts and virosomes were added to a quartz microcuvette containing HBS. After a 2-min incubation period, the medium was acidified to pH 5.6 by the injection of 30 μl of 0.10 m acetic acid and 0.10 m 2-[N-morpholino]ethanesulfonic acid (pH 4.2). The final volume was 0.90 ml and the final ghost and virosome concentrations were 50 μm and 1.0 μm phospholipid, respectively.

Fusion was continuously monitored at 37°C by the decrease of pyrene excimer fluorescence[21] at excitation and emission wavelengths of 345 nm (bandpass 0.5 nm) and 490 nm (bandpass 20 nm), respectively. A 475 nm cut-off filter was placed in the emission beam. The measurements were carried out with a SPF-500C spectrofluorometer (SLM Instruments, Urbana, IL, USA) equipped with a thermostatted cuvette holder and a magnetic stirring device. Extents of fusion were obtained by calculating $100 \times (E_0 - E_t)/(E_0 - E_\infty)$, where E_0 and E_t represents the excimer fluorescence at time zero and at time t during fusion, respectively. E_∞, which represents background intensity at 490 nm at infinite dilution of pyrPC, was obtained by adding 20 μl of 0.20 m C12E8 after fusion had proceeded for 3–4 min.

Fusion of virosomes with BHK cells: Cells grown in normal growth medium (DMEM-FBS) to 80–100% confluent (10^5 cells/cm^2) were washed twice with phosphate-buffered saline (PBS; 0.154 m NaCl in 0.88 mm KH_2PO_4 and 3.37 mm Na_2HPO_4, pH 7.4) and detached from the plates with 0.25 mm trypsin. Following detachment an equal volume of DMEM-FBS was added and the cells washed by centrifugation (5 min at 150 *g*; Beckman GS-6 centrifuge; Beckman, Palo Alto, CA, USA). The cells were resuspended in DMEM-FBS and incubated for 1 h at 37°C with agitation to prevent reattachment. The cells (10^6) were centrifuged (5 min at 150 *g*) and resuspended in 2 ml HBSS-BSA (Hanks buffer saline with 10 mm HEPES, pH 7.4 and 1% bovine serum albumin). Virosomes were added to a final concentration of 0.2–0.5 μm and pyrene fluorescence monitored for up to 1 h at 37°C as indicated above. Fluorescence for infinite dilution was determined after addition of 100 μl 1 m octylglucoside. For the fusion assay in the presence of NH_4Cl, the cells were resuspended in HBSS-BSA containing 50 mm NH_4Cl and incubated for 15 min before the addition of virosomes.

DNA binding to virosomes containing cationic lipid (DODAC)

Virosomes containing 30 mol% DODAC (20 nmol phospholipid) were incubated in 100 μl HBS with 1–10 μg radiolabeled plasmid DNA (pCMV β-gal spiked with ^{3}H-pCMV β-gal to give a specific activity of 8×10^3 dpm/μg plasmid) for 15 min at room temperature. Following incubation the virosome/DNA complexes were pelleted by centrifugation (5 min in a tabletop TL100 ultracentrifuge, Beckman, at 100 000 *g*), the supernatant was removed and the pellet washed twice with 100 μl HBS. The pellet was dissolved in 40 μl 0.1 m C12E8. The amounts of DNA recovered in the pellet and in the first supernatant were determined by liquid scintillation counting.

Transfections

As model target cells for the transfection studies, we used a mammalian cell line derived from baby hamster kidney cells (BHK-21 cells), obtained from the American Type Culture Collection (Rockville, MD, USA). These cells were grown in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Grand Island, NY, USA) supplemented with 5% fetal bovine serum (FBS; Intergen, Purchase, NY, USA) at 37°C and 5% CO_2. Subconfluent BHK-21 cells, grown in 75-cm^2 tissue culture flasks (Falcon, Lincoln Park, NJ, USA), were detached by brief trypsinization with 0.25% trypsin in citrate saline (StemCell Technologies, Vancouver, BC, Canada). After counting, cells were seeded in 48-well plates (Falcon, Lincoln Park, NJ, USA). Each well contained 2.5×10^4 cells in 1 ml of DMEM/FBS. The next day the medium was replaced by 0.5 ml DMEM/FBS and aliquots (not more than 50 μl) of mixtures of (DODAC-containing) virosomes and plasmid DNA were added. The suspension was mixed well after each addition and incubated for 30 min at 37°C and 5% CO_2. Thereafter 0.5 ml DMEM/FBS was added and the cells were grown further for 48 h. Virosome-DNA mixtures were prepared by incubating pCMV β-gal with (DODAC-containing) virosomes in HBS during 30 min. In most cases these mixtures contained 6.0 μg DNA and 20 nmol virosomal phospholipid.

Assays for determination of β-galactosidase activity

Cells expressing β-galactosidase were cytochemically stained with the substrate 5-bromo-4-chloro-3-indolyl β-d-galactopyranoside (X-gal). Briefly, after washing with phosphate-buffered saline (PBS; 0.154 m NaCl in 0.88 mm KH_2PO_4 and 3.37 mm Na_2HPO_4, pH 7.4), cells were fixed with 1% paraformaldehyde in PBS. After 5 min incubation at room temperature, cells were washed again with PBS and subsequently incubated for 2–4 h at 37°C with 0.2 mg/ml X-gal in PBS containing 2.0 mm $MgCl_2$, 5.0 mm $K_3[Fe(CN)_6]$ and 5.0 mm $K_4[Fe(CN)_6]$.

For quantitative determination of the β-galactosidase formed and the amounts of extractable protein in cell lysate, cells were washed with PBS and then lysed by incubation in 0.20 ml of 0.25% Triton X-100 in 5.0 mm Hepes (pH 8.0) and 1.0 mm $MgCl_2$ for 10 min at 37°C. The lysate was centrifuged at room temperature for 1 min at 2000 *g*, and the supernatant collected. The amount of β-galactosidase formed was assayed with the chromogenic substrate *o*-nitrophenyl β-d-galactopyranoside (ONPG). Aliquots of the supernatants (25 μl) were transferred to wells on 96-well trays and 125 μl of reagent was added. The reagent consisted of 15 mg ONPG in 25 mm Hepes (pH 8.0) containing 0.15 m NaCl, 1.0 mm $MgCl_2$, 0.25% Triton X-100 and 0.30% 2-mercaptoethanol. After incubation of 1–4 h at 37°C the color development was read at 405 nm. The absorbance was converted to amount of β-galactosidase formed using standards prepared with *E. coli* β-galactosidase (Grade VIII; Sigma Chemical, St Louis, MO, USA) run on the same tray, under the same conditions. The amount of protein present was determined with a commercial assay (DC Protein Assay; Bio-Rad, Hercules, CA, USA) in 96-well plates with bovine serum albumin (Fraction V; Sigma) as reference. Sample

volumes were 25 μl, and the albumin standard was diluted in the same buffer as used above for lysis of the cells.

Freeze-fracture electron microscopy

Freeze-fracture was performed on a Balzers Freeze-Etching system BAF 400D (Balzers AG, Balzers, Liechtenstein). Samples were cryofixed in presence of 25% glycerol by plunging into liquid freon 22. The fractured surface was shadowed unidirectional with platinum/carbon (45°) and coated with carbon (90°) immediately after fractioning. Replicas were analyzed using a JEOL Model JEM 1200 EX electron microscope (Jeol, Soquelec, Montreal, QC, Canada).

DNA condensation monitored by ethidiumbromide intercalation

Constant amounts of DNA (6 nmol) were mixed with various amounts of DODAC containing virosomes and DODAC:DOPE (1:1 molar ratio) liposomes, respectively, in a final volume of 0.5 ml to give the desired charge ratio. After 15 min incubation, 40 μl of 3 μm EtBr was added and vortexed. EtBr fluorescence was monitored on a SLM Aminco fluorometer (SLM Instruments, Urbana, IL, USA) at excitation and emission wavelength of 200 and 600 nm, respectively.

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