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# Sendai Virosomes Revisited: Reconstitution with Exogenous Lipids Leads to Potent Vehicles for Gene Transfer

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A reliable new procedure is described for the reconstitution of Sendai viral envelopes suitable for gene transfer. Both fusion and hemagglutinin-neuraminidase glycoproteins were extracted from purified Sendai virus and reconstituted together with DNA in the presence of cholesterol:sphingomyelin:phosphatidylcholine:phosphatidylethanolamine (Chol:SM:PC:PE) in a molar ratio of 3.5:3.5:2:1. Before reconstitution, the DNA to be transferred was condensed by pretreatment with polylysine. Exogenous lipid addition and the DNA-condensation step were essential for maximal size as well as for fusogenic activity of the resulting virosomes, the analysis of which revealed (1) the absence of any genomic material originating from Sendai virus, (2) the presence of fusogenic spikes in a functional orientation, (3) the encapsulation of reporter genes, and (4) high-transfer activity for plasmids carrying the green fluorescent protein (GFP) gene and double-stranded nucleotides into different mammalian cells. Transfer rates were up to 10-fold higher than those obtained with different cationic lipids. Gene delivery by means of our lipid-enriched Sendai virosomes extends the known gene transfer strategies, including those based on Sendai virus previously published. 

## INTRODUCTION

The development of methods for the efficient introduction of foreign genes into living cells aims to provide tools to elucidate intracellular processes at the molecular level. In addition, the development of suitable delivery vectors for in vivo gene transfer is essential for the clinical application of therapeutic genes. The majority of the current protocols for gene transfer have involved nonviral as well as viral methods. Among the former, liposome-mediated gene transfer has often been used for DNA delivery into living cells (Felgner *et al.,* 1987; Ostro and Cullis, 1989; Zenke *et al.,* 1990; Thierry *et al.,* 1997; for review, see Ledley, 1995). Generally there are two types of liposome-mediated methods. Electrostatic transfer includes the formation of ill-defined complexes between cationic lipids and the negatively charged DNA. This system allows efficient transfection *in vitro*, but gene transfer *in vivo* is often connected with serious disadvantages such as cytotoxicity caused by the use of cationic lipids, which are incompatible with the biological environment (Behr *et al.,* 1989; for review, see Gao and Huang, 1995). In the internal method, on the other hand, macromolecules are encapsulated into closed lipid bilayer vesicles prior to transfer (Szoka and Papahadjopoulos, 1980; Ostro and Cullis,1989; Reszka *et al.,* 1995). Although such liposomes may be more stable *in vivo*, they have been less efficient than cationic lipids and were often found to be subject to lysosomal degradation (Bertling *et al.,* 1991; Legendre and Szoka, 1992).

Additionally, a number of viral gene transfer methods have been developed. Viral vectors make use of the infection machinery of their complete parent viruses (including the viral genome) to transfer DNA into living cells. This strategy has been employed in the design of pseudotype retrovirus vectors (Hopkins, 1993), adenovirus-associated vectors (Yang *et al.,* 1994), and lentivirus vectors (Miyoshi *et al.,* 1999). More recently, a baculovirus vector has been found that appears to hold promise for transducing mammalian cells (Condreay *et al.,* 1999). Viral transfection has a higher transformation efficiency and exhibits stable transduction of nondividing cells. Because of their small size, however, viral vectors have a limited capacity for the DNA to be transferred. In addition, with these vectors safety hazards are not completely excluded because of the oncogenic potential of some viruses employed. The risk of random insertion of genetic elements of the parent virus, possible expression of undesirable viral genes, and alterations of the host genome structure cannot be fully avoided.

To improve viral delivery systems, we have focused our efforts on the development of reconstituted Sendai

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viral envelopes, which can be regarded as hybrid vehicles since they are based on both viral and nonviral strategies. Sendai virus, a member of the Paramyxovirus family, contains two virus-encoded glycoproteins: the fusion (F) protein and the hemagglutinin-neuraminidase (HN) protein in its envelope, which are both essential for initiating infection of the host cell. While HN provides viral binding to host cell receptors consisting of sialoglycoproteins or sialolipids, F protein interacts with the lipid bilayer of the host cell membrane to induce cell fusion (Galinski and Wechsler, 1991) at neutral pH. In Sendai virosomal gene transfer this latter property is exploited, because it introduces DNA directly into the cytoplasm, thereby avoiding endocytotic pathways and lysosomal degradation. Several investigators have previously incorporated DNA or proteins into Sendai virus envelopes (Gitman and Loyter, 1984; Gitman *et al.,*1985) or into the fusion products of Sendai virus with red blood cell ghosts (Sugawa *et al.,* 1985) for transfer into target cells. To date, none of these early methods has developed into a routine application in gene therapy.

A related but more recent delivery system is based on viral envelope-modified liposomes, so-called Sendai virus-liposomes (Kaneda *et al.,* 1987), in which varieties of macromolecules have been transferred from a mixture of anionic liposomes and UV-inactivated complete Sendai virus (Saeki *et al.,* 1997). While apparently working *in vivo*, transgene expression mediated by such nondefined Sendai-liposomes in cultured cells is low (Reszka and Schmidt, unpublished observations). In addition, such Sendai-liposomes contain complete viral genomes, which are transfected into the cells along with the reporter gene. This does not seem desirable, especially during *in vivo* application, during which the viral genes could potentially produce unexpected side effects. Gene delivery based on Sendai virus was also described by Ramani *et al.* (1998), who used reconstituted Sendai viral envelopes containing only F protein. However, their application is restricted exclusively to human parenchymal cells (hepatocytes), which carry the asialoglycoprotein receptor, to which F protein can bind, and no other tissues can be targeted.

Here we describe a novel way of producing transfer vehicles by which polylysine-condensed DNA is efficiently and reproducibly entrapped into Sendai viral envelopes during reconstitution of both F and HN protein in the presence of empirically defined exogenous lipid mixtures. This method yields virosomes of a practical size (capacity) and with high fusogenic activity. The procedure is simple and highly reproducible. When compared to conventional cationic liposomal vectors, the virosomes described here are quite efficient and may well be advantageous for gene transfer in particular cell types as for instance endothelial cells.

## RESULTS

### Preparation and characterization of reconstituted Sendai virosomes

Largely following the classical procedure introduced by Loyter and coworkers (Gitman and Loyter, 1984; Gitman *et al.,* 1985), we initially prepared Sendai virosomes from viral extracts that contained spike proteins and endogenous viral lipids. SDS–PAGE under reducing conditions of such reconstituted envelopes revealed the polypeptide pattern after Coomassie blue staining, as shown in Fig. 1A. Unlike the parent Sendai virus, virosomes contain only two prominent bands corresponding to F and HN protein. To examine the protein identity of virosomes as well as Sendai virus, proteins were analyzed by immunoblotting with the use of established monoclonal antibodies against the individual proteins (a kind gift from W. J. Neubert, MPI, Munich). The results in Fig. 1B confirm that the two protein bands from virosomes truly represent glycoproteins HN and F, and the absence of spike proteins in the sediments after envelope extraction demonstrated the efficiency of spike-solubilization with 1% Triton X-100 (results not shown). Although the extracted viral glycoproteins remain in the supernatant after centrifugation, the viral M, NP, P, and L proteins are all found in the sediment. Since NP, P, and L proteins are known to be associated with the viral RNA-genome, no genetic information should be present in the supernatant extract used for reconstitution (see below).

Electron microscopy after negative staining of reconstituted envelopes revealed the presence of different particle populations (Fig. 2A, upper panel). Only a few of the analyzed particles showed spherical shape with sizes varying between 40 and 60 nm (Fig. 2A, lower panel) and external spikes protruding from their membranes. However, most of the protein spikes did not appear to be incorporated into the envelopes; instead, they tended to assemble into bulky (50–100 nm) protein-aggregates (arrows in Fig. 2A). Judging from previous reports it seems possible that the hydrophobic parts of F and HN are associated to form the core of an oligomere with the hydrophilic heads oriented outward (Shimizu *et al.,* 1974; Hsu *et al.,* 1981).

To examine whether the addition of exogenous lipids can augment particle size and/or form of the virosomes, we prepared a series of lipid mixtures for reconstitution (Table 1) which contained cholesterol (Chol), sphingomyelin (SM), phosphatidylcholine (PC), and phosphatidylethanolamine (PE) in varying relative amounts. As apparent from Table 1 the molar ratios between Chol and SM (1:1) and between PC and PE (2:1) were kept constant, whereas the total content of Chol and SM was continually increased from 10 to 100%. All lipid mixtures were hydrated and added to viral extracts before reconstitution was initiated. Quantitative electron microscopy of the resulting virosomes revealed that the use of ex-

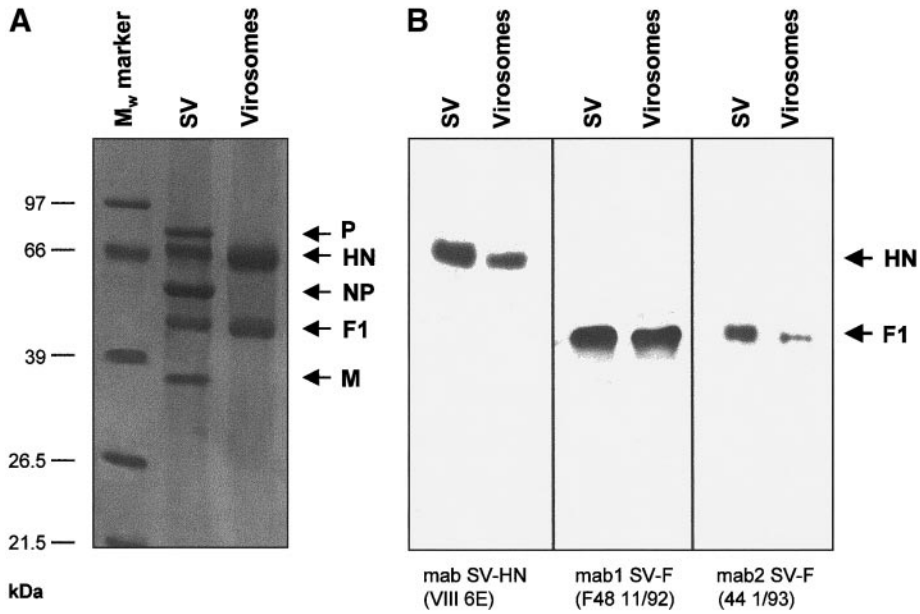


FIG. 1. Electrophoretic analysis of reconstituted Sendai virus envelopes (virosomes). (A) Coomassie-stained gel following SDS–PAGE of intact Sendai virus (SV) particles and of Sendai virus envelopes (virosomes) reconstituted from endogenous viral lipids. Molecular weight markers are shown on the left. (B) For the detection of F and HN protein Sendai virus and reconstituted Sendai virosomes were immunoblotted with the use of monoclonal antibodies directed against HN or F protein. (Monoclonal antibodies were kindly provided by Wolfgang Neubert, MPI, Martinsried.)

ogenous lipids indeed had a significant effect on the morphology of the reconstituted envelopes (Fig. 2B). Particle size increased with rising contents of Chol and SM, reaching a maximum when these two equimolar lipid species represented 70% of total lipid. Reconstitution with Chol:SM:PC:PE in a molar ratio of 3.5:3.5:2:1 (sample 9, Table 1) resulted in spherical vesicles which resemble intact Sendai virus particles in their size and especially by the appearance of a densely and evenly packed fringe of spikes on their surface (Fig. 2B, upper panel). The absence of negative stain in the vesicles also indicates that optimized virosomes are intact and not leaky. The presence of correctly oriented F and HN glycoproteins in the membrane is apparent from immunogold labeling of reconstituted virosomes with polyclonal antibodies against Sendai virus following electron microscopy (Fig. 2C).

Similar results were also obtained after labeling with monoclonal antibody directed against either F or HN protein (not shown). About 50% of the virosomes prepared with the above-mentioned optimized lipid composition were about 100 nm in diameter (Fig. 2B, lower panel). PAGE analysis resulted in the same protein pattern as shown in Fig. 1A for virosomes prepared just from endogenous viral lipids. The complete absence of a functional RNA-genome in the lipid-enriched virosomes depicted in Fig. 2B was confirmed by infectivity assays which failed to yield any virus even after four passages through embryonated eggs (not shown). Both HN and F of the Sendai virus envelope are normally required to induce either virus-cell fusion or hemolysis of red blood cells. Our data compiled in Table 1 show that reconstituted Sendai virosomes are able to hemolyze human erythrocytes with different yields depending on the reconstitution protocol. Hemolytic activity was very low when virosomes were prepared without exogenous lipids; however, the addition of exogenous lipids during reconstitution markedly enhanced hemolytic activity, both in rate (not shown) and extent. This increased hemolytic potential was particularly pronounced with virosomes containing Chol:SM:PC:PE in a molar ratio of 3.5:3.5:2:1 (sample 9, Table 1).

Since hemolysis has been treated as a debatable marker for fusogenic potential in the literature, we characterized the functional features of virosomes in more detail and chose to measure dequenching kinetics of human erythrocyte ghosts (HEG) with different virosome preparations or intact Sendai virions labeled with the lipidic fluorophore R18. The data in Fig. 3 show that virosomes prepared without any addition of exogenous lipids (designated virosomes without lipids) induce only a slow increase of R18 fluorescence reaching only about one-fifth of the extent measured for native Sendai virions (designated SV). However, the presence of exogenous lipids (Chol:SM:PC:PE = 3.5:3.5:2:1) during virosome preparation resulted in a significant increase of fluorescence dequenching (FDQ; designated virosomes with lipids), almost approaching the levels obtained with native Sendai virus. Since FDQ is widely accepted as a measure for membrane fusion, it appears from the results in Fig. 3 that the fusogenic activity of virosomes reconstituted with exogenous lipids in the above-men-

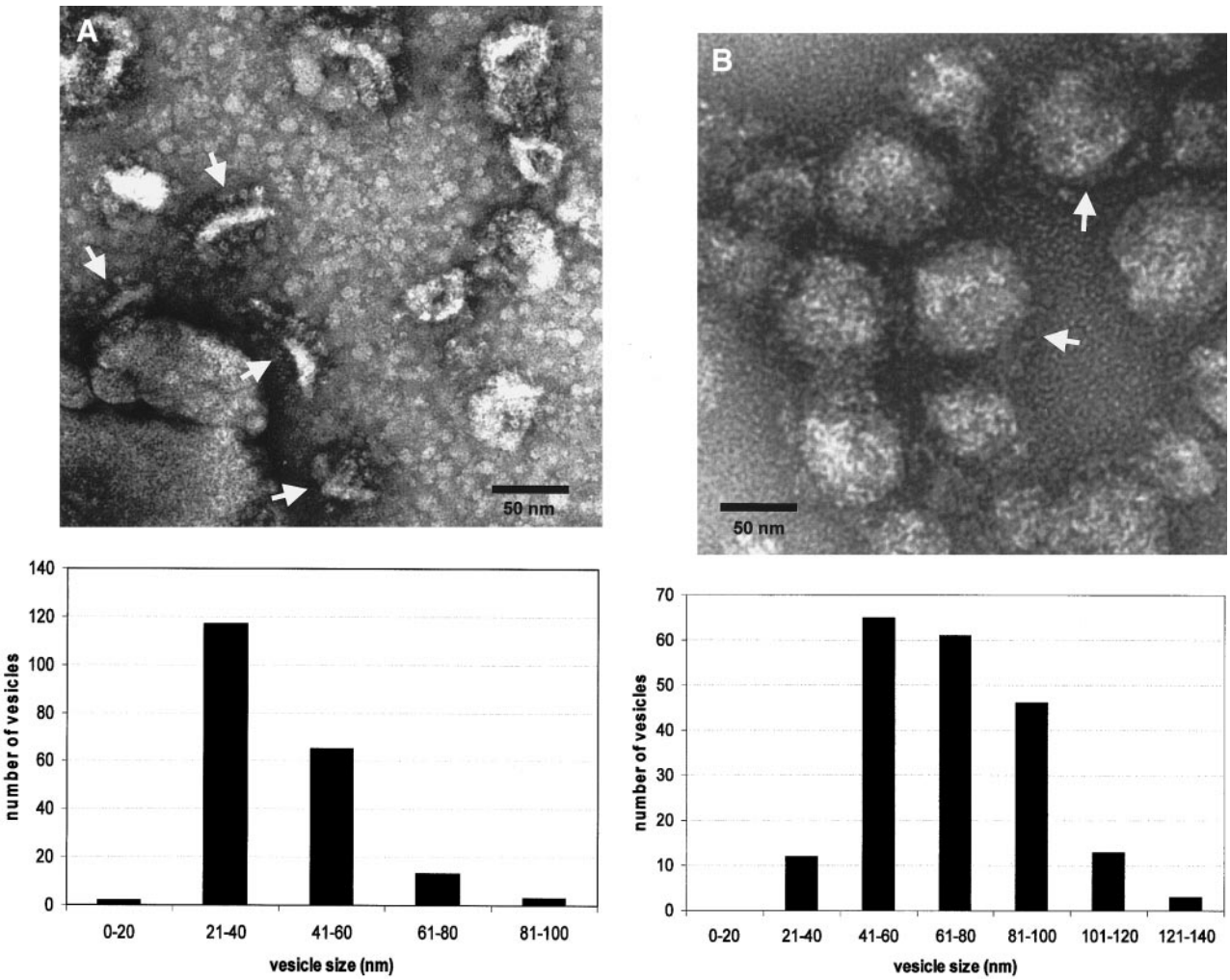


FIG. 2. Electron microscopy and size analysis of Sendai virosomes. (A) Upper panel: Negative staining of Sendai virosomes reconstituted in the absence of exogenous lipids (as in Fig. 1). Arrows show aggregated spikes. Lower panel: Size distribution of 200 analyzed virosomes. (B) Upper panel: Virosomes reconstituted in the presence of the optimized mixture of exogenous lipids (compare sample 9 in Table 1). Arrows mark the spikes protruding from the envelopes of reconstituted virosomes. Lower panel: Size distribution of 200 analyzed virosomes. (C) Immunoelectron microscopy of the same type of virosomes incubated with immune serum directed against Sendai virus. Bound antibodies were detected using Protein-A 15-nm gold conjugates as described under Materials and Methods. The density of gold marker on the virosomes is much higher that the background labeling. All cases shown represent the results from one of at least three independent experiments which yielded similar distribution patterns.

tioned composition is very similar to that expressed by native virions. Optimized virosomes with their spikes blocked by heat denaturation (designated heated virosomes) or by antibodies directed against Sendai envelopes or particular spike proteins fail to induce fusion (data not shown). Such control experiments indicate that virosomal fusion induction is indeed mediated by the viral glycoprotein spikes protruding from the virosomal surface (compare Figs. 2B and 2C).

## Encapsulation of DNA into virosomes

To examine the transfection potential of virosomes we added a plasmid encoding for green fluorescence protein (pCX-GFP) during reconstitution in the presence of exogenous lipids. Since unsupported encapsulation of DNA into virosomes (or liposomes) often results in poor entrapment because of the large size and negative charge of the uncondensed DNA (Sternberg *et al.,* 1994), we added a condensation step prior to reconstitution. Plasmid DNA and poly-L-lysine (PLL) in a weight ratio 1:1.5 in deionized water were rapidly mixed before reconstitution. Incorporation of DNA/polylysine-complexes into virosomes was eventually assayed by visualizing DNase-resistant DNA recovered from the reconstitution products after their treatment with SDS. Figure 4 shows that only virosomes prepared with the optimized lipid mixture (compare Table 1) effectively trap PLL-condensed DNA. More importantly, only when pCX-GFP was PLL treated prior to reconstitution does it become DNase I resistant. This indicates that the plasmid is probably entrapped into the virosomes rather than sticking to their surface (Fig. 4, lanes 5 and 6). When virosomes are

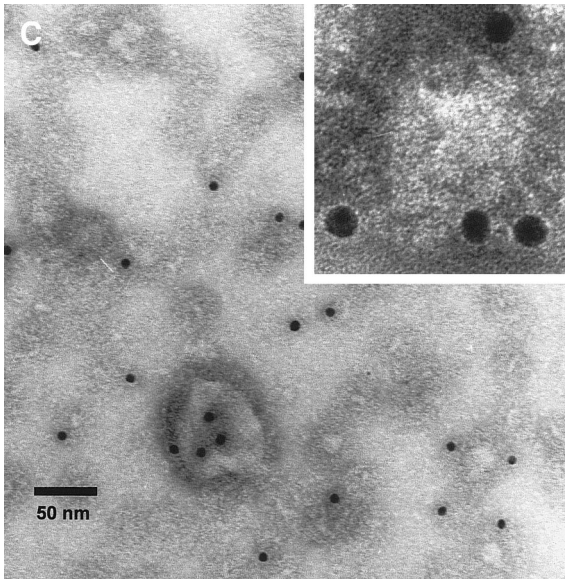


FIG. 2—*Continued*

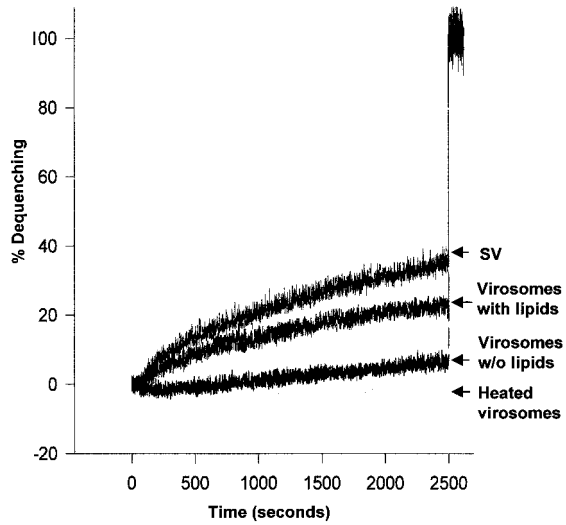


FIG. 3. Kinetics of fluorescence dequenching (FDQ) of R18-labeled Sendai virus (SV) and different Sendai virosomes with human erythrocyte ghosts (HEG). HEG were incubated with virosomes reconstituted in the absence (without lipids) or in the presence (with lipids) of the optimized lipid mixture. Heat-inactivated optimized virosomes were run as negative control. Sendai virus or virosomes were added to the HEG suspension in the cuvette and fusion monitoring started immediately ($t = 0$). At $t = 2500$ s, Triton X-100 was added for infinite dilution of the fluorophore and this fluorescence intensity was set to 100% FDQ. Typical time courses of fusion at 37°C are shown. The kinetics were normalized as described under Materials and Methods.

produced without the addition of exogenous lipids, polylysine-condensed DNA was also found to be associated with the products (Fig. 4, lane 4). This DNA, however, was completely digested after treatment of such virosomes with DNase I (Fig. 4, lane 3), suggesting that the plasmid was externally adsorbed rather than encapsulated into virosomes. Condensing DNA with polylysine appears essential for effective encapsulation, since the use of uncondensed DNA during reconstitution with optimized exogenous lipids resulted mainly in empty virosomes (Fig. 4, lanes 1 and 2). The nature of fast-migrating material, as seen in Fig. 4, is unknown at present; however, it is unlikely that it represents RNA, since these bands are resistant to RNaseA treatment (results not shown). Routine analysis by protein assays and densitometry after agarose gel electrophoresis of encapsulated DNA into optimized virosomes revealed a DNA/protein ratio of 1 to 2 $\mu$g of intact plasmid DNA per mg total protein. No detectable leakage of DNA was observed when virosomes were left at 4°C in PBS or DMEM for up to 48 h. We also tested for protein composition, hemolytic activity, and morphology of optimized virosomes loaded with PLL/DNA and found that all these parameters were almost identical to those shown for virosomes without DNA (compare Figs. 1, 2, and 3).

TABLE 1

Effect of Exogenous Lipids Added during Reconstitution of Sendai Virosomes on Hemolytic Activity

| Sample | CHOL (mol%) | SM (mol%) | PC (mol%) | PE (mol%) | Hemolytic activity (%)[a] |
|---|---|---|---|---|---|
| 1 | 0 | 0 | 0 | 0 | 5 |
| 2 | 0 | 0 | 67 | 33 | 6 |
| 3 | 5 | 5 | 60 | 30 | 8 |
| 4 | 10 | 10 | 53 | 27 | 11 |
| 5 | 15 | 15 | 47 | 23 | 12 |
| 6 | 20 | 20 | 40 | 20 | 13 |
| 7 | 25 | 25 | 33 | 17 | 15 |
| 8 | 30 | 30 | 27 | 13 | 19 |
| 9 | 35 | 35 | 20 | 10 | 27 |
| 10 | 40 | 40 | 13 | 7 | 17 |
| 11 | 45 | 45 | 7 | 3 | 14 |
| 12 | 50 | 50 | 0 | 0 | 12 |
| 13 | 0 | 0 | 67 | 33 | 0 |

[a] Hemolytic activity of intact parent Sendai virions was set as 100%. Mean hemolytic activity was calculated from at least three independent experiments, which deviated by 3% maximally.

## *In vitro* gene delivery activity of optimized Sendai virosomes

Coming back to the key question of gene delivery, we tested the transfection efficiency of virosomes prepared with exogenous Chol:SM:PC:PE (3.5:3.5:2:1) and with the PLL-condensed plasmid pCX-GFP and compared it to that of cationic lipids. Cultured CV.1, F98, and HUVEC endothelial cells were transfected as described under Materials and Methods and subjected to fluorescence microscopy 48 h later. Parallel cultures of the same cells were transfected with pCX-GFP by use of specific cationic lipids. Initially we compared the transfection efficiency of different commercially available as well as self-prepared cationic lipids to select the most potent

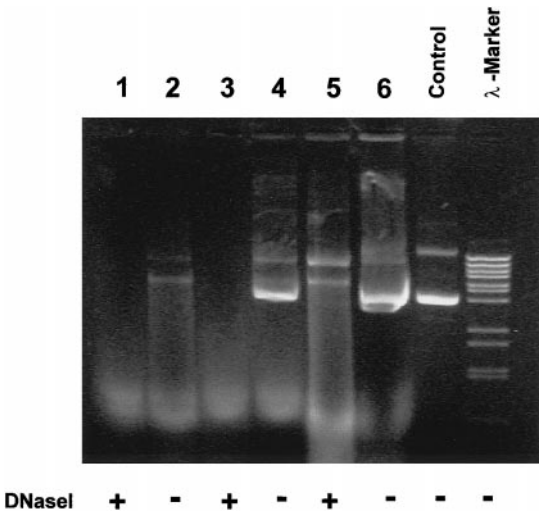


FIG. 4. DNase I sensitivity of DNA associated with Sendai virosomes. Differently prepared virosomes (15 $\mu$g of total protein) were lysed with 2% SDS and analyzed by electrophoresis on a 1% agarose gel after treatment with (+) or without (−) DNase I. A molecular weight marker (phage λ DNA digested with *Bst*EII) and untreated pCX-GFP DNA (50 ng) were run as controls (lanes λ Marker and Control). Lanes 1 and 2: uncondensed DNA, virosomes prepared with exogenous lipids; lanes 3 and 4: polylysine-condensed DNA, without exogenous lipids present during reconstitution; lanes 5 and 6: polylysine-condensed DNA, with exogenous lipid present during reconstitution.

formula for each cell type used. For CV.1 cells lipofectin was found to yield the highest transfection level. Rat glioblastoma cell line F98 was most effectively transfected with cationic lipid mix DC30 (Reszka *et al.,* 1995) and human umbilical vein endothelial cells (HUVECs) with Lipofectamin. As shown in Fig. 5, virosomal GFP expression was detected in all of the cell types investigated (right panels). More importantly, the same figure demonstrates that virosomal transfer was as efficient (CV.1 cells) as or even better (F98 and HUVECs) than the transfer induced by the most potent cationic lipids (left panel). Transfection efficiency normalized to 1 $\mu$g of pCX-GFP DNA was 4% for CV.1 and 2% for endothelial cells when these were transfected with cationic lipids, and 7% for CV.1 and 25% for HUVECs after transfection with optimized virosomes. When transfection was done with virosomes lacking exogenous lipids, with uncondensed plasmid or with naked plasmid DNA treated with PLL, no expression of GFP was detected in any of the cell types tested (data not shown). When virosomes were heat-treated or incubated with polyclonal antibodies against Sendai virus prior to transfection, the number of cells expressing GFP was reduced to values below 5 and 30%, respectively, when compared to the corresponding control virosomes (data not shown).

To test the influence of the size of the DNA to be transferred we encapsulated 20-bp-long double-stranded oligonucleotides instead of the GFP-plasmid and compared the efficiency of delivering double-stranded nucleotides *in vitro* using virosomes or cationic lipids. FITC-labeled and poly-L-lysine-treated double-stranded nucleotides were transfected to cultured HUVE cells in parallel to cationic transfection and the transfer monitored by FACS analysis as well as by fluorescence microscopy (Fig. 6). Fluorescence-labeled double-stranded nucleotides were reconstituted with the above-mentioned exogenous lipids and the resulting Sendai virosomes prepared and characterized as described earlier. Representative data from multiple experiments in Fig. 6 revealed that FITC-labeled double-stranded nucleotides delivered by virosomes were detected in most of the cells, while cationic lipid transfer was successful in only a fraction of the endothelial cells. It is noteworthy that, in contrast to cationic transfer, transfection with virosomes yielded high concentrations of double-stranded nucleotides in the transfected cells and that double-stranded nucleotides were accumulated predominantly in the nuclei (Fig. 6, lower left panel).

## DISCUSSION

The present work describes a new rational and quantitative approach for the reconstitution of highly fusogenic vesicles derived from Sendai virus envelopes for delivery of a variety of macromolecules (plasmid DNA and double-stranded nucleotides) into different mammalian cells. When compared with the pioneering protocols of Loyter and coworkers (Gitman and Loyter, 1984; Gitman *et al.,*1985), the novelty of our method includes the addition of particular exogenous lipids during reconstitution and a poly-L-lysine (PLL) condensation step for the nucleic acid to be transferred. These measures increase the transfection efficacy of final virosomes for various reasons as, for instance, by protecting the entrapped material from degradation. The advantage over the virosomal-transfer system propagated by Kaneda and coworkers (Kaneda *et al.,* 1987; Saeki *et al.,* 1997) is that the virosomes reported here are active *in vitro* and fully characterized. Most importantly, they are devoid of any viral genomic material, which represents a potential hazard during application in gene therapy. SDS–PAGE analysis and immunoblotting of the virosomes with monoclonal antibodies demonstrate that only F and HN proteins are presented in the virosomal preparation (Fig. 1), whereas the nucleocapsid- and M proteins are discarded after centrifugation. The complete absence of viral genomic material in our virosomes has been substantiated by the most sensitive procedure available: serial passage through embryonated eggs.

Since morphological characteristics of reconstituted particles are often helpful in understanding and predicting functional activities, we first examined the fine structure of reconstituted virosomes. Figure 2A shows that such virosomes reconstituted without the addition of exogenous lipids are significantly smaller (40–60 nm) than intact Sendai virions and that many of the protein

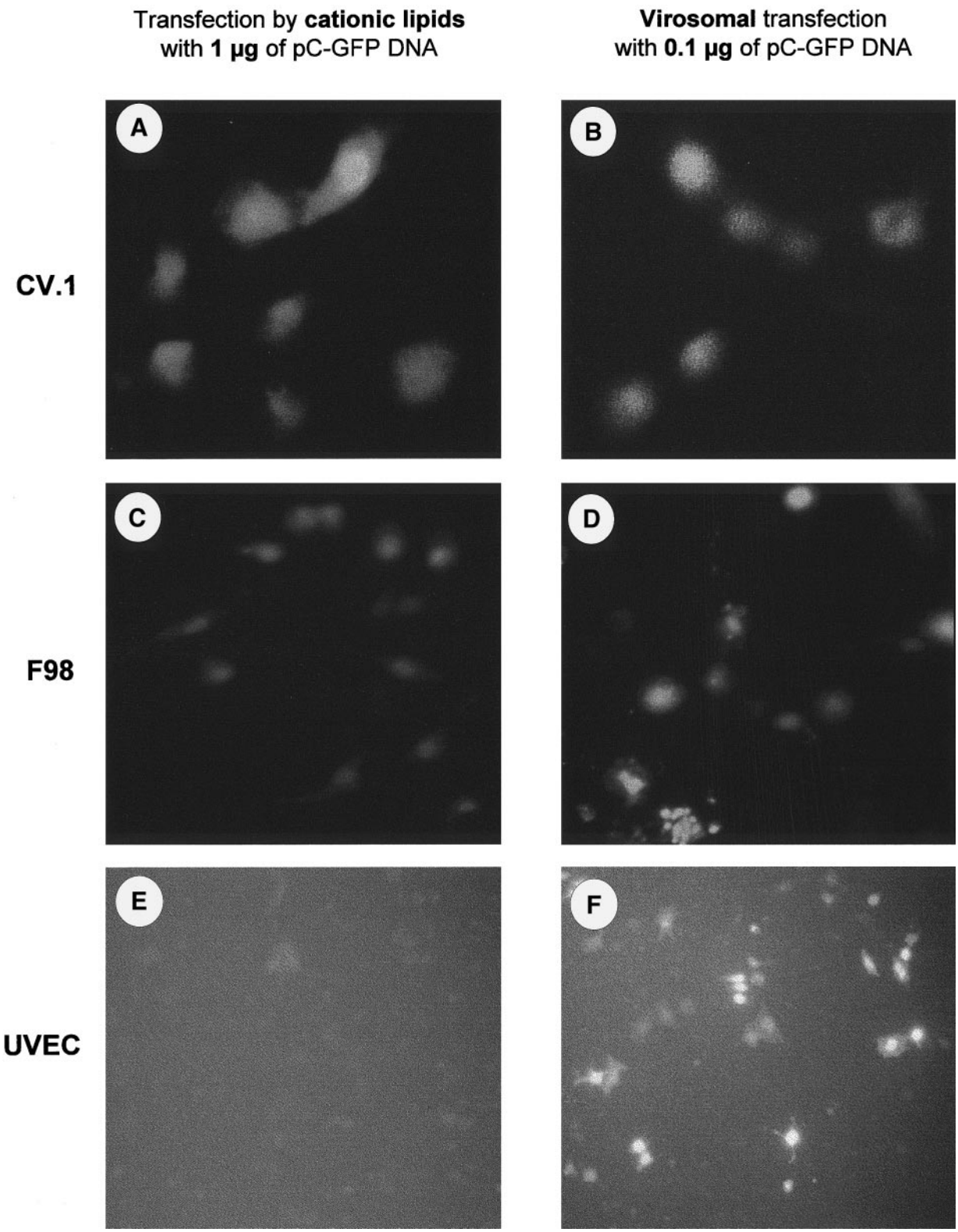


FIG. 5. Expression of green fluorescent protein (GFP) in different cell types transfected with Sendai virosomes or by using Lipofectin. Optimized Sendai virosomes containing 100 ng of polylysine-condensed pCX-GFP DNA were directly inoculated into CV.1 (B), F98 (D), or human umbilical vein endothelial cells (F) cultured in 35-mm dishes ($5 \times 10^5$ cells) as described under Materials and Methods. After incubation at 37°C for 1 h (for CV.1 cells) or for 4 h (for F98 and endothelial cells), the virosome suspension was replaced with DMEM/5% FCS. For comparison the cells were each transfected with 1 $\mu$g of reporter plasmid by using Lipofectin for CV.1 (A), DC30 for F98 (C), and Lipofectamin for HUVEC (E). After 48-h incubation at 37°C, cells were subjected to fluorescence microscopy to visualize the expression of GFP by counting the fluorescent cells.

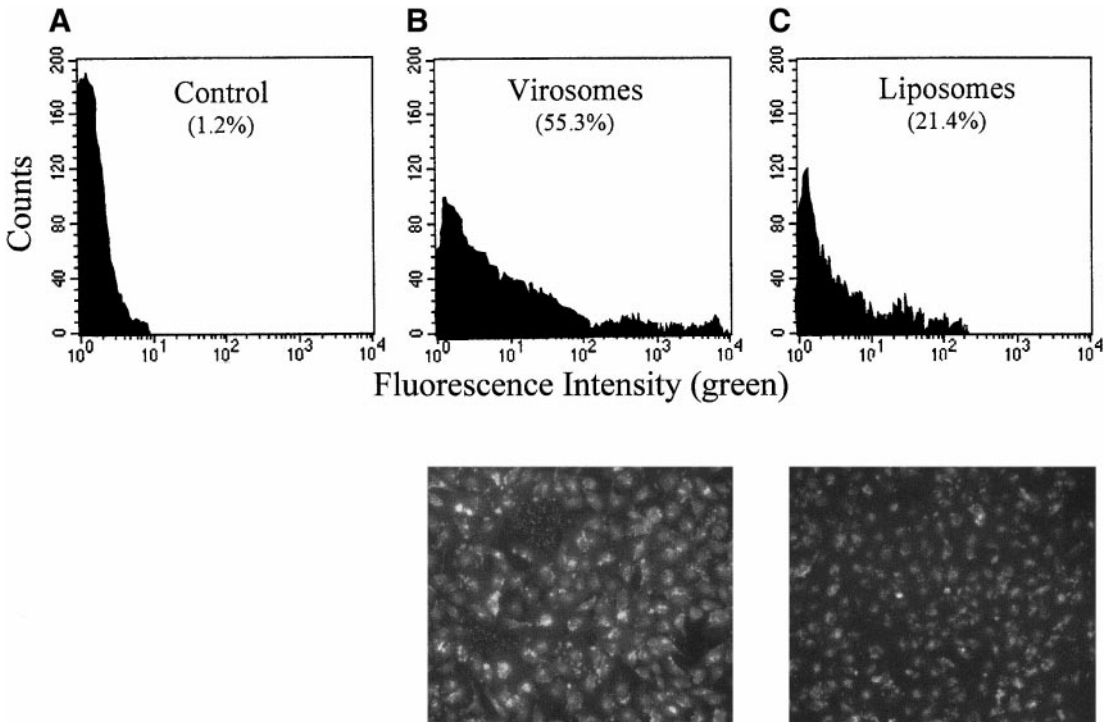


FIG. 6. Histograms of the FACS analysis (upper panel) and of fluorescence microscopy (lower panel) of human umbilical vein endothelial cells (HUVECs) after transfer of FITC-labeled double-stranded nucleotides. (B) Sendai virosomes prepared with the optimized exogenous lipid mixture and containing polylysine-treated FITC-labeled double-stranded nucleotides were transfected into HUVE cells as described under Materials and Methods and compared with transfection either by naked double-stranded nucleotides (A) or by use of Lipofectamin (C). After incubation for 4 h at 37°C, the virosome suspension was replaced with medium and the incubation continued for 1 h prior to FACS analysis (upper panel) or fluorescence microscopy (lower panel). Numbers of cells (counts) are plotted against fluorescence intensity. About 50,000 cells were analyzed in each experiment and transfection efficiency calculated (insert in brackets).

spikes are aggregated. It has been previously reported that the detergent-extractability profile for cellular lipids depends on the lipid composition of the membranes (Davies *et al.,* 1984; Banerjee *et al.,* 1995). For example, Triton X-100 that was used in our study for solubilization of Sendai virions has only a limited capacity to extract sphingomyelin and cholesterol (Davies *et al.,* 1984; Brown and Rose, 1992). Thus, during extraction of the viral lipid bilayer by TX-100, these lipid species are partially insoluble and therefore lost from the endogenous lipids present in the reconstitution mix during centrifugation in the form of insoluble vesicles. Electron microscopic inspection of sediments after TX-100 extraction revealed the existence of such multilamellar vesicles (not shown); thus, solubilization of Sendai virions with TX-100 leads to a selective loss of particular lipids. In addition, some of the viral envelope phospholipids could be lost by absorption to the BioBeads used to remove TX-100 from the system. Altogether, it appears that during reconstitution some of the viral envelope lipids are selectively reduced, thereby leading to a suboptimal protein/lipid ratio for reconstitution, which results in nonfunctional glycoprotein aggregates and in reconstituted virosomes of only small size (Fig. 2A). Accordingly, the hemolysis and R-18 fusion assays (Table 1 and Fig. 3) revealed only low activities for virosomes reconstituted exclusively from endogenous lipids.

To overcome this problem we added exogenous phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (SM), as well as cholesterol (Chol) in various proportions during reconstitution. These lipids were selected because plasma membranes of animal cells are known to contain PC, PE, and SM in a similar range of molar ratios. It has also been demonstrated that the membrane lipid composition influences the conformation of integral membrane proteins (Stamatatos and Silvius, 1987; Dracheva *et al.,* 1996), which could be an important factor in membrane fusion. From liposomal research it is known that a high cholesterol content makes liposomal membranes rigid, reduces lipid fluidity, and facilitates membrane fusion (Stamatatos and Silvius, 1987; McIntosh *et al.,* 1989). In addition, envelopes of RNA viruses such as HIV, NDV, Sendai virus, and VSV were reported to contain similar amounts of Chol and phospholipids (Blough and Lawson,1968; Aloia *et al.,* 1988) and the biological activities of these viruses (e.g., fusion) were diminished when the content of Chol was reduced (Moore *et al.,* 1978; Citovsky *et al.,* 1986). In our empirical approach to add exogenous lipids for maximal hemolysis, fusogenicity in the R-18 assay, and GFP ex-

pression after transfection, we found the enrichment of the reconstitution mixture by Chol:SM:PC:PE in a molar ratio of 3.5:3.5:2:1 to result in highly efficient virosomes (Figs. 2, 3, and 5, Table 1). As discussed earlier, the requirement for a high content of SM and Chol in the exogenous lipid mixture (35% of each) can be explained best by the selective insolubility (and loss) of these lipids during the initial Triton X-100 extraction of purified Sendai virus. Our results also show that both the phospholipid composition and the cholesterol/phospholipid ratio have a pronounced effect on morphology and on the functional properties of reconstituted virosomes.

Another important parameter that has a considerable influence on transfection yields is the amount of DNA encapsulated during reconstitution. Electron microscopic studies on DNA containing liposomes by Sternberg *et al.* (1994) had revealed that DNA molecules are not well condensed, but may exist in an extended conformation reaching up to 600 nm in length. These data can explain our observation that passive encapsulation of pCX-GFP DNA into optimized virosomes (which are ~80–100 nm in diameter) results only in poor entrapment (Fig. 4). Cationic polymers such as polylysine, histones, and protamine are known to complex and condense DNA from its extended conformation into highly compact structures of about 60 nm (Gao and Huang, 1996; Vitiello *et al.,* 1996). Therefore we included a poly-L-lysine (PLL) DNA condensation step to our reconstitution protocol. PLL possesses a high capacity for DNA condensation and is biodegradable. Since it also resembles a nuclear localization signal (Dingwall and Laskey, 1991; Gao and Huang, 1996), it might facilitate active nuclear uptake of the PLL-DNA complex. Preincubation of DNA with PLL during reconstitution results in the association of condensed DNA with virosomes. However, only the virosomes prepared in the presence of exogenous lipids (designated optimized virosomes) seem to fully encapsulate DNA, thereby making it DNase I-resistant.

In contrast, with virosomes formed exclusively from endogenous lipids, DNA was completely digestable by DNase I, indicating that the plasmid DNA remained largely accessible, perhaps by sticking to the outer virosomal membrane (Fig. 4). These results demonstrate that both the addition of exogenous lipids as well as the condensation of DNA with PLL prior to reconstitution are required to produce the highly effective gene delivery system. Despite the special features required to guarantee the reliable and high transfer potential of the products, the reconstitution protocol is quite simple and allows the production of optimized virosomes within 2 days (for schematized flow sheet of procedure, see Fig. 7) from Sendai virus concentrates. In attempting to avoid virus production, we are presently testing reconstitution protocols that use the recombinant spike proteins HN and F from insect cells (Ponimaskin and Schmidt, unpublished data). Despite the recent observation that F protein may be sufficient for virosomal fusion under certain conditions (Ramani *et al.,* 1998), we plan to maintain the use of both F and HN glycoproteins in our virosomes during future experiments, because there is evidence that HN actively participates in virus-cell fusion (Gitman and Loyter, 1984; Bousse *et al.,* 1994; Ponimaskin and Schmidt, unpublished data).

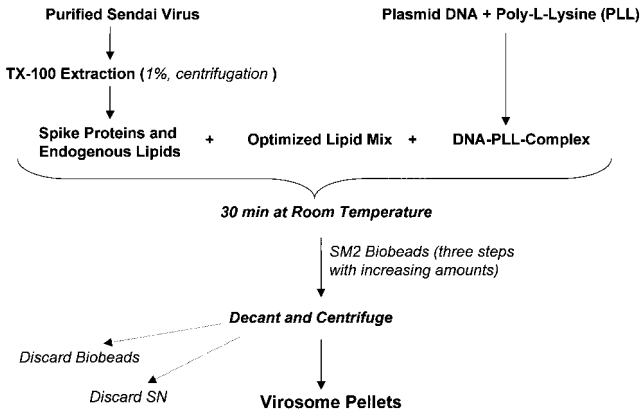


FIG. 7. Flow-sheet protocol for reconstitution of optimized Sendai virosomes. This schematic summarizes the procedure for the preparation of optimized Sendai virosomes loaded with DNA. The resulting virosomes are highly fusogenic and capable of efficient gene transfer into different mammalian cells. For details see Materials and Methods and Results sections.

As apparent from Fig. 5, incubation of such loaded virosomes with the different cell types resulted in the expression of GFP, demonstrating that virosomes had fused with and released their contents into the monolayered cells (Fig. 5). Heat-treated virosomes as well as virosomes that had been preincubated with polyclonal antibodies against Sendai virus are inefficient in terms of GFP expression. This suggests that delivery is a fusion-mediated process initiated by the viral spikes located on the outer surface of virosomes. The competence of optimized virosomes in the transfection of glioblastoma cells (F98) may be exploited for gene delivery into tumor cells also, and our *in vivo* experiments are supporting this possibility (Reszka *et al.,* manuscript in preparation). The difference in transfer efficiency observed between virosomal and cationic lipid-mediated delivery is most striking for endothelial cells, which were only weakly transfected with the best of all tested cationic lipids, Lipofectamin (Fig. 5). Likewise, FITC-labeled double-stranded nucleotides were delivered by virosomes to endothelial cells with an efficacy of 50%, whereas it was only 20% with the best of all cationic lipids tested. These results emphasize the potential advantage of our optimized virosomes.

The double-stranded nucleotides used in our ongoing study possess a high affinity for nuclear factor-$\kappa$B (NF$\kappa$B). This factor plays a pivotal role in the transactivation of genes for certain cytokines and adhesion molecules (for review, see Cramer and Muller, 1999) and is

probably involved in the regulation of the expression of proteins relevant to myocardial infarction (Morishita *et al.,* 1997). Our preliminary results along these lines demonstrate that transfer of NFκB oligonucleotides as decoy double-stranded nucleotides into endothelial cells by use of optimized virosomes is a promising method to specifically inhibit the activation of NFκB (Gawaz *et al.,* 1998; Gawaz *et al.,* manuscript in preparation).

All these data together encourage us to pursue our studies. Considering the high levels of GFP expression and the efficient transfer of double-stranded nucleotides achieved *in vitro,* particularly with endothelial cells, as well as the ease and the high reproducibility of our reconstitution procedure, we believe that the Sendai virosomes reported here are well suited for a wider application in therapeutic gene and drug delivery. In contrast to cationic lipids as a delivery system, our highly fusogenic virosomes bear the advantageous potential of exploiting antibodies for tissue-specific targeting.

## MATERIALS AND METHODS

*Reagents.* Fluorophore octadecyl rhodamine B chloride (R18) was obtained from Molecular Probes. SM-2 BioBeads were purchased from Bio-Rad. Triton X-100 (TX-100) was obtained from Boehringer Mannheim (Germany). Dulbecco's modified Eagle's medium (DMEM), Lipofectin, and Lipofectamin reagents were purchased from Life Technologies (Eggenstein, Germany). Fetal calf serum (FCS) and trypsin-EDTA were obtained from BioWhittaker (Belgium). PIPES, poly-L-lysine, and sphingomyelin were from Sigma (St. Louis, MO). Protein A 15-nm gold conjugates were purchased from Plano (Germany).

*Virus.* Sendai virus (Z strain), initially obtained from R. Rott (Justus-Liebig-University, Gieβen), was propagated on chorioallantoic membranes of 11-day-old embryonated chicken eggs. Diluted viral stock (200 μl) containing 30 hemagglutinating units per 1 μg protein in PIPES-buffer (5 mM PIPES [pH 7.5], 145 mM NaCl) was used to infect the eggs, which were then incubated at 37°C for 48 h. The infection process was terminated by placing the eggs at 4°C overnight. The allantoic fluid was harvested and centrifuged at 1500 *g* for 30 min at 4°C and the clear supernatant was collected. Virus was sedimented at 25,000 *g* for 2.5 h at 4°C in a Beckman L7 ultracentrifuge using a Beckman type 19 rotor. The virus sediment was suspended in a small volume of PIPES-buffer. The viral yield from 100 eggs was 10–40 mg of total viral protein. The purified virus was adjusted to a final protein concentration of 2 mg/ml by adding PIPES-buffer and 2.5-ml aliquots (5 mg total viral protein) were stored at −70°C until further use.

*Plasmid and double-stranded nucleotides.* The plasmid pCX-GFP, containing cytomegalovirus promotor-enchancer element and β-actin/β-globin promoter linked to the green fluorescence protein (GFP) gene was obtained from Drs. M. Okabe and J. Miyazaki (Osaka University, Japan) (Miyazaki *et al.,* 1989). The 20-bp-long phosphorothioate double-stranded nucleotides derived from the second κB site within the human MCP-1 promoter and labeled with fluorescein isothiocyanate (FITC) at the 5′ end (5′-FITC-AGA GTG GGA ATT TCC ACT CA-3′) were purchased from MWG (Ebersberg, Germany). The double-stranded nucleotides were dissolved in balanced salt solution (135 mM NaCl, 5.4 mM KCl, 10 mM Tris–HCl [pH 7.6]) at a concentration of 50 μM. Supercoiled plasmid pCX-GFP was isolated from *E. coli* strain TG1 (Stratagen) by QIAGEN-Maxi plasmid purification kit according to the manufacturer's protocol.

*Preparation of lipids.* Lipid mixtures from cholesterol, sphingomyelin, phosphatidylcholine, and phosphatidylethanolamine in various combinations were prepared by dissolving in 2 ml of chloroform. They were then dried as a thin film to the walls of glass tubes by evaporation of the solvent. The dried lipid mixture was hydrated in 600 μl of PIPES-buffer containing 0.5% of Triton X-100 by intensive rocking immediately before reconstitution.

*Preparation of Sendai virosomes loaded with pCX-GFP or double-stranded nucleotides.* A suspension of Sendai virus in PIPES-buffer (5 mg total protein) was centrifuged at 80,000 *g* for 30 min at 4°C in a Beckman ultracentrifuge using a SW35 rotor and the viral pellet resuspended in 300 μl of PIPES-buffer containing 1% of Triton X-100. After incubation at 4°C for 1 h, the suspension was centrifuged at 100,000 *g* for 50 min at 4°C to remove detergent-insoluble material. While this centrifugation was in progress, DNA-PLL complexes were prepared by mixing aliquots of pCx-GFP plasmid DNA (100 μg) or double-stranded nucleotides (50 μg) with varying amounts of PLL followed by incubation at 20°C for 10 min. From the previously described suspension in PIPES-buffer with 0.5% TX-100, 600 μl of lipid were then added and the incubation continued for an additional 30 min at 20°C. The mixture of PLL-treated DNA (65 μg of DNA/mg of viral input protein) and lipids (275 nmol/mg of viral input protein) was added to the clear supernatant from the previous centrifugation, which contained the Triton X-100 solubilized fraction (i.e., the spike glycoproteins F and HN of Sendai virus). Virosomes were reconstituted by stepwise removal of detergent with the use of SM-2 BioBeads. Briefly, 30 mg of methanol-washed SM-2 BioBeads were added to the above-mentioned suspension and incubated at 20°C with gentle rocking. After 2 h an additional 60 mg of SM-2 was added and the incubation continued for 4 h. Finally, 90 mg of SM-2 BioBeads was added to the suspension and reconstitution extended for another 16 h. The virosome suspension was then carefully aspirated from BioBeads with the use of a 26-gauge needle and centrifuged at 100,000 *g* at 4°C for 45 min in a TLA-100.2 rotor with the use of a Beckman TL-100 ultracentrifuge. The virosome sediment was resuspended in 3 ml DMEM medium to a final protein

concentration of 0.1 to 0.5 mg/ml and stored at 4°C until further use.

*Hemolysis assay.* RBCs were drawn from a healthy chicken into heparin-coated tubes and washed three times with PBS. Washed RBCs were resuspended at a concentration of 1% (v/v) in PBS. RBCs (1%, 600 $\mu$l) were mixed with 200 $\mu$l of Sendai virus or virosomes (1 $\mu$g total protein) and this mixture was left on ice for 30 min, followed by a 60-min incubation at 37°C. The reaction was terminated by chilling on ice and the mixture spun at 1000 *g* for 15 min. Hemoglobin released into the supernatant was measured at 540 nm in a spectrophotometer. The amount of hemoglobin released in the presence of 1% TX-100 was taken as the reference value for 100% lysis.

*R-18 fusion assay.* For labeling Sendai virus or virosomes with the lipid probe octadecyl rhodamine B chloride (R18), samples containing 1 mg protein were mixed with 10 nM R18 in ethanol under vortexing. After incubation for 30 min at room temperature in the dark, the preparation was centrifuged and the sediment resuspended in PIPES-buffer. After washing four times the labeled samples were kept in PIPES containing 1 mM each of $CaCl_2$ and $MgCl_2$. Ghosts were prepared from human erythrocytes by hypotonic lysis in 5 mM sodium phosphate in the presence of BSA as previously described (Schwoch and Passow, 1973). To initiate the assay 20 $\mu$l of the R18-labeled sample (containing 20 $\mu$g total protein) was added to a cuvette containing 1.93 ml of prewarmed PIPES-buffer (37°C) containing 1 mM each of $Ca^{2+}$ and $Mg^{2+}$, and 50 $\mu$l of ghost suspension (50 $\mu$g protein). FDQ of R18 was measured continuously by using a spectrofluorimeter (SLM Aminco-Bowman, series 2) with 0.5-s time resolution at excitation and emission wavelengths of 560 and 595 nm, respectively. A 570-nm cutoff filter was placed in the emission optical pathway to reduce scattering. To normalize the data, the percentage FDQ (%FDQ) at any time point was calculated from the equation: $\%FDQ = 100[F(t) - F_0]/(F_T - F_0)$, where $F_0$ and $F(t)$ are fluorescence intensities at time 0 and at a given time point $t$, and $F_T$ is the fluorescence intensity in the presence of 0.5% Triton X-100, defined as the fluorescence at "infinite" dilution of the probe (Morris *et al.,* 1989).

*Electron microscopy.* Virosome samples were placed on formovar/carbon-coated copper grid and then stained either with 2% uranyl acetate for 2 min or with 2% ammonium molybdate for 1 min. Grids were dried and examined by JEM 100CX transmission electron microscope (Jeol, Tokyo, Japan), operating at an accelerating voltage of 80 kV with a magnification from 15,000 to 100,000×. Results were documented on Agfa-EM film (Plano, Wetzlar). For quantification 200 to 800 virosomes were analyzed for each lipid composition and mean sizes were then determined. For immunoelectron microscopy a protein A-gold technique was used as previously described (Slot and Geuze, 1985), with minor modifications. After absorption of virosomes on a formvar/carbon-coated nickel grid for 20 min, the specimen was treated with 50 mM glycine in phosphate-buffered saline (PBS) and washed twice with PBS containing 0.2% gelatin and 0.5% bovine serum albumin (PBG). The sample was incubated with diluted polyclonal rabbit antibodies directed against Sendai virus or with monoclonal antibodies directed against either F or HN protein (1/100 or 1/1000 in PBG) for 1 h and then washed with PBG, PBS, and Aqua bidest, followed by incubation with protein A-gold conjugates (1/100 or 1/200 in PBG) for 1 h. Finally, the grids were stained either with 2% uranyl acetate or with 2% ammonium molybdate and examined by transmission electron microscopy as described earlier.

*Cell culture and transfection of cultured cells.* CV.1 and F98 cells were maintained as monolayer cultures in DMEM supplemented with 5% FCS. Primary HUVECs were harvested by use of collagenase digestion as described (Gawaz *et al.,* 1998). Cells were pooled from three to six prepared umbilical veins and were grown in complete medium composed of M199 (Sigma), 10% FCS, 2 mM glucose, 100 U/ml penicillin, and 100 mg/l streptomycin and used as confluent monolayers after one to two passages. All cell types were grown in 35-mm dishes ($5 \times 10^5$ cells), washed twice with appropriate medium without FCS, and transfection was initiated by adding 300 $\mu$l of virosomes (150 $\mu$g total protein) in medium. After 1 h for CV.1 cells or 4 h for F98 and HUVECs, incubation at 37°C in 5% $CO_2$ virosome suspension was replaced with a complete medium and cells were further incubated for 48 h. For a comparative assessment of gene transfer, all cell types were transfected in parallel with 1 $\mu$g of pCX-GFP DNA with the use of Lipofectin (Life Techologies) for CV.1 cells, Lipofectamin (Life Technologies) for HUVE cells, and DC30 for F98. Transfection protocols were as described by the manufacturer (Lipofectin, Lipofectamin) or as previously reported (DC30) (Reszka *et al.,* 1995). The total amount of virosomal DNA applied to the cells in all other experiments is specified with the respective results.

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