Effective Transfection of Rabies DNA Vaccine in Cell Culture Using an Artificial Lipoprotein Carrier System

Fars Alanazi,[1] Zhen F. Fu,[2] and D. Robert Lu[3,4]

Received November 23, 2003; accepted December 24, 2003

Purpose. To evaluate the transfection efficiency in cell culture of rabies plasmid DNA vaccine carried by a novel artificial lipoprotein system.

Methods. Phospholipid nanoemulsions resembling the lipid core of natural lipoproteins were prepared. The artificial lipoprotein carrier system for DNA was constructed by assembling of the nanoemulsion (NE)–palmitoyl-poly-L-lysine (p-PLL)–rabies DNA complex. Agarose gel electrophoresis, zeta potential, and mobility measurement were conducted to determine the surface charge balance in various complex compositions. Transfection and transfection efficiency were examined by fluorescence microscopy and flow cytometry, respectively.

Results. The artificial lipoprotein system was successfully constructed and the rabies DNA vaccine was effectively transfected in glioma cell line SF-767. The amount of p-PLL incorporated into the artificial lipoprotein formulations had a significant effect on transfection efficiency. The new system also proved to be more efficient in cellular transfection of rabies DNA vaccine than the commercial lipofectamine formulation.

Conclusions. Effective transfection of rabies DNA vaccine in cell culture can be achieved using the novel artificial lipoprotein carrier system, and the charge balance of the NE–p-PLL–DNA complex appears an important factor.

KEY WORDS: artificial lipoprotein; cell culture; nanoemulsion; palmitoyl poly-L-lysine; rabies plasmid DNA; transfection efficiency; vaccine delivery.

INTRODUCTION

DNA vaccine is a simple ring of DNA containing a gene encoding an antigenic protein. DNA vaccine uses DNA sequences from the virus that encode specific proteins against which a protective or therapeutic immune response is desired (1–3). Because DNA vaccines only encode selected components of a pathogen, rather than the entire pathogen, they place the vaccinated host at no risk for infection. DNA vaccine can also initiate both humoral and cellular immune responses without being pathogenic and thus behaves like the attenuated organisms. The early work by Wolff *et al.* (2) found that injection of naked DNA into muscle of mice resulted in transfection and expression of the reporter gene. Since then, researchers have explored DNA-encoded antigens as a novel vaccination covering a large number of pathogens (1,3).

Similar to other types of gene therapy, DNA vaccine requires a carrier or vector to deliver the exogenous genes. Use of live or attenuated organisms like bacteria (4,5) or viruses (6) as DNA vectors are considered risky due to their poor safety profiles (3). The liposome-based DNA carriers mostly involve cationic lipids that electrostatically interact with negatively charged DNA (7), but significant cellular toxicity has been found associated with these systems (8–11). Concerns about the viral-induced immune responses, the risk associated with replication-competent viruses, and the cellular toxicity of cationic liposomes have stimulated efforts toward the development of lipoprotein-based gene delivery systems (11–14). Recently, our laboratory has developed an artificial lipoprotein delivery system as a novel gene carrier (11). Compared to lipofectamine system (a commercial liposomal gene transfection system), our new delivery system, carrying a model plasmid DNA containing the reporter gene for β-galactosidase, demonstrated similar transfection efficiency but with a much lower cytotoxicity. In the experiment, the cell viability was up to 75% using our system compared to only 24% when using lipofectamine system.

Our previous work, however, has not examined any therapeutic gene, especially the DNA vaccine, carried by the artificial lipoprotein. DNA vaccines expressing rabies virus glycoprotein have been constructed and tested for immunogenicity and protection against challenge (15–17). Although these constructs are capable of inducing a protective immunity, large amount of the DNA is required (17). Furthermore, it takes more than six weeks to develop measurable responses (17). Thus, it is necessary to improve the efficiency of the DNA vaccines. In this paper, we attempted to use artificial lipoproteins as a carrier to deliver a DNA vaccine expressing rabies virus glycoprotein into cell culture. Similar to the structure of natural lipoproteins, the artificial lipoprotein delivery system consists of nanoemulsion cores made of natural lipids and surface lipidized poly-L-lysine, which replaces the surface protein as in natural lipoproteins (Fig. 1). The gene encoding rabies virus glycoprotein (18) was constructed in plasmid and used as the rabies DNA vaccine. The surface charge parameters (zeta potential and mobility) of the artificial lipoprotein–plasmid DNA complex was determined. The effect of varying the ratio of lipidized poly-L-lysine to the nanoemulsion on transfection efficiency (expressing the rabies virus glycoprotein in human glioma cell line SF767) was studied. In addition, a comparison study of the capability of transfecting human glioma SF-767 cell line between the artificial lipoprotein and Lipofectamine 2000 was conducted.

MATERIALS AND METHODS

Materials

Egg yolk phosphatidylcholine (99%), cholesterol (99%), triolein (99%), cholesterol oleate (98%), L-α-lysophosphatidylcholine (99%), poly-L-lysine hydrobromide (MW 48,100 Da based on viscosity), and palmitoyl chloride (98%) were purchased from Sigma (St. Louis, MO, USA). Electrophoretic grade agarose was purchased from Bio-Rad (Hercules, CA, USA). Lipofectamine 2000 was purchased from Invitrogen

[1] Department of Pharmaceutical and Biomedical Sciences, University of Georgia, Athens, Georgia 30602.
[2] Department of Pathology, University of Georgia, Athens, Georgia 30602.
[3] Department of Pharmaceutical Sciences, School of Pharmacy, Temple University, Philadelphia, Pennsylvania 19140.
[4] To whom correspondence should be addressed. (e-mail: rlu@temple.edu)

0724-8741/04/0400-0675/0 © 2004 Plenum Publishing Corporation

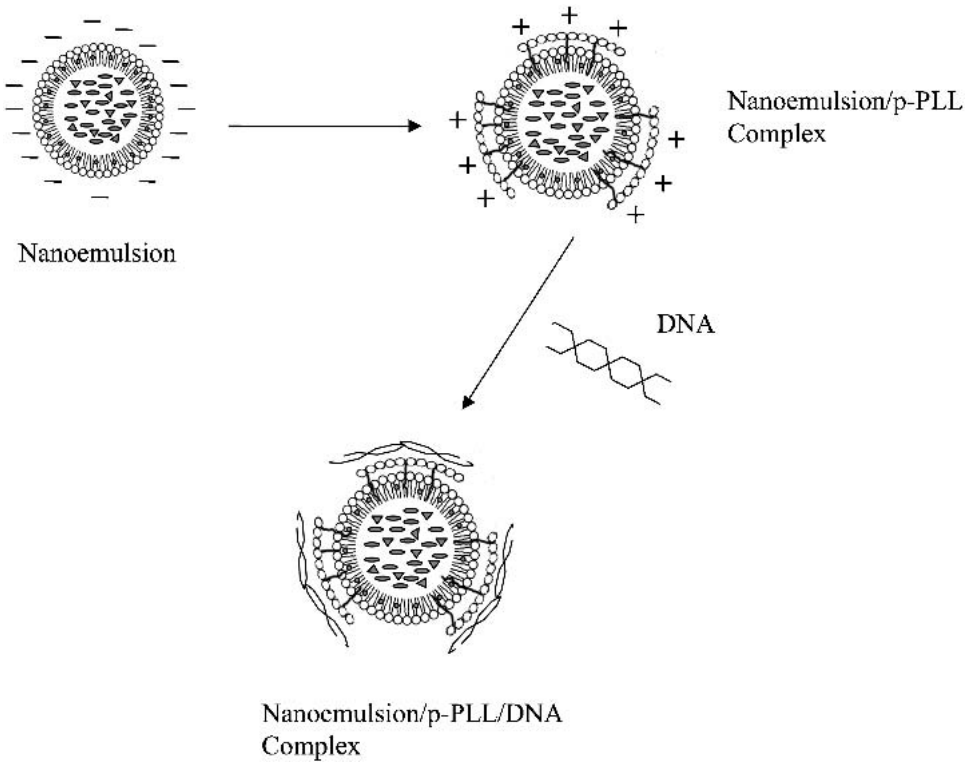

Fig. 1. Construction of NE–p-PLL–DNA vaccine complex.

Corporation (Carlsbad, CA, USA). All others chemical were analytical grade obtained from Sigma or J. T. Baker (Philipsburg, NJ, USA).

Preparation of Nanoemulsion and Synthesis of Palmitoyl Poly-L-Lysine

The nanoemulsion (NE) was prepared as described by Shawer *et al.* (19). In brief, the lipid components of oily phase (triolein, 70%; egg yolk phosphatidylcholine, 22.7%; lysophosphatidylcholine, 2.3%; cholesterol oleate, 3%; and cholesterol, 2%) were dissolved separately in chloroform and mixed thoroughly. Organic solvent was removed, and NaCl solution (2.4 M) was added to dried lipids in ratio 0.01:1 (w/v, total weight of lipids in gram vs. ml of 2.4 M NaCl solution) (11,19). The mixture was sonicated using a Model 450 Sonifier (Branson Ultrasonics Corporation, Danbury, CT, USA) and passed through an Emulsiflex B3 device (Avestin, Ontario, Canada). Submicrometer particle Sizer Model 370 (Nicomp Particle Sizing System, San Barbara, CA, USA) was used to measure particle size distribution of nanoemulsion.

Lipidization of poly-L-Lysine (PLL) was performed as described by Kim *et al.* (20). Palmitoyl fatty chain was linked to the ε-amino group of PLL. The resulting polymer, palmitoyl poly-L-lysine (p-PLL), was verified by H-NMR (d-DMSO) and characterized by agarose gel electrophoresis using Nile Red as the fluorescent dye (Nile Red stains the palmitoyl chains). Agarose gel (0.4%) was prepared in TAE buffer (40 mM Tris-acetic acid, 1 mM EDTA, pH 8.0). Twenty microliters of PLL solution (1 mg/ml) and 20 μl of p-PLL solution (1 mg/ml) were added to dried Nile Red dye and incubated for 30 min, respectively. Six microliters of glycerin was added to increase the density of the sample, and the samples were then loaded into the wells of the agarose gel. Horizontal mini-gel system (CBS Scientific Company Inc, CA, USA) was used to run the electrophoresis for 1 h at 90 volts (20 mA) at room temperature. The mobility of the samples in the electric field was visualized by ChemiImager System (Alpha Innotech Corporation, San Leandro, CA).

Assembly of Nanoemulsion, p-PLL, and Rabies Plasmid DNA Complex

Fifty microliters of NE was diluted with 0.2 ml phosphate-buffered saline (PBS) solution. Various amounts of p-PLL were incubated for 1 h at 37°C with diluted NE based on the weight ratio of p-PLL to triolein (triolein was a major component of NE). The weight ratios were 0:1, 0.125:1, 0.25:1, 0.5:1, 1:1, and 2:1 (p-PLL to triolein). After the incubation, 2 μg of pCDNA-RG (rabies glycoprotein plasmid DNA) was added and incubated for 15 min at room temperature. The electrophoretic mobility of NE–p-PLL and NE–p-PLL–pCDNA-RG were examined using agarose gel (0.4%) as described above. Zeta potential and mobility of the NE–p-PLL–pCDNA-RG complexes were measured by Submicron Particle Size Analyzer 90Plus (Brookhaven Instrument Corporation, Holtsville, NY, USA).

Construction and Transfection of Rabies Plasmid DNA

Plasmid DNA expressing rabies virus glycoprotein was constructed in pCDNA-3 vector. Rabies virus glycoprotein cDNA (18) was cloned into pCDNA-3 vector using standard molecular cloning technique. After confirming the sequence and orientation of the transgene in the vector, the pCDNA-RG was obtained. Transfection study was carried out on hu-

man glioma cell line SF-767 obtained from the tissue bank of brain tumor research center (University of California-San Francisco, CA, USA). The glioma cell line was used because of its characteristics of aggressive growth (11). The cells were grown in 75 cm^2 plastic cell culture flasks containing 13 ml of Eagle's Minimum Essential Media (EMEM) (Cambrex Co., Walkersville, MD, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma Chemicals, St. Louis, MO, USA). Cells were allowed to grow for 10 days to maintain the exponential growth. Transfection was performed in 6-well culture plates and 3×10^5 cells were seeded in each well. Before cells were transfected, they were allowed to grow for 24 h.

For transfection, NE (50 μl) was diluted with 0.2 ml PBS solution and incubated with various amount of p-PLL (p-PLL:triolein ratio of 0.125:1, 0.25:1, 0.5:1, and 1:1) for 1 h at 37°C. After the incubation, 2 μg of pCDNA-RG was added and incubated for 15 min at room temperature. One milliliter of EMEM (without FBS) was added to each well following by addition of NE–p-PLL–pCDNA-RG complex and incubated for 12 h at 37°C and 5% CO_2. Afterward, incubation media was discarded and 1 ml of EMEM containing 20% FBS was added to each well and incubated for additional 36 h. The transfected cells were rinsed three times with PBS and then fixed in acetone solution (80% v/v, acetone:water) for 10 min at –20°C. Fixed cells were air-dried and stored in refrigerator pending indirect immunofluorescence assay (IFA). For IFA, cells were rinsed three times with PBS and a 1:50 dilution of a rabies glycoprotein-specific mouse monoclonal anti-G-antibody (Accurate Chemical and Scientific Corporation, Westbury, New York, USA) was added (18). Cells were incubated for 1 h at 37°C and rinsed three times with PBS (3–4 min each). A 1:100 dilution of a secondary goat anti-mouse fluorescein (FITC)-conjugated antibody (Accurate Chemical and Scientific Corporation) was added, and cells were incubated for 1 h at 37°C. Cells were rinsed three times with PBS, air-dried, and examined using an Olympus fluorescence microscopy (Olympus American Inc, Melville, NY, USA).

Lipofectamine 2000 was used as the positive control. The ratios of DNA to lipofectamine (w/v) were 1:0.5, 1:2, 1:3, and 1:5 according to the manufacturer's protocol for optimization of the transfection. NE–p-PLL–pCDNA-3 (plasmid only without rabies gene) was used as the negative control. In addition, p-PLL–pCDNA-RG (similar amount of p-PLL but with no nanoemulsion) was also used as the formulation control.

Evaluation of Transfection Efficiency

The transfection efficiency of transient expression of rabies glycoprotein in the glioma cells was determined by flow cytometric evaluation. Binding of rabies glycoprotein-specific mouse monoclonal anti-G-antibody was revealed by secondary staining using secondary goat anti-mouse fluorescein (FITC)-conjugated antibody. The staining procedure was similar to that for the above-mentioned microscopic examination but with some modifications. Because flow cytometry runs cells in suspension, the cells were washed and suspended in PBS. Suspended cells were incubated with the first antibody (1:50 dilution) for 1 h. Cells were centrifuged, media was discarded, and cells washed with PBS. Cells were incubated with the secondary antibody (1:100) for 1 h, centrifuged, washed with PBS, and resuspended in PBS. Twenty thousand cells for each sample were examined in FACS Calibur flow cytometry device (Becton Dickimson, San Jose, CA, USA) using FLOWJO software (Tree Star Inc, San Carlos, CA, USA). Clumps and debris were excluded using forward and side-scatter windows. Transfection efficiency was calculated based on the percentage of the positive cells (that expressed rabies glycoprotein) in total number of cells.

RESULTS

Preparation of the Artificial Lipoprotein as DNA Carrier

Milky transparent emulsion was produced through the sonication procedure, when temperature was kept at 55°C, above the phase transition temperature of lipids to ease emulsification. Sonication was conducted under a nitrogen environment to reduce lipid oxidation during emulsification. The size of the emulsion was further reduced using the Emulsiflex B3 device. The mean particle size (measured as number weighted) was reduced to 54.3 ± 24.8 nm (Fig. 2). Zeta potential of nanoemulsion was –43.67 ± 1.38 mV and its mobility was –3.4 ± 0.11 [μ/s] [V/cm].

Characterization of the p-PLL was performed by ^{1}H-NMR using a Brucker-400 AMX spectrometer and the result was as follows. ^{1}H-NMR (400 Hz, d-DMSO): δ 0.82 (d, 3H, CH_2-$\underline{CH_3}$, J = 5.6 Hz), δ 2.14 (CH_2-$\underline{CH_2}$ –CO_2-). Zeta potential of PLL and modified p-PLL were 37.65 ± 3.43 and 40.64 ± 3.51, respectively. The chemical modification did not significantly change the surface charge of the polymer, maintaining its suitability of carrying DNA. The electrophoretic data indicated that in the presence of Nile Red, the p-PLL showed fluorescence emission while unmodified PLL did not emit fluorescence (Fig. 3).

The phospholipid nanoemulsion particles had a negatively charged surface, and the agarose gel electrophoresis showed that it moved toward the anode (Fig 4, lane 1). Incorporation of p-PLL into the nanoemulsion particles resulted in a change of the surface charge, observed through the retardation of the electrophoretic mobility in electrical filed (lane 2 to Lane 6). The result indicated that p-PLL was successfully incorporated into the phospholipid nanoemulsion particles.

Assembly of the Complex of Nanoemulsion, p-PLL, and Rabies Plasmid DNA

Because the net charge on DNA is negative, there exists electrostatic repulsion between DNA and NE. The incorporation of p-PLL into NE made the surface charge positive, and the complex became electrostatically attractive with DNA. It can be seen in Fig. 5 that pCDNA-RG (lanes 1 and 8) migrated toward the anode due to its negative charge. In lane 2, pCDNA-RG was incubated with p-PLL alone and both were well interacted, resulting in the disappearance of the DNA band (because p-PLL condensed DNA molecules and thus ethidium bromide could not intercalate into DNA molecules resulting in no fluorescence emission). In lane 3, pCDNA-RG was incubated with NE only, and DNA molecules migrated toward the anode, indicating unfavorable interaction between DNA and NE due to the electrostatic repulsion. Lanes 4 to 7 were the complexes of NE with different

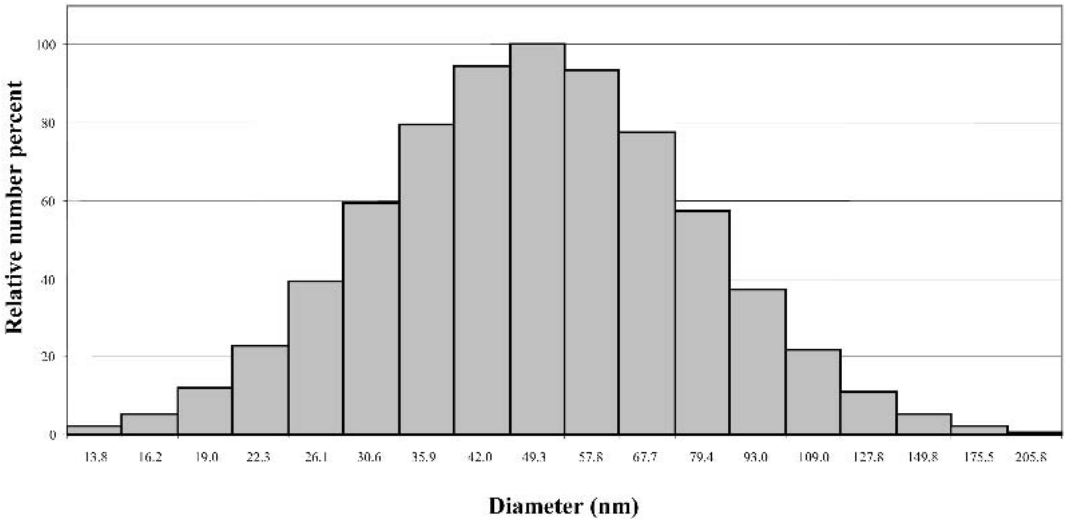

Fig. 2. Size distribution of nanoemulsion by photon correlation spectroscopy. The number weighted mean particle size was 54.3 ± 24.8 nm (n = 5).

amounts of p-PLL and a fixed amount (2 μg) of pCDNA-RG (the ratio of p-PLL to triolein was 0.125:1, 0.25:1, 0.5: 1, and 1:1, respectively). Similar to lane 2, no DNA band was observed, again indicating that DNA molecules were condensed by NE–p-PLL complexes.

Because of its importance to cellular transfection, the surface charge of the NE–p-PLL–pCDNA-RG complex was further determined. Zeta potential of these complexes was –43.67, –18.35, 12.51, 16.86, and 21.29 (Fig. 6), and its mobility was –3.41, –1.43, 0.98, 1.32, and 1.66 (Fig. 7) for p-PLL:triolein ratio of 0:1, 0.125:1, 0.25:1, 0.5:1, and 1:1, respectively (the amount of pCDNA-RG was kept constant at 2 μg/50 μl of NE).

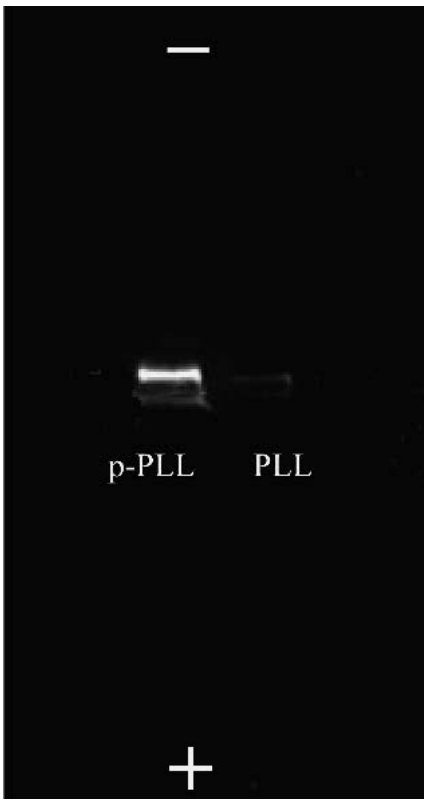

Fig. 3. Agarose gel electrophoresis of PLL and p-PLL stained with Nile Red dye.

Transfection of Rabies Plasmid DNA

The expression of rabies glycoprotein was monitored by using antibody against the rabies virus glycoprotein as described (18). The transfected cells were fixed with acetone

Fig. 4. Agarose gel electrophoresis of the complex of NE and p-PLL stained with Nile Red dye (lane 1: NE; lane 2 to lane 6 were NE–p-PLL complexes with p-PLL to triolein ratio of 0.125:1, 0.25:1, 0.5:1, 1:1, and 2:1, respectively).

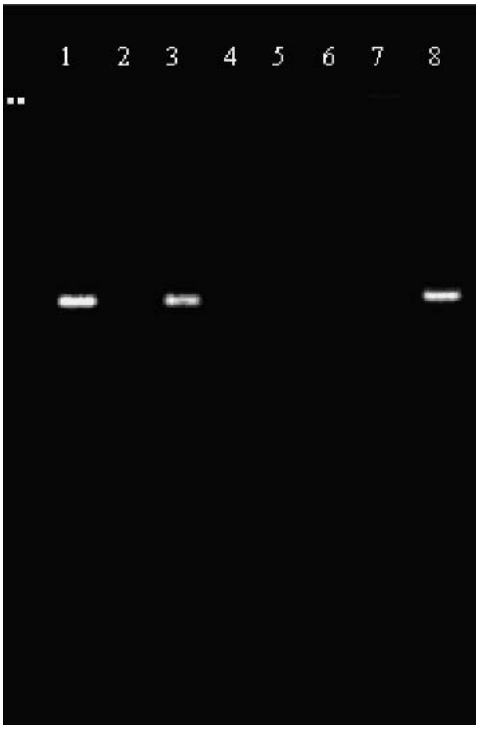

Fig. 5. Agarose gel electrophoresis of the complex of NE and p-PLL with rabies plasmid DNA vaccine stained with ethidium bromide (lanes 1 and 8: pure pCDNA-RG; lane 2: p-PLL–pCDNA-RG; lane 3: NE–pCDNA-RG; lanes 4 to 7: the complex of NE with different amount of p-PLL and pCDNA-RG. The p-PLL to triolein ratio was 0.125:1, 0.25:1, 0.5:1, and 1:1, respectively).

and reacted with polyclonal antibodies against rabies virus glycoprotein, followed by FITC-conjugated anti-mouse secondary antibody. The expression of rabies virus glycoprotein was then visualized under fluorescence microscopy (Fig. 8). Binding of FITC antibody with glycoprotein on the cell membrane of transfected cells made the cells show light green fluorescence. The non-transfected cells, however, lacked of antibody specific affinity (i.e., absence of rabies glycoprotein) and exhibited a black background. The transfection can

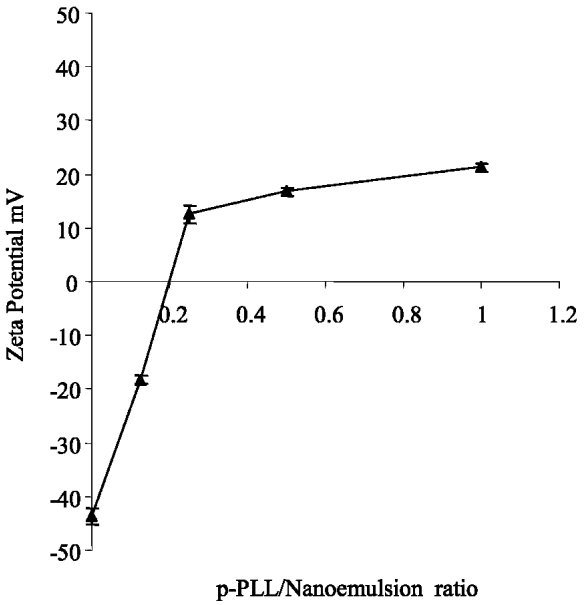

Fig. 6. Zeta potential of the NE particles and their complexes with different amount of p-PLL (pCDNA-RG was kept constant) (n = 5).

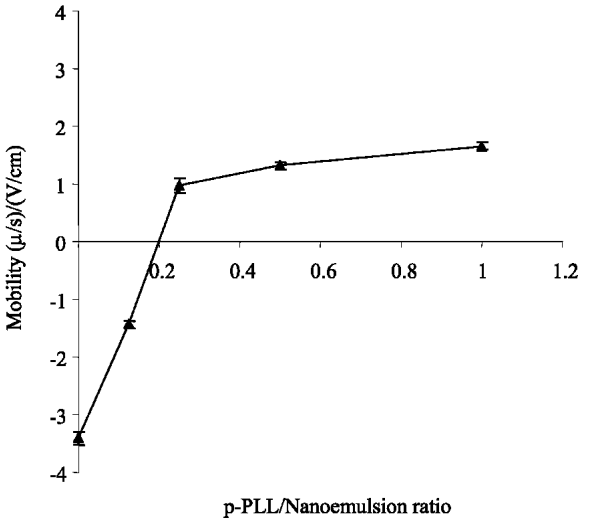

Fig. 7. The mobility of the NE particles and their complexes with different amounts of p-PLL (pCDNA-RG was kept constant) (n = 5).

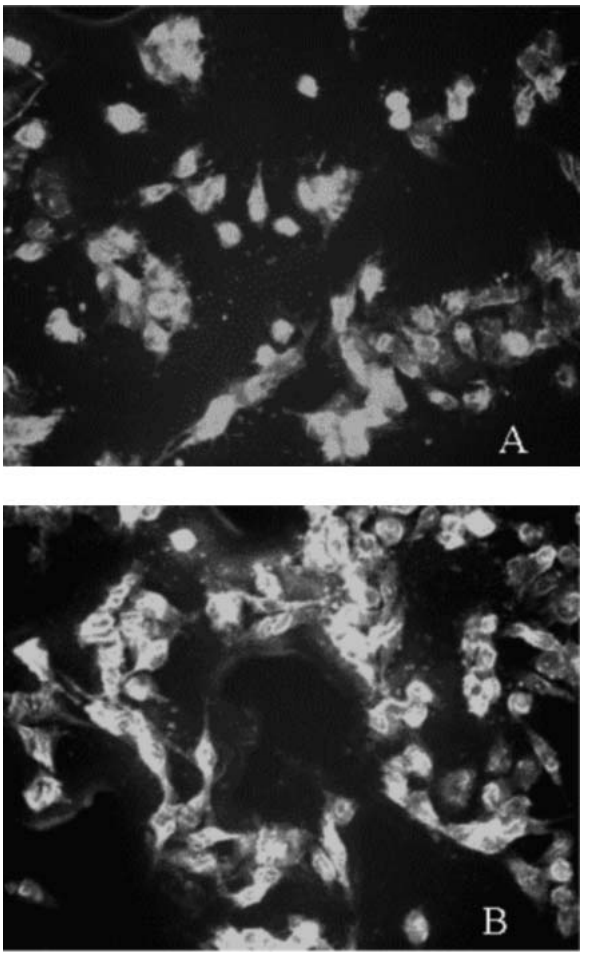

Fig. 8. Picture of transfected cells using NE–p-PLL–pCDNA-RG complex under fluorescence microscopy. (A) and (B) represent the formulation with p-PLL to triolein ratio of 0.5:1 and 1:1, respectively.

clearly be observed from the cells that expressed rabies glycoprotein and were tagged with the fluorescent probe. Transfection efficiency of the rabies glycoprotein plasmid DNA carried by the artificial lipoprotein formulation and lipofectamine 2000 in human glioma SF-767 cell line is presented in Fig. 9. The transfection efficiency of the negative control (NE–p-PLL–pCDNA-3) was 0.30 ± 0.23. The transfection efficiency of the positive controls (p-PLL–pCDNA-RG) was 6.86 ± 0.1 (L0.5) and 8.67 ± 0.51(L1), respectively. The transfection efficiency of rabies DNA carried by lipofectamine 2000 was 4.46 ± 0.23, 5.39 ± 0.43, 23 ± 3.85, and 3.5 ± 0.59 when the ratio of pCDNA-RG–lipofectamine was formulated as 1:0.5, 1:2, 1:3, and 1:5, respectively. However, the transfection efficiency of rabies DNA carried by our artificial lipoprotein system (NE–p-PLL–pCDNA-RG complex) was 6.76 ± 0.32, 6.25 ± 0.21, 36.33 ± 8.08, and 96.30 ± 0.14 for the formulations with p-PLL:triolein ratio of 0.125:1, 0.25:1, 0.5:1, and 1:1, respectively. The transfection using the artificial lipoprotein system was effective, and the transfection efficiency of the new system was significantly higher than that of lipofectamine 2000.

DISCUSSION

DNA-based vaccine is composed of nucleic acid material encoding an antigenic protein, which can be expressed *in vivo* for induction of antigen-specific immune responses. Experiments in animal models have shown the feasibility of producing effective DNA vaccines against various viruses, bacteria, and parasites (21). However, successful DNA vaccine immunization still depends largely on development of delivery systems that can efficiently and safely introduce the exogenous genetic material into the target hosts.

Owing to the safety concerns of using viruses and bacteria as DNA carriers (3) and the cytotoxicity of DNA–cationic liposome complexes (8–11), many attempts have been made to search for efficient nonviral DNA vaccine carriers with less cellular toxicity. Kim *et al.* developed a Terplex system, containing natural low-density lipoprotein and stearyl-poly-L-lysine, as a novel gene delivery vector and have shown promising results (12,20,22). Hara *et al.* (1997) used the hydrophobic core of the chylomicron remnant to solubilize DNA–cationic lipids complex to deliver DNA to liver (14). Recently, artificial lipoprotein system was developed in our laboratory as a novel gene delivery system (11). The artificial lipoprotein system mimics the natural lipoproteins and is composed of 70% triglyceride (triolein), 25% phospholipid (22.7% egg yolk phosphatidylcholine and 2.3% lysophosphatidylcholine), 3% cholesterol ester (cholesterol oleate), and 2% cholesterol. The new delivery system was capable of transfecting human glioma cell line *in vitro* with much lower toxicity compared to lipofectamine. To evaluate further its use for therapeutic genes and its transfection efficiency, this research examined the utilization of the artificial lipoprotein system to delivery rabies DNA vaccine in cell culture.

The artificial lipoprotein system consists of an oil-in-water nanoemulsion stabilized by an interfacial layer of natural phospholipids (Fig. 1). Results from gel electrophoresis (Fig. 4, lane 1) and measurement of zeta potential (–43.67 ± 1.38 mV) and mobility (–3.4 ± 0.11 [μ/s][V/cm]) confirmed that the net charge of the NE was negative. DNA carries a negative charge, and thus the NE alone is an unsuitable DNA

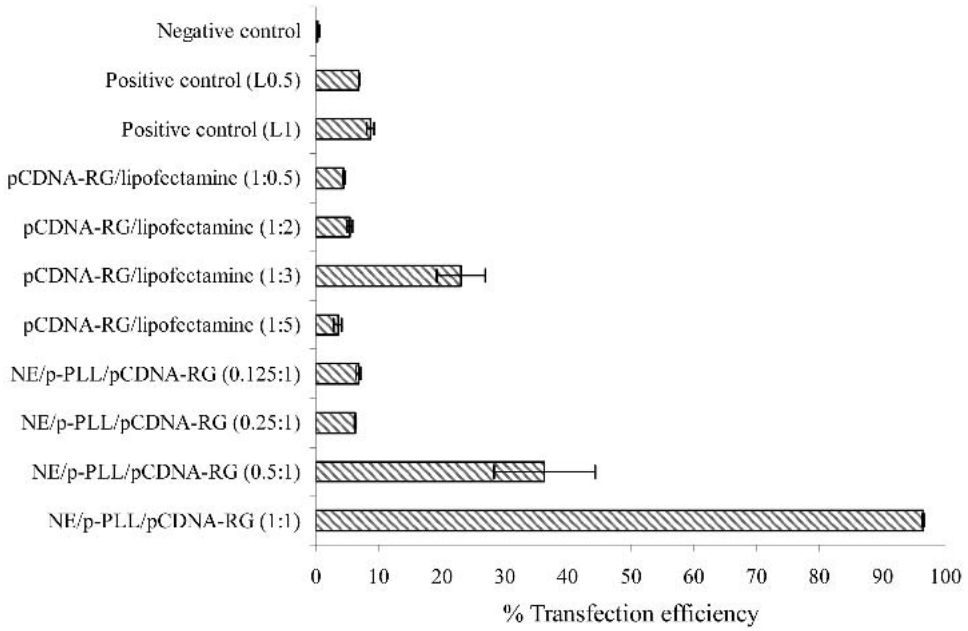

Fig. 9. Percentage transfection efficiency in human glioma SF-767 cell line with the artificial lipoprotein formulation and lipofectamine 2000. Lane 1 (top) is the negative control (NE–p-PLL–pCDNA-3). Lanes 2 (L0.5) and 3 (L1) are the positive controls (p-PLL–pCDNA-RG) and the quantity of p-PLL added was equivalent to that in lanes 10 and 11, respectively. Lanes 4, 5, 6, and 7 refer to the transfection using lipofectamine 2000 with pCDNA-RG–lipofectamine ratio of 1:0.5, 1:2, 1:3, and 1:5, respectively (pCDNA-RG quantity was kept constant). Lanes 8, 9, 10, and 11 refer to the transfection using artificial lipoprotein system (NE–p-PLL–pCDNA-RG) with the p-PLL to triolein ratio of 0.125:1, 0.25:1, 0.5:1, and 1:1, respectively (pCDNA-RG quantity was kept constant) (n = 3 for all experiments).

carrier (Fig. 5, lane 1, DNA was not held by NE). Therefore, poly-L-lysine was chemically lipidized and incorporated into the nanoemulsion through hydrophobic interactions (11) to condense and carry DNA molecules on the complex surface. Incorporation of p-PLL into NE was successfully achieved, and the addition of p-PLL in amounts ranging from 0.125 to 2, as the p-PLL:triolein ratios, was capable of retaining the electrophoretic mobility of the NE indicating the neutralization of the NE surface charge (Fig. 4).

The assembly of the artificial lipoprotein carrier with DNA can be represented by Fig. 1. The schematic illustration showed that free cationic polyamine group of p-PLL interacts with negatively charged DNA. When DNA binds to polyamine group, the electrostatic repulsion among the nucleic acid phosphates are neutralized leading to DNA collapse into a compact state, reducing its volume four to six orders of magnitude less than that of uncondensed DNA (23). Condensed DNA was shown to not emit fluorescence with ethidium bromide because the condensation prevents ethidium bromide from intercalating into the DNA molecules (11). Results from gel electrophoresis (Fig. 5) showed that DNA was condensed by p-PLL (lane 2) as well as NE–p-PLL complex (lanes 4–7) but not by NE alone (lane 3). DNA molecules were held by NE–p-PLL complex when a sufficient amount of p-PLL was added. Furthermore, because cell membrane has a negatively charged surface, it is important to mask the negative charge of the DNA–carrier complex. The zeta potential and mobility of the NE–p-PLL–pCDNA-RG complexes are shown in Figs. 6 and 7, respectively, indicating that increasing the p-PLL amount can lead to reversal of zeta potential and mobility from negative to positive values while maintaining its DNA carrying capability.

Transient expression of rabies glycoprotein was visualized under fluorescence microscopy, and Fig. 8 indicates that a significant number of cells were transfected and expressed the rabies glycoprotein. The percentage transfection efficiency (%TE) was obtained through flow cytometric assay (Fig. 9). The highest transfection efficiency was achieved when the cells were incubated with the artificial lipoprotein complex composed of NE–p-PLL–pCDNA-RG at 1:1 p-PLL:triolein ratio, when the amount of pCDNA-RG was kept constant at 2 μg/50 μl of NE. The %TE of the negative control was detected at very low level (0.30 ± 0.23), indicating the sensitivity of the detection method. Increase of p-PLL amount in the artificial lipoprotein formulation resulted in an increase in the %TE. This phenomenon could be attributed to the increase in net surface positivity of the NE–p-PLL–pCDNA-RG complex, resulting in a greater affinity to interact with the negatively charged cell membrane (24). The NE–p-PLL–pCDNA-RG complexes (p-PLL:triolein ratios of 0.5:1 and 1:1) showed up to 5.3-fold and 11-fold increase in the %TE compared to p-PLL–pCDNA-RG complex when equivalent amounts of p-PLL and pCDNA-RG were used, respectively. The results clearly demonstrated the important role of the artificial lipoprotein carrier system. The highest %TE with lipofectamine 2000 was 23 ± 3.85 and it was achieved with 1:3 ratio (pCDNA-RG:lipofectamine) according to the manufacturer's recommended protocol. Compared to the lipofectamine system, the artificial lipoprotein carrier system for DNA illustrated about a 4.2-fold increase in %TE based on the highest %TE, indicating the high effectiveness of the new carrier system.

In conclusion, a novel artificial lipoprotein system has been developed as a new carrier for DNA vaccines. The amount of p-PLL incorporated into the artificial lipoprotein formulations had a significant effect on transfection efficiency. The new system demonstrated a highly effective transfection capability of rabies DNA vaccine in cell culture. The new system also proved to be more efficient in cellular transfection of rabies DNA vaccine than the commercial lipofectamine 2000 formulation.

ACKNOWLEDGMENT

The author would like to thank Chakravathy Chennareddy (Department of Veterinary Pathology, University of Georgia) for his assistance in amplification of plasmid DNA encoding rabies glycoprotein.

REFERENCES

1. Z. Cui and R. J. Mumper. Microparticles and nanoparticles as delivery systems for DNA vaccines. *Crit. Rev. Ther. Drug Carrier Syst.* **20**:103–137 (2003).
2. J. A. Wolff, R. W. Malone, P. Williams, W. Chong, G. Acsadi, A. Jani, and P. L. Felgner. Direct gene transfer into mouse muscle in vivo. *Science* **247**:1465–1468 (1990).
3. S. Liljeqvist and S. Stahl. Production of recombinant subunit vaccines: protein immunogens, live delivery systems and nucleic acid vaccines. *J. Biotechnol.* **73**:1–33 (1999).
4. T. Beyer, M. Herrmann, C. Reiser, W. Bertling. and J. Hess. Bacterial carriers and virus-like-particles as antigen delivery devices: role of dendritic cells in antigen presentation. *Curr. Drug Targets Infect. Disorders* **1**:287–302 (2001).
5. C. C. Guo, J. Ding, B. R. Pan, Z. C. Yu, Q. L. Han, F. P. Meng, N. Liu, and D. M. Fan. Development of an oral DNA vaccine against MG7-Ag of gastric cancer using attenuated salmonella typhimurium as carrier. *World J. Gastroenterol.* **9**:1191–1195 (2003).
6. K. Lundstrom. Alphavirus vectors for gene therapy applications. *Curr. Gene Ther.* **1**:19–29 (2001).
7. J. Navarro, N. Oudrhiri, S. Fabrega, and P. Lehn. Gene delivery systems: Bridging the gap between recombinant viruses and artificial vectors. *Adv. Drug Deliv. Rev.* **30**:5–11 (1998).
8. C. R. Dass. Cytotoxicity issues pertinent to lipoplex-mediated gene therapy in-vivo. *J. Pharm. Pharmacol.* **54**:593–601 (2002).
9. H. Madry, R. Reszka, and J. Bohlender. and J. Wagner. Efficacy of cationic liposome-mediated gene transfer to mesangial cells in vitro and in vivo. *J. Mol. Med.* **79**:184–189 (2001).
10. M. C. Filion and N. C. Phillips. Toxicity and immunomodulatory activity of liposomal vectors formulated with cationic lipids toward immune effector cells. *Biochim. Biophys. Acta* **1329**:345–356 (1997).
11. G. Pan, M. Shawer, S. Øie, and D. R. Lu. In vitro gene transfection in human glioma cells using a novel and less cytotoxic artificial lipoprotein delivery system. *Pharm. Res.* **20**:738–744 (2003).
12. D. A. Bull, S. H. Bailey, J. J. Rentz, J. S. Zebrack, M. Lee, S. E. Litwin, and S. W. Kim. Effect of Terplex/VEGF-165 gene therapy on left ventricular function and structure following myocardial infarction. VEGF gene therapy for myocardial infarction. *J. Controll. Rel.* **93**:175–181 (2003).
13. J. M. Benns and S. W. Kim. Tailoring new gene delivery designs for specific targets. *J. Drug Target.* **8**:1–12 (2000).
14. T. Hara, Y. Tan, and L. Huang. In vivo gene delivery to the liver using reconstituted chylomicron remnants as a novel nonviral vector. *Proc. Natl. Acad. Sci. U. S. A.* **94**:14547–14552 (1997).
15. Z. Q. Xiang, S. L. Spitalnik, J. Cheng, J. Erikson, B. Wojczyk, and H. C. Ertl. Immune responses to nucleic acid vaccines to rabies virus. *Virology* **209**:569–579 (1995).
16. N. B. Ray, L. C. Ewalt, and D. L. Lodmell. Nanogram quantities of plasmid DNA encoding the rabies virus glycoprotein protect mice against lethal rabies virus infection. *Vaccine* **15**:892–895 (1997).
17. J. E. Osorio, C. C. Tomlinson, R. S. Frank, E. J. Haanes, K. Rushlow, J. R. Haynes, and D. T. Stinchcomb. Immunization of

dogs and cats with a DNA vaccine against rabies virus. *Vaccine* **17**:1109–1116 (1999).
18. Z. F. Fu, C. E. Rupprecht, B. Dietzschold, P. Saikumar, H. S. Niu, I. Babka, W. H. Wunner, and H. Koprowski. Oral vaccination of raccoons (Procyon lotor) with baculovirus-expressed rabies virus glycoprotein. *Vaccine* **11**:925–928 (1993).
19. M. Shawer, P. Greenspan, S. Øie, and D. R. Lu. VLDL-resembling phospholipid-submicron emulsion for cholesterol-based drug targeting. *J. Pharm. Sci.* **91**:1405–1413 (2002).
20. J. S. Kim, A. Maruyama, T. Akaike, and S. W. Kim. In vitro gene expression on the smooth muscle cells using a terplex delivery system. *J. Controll. Rel.* **47**:51–59 (1997).
21. A. L. Davis. Gene based vaccine. In A. Rolland (ed.), *Advance Gene Delivery Book*, Amsterdam: Harwood academic, 1999, pp. 213–233.
22. L. Yu, H. Suh, J. J. Koh, and S. W. Kim. Systemic administration of TerplexDNA system: pharmacokinetics and gene expression. *Pharm. Res.* **18**:1277–1283 (2001).
23. J. G. Duguid and R. H. Durland. DNA packing in non-viral systems. In A. Rolland (ed), *Advance Gene Delivery Book*, Amsterdam: Harwood academic, 1999, pp. 45–63
24. S. Han, R. I. Mahato, Y. K. Sung, and S. W. Kim. Development of biomaterials for gene therapy. *Mol. Ther.* **2**:302–317 (2000).