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In vitro and in vivo Transient Expression in Insect Cells Mediated by the Cationic Liposome DDAB/DOPE

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Cationic liposomes complexed with DNA have been extensively utilized for the delivery of reporter or therapeutic genes both in culture and in vivo. We investigated and determined the optimum conditions of a cationic liposome, composed of dimethyldioctadecylammonium bromide (DDAB) and dioleoyl phosphatidylethanolamine (DOPE), mediated a reporter plasmid expressing luciferase into insect cell lines (Sf-21 and Bm-N) and silkworm larvae. Together the data demonstrated that *Bombyx mori* nuclear polyhedrosis virus (BmNPV) genomic DNA (128 kb) was successfully transfected into Bm-5 cells using this liposome. These results suggest that DDAB/DOPE liposome will be useful as delivery agents for gene transfer to insect cells both in vitro and in vivo.

Key words : Cationic liposome, Transient transfection, Insect cells, Silkworm larvae

Introduction

In gene expression regulation and gene therapy studies, transfection method has been commonly used to delivery functional genes into cells. Since Felgner *et al.* (1987) reported the effective transfection of mammalian cells mediated by cationic liposomes, which have been successfully used to delivery DNA (Caplen *et al.*, 1995; Fortunati *et al.*, 1996; Jarnagin *et al.*, 1992; Liu *et al.*, 1995; Murray *et al.*, 1999; Stewart *et al.*, 1992), RNA (Kariko *et al.*, 1998; Lu *et al.*, 1994; Malone *et al.*, 1989), oligonucleotides (Bennett *et al.*, 1992; Geromel *et al.*, 2001) and proteins (Debs *et al.*, 1990; Walker *et al.*, 1992) into various cells both in vitro and in vivo. DNA could interact spontaneously with cationic liposomes to form lipid-DNA complexes, and this complex formation was presumably due to ionic interactions between the positively charged group on the cationic lipid molecule and the negatively charged phosphate groups on the DNA (Felgner *et al.*, 1987). Several cationic liposomes generally prepared by evaporation and sonication, are now commercially available; however, their large-scale use is confined for the great expense. Recently, Campbell (1995) reported using the solvent-injection method to prepare cationic liposomes for DNA transfection. The efficiency of these cationic liposomes in delivering DNA into a variety of mammalian cell lines was confirmed.

In the present study, a DNA delivery reagent, composed of a commercial available cationic lipid dimethyldioctadecylammonium bromide (DDAB) and a neutral helper lipid dioleoyl phosphatidylethanolamine (DOPE), was developed via the injection method. The efficacy of this cationic liposome reagent mediated DNA transfer into insect cells both in culture and in vivo was demonstrated.

Materials and Methods

Lipids and preparation of liposome

DDAB and DOPE were purchased from Sigma Chemical Co. (MO, USA). Lipofectin Reagent was obtained from Life Technologies (MD, USA). DDAB/DOPE liposome (1:2 molar ratio) was prepared as described (Campbell, 1995) with a slight modification. DDAB (6.6 μmol) and DOPE (13.4 μmol) were dissolved in 1.0 ml of absolute ethanol, then 142 μl of this solution was rapidly injected into 858 μl of sterile distilled water while vortex mixing.

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The final concentration was 2 mg/ml.

Cell lines and silkworm variety

The *Spodoptera frugiperda* cell line (Sf-21) and the *Bombyx mori* cell line (Bm-N, Bm-5) were maintained in TC-100 medium (Gibco, USA) with 10% fetal bovine serum (FBS) and incubated at 27°C. The JY1 strain of silkworm *B. mori* was used. Larvae were routinely fed on mulberry leaves as described (Lu, 1991).

Preparation of liposome-DNA complexes

The transient expression plasmid pBmIE1.luc, containing a firefly luciferase reporter gene driven by an immediately-early *(ie-1)* gene promoter of *Bmbyx mori* nuclear polyhedrosis virus (BmNPV), was constructed in our laboratory. BmNPV genomic DNA was extracted from the hemolymph of virus-infected 5th instar silkworm larvae as described (Maeda, 1989). Complexes were made by adding varying amounts of liposome to 100 μl of sterile distilled water containing 1 μg of pBmIE1.luc plasmid DNA. This solution was mixed gently and incubated at room temperature for 15 min, allow binding of the DNA to the liposome. These samples were then electrophoresed on a 1% agarose gel and complexes was demonstrated by a gel retardation assay.

Transfection of cultured insect cells and silkworm larvae

Cells to be transfected were seeded at a density of 5×10^5 cells per 15 cm^2 flask in TC-100 medium containing 10% FBS. On the following day, the medium was removed and replaced with 1.5 ml of TC-100 without serum plus 100 μl of transfection solution. After incubating the cells for 4 hrs at 27°C, the basal medium was removed and 3 ml of TC-100 medium containing 10% FBS was added. The cells were cultured for an additional 48 hrs. Similarly, 20 μl of transfection solution containing 1 μg of plamid DNA and varying amounts of liposome was microinjected into each 5th instar silkworm larvae at 48 hrs after molting. The larvae were fed for an additional 48 hrs.

Luciferase reporter gene assay

Cultured cells or the hemolymph of silkworm larvae were harvested at 48 hrs post transfection, centrifuged at 4°C, washed in PBS and resuspended in 500 μl of Reporter Lysis Buffer (Promega, USA). The cells were lysed by a single freeze-thaw, and the debris was removed by a brief centrifugation. Twenty microliters of cell lysate were assayed for luciferase activity in a micro test tube containing Luciferase Assay Reagent (Promega, USA) using a Liquid Scintillation Spectrometer (Beckman, USA) as described (Idahl *et al.*, 1986). The amount of protein in the lysate was determined using the bradford method as described (Smith, 1995).

Results

Formation of liposome-DNA complexes

Using a simple and rapid injection method, we prepared a liposome reagent composed of the cationic lipid DDAB and a neutral lipid DOPE, in a 1:2 molar ration. This ratio of DDAB to DOPE was demonstrated to be efficient in mediating transfection of several mammalian cell lines (Rose *et al.*, 1991). The liposome-DNA formation was examined by an agarose gel electrophoresis. As shown in Fig. 1, in the absence of liposome, DNA migrated into the gel (lane 1). However, with the increasing amounts of liposome, the plasmid DNA was partially or entirely retained in the sample well. At a 10 nmol of liposome:1 μg of DNA (lane 3) ratio, the DNA was nearly bound by liposome. When the ratio was 15:1 (lane 4) and 20:1 (lane 5), DNA was completely bound and, failed to migrate into the gel.

Optimization of the transfection protocols

DDAB/DOPE liposome was next tested for their ability to transfect insect cells. Cells transfected with the plasmid pBmIE1.luc expressing Luciferase activity were measured at 48 hrs post transfection. The transfection technique was optimized for the transient expression assay, using two insect cell lines, Sf-21 and Bm-N. These cell lines are very useful for the production of heterologous proteins by baculovirus expression vector systems. Silkworm has

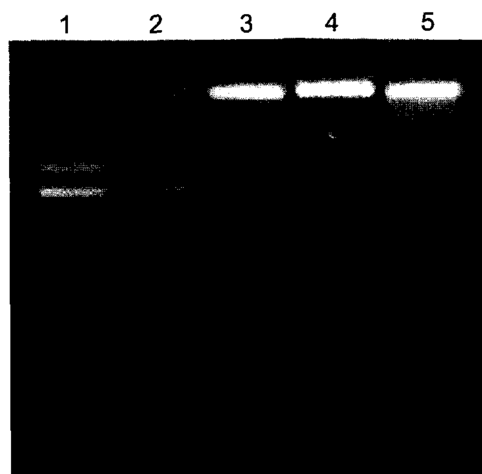

Fig. 1. Electrophoretic mobility of liposome-DNA complexes. Various DDAB/DOPE liposomes were complexed with 1 μg of plasmid DNA as described in the text. Lane 1, DNA alone; lane 2 to lane 5, 5, 10, 15, 20 nmol of liposome, respectively.

been domesticated for thousands of years because of its economic importance for the silk production. In addition, silkworm together with fruitfly traditionally has been utilized as a model organism for the genetic and physiological studies. Recently, silkworm has also been used as "bio-factory" to produce useful proteins via vector systems, such as nuclear polyhedrosis virus (Choudary *et al.*, 1992; Maeda *et al.*, 1985). Thus, we examined the transient expression of the reporter plasmid in the silkworm larvae mediated by DDAB/DOPE liposome prepared by injection. Under the condition with 10 nmol of liposome, only 1 μg of pBmIE1.luc was required for optimal transfection of Sf-21 cells. With the increasing amounts of adding DNA, the luciferase activity decreased (Fig. 2A). There was a significant decrease of enzyme activity with lower DNA concentration (0.25 μg), whereas high DNA levels also suppressed the transfection efficiency. The optimal liposome concentrations were determined using Sf-21, Bm-N cell lines and Silkworm larvae (Fig. 2B). One microgram of plasmid DNA was employed in each experiment. Optimal amounts of DDAB/DOPE liposome were different for the above two cell lines, 10 nmol for Sf-21 cells while 15 nmol for Bm-N cells. Transfection efficiency in Sf-21 cells was relatively insensitive to liposome concentration, only a 3- to 4-fold difference in luciferase activity was observed when 5-20 nmol of DDAB/DOPE was used. However, there was more than a 10-fold difference between the highest and the lowest expression level in Bm-N cells. Similar to Sf-21 cells, the most satisfactory transfection in silkworm larvae was obtained with 10 nmol of liposome. High amounts of DDAB/DOPE appeared to be toxic in transfected cells, by which cell death increased.

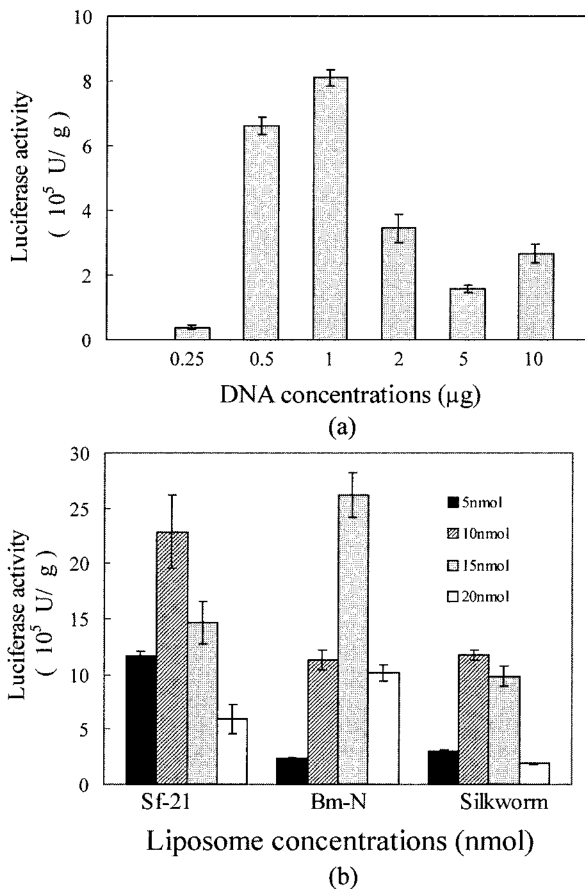

Fig. 2. Transfection of insect cell lines and silkworm larvae with plasmid pBmIE1.luc and DDAB/DOPE. (A) Sf-21 cells were transfected with various concentrations of DNA and 10 nmol of liposome. Each reaction contained 1 μg of cell protein. (B) Sf-21, Bm-N cell lines and silkworm larvae were transfected with various concentrations of liposome and 1 μg of DNA. Each reaction contained 1 μg of protein from Sf-21, Bm-N cell extract or 4 μg of protein from the hemolymph cell extract of silkworm larvae. The results represented averages from triplicate transfections.

DDAB/DOPE liposome vs. Lipofectin Reagent for transient transfection of Sf-21 cells and silkworm larvae

The ability of DDAB/DOPE to facilitate functional gene expression was compared with a commercial liposome, Lipofectin Reagent in Fig. 3. At a 10 nmol of liposome:1 μg of DNA ratio, DDAB/DOPE yielded an 8- to 10-fold increase in luciferase activity relative to transient transfection with an optimized dose of Lipofectin Reagent in both Sf-21 cells and silkworm larvae.

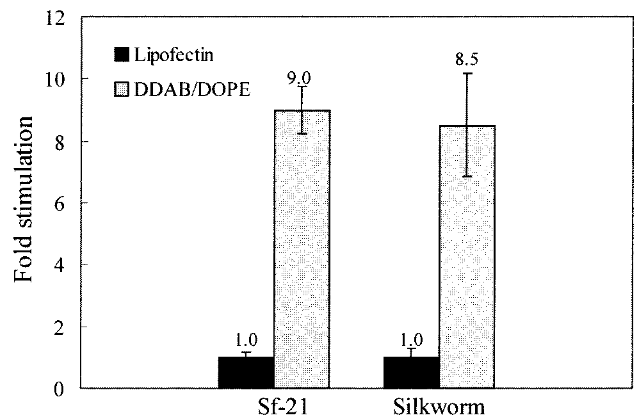

Fig. 3. Comparison of transfection mediated by DDAB/DOPE and Lipofectin Reagent. Sf-21 cells and silkworm larvae were transfected with 1 μg of plasmid pBmIE1.luc and 10 nmol of DDAB/DOPE, or with the optimum dose of Lipofectin Reagent. Luciferase activity was presented as the fold stimulation over Lipofectin Reagent-mediated transfection that was arbitrarily set as a 1.0. The results represented averages from triplicate transfections.

DDAB/DOPE liposome mediated BmNPV DNA transfer into Bm-5 cells

To demonstrate that DDAB/DOPE could be used to introduce larger DNA molecule into insect cells, we examined the transfection of Bm-5 cells with 128 kb of BmNPV genomic DNA. During a late stage of infection, BmNPV produce inclusion bodies called polyhera in the infected nucleus. The major component of polyhedra is a hyper-expressed protein called polyhedrin. The polyhedrin gene is nonessential for viral replication. Using the polyhedrin promoter, BmNPV has been utilized as an efficient vector for the high-level expression of foreign genes in *B. mori* cell lines and silkworm larvae (Choudary *et al.*, 1992; Maeda, 1989). Polyhedra have the crystalline proteinaceous structures, which are easily recognized under the optical microscope. As shown in Fig. 4, a large quantity of polyhedra appeared in Bm-5 cells post transfection with BmNPV DNA mediated by DDAB/DOPE liposome. Bm-5 cells exhibited virus-infected symptom at 3 days post transfection, followed by polyhedra were found in most cells under the examination on an inverted microscope. The result indicated that DDAB/DOPE could efficiently mediate large viral genome transfer into insect cells.

Discussion

Cationic liposomes have been successfully used for transfection of various cell lines. In this study, we described the use of DDAB/DOPE liposome to facilitate the functional delivery of DNA into cultured insect cells and silkworm larvae. It was reported that DOTMA could entrap the polynucleotide in the complex interior (Felgner and Ringold, 1989); however, DNA was bound to the surface of DDAB/DOPE prepared by injection technique, rather than being entrapped within the vesicles during formation (Campbell, 1995).

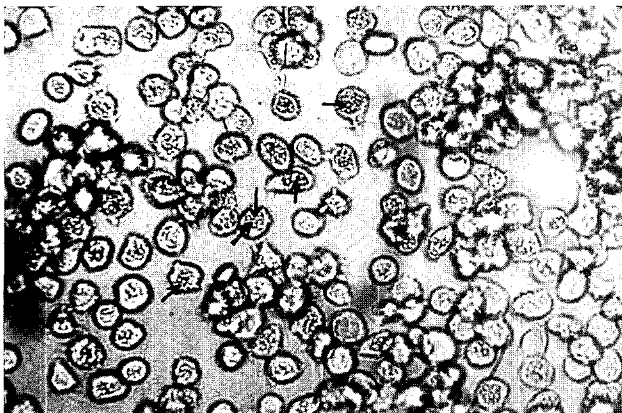

Fig. 4. BmNPV polyhedra produced in Bm-5 cells transfected with DDAB/DOPE liposome. Polyhedra were marked with arrows.

The factors controlling cationic liposome-DNA complex-based gene transfer in cells and in animals have been understood to a certain extent (Mounkes *et al.*, 1998; Sakurai *et al.*, 2001). To examine the optimal conditions for DDAB/DOPE-mediated transfections of insect cells, complexes with different amounts of reporter plasmid expressing luciferase and appropriate liposome were used to transfect Sf-21 cells. Only 1 μg of plasmid was required for an optimal signal in transient transfections. We then tested the liposome concentration for the satisfactory transfections of Sf-21 cells, Bm-N cells and silkworm larvae in the presence of 1 μg of DNA. It was found that the optimal amounts of DDAB/DOPE were different for the above two cell lines, 10 nmol for Sf-21 and 15 nmol for Bm-N, respectively. This was in agreement with that the concentration of lipid used in a transfection depended on the cell line (Felgner *et al.*, 1987; Peters *et al.*, 1999). DDAB/DOPE liposome could achieve high transfection levels in insect cell lines, we therefore wished to assess transfection efficiency in silkworm larvae with the reporter plasmid pBmIE1.luc in order to mimic an in vivo model. A simple and effective liposome-DNA complex administration, microinjection of DDAB/DOPE and plasmid DNA into the hemolymph of silkworm larvae, was evaluated. The luciferase activity efficiently expressed in the hemolymph cells was detected, and the optimal liposome:DNA ratio was 10 nmol:1 μg. The ratio of liposome to DNA is critical to the reagents efficacy (Felgner *et al.*, 1989). In our study, The optimum ratio of DDAB/DOPE:DNA was 10-15 nmol:1 μg, and this result correlated with the previous finding of gel retardation (Fig. 1). At these ratios, the plasmid DNA was bound by liposome on the whole. Although DNA was completely bound to the liposome at the ratio of 20:1 (20 nmol of liposome:1 μg of DNA), there was a decrease of luciferase activity in transient transfections. It was indicated that high levels of liposome appeared to be toxic to cells. Theoretically, the liposome-DNA complexes should have a net positive charge to bind to target cells and fuse with the negatively charged plasma membrane (Felgner *et al.*, 1987, 1989). However, Pires *et al.* (1999) reported that fusion of the cationic liposomes with cells occurred mainly at the plasma membrane level, and no correlation between fusion of the lipid-DNA complexes with the plasma membrane and the levels of transfection. In addition, the previous study indicated that endocytosis seemed to be the main uptake pathway of DNA-cationic liposome complexes, while fusion between cationic liposomes and the cell membrane played a secondary role in determining transfection efficiency (Hui *et al.*, 1996). It was also reported that the topology of the cationic liposome-DNA complexes might not allow the entry of DNA

into cells through a fusion process at the plasma membrane (De Lima *et al.*, 1999). Thus, the relationship between the features of the liposome-DNA complexes and their mode of interaction with cells, the efficiency of gene transfer and gene expression remain to be clarified. Based on previous study and our experience, it is best to optimize the concentration for each specific system to be studied. Furthermore, We found here that the transfection efficiency of cultured insect cells in the presence of 10% FBS, was less 1% than that of serum-free (data not shown). This suggested that there were some transfection inhibitors in serum, and which was also demonstrated by the previous reports (Crook *et al.*, 1998; Pires *et al.*, 1999; Yang and Huang, 1997). This inhibitory effect could be overcome by increasing the charge ratio of cationic liposome to DNA (Crook *et al.*, 1998). De Lima *et al.* (1999) reported that the ternary complexes of cationic liposomes, DNA, and protein or peptide had the advantages of being active in the presence of serum and non-toxic to cells.

In the present study, we found that the transfection efficiency of DDAB/DOPE liposome, prepared by the injection method, was 8- to 10-fold than that of a commercial Lipofectin Reagent in insect cells both in vitro and in vivo. Moreover, this liposome could successfully mediated 128 kb of BmNPV DNA transfer into Bm-5 cells. All of these findings indicate that DDAB/DOPE liposome is an inexpensive, highly efficient and reproducible alternative for the transfection of insect cells and is well suited for systemic gene transfer to insect individuals.

Acknowledgments

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