ELSEVIER

International Journal of Pharmaceutics 206 (2000) 97–104

international journal of pharmaceutics

www.elsevier.com/locate/ijpharm

High-efficiency gene transfection of macrophages by lipoplexes

Sujatha Dokka [a], David Toledo [b], Xianglin Shi [c], Jianping Ye [c], Yon Rojanasakul [a,*]

[a] *Department of Basic Pharmaceutical Sciences, West Virginia University, P.O. Box 9530, Morgantown, WV 26506, USA*
[b] *Rhone-Poulenc Rorer Central Research, Collegeville, PA 19426, USA*
[c] *Pathology and Physiology Research Branch, National Institute for Occupational Safety and Health, Morgantown, WV 26505, USA*

Received 21 December 1999; received in revised form 18 July 2000; accepted 18 July 2000

Abstract

Macrophage transfection studies are crucial for understanding gene regulation and expression. However, gene transfection in macrophages is difficult. We have shown here that macrophages are more resistant to gene transfection compared with other cell types. To further develop an efficient gene delivery system for macrophages, we evaluated various liposomal and non-liposomal agents including LipofectAMINE®, Lipofectin®, DOTAP, DEAE-dextran, and the DNA condensing agent protamine sulfate for their ability to promote gene transfection. CMV-luciferase was used as a reporter plasmid. Macrophage transfection was maximal at the DNA:LipofectAMINE:protamine ratio of 1:12:1 μg/ml. The LipofectAMINE formulation showed a 10–12-fold increase in transfection efficiency over DOTAP and a 4–5-fold increase over Lipofectin. This transfection method showed minimal toxicity at the concentrations tested and was at least 20–25-fold superior to the most frequently used DEAE-dextran method for macrophage transfection.

Keywords: Macrophages; Gene transfection; Liposomes; DEAE-dextran; Protamine

1. Introduction

Macrophages play an important role in host defense against noxious substances and are involved in a variety of disease processes including autoimmune diseases, infections and inflammatory disorders (Pierce, 1990). Molecular analysis of macrophage functions can be accomplished by gene transfection assays. However, gene transfection in macrophages has proven difficult. A number of non-viral gene transfer methods have been developed to improve macrophage transfection, including calcium-phosphate precipitation (Roussel et al., 1988; Stief et al., 1989), DEAE-dextran (Hess and Clements, 1985; Rupprecht and Cole-

* Corresponding author: Tel.: +1-304-2931476; fax: +1-304-2935483.
E-mail address: yrojanasakul@hsc.wvu.edu (Y. Rojanasakul).

0378-5173/00/$ - see front matter
PII: S0378-5173(00)00531-7

man, 1991; Mack et al., 1998), particle bombardment (Burkholder et al., 1993), and electroporation (Daumler and Zimmermann, 1989; Economou et al., 1989). These methods are, however, associated with low transfection efficiency or high cellular toxicity. Being non-dividing cells, macrophages are also resistant to viral-mediated gene transfection, e.g. retroviruses. Adenoviral vectors, although capable of transfecting non-dividing cells, are associated with immunogenicity, and thus their use has been limited.

The DEAE-dextran method of transfection has been described as the most efficient gene transfer method for macrophages developed thus far (Mack et al., 1998). This method was shown to be more efficient than the other existing methods including the calcium phosphate, electroporation and lipofection (Mack et al., 1998). In our experience, this method provides a reasonable but still relatively low transfection efficiency. In this study, we further developed gene transfection systems for macrophages using a variety of liposomal agents. We optimized the transfection efficiency of these systems and compared them with the DEAE-dextran method. In addition, we tested the effect of protamine sulfate on liposome-mediated gene transfer. Protamine is a major component of sperm nucleus with a role in DNA stabilization. This compound has been shown to cause condensation of DNA which promotes the cellular entry and gene expression of plasmid DNA (Gao and Huang, 1996; Sorgi et al., 1997). Our results showed that the optimized liposomal systems were more effective and less toxic than the DEAE-dextran and that protamine greatly enhanced the level of transgene expression mediated by liposomes.

2. Materials and methods

2.1. Cell culture

All cell lines including the macrophage RAW 264.7, alveolar epithelial A549, kidney embryonic 293, and liver HEPG2 were obtained from the American Type Cell Culture Collection (Rockville, MD). The cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 100 U/ml penicillin–streptomycin. They were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Prior to use, cells were briefly trypsinized or mechanically scraped and centrifuged. They were plated at $\sim 1 \times 10^6$ cells/ml in 12-well tissue culture plates one day before transfection studies.

2.2. Plasmid DNA

The expression vector CMV-luciferase contains the promoter-enhancer region of Cytomegalovirus upstream from the luciferase gene (kindly provided by Dr Leaf Huang, University of Pittsburgh). The plasmid was purified using the Qiagen Endofree plasmid kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions.

2.3. Liposomal transfection

Approximately 1×10^6 cells were plated on a 12-well plate and allowed to grow for 24 h before the transfection. The plasmid DNA (0.5–2 μg/ml) was diluted in 200 μl of DMEM. In some studies, protamine sulfate (0.5–2 μg/ml) was also added to the DNA. The liposome (3–15 μg/ml) was diluted in 200 μl of DMEM. The diluted DNA and liposome were combined and incubated at room temperature for 15–20 min. Cells with transfection reagents were incubated for 4 h according to the manufacturer's instructions. This incubation time was also found to provide optimum transgene expression in our system. Transfection medium was then replaced with growth medium containing 10% FBS. Cells were cultured for an additional 48 h before the level of gene expression was determined. All transfections were conducted under sterile conditions and duplicate plates were tested for each condition.

2.4. DEAE-dextran transfection

Approximately 1×10^6 cells were plated on a 12-well plate and allowed to grow for 24 h before the transfection. The plasmid DNA (1 μg/ml) was mixed with varying concentrations of diethyl-

aminoethyl-dextran (0–200 μg/ml) (Sigma, St. Louis, MO) in DMEM. The diluted DNA and DEAE-dextran were added to pre-washed cells. Wrapped plates were incubated at 37°C for 2 h. Longer incubation times, e.g. 4 h, resulted in lower transgene expression and increased cellular toxicity. After incubation, the cells were washed and incubated with growth medium containing 10% FBS. The cells were then cultured for an additional 48 h before the level of gene expression was determined. All transfections were conducted under sterile conditions and duplicate plates were tested for each condition.

2.5. Measurement of luciferase activity

Luciferase synthesized during the in vitro translation was quantitated by the assay of enzyme-dependent light production using a luciferase assay kit (Promega, Madison, WI). Cells were washed twice with PBS and incubated at room temperature for 10 min in the presence of 250 μl of lysis buffer (Promega) and then centrifuged at $12\,000 \times g$. Ten microliters of each sample was placed in a 5-ml polystyrene test tube and the tubes were then loaded into an automated luminometer (BioRad, Hercules, CA). At the time of measurement, 100 μl of luciferase substrate was automatically injected into each sample, and total luminescence was measured over a 20-s time interval. Output is quantitated as relative light units (RLU). Protein concentration in the supernatant was determined by BCA protein assay reagent (Pierce, Rockford, IL). Luminescence detected was standardized per μg protein present in the supernatant.

2.6. Measurement of LDH activity

LDH assay was performed to assess the effect of test agents on cellular toxicity. Cells were treated with plasmid DNA and transfecting agents, either individually or in combination as indicated. After the treatments, the cell supernatants were collected and assayed for LDH activity. LDH activity was determined by monitoring the oxidation of pyruvate coupled with the reduction of NAD at 340 nm using an LDH assay kit (Roche Diagnostic Systems, Montclair, NJ). The assay was performed on Cobas Fara II Analyzer (Roche Diagnostic Systems). One unit per liter of LDH activity is defined as the amount of enzyme that converts 1 μmol of lactate to 1 μmol of pyruvate with the concomitant reduction of 1 μmol of NAD to 1 μmol of NADH per minute per liter of sample in the assay procedure.

2.7. Measurement of particle size

Particle size measurements were performed using dynamic laser scattering (Coulter N4SD particle sizer, Hialeah, FL). DNA samples (1 μg/ml) containing LipofectAMINE (12 μg/ml) with or without protamine (1 μg/ml), or DEAE-dextran (100 μg/ml) were prepared as described above. Particle size was determined after a 15-min incubation period at room temperature. The data are represented as the mean ($n = 5$) diameter with standard deviation (S.D.).

3. Results and discussion

3.1. Comparison of gene transfection in different cell lines

Gene transfection is known to be cell type-dependent. In our experience we found that macrophages are difficult to transfect. To substantiate this point, we carried out a comparison study in which various cell lines from different origins including the macrophage RAW 264.7, kidney embryonic 293, alveolar epithelial A549 and liver HEPG2 were transfected with the CMV-luciferase reporter plasmid. Gene transfection was carried out under the same transfection conditions using LipofectAMINE as a transfecting agent. Fig. 1 shows that all the four cell lines were minimally transfected in the absence of liposome. With liposome, the transfection efficiency was greatly enhanced in all cell lines tested. However, the level of enhancement was most pronounced in the HEPG2 cells, followed by the embryonic 293, epithelial A549, and macrophage RAW 264.7 cells, respectively. These results supported previous observations that different cell lines exhibit

different levels of transfection and that macrophages are relatively difficult to transfect compared with other cell types. The basis for the low transfection in macrophages is not known but probably due to the presence of high enzymatic activities and the non-proliferating nature of this cell type. Macrophages are scavenging cells possessing a high level of digestive enzymes which could inactivate DNA and result in low transfection efficiency.

3.2. DEAE-dextran and LipofectAMINE-mediated gene transfer

To develop an efficient system for transfecting macrophages, we evaluated DEAE-dextran and LipofectAMINE for their transfection efficiency. The DEAE-dextran method of transfection is a well-established protocol that has been shown to be superior than most other methods of transfection for macrophages (Rupprecht and Coleman, 1991; Mack et al., 1998). In the present study, we further tested DEAE-dextran in our system and compared its transfection efficiency with that of the LipofectAMINE. We optimized the two transfection methods by varying the DNA-transfecting agent ratios (Fig. 2A). The DEAE-dextran gave optimal transfection at the concentration of about 100–200 μg/ml per μg DNA whereas the LipofectAMINE showed optimal transfection at 12 μg/ml per μg DNA. At their optimal concentrations, LipofectAMINE was approximately 8-fold more effective than DEAE-dextran and showed minimal cellular toxicity as demonstrated by LDH assay. Higher concentrations of DEAE-dextran, i.e. >300 μg/ml, substantially increased cellular toxicity without significantly improving gene transfer efficiency (Fig. 2B). In all the studies, no significant cytotoxicity was observed when DNA was used alone in the absence of transfecting agents. i.e. $<1\%$. It is also interesting to note that the polycationic head group of LipofectAMINE (i.e. spermine) alone when used at similar concentrations had no promoting effect on gene transfer efficiency. This result suggests that the lipid portion of LipofectAMINE is required for efficient gene transfection.

The observation that LipofectAMINE was more effective than DEAE-dextran is contradictory to a recent report by Mack et al. (1998) who showed that LipofectAMINE was toxic to the cells and thus was less effective than DEAE-dextran. The basis for this discrepancy is not clear but may be due to the differences in transfection conditions and quality of the transfecting agents, as well as the assay methods used. In the previous

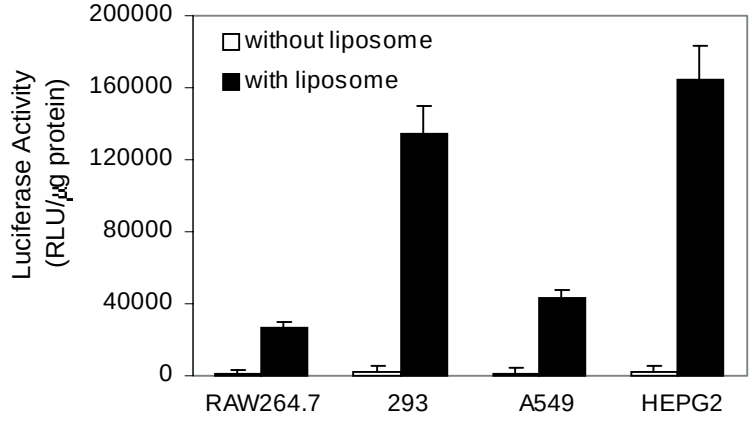

Fig. 1. Comparison of gene transfection in different cell lines. Cells (1×10^6/ml) were transfected for 4 h at 37°C with reporter gene CMV-luc (1 μg/ml) in the presence or absence of LipofectAMINE (12 μg/ml). This amount of LipofectAMINE was optimized for macrophage transfection (see Fig. 2). Two days post-transfection, the cells were washed, lysed, and measured for luciferase activity as described in Section 2. Each data point represents the mean (with S.D.) of quadruplicate samples and the data are normalized to protein content.

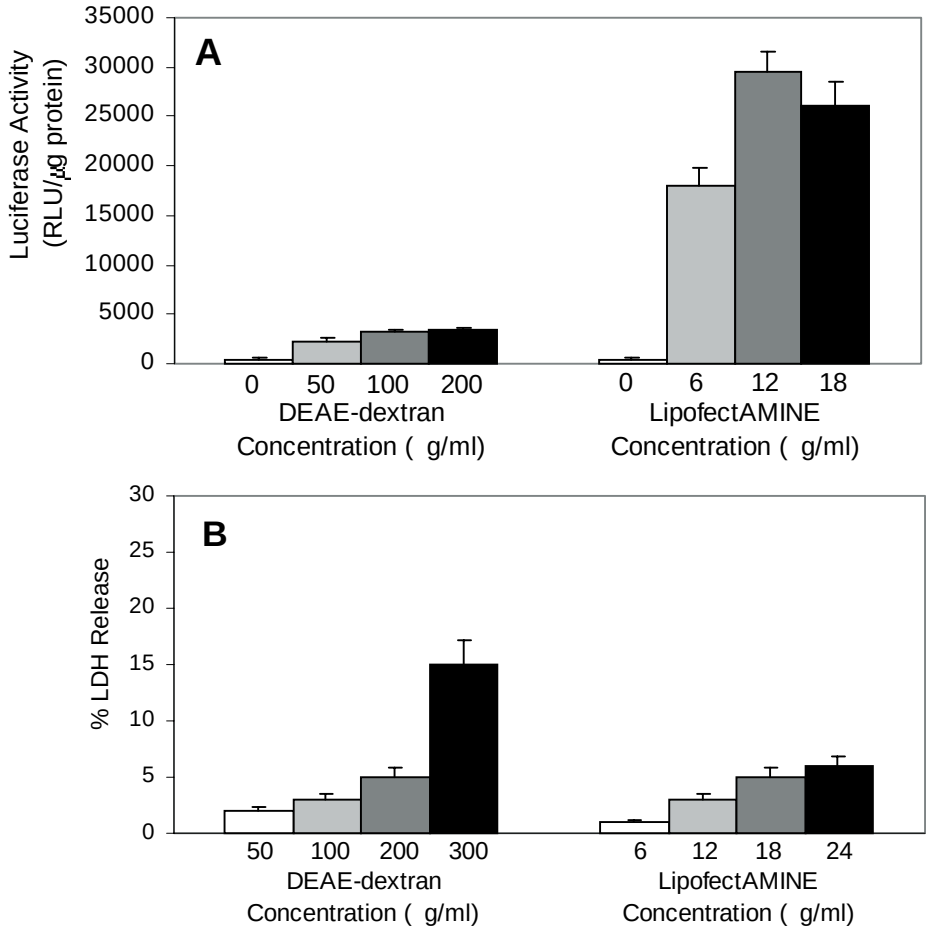

Fig. 2. Effects of DEAE-dextran and LipofectAMINE concentration on (A) transfection efficiency and (B) cellular toxicity of macrophage RAW 264.7 cells. Cells (1×10^6/ml) were incubated with transfection media that contained increasing amounts of DEAE-dextran (0–200 μg/ml) or LipofectAMINE (0–18 μg/ml) in the presence of pCMV-luc (1 μg/ml). Two days post-transfection, the cells and supernatants were collected and analyzed for luciferase and LDH activities as noted in Section 2. Each data point represents the mean (with S.D.) of quadruplicate samples.

study, Mack et al. did not report how cellular toxicity was measured. It appears that a direct morphologic examination of cell death was used. In our study, a sensitive LDH assay was used to assess cellular toxicity. At the level of cellular toxicity reported in this study, no apparent cell death could be directly observed. Other possibilities that could contribute to the observed discrepancy are the difference in cell type used in the two studies and the difference in particle size of the DNA complexes prepared. Previous study by Mack et al. utilized primary cultures of macrophages while our study utilized continuous macrophage cultures. While both the cells are of macrophage origin, they may respond differently to gene transfection. The particle size of DNA complex has long been recognized as a key determinant of gene transfection efficiency. Compact DNA structures have been reported to have greater transfection efficiency due to improved DNA cellular uptake and enzymatic stability (Sorgi et al., 1997). The role of size was further tested in this study by using dynamic light scattering. Under optimized conditions, the DNA/Lipo-

fectAMINE complex (1/12 μg/ml) had a mean diameter of 376 ± 42 nm. In the presence of the condensing agent protamine sulfate (1 μg/ml), the size of the complex was reduced to 124 ± 18 nm. The DNA/DEAE-dextran complex (1/100 μg/ml) had a greater mean diameter of 743 ± 82 nm. These results are consistent with our transfection data and support the findings in this study. In the previous study, Mack et al. did not report the data on particle size of the complexes and therefore direct comparison of the two data could not be made.

3.3. Effect of protamine sulfate on transfection

Protamine sulfate has been shown to promote lipid-based gene transfer in different cell types (Gao and Huang, 1996; Sorgi et al., 1997). However, its effect on macrophages has not been reported. In this study, we tested whether protamine can also enhance gene transfer efficiency in macrophages. Fig. 3B shows a dose-dependent increase in macrophage transfection by protamine. Maximum transfection was observed at the protamine concentration of about 1 μg/ml and LipofectAMINE concentration of 12 μg/ml per μg DNA. This enhanced transfection was about 23-fold more effective than DEAE-dextran-mediated gene transfer (Fig. 3A). Protamine by itself or in combination with DEAE-dextran had no significant effect on gene transfection (results not shown). At the concentrations tested, protamine caused no significant toxic effect to the cells.

Protamine has been proposed to increase lipid-mediated gene transfection by condensing DNA into a compact structure, which promotes DNA cellular entry and enzymatic stability (Kabanov and Kabanov, 1995). Other polycationic condensing agents such as polylysine have also been shown to possess this property. However, protamine appears to be more effective than other condensing agents despite of the fact that they possess a similar condensing activity (Kabanov and Kabanov, 1995). It has been suggested that protamine, due to the presence of nuclear localization signals in its amino acid sequence, can potentiate gene expression by increasing the nuclear translocation of DNA (Sorgi et al., 1997). While we did not demonstrate such an effect of protamine in this study we found that protamine can effectively enhance transfection activities of all liposomal agents tested (see below). Because of

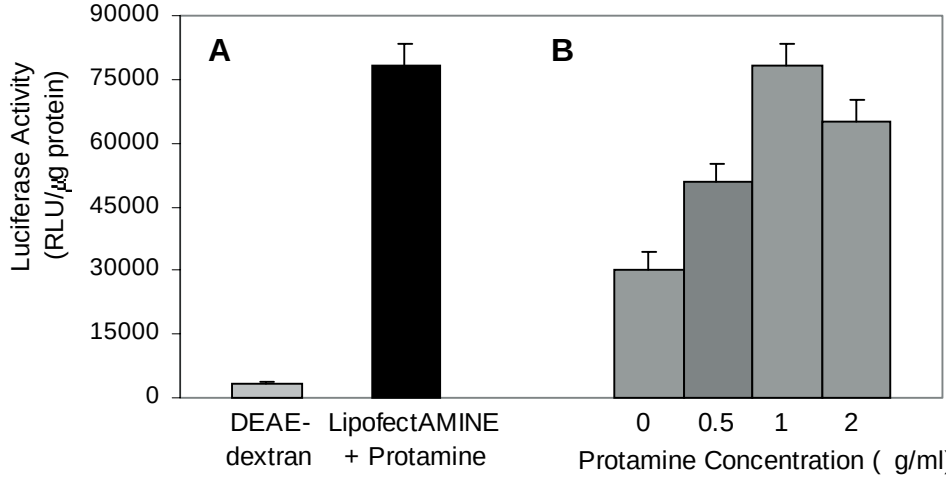

Fig. 3. Effect of protamine sulfate on LipofectAMINE-mediated gene transfection. (A) Comparison of gene transfection mediated by DEAE-dextran (100 μg/ml) and LipofectAMINE/protamine (12/1 μg/ml) in macrophage RAW 264.7 cells. (B) Varying amounts of protamine sulfate (0–2 μg/ml) were added to transfection media containing LipofectAMINE (12 μg/ml) and pCMV-luc (1 μg/ml). Analysis of luciferase gene expression was performed as described in the Section 2. Each data point represents the mean (with S.D.) of quadruplicate samples and the data are normalized to protein content.

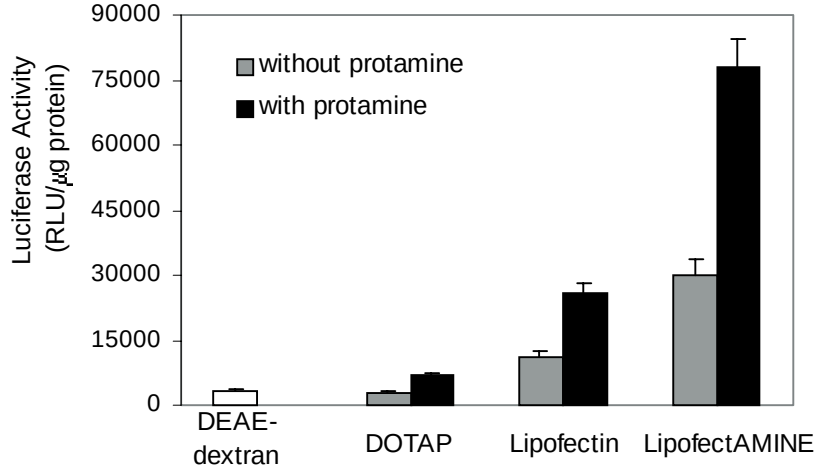

Fig. 4. Comparison of transfection activity of different liposomal formulations in macrophage RAW 264.7 cells. Each liposomal formulation was optimized for their transfection efficiency. The optimized conditions (DNA:liposome:protamine) for each formulation were: DOTAP, 1:10:1; Lipofectin, 1:6:1; and LipofectAMINE, 1:12:1. The data represent the maximum values of gene expression for each formulation. Transfection and luciferase assay were performed as noted in Section 2. Each data point represents the mean (with S.D.) of quadruplicate samples and the data are normalized to protein content.

its simplicity, versatility, and ease of use, this compound could be readily used as an adjuvant in a variety of gene transfer protocols.

3.4. Gene transfer mediated by different liposomal formulations

Different liposomal formulations including LipofectAMINE, Lipofectin, and DOTAP were further tested for their gene transfer efficiency in an attempt to determine the most efficient system for transfecting macrophages. All the liposomal formulations were optimized to their best efficiency both in the presence or absence of protamine (Fig. 4). At their optimal concentrations and in the absence of protamine, LipofectAMINE was found to be most effective, followed by Lipofectin and DOTAP. DOTAP was as equally effective as the DEAE-dextran while Lipofectin was 3-fold more effective. In the presence of protamine, LipofectAMINE showed a 4–5-fold increase in transfection efficiency over Lipofectin and at least 10–12-fold increase over DOTAP (Fig. 4). The increased efficiency of LipofectAMINE over other cationic liposomes may be attributed to its greater number of positively charged groups which could condense DNA more efficiently than the monovalent lipid Lipofectin and DOTAP.

4. Conclusion

We have developed a simple, efficient and reproducible method of transfection for macrophages. Transient transfection with our optimized delivery system resulted in a high transgene expression compared to other existing methods of transfection. The macrophages transfected with this system were $>95\%$ viable as determined by LDH assay. We have successfully applied this technique for the stable transfection of macrophages. The transfection protocol permits the study of gene regulation in adherent macrophage cultures and may be applicable to in vivo macrophage gene transfer.

Acknowledgements

This work was supported in part by the National Institutes of Health Grant HL62959 and by

the National Institute for Occupational Safety and Health.

References

Burkholder, J.K., Decker, J., Yang, N.S., 1993. Rapid transgene expression in lymphocyte and macrophage primary cultures after particle bombardment-mediated gene transfer. J. Immunol. Methods 165, 149–156.

Daumler, R., Zimmermann, U., 1989. High yields of stable transformants by hypo-osmolar plasmid electroinjection. J. Immunol. Methods 122, 203–210.

Economou, J.S., Rhoades, K., Essner, R., McBride, W.H., Gasson, J.C., Morton, D.L., 1989. Genetic analysis of the human tumor necrosis factor/cachectin promoter region in a macrophage cell line. J. Exp. Med. 170, 321–326.

Gao, X., Huang, L., 1996. Potentiation of cationic liposome-mediated gene delivery by polycations. Biochemistry 35, 1027–1036.

Hess, J.L., Clements, J.E., 1985. Cis- and trans-acting transcriptional regulation of Visna virus. Science 238, 482–485.

Kabanov, A.V., Kabanov, V.A., 1995. DNA complexes with polycations for the delivery of genetic material into cells. Bioconj. Chem. 6, 7–20.

Mack, K.D., Wei, R., Elbagarri, A., Abbey, N., McGrath, M.S., 1998. A novel method for DEAE-dextran mediated transfection of adherent primary cultured human macrophages. J. Immunol. Methods 211, 79–86.

Pierce, G.F., 1990. Macrophages: Important physiologic and pathologic sources of polypeptide growth factors. Am. J. Respir. Cell Mol. Biol. 2, 233–234.

Roussel, M.F., Rettenmier, C.W., Sherr, C.J., 1988. Introduction of a human colony stimulating factor-1 gene into a mouse macrophage cell line induces CSF-1 independence but not tumorigenicity. Blood 71, 1218–1225.

Rupprecht, A., Coleman, D., 1991. Transfection of adherent murine peritoneal macrophages with a reporter gene using DEAE-dextran. J. Immunol. Methods 144, 157–163.

Sorgi, F.L., Bhattacharya, S., Huang, L., 1997. Protamine sulfate enhances lipid-mediated gene transfer. Gene Ther. 4, 961–968.

Stief, A., Winter, D.M., Stratling, W.H., Sippel, A.E., 1989. A nuclear DNA attachment element mediates elevated and position-independent gene activity. Nature 341, 343–345.