Oxygen Radical-Mediated Pulmonary Toxicity Induced by Some Cationic Liposomes

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Purpose. The objectives of this study are to investigate the toxicity associated with polycationic liposomes and to elucidate the underlying mechanism. We tested the hypothesis that the positive charge of liposomes is a key determinant of toxicity by testing differently charged liposomes in mice.
Methods. Differently charged liposomal systems including cationic liposomes, LipofectAMINE and DOTAP, and neutral and negative liposomes were evaluated for their toxicity after pulmonary administration in mice. LDH assay and differential cell counts were performed to measure toxicity and pulmonary inflammation, respectively. Reactive oxygen intermediates (ROI) were assessed by chemiluminescence.
Results. Instillation of cationic liposomes elicited dose-dependent toxicity and pulmonary inflammation. This effect was more pronounced with the multivalent cationic liposome LipofectAMINE as compared to the monovalent cationic DOTAP. Neutral and negative liposomes did not exhibit lung toxicity. Toxicity associated with cationic liposomes correlated with the oxidative burst induced by the liposomes. LipofectAMINE induced a dose-dependent increase in ROI generation. This effect was less pronounced with DOTAP and absent with neutral and negative liposomes.
Conclusions. ROI play a key role in cationic lipid-mediated toxicity. Polyvalent cationic liposomes cause a release of ROI which are responsible for the pulmonary toxicity.

KEY WORDS: cationic liposomes; charge; toxicity; reactive oxygen intermediates.

INTRODUCTION

With our increasing knowledge of human inherited and acquired diseases at a molecular level, the use of gene therapy to restore a missing function or to treat certain diseases has become a clinical reality. Currently, however, the rate-limiting step in its successful application is the development of safe and efficient gene delivery vehicles. Vectors proposed for gene delivery fall into two categories, viral and nonviral. Viral vectors have provided high gene transfection. However, the safety concerns and the immunological profile of viral vectors have steered research towards the development of efficient non-viral carriers. Cationic lipids represent a class of nonviral vectors that have shown the greatest success and promise so far. Their lack of immunogenicity, simplicity and ease of production make them attractive vectors for gene therapy (1). Cationic liposomes have been reported to successfully transfect cells both *in vitro* and *in vivo* (1–3). Cationic lipid based gene delivery protocols have also been used in clinical trials (4,5). However, their relatively low transfection efficiency compared to that of the viral vectors makes them far from ideal.

Another concern regarding the use of cationic liposomes is their toxicity. Cationic lipids were considered to be safe initially, however, increasing evidence has suggested that they may cause some toxic effects *in vivo* (6–8). Experimental data indicate that *in vivo* toxicity does not correlate well with *in vitro* toxicity. In general, cationic liposomes are better tolerated *in vitro* suggesting that other factors involved *in vivo* are responsible for the toxicity. Scheule *et al.* (6) have shown significant lung toxicity following a nasal instillation of up to 300 nmol of lipid complexed with 400 nmol of pDNA. They observed a dose-dependent lung inflammation associated with cationic lipids. Song *et al.* (8) have demonstrated acute toxicity at high positive charge ratios of cationic lipid to plasmid DNA. Freimark *et al.* (7) reported that intratracheal instillation of lipid alone or plasmid/lipid complex induced cytokine production and cellular influx in the lung airway. We have also found in this study that cationic liposomes cause an inflammatory response in a dose-dependent manner when administered intratracheally.

The mechanism by which cationic liposomes facilitate gene transfection is still not completely understood. Since biological membranes are negatively charged (9), it has been suggested that liposomes interact with cells through a nonspecific charge interaction. Cationic liposomes form complexes with DNA through electrostatic interactions. The excess positive charge of the complexes allows non-specific interactions with cell membranes. This property which results in efficient transfection, may be also responsible for causing cellular toxicity. Cationic lipids are also excellent surfactants with a potential of causing membrane solubilization, poration, and lysis. They can also change the membrane properties by interacting with membrane proteins such as protein kinase C (10) which play an important role in signal transduction and gene regulation.

The origin of toxicity caused by cationic liposomes is not well understood. We hypothesized that the positive charge of liposomes plays an important role in lung toxicity. Using differently charged liposomes, we found that the multivalent cationic liposome LipofectAMINE was much more toxic than the monovalent cationic liposome DOTAP, and that neutral and negative liposomes were not toxic at the relevant concentrations.

Reactive oxygen intermediates (ROI) have been identified as key mediators of pulmonary damage induced by various stimuli (11). To further identify the cause of toxicity, we studied the effect of different liposomes on free radical generation. We observed that the highly charged cationic liposome LipofectAMINE caused a marked inflammatory response as determined by neutrophil influx and oxidative burst of lung cells. This effect is charge related and can be reversed by pretreatment of mice with the free radical scavenger PBN (N-ter-butyl-α-phenylnitrone). Hence, our results suggest that ROI play a key role in cationic lipid-mediated toxicity.

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METHODS

Animals

Male Balb/c mice (4–6 weeks old) were obtained from Jackson Laboratories (Bar Harbor, ME). The mice were fed water and food *ad libitum*. Intratracheal instillations of test agents were performed by anesthetizing the animals with a mixture of ketamine and xylazine (45 and 8 mg/kg, i.p., respectively) and challenged by aspiration. The animals were placed on a board in a supine position. The animal's tongue was extended with lined forceps and 50–100 μl of the test solution was placed on the back of the tongue.

Liposomes

Cationic liposomes used in this study were LipofectAMINE (Gibco BRL, Gaithersburg, MD) and DOTAP (Boehringer Mannheim, Indianapolis, IN). LipofectAMINE is a 3:1 mixture of cationic lipid DOSPA and neutral lipid DOPE. The neutral liposome used in this study was palmitoyl-oleoyl phosphatidylcholine (Sigma, St. Louis, MO) and the negative liposome was a mixture of palmitoyl-oleoyl phosphatidylcholine and dioleoyl phosphatidylglcyerol (Sigma). The neutral and negative liposomes were reconstituted in sterile water according the manufacturer's instructions. The neutral and negative liposomes were extruded through polycarbonate membranes to achieve the same size as that of LipofectAMINE and DOTAP. Briefly, the lipid solution was briefly vortexed, followed by incubation at 50°C for 10 min and then sequentially extruded through polycarbonate membranes with the following pore sizes: 1.0, 0.6 and 0.2 μm. The size of the liposomes were measured by dynamic laser scattering using Coulter N4SD particle sizer (Hialeah, FL). The mean particle size of different liposomes are as follows: LipofectAMINE, 160 nm; DOTAP, 189 nm; neutral, 171 nm; and negative, 174 nm.

Bronchoalveolar Lavage

At selected time intervals, treated mice were euthanized with an intraperitoneal injection of 0.25 mL sodium pentobarbital (Western Medical Supply, Arxadia, CA). A tracheal canula was inserted and the lungs were lavaged through the canula using ice-cold PBS. Five lavages (0.8 mL each) were collected. The first lavage was separated from the consecutive lavages. Bronchoalveolar lavage cells (BALC) were isolated by centrifugation (500*g*, 10 min, 4°C). The cell free supernatants were collected and used for biochemical measurements. For each animal, the cell pellet was resuspended in 1 mL of HEPES buffer, centrifuged (500*g*, 10 min, 4°C), and the supernatant was decanted and discarded. The BALC pellet was then resuspended in 1 mL HEPES buffer and cell counts and differentials were determined using a Coulter Multisizer II (Coulter Electronics, Hialeah, FL).

Lactate Dehydrogenase (LDH) Assay

LDH assay was performed to assess the effect of liposomes on cellular toxicity. LDH is a cytoplasmic enzyme that is released when the cell membrane is damaged. Mice were instilled with the test agents. After the treatments, bronchoalveolar lavage was performed and the supernatant from the first lavage was 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).

Administration of PBN

For this procedure, the spin trapping agent PBN was administered *in vivo* by i.p. injection at a dose of 300 mg/kg, 20 min before instillation of liposomes after dissolving it in PBS.

Chemiluminescence

Chemiluminescence (CL) assay of BALC was conducted in a total volume of 0.25 mL HEPES buffer. CL is a measurement of ROI generated by BALC during respiratory burst (12). Zymosan was added to the test medium to initiate the metabolic burst of the cells that produce CL. Resting CL was determined by incubating 0.25×10^6 BALC at 37°C for 5 min with 0.04% (w/v) luminol (Sigma) followed by the measurement of CL for 15 min. To determine zymosan-stimulated CL, the assay was modified to include 1 mg unopsonized zymosan (Sigma), which was added to the assay immediately prior to measurement of CL. CL measurement was conducted with an automated luminometer (Wallace, Inc., Gaithersburg, MD) at 390–620 nm for 15 min.

RESULTS

Charge Dependent Toxicity of Liposomes

To test the hypothesis that the charge of the liposome is responsible for toxicity, we tested differently charged liposomes on lung inflammation and damage. Cationic liposomes used in this study were LipofectAMINE and DOTAP. LipofectAMINE is a commercially available liposome that has been successfully used *in vitro* (13). In our experience, we have found that LipofectAMINE is superior to most other commercially available liposomes for *in vitro* gene transfection of lung cells. The cationic lipid present in LipofectAMINE has 4 protonatable amines on its headgroup at physiological pH. DOTAP is a monovalent cationic lipid. The neutral liposome used in this study is phosphatidylcholine and the negative liposome is a mixture of phosphatidylcholine and phosphatidylglycerol.

Intratracheal instillations of differently charged liposomes were performed in mice lungs. One day after instillation, mice were lavaged and bronchoalveolar lavage fluid (BALF) was tested for LDH activity and cell counts. LDH activity is a measure of toxicity. Inflammation is characterized by an increase in polymorphonuclear leukocytes (PMN) and hence, the cell counts are an indication of inflammation. There was a charge dependent toxicity of liposomes as determined by LDH (Fig. 1A). For a fair comparison, the dose of all liposomes was fixed at a dose that is commonly used in gene transfection protocols *in vivo* (14). The polycationic LipofectAMINE was found to be most toxic, followed by DOTAP, which is monocationic. Neutral and anionic liposomes did not exhibit significant toxicity over PBS control ($p < 0.05$). This toxicity correlated with the inflammation induced. LipofectAMINE-induced inflammation, as determined by PMN count, was significantly greater than that induced by other liposomes ($p < 0.01$) (Fig.

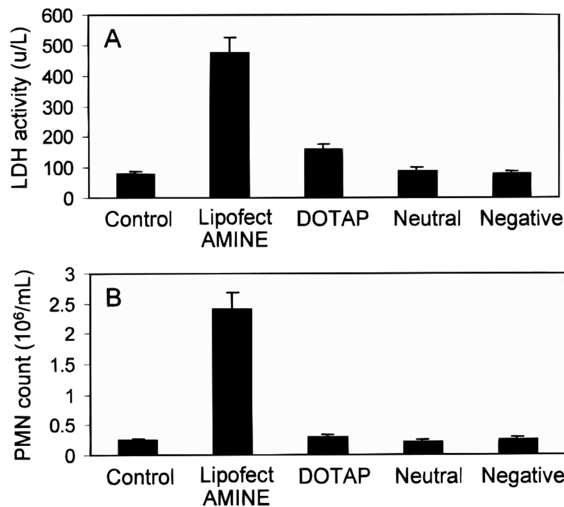

Fig. 1. Comparison of toxicity induced by differently charged liposomes. Intratracheal instillations were performed on mice with LipofectAMINE, DOTAP, neutral and negative liposomes at a fixed dose of 200 nmol/mouse. Bronchoalveolar lavage was performed 24 h after instillation. The BAL was tested for (A) LDH activity and (B) PMN cell count as described in the *Methods* section. The values are the mean ± SD of 3 animals.

1B). Further studies have shown that there is a dose-dependent increase in toxicity (Fig. 2A), as well as in the induction of inflammation (Fig. 2B) associated with instillation of LipofectAMINE. These results suggest that toxicity of liposomes is dependent on the charge of the liposome and that toxicity is seen only with cationic liposomes.

The positively charged headgroup of the liposome is responsible for its cationic nature. To identify whether this component of the cationic liposome is responsible for the observed toxicity, we instilled mice with relevant concentrations of spermine. Spermine is the headgroup of the cationic lipid present in LipofectAMINE. However, administration of spermine elicited neither a toxicity (Fig. 2A) nor an inflammatory response (Fig. 2B) in comparison to the administration of equivalent dose of cationic liposome ($p < 0.05$). These results suggest that both the cationic headgroup and lipophilic tail of the liposome are required for toxicity induction, presumably through its surfactant action.

Oxygen Radical-Mediated Toxicity of Cationic Liposomes

Reactive oxygen intermediates (ROI) have been identified as key mediators of pulmonary damage (11). We hypothesized that the cationic liposome mediated toxicity was in part due to the production of ROI by lung cells. To study the effect of liposomal charge on ROI production, we instilled mice with differently charged liposomes and lavaged the animals 24 h after instillation. The lavaged cells were then tested for their basal and zymosan-induced oxidative burst. Consistent with our toxicity data, we observed that cationic liposomes triggered an oxidative burst (Fig. 3A). Neutral and negative liposomes did not exhibit a zymosan-induced CL response. Multivalently charged cationic liposome, LipofectAMINE, showed a greater induction of ROI than the monovalently charged DOTAP. LipofectAMINE-induced ROI production was found to be dose-dependent (Fig. 3B). Stimulation of lavaged cells with zymosan in cationic liposome-instilled mice resulted in about 25–100 times enhancement of zymosan-triggered peak CL intensity over the controls.

The role of ROI in cationic lipid-mediated toxicity was further studied by administering a free radical trapper, PBN. PBN has been used in previous studies as a spin trap for ROI (11). It would be reasonable to assume that if oxygen radicals produced by LipofectAMINE are responsible for the toxicity,

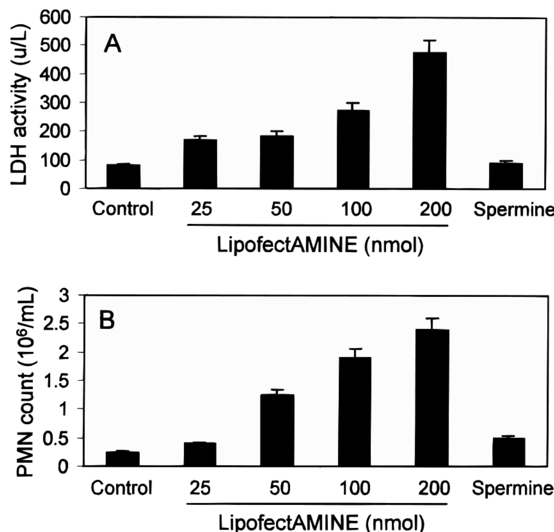

Fig. 2. Effects of LipofectAMINE and spermine on (A) LDH activity and (B) PMN cell count. Intratracheal instillations were performed on mice with LipofectAMINE (25–200 nmol/mouse) or spermine (200 nmol/mouse). Bronchoalveolar lavage was performed 24 h after instillation. The values are the mean ± SD of 3 animals.

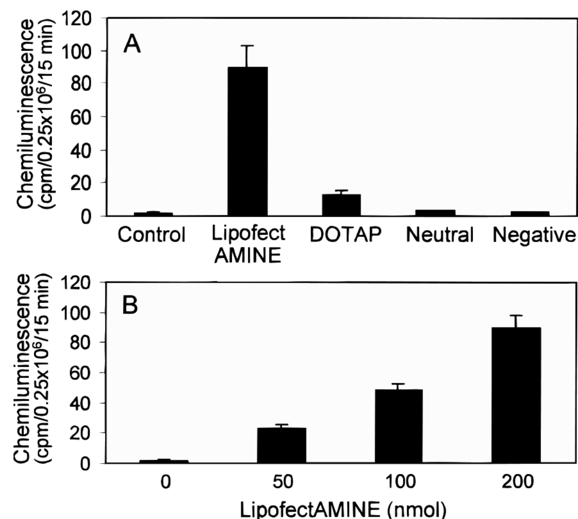

Fig. 3. (A) Comparison of ROI production by differently charged liposomes. (B) Dose-dependent induction of ROI by LipofectAMINE. Intratracheal instillations were performed on mice with LipofectAMINE, DOTAP, neutral and negative liposomes (200 nmol/mouse). Bronchoalveolar lavage was performed 24 h after instillation and was analyzed by chemiluminescence. The values are the mean ± SD of 3 animals.

then scavenging these radicals would reverse the toxicity. To test this hypothesis, we pretreated the mice with PBN (i.p., 300 mg/kg) 20 min before liposomal instillation. One day after instillation, mice trachea were lavaged and the lavaged cells were tested for oxidative burst, toxicity and inflammation. As can be seen in Fig. 4A, PBN decreases oxidative burst induced by liposome treatment. PBN by itself does not cause any production of ROI. It also decreases the toxicity (Fig. 4B) and inflammation (Fig. 4C) induced by LipofectAMINE ($p < 0.01$). These results support the role of ROI in LipofectAMINE-mediated lung toxicity.

DISCUSSION

Cationic lipids have been used successfully in both *in vitro* and *in vivo* gene transfer (1–3). However, there have been some recent reports of toxicity associated with their use *in vivo* (6,7). The principle objective of this study is to understand the influence of charge of the liposome on its *in vivo* toxicity profile. We have tested the toxicity of differently charged liposomes *in vivo* in mice. This study has revealed four significant observations: (1) charge of liposome is a key determinant of toxicity; (2) cationic liposomes are more toxic than neutral/negative liposomes and multivalent cationic liposomes are more toxic than monovalent cationic liposomes; (3) the cationic headgroup of liposome by itself is not toxic but its presence in the cationic liposome causes toxicity; and (4) ROI are responsible for cationic liposome-mediated toxicity.

Cationic lipids are composed of a hydrophilic anchor connected via a linker to a positively charged headgroup (1,2). It has been shown that although all components of the cationic lipid contribute to its transfection efficiency, the nature of the headgroup plays a major role in transfection efficiency of the lipid. The positively charged headgroup of most cationic lipids consists of one or more amine groups with various degrees of substitution. This domain facilitates both electrostatic interactions with negatively charged DNA to form the lipoplex as well as interactions with target cells. Polyvalent headgroups have been reported to be superior to their monovalent counterparts due to their capacity for greater DNA compaction (13) and non-specific cell interactions. However, for the same reason, they are also toxic. We and others (6,7) have observed that direct instillation of cationic lipid into the lungs elicits an inflammatory response which is dose-dependent. Forming a complex with plasmid DNA did not decrease the toxicity even when the net charge of pDNA/lipid complexes was neutral.

The mechanism of toxicity of cationic liposomes has not been well characterized. It has been suggested that the amphipathic nature of cationic lipid is responsible for toxicity because they are excellent detergents. This hypothesis is supported by our results. Spermine, the headgroup of lipid responsible for the positive charge, is not toxic by itself at the relevant concentrations. Scheule *et al.* (6) have suggested that introducing a larger headgroup increases water solubility resulting in a greater tendency to form micelles and hence, a potential for greater toxicity.

Cationic liposomes have been reported to cause cellular influx and lung inflammation (6). Since inflammation is associated with an elevated level of ROI, we tested whether positively charged liposomes can induce ROI production. Using luminol dependent CL assay, we demonstrated that cationic liposomes did induce ROI production by lung cells. The role of ROI in cationic liposome-induced lung inflammation was confirmed by experiments using the spin trapping agent PBN. PBN has been shown to reduce endotoxin-induced rat mortality through its ability to trap intermediate oxygen and carbon-centered radicals (15). We have shown here that *in vivo* pretreatment with PBN decreases the ability of lung cells to release ROI in response to stimuli. This pretreatment also decreased pulmonary inflammation and toxicity induced by cationic liposomes.

We report here, for the first time, the role of ROI in cationic lipid mediated toxicity. The data presented here suggest that cationic liposomes cause inflammation and toxicity via a pathway that involves the generation of oxygen free radicals because scavenging these radicals decreases toxicity.

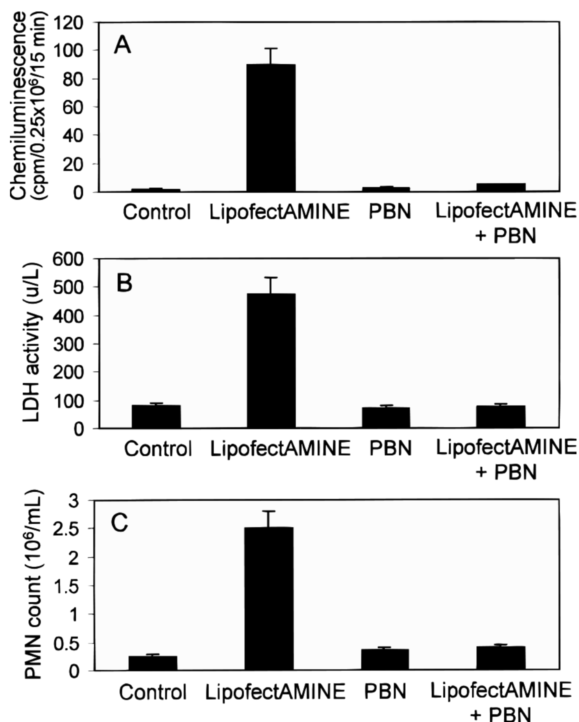

Fig. 4. Effects of PBN on (A) chemiluminescence, (B) LDH activity, and (C) cell count. Mice were pretreated with free radical scavenger, PBN, (i.p., 300 mg/kg) 20 min before intratracheal instillations with LipofectAMINE (200 nmol/mouse). Bronchoalveolar lavage was performed 24 h after instillation. The values are the mean ± SD of 3 animals.

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REFERENCES

1. X. Gao and L. Huang. Cationic liposome-mediated gene transfer. *Gene Ther.* **2**:710–722 (1995).
2. X. Gao and L. Huang. A novel cationic liposome reagent for efficient transfection of mammalian cells. *Biochim. Biophys. Res. Commun.* **179**:280–285 (1991).
3. K. L. Brigham, B. Meyrick, B. Christman, M. Magnusson, G. King, and L. Berry. In vivo transfection of expression of murine lungs with a functioning prokaryotic gene using a liposome vehicle. *Am. J. Med. Sci.* **298**:278–281 (1989).
4. N. J. Caplen, E. W. Alton, P. G. Middleton, J. R. Dorin, B. J.

Stevenson, X. Gao, S. R. Durham, P. K. Jefferey, M. E. Hodson, and C. Coutelle. Liposome-mediated CFTR gene transfer to the nasal epithelium of patients with cystic fibrosis. *Nature Med.* **1**:39–46 (1995).
5. G. J. Nabel, E. G. Nabel, Z. Y. Yang, B. A. Fox, G. E. Plautz, X. Gao, L. Huang, S. Shu, D. Gordon, and A. E. Chang. Direct gene transfer with DNA-liposome complexes in melanoma: Expression, biologic activity, and lack of toxicity in humans. *Proc. Natl. Acad. Sci. USA* **90**:11307–11311 (1993).
6. R. K. Scheule, J. A. St. George, R. G. Bagley, J. Marshall, J. M. Kaplan, G. Y. Akita, K. X. Wang, E. R. Lee, D. J. Harris, C. Jiang, N. S. Yew, A. E. Smith, and S. H. Cheng. Basis of pulmonary toxicity associated with cationic lipid-mediated gene transfer to the mammalian lung. *Human Gene Ther.* **8**:689–707 (1997).
7. B. D. Freimark, H. P. Blezinger, V. J. Florack, J. L. Nordstrom, S. D. Long, D. S. Deshpande, S. Nochumson, and K. L. Petrak. Cationic lipids enhance cytokine and cell influx levels in the lung following administration of plasmid: Cationic lipid complexes. *J. Immunol.* **160**:4580–4586 (1998).
8. Y. K. Song, F. Liu, S. Chu, and D. Liu. Characterization of cationic liposome-mediated gene transfer in vivo by intravenous administration. *Human Gene Ther.* **8**:1585–1594 (1997).
9. J. P. Vigneron, N. Oudrhiri, M. Fauquet, L. Vergely, J. C. Bradley, M. Basseville, P. Lehn, and J. M. Lehn. Guanidium-cholesterol cationic lipids: efficient vectors for the transfection of eukaryotic cells. *Proc. Natl. Acad. Sci. USA* **93**:9682–9686 (1996).
10. R. Bottega and R. M. Epand. Inhibition of protein kinase C by cationic amphiphiles. *Biochemistry* **31**:9025–9030 (1992).
11. S. Gossart, C. Cambon, C. Orfila, M. H. Seguelas, J. C. Lepert, J. Rami, and B. Pipy. Reactive oxygen intermediates as regulators of TNF-α production in rat lung inflammation induced by silica. *J. Immunol.* **156**:1550–1548 (1996).
12. J. M. Antonini, K. Vandyke, Z. Ye, M. DiMatteo, and M. J. Reasor. Introduction of luminol-dependent chemiluminescence as a method to study silica inflammation in the tissue and phagocytic cells of rat lung. *Environ. Health Persp.* **102**:37–42 (1994).
13. P. Hawley-Nelson, V. Ciccarone, G. Gebeyehu, and J. Jessee. Lipofectamine reagent: A new, higher efficiency polycationic liposome transfection reagent. *Focus* **15**:73–79 (1993).
14. D. Liu, J. E. Knapp, and Y. K. Song. Mechanism of cationic liposome-mediated transfection of the lung endothelium. In L. Huang, M. C. Hung, and E. Wagner (eds.), *Nonviral Vectors for Gene Therapy*, Academic Press, New York, 1999, pp. 313–335.
15. H. W. Pogrebniak, M. J. Merino, S. M. Hahn, J. B. Mitchell, and H. I. Pass. Spin trap salvage from endotoxemia: the role of cytokine down-regulation. *Surgery* **112**:130–135 (1992).