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Biochimica et Biophysica Acta 1329 (1997) 345–356

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

# Toxicity and immunomodulatory activity of liposomal vectors formulated with cationic lipids toward immune effector cells

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Received 2 April 1997; accepted 19 May 1997

**Abstract**

Liposomal vectors formulated with cationic lipids (cationic liposomes) and fusogenic dioleoylphosphatidylethanolamine (DOPE) have potential for modulating the immune system by delivering gene or antisense oligonucleotide inside immune cells. The toxicity and the immunoadjuvant activity of cationic liposomes containing nucleic acids toward immune effector cells has not been investigated in detail. In this report, we have evaluated the toxicity of liposomes formulated with various cationic lipids towards murine macrophages and T lymphocytes and the human monocyte-like U937 cell line. The effect of these cationic liposomes on the synthesis of two immunomodulators produced by activated macrophages, nitric oxide (NO) and tumor necrosis factor-$\alpha$ (TNF-$\alpha$), has also been determined. We have found that liposomes formulated from DOPE and cationic lipids based on diacyltrimethylammonium propane (dioleoyl-, dimyristoyl-, dipalmitoyl-, disteroyl-: DOTAP, DMTAP, DPTAP, DSTAP) or dimethyldioctadecylammonium bromide (DDAB) are highly toxic in vitro toward phagocytic cells (macrophages and U937 cells), but not towards non-phagocytic T lymphocytes. The rank order of toxicity was DOPE/DDAB > DOPE/DOTAP > DOPE/DMTAP > DOPE/DPTAP > DOPE/DSTAP. The $ED_{50}$'s for macrophage toxicity were < 10 nmol/ml for DOPE/DDAB, 12 nmol/ml for DOPE/DOTAP, 50 nmol/ml for DOPE/DMTAP, 400 nmol/ml for DOPE/DPTAP and > 1000 nmol/ml for DOPE/DSTAP. The incorporation of DNA (antisense oligonucleotide or plasmid vector) into the cationic liposomes marginally reduced their toxicity towards macrophages. Although toxicity was observed with cationic lipids alone, it was clearly enhanced by the presence of DOPE. The replacement of DOPE by dipalmitoylphosphatidylcholine (DPPC) significantly reduced liposome toxicity towards macrophages, and the presence of dipalmitoylphosphatidylethanolamine-$PEG_{2000}$ (DPPE-$PEG_{2000}$: 10 mol%) in the liposomes completely abolished this toxicity. Cationic liposomes, irrespective of their DNA content, downregulated NO and TNF-$\alpha$ synthesis by lipopolysaccharide (LPS)/interferon-$\gamma$ (IFN-$\gamma$)-activated macrophages. The replacement of DOPE by DPPC, or the addition of DPPE-$PEG_{2000}$, restored NO and TNF-$\alpha$ synthesis by activated macrophages. Since macrophages constitute the major site of liposome localization after parenteral administration and play an important role in the control of the immune system, cationic liposomes should be used with caution to deliver gene or antisense oligonucleotide to mammalian cells.

Abbreviations: DOPE, dioleoylphosphatidylethanolamine; DPPC, dipalmitoylphosphatidylcholine; DOTAP, dioleoyl-, DMTAP, dimyristoyl-, DPTAP, dipalmitoyl-, DSTAP, distearoyl-diacyltrimethyammonium propane; DDAB, dimethyldioctadecylammonium bromide; DPPE-$PEG_{2000}$, dipalmitoylphosphatidylethanolamine-polyethylene $glycol_{2000}$; NBD-PE, 7-nitro-2-1,3-benzoxadiazol-4-yl-phosphatidylethanolamine; FCS, fetal calf serum; LDH, lactate dehydrogenase; NO, nitric oxide; TNF-$\alpha$, tumor necrosis factor-$\alpha$; MDP, *N*-acetylmuramyl-L-alanyl-D-isoglutamine; LPS, lipopolysaccharide; IFN-$\gamma$, interferon-$\gamma$

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*PII* S0005-2736(97)00126-0

Cationic lipids show in vitro toxicity toward phagocytic cells and inhibit in vitro and in situ NO and TNF-$\alpha$ production by activated macrophages. 

*Keywords:* Cationic liposome; Cationic lipid; Antisense oligonucleotide; Toxicity; Immunoadjuvant

## 1. Introduction

Liposomes formulated with cationic lipids (cationic liposomes) are usually used to deliver DNA or RNA inside mammalian cells [1]. Most liposomes used for this purpose are composed of cationic lipids which can form stable complexes with the anionic nucleic acids, and dioleoylphosphatidylethanolamine (DOPE) which can destabilize the endosome compartment and cause the release of nucleic acids into the cytoplasm [2]. Lipofection, or liposomal nucleic acid transfer, offers a number of advantage over current methods of transfection such as calcium phosphate precipitation, electroporation, direct injection or retroviral-mediated transfer. Cationic liposomes are very efficient in gene delivery in vitro, do not disrupt the cell membrane, are capable of transfecting many cell types, are metabolizable and have the potential to target specific tissues in vivo [3]. Cationic liposomes may be useful in the treatment of a number of diseases, including DNA delivery for gene therapy. An example is the use of DOPE/dimethylaminoethanecarboyl–cholesterol (DC–Chol) liposomes that are currently being evaluated for the delivery of the chloride transporter gene to the lungs of individuals with cystic fibrosis [4]. Cationic liposomes may also be useful for the delivery of antisense oligonucleotides capable of inhibiting tumor cell propagation [5]. Cationic liposomes containing appropriate nucleic acids (antisense DNA or plasmid vector) have the potential to act as immunomodulators to improve current anti-microbial, anti-viral or anti-inflammatory therapy. However a lack of toxicity and immunomodulatory studies precludes any generalization as to their innocuity toward the immune system.

The limited number of toxicity studies on liposomes formulated with cationic lipids have used non-immune, non-phagocytic cells [6,7], or non-phagocytic immune cells such as T lymphocytes [8]. Prior to evaluating the potential of cationic liposomes as a delivery vehicle for nucleic acid immunotherapy we felt it prudent to examine the toxicity of different formulations towards two major immune effector cell populations, macrophages and T lymphocytes. Macrophages constitute the major site of liposome localization after parenteral administration [9]. Macrophages, which are known to present antigens, have the capacity to phagocytosis a large quantity of antigen or liposomes, while T lymphocytes do not have this capacity [10]. We have further evaluated the effect of liposomes containing cationic lipids on the production of nitric oxide (NO) and tumor necrosis factor-$\alpha$ (TNF-$\alpha$), two important immunomodulator secreted by activated macrophages. The production of NO by activated macrophages provides an early non-specific defence against pathogens prior to the development of a specific immune response, can regulate the development of T-helper 1 and T-helper 2 immunity and is implicated in the inflammatory process [11]. TNF-$\alpha$, which is produce predominately by macrophages, has an anti-viral and an anti-tumoral activity, enhances T and B lymphocyte responsiveness and also plays an important role in inflammatory reactions [12].

Our results show that liposomes formulated with DOPE and cationic lipids are highly toxic for phagocytic macrophages and monocyte-like U937 cells, but not for non-phagocytic T lymphocytes. Toxicity and downregulation of NO and TNF-$\alpha$ was related to the presence of cationic lipid and was enhanced by DOPE. The incorporation of DNA, as either an antisense oligonucleotide or as the plasmid vector pBR322, marginally reduced cationic liposomes toxicity toward macrophages, but did not reduced the ability of cationic lipids to non-specifically downregulate NO and TNF-$\alpha$ synthesis by activated macrophages.

## 2. Materials and methods

### 2.1. Liposomes preparation

Glassware was treated at 180°C for 4 h to inactivate endotoxin. Sterile pyrogen-free NaCl (0.85%

w/v) was used to prepare all solutions and all manipulations were carried out in a class 100 horizontal laminar flow cabinet. Cationic lipids (dioleoyl-[DOTAP], dimyristoyl-[DMTAP], dipalmitoyl-[DPTAP], distearoyl-[DSTAP] diacyl trimethylammonium propane; Avanti Polar Lipids, Alabaster, AL or dimethyldioctadecylammonium bromide [DDAB]; Sigma, St.-Louis, MO) were dissolved in chloroform (10 mg/ml), mixed at a molar ratio of 1:1 with DOPE or with dipalmitoylphosphatidylcholine (DPPC) (20 μmol of each) (Avanti Polar Lipids) and evaporated to dryness at 60°C in a round-bottomed flask using a rotary evaporator. Amphiphilic dipalmitoylphosphatidylethanolamine-$PEG_{2000}$ (DPPE-$PEG_{2000}$), 10 mol% final concentration (Shearwater Polymers, Huntsville, AL) or fluorescent 7-nitro-2-1,3-benzoxadiazol-4-yl-phosphatidylethanolamine (NBD-PE), 5 μg/μmol (lipid/phospholipid) (Avanti Polar Lipids) was dissolved in chloroform and mixed with cationic lipid/phospholipid prior to rotary evaporation. Multilamellar liposomes (non-sonicated) were prepared by adding the required volume of 0.85% w/v NaCl (1 ml/10 μmol cationic liposomes) followed by agitation. Unilamellar liposomes (< 0.12 μm) were obtained by sonication.

### 2.2. Incorporation of DNA into cationic liposomes

The antisense oligonucleotide [5′ GAC TTT GAA GAG GAG AAA 3′] was synthesized using an automated DNA synthesizer (Beckman Oligomer 1000, Columbia, MD) by the Department of Biochemistry, Université de Montréal. The antisense DNA was purified by several cycles of ethanol precipitation, and was resuspended in DNAse-free water before use. Purified pBR322 plasmid (4363 bp) was kindly provided by Dr. Philippe Raymond (Institut Armand-Frappier, Laval, Québec, Canada). Antisense oligonucleotide (2.0, 10.0 and 25.0 mg) or plasmid (0.1, 0.5 and 2.5 μg) were mixed with 0.01, 0.1 or 1.0 μmol/ml of uni lamellar cationic liposomes composed of DOPE/DOTAP (1:1 mol ratio) and incubated for 30 min at room temperature before use.

### 2.3. Liposomes characterization

The size of reconstituted liposomes was determined by photo correlation spectroscopy in a Coulter 4N Plus submicron particle analyser (Coulter, Miami, FL). Liposomal charge, measured as the zeta (ζ) potential, was determined in 0.85% w/v NaCl (buffered to pH 7.4 with 10 mM sodium phosphate buffer) by Doppler electrophoretic light scattering using a Coulter DELSA 440 SX.

### 2.4. Cells

Macrophages were obtained by i.p. injection of female CD1 mice (Charles River, St-Constant, Canada) with 1.5 ml sterile Brewer's thioglycollate broth (Difco, Detroit, USA). The peritoneal exudate (> 85% macrophages) was harvested 4 days later, washed by centrifugation in Hank's balanced salt solution (HBSS) and seeded in 96-well flat-bottom micro plates at $1.0 \times 10^5$ macrophages/well in RPMI-1640 containing 10% heat-inactivated fetal calf serum (FCS), 50 μg/ml gentamycin sulphate and 20 mM of HEPES (all from Gibco Life Science, Burlington, Canada). T cells were isolated from female CD1 spleen cells. Spleens were aseptically removed and single cell suspension were prepared by gentle teasing through sterile stainless steel screens. Cell suspensions (10 ml) were layered on Lympholyte-M cell separation media (5 ml; CedarLane, Hornby, Ontario, Canada) and centrifuged at 2200 rpm for 30 min to remove red blood cells and dead cells. The cells were washed by centrifugation (15 ml medium, 1500 rpm for 10 min). T cells were purified by Thy1.2 positive selection using magnetic micro beads (MiniMacs cell sorter, Miltenyi Biotec, Bergish Gladbach, Germany) and seeded at $1.0 \times 10^5$ T cells/well in 200 μl RPMI 1640/10% FCS. Activated T lymphocytes were obtained by adding $10^5$ spleen feeder cells/well and 1% of phytohaemagglutinin-A (PHA) (Gibco Life Science) for 24 h as previously described [13]. Human monocyte-like U937 cells were obtained from the American Type Culture Collection (Rockville, MD) and cultured in RPMI 1640/10% FCS at 37°C, 5% $CO_2$.

### 2.5. Toxicity assay

Liposome toxicity towards immune effector cells was determined as previously described [14]. Briefly, $1.0 \times 10^5$ cell/well (macrophages, T cells or U937 cell) in 100 μl medium were incubated with the indicated liposome formulation at different concentra-

tions at 37°C, 5% $CO_2$ for 24 h. Lactate dehydrogenase (LDH) activity in the medium was used as an indicator of cell death, and was determined by means of a commercial kit (Sigma Chemical). Total LDH activity was determined by incubating the cells with 1.0% v/v Triton X-100 in water to induce lysis, followed by vigorous agitation.

### 2.6. NO and TNF-α determination

Macrophages ($1 \times 10^5$ cells/well) in 100 μl medium were incubated with the indicated liposomes for 3 h at 37°C, 5% $CO_2$ and washed 3 times with 200 μl warm RPMI-1640/10%FCS. The macrophages were then incubated with *E. coli* 011:B4 lipopolysaccharide (LPS)/interferon-γ (IFN-γ) (1.0 μg/ml and 500 U/ml respectively) (LPS from Sigma, IFN-γ from Gibco Life Science) for 48 h in a final volume of 200 μl. $NO_2^-$, one of the end products of NO synthesis, was measured after 48 h incubation by mixing 50 μl cell supernatant with 50 ml of Griess Reagent (1% sulfanilamide in 2.5% $H_3PO_4$ mixed with an equal volume of 0.1% *N*-(1-naphthyl)ethylenediamine HCl in $H_2O$) and determining the absorbance at 550 nm against $NaNO_2$ standards. TNF-α synthesis was measured by means of a commercial ELISA kit (BioSource, Camarillo, CA) using 50 μl supernatant. LDH was determined as described earlier (Section 2.5) at 3, 24 and 48 h using 2 μl supernatant. For in situ macrophage treatment, cationic liposomes were injected i.p. (60 mg/kg) in a volume of 500 μl on day 3 following the injection of thioglycollate broth. The macrophages were harvested on day 4 and NO and TNF-α synthesis measured after LPS/IFN-γ as described above.

### 2.7. Liposomes endocytosis assays

Macrophages and monocyte-like U937 cells ($5 \times 10^5$ cells/well) were incubated in 6-well flat-bottom micro plate (1.0 ml) with the indicated fluorescent NB-PE liposomes (10 μmol/ml) for 4 h at 37°C, 5% $CO_2$. At the end of the incubation the cells were washed twice with ice-cold HBSS (3.0 ml) and then lysed in HBSS containing 0.5% Triton X-100 (1.0 ml). Fluorescence was measured using excitation and emission wavelengths of 460 nm and 534 nm respectively. Experiments were also carried out in parallel at 4°C and endocytosis was calculated from the results obtained at 37°C minus those at 4°C [15]. Results were expressed as nmol of lipid/$5 \times 10^5$ cells/4 h.

## 3. Results

### 3.1. Liposomes diameter and ζ potential

Two populations of cationic liposomes with different sizes were used in this study: unilamellar liposomes with a diameter $< 0.12$ μm and multilamellar liposomes with a diameter $> 1.00$ μm. There was no evidence of liposomes aggregation on storage at 4°C over a period of 24 h. The liposomal charge, measured as the ζ potential, was also determined (Table 1). The rank order of liposomal ζ potential was DOPE/DDAB = DOPE/DOTAP = DOPE/DMTAP > DOPE/DPTAP > DOPE/DSTAP (1:1 mol ratio), and was found to be independent of liposomes diameter.

Table 1
Liposome characterization

| Liposome formulations | ζ potential (mV) | Size (μm) non-sonicated | Size (μm) sonicated |
|---|---|---|---|
| DOPE/DDAB | $+40.0 \pm 9.0$ | 1.05–3.20 | 0.10–0.12 |
| DOPE/DOTAP | $+42.0 \pm 10.9$ | 1.00–1.33 | 0.08–0.11 |
| DOPE/DMTAP | $+42.9 \pm 12.5$ | 1.75–3.19 | 0.10–0.12 |
| DOPE/DPTAP | $+22.4 \pm 3.0$ | 3.00–5.87 | 0.09–0.12 |
| DOPE/DSTAP | $+15.0 \pm 6.3$ | 5.31–10.00 | 0.11–0.12 |
| DPPC/DOTAP | $+42.6 \pm 6.2$ | 1.09–1.46 | 0.08–0.10 |
| DOPE/DOTAP + DPPE-$PEG_{2000}$ | $+3.2 \pm 3.0$ | 1.15–1.46 | 0.08–0.10 |

Liposome charge, measured as the ζ potential, and liposome size were determined in NaCl (0.85% w/v) at 25°C. ζ potential is expressed as the mean ± S.D. of 3 independent experiments. Liposome size is expressed as the range of 3 independent experiments.

### 3.2. Toxicity of cationic liposomes toward macrophages and T lymphocytes

Liposomes formulated at a 1:1 mol ratio (phospholipid/cationic lipid) were highly toxic toward macrophages as determined by the release of LDH into the cell supernatant (Fig. 1). The rank order of toxicity was DOPE/DDAB > DOPE/DOTAP > DOPE/DMTAP > DOPE/DPTAP > DOPE/DSTAP. Similar results were obtained with monocyte-like U937 cells, and these results were confirmed by trypan blue exclusion tests (data not shown). The $ED_{50}$'s for macrophage toxicity were $< 10$ nmol/ml for DOPE/DDAB, 12 nmol/ml for DOPE/DOTAP, 50 nmol/ml for DOPE/DMTAP, 400 nmol/ml for DOPE/DPTAP and $> 1000$ nmol/ml for DOPE/DSTAP. Toxicity was not influenced by the diameter of liposomes ($< 0.12$ $\mu$m vs. $> 1.00$ $\mu$m) but was influenced by the presence of 10% FCS during the first 3 h of incubation (Fig. 1). The absence of FCS during the first 3 h of a 48 h incubation in complete medium containing 10% FCS increased the toxicity of cationic liposomes toward macrophages. However, incubation of macrophages in the absence of cationic liposomes and FCS during the first 3 h of incubation did not increase macrophages death as measured by the LDH release. As illustrated in Fig. 1 for DOPE/DOTAP liposomes, none of the cationic liposomes tested showed toxicity toward purified T lymphocytes in the presence or absence of FCS during the first 3 h of incubation. Furthermore, the liposomes showed no toxicity towards T cells activated by the mitogenic agent PHA (Fig. 1).

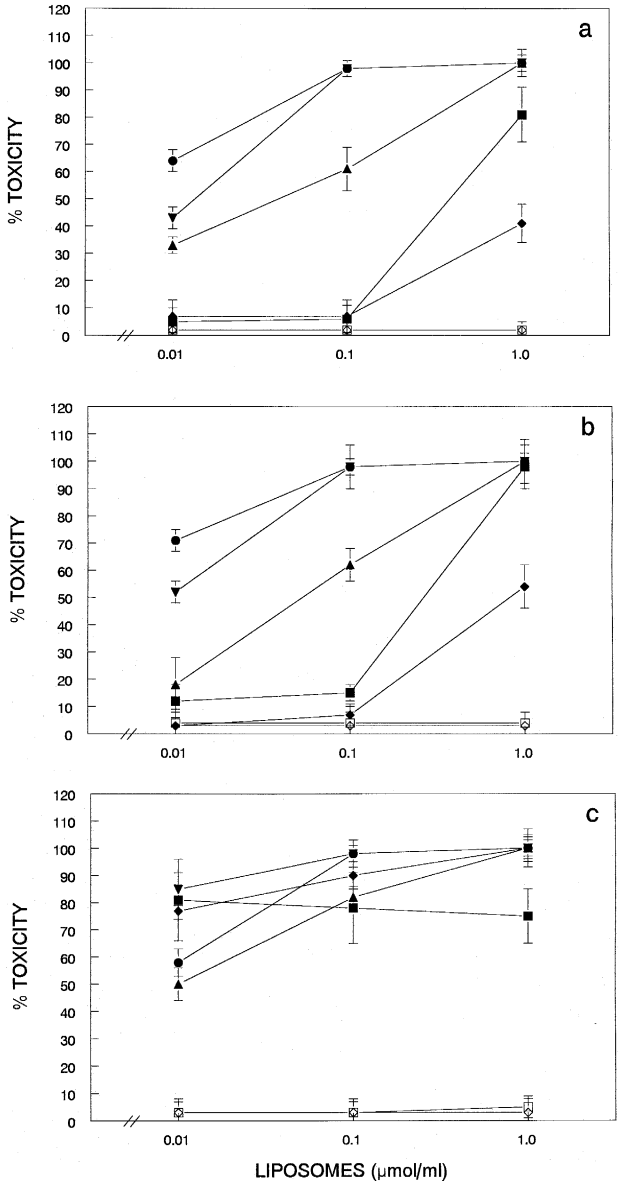


Fig. 1. Cationic liposomes were toxic for macrophages but not for T lymphocytes. Macrophages ($1\times10^5$/well) were incubated with the indicated cationic liposomes (DOPE/DDAB ●, DOPE/DOTAP ▼, DOPE/DMTAP ▲, DOPE/DPTAP ■, DOPE/DSTAP ◆) for 24 h at 37°C, 5% $CO_2$ in RPMI-1640/10% FCS. Resting (◇) or activated T cells by 1% of PHA (□) were incubated with DOPE/DOTAP liposomes for 24 h. at 37°C, 5% $CO_2$ in RPMI-1640/10% FCS. The diameter of the liposomes used was 0.08–0.12 $\mu$m (unilamellar) (a) and 1.00–10.00 $\mu$m (multilamellar) (b). In (c), macrophages or T cells were incubated with unilamellar liposomes in 100 $\mu$l of RPMI-1640 without FCS for 3 h at 37°C, 5% $CO_2$. Then, 100 $\mu$l of complete medium containing 20% FCS (final concentration: 10% FCS) was added to the corresponding well and incubated for a further 21 h. Toxicity was determined by the release of LDH activity into the supernatant after 24 h incubation. Data represent the mean ± S.D. of two replicates of three independent experiments.

### 3.3. The incorporation of DNA in cationic liposomes does not significantly reduce toxicity toward macrophages

We have evaluated whether the incorporation of DNA, as either a single stranded antisense oligonucleotide (15 mers) or as the double stranded plas-

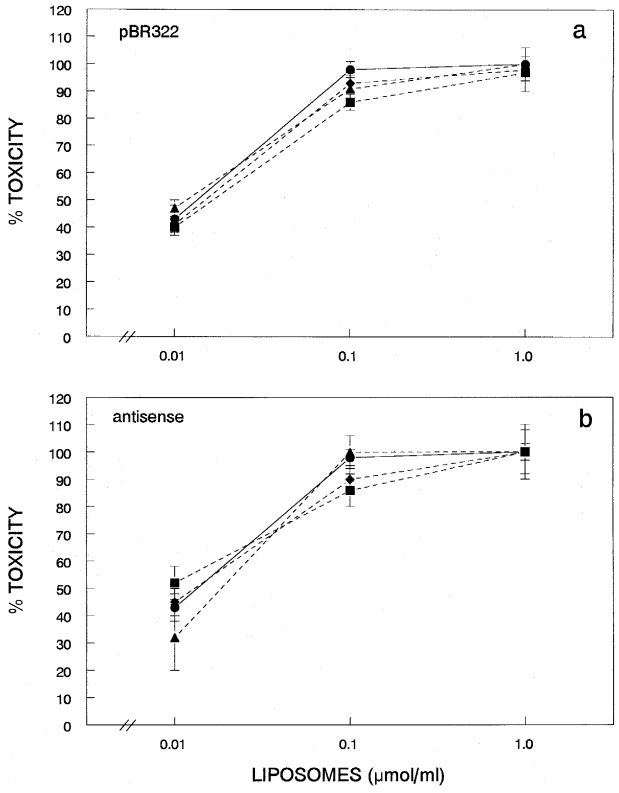


Fig. 2. The incorporation of DNA in cationic liposomes marginally reduced the toxicity observed towards macrophages. In (a) plasmid pBR322 [0.1 (♦), 0.5 (▲) and 2.5 μg (■)] or in (b) antisense oligonucleotides [2.0 (♦), 10.0 (▲) and 25.0 μg (■)] were mixed with 0.01, 0.1 and 1.0 μmol/ml of unilamellar DOPE/DOTAP liposomes (1:1 mol ratio) and incubated for 30 min at room temperature. The diameter of plasmid pBR322/cationic liposomes was between 0.3–3.8 μm and was between 0.1–1.0 μm for antisense DNA/cationic liposomes. Macrophages ($1\times10^5$/well) were incubated with the indicated cationic liposomes for 24 h at 37°C, 5% $CO_2$ in RPMI-1640/10% FCS. Toxicity was determined by the release of LDH activity into the supernatant after 24 h incubation. Data represent the mean ± S.D. of two replicates of three independent experiments.

mid vector pBR322 (4363 bp), in cationic liposomes can reduced the observed toxicity toward macrophages. The incorporation of different amounts of DNA by different concentrations of unilamellar DOPE/DOTAP liposomes, some of which represent the optimal cationic liposomes/DNA ratio that is currently used for in vitro transfection [16], did not significantly reduce the toxicity observed toward macrophages (Fig. 2). Nevertheless, the incorporation of antisense or plasmid DNA into cationic liposomes significantly reduced the ζ potential (Table 2).

### *3.4. The addition of DPPE-$PEG_{2000}$ abolished the toxicity toward macrophages*

The fact that cationic liposomes are highly toxic toward macrophages but not toxic toward T lymphocytes could be explained by the enhanced relative phagocytic activity of macrophages compared to T cells. To test this hypothesis we have incorporated into DOPE/DOTAP liposomes 10 mol% of DPPE-$PEG_{2000}$. The presence of DPPE-$PEG_{2000}$ in liposomes blocks endocytosis but not pinocytosis [15]. The toxicity of the liposomes toward macrophages and monocyte-like U937 cells was completely abolished by the incorporation of DPPE-$PEG_{2000}$ into DOPE/DOTAP liposomes (Fig. 3). The addition of DPPE-$PEG_{2000}$ significantly decreased the ζ potential of DOPE/DOTAP liposomes (DOPE/DOTAP: 42.0 ± 10.9 mV vs. DOPE/DOTAP +10 mol% DPPE-$PEG_{2000}$: 3.2 ± 3.0 mV).

### *3.5. The replacement of DOPE by DPPC reduced the toxicity toward macrophages*

DOPE is a pH-sensitive phospholipid that can destabilize the endosomal membrane at acidic pH and assist liposomes in delivering their contents into the cytoplasm [2]. The association of DOPE with cationic

Table 2
The incorporation of DNA by cationic liposomes influences the ζ potential

| DNA | ζ potential (mV) DOPE/DOTAP (μmol) | | |
|---|---|---|---|
| | 0.01 | 0.1 | 1.0 |
| pBR322, 0.1 μg | +3.2 ± 2.9 | +25.6 ± 23.5 | +42.2 ± 18.5 |
| pBR322, 0.5 μg | +1.9 ± 0.7 | +13.3 ± 19.7 | +25.0 ± 23.2 |
| pBR322, 2.5 μg | +0.7 ± 6.5 | +10.6 ± 24.1 | +17.1 ± 21.3 |
| antisense, 2.0 μg | +2.0 ± 14.1 | +21.7 ± 10.0 | +41.1 ± 12.3 |
| antisense, 10.0 μg | −8.8 ± 12.3 | −3.9 ± 15.7 | −2.3 ± 13.6 |
| antisense, 25.0 μg | −11.2 ± 16.0 | −10.4 ± 24.0 | −9.0 ± 18.1 |

Plasmid pBR322 (0.1, 0.5 and 2.5 μg) or antisense DNA (2.0, 10.0 and 25.0 μg) were mixed with 0.01, 0.1 or 1.0 μmol/ml of unilamellar DOPE/DOTAP liposomes (1:1 mol ratio) and incubated for 30 min at room temperature. Liposome charge, measured as the ζ potential, was determined in NaCl (0.85% w/v) at 25°C. The ζ potential is expressed as the mean ± S.D. The diameter of plasmid pBR322/cationic liposomes was between 0.3–3.8 μm and was between 0.1–1.0 μm for antisense DNA/cationic liposomes.

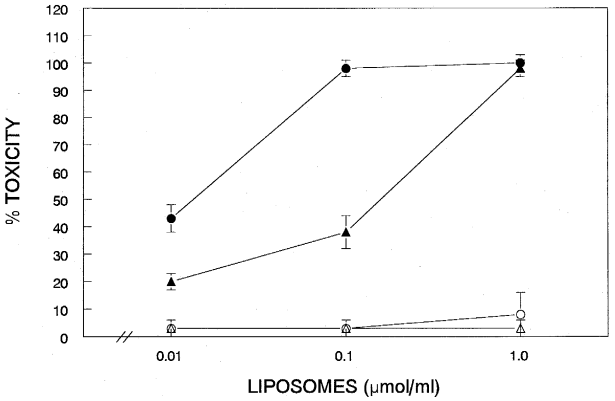


Fig. 3. Toxicity was abolished by the incorporation of DPPE-$PEG_{2000}$ (10 mol%) into DOPE/DOTAP cationic liposomes. Macrophages or U937 monocyte-like cells ($1\times10^5$/well) were incubated with DOPE/DOTAP (●, ▲) or with DOPE/DOTAP + 10 mol% DPPE-$PEG_{2000}$ (○, △) (diameter $< 0.11$ μm) for 24 h at 37°C, 5% $CO_2$, in RPMI-1640/10% FCS. Toxicity was determined by the release of LDH activity into the supernatant after 24 h incubation. Data represent the mean ± S.D. of two replicates of three independent experiments.

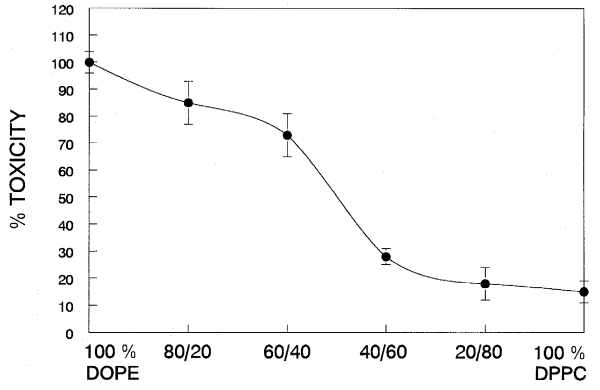


Fig. 4. Toxicity of DOTAP-based cationic liposomes was reduced by the replacement of DOPE with DPPC. Macrophages ($1\times10^5$/well) were incubated for 24 h at 37°C, 5% $CO_2$ in RPMI-1640/10% FCS with 5.0 μmol/ml of DOPE/DOTAP liposomes in which DOPE had been replaced by DPPC. The liposome diameter was $< 0.12$ μm. Toxicity was determined by the release of LDH activity into the supernatant after 24 h incubation. Data represent the mean ± S.D. of two replicates of three independent experiments.

lipids may therefore be responsible for the toxicity observed toward macrophages. To test this hypothesis we have replaced DOPE with DPPC. DPPC is a pH-insensitive phospholipid that does not destabilize the endosomal membrane. The replacement of DOPE by DPPC in liposomes containing cationic DOTAP did not change the $\zeta$ potential (DOPE/DOTAP: $42.0 \pm 10.9$ mV, DPPC/DOTAP: $42.6 \pm 6.8$ mV). However, as shown in Fig. 4, the progressive replacement of DOPE by DPPC significantly reduced toxicity towards macrophages. The replacement of DOPE by DPPC did not completely abolished the toxicity (as was observed for the addition of DPPE-$PEG_{2000}$), but the toxicity never exceed 15% at the concentrations used.

### *3.6. DOPE / DOTAP and DPPC / DOTAP are taken up at similar rates by cells*

It is possible that the reduction of toxicity observed following the replacement of DOPE by DPPC was due to a decrease in endocytosis by macrophages, rather than a reduction in endosomal membrane destabilization. To test this hypothesis, we have measured the uptake by adherent macrophages and non-adherent monocyte-like U937 cells of cationic liposomes containing the fluorescent phospholipid NB-PE. We have observed that DOPE/DOTAP and DPPC/DOTAP were taken up at similar rates by both macrophages and monocyte-like U937 cells (Table 3). However, liposomes were endocytosed by U937 cells as approximately a 2-fold lower rate than

Table 3
Cationic liposome uptake by macrophages and monocyte-like U937 cells

| Liposome formulations | Liposome endocytosis rate (nmol/$5\times10^5$cells/4 h) | |
|---|---|---|
| | Macrophages | Monocyte-like U937 cells |
| DPPC/DOTAP | 27.1 ± 3.5 | 13.5 ± 6.7 |
| DOPE/DOTAP | 28.1 ± 6.0 | 11.2 ± 1.5 |
| DOPE/DDAB | 34.5 ± 8.0 | 9.8 ± 4.5 |
| DOPE/DMTAP | 28.8 ± 4.4 | 13.4 ± 0.9 |
| DOPE/DPTAP | 27.3 ± 5.1 | 21.0 ± 2.4 |
| DOPE/DSTAP | 28.8 ± 5.6 | 2.2 ± 1.2 |
| DOPE/DOTAP + DPPE-$PEG_{2000}$ | 1.2 ± 0.6 | 0.8 ± 0.6 |
| DOPE/DOTAP (w/o FCS) * | 45.9 ± 5.4 | n.d. |

Fluorescent cationic liposomes (10 μmol/ml; $< 0.12$ μm) containing 5 μg/mmol of NBD-PE were incubated for 4 h with macrophages or with U937 monocyte-like cells ($5.0\times10^5$ cells/ml) at 4°C and at 37°C, 5% $CO_2$ in RPMI-1640/10% FCS, or in RPMI-1640 without FCS(*). Endocytosis was calculated from the results obtained at 37°C minus those at 4°C. Data represent the mean ± S.D. of duplicate determinations of two independent experiments.

the macrophages (Table 3). All the cationic liposomes tested had comparable macrophages uptake rates (27.1–34.5 nmol/$5 \times 10^5$/4 h.), while the uptake of cationic liposomes by monocyte-like U937 cells was more variable (2.2–21.0 nmole nmol/$5 \times 10^5$/4 h.). The absence of FCS during the incubation of cationic liposomes with macrophages increased by 1.7-fold the amount of internalized cationic liposomes (Table 3). Furthermore, we have found that the addition of DPPE-$PEG_{2000}$ to DOPE/DOTAP liposomes decreases the binding and the endocytosis of these liposomes by macrophages and by U937 monocyte-like cells (Table 3). The inhibition of binding and uptake by DPPE-$PEG_{2000}$ was correlated with the abolition of the toxicity shown in Fig. 3.

### *3.7. Cationic lipids are toxic toward macrophages*

Our results show that the association of DOPE and cationic lipids is highly toxic toward macrophages but not the association of DPPC and cationic lipid. To evaluate if the association of DOPE and cationic lipids is strictly necessary for the induction of toxicity, we have used DOPE alone or cationic lipid alone in our assays. DOPE or cationic lipids alone do not form liposomes but form aggregates having diameters $> 10$ $\mu$m. Cationic lipids were nevertheless toxic toward macrophages at concentrations equivalent to those used in the liposomes, while DOPE alone was not toxic (Fig. 5). The addition of DOPE synergistically enhanced the toxicity of DDAB, DOTAP and DMTAP (Fig. 5).

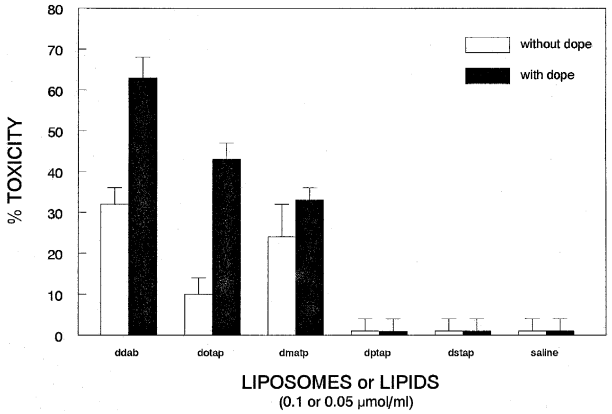


Fig. 5. Cationic lipids were toxic toward macrophages. Macrophages ($1 \times 10^5$/well) were incubated with the indicated cationic liposomes (0.10 $\mu$mol/ml; diameter $< 0.12$ $\mu$m), cationic lipids or DOPE (concentration equivalent for the cationic lipids or DOPE: 0.05 $\mu$mol/ml; diameters $> 10$ $\mu$m) for 24 h at 37°C, 5% $CO_2$ in RPMI-1640/10% FCS. Toxicity was determined by the release of LDH activity in the supernatant. Data represent the mean ± S.D. of two replicates of three independent experiments.

### *3.8. Cationic liposomes and cationic lipids downregulate NO and TNF-α synthesis*

The incubation of macrophages with 0.1 $\mu$mol/ml cationic liposomes for 3 h in the presence of FCS was not sufficient to induce significant toxicity, but was sufficient for the modulation of NO and TNF-$\alpha$ production by LPS/IFN-$\gamma$ activated macrophages (Fig. 6a, b, c). The synthesis of NO was strongly reduced in vitro by DOPE/DOTAP (88%) while TNF-$\alpha$ synthesis was reduced by 62%. After i.p. administration of DOPE/DOTAP (5 $\mu$mol), in vitro NO synthesis was inhibited by 74% and TNF-$\alpha$ synthesis by 81% in LPS/IFN-$\gamma$ activated macrophages. Similar results were obtained with DOPE/DMTAP and DOPE/DDAB at different concentration (data not shown). The addition of DPPE-$PEG_{2000}$ to DOPE/DOTAP or to DOPE/DMTAP liposomes, or the replacement of DOPE by DPPC restored NO and TNF-$\alpha$ production by activated macrophages to normal levels (Fig. 6a, b). The downregulation of NO and TNF-$\alpha$ by activated macrophages was not influenced by the size of the liposome ($< 0.12$ $\mu$m vs. $> 1.00$ $\mu$m) (data not shown). The incorporation of DNA, either as antisense oligonucleotide or as the plasmid pBR322, in DOPE/DOTAP liposomes did not reduce their ability to downregulate NO and TNF-$\alpha$ synthesis by activated macrophages. As illustrated in Fig. 6c, the in vitro synthesis of NO and TNF-$\alpha$ was not restored by the incorporation of 0.5 $\mu$g of plasmid pBR322 or 10.0 $\mu$g of antisense oligonucleotides to 0.1 $\mu$mol/ml of DOPE/DOTAP liposomes. Similar results were obtained with different combination of DNA/cationic liposome in vitro and in situ (data not shown). As shown in Fig. 6d, this downregulation is not only due to the association of DOPE with cationic lipids but could be obtained with cationic lipids alone. The downregulation of NO and TNF-$\alpha$ synthesis was not permanent. The inhibition of NO and TNF-$\alpha$ was reversed if macrophages initially incu-

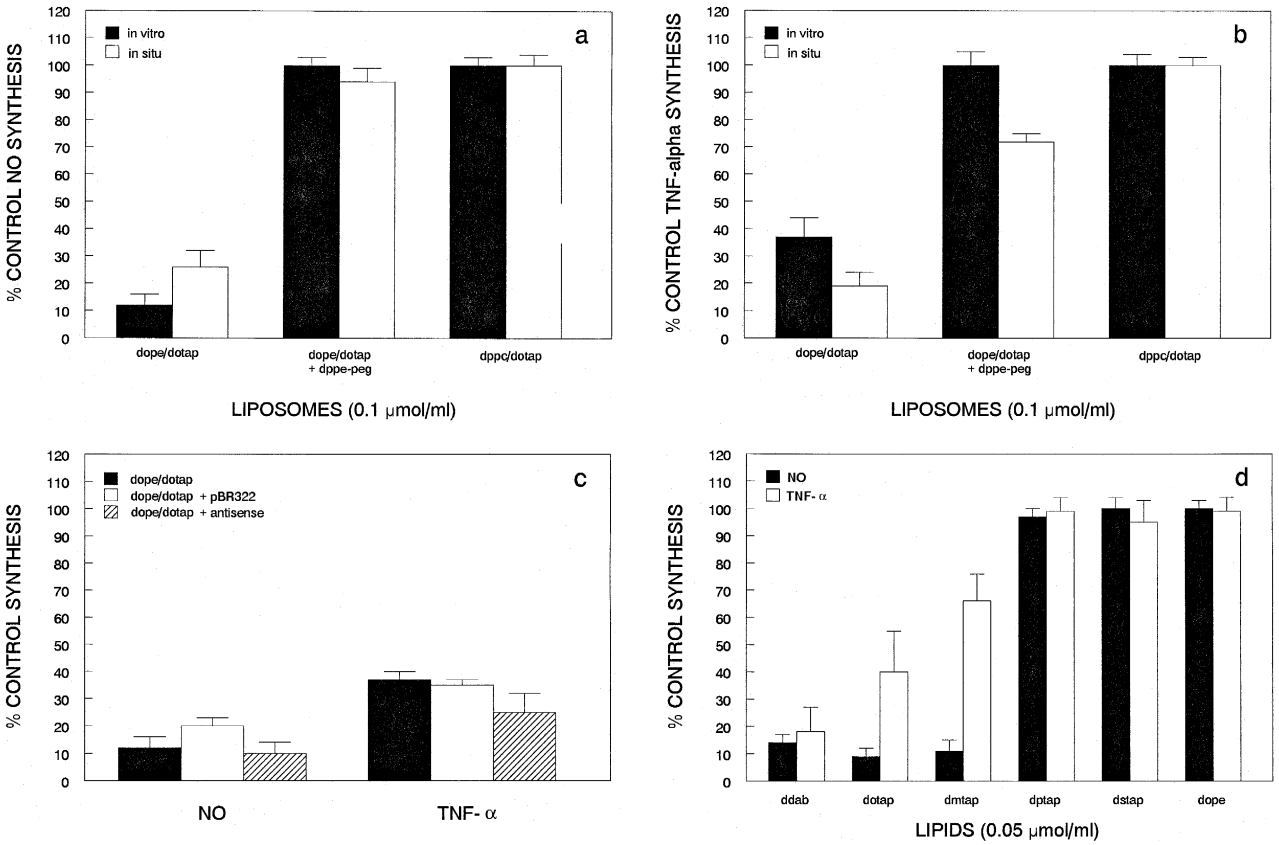


Fig. 6. DNA does not influence the ability of cationic liposomes and cationic lipids to downregulate in vitro and in situ NO and TNF-$\alpha$ synthesis. Macrophages ($1 \times 10^5$/well) were incubated with the indicated cationic liposomes [free (a, b) or complexed to DNA (c)] or cationic lipids (d) for 3 h at 37°C, 5% $CO_2$ in RPMI-1640/10% FCS. The macrophages were then extensively washed and incubated with LPS/IFN-$\gamma$ (b) (1.0 $\mu$g/ml and 500 U/ml respectively) for 48 h. NO was measured after 48 h in the supernatant by reaction with Griess reagent (a, c, d) and TNF-$\alpha$ by commercial ELISA (b, c, d). For in situ treatment in (a) and (b), cationic liposomes (60 mg/kg) were injected i.p. in a volume of 500 $\mu$l on day 3 following the injection of thioglycollate broth. The macrophages were harvested on day 4 and NO and TNF-$\alpha$ synthesis measured after LPS/IFN-$\gamma$ stimulation as described above. Cationic liposome/DNA preparations were obtained by incubating plasmid pBR322 (0.5 $\mu$g) or antisense DNA (10.0 $\mu$g) with DOPE/DOTAP (0.1 $\mu$mol/ml) for 30 min at room temperature. The diameter of plasmid pBR322/cationic liposomes was between 0.8–1.6 $\mu$m and was between 0.3–0.9 $\mu$m for antisense DNA/cationic liposomes. Results are expressed as % of NO or TNF-$\alpha$ reduction vs LPS/IFN-$\gamma$ activated macrophages without liposomes. No toxicity was observed. Data represent the mean $\pm$ S.D. of two replicates of three independent experiments.

bated for 3 h with cationic liposomes were activated by LPS/IFN-$\gamma$ after a minimum further 72 h of cell culture without liposomes (data not shown).

## 4. Discussion

The influence of liposome lipid/phospholipid composition has so far been assumed to be relatively unimportant because of the presumed inert nature of lipids [17–19] and their lack of toxicity toward non-phagocytic cells [6–8]. In this study we have shown that all of the cationic liposomes tested were toxic towards phagocytic macrophages and monocyte-like U937 cells but were not toxic for non-phagocytic T lymphocytes. The rank order of toxicity was DOPE/DDAB > DOPE/DOTAP > DOPE/DMTAP > DOPE/DPTAP > DOPE/DSTAP. The $ED_{50}$'s for macrophage toxicity were $< 10$ nmol/ml for DOPE/DDAB, 12 nmol/ml for DOPE/DOTAP, 50 nmol/ml for DOPE/DMTAP, 400 nmol/ml for DOPE/DPTAP and $> 1000$ nmol/ml for DOPE/DSTAP. The addition of DNA, either as antisense oligonucleotide or as plasmid vector, did

not reduce this toxicity. Furthermore, we have shown that these liposomes, irrespective of whether or not they are complexed with DNA, are capable of down-regulating both in vitro and in situ the synthesis of NO and TNF-$\alpha$ by activated macrophages.

The difference observed between macrophage and T lymphocyte toxicity may be explained by the relative phagocytic activity of macrophages in comparison with T cells. Macrophages have an innate capacity to non-specifically phagocytosis large quantities of particles such as liposomes while T cells do not have this characteristic [20]. It has been shown that macrophages can internalize liposomes containing protein antigens, for example liposomes formulated with DOPE and palmitoyl homocysteine or dioleoylphosphatidylcholine and dioleoylphoshatidylserine, and present their contents to T lymphocytes to initiate an immune response [10,21]. The addition of DPPE-$PEG_{2000}$ to cationic liposomes, which blocks the endocytosis of liposomes by macrophages and monocyte-like U937 cells (Table 3), abolished their toxicity. These results strongly indicate that the high phagocytic activity of macrophages is responsible for the high degree of toxicity. Phagocytic activity, which is less pronounced in monocyte-like U937 cells than in macrophages, as demonstrated by us (Table 3) and by others [22], correlated with the lower level of toxicity observed toward monocyte-like U937 cells in comparison to peritoneal macrophages.

The presence of pH-sensitive DOPE in the cationic liposomes appears to be implicated in the toxicity of cationic lipids toward macrophages. We have found that while all of the cationic lipids tested were toxic in the absence of DOPE, the addition of DOPE clearly enhanced this toxicity in a synergistic manner. The replacement of DOPE by DPPC reduced this toxicity. DOPE may enhance cationic lipid toxicity by destabilizing the endosome membrane (due to the formation of an inverted hexagonal phase at acidic pH) and thus releasing cationic lipids into the cytoplasm. DPPC does not possess this characteristic [23,24]. Non-cationic liposomes are also toxic towards phagocytic cells but to a lesser extent than cationic liposomes. We have shown previously that liposomes composed of dipalmitoylphosphatidylcholine (DPPC)/dipalmitoylphosphatidylethanolamine (DPPE), DPPC/dimyristoylphosphatidylglycerol (DMPG) and DPPC/phosphatidylserine (PS) phospholipids (final concentration 0.5 $\mu$mol phospholipids/ml) were toxic towards macrophages, although the maximum toxicity never exceeded 15% for any liposome preparations [25]. In the present study we have also shown that the toxicity of DPPC/DOTAP liposomes is comparable to the toxicity observed with anionic or neutral liposomes ($<$ 15%).

Positively charged molecules have been implicated in cellular toxicity in other models [26]. Our results show that liposome charge, as measured by the $\zeta$ potential, is not an important factor in explaining this toxicity. Although DOPE/DDAB, DOPE/DOTAP, DOPE/DMTAP and DPPC/DOTAP liposomes have comparable positive $\zeta$ potentials, they differ significantly in their toxicity. The incorporation of DNA in cationic liposomes, which gave rise to a negative $\zeta$ potential in several formulations (see Table 2), did not abolished the toxicity observed toward macrophages. Furthermore, the $\zeta$ potential of cationic liposomes incubated overnight with RPMI-1640 containing 10% FCS is slightly negative (e.g., DOPE/DOTAP: $-8.0$ mV) but these liposomes are still highly toxic towards macrophages. The differences in toxicity observed in the absence and presence of FCS may be explained by the fact that FCS interferes with cationic liposomes binding and endocytosis (this study). Litzinger et al. have reported similar results for antisense oligonucleotide delivery using DOPE/DC–Chol cationic liposomes [27]. The observation that DOPE/DPTAP and DOPE/DSTAP have toxicity comparable to DOPE/DDAB, DOPE/DOTAP and DOPE/DMTAP in the absence of FCS, strongly suggests that DOPE/DPTAP and DOPE/DSTAP interact differently with serum proteins in a qualitative and/or quantitative manner. Membrane fluidity could explained their different toxicity in presence of FCS. It is highly likely that DOPE/DPTAP, DPPC/DOTAP and DOPE/DSTAP liposomes do not possess a fluid membrane at 37°C. DPPC, DPTAP and DSTAP have liquid-crystalline transition temperatures ($T_g$) of $+41.4$°C, $+44.5$°C and $+55.4$°C respectively, while DOTAP and DMTAP have $T_g$'s of $-16.5$°C and $+34.5$°C respectively (Pham and Phillips, unpublished observations). It is clear that DDAB, which has a $T_g$ of $+46.0$°C, does not follow this pattern, but its chemical structure is different from the 'tri-

methylammonium propane' series. We are currently evaluating the $T_g$ of cationic liposomes in the absence and presence of DNA.

It is clear that under non-toxic condition, cationic liposomes are not inert. Cationic liposomes, via their cationic lipid component, can downregulate at least two immunomodulators, NO and TNF-$\alpha$, produced by activated macrophages. The downregulation of NO and TNF-$\alpha$ synthesis by cationic liposomes may be explained by the fact that cationic lipids modulate the activity of protein kinase C (PKC) activity. Bottega and Epand [28] have shown that positively charged amphiphiles inhibit PKC activity, while Farhood et al. [29] have obtained similar results with cationic derivatives of cholesterol that are currently used in cationic liposomes. The inhibition of PKC activity has been shown to inhibit the transfection activity of nucleic acids [29]. The biosynthesis of NO and TNF-$\alpha$ are PKC-dependent processes [30,31]. The downregulation of NO and TNF-$\alpha$ synthesis by the cationic liposomes used in this study can be correlated with their ability to inhibit PKC activity (Filion and Phillips, manuscript submitted). The use of cationic liposomes to deliver antisense oligonucleotides to macrophages or other phagocytic cells may therefore not be appropriate. Non-specific modulation by cationic lipids of the level of expression of several molecules that depend directly or indirectly on PKC activity, as illustrated here by the downregulation of NO and TNF-$\alpha$ synthesis, may lead to a misinterpretation of the data obtained. Furthermore, NO and TNF-$\alpha$ have significant roles in immune and inflammatory processes [11,12]. In fact, we have shown that cationic liposomes, irrespective of whether or not they are complexed to DNA, have potent anti-inflammatory activity in vivo (Filion and Phillips, manuscript submitted).

Cationic liposomes should perhaps be used with caution to deliver DNA in vivo, especially in antisense oligonucleotides therapy which requires the long-term administration of large amounts of DNA for therapeutical efficacy. For example, the administration of 6 mg/kg/day of antisense oligonucleotide directed against PKC-$\alpha$ mRNA for at least 24 days is necessary to completely inhibit the growth of tumors in mice [32]. In humans, clinical studies with 0.1 mg/kg of antisense oligonucleotides complementary to *gag* gene of HIV-1 has been carried-out [33]. In such cases, the adoption of a cationic liposome delivery strategy to increase the stability of the antisense oligonucleotides represents the administration of approximately 1–60 mg/kg/day of cationic liposomes using a cationic liposome/DNA ratio of 10:1. In this study we have shown than in vivo treatment with 3.75, 15.0 or 60.0 mg/kg of cationic liposomes significantly decreases the synthesis of NO and TNF-$\alpha$ by activated macrophages is associated with anti-inflammatory activity (Fig. 6 and Filion and Phillips, manuscript submitted). Furthermore it has been shown that the association of oligonucleotides and cationic liposomes formulated with DC–Chol forms aggregates that are capable of accumulating within pulmonary capillaries and provoking the formation of emboli [27].

The use of cationic liposomes to deliver gene therapy via plasmid vector is less problematic. Stable gene transfer can be achieved with a single dose of DNA-liposome complex. For example, gene expression following the i.v. administration of chloramphenicol acetyltransferase reporter plasmid complexed with liposomes formulated from DOPE and dioctadecemyloxypropyltrimethylammonium chloride (DOTMA) was detected for up to 9 weeks [34]. However, we feel it prudent to highlight the fact that cationic liposomes have been reported to have several adverse effects other than the downregulation of NO and TNF-$\alpha$ synthesis. Liposomal vectors formulated with positively-charged stearylamine (SA), DOTMA or dihexadecyloxylpropyltrimethylammonium chloride (BisHOP) have been shown to interact with serum proteins and red blood cells, inducing a strong clotting response and haemolysis respectively [26,35]. The presence of a positive charge at the surface of liposomes formulated with SA or BisHOP is also associated with an enhancement of their blood clearance rates [36]. Liposomes based on SA or DOTAP cationic lipids can also activate complement via the alternative pathway [37]. These complement-activating liposomes have C3b associated with their membrane which enhances the recognition of liposomes by the immune system as foreign particles. Furthermore, it has been shown that cationic DOPE/DOTAP liposomes can induce epithelial necrosis at terminal and respiratory bronchi, alveolar ducts and alveoli following pulmonary administration [38].

It is clear from the present study that cationic

liposomes should be used with caution for the intracellular delivery of DNA to macrophages. Cationic liposomes, whether or not they are complexed with DNA, are highly toxic in vitro toward macrophages, but not toward non-phagocytic T cells. The downregulation by cationic liposomes under non-toxic conditions of (at least) two of the immunomodulators produced by activated macrophages should also be take in consideration. The impact of cationic lipid-induced macrophage downregulation on gene expression or on macrophage-related host defence is at present unknown.

## Acknowledgements

This work was supported by a grant to N.C.P. from Pasteur-Mérieux-Connaught Laboratories, North York, Ontario, Canada.

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