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Major limitations in the use of cationic liposomes for DNA delivery

Mario C. Filion, Nigel C. Phillips *

Faculté de Pharmacie, Université de Montréal, C.P. 6128, Succ. Centre-Ville, Montréal, Québec H3C 3J7, Canada

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Abstract

Liposomal vectors formulated with cationic lipids and the fusogenic phospholipid dioleyolphosphatidylethanolamine (DOPE) are usually used to target DNA inside mammalian cells. Since macrophages constitute the major site of liposome localisation after parenteral administration we felt it prudent to examine the effect of cationic liposomes on the production of several important immuno-inflammatory modulators secreted by activated macrophages. In addition, we have evaluated the toxicity of different cationic liposome formulations towards phagocytic macrophages and non-phagocytic T-lymphocytes. Our results indicate that cationic liposomes are able to down-regulate the synthesis of the protein kinase C (PKC)-dependent mediators nitric oxide (NO), tumour necrosis factor-α (TNF-α) and prostaglandin E_2 (PGE_2) by activated macrophages after in vitro incubation under non-toxic conditions or after in vivo treatment, while the production of PKC-independent IL-6 is not modified. We have shown that cationic lipids possess potent anti-inflammatory activity in vivo. Prolonged incubation (>3 h) of macrophages with cationic liposomes induced a high level of toxicity ($ED_{50} < 50$ nmol/ml) that was not seen with non-phagocytic T-cells ($ED_{50} > 1000$ nmol/ml). The rank order of toxicity was DOPE/dimethyldioctacylammonium bromide (DDAB) > DOPE/dioleyoltrimethylammonium propane (DOTAP) = DOPE/dimethylaminoethanecarbamoyl cholesterol (DC-Chol) > DOPE/dimyristoyltrimethylammonium propane. The replacement of DOPE by dipalmitoylphosphatidylcholine (DPPC) or the incorporation of dipamitoylphosphatidylethanolamine-PEG_{2000} (DPPE-PEG_{2000}) in DOPE/cationic lipids reduced the toxicity toward macrophages and restored the synthesis of PKC-dependent modulators. The incorporation of DNA, either as an antisense oligonucleotide (15-mers) or as the plasmid vector pBR322 (4363 bp), in cationic liposomes did not reduce these adverse effects. These results, in addition to the observation that cationic liposomes are extremely toxic following oral administration, indicate that DOPE/cationic lipid liposomes are not appropriate for DNA (or drug) delivery.

Keywords: Cationic liposomes; DNA delivery; Toxicity; Inflammation; Protein kinase C

* Corresponding author. Tel.: +1 514 3435851; fax: +1 514 3432102; e-mail: phillipn@.mistrale.ere.umontreal.ca

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1. Introduction

The influence of liposome phospholipid composition on the activity of incorporated immunomodulatory drugs has so far been assumed to be relatively unimportant because of the presumed inert nature of phospholipids towards the immune system (van Roijeen and Nieuwmegen, 1983; Storm et al., 1993). There is, however, ample evidence of intrinsic immunomodulatory activity for several phospholipids. For example, phosphatidyl serine has been shown to stimulate macrophage growth (Yui and Yamazaki, 1987) and to inhibit the production of IL-2 and IL-2 receptor expression in T-lymphocytes (Caselli et al., 1992) while phosphatidylethanolamine can stimulate the synthesis of prostaglandin E_2 by macrophages (Bakouche et al., 1987). In this report we have evaluated the toxicity and the immunomodulatory activity of different cationic lipids toward murine macrophages and T-lymphocytes. Cationic lipids are usually used in association with dioleyolphosphatidylethanolamine (DOPE) to deliver nucleic acids inside mammalian cells for antisense oligonucleotide or gene therapy (Farhood et al., 1995).

We have found that liposomes formulated with DOPE and cationic dioleyoldiacyltrimethylammonium propane (DOTAP) or dimethylaminoethanecarbamoyl cholesterol (DC-Chol) lipids down-regulate, in vitro and in situ, the synthesis of NO, TNF-α and PGE_2 by activated macrophages while the synthesis of IL-6 is not affected. The incorporation of DNA, either as an antisense oligonucleotide (15-mers) or as the plasmid vector pBR322 (4363 bp), into cationic liposomes does not modify the ability of cationic liposomes to inhibit NO, TNF-α and PGE_2 synthesis. This down-regulation was due to the cationic lipid component of the liposome. The biosynthesis of NO, TNF-α and PGE_2 synthesis, but not IL-6, is dependent on protein kinase C (PKC) activity (Kovacs et al., 1988; Severn et al., 1992; Blanco et al., 1995; Tremblay et al., 1995). We have therefore determined whether the cationic lipids can inhibit PKC activity. The cationic lipids have the ability to dramatically inhibit the activity of PKC, but not the activity of a non-related protein kinase, PKA. The PKC IC_{50} was 115 nM for DOTAP and 506 nM for DC-Chol. The replacement of fusogenic DOPE lipids by non-fusogenic dipalmitoylphosphatidylcholine (DPPC), which prevents the release of cationic lipid into the cytoplasm or the incorporation of dipalmitoylphosphatidylethanolamine-PEG_{2000} (DPPE-PEG_{2000}; 10 mol%), which prevents the interaction between liposomes and the macrophage cell surface, restored the synthesis of NO and TNF-α synthesis, suggesting that the release of cationic lipids into the macrophage cytoplasm is essential for the inhibition of these immunomodulators.

Prolonged incubation of macrophages (>3 h) with cationic DOPE/DOTAP, DOPE/DC-Chol, DOPE/dimethyldioctacylammonium bromide (DDAB) or DOPE/dimethyl dioleyoldiacyltrimethylammonium propane (DMTAP) induced a high level of toxicity ($ED_{50} < 50$ nmol/ml) while the incubation with non-phagocytic T-lymphocytes did not induce toxicity ($ED_{50} > 1000$ nmol/ml). The rank order of toxicity towards macrophage was DOPE/DDAB > DOPE/DOTAP = DOPE/DC-Chol > DOPE/DMTAP. Although toxicity was observed with cationic lipids alone it was clearly enhanced by the presence of DOPE in a synergetic manner. The incorporation of DNA, either as an antisense oligonucleotide or as the plasmid pBR322, in cationic liposomes had little effect on toxicity. The replacement of DOPE by DPPC reduced cationic liposomes toxicity toward macrophages and the presence of DPPE-PEG_{2000} (10 mol%) abolished this toxicity. These data strongly suggest that the liposome is endocytosed by the macrophages and subsequently released into the cytoplasm to induce toxicity.

It is clear from our studies that the use of cationic liposomes for in vitro nucleic acids transfection to macrophages is not appropriate. They show high toxicity toward macrophages and under non-toxic condition downregulate (at least) three of the immunomodulators produced by activated macrophages. Since the macrophage constitutes the major site of liposome localisation after parenteral administration (Velinova et al., 1996)

and is a major contributor in the initiation and maintenance of immune and inflammatory responses (Adams and Hamilton, 1984), cationic liposomes should be used with caution in vivo. The synthesis of NO, TNF-α and PGE_2 by activated macrophages, which play an important role in immune and inflammatory responses (Vassalli, 1992; Dugas et al., 1995), was inhibited following the i.p. administration of cationic liposomes. In vivo, cationic liposomes have a strong anti-inflammatory activity in immune and non-immune models of inflammation (Filion and Phillips, 1997a). We have also noted that cationic liposomes are highly toxic in mice following oral administration, inducing a profound and lethal hypothermia.

The supposition that all liposomes are biologically compatible or acceptable (van Roijeen and Nieuwmegen, 1983; Storm et al., 1993) appears to have erroneously applied to cationic liposomes. A number of other studies demonstrating adverse effects associated with the use of cationic lipids and/or cationic liposomes will be discussed in addition to the results obtained in our own studies. Together, these data strongly suggest that cationic liposomes are not appropriate for DNA (or drug) delivery.

2. Experimental procedures

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. All manipulations were carried out in a class 100 horizontal laminar flow cabinet. The cationic lipids DOTAP and DMTAP were obtained from Avanti Polar Lipids (Alabaster, AL), while DDAB and DC-Chol were obtained from Sigma (St. Louis, MO). The cationic lipids were dissolved in chloroform, mixed in a 1:1 mol ratio with DOPE or with DPPC (Avanti Polar Lipids) and evaporated to dryness at 60°C in a round-bottomed flask using a rotary evaporator. Amphiphilic DPPE-PEG_{2000}, 10 mol% final concentration (Shearwater Polymers, Huntsville, AL) was dissolved in chloroform and mixed with the cationic lipid/phospholipid prior to rotary evaporation. Liposomes were prepared by adding the required volume of 0.85% w/v NaCl followed by agitation and sonicated to obtain unilamellar liposomes (< 0.12 μm). The size of the liposomes was determined by photo correlation spectroscopy in a Coulter 4N Plus submicron particle analyser (Coulter, Miami, FL). Liposomal charge, determined as the 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.

2.2. 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 OligoM1000, Columbia, MD) by the Department of Biochemistry, Université de Montréal. The antisense DNA was purified by several cycles of ethanol precipitation and resuspended in DNase-free water before use. Purified pBR322 plasmid (4363 bp) was kindly provided by Dr Philippe Raymond (Institut Armand-Frappier, Laval, Qc., Canada). Plasmid (0.1, 0.5 and 2.5 μg) or antisense oligonucleotide (2.0, 10.0 and 25.0 μg) were mixed with 0.01, 0.1 or 1.0 μmol/ml of unilamellar cationic liposomes composed of DOPE/DOTAP (1:1 mol ratio) and incubated for 30 min. at room temperature before use.

2.3. NO, TNF-α, IL-6 and PGE_2 determination

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) and were harvested 4 days later as previously described (Phillips and Gagné, 1995). Macrophages were incubated at 1×10^5 cells/well with the indicated liposomes for 3 h at 37°C, 5% CO_2 and washed extensively with warm RPMI-1640/10%FCS (Gibco Life Science, Burlington, Canada). The macrophages were then incubated with *E. Coli* 011:B4 lipopolysaccharide (LPS) (1.0 μg/ml, obtained from Sigma)/interferon-γ (IFN-γ) (500 U/ml, obtained from Gibco

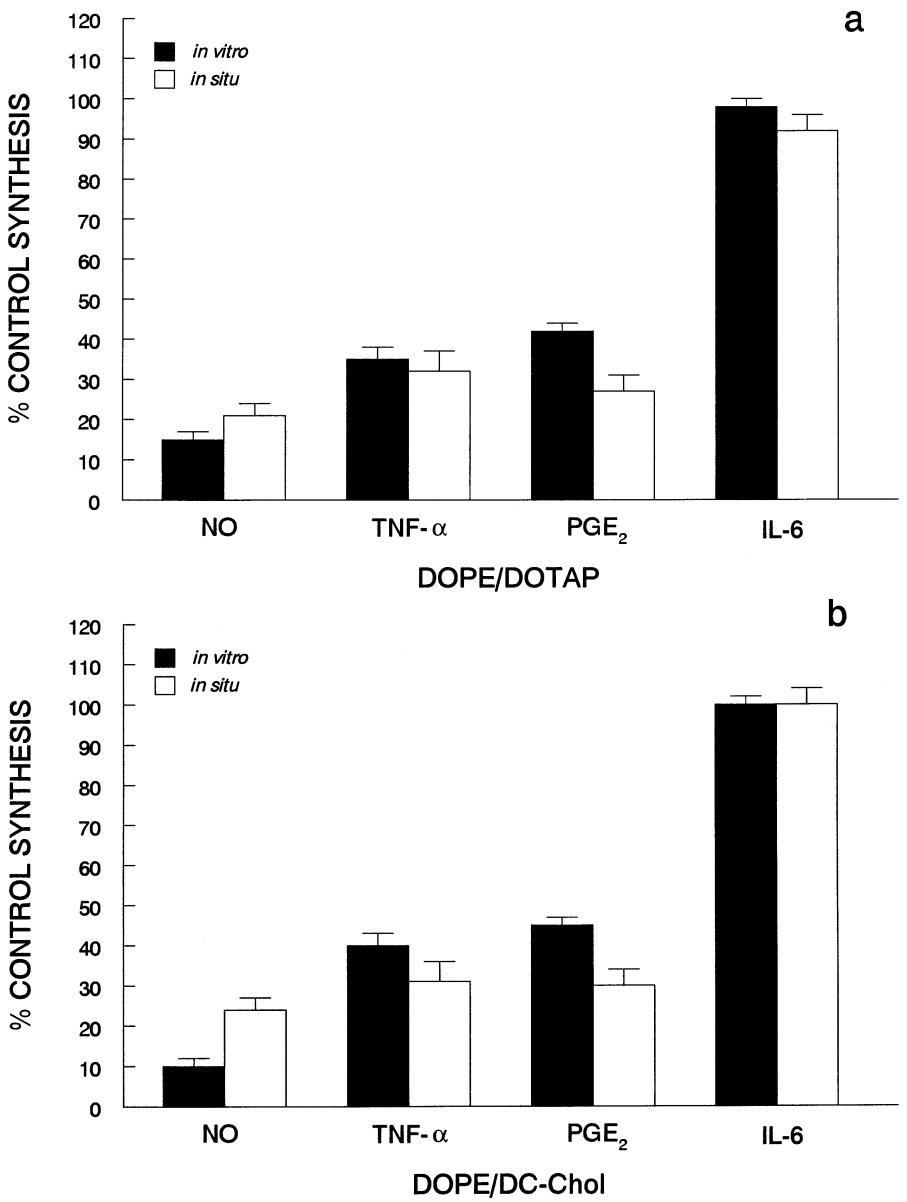

Fig. 1. Cationic liposomes down-regulated NO, TNF-α and PGE_2 synthesis. Macrophages (1×10^5/well) were incubated (a) with DOPE/DOTAP (0.1 μmol/ml) or (b) with DOPE/DC-Chol (0.1 μmol/ml) for 3 h at 37° C, 5% CO_2 in RPMI-1640/10% FCS. The macrophages were then washed extensively and incubated with LPS/IFN-γ (1.0 μg/ml and 500 U/ml respectively) for 48 h. NO was measured in the supernatant after 48 h by reaction with Griess reagent while TNF-α, IL-6 and PGE_2 were measured by commercial ELISA. For in situ treatment, cationic liposome (60 mg/kg) were injected i.p. in a volume of 500 μl on day 3 following the injection of thioglycollate broth. The macrophages were harvested on day 4 and NO, TNF-α, IL-6 and PGE_2 measured after LPS/IFN-γ stimulation as described above. Results are expressed as a percentage of NO, TNF-α, IL-6 and PGE_2 reduction versus LPS/IFN-γ activated macrophages without liposomes. No toxicity was observed under these conditions. The diameter of the liposome used was between 0.08–0.12 μm. Data represent the mean $\pm$ S.D. of two replicates of three independent experiments.

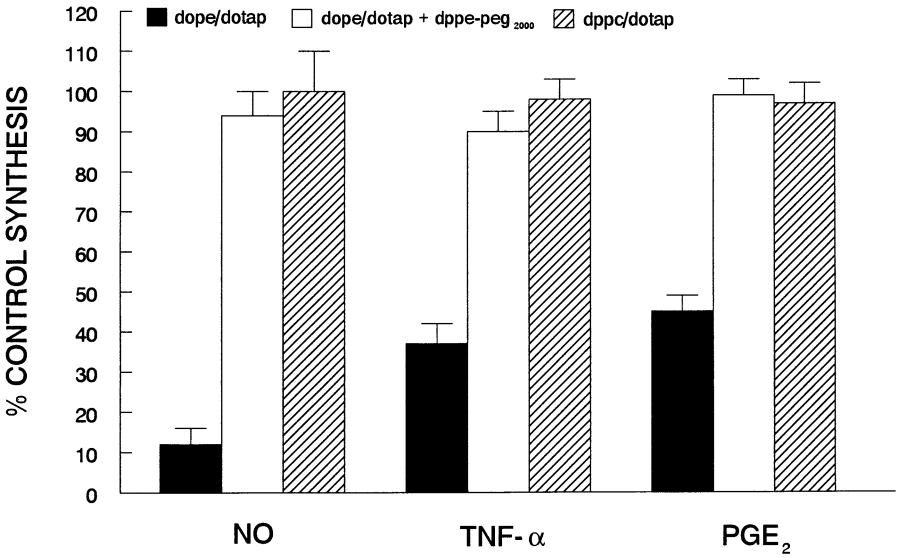

Fig. 2. The incorporation of DPPE-PEG_{2000} (10 mol%) into DOPE/DOTAP liposomes or the replacement of DOPE by DPPC abolished the down-regulation of NO, TNF-α and PGE_2 synthesis. Macrophages (1×10^5/well) were incubated with 0.1 μmol/ml of DOPE/DOTAP, DPPC/DOTAP or DOPE/DOTAP/DPPE-PEG_{2000} (10 mol%) for 3 h at 37° C, 5% CO_2 in RPMI-1640/10% FCS. The macrophages were then washed extensively and incubated with LPS/IFN-γ (1.0 μg/ml and 500 U/ml respectively) for 48 h. NO was measured after 48 h. in the supernatant by reaction with Griess reagent while TNF-α, IL-6 and PGE_2 were measured by commercial ELISA. Results are expressed as a percentage of NO, TNF-α, IL-6 and PGE_2 reduction versus LPS/IFN-γ activated macrophages without liposomes. No toxicity was observed under these conditions. The diameter of the liposome used was between 0.08–0.12 μm. Data represent the mean $\pm$ S.D. of two replicates of three independent experiments.

Life Science) for 48 h. NO_2^-, one of the end products of NO synthesis, was measured after 48 h incubation by mixing 50 μl supernatant with 50 μl of Griess Reagent (1% sulphanilamide in 2.5% H_3PO_4 mixed with an equal volume of 0.1% *N*-(1-naphtyl)ethylenediamine HCl in H_2O) and determining the absorbance at 550 nm against $NaNO_2$ standards. TNF-α, IL-6 and PGE_2 synthesis were measured by means of commercial ELISA kits (TNF-α and IL-6: BioSource, Camarillo, CA; PGE_2: Amersham, Oakville, Ontario, Canada) using 50 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, TNF-α and PGE_2 synthesis measured after LPS/IFN-γ treatment as described above. Toxicity was determined as described in Section 2.4 at 3, 24 and 48 h. using 2 μl supernatant.

2.4. Toxicity assay

Liposome toxicity towards immune effector cells was determined as previously described (Phillips and Gagné, 1995). Briefly, 1.0×10^5 cells/well (macrophages or T-cells) were incubated with the indicated liposome formulation at different concentrations 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). Total LDH activity was determined by incubating the cells in 1.0% v/v Triton X-100 to induce lysis, followed by vigorous agitation.

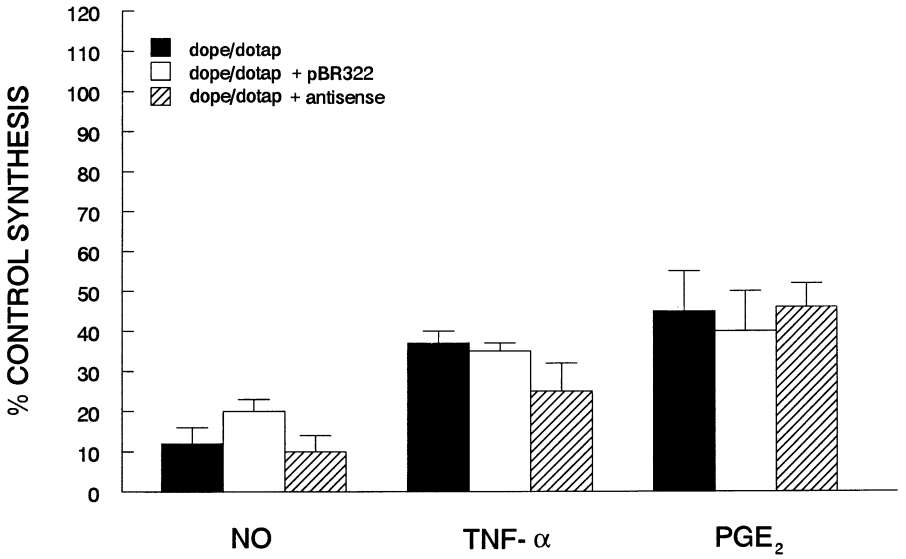

Fig. 3. The incorporation of DNA by DOPE/DOTAP liposomes does not reduce the ability of cationic DOPE/DOTAP to down-regulate NO, TNF-α and PGE_2 synthesis. Macrophages (1×10^5/well) were incubated with the indicated cationic liposomes/DNA complex for 3 h at 37° C, 5% CO_2 in RPMI-1640/10% FCS. Cationic liposome/DNA preparations were obtained by incubating plasmid pBR322 (0.5 μg) or antisense oligonucleotide (10.0 μg) with DOPE/DOTAP (0.1 μmol/ml) for 30 min. at room temperature. The diameter of pBR322/cationic liposomes was between 0.8–1.6 μm and was between 0.3–0.9 μm for antisense DNA/cationic liposomes. Results are expressed as a % of NO, TNF-α and PGE_2 reduction versus LPS/IFN-γ activated macrophages without liposomes. No toxicity was observed under these conditions. Data represent the mean $\pm$ S.D. of two replicates of three independent experiments.

3. Results and discussion

3.1. Cationic liposomes inhibited PKC-dependent immunodulator synthesis by activated macrophages

The incubation of macrophages with positively charged DOPE/DOTAP or DOPE/DC-Chol liposomes (0.1 μmol/ml) for 3 hours did not induce toxicity (data not shown) but significantly down-regulated the in vitro synthesis of NO, TNF-α and PGE_2 by LPS/IFN-γ-activated macrophages (Fig. 1). The production of IL-6 was not affected (Fig. 1). Similar results were obtained with LPS/IFN-γ-activated macrophages following i.p. administration of cationic DOPE/DOTAP or DOPE/DC-Chol liposomes (60 mg/kg) (Fig. 1). This down-regulation was mediated by the cationic lipid component. The addition of DOTAP or DC-Chol to macrophages at concentrations equivalent to those used in the liposomes formulated with DOPE down-regulated the synthesis of NO, TNF-α and PGE_2 by activated macrophages. The addition of DOPE, which does not form liposomes but forms aggregates having a diameter > 10 μm, to macrophages did not affect immunomodulator synthesis (Filion and Phillips, unpublished).

The biosynthesis of TNF-α, NO and PGE_2 is a PKC-dependent process (Kovacs et al., 1988; Severn et al., 1992; Blanco et al. 1995), while the regulation of IL-6 production in cells of the monocyte lineage is a PKC-independent process (Gross et al., 1993; Tremblay et al., 1995). Previously, it has been shown that some cationic amphiphilic compounds can inhibit PKC activity (Bottega and Epand, 1992; Farhood et al., 1992). We have therefore determined whether the cationic lipids used in our study can inhibit PKC. The cationic lipids DOTAP and DC-Chol lipids dramatically inhibited the activity of PKC (IC_{50} of 115 nm for DOTAP and 506 nm for DC-Chol), while the activity of a non-related protein kinase, PKA, is not modified (Filion and Phillips, 1997a).

Table 1
Charge neutralisation of cationic lipids with anionic DNA does not abrogate toxicity or down-regulation of NO, TNF-α and PGE_2 by activated macrophages

Liposomes	ζ potential (mV ± S.D.)	Diameter (μm ± S.D.)	Toxicity	Inhibition of immunomodulators
DOPE/DOTAP (0 01 μmol/ml)+saline	+42.0 ± 10.9	0.08–0.11	Yes[a]	Yes
DOPE/DOTAP (0.01 μmol/ml)+pBR322 (2.5 μg)	+0.7 ± 6.5	3.35–3.80	Yes	Yes
DOPE/DOTAP (0.01 μmol/ml)+antisense DNA (10.0 μg)	−8.8 ± 12.3	0.76–0.95	Yes	Yes

Plasmid pBR322 (2.5 μg) or antisense DNA (10.0 μg) were mixed with 0.01 μmol/ml of unilamellar DOPE/DOTAP liposomes (1:1 mol ratio) and incubated 30 min at room temperature. Liposome charge, measured as the ζ-potential and liposome size were determined in NaCl (0.85% w/v) at 25° C.

[a] Toxicity was determined by the release of LDH into the medium supernatant after 24 h incubation.

[b] Immunomodulatory activity was determined by the production of NO, TNF-α and PGE_2 by LPS/IFN-γ activated macrophages.

The inhibition of PKC by cationic amphiphile compound has been shown to down-regulate inflammation through the reduction of PKC-dependent immunomodulators (Misiewicz et al., 1996; Sugunuma et al., 1996; Koga et al., 1995). The cationic DOTAP and DC-Chol lipids used in our study, which are widely used for gene therapy, also possess this anti-inflammatory activity. The parenteral administration (i.p. or s.c.) or the local injection of cationic DOTAP and DC-Chol, as well as DDAB and DMTAP lipids, reduce dramatically the footpad inflammation induced by carrageenan or by sheep red blood cell challenge in mice (Filion and Phillips, 1997a).

The incubation of macrophages with positively charged liposomes formulated with fusogenic DOPE down-regulated NO, TNF-α and PGE_2 synthesis by activated macrophages, while the incubation of liposomes formulated with DOTAP and non-fusogenic DPPC did not inhibit the production of NO, TNF-α and PGE_2. These results suggest that the presence of DPPC prevents the down-regulation of these modulators by blocking the release of cationic DOTAP lipids in the cytoplasm following endocytosis (Fig. 2). We have previously shown that the replacement of DOPE by DPPC in cationic liposomes formulated with DOTAP does not reduce the quantity of cationic liposomes bound at the cell surface of or endocytosed by macrophages (Filion and Phillips, 1997b). The inhibition of NO, TNF-α and PGE_2 by activated macrophages was also abrogated by the addition of DPPE-PEG_{2000} (Fig. 2), an amphiphilic compound which is able to block the interaction between liposomes and the macrophage cell surface (Vertut-Doï et al., 1996; Filion and Phillips, 1997b). The internalization by the macrophages of the cationic liposomes and the release of cationic lipids into the macrophage cytoplasm would appear to be essential for the inhibition of PKC-dependent immunomodulators. Furthermore, we have found that the in vivo administration of DPPC/DOTAP or DOPE/DOTAP/DPPE-PEG_{2000} abrogated the anti-inflammatory activity observed with DOPE/DOTAP liposomes (Filion and Phillips, 1997a).

Liposomal vectors formulated with cationic lipids are usually used to deliver antisense oligonucleotides or plasmid vector inside non-phagocytic mammalian cells (Farhood et al., 1995). We have further evaluated whether the incorporation of DNA, either as a single stranded antisense oligonucleotide (15-mers) or as the double stranded plasmid vector pBR322 (4363 bp), in cationic liposomes can abrogate this non-specific inhibition of NO, TNF-α and PGE_2 by cationic lipids. As illustrated in Fig. 3, the incorporation of 10.0 μg of antisense DNA or 0.5 μg of plasmid vector pBR322 in cationic DOPE/DOTAP liposomes (0.1 μmol/ml) did not restore the production of NO, TNF-α and PGE_2 by activated macrophages. Similar results were obtained with different concentration of DNA complexed to various quantities of liposomes (Filion and Phillips, unpublished). These results clearly show that charge neutralisation of cationic lipid with

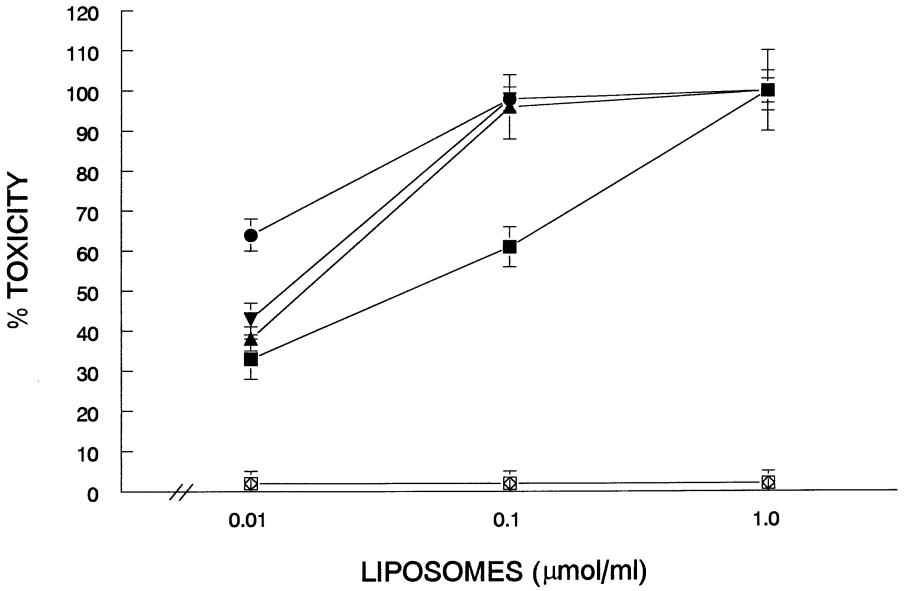

Fig. 4. Cationic liposomes were toxic for macrophages but not for T-lymphocytes. Macrophages (1×10^5/well) were incubated with the indicated cationic liposomes (DOPE/DDAB, ●; DOPE/DOTAP, ▼; DOPE/DC-Chol, ▲; DOPE/DMTAP, ■) for 24 h at 37°C, 5% CO_2 in RPMI-1640/10% FCS. Resting (◇) or activated T-cells 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 μm (unilamellar). Toxicity was determined by the release of LDH activity at 24 h. Data represent the mean ± S.D. of two replicates of three independent experiments.

anionic DNA is not sufficient to abrogate the down-regulation of NO, TNF-α and PGE_2 by activated macrophages (Table 1).

3.2. Cationic liposomes were toxic toward phagocytic macrophages

Prolonged in vitro incubation (>3 h) of macrophages with different formulations of cationic liposomes induced a high level of toxicity. The rank order of toxicity was DOPE/DDAB > DOPE/DOTAP = DOPE/DC - Chol > DOPE/DMTAP (Fig. 4). The ED_{50} for macrophage toxicity were <10 nmol/ml for DOPE/DOTAP, 12 nmol/ml for DOPE/DOTAP, 16 nmol/ml for DOPE/DC-Chol and 50 nmol/ml for DOPE/DMTAP. Cationic liposomes were not toxic toward resting or T-cells activated by 1% PHA ($ED_{50} >$ 1000 nmol/ml) (Fig. 4).

The difference in toxicity observed between macrophages and T-lymphocytes may be explained by the relative phagocytic activity of macrophages compared to T-cells. Macrophages, which are known to present antigens, have the capacity to phagocytosis a large quantity of particles such as liposomes, while T-cells do not have this capacity (Filion et al., 1996). The presence of $DPPE\text{-}PEG_{2000}$ in liposomes, which blocks liposome endocytosis (Vertut-Doï et al., 1996; Filion and Phillips, 1997b) abolishes the toxicity toward macrophages, indicating that the cationic liposomes must be internalized to induce toxicity. The replacement of DOPE by non-fusogenic DPPC lipids also abolished this toxicity, indicating that the destabilisation of the endosomic membrane and subsequently release of cationic lipid into the cytoplasm is necessary for the induction of toxicity (Filion and Phillips, 1997b).

The incorporation of different amounts of DNA, either as antisense oligonucleotide or the plasmid pBR322, in unilamellar DOPE/DOTAP liposomes, some of which represent the optimal cationic liposomes/DNA ratios currently used for in vitro transfection (Whitt et al., 1995), did

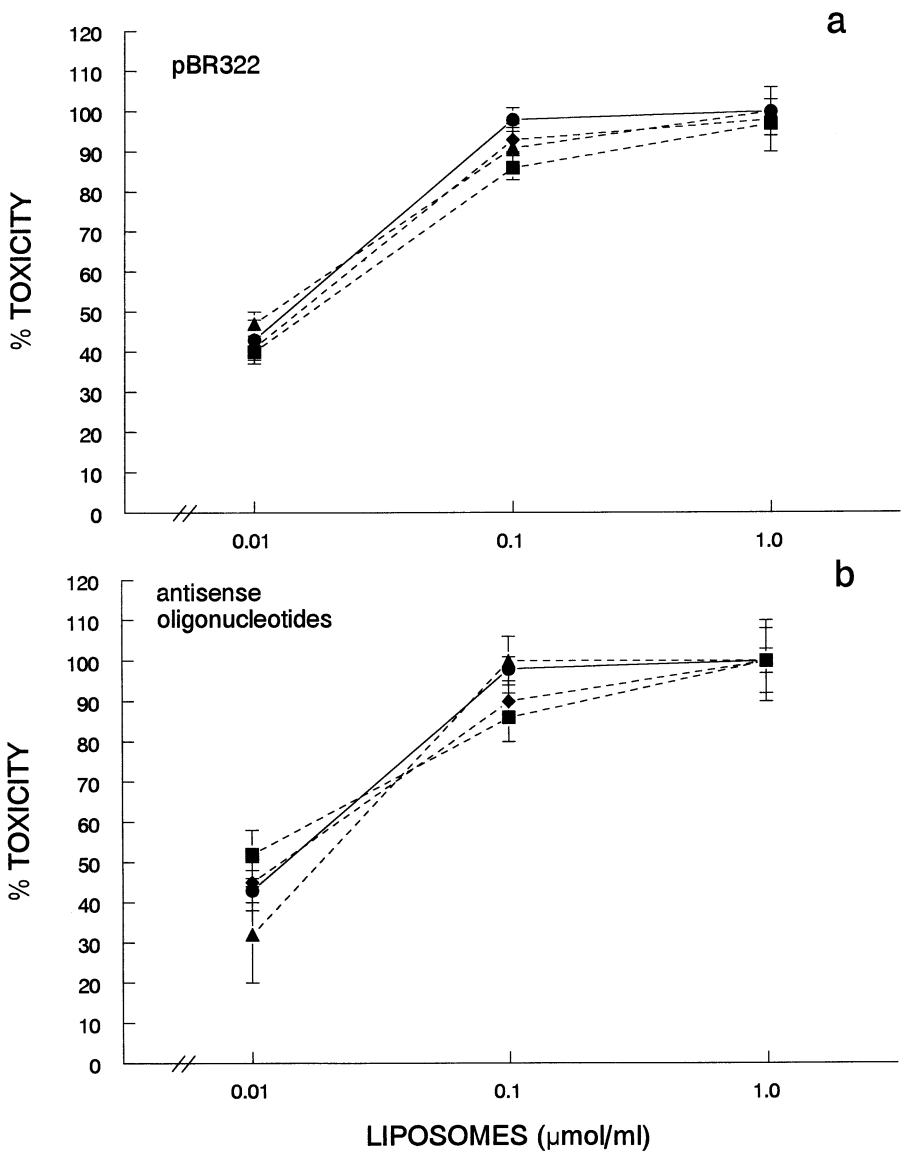

Fig. 5. The incorporation of DNA in cationic liposomes marginally reduced the toxicity observed towards macrophages. In (a) plasmid pBR322 (0.1 (◆), 0.5 (▲) and 2.5 μg (■)) or in (b) antisense oligonucleotides (2.0 (◆), 10.0 (▲) and 25.0 μg (■)) were mixed with 0.01, 0.1 and 1.0 μmol/ml of unilamellar 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 (1×10^5/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 at 24 h. Data represent the mean $\pm$ S.D. of two replicates of three independent experiments.

not significantly reduced the toxicity of DOPE/DOTAP liposomes towards macrophages (Fig. 5). These results clearly show that charge neutralisation of cationic lipid with anionic DNA is not sufficient to abrogate the toxicity (Table 1).

Table 2
Adverse effects associated with the use of cationic lipids or cationic liposomes

In vitro
Induction of chromosome aberrations in human culture cells (Nuzzo et al., 1985)
Induction of haemolysis (Yoshira and Nakae, 1986; Senior et al., 1991)
Inhibition of the respiratory burst of neutrophils (Ferencick et al., 1990)
Enhanced superoxide production by neutrophils (Ferencick et al., 1990)
Inhibition of PKC activity (Farhood et al., 1992; Filion and Phillips, 1997a)
Toxic for non-phagocytic cells (Parhham and Wetzig, 1993; Lappalainen et al., 1994; Cortesi et al., 1996)
Down-regulation of the production of IgG and IgM by human peripheral blood mononuclear cells (Jahnova et al., 1994)
Aggregation of cationic liposomes by albumin, IgG or salivary glycosaminoglycan (Phillips and Heydari, 1996) Phillips and Heydari,
Highly toxic for phagocytic cells (Filion and Phillips, 1997b)
Down-regulation of NO, TNF-α and PGE_2 synthesis (Filion and Phillips, 1997b)
Inhibition of DNase activity (Khalifé, Filion and Phillips, unpublished)
In vivo
Induction of eyes inflammation in rabbits when instilled intra-ocularly (Adams et al., 1977)
Affects immune response to antigens in mice; dependence upon type of antigen, dose and time of administration (Hilgers et al., 1984)
Neurotoxic after i.c. injection; produces epileptic seizure and death in mice (Taniguchi et al., 1988)
Activation of complement via the alternative pathway (Chon et al., 1991)
Toxic when administered intra-articularly into knee joints (Nita et al., 1996)
Induction of acute pulmonary inflammation reaction (Malone, 1996)
Induction of emboli after i.v. injection (Litzinger et al., 1996)
Highly toxic when administered orally; provoking a profound and lethal hypothermia (Filion and Phillips, 1997a)
Down-regulation of NO, TNF-α and PGE_2 synthesis (in situ) (Filion and Phillips, unpublished)
Strong anti-inflammatory activity in carrageenan and in sheep red blood cell challenge inflammatory models (Filion and Phillips, 1997a)
Induction of anti-single strand DNA antibodies (unpublished observation)

3.3. Cationic liposomes induce a large number of adverse effects

It is clear from this study that the use of cationic liposomes to deliver nucleic acids to phagocytic cells is inappropriate. In vitro, cationic liposomes, irrespective of whether or not they are complexed to DNA, have been shown to be extremely toxic toward phagocytic macrophages. Even under non-toxic conditions cationic liposomes non-specifically down-regulate the synthesis of NO, TNF-α and PGE_2 in a dose-dependent manner. More importantly, these pro-inflammatory modulators can be down-regulated in vivo by the administration of cationic liposomes. A single dose of cationic liposomes, DOPE/DDAB, DOPE/DOTAP, DOPE/DC-Chol or DOPE/DMTAP (ED_{50}: approximately 2 mg/kg), administrated i.p. is sufficient to inhibit the inflammation induced in mice by carrageenan or by sheep red blood cell challenge (Filion and Phillips, 1997a), demonstrating that the down-regulation of NO, TNF-α and PGE_2 production observed in vitro and in situ can be correlated with inhibitory activity in vivo. The impact of cationic-lipid induced macrophage down-regulation on gene expression or on macrophage-related host defence mechanisms is at present unknown.

Cationic liposomes should almost certainly be used with caution to deliver DNA especially, in antisense DNA therapy which requires repeated administration of large amounts of nucleic acids to obtain the expected effect. For example, the long-term administration (>24 days) of 6 mg/kg per day of antisense DNA complementary to PKC-α mRNA is necessary to block tumour proliferation in mice (Dean et al., 1996). In humans, clinical trials have been attempted with 0.1 mg/kg of antisense DNA directed against the gag gene of HIV-1 (Zhang et al., 1995). Using the commonly used cationic liposome/DNA ratio of 10:1, the adoption of a cationic liposome delivery strategy to increase the stability of antisense oligonucleotide would represent the administration of approximately 1–60 mg/kg per day of cationic liposomes, a dose which we have found capable of down-regulating NO, TNF-α and PGE_2 synthesis

and inhibit inflammation (Fig. 3 and Filion and Phillips, unpublished). Furthermore, we have shown that the use of cationic liposomes to target DNA to the gastrointestinal tract is inappropriate. Cationic DOPE/DOTAP liposomes are extremely toxic to CD1 mice following the administration of a single dose, provoking a profound and lethal hypothermia.

Complementary to our results, we have identified a range of adverse effects associated with the use of cationic lipids or cationic liposome (Table 2). This non-exhaustive list demonstrates very clearly that cationic liposomes must be used with caution for DNA (or drug) delivery. We believe that alternatives to cationic liposomes for DNA therapy should be considered in order to avoid these dose-limiting and often fatal adverse effects.

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