British Journal of Pharmacology (1997) 122, 551–557

Anti-inflammatory activity of cationic lipids

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1 The effect of liposome phospholipid composition has been assumed to be relatively unimportant because of the presumed inert nature of phospholipids.

2 We have previously shown that cationic liposome formulations used for gene therapy inhibit, through their cationic component, the synthesis by activated macrophages of the pro-inflammatory mediators nitric oxide (NO) and tumour necrosis factor-α (TNF-α).

3 In this study, we have evaluated the ability of different cationic lipids to reduce footpad inflammation induced by carrageenan and by sheep red blood cell challenge.

4 Parenteral (i.p. or s.c) or local injection of the positively charged lipids dimethyldioctadecylammomium bromide (DDAB), dioleyoltrimethylammonium propane (DOTAP), dimyristoyltrimethylammonium propane (DMTAP) or dimethylaminoethanecarbamoyl cholesterol (DC-Chol) significantly reduced the inflammation observed in both models in a dose-dependent manner (maximum inhibition: 70–95%).

5 Cationic lipids associated with dioleyol- or dipalmitoyl-phosphatidylethanolamine retained their anti-inflammatory activity while cationic lipids associated with dipalmitoylphosphatidylcholine (DPPC) or dimyristoylphosphatidylglycerol (DMPG) showed no anti-inflammatory activity, indicating that the release of cationic lipids into the macrophage cytoplasm is a necessary step for anti-inflammatory activity. The anti-inflammatory activity of cationic lipids was abrogated by the addition of dipalmitoylphosphatidylethanolamine-poly(ethylene)glycol-2000 (DPPE-PEG_{2000}) which blocks the interaction of cationic lipids with macrophages.

6 Because of the significant role of protein kinase C (PKC) in the inflammatory process we have determined whether the cationic lipids used in this study inhibit PKC activity. The cationic lipids significantly inhibited the activity of PKC but not the activity of a non-related protein kinase, PKA. The synthesis of interleukin-6 (IL-6), which is not dependent on PKC activity for its induction in macrophages, was not modified *in vitro* or *in situ* by cationic lipids. The synthesis of NO and TNF-α in macrophages, both of which are PKC-dependent, was downregulated by cationic lipids.

7 These results demonstrate that cationic lipids can be considered as novel anti-inflammatory agents. The downregulation of pro-inflammatory mediators through interaction of cationic lipids with the PKC pathway may explain this anti-inflammatory activity. Furthermore, since cationic lipids have intrinsic anti-inflammatory activity, cationic liposomes should be used with caution to deliver nucleic acids for gene therapy *in vivo*.

Keywords: Inflammation; anti-inflammatory drug; carrageenan; protein kinase C; nitric oxide; TNF-α; interleukin-6; liposome; gene therapy

Introduction

Liposomal phospholipids have been generally believed to be non-toxic, non-inflammatory and biocompatible (Van Rooijen & Nieuwmegen, 1983; Alving, 1992). However, there is ample evidence for an intrinsic pharmacological and immunological activity for several phospholipids. For example, phophatidylserine has been shown to inhibit the production of interleukin-2 (IL-2) and IL-2 receptor expression in T lymphocytes (Caselli *et al*., 1992), to enhance the ability of epidermal Langherans cells to induce contact hypersensitivity (Girolomoni *et al*., 1993), to stimulate cell growth (Yui & Yamazaki, 1987) and to decrease antibody production (Phillips *et al*., 1996). Phosphatidylethanolamine can enhance the anti-coagulant activity of activated protein C (Smirnov *et al*., 1995) and inhibit immune response against incorporated Gross virus surface antigen via activation of macrophage prostaglandin E_2 synthesis (Bakouche *et al*., 1987).

We have previously shown that cationic liposome formulations, usually used to target plasmid DNA or antisense oligonucleotides inside mammalian cells, can inhibit the synthesis of nitric oxide (NO) and tumour necrosis factor-α (TNF-α) *in vitro* by activated macrophages (Filion & Phillips, unpublished observations). NO and TNF-α are two important pro-inflammatory modulators secreted predominately by activated macrophages (Vassalli, 1992; Dugas *et al*., 1995). NO is generally the result of either a diffuse or a localized inflammatory response resulting from infection or tissue injury. The release of NO has been associated with the acute inflammatory response following footpad injection with carrageenan (Ialenti *et al*., 1992) and with inflammatory disorders (Ialenti *et al*., 1993; St-Clair *et al*., 1996). Furthermore, it has been shown that inhibition of NO synthesis can suppress the inflammation induced by carrageenan (Ianaro *et al*., 1994) or the development of adjuvant-induced arthritis (McCartney-Francis *et al*., 1993; Connor *et al*., 1995). Elevated levels of TNF-α have also been demonstrated in acute and chronic models of inflammation (Buchan *et al*., 1988; Sekut *et al*., 1994). The downregulation of TNF-α significantly inhibits paw inflammation in carrageenan and in adjuvant-arthritis inflammatory models (Sekut *et al*., 1995).

The observation that cationic liposomes used for nucleic acid delivery can downregulate the synthesis of NO and TNF-α, *in vitro*, by activated macrophages suggested that their cationic lipid component may suppress inflammatory responses. We have therefore evaluated the ability of a number of different positively charged lipids (dimethyldioctadecylammomium bromide [DDAB], dioleyoltrimethylammonium propane [DOTAP], dimyristoyltrimethylammonium propane [DMTAP] or dimethylaminoethanecarbamoyl cholesterol [DC-Chol] to inhibit footpad inflammation induced by carrageenan or by sheep red blood cell (SRBC) challenge.

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Our results show that all the cationic lipids tested reduced significantly inflammation in both models. The addition of dipalmitoylphosphatidyl-poly (ethylene)glycol (DPPE-PEG_{2000}), which can block the interaction between phagocytic cells and liposomes (Vertut-Doï *et al*., 1996; Filion & Phillips, unpublished observations), abrogated the anti-inflammatory activity of the cationic lipids. The association of cationic lipids with dipalmitoylphosphatidylcholine (DPPC) or with dimyristoylphosphatidylglycerol (DMPG), which prevents the release of cationic lipids into the cytoplasm (Zhou & Huang, 1994), also suppressed the anti-inflammatory activity of cationic lipids. The association of dioleyol- or dipalmitoyl-phosphatidylethanolamine (DOPE or DPPE) with cationic lipids, which destabilizes the endosomal compartment at acidic pH and releases cationic lipids into the cytoplasm (Farhood *et al*., 1995), did not modify the anti-inflammatory activity.

Methods

Cationic lipids and cationic 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. All lipids were obtained from Avanti Polar Lipids (Alabaster, AL) except DDAB and DC-Chol which were obtained from Sigma Co. (St-Louis, MO). Cationic lipids were dissolved in chloroform and evaporated to dryness at 60°C in a round-bottomed flask by a rotary evaporator. In some experiments cationic lipids were dissolved in chloroform and mixed in 1:1 mol ratio with DOPE, DPPE, DPPC or DMPG, or with amphiphilic DPPE–PEG_{2000} (10 mol% final concentration; obtained from Shearwater Polymers inc., Huntsville, AL) before rotary evaporation. Lipids or liposomes were prepared by adding the required volume of 0.85% w/v NaCl followed by agitation.

Cationic lipids and cationic liposomes characterization

The size of reconstituted liposomes was determined by photon correlation spectroscopy in a Coulter 4N Plus submicron particle analyzer (Coulter Corp., Miami, FL). Liposomal or lipid charge, determined as the zeta (ζ) potential, was determined in 0.85% NaCl (buffered to pH 7.4 with 10 mM sodium phosphate buffer) by Doppler elcctrophoretic light scattering with a Coulter DELSA 440 SX.

Carrageenan- and sheep red blood cell-induced footpad inflammation

Female CD1 mice (Charles River, St-Constant, Québec) were injected in the hind paw with a 1% solution of carrageenan (Sigma) in a final volume of 50 μl. For the SRBC model, mice were immunized s.c. with 1×10^8 washed SRBC in a volume of 500 μl. The mice were challenged on day 5 after the initial sensitization by injecting 1×10^8 SRBC in a final volume of 50 μl into the right hind paw. Control animals were injected with the same volume of 0.85% w/v NaCl solution. Footpad swelling was measured by water-displacement. Cationic lipids or cationic liposomes were injected i.p. or s.c. in a final volume of 500 μl or were injected in the footpad in a final volume of 50 μl 2 h before the induction of the inflammation.

Protein kinase assays

Purified PKC and PKA were obtained from Boehringher Mannheim (Laval, Québec). PKC and PKA activity was determined by use of a commercial ELISA kit (Pan Vera Corp., Madison, WI). Briefly, cationic lipids (0.4–6.5 μg) were incubated with purified PKC (12 μu) or with purified PKA (200 μu) in the presence of the peptide RFARKGSLRQKNV for 20 min at 25°C. Protein kinase activity was measured with the biotinylated monoclonal antibody 2B9 which recognizes the phosphorylated form of this peptide, and was revealed with streptavidin conjugated to peroxidase with *o*-phenylenediamine as substrate. The O.D. at 492 nm was read by means of a microplate reader (MR 5000, Dynatech, Chantilly, VA).

NO, TNF-α and IL-6 determination

Macrophages were obtained from female CD1 mice injected i.p. with 1.5 ml sterile Brewer's thioglycollate broth for 4 days (Difco, Detroit, MI). The peritoneal exudate (>85% macrophages) was harvested at day 4, washed by centrifugation in Hank's balanced salt solution (HBSS) and seeded in 96-well flat bottom microplates at 1.0×10^5 macrophages/well in RPMI-1640 containing 10% heat-inactivated foetal calf serum (FCS), 50 μg of gentamycin sulphate and 20 mM of HEPES (all from Gibco Life Science, Burlington, Ontario). The cells were allowed to adhere for 18 h at 37°C in an atmosphere of 5% CO_2 after which non-adherent cells were removed by gentle washing with warm medium. The macrophage monolayer was treated with 87 μg ml^{-1} cationic lipids for 3 h, washed extensively with warm medium and activated for 48 h with 1.0 μg ml^{-1} *E. Coli* 011:B4 lipopolysaccharide (LPS). NO_2^-, one of the end products of NO synthesis, was measured after 48 h incubation, by mixing 50 μl supernatant with Griess reagent as previously described (Phillips & Gagné, 1995). IL-6 and TNF-α synthesis were measured by ELISA (BioSource, Camarillo, CA) with 50 μl or 100 μl supernatant, respectively. For *in situ* macrophage treatment, lipids (60.0 mg kg^{-1}) 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 IL-6, NO and TNF-α synthesis measured after LPS stimulation as described above.

Statistical analysis

The relative activity of DOTAP administered in the footpad or i.p. was determined by use of Pharm/PCS (version 4) software (Microcomputer Specialists, Philadelphia, PA).

Results

Cationic lipids reduce inflammation induced by carrageenan

The injection of carrageenan into the hind paw produced an inflammatory response which was quantified by measuring changes in footpad volume. Swelling induced by carrageenan was detected at 3 h, peaked at 48 h, and began to decrease at 72 h. In initial experiments 60 mg kg^{-1} of the cationic lipids DDAB, DOTAP, DMTAP or DC-Chol were administered i.p. 2 h before the injection of carrageenan. The injection of cationic lipids significantly reduce the footpad volume at 3 h. with maximum inhibition being seen at 48 h (Figure 1). The footpad volume reduction persisted for at least 72 h. The administration of cationic lipids 24 h. after the induction of the inflammation did not extend the anti-inflammatory activity (data not shown). An i.p. injection of saline alone did not reduce the observed paw inflammation (Figure 1), while the injection of cationic lipids into non-inflamed hind paws at different concentrations did not induce inflammation (data not shown).

The dose-dependent anti-inflammatory activity of cationic lipids was evaluated by use of DOTAP. As shown in Figure 2, DOTAP had significant dose-related anti-inflammatory activity when administered i.p. Direct injection of DOTAP into the footpad 2 h. before the administration of carrageenan into the same hind paw also inhibited inflammation in a dose-dependent manner, although reduced activity was seen with the highest dose used (Figure 2). Comparable results were obtained when DOTAP was administered s.c. in the flank or at the base of the neck (data not shown), indicating that the route

of cationic lipid injection did not affect the observed anti-inflammatory activity. The relative potency obtained with DOTAP injected i.p. was 1.0 while the relative potency obtained with DOTAP administered into the footpad was 2.5 (95% confidence limits, 0.23–8.53, $P<0.05$ versus i.p. injection).

Cationic lipids reduce inflammation induced by SRBC challenge

Anti-inflammatory activity was also examined in a SRBC-induced model of delayed-type hypersensitivity. Mice were sensitized by the s.c. injection of SRBC and were subsequently challenged with SRBC in the footpad 5 days later. Swelling in the SRBC-challenged footpad of mice was significantly reduced by the administration of DOTAP (60 mg kg^{-1}, i.p.) but to lesser extent than the reduction obtained in the carrageenan model (Figure 3).

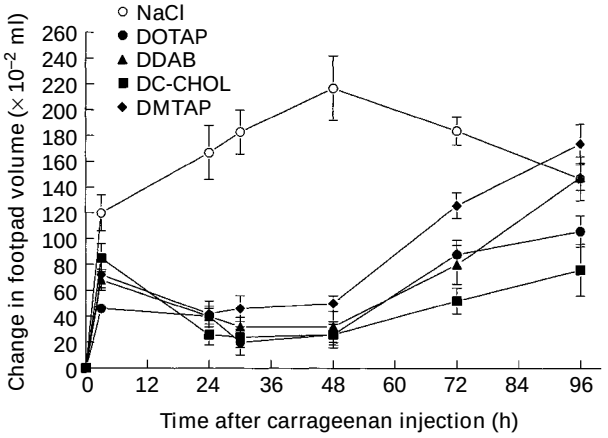

Figure 1 Inhibition of carrageenan-induced footpad inflammation by different cationic lipids. Female CD1 mice (>5 per group) were injected into the right hind paw with a 1% solution of carrageenan in a final volume of 50 μl to induce inflammation; 60 mg kg^{-1} of cationic lipids, DOTAP, ● DDAB, DC-Chol, (■) DMTAP (◆) or 0.85% w/v NaCl solution was administered (i.p.) in a volume of 500 μl 2 h, before the induction of inflammation. Changes in footpad volume were measured by water-displacement 3, 24, 30, 48, 72 and 96 h following injection of carrageenan. Data are expresssed as the mean of three independent experiments; vertical lines show s.d.

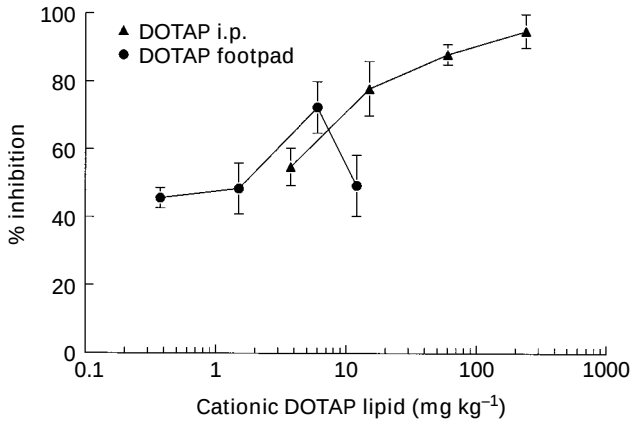

Figure 2 Intraperitoneal or footpad injection of cationic DOTAP lipid inhibited in a dose-dependent manner the inflammation induced by carrageenan. Female CD1 mice (>5 per group) were injected into the right hind paw with a 1% solution of carrageenan in a final volume of 50 μl to induce inflammation. Then either 3.75, 15.0, 60.0 and 240.0 mg kg^{-1} DOTAP was administered i.p. in a volume of 500 μl, or 0.375, 1.5, 6.0 and 24.0 mg kg^{-1} DOTAP was administered into the right footpad in a volume of 50 μl, 2 h before the induction of the inflammation. Results are expressed as % of inhibition vs control mice injected i.p. with 0.85% w/v NaCl solution in a volume of 500 μl or vs control mice injected into the right footpad with 50 μl of 0.85% w/v NaCl solution before the induction of the inflammation, respectively. Changes in footpad volume were measured by water-displacement 48 h following injection of carrageenan. Data are expressed as the mean of three independent experiments; vertical lines show s.d.

The anti-inflammatory activity of different formulations of cationic liposomes

Having established the anti-inflammatory activity of cationic lipids in the carrageenan-induced inflammation model, we tested the ability of cationic liposomal vectors used for nucleic acid delivery to inhibit the inflammatory response non-specifically. Cationic liposomes used for gene or antisense oligonucleotide delivery inside mammalian cells are usually composed of cationic lipids which can form a stable complex with anionic nucleic acids and pH-sensitive DOPE, which destabilizes the endosome compartment and releases nucleic acids into the cytoplasm (Smith *et al*., 1993). The widely used cationic liposome DOPE/DOTAP formulation was able to inhibit the inflammation induced by carrageenan, giving similar results to those obtained with DOTAP alone (Figure 4).

The replacement of DOPE by DPPE phospholipids in the cationic liposomes formulation did not modify cationic lipid anti-inflammatory activity. The replacement of DOPE by DPPC or by DMPG, two phospholipids which do not destabilize the endosomal membrane, abrogated the capacity to inhibit inflammation (Figure 4). The liposomal surface charge resulting from the association of positively charged DOTAP with different types of phospholipids could explain why certain liposomal formulations show no anti-inflammatory activity. We therefore measured the liposomal surface charge, determined as the zeta (ζ) potential, for each formulation (Table 1). Although DOPE/DOTAP, DPPE/DOTAP and DPPC/DOTAP have comparable positive ζ potentials, they differ significantly in their ability to inhibit inflammation. Furthermore, we observed that the binding of these liposomes to the cell surface and their uptake by macrophages were similar and found no difference in the ability of unilamellar liposomes (< 0.12 μm) or multilamellar liposomes (> 1.00 μm) to inhibit inflammation (data not shown).

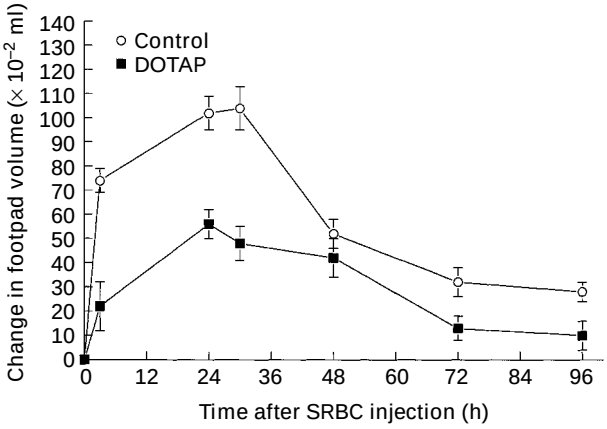

Figure 3 Inhibition of SRBC-induced footpad inflammation by cationic DOTAP. Female CD1 mice (>5 per group) were immunized s.c. with 1×10^8 SRBC in a volume of 500 μl. The mice were challenged on day 5 after the initial sensitization by injecting 1×10^8 SRBC in a final volume of 50 μl into the right hind paw. Then 60 mg kg^{-1} DOTAP lipid or vehicle (control) was administered i.p. in a volume of 500 μl 2 h before the induction of the inflammation. Changes in footpad volume were measured by water-displacement at 3, 24, 30, 48, 72 and 96 h following injection of carrageenan. Data are expresssed as the mean of two independent experiments; vertical lines show s.e.mean.

The addition of DPPE-PEG_{2000} molecules to different liposome formulations has been shown to inhibit the binding and subsequently endocytosis of these liposomes by phagocytic cells (Vertut-Doï *et al*., 1996; Filion & Phillips, unpublished observations). As shown in Figure 5, the addition of DPPE-PEG_{2000} to cationic DOTAP or to DOPE/DOTAP liposomes reduced the anti-inflammatory activity observed by 3.5 and 4.0 fold, respectively.

Cationic lipids inhibit PKC activity

PKC plays a significant role in the signal transduction leading to an inflammatory response (Parker *et al*., 1987; Zor *et al*., 1990). We have previously shown that cationic liposomes can downregulate the synthesis *in vitro* of NO and TNF-α by activated macrophages (Filion & Phillips, unpublished observations). Since the biosynthesis of NO and TNF-α is a PKC-dependent process (Kovacs *et al*., 1988; Severn *et al*., 1992), we determined, by use of DOTAP and DC-Chol, whether cationic lipids can inhibit PKC. The cationic lipids DOTAP and DC-Chol were able to inhibit the activity of PKC dramatically, but not the activity of a non-related protein kinase, PKA (Figure 6). The phospholipids DOPE, DPPE, DPPC and DMPG were not able to block the activity of PKC or PKA (data not shown).

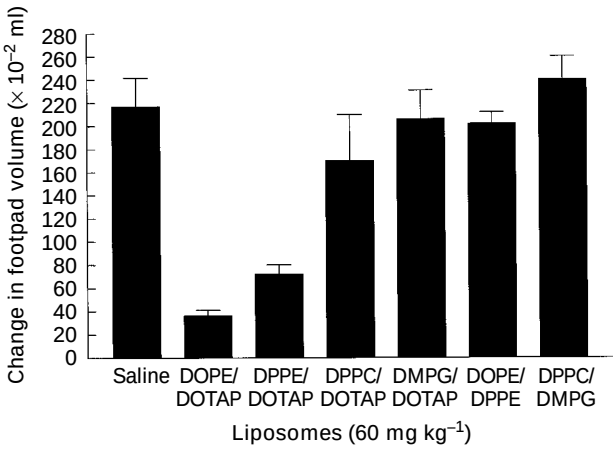

Figure 4 Only liposomes formulated with cationic lipids and DOPE or DPPE inhibited carrageenan-induced footpad inflammation. Female CD1 mice (>5 per group) were injected into the right hind paw with a 1% solution of carrageenan in a final volume of 50 μl to induce inflammation. Then 120 mg kg^{-1} of different cationic liposomes formulated at 1:1 mol ratio (cationic lipid and phospholipid 60 mg kg^{-1} each, respectively) was administered i.p. in a volume of 500 μl 2 h before the induction of the inflammation. The diameter of the liposomes used was between 1.0 and 3.0 μm. Results are expressed as changes in footpad volume measured by water-displacement 48 h following injection of carrageenan. Data are expressed as the mean of three independent experiments; vertical lines show s.d.

Table 1 Liposome or lipid charge, measured as ζ potential

Liposomes	ζ potential (mV)
DOPE/DOTAP	+42.0±10.9
DPPE/DOTAP	+39.8±9.7
DPPC/DOTAP	+42.6±6.2
DMPG/DOTAP	−21.7±11.0
DOPE/DPPE	−8.2±4.1
DPPC/DMPG	−24.1±6.3
DOTAP lipids	+42.5±9.0

Liposome or lipid charge, measured as ζ potential, was determined in NaCl (0.85% w/v) at 25°C. ζ potential is expressed as the mean±s.d. of three independent experiments.

The PKC IC_{50} was 115 nM for DOTAP and 506 nM for DC-Chol.

Cationic lipids do not downregulate IL-6 synthesis

To confirm that the downregulation of cytokine synthesis observed in LPS-activated macrophages was specific to the inhibition of PKC activity, we measured, by ELISA, the production of IL-6. It has been shown that the regulation of IL-6 synthesis in cells of the monocyte lineage is PKC-independent (Gross *et al*., 1993; Tremblay *et al*., 1995). Our results showed

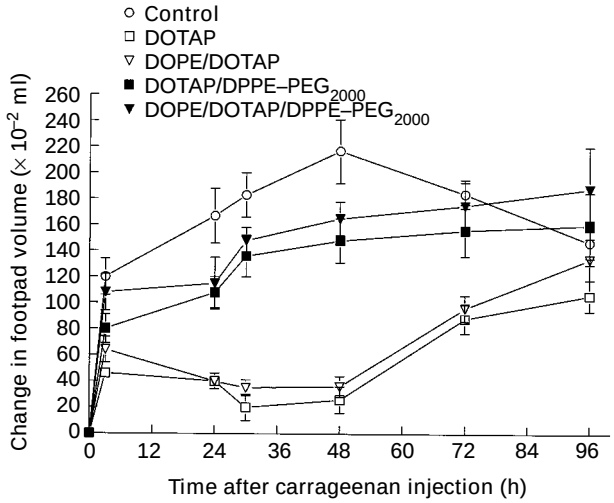

Figure 5 The anti-inflammatory activity of DOTAP or DOPE/DOTAP liposomes was abrogated by the addition of DPPE-PEG_{2000}. Female CD1 mice (>5 per group) were injected into the right hind paw with a 1% solution of carrageenan in a final volume of 50 μl to induce inflammation; 60 mg kg^{-1} of cationic DOTAP lipids or 120 mg kg^{-1} of DOPE/DOTAP liposomes formulated at 1:1 mol ratio (DOPE and DOTAP 60 mg kg^{-1} each respectively) associated with 10 mol% DPPE-PEG_{2000} was administered i.p. in a volume of 500 μl for 2 h. The diameter of DOTAP vesicles associated with DPPE-PEG_{2000} was >5.0 μm while the diameter of the DOPE/DOTAP liposomes associated with DPPE-PEG_{2000} was between 1.0 and 3.0 μm. Changes in footpad volume were measured by water-displacement 3, 24, 30, 48, 72 and 96 h following injection of carrageenan. Data are expresssed as the mean of three independent experiments; vertical lines show s.d.

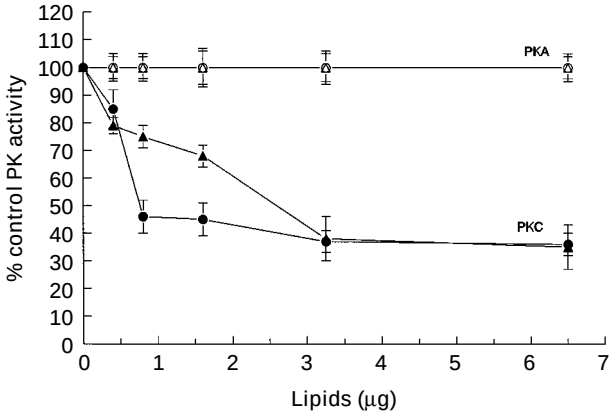

Figure 6 PKC (12 μu) or PKA (200 μu) were incubated with DOPE or DC-Chol (0.4–6.5 μg) and the peptide RFARKGSLRQKNV for 20 min at 25°C. The phosphorylation activity was detected by the biotinylated 2B9 monoclonal antibody recognizing phosphorylated form of this peptide and was revealed with streptavidin conjugated to peroxydase. Data represent the mean of two replicates of four independent experiments; vertical lines show s.d. The IC_{50} was 115 nm for DOTAP lipids and 506 nm for DC-Chol lipids.

that IL-6 synthesis by activated macrophages *in vitro* was not modified by the addition of DOTAP, while NO and TNF-α synthesis were strongly downregulated (Figure 7). Similar results were obtained with LPS-activated macrophages following *in situ* treatment with cationic lipids (Figure 7). The cationic lipids DDAB, DMTAP and DC-Chol also inhibited the *in vitro* and *in situ* synthesis of NO and TNF-α by activated macrophages, while the production of IL-6 was not affected (data not shown).

Discussion

In the present study a new class of compound having anti-inflammatory activity was identified. The cationic lipids DDAB, DOTAP, DMTAP and DC-Chol demonstrated the ability to reduce dramatically inflammation induced by carrageenan or by SRBC challenge.

The ability of cationic lipid or cationic liposomes to inhibit inflammation was surprising since the injection of positively charged proteins or biomaterials locally into articular joints, muscles or lung, has been shown to induce inflammatory responses (Phillips & Page-Thomas, 1979; Van de Berg *et al*., 1987; Van Lent *et al*., 1987; Hunt *et al*., 1996). The anti-inflammatory activity of cationic lipids may be related to their particular chemical structure. DDAB, DOTAP and DMTAP are amphiphilic compounds containing a permanent positively charged quaternary amino head group, while DC-Chol is a positively charged amphiphile with a tertiary amino head group (Düzgüines *et al*., 1989; Gao & Huang, 1991). It has previously been shown that cationic amphiphilic compounds can inhibit PKC activity (Bottega & Epand, 1992; Farhood *et al*., 1992). It is probable that they function as analogues of the naturally occuring cationic lipid spingosine, an endogenous negative effector of PKC (Reston *et al*., 1991). Our findings are in accordance with these results. The cationic lipids used in our study were able to inhibit PKC activity at low concentrations, DOTAP having an IC_{50} of 115 nM and DC-chol having an IC_{50} of 506 nM.

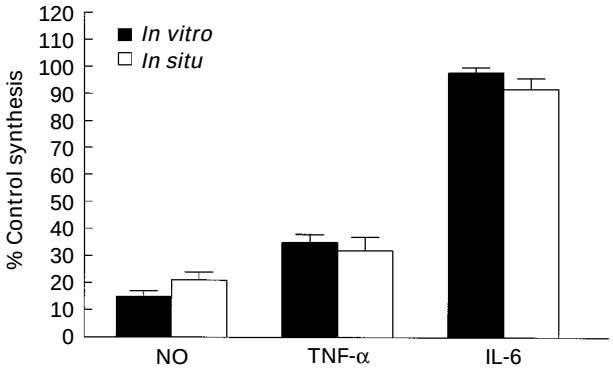

Figure 7 Cationic lipids downregulate NO and TNF-α synthesis while IL-6 synthesis is not affected. For *in vitro* treatment, macrophages obtained after i.p. injection of female CD1 mice with Brewer's thioglycollate broth were incubated at 1×10^5 cells/well with DOTAP lipids (87 μg ml^{-1}) for 3 h at 37°C, 5% CO_2 in RPMI-1640/10% FCS. The macrophages were then washed extensively and incubated with LPS (1.0 μg ml^{-1}) for 48 h. For *in situ* treatment, DOTAP (60 mg kg^{-1}) was injected i.p. in a volume of 500 μl on day 3 following the injection of Brewer's thioglycollate broth. Then macrophages were harvested after 18 h on day 4 and activated by LPS (1.0 μg ml^{-1}) for 48 h. NO in the supernatant after 48 h of incubation was measured by reaction with Griess reagent, while TNF-α and IL-6 were detected by means of commercial ELISA kits. Results are expressed as % of NO, TNF-α and IL-6 reduction vs LPS activated macrophages without lipids. No toxicity was obsereved toward macrophages. Data represent the mean of two replicates of three independent experiments; vertical lines show s.e.mean.

The ability of cationic lipids to inhibit PKC activity may explain why the synthesis of NO and TNF-α was downregulated in activated macrophages, while IL-6 production was not affected. It has been demonstrated that NO and TNF-α require PKC activity for their expression (Kovacs *et al*., 1988; Severn *et al*., 1992), while IL-6 production has been shown to be PKC-independent in cells of the monocyte lineage (Gross *et al*., 1993; Tremblay *et al*., 1996). NO and TNF-α are two important regulators of the inflammatory process (Buchan *et al*., 1988; Ialenti *et al*., 1992; Sekut *et al*., 1994; St-Clair *et al*., 1996). The inhibition of NO or TNF-α in carrageenan or adjuvant-arthrithis induced models has been associated with a dramatic reduction of inflammation (McCartney-Francis *et al*., 1993; Ianaro *et al*., 1994; Sekut *et al*., 1995; Connor *et al*., 1995). A number of other pro-inflammatory mediators such as IL-1β and cyclo-oxygenase (COX-2) (Shapira *et al*., 1994; Blanco *et al*., 1995) have been shown to be dependent on PKC activity for their expression. Although we have not yet tested the ability of cationic lipids to downregulate IL-1β and COX-2 expression, it seemed reasonable to assume that they will be affected in a manner similar to those seen with NO and TNF-α. Inhibition of PKC, which appears to play a central role in inflammation, offers considerable potential for the treatment of a number of inflammatory disorders and several selective inhibitors of PKC are under development for the treatment of inflammation (Nixon *et al*., 1991; Jacobson *et al*., 1995; Lang *et al*., 1995).

Other compounds, which have structural similarities to cationic lipids, possess intrinsic anti-inflammatory activity even though they were not designed to block PKC activity specifically or the production of pro-inflammatory mediators. For example tamoxifen, an oestrogen antagonist drug commonly used in the treatment of oestrogen-dependent breast and endometrial cancers (Legha, 1988) inhibits TNF-α mRNA expression and TNF-α release in BALB/3T3 cells (Suganuma *et al*., 1996) and can supress the inflammation induced by carrageenan in female LEW/N rats (Misiewicz *et al*., 1996). Tamoxifen is a cationic amphiphile with a tertiary amine head group, and can downregulated PKC activity (Bottega & Epand, 1992). Ketamine, an anaesthetic also having a cationic amphiphile structure, is able to downregulate the synthesis of TNF-α and can suppress mortality in a carrageenan-sensitized endotoxin shock model of male Wistar rats (Koga *et al*., 1995). Furthermore, a number of positively charged quaternary amine ether lipids have been shown to inhibit proliferation of the HL-60 promyelocytic leukemia cell line through the specific inhibition of PKC activity (Morris-Natschke *et al*., 1990).

Our results obtained with the carrageenan-induced footpad inflammation model indicate that the route of cationic lipids injection was not important in the anti-inflammatory activity observed, suggesting a common mechanism of action. The anti-inflammatory activity of cationic lipids could be mediated by the rapid interaction of these cationic lipids with phagocytic cells, such as monocytes, macrophages or Kuppfer cells (Litzinger *et al*., 1996; Filion & Phillips, unpublished observations). Macrophages play a very important role in the inflammatory processses in producing a vast array of mediators (nitric oxide, interleukins, chemokines, prostaglandins), in secreting tissue-degrading enzymes and by their immunoregulatory functions in the course of antigen-driven responses (Adams & Hamilton, 1984; Nathan, 1987). The finding that the addition of DPPE-PEG_{2000} to the cationic lipid, which blocks the binding and the endocytosis of liposomes by macrophages (Vertut-Doï *et al*., 1996; Filion & Phillips, unpublished data), abrogated the anti-inflammatory activity of these cationic lipids indicates that macrophages play a pivotal role in this process. Furthermore, we found that macrophages isolated after i.p. injection of cationic lipids showed defective secretion of NO and TNF-α when activated by LPS. Hilgers *et al*. (1984) have also shown that the i.p. injection of positively charged liposomes can modulate the immune system. This immunomodulating effect was restricted to thymus-dependent antigens indicating that macrophages are also involved in this process (Hilgers *et al*., 1984).

The association of cationic lipids with DPPC or DMPG phospholipids, which prevents the release of cationic lipids into the cytoplasm (Zhou & Huang, 1994), blocked their anti-inflammatory activity, while the association of cationic lipids with DOPE and DPPE phospholipids, which can destabilize the endosomal compartment at acidic pH and release cationic lipids into the cytoplasm (Farhood *et al*., 1995), resulted in a retention of anti-inflammatory activity. These results suggest that the release of cationic lipids into the macrophage cytoplasm may be essential for the induction of anti-inflammatory activity and provide evidence for a role of macrophages in this anti-inflammatory activity.

Although footpad injection of cationic lipid was more effective than i.p. injection, systemic anti-inflammatory mechanisms cannot be excluded. Usually, following i.p. injection of cationic liposomes a significant percentage of liposomal lipid is drained by the lymphatic systems, probably via phagocytic cells, and is found in the regional lymph nodes and the spleen (Philips *et al*., 1993). Although there are no data on the localization of cationic lipids after footpad injection, it has been shown that liposomal phospholipid is localized in the draining lymph nodes after footpad administration through macrophage migration (Velinova *et al*., 1996). Cationic liposomes can also be captured by phagocytic Kupffer cells in the liver and may exert their anti-inflammatory via these cells (Litzinger *et al*., 1996). It is also possible that liposomes injected i.p. or s.c. will go directly to the site of inflammation and block the recruitment of cells implicated in this inflammation. Love *et al*., (1990) have shown that liposomes injected i.v. accumulate in inflamed joint tissue sites of rats with adjuvant arthritis. Whether cationic lipids are capable of accumulating at the site of inflammation following i.p., s.c. or i.v. administration remains to be determined. The downregulation of NO and TNF-α synthesis either at the sites of injection, in the lymph nodes, in the liver, at the site of inflammation or systemically can interfere with the migration of immune cells from the circulation to sites of tissue injury, through the downregulation of integrin molecules or through the inhibition of chemokines and chemokine receptor expression (Picker & Butcher, 1992). For example, it has been demonstrated that lymphocytes trapping at inflammatory sites is mediated through the LFA-1/ICAM-1 homing receptor which is controlled by the level of TNF-α expression (Paleolog *et al*., 1996). The inhibition of NO and TNF-α synthesis by cationic lipids may also modify intercommunication between the immune, endocrine and central nervous system, which has been shown to play an important role in several inflammatory disorders (Wilder, 1995). The pharmacokinetic profile and biodistribution of cationic lipids following their administration via the i.p., s.c., and footpad routes is currently being determined in order to obtain a better understanding of their potential sites of activity.

Finally, we feel it prudent to highlight the fact that cationic liposomes should be used with caution to deliver nucleic acids *in vivo* in gene or antisense therapy, in view their intrinsic anti-inflammatory activity and potential immunomodulatory activity. DOPE/DOTAP and DOPE/DC-Chol cationic liposomes, two formulations which are widely used for gene or antisense therapy (Smith *et al*., 1993; Farhood *et al*., 1995), are able to inhibit dramatically the inflammation induced by carrageenan and by SRBC challenge. The impact of macrophage downregulation on gene expression or on macrophage-related host defence is, at present, unknown.

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

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(Received January 24, 1997
Accepted June 25, 1997)