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International Journal of Pharmaceutics 284 (2004) 31–41
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

Surfactant vesicle-mediated delivery of DNA vaccines via the subcutaneous route

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Received 4 January 2004; received in revised form 21 June 2004; accepted 6 July 2004

Abstract

Compared to naked DNA immunisation, entrapment of plasmid-based DNA vaccines into liposomes by the dehydration–rehydration method has shown to enhance both humoural and cell-mediated immune responses to encoded antigens administered by a variety of routes. In this paper we have compared the potency of lipid-based and non-ionic surfactant based vesicle carrier systems for DNA vaccines after subcutaneous immunisation. Plasmid pI.18Sfi/NP containing the nucleoprotein (NP) gene of A/Sichuan/2/87 (H3N2) influenza virus in the pI.18 expression vector was incorporated by the dehydration–rehydration method into various vesicle formulations. The DRV method, entailing mixing of small unilamellar vesicles (SUV) with DNA, followed by dehydration and rehydration, yielded high DNA vaccine incorporation values (85–97% of the DNA used) in all formulations. Studies on vesicle size revealed lipid-based systems formed cationic submicron size vesicles whilst constructs containing a non-ionic surfactant had significantly large z-average diameters (>1500 nm). Subcutaneous vesicle-mediated DNA immunisation employing two DRV(DNA) formulations as well as naked DNA revealed that humoural responses (immunoglobulin total IgG, and subclasses IgG_1 and $1gG_{2a}$) engendered by the plasmid encoded nucleoprotein were substantially higher after dosing twice, 28 days apart with 10 μg DRV-entrapped DNA compared to naked DNA. Comparison between the lipid and non-ionic based vesicle formulations revealed no significant difference in stimulated antibody production. These results suggest that, not only can DNA be effectively entrapped within a range of lipid and non-ionic based vesicle formulations using the DRV method but that such DRV vesicles containing DNA may be a useful system for subcutaneous delivery of DNA vaccines.

Keywords: Liposomes; Non-ionic surfactant vesicles; Niosomes; DNA vaccines; Subcutaneous vaccination

1. Introduction

The ability of naked DNA immunisation to induce both humoural and cell-mediated immune responses to the encoded antigen in animal mod-

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doi:10.1016/j.ijpharm.2004.07.012

els is well documented (e.g., Ulmer et al., 1993; Fooks et al., 1996; Mohamed et al., 2003). However, as DNA immunisation appears less effective in human trails (Ulmer, 1999), there is a need to increase the potency of DNA vaccines via formulation. To that end, liposome-entrapped DNA prepared by the dehydration–rehydration method (DRV liposomes) (Kirby and Gregoriadis, 1984; Perrie and Gregoriadis, 2000) has been shown to promote greater humoural and cell-mediated immune responses against the encoded antigen in immunised mice (Gregoriadis et al., 1997, 1999; Perrie et al., 2001) than naked DNA or DNA complexed to identical preformed liposomes. Similar observations have been made with plasmid DNA-entrapped within non-ionic surfactant based vesicles prepared by the same method (Obrenovic et al., 1998; Obrenovic and Gregoriadis, 1999).

In contrast to naked DNA, DNA entrapped within multilamellar vesicles such as those obtained by the dehydration–rehydration method, is protected from nuclease attack in biological milieu. Furthermore, after administration liposomes are likely to deliver their DNA content to antigen presenting cells (APC) infiltrating the site of injection or in the draining lymph nodes (Tuner et al., 1983; Velinova et al., 1996; Perrie et al., 2001). One other advantage that may be offered by vesicle-mediated DNA delivery is the choice of the subcutaneous route, which for naked DNA, has so far failed to elicit immune responses (Gregoriadis, 1998). Indeed, recently subcutaneous liposome-mediated DNA immunisation employing two DRV(DNA) liposome formulations as well as naked DNA revealed that humoural responses engendered by the plasmid encoded nucleoprotein were substantially higher after two 10 μg doses of liposome-entrapped DNA compared to equal doses of naked DNA (Perrie et al., 2003). These results suggests that, not only can DNA be effectively entrapped within liposomes using the DRV method but that such DRV liposomes containing DNA may be a useful system for subcutaneous delivery of DNA vaccines.

2. Materials and methods

2.1. Materials

Egg phosphatidylcholine, Grade I, (PC) was purchased from Lipid Products, Nutfield, Surrey, UK. Dioleoyl phosphatidylethanolamine (DOPE) and 1,2-dioleoyl-3-(trimethylammonium) propane (DOTAP) were purchased from Avanti Polar Lipids, Alabama, USA. Cholesterol (Chol), monopalmitoyl-rac-glycerol (C16:0) (Monopal) and cholesteryl 3-*N*-(dimethyl amino ethyl) carbamate (DC-Chol) were purchased from Sigma, Pool, Dorset, UK. The attenuated influenza virus (A/Sichuan/2/87) was a gift from Dr. R. Newman from The National Institute for Biological Standards and Control, Herts, UK. Horseradish peroxidase-conjugated goat anti-mouse immunoglobulin total IgG (IgG_T) was obtained from Sigma-Aldrich Company Ltd., Pool, UK and horseradish peroxidase-conjugated goat anti-mouse immunoglobulin IgG_1 and IgG_{2a}, together with foetal calf serum were obtained from Sera-Lab, Crawly Down, Sussex, UK. Ninety-six well flat-bottomed microtiter plates (Immulon IB) were purchased from Dynatech Labs, Billingshurst, Sussex, UK. All other reagents were of analytical grade.

2.2. Plasmids

Two plasmids were used in this study; pRc/CMV HBS and pI.18Sfi/NP. The pRc/CMV HBS (also referred to as pCMV-HBs(S)) cloned by Robert Whalen, Pasteur Institute (Davis et al., 1993) expressing the sequence coding for the S (small) region of HBsAg (subtype ayw) was supplied by Aldeveron, Fargo, USA. Plasmid pI.18Sfi/NP containing the full length nucleoprotein (NP) gene of A/Sichuan/2/87 (H3N2) in the pI.18 expression vector was a gift from Dr. J. Roberson from The National Institute for Biological Standards and Control, Herts, UK. The backbone of this plasmid is pUC-based encoding a bacterial origin of replication and ampicillin-resistance gene for growth and selection. It also carries a cytomegalovirus (CMV) promoter and terminator sequence (Cox et al., 2002).

2.3. Preparation of DRV-entrapped plasmid DNA

The dehydration–rehydration procedure (Kirby and Gregoriadis, 1984; Perrie and Gregoriadis, 2000; Perrie et al., 2002, 2003) was used for the incorporation of plasmid DNA (pRc/CMV-HBS, or pI.18Sfi/NP; 100–150 μg) into surfactant vesicles. In brief, 2 ml of small unilamellar vesicles (SUV) prepared by sonication and composed of 16 μmol PC or Monopal, 8 μmol

of DOPE, 4 μmol of Chol, and 4 μmol of either the cationic lipid DOTAP or DC-Chol. These SUV were mixed with 100–150 μg of plasmid DNA, frozen at −20 °C and freeze-dried overnight. Controlled (Kirby and Gregoriadis, 1984) rehydration of the dry powders led to the formation of DNA-containing multilamellar (Perrie and Gregoriadis, 2000; Perrie et al., 2001) dehydration–rehydration vesicles (DRV(DNA)). DRV preparations were then centrifuged twice at 25,000 × *g* for 40 min to remove non-entrapped or -complexed DNA and re-suspended in 0.01 M sodium phosphate containing 0.15 M NaCl, pH 7.4 (PBS) to the required volume. DNA incorporation with the various vesicle preparations was estimated on the basis of PicoGreen analysis of plasmid DNA recovered in the suspended pellets and the supernatants after treatment with 0.1% Triton X-100 (Fenske et al., 2002) in according to manufacturers protocol.

2.4. Determination of vesicle size and zeta potential

The z-average vesicle diameter of the surfactant vesicles was measured on a ZetaPlus (Brookhaven Instruments) at 25 °C by diluting 20 μl of the dispersion to the appropriate volume with doubly-filtered (0.22 μm pore size) distilled water. The zeta potential (an indirect measurement of the vesicle surface charge) of the various vesicle formulations was measured in 0.001 M PBS at 25 °C again on a ZetaPlus (Brookhaven Instruments).

2.5. Agarose gel electrophoresis studies

Samples of vesicle-entrapped DNA were subjected to agarose gel (1.0%) electrophoresis to determine the retention of DNA by the surfactant vesicles under various conditions. In brief, 8 μl (1.6–2.0 μg DNA) of DRV or SUV suspension were mixed with gel loading buffer (sucrose, 40% w/v; EDTA, 0.1 M pH 8) and subjected to agarose gel electrophoresis in the presence of ethidium bromide (0.5 μg/ml) for 1 h at 90 V. In some experiments the gel loading buffer was supplemented with sodium dodecyl sulphate to a concentration below (0.05% w/v) the critical micelle concentration (Florence and Attwood, 1998) to measure the effect of anionic competition on the liposome formulations (Perrie and Gregoriadis, 2000).

2.6. Immunisation protocol

Female B57 black mice (6–8 weeks old), in groups of five, were given two subcutaneous injections, 28 days apart, of 10 μg > naked (pI.18Sfi/NP plasmid or 10 μg pI.18Sfi/NP entrapped in cationic liposomes composed of PC, DOPE and DC-Chol (4:2:1 molar ratio) or cationic Monopal-based vesicles (Monopal, DOPE, Chol and DC-Chol; 4:2:1:1 molar ratio). All animal experiments were conducted in accordance with the Animal (Scientific Procedures) Act 1986.

Humoural responses (total IgG (IgG_T), IgG_1, and IgG_{2a}) in immunised mice engendered by the plasmid encoded nucleoprotein were detected by ELISA using the whole influenza virus (A/Sichuan/2/87). This ELISA method was developed based on similar ELISA protocols where the pI-18Sfi/NP plasmid was used in the immunisation procedure (Cox et al., 2002). Maxisorb 96-well plates (Dynatech) were coated for 16 h at 4 °C with the whole influenza virus (100 μl, 1 μg/ml) in 0.05 M sodium carbonate-bicarbonate buffer (pH 9.6). After blocking with bovine serum albumin (1% w/v) in PBS containing Tween 20 (0.05% v/v; PBS-Tween buffer) for 1 h at 37 °C the plates were washed three times with PBS-Tween buffer. Sera collected at time intervals from immunised and non-immunised (control group) were two-fold serially diluted in PBS and 100 μl/well applied and again the plates were incubated for 1 h at 37 °C and after which washed with PBS-Tween buffer. Peroxidase-conjugated goat anti-mouse IgG_T, IgG_1, or IgG_{2a} were diluted (1 in 4000) in blocking buffer supplemented with 5% fetal calf serum and 100 μl/well dispensed. Finally, after incubating for 1 h at 37 °C and washing with PBS-Tween buffer, 200 μl of substrate (40 mg *O*-phenylenediamine in 100 ml 2.8 mM sodium phosphate/1.4 mM citric acid buffer, pH 5, into which 40 μl of 30% H_2O_2 had been added immediately before dispensing) was added. The reaction was stopped after 30 min by the addition of 50 μl 2.5 M H_2SO_4 and the colour change read at 492 nm. To validate the protocol and to provide a positive control a sample of mouse anti-nucleoprotein sera (generated using A/Sichuan/2/87 virus) was used. Immune responses are expressed as the $\log_{10}$ of the reciprocal of serial (two-fold) serum dilution required for the optical density to reach a reading of about 0.200 (end point dilution). The mean of the $\log_{10}$ serial dilution end points and their standard deviation were calcu-

lated for each group and significance levels determined using the student *t*-test for unpaired dilutions.

3. Results and discussion

3.1. Incorporation of DNA into DRV

As previously reported with other plasmids and DRV liposome formulations (Gregoriadis et al., 1996; Perrie and Gregoriadis, 2000; Perrie et al., 2001, 2002, 2003), plasmid (pRc/CMV-HBS or pI.18SFi/NP) entrapment (% of total used) in all PC and Monopal-based systems successfully prepared was considerable (85–97%; Tables 1 and 2) and can be attributed in part to the electrostatic interaction between the anionic DNA and the cationic vesicles as demonstrated by their measured cationic zeta potential (40–53 mV; Tables 1 and 2). In this present study DNA incorporation was based on PicoGreen analysis (Fenske et al., 2002). PicoGreen is an intercalating cyanine dye which selectively binds to double stranded plasmid DNA with a linear detection range extending over more than four orders of magnitude of DNA concentration and therefore can be effectively used to determine double stranded liposomal DNA loading. The entrapment values measured for DNA incorporation into the PC and Monopal formulations using this intercalating dye strongly correlate with entrapment values based on ^{35}S-radioassay previously reported (Gregoriadis et al., 1996; Arunothayanun et al., 1998; Perrie and Gregoriadis, 2000; Perrie et al., 2001). Such entrapment values, based on the assay of ^{35}S radioactivity have been shown (Perrie and Gregoriadis, 2000) to be reproducible and to predominantly reflect actual DNA entrapment within the bilayers as-opposed to complexation of DNA with the vesicle surface.

Results in Table 1 also demonstrate that, whilst all PC-based liposome formulations produced submicron sized vesicles, attempts to prepare formulations containing a combination of Monopal, DOPE and DOTAP were unsuccessful, and resulted in the formation of a precipitate. The inability of this combination to form a stable bilayer structure appears to be attributed to the combined presence of Monopal and DOPE since substitution of either PC for Monopal, or DOPE for cholesterol both resulted in the formulation of stable DRV(DNA) approximately 2 μm in size or below (Table 1). Attempts to formulate a Monopal/DOTAP system containing DOPE by adding cholesterol to the system (Monopal:DOPE:Chol:DOTAP; 16:8:4:4 molar ratio; Table 1) were also unsuccessful. The preference to include DOPE within the Monopal formulation was supported by previous in vitro (e.g., Felgner et al., 1994) investigations, which demonstrate the presence of DOPE within vesicle bilayers enhances liposome mediated gene expression. Similarly, in vivo the advantage of the presence of DOPE in both liposomal (Perrie et al., 2002) and Monopal (Obrenovic et al., 1998) vesicles which mediated DNA immunisation via the intramuscular route has also been demonstrated. Since the original report (Felgner et al., 1987) in which cationic liposomes composed of the cationic lipid DOTMA (*N*-[1-(2,3-dioleyloxy)propyl]-

Table 1
DOTAP based cationic DRV: the effect of bilayer composition on DNA incorporation, vesicle size, and zeta potential

Vesicle composition	DNA incorporation (% of used ±S.D.)	Size (nm ± S.D.)	Zeta potential (mV ± S.D.)
PC:DOPE:DOTAP (16:8:4 μmol)	94.0 ± 2.8	775 ± 68	42.1 ± 1.3
Monopal:DOPE:DOTAP (16:8:4 μmol)	ND	PPT	ND
PC:Chol:DOTAP (16:8:4 μmol)	90.0 ± 3.0	932 ± 123	53.9 ± 1.5
Monopal:Chol:DOTAP (16:8:4 μmol)	85.5 ± 9.7	2021 ± 399	45.6 ± 2.1
PC:DOPE:Chol:DOTAP (16:8:4 μmol:4 μmol)	94.8 ± 4.4	721.8 ± 56	43.0 ± 2.0
Monopal:DOPE:Chol:DOTAP (16:8:4:4 μmol)	ND	PPT	ND

Plasmid DNA (pRc/CMV HBS or pI.18Sfi/NP; 100 μg) was incorporated into cationic DRV to produce DRV(DNA) of various lipid compositions and lipid molar ratios as shown. In addition each formulation contained 4 μmol of the cationic surfactant DOTAP. Incorporation values were based on PicoGreen Analysis and were not significantly different for each plasmid therefore results were pooled. The vesicle z-average diameter and zeta potential of the DRV was measured in double-distilled water or 0.001 M PBS respectively at 25 °C using a Brookhaven ZetaPlus. ND: not determined; PPT: precipitate. Results represent means ± S.D., n = 3–5.

Table 2
DC-Chol based DRV: the effect of bilayer composition on DNA incorporation, vesicle size, and zeta potential

Vesicle composition	DNA incorporation (% of used ± S.D.)	Size (nm ± S.D.)	Zeta potential (mV ± S.D.)
PC:DOPE:DC-Chol (16:8:4 μmol)	93.3 ± 3.3	856 ± 89	41.4 ± 2.8
Monopal:DOPE:DC-Chol (16:8:4 μmol)	ND	PPT	ND
PC:DOPE:Chol:DC-Chol (16:8:4:4 μmol)	95.8 ± 4.1	703 ± 40	40.4 ± 1.6
Monopal:DOPE:Chol:DC-Chol (16:8:4:4 μmol)	90.0 ± 7.0	1755 ± 345	40.3 ± 2.8
PC:DOPE:Chol:DC-Chol (16:4:8:4 μmol)	97.0 ± 2.6	799 ± 43	44.9 ± 1.3
Monopal:DOPE:Chol:DC-Chol (16:4:8:4 μmol)	93.6 ± 3.1	1624 ± 350	42.9 ± 1.2

Plasmid DNA (pRc/CMV HBS or pI.18Sfi/NP; 100 μg) was incorporated into cationic DRV to produce DRV(DNA) of various lipid compositions and lipid molar ratios as shown. In addition each formulation contained 4 μmol of the cationic surfactant DC-Chol. For other details see Table 1. ND: not determined; PPT: precipitate. Results represent means ± S.D., n = 3–5.

N,*N*,*N*-trimethylammonium chloride) and the neutral lipid DOPE were shown to be effective transfection agents, many authors (e.g., Legendre and Szoka, 1993) have reported improved transfection efficiency when DOPE (often referred to as a 'helper lipid') is incorporated in cationic lipid transfection systems compared to formulations omitting DOPE or replacing it with other lipids. Phosphatidylethanolamines such as DOPE have a relatively small head group and two bulky fatty acyl chains, which give the molecule an inverted cone shape (Israelachvili et al., 1977). Due to this molecular cone shape, DOPE or membranes enriched with DOPE have a strong tendency to form an inverted hexagonal phase, and promote membrane destabilisation (Cullis et al., 1986). This ability of DOPE to promote membrane destabilisation has been suggested to facilitate gene transfection (Szoka et al., 1996) and liposome-mediated DNA immunisation (Perrie et al., 2001) by promoting the disruption of the endosomal membrane after endocytosis/phagocytosis of liposomal-DNA systems and ensuring escape of the plasmid DNA into the cytoplasm. Therefore, in an effort to prepare a Monopal-based DRV(DNA) containing DOPE within the bilayer a second series of formulations were prepared using the cationic lipid DC-Chol (which contains a cholesterol backbone) to replace DOTAP which has dioleoyl hydrocarbon chains similar to DOPE. Table 2 demonstrates that by substituting DC-Chol for DOTAP, Monopal formulations containing DOPE were successfully formulated but only when additional cholesterol was present within the bilayer composition.

Whilst there were no notable formulation problems with any of the PC-based liposome compositions tested, recent studies (Perrie et al., 2003) have demonstrated that the combination of DOPE and DOTAP can result in a small decrease in DRV(DNA) stability. Investigating the DNA retention capability of various DRV(DNA) in the presence of competitive anions, it was noted that DRV(DNA) compositions containing high dioleoyl chain concentration (resulting from the combination of DOPE and DOTAP) were more susceptible to DNA loss under such conditions compared with similar formulations where DOPE was substituted for Chol, or where DC-Chol replaced DOTAP (Perrie et al., 2003). This again may be contributed to the reduced stability of DOPE containing membranes already discussed.

Notable from both Tables 1 and 2 is the almost two-fold increased vesicle size of Monopal vesicles compared with their PC counterparts. This increased vesicle size may be, to a degree, the result of increased vesicle aggregation in the presence of electrolytes in the buffered solutions used in the vesicle suspensions. Indeed both Monopal-based SUV (Fig. 1a) and DRV(DNA) (Fig. 1b) z-average diameter increased significantly ($P < 0.001$–0.05) when suspended in phosphate buffered saline and 0.9% saline compared to when suspended in distilled water. In contrast, the equivalent SUV and DRV(DNA) liposome formulations demonstrated no significant change in z-average diameter when suspended in each of the three media. However even in the absence of electrolytes, the Monopal DRV(DNA) formed were significantly larger in size compared to their equivalent liposomal DRV(DNA) (1160 nm compared with 769 nm, respectively; Fig. 1b) suggesting again this increase in vesicle size can be attributed to the inclusion of the non-ionic surfactant Monopal in the DRV(DNA) formulation.

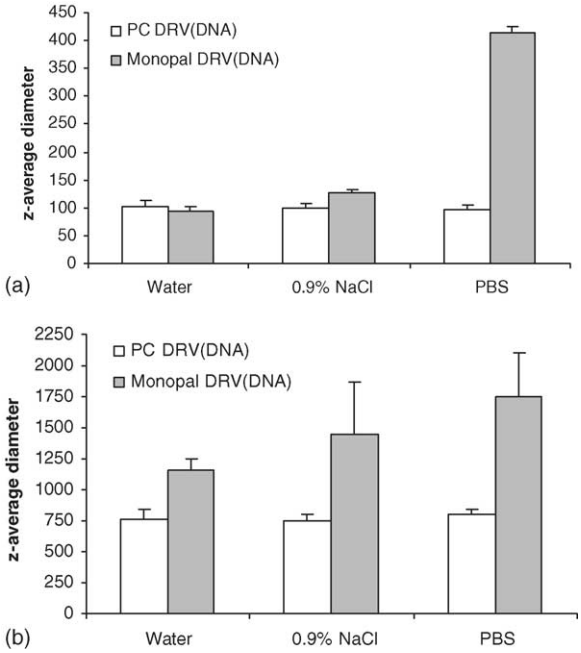

Fig. 1. z-Average diameter of PC DRV(DNA) (16 μmol PC, 8 μmol DOPE, 4 μmol Chol, and 4 μmol DC-Chol) and Monopal DRV(DNA) (16 μmol Monopal, 8 μmol DOPE, 4 μmol Chol, and 4 μmol DC-Chol) prepared as SUV (a) and DRV(DNA) (b) in double distilled water, 0.9% saline and phosphate buffered saline (pH 7.4). Vesicle z-average diameter was determined using a Brookhaven ZetaPlus at 25 °C. Values denote mean ± S.D. (n = 3–5).

3.2. Gel electrophoresis studies

To investigate the DNA spatial location within the various cationic DRV, preparations were subjected to gel electrophoresis in the presence of sodium dodecyl sulphate (SDS) at a concentration (0.05%) below the critical micelle concentration of the surfactant (Florence and Attwood, 1998). It has previously been shown (Perrie and Gregoriadis, 2000) that at this concentration the anionic SDS, although unable to solubilise the DRV surfactants, is able to electrostatically compete with the DNA bound to the cationic surface charges and release it into the medium. Released DNA would then be expected to migrate to the cathode, with DNA unavailable (presumably entrapped) to SDS displacement remaining at the site of application.

Fig. 2a shows that, on gel electrophoresis of all six DRV preparations in the absence of anionic molecules, DNA remains at the site of application, bound to

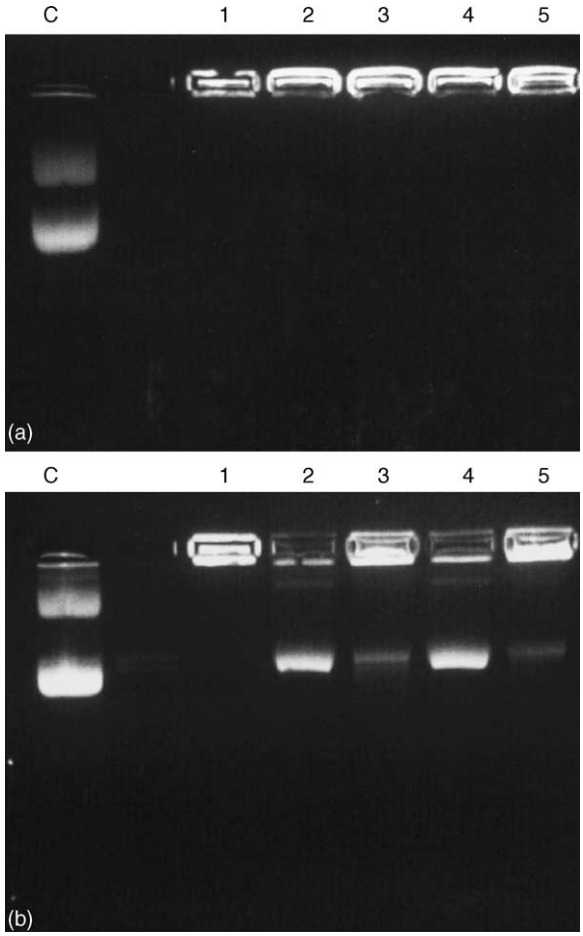

Fig. 2. (a) Gel electrophoresis of DRV(DNA) composed of – lane C: naked DNA; lane 1: 16 μmol Monopal, 8 μmol DOPE, 4 μmol Chol, and 4 μmol DC-Chol; lane 2: 16 μmol PC, 8 μmol DOPE, and 4 μmol DOTAP; lane 3: 16 μmol PC, 8 μmol DOPE, and 4 μmol DC-Chol; lane 4: 16 μmol PC, 8 μmol DOPE, 4 μmol Chol, and 4 μmol DOTAP; lane 5: 16 μmol PC, 8 μmol DOPE, 4 μmol Chol, and 4 μmol DC-Chol. (b) As in (a) but in the presence of anionic SDS.

the cationic charges of the preparations. Similarly, following electrophoresis in the presence of SDS (Fig. 2b) no measurable DNA is displaced from the Monopal/DC-Chol preparation (Fig. 1b, lane 1). However from Fig. 2b it appears that despite the similar cationic nature and zeta potential of the formulations tested (Tables 1 and 2) the bilayer composition of the DRV(DNA) can influence the amount of DNA displaced from DRV(DNA) in the presence of competitive anions. DRV(DNA) formulations containing DOTAP

(Fig. 2b, lanes 2 and 4) displayed more DNA loss under the described conditions compared with their DC-Chol containing DRV(DNA) counterparts (Fig. 2b, lanes 3 and 5, respectively). Therefore from this data it becomes apparent that by substituting DOTAP for DC-Chol or replacing Monopal with PC within the vesicle formulation results in an increased displacement of DNA from the DRV(DNA) formulations. Since it is proposed that DNA is predominantly entrapped within the bilayers of DRV, presumably bound to the inner cationic charges with only a minor portion of DNA interacting with surface cationic charge of the vesicles, it is apparent any change in the surface zeta potential (an indirect measure of surface charge) of the DRV(DNA) would influence the ability of the DRV(DNA) to retain this surface bound DNA. However from Tables 1 and 2 it is clear that there is no apparent difference between the analogous DOTAP and DC-Chol formulations suggesting that other factors may be involved. It is unclear, at present why the high dioleoyl chain concentration (resulting from the combination of DOPE and DOTAP as in Fig. 2b, lanes 3 and 5) of the DRV(DNA) results in an increased displacement of DNA from the DRV(DNA) formulations however this may be contributed to the reduced stability of DOPE containing membranes (Farhood et al., 1995) which can promote membrane mixing and fusion (Stamatataos et al., 1988; Wrobel and Collins, 1995). Further, increasing the quantity of DOPE in cationic lipid-complexes appears (Wong et al., 1996) to increase dissociation of DNA from cationic liposomes. Therefore it may be that the combination of DOPE and DOTAP within the DRV(DNA) results in bilayer systems which are less resistant to destabilisation in the presence of competitive anions compared with vesicles containing a combination of DOPE and DC-Chol. Further, replacing PC with non-ionic surfactant monopal (Fig. 2b, lane 2), results in a formulation, which appears resistant to the induced destabilisation and resultant DNA loss. Conformation of the enhanced stability of Monopal DRV(DNA) can be seen with a similar monopal and PC analogous vesicle pair which contain an increased cholesterol content (Fig. 3). Again, on gel electrophoresis of both formulations in the absence of anionic molecules, DNA remains at the site of application, bound to the cationic charges of the preparations with no measurable DNA displacement under the described conditions (Fig. 3, lanes 1 and 2, respectively). However in the presence of SDS, again DNA displacement is only detected from the liposome preparation (Fig. 3, lanes 3 and 4) and not the Monopal preparation (Fig. 3, lanes 5 and 6).

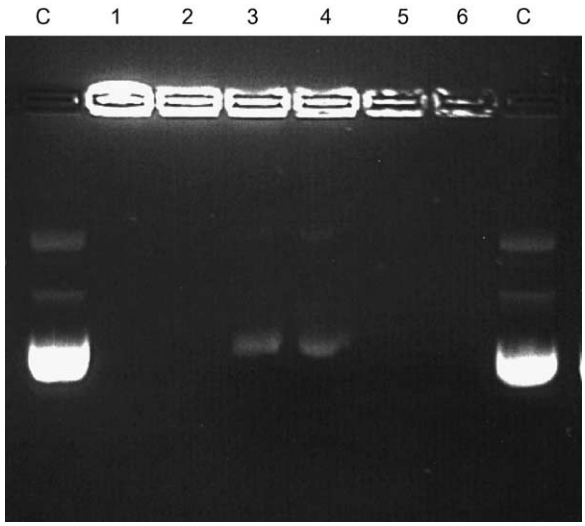

Fig. 3. Gel electrophoresis of DRV(DNA) composed of – lane C: naked DNA; lane 1: 16 μmol PC, 4 μmol DOPE, 8 μmol Chol, and 4 μmol DC-Chol; lane 2: 16 μmol Monopal, 4 μmol DOPE, 8 μmol Chol, and 4 μmol DC-Chol; lanes 3 and 4: as in lane 1 in the presence of SDS; lanes 5 and 6: as in lane 2 in the presence of SDS.

3.3. Immunisation with DRV-entrapped DNA

On the basis of previous findings that intramuscular injection of DNA entrapped in liposomes (Gregoriadis et al., 1997, 1999; Perrie et al., 2001) and niosomes (Obrenovic et al., 1998; Obrenovic and Gregoriadis, 1999) is more effective at inducing immune responses against the encoded antigen than naked DNA, further work was carried out to investigate the efficiency of such preparations after subcutaneous immunisation. To allow the detection of surfactant vesicle-mediated improvement (if any) of immune responses to encoded antigen, doses of plasmid DNA were, as previously (Gregoriadis, 1998; Gregoriadis et al.,1997; Perrie et al., 2001), low enough (10 μg) for naked DNA to fail to induce responses under the present conditions. Two formulations were tested: liposomes composed of 16 μmol PC, 8 μmol of DOPE and 4 μmol of DC-Chol (Table 2; Formulation 7) were compared with similarly formulated vesicles where Monopal replaced the

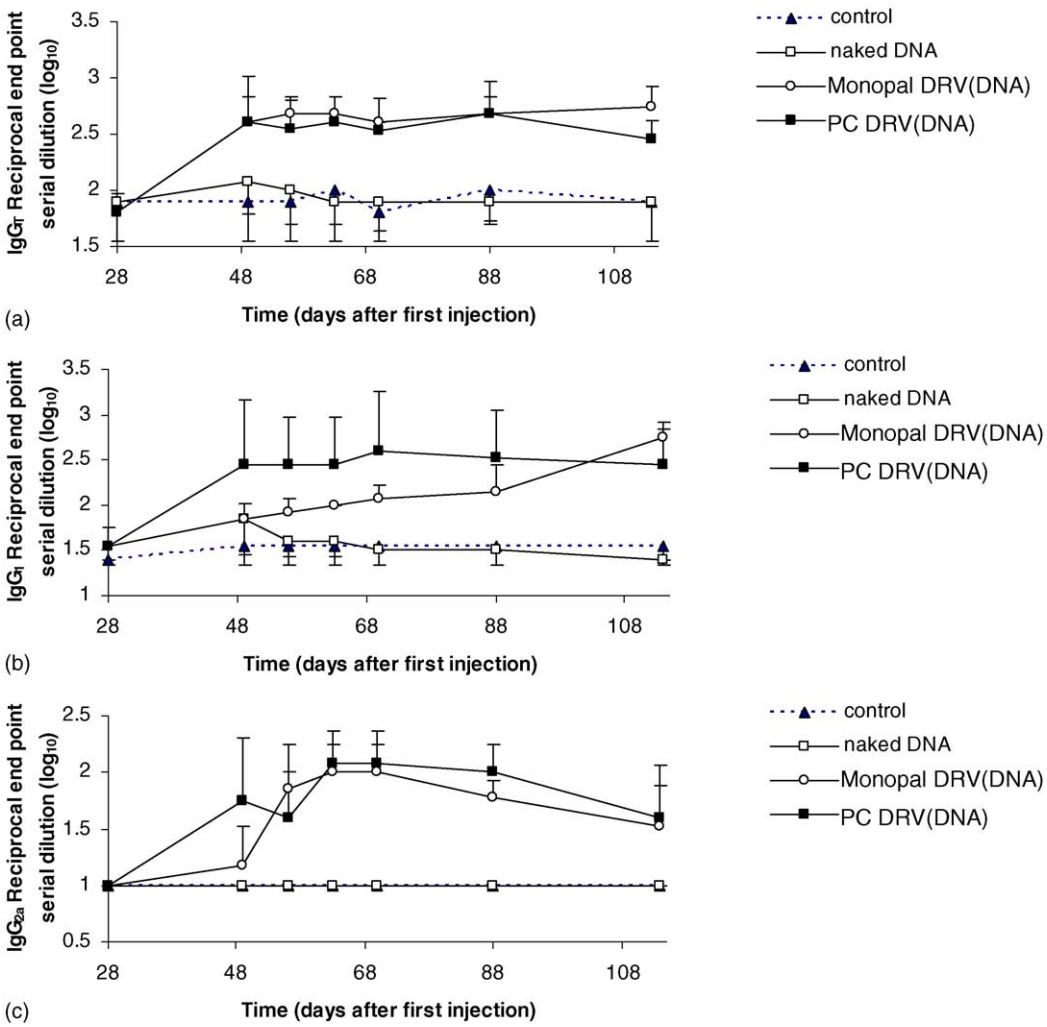

Fig. 4. Time course of immune responses in mice subcutaneously immunised with 10 μg of naked, liposome or niosome-entrapped pI.18Sfi/NP. Female B57 black mice (6–8 weeks old) in groups of five were given two subcutaneous injections 28 days apart either with 10 μg > naked (pI.18Sfi/NP plasmid DNA (open square) or with 10 μg pI.18Sfi/NP entrapped in cationic DRV composed of Monopal, DOPE, Chol and DC-Chol (4:2:1:1 molar ratio; open circle; Monopal DRV(DNA)) or composed of PC, DOPE and DC-Chol (4:2:1 molar ratio; closed square; DRV(DNA)). Values at various time points after the first injection are mean ± S.D. ($n = 5$) of $\log_{10}$ of the reciprocal end-point two-fold serum dilutions required for OD readings to reach a value of about 0.200. Values from untreated control mice are also shown (closed triangle). (a) Represents antibody IgG_T responses; (b and c) represents antibody IgG_1, and IgG_{2a} responses, respectively.

PC component of the liposome formulation (Table 2; Formulation 10). In addition 4 μmol of cholesterol was added to the Monopal formulation to avoid precipitation. The liposome formulation employed within this immunisation was chosen on the basis it had been identified as the lead liposome formulation for previous investigations (Gregoriadis et al., 1997; Perrie et al., 2001, 2003). Fig. 4a shows IgG_T responses in mice at various time points after two injections, 28 days apart of 10 μg of pI.18Sfi/NP, either naked or entrapped in PC or Monopal DRV (Table 2; Formulations 7 and 10, respectively). Results reveal that at all time points measured, mice immunised with naked DNA showed no greater engendered antibody immune response compared to the control, non-immunised group (Fig. 4). In contrast, by day 49, all IgG_T responses from mice

immunised with either DRV formulation were significantly higher ($P < 0.05$–0.005). Moreover, comparison of antibody responses between the Monopal and PC DRV(DNA) revealed no significant difference at all time points measured. Similar results were found when the IgG_1 and IgG_{2a} subclass responses were determined (Fig. 4b and c) respectively): by day 56 both the groups immunised with DRV formulations entrapping DNA provoked higher end point sera dilutions ($P < 0.05$–0.0001) than mice given free DNA. Whilst IgG_1 values reported in Fig. 4b suggest lower responses were obtained from the Monopal DRV immunisation compared with liposomes, statistical analysis of antibody responses from the PC and Monopal immunisation groups revealed no significant difference ($P > 0.05$) between either IgG_1 or IgG_{2a} levels after day 56. As with IgG_T, antibody responses for both IgG_1 and IgG_{2a} subclasses in mice immunised with naked DNA were no greater than those in naive mice. In agreement, previous work using a plasmid encoding the Hepatitis B surface antigen (ayw subtype) (Gregoriadis et al., 1998) and the pI.18sFi/NP (Perrie et al., 2003) also revealed responses for liposome-entrapped and naked DNA indicated greater antibody responses against the encoded antigen for the former when given via the subcutaneous route. Moreover, T-cell responses measured by analysis of interferon-γ and interleukin-4 levels in the spleens of mice treated with DRV(DNA) were significantly higher than those measured in mice treated similarly with naked DNA (Gregoriadis et al., 2002).

Liposomes (and entrapped drugs) injected locally are known (Tuner et al., 1983; Velinova et al., 1996) to be taken up by antigen presenting cells (APC) infiltrating the site of injection or in the lymph nodes draining the injected site, an event that has been implicated (Gregoriadis, 1990) in their immunoadjuvant activity. Clearance of liposomes from the site of injection has been shown (Segal et al., 1975) to be inversely related to vesicle size, presumably because of the slower rate with which large liposomes pass through anatomical barriers (e.g., those of the lymphatic capillaries (Segal et al., 1975)). The Monopal DRV used here were about twofold larger than the liposomes (1755 nm versus 856 nm; Table 2) yet, comparison of the IgG immune responses obtained revealed no significant difference between the two formulations of vesicles tested. However the trend of reduced IgG_1 responses (Fig. 4b) and the slight delay in the enhancement of IgG_{2a} (Fig. 4c) responses may be attributed to the enhanced Monopal vesicle size and the delay in these vesicles reaching the lymphatics.

Previous (Velinova et al., 1996) morphological observations revealed after subcutaneous injection the majority of liposomes arrived at the subcapsular sinuses, probably via afferent lymphatic vesicles, in the non-bound form (Velinova et al., 1996). Subsequently, liposomes were dispersed throughout the lymph node either by permeation as free vesicles along the sinuses or by cells involved in vesicle uptake. The majority of such cells were free macrophages, littoral cells and fixed macrophages. Once within cells, liposomes were seen digested by the lysosomal apparatus with varying loss of their lamellar structure, leaving the entrapped moiety (in the above studies gold particles) within the lysosomes (Velinova et al., 1996). In the case of DRV(DNA), for transfection to occur the plasmid DNA must escape degradation within the lysosome. As already discussed, it has been proposed that before reaching the lysosome, plasmid DNA complexed to preformed cationic liposomes (lipoplexes), may escape the endosome via the >flip-flop (mechanism of Szoka et al. (Szoka et al., 1996; Zelphati and Szoka, 1998). This relies on the combination of a cationic lipid component and the fusogenic lipid DOPE which after endocytosis of the lipoplex, is responsible for destabilising the endosomal membrane whereupon, through lateral diffusion of anionic lipids from the cytoplasm-facing endosomal monolayer, DNA is displaced from the complex and released into the cytosol for eventual episomal transfection (Szoka et al., 1996; Zelphati and Szoka, 1998). With this in mind, it is conceivable that after phagocytosis of the DRV(DNA) a similar mechanism may be responsible for the escape of the entrapped DNA thus allowing its release from the endocytic vacuoles prior to fusion with the lysosomes. Indeed, the presence of a cationic lipid (Gregoriadis et al., 1997) and DOPE (Perrie et al., 2001) has been shown to be beneficial in promoting liposome- and niosome-mediated (Obrenovic et al., 1998) DNA immunisation via the intramuscular route.

4. Conclusions

Comparison of the physico-chemical characteristics (zeta potential and vesicle size) of DRV contain-

ing PC or Monopal suggests that despite the similar cationic surfactant content and hence zeta potentials of comparable formulations, DRV(DNA) containing the non-ionic surfactant monopalmitoyl-rac-glycerol had measured z-average diameters approximately twice that of their PC-based liposome counterparts. In addition, such vesicles were shown to display an increased stability and increased DNA retention in the presence of competitive anions when compared to similarly formulated liposomes. Despite these differences in physico-chemical characteristics, both DRV(DNA) formulations were shown to effectively enhance subcutaneous DNA immunisation despite the requirement of cholesterol (to facilitate vesicle formation) within the Monopal DRV(DNA) formulation. In this respect, it will be of interest in a future study to see whether supplementation of the liposome formulation with similar cholesterol concentrations has an influence in immune responses. Vesicle-mediated DNA immunisation via the subcutaneous route revealed that humoural responses engendered by the plasmid encoded nucleoprotein were substantially higher after dosing with vesicle-entrapped DNA compared to naked DNA. Unlike the influenza heamagglutinin antigen molecule which is notoriously polymorphic in nature (Montgomery et al., 1983), the nucleoprotein antigen expressed by the plasmid used in the present immunization studies is conserved in different viral isolates (Montgomery et al., 1983; Yankauckas et al., 1993) thus potentially allowing the development of an influenza vaccine that would be independent of strain variations. Our results suggest that formulation of the nucleoprotein plasmid in DRV enhances engendered humoural immune responses after subcutaneous immunization and that such DRV containing DNA may be a useful system for subcutaneous delivery of DNA vaccines. This can be attributed to the ability of vesicles to not only protect their DNA content from nuclease attack (Gregoriadis et al., 1996; Obrenovic et al., 1998) but also deliver it to APC infiltrating the site of injection or in the lymphatics.

Acknowledgements

The authors gratefully acknowledge the financial support for this work provided in part by Lipoxen Technologies Ltd., School of Pharmacy, University of London and by the Engineering and Physical Sciences Research Council.

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