

Liposome-mediated DNA vaccination: the effect of vesicle composition

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Abstract

Liposome-entrapped DNA has been shown to enhance the potency of DNA vaccines, possibly by facilitating uptake of the plasmid by antigen-presenting cells (APC). In this paper, we have investigated the influence of the liposomal composition and surface charge on such potency. Plasmid DNA pRc/CMV HBS encoding the S (small) region of hepatitis B surface antigen was entrapped within cationic liposomes of various compositions and surface charges with high efficiency (88–97% of the amount used) by the dehydration–rehydration method that generates dehydration–rehydration vesicles (DRV). Cryo-electron microscopy revealed that DNA-containing DRV (DRV(DNA)) were multilamellar. In immunisation studies, female Balb/c mice were given two to four intramuscular injections of 10 µg naked or liposome-entrapped pRc/CMV HBS and bled at time intervals. Results indicate that the lipid composition of the DRV(DNA) influences the strength of the humoral response (immunoglobulin (Ig)G subclasses) with inclusion of dioleoyl phosphatidylethanolamine (DOPE) or phosphatidylethanolamine (PE) in the liposomal structure contributing to greater responses. DRV(DNA) in which the DOPE or PE were omitted or substituted with cholesterol led to significant reduction of humoral responses against the encoded antigen. Replacing phosphatidylcholine (PC) in the DRV(DNA) with the high-melting distearoyl phosphatidylcholine also contributed to lower responses. In other experiments, IgG responses were monitored in mice immunised with pRc/CMV HBS entrapped in DRV composed of PC and DOPE as before but incorporating increasing amounts of DOTAP (1–16 µmol). Maximal IgG responses were observed at 10 weeks after the first of four injections and suggested a trend of higher responses when 4 or 8 µmol DOTAP was present in the DRV(DNA) formulation. Cell-mediated immunity (measured in terms of endogenous antigen-specific splenic interferon-γ) in mice immunised with pRc/CMV HBS entrapped in liposomes composed of PC, DOPE and DOTAP (16:8:4 molar ratio) was much greater than in animals treated with naked plasmid. These results indicate that liposome-mediated DNA immunisation is more effective than the use of naked DNA, and also suggest that the presence of fusogenic phosphatidylethanolamine in DRV in conjunction with a low-melting phosphatidylcholine and an appropriate content of cationic lipid might contribute to more effective liposomal DNA vaccines. The notion that liposomes improve immune responses to the plasmid-encoded vaccine by facilitating the latter's uptake by APC was supported by the observation that in Balb/c mice injected intramuscularly with liposome-entrapped pCMV. Enhanced green fluorescent protein, expression of the gene in terms of fluorescence intensity in the draining lymph nodes, was much greater than in animals treated with the naked plasmid. © 2001 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Numerous animal studies [1–5] have shown that DNA immunisation, a promising recent approach to vaccination, induces potent cell-mediated protective immune responses and, in certain cases, potent antibody responses against the encoded antigen [1–5]. In most of these studies, naked DNA was administered by the

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intramuscular route, although other routes (e.g. intradermal, intranasal and intravaginal) have also been used [2,3]. It is likely [2] that, following intramuscular injection, much of the DNA is degraded by interstitial nucleases with the surviving material being taken up by cells that are transfected episomally [2–4]; for instance, myocytes [4]. These, however, lack co-stimulatory molecules and are therefore unable to act as professional antigen-presenting cells (APC). There is, however, evidence [6] that some of the injected DNA transfects APC directly. These cells are then activated and migrate to the draining lymph nodes for antigen presentation [2,6]. A number of approaches [2,3] to more effective use of DNA vaccines in terms of potency or type of immune responses have been proposed, and include 'gene gun' delivery [7] that appears to favour the development of the immunoglobulin (Ig)G₁ isotype, cationic lipid complexes reported [8,9] to function as adjuvants, delivery via cochleates that are claimed [10] to promote cytotoxic T lymphocyte responses, a variety of microparticles [11,12] that can control the type of immunity to the expressed antigens, the use of attenuated bacteria [2] (for instance, *Shigella flexneri*) transformed with a plasmid DNA vaccine that is delivered and expressed in mucosal surfaces [13] and, more recently, the use of polyoma virus replicon-based DNA vaccine that allows the latter to replicate [14].

In a recent approach pursued in this laboratory [15–17], DNA vaccines are entrapped within cationic liposomes as opposed to being complexed externally with such vesicles to form lipoplexes [8,9]. In addition to protecting DNA from nuclease degradation [18,19], DNA-containing liposomes would be a suitable means to deliver their contents directly to APC [3,15]: after local injection, liposomes are endocytosed rapidly by APC infiltrating the site of injection or in the draining lymph nodes [20,21]. Moreover, depending on their size, some of the liposomes may break down locally to release their vaccine content slowly. These events have formed the basis for the use of liposomes as immunological adjuvants for protein and peptide vaccines [22]. Indeed, work [15–17] with pRc/CMV HBS (encoding the S region of the hepatitis B surface antigen (HbsAg)) has shown already that intramuscular injection of mice with small amounts (e.g. 2–10 µg) of the plasmid entrapped in cationic liposomes evoked much greater humoral and cell-mediated immune responses against the encoded antigen than similar amounts of naked DNA or DNA complexed to identical, preformed liposomes. In this paper, we have investigated the effect of lipid composition of liposomal DNA constructs on immune responses. Results confirm previous findings [15–17] of liposome-enhanced humoral immunity to the encoded antigen. Furthermore, it appears that optimal enhancement of immunity is dependent on the inclusion into the liposomal bilayers of the fusogenic

phosphatidylethanolamines, a low-melting phosphatidylcholine and a certain proportion of cationic lipid. Work with intramuscularly injected naked or liposome-entrapped plasmid DNA encoding enhanced green fluorescent protein (pCMV.EGFP) showed that liposomes contributed to greater expression of the plasmid both in the injected muscle and the draining lymph nodes. This finding supports the notion that liposomes promote immune responses to DNA vaccines by facilitating their uptake by APC in the lymphoid tissues.

2. Materials and methods

2.1. Materials

The sources and grades of egg phosphatidylcholine (PC), distearoyl phosphatidylcholine (DSPC), phosphatidylethanolamine (PE), dioleoyl phosphatidylethanolamine (DOPE), cholesterol (CHOL) and 1,2-dioleoyl-3-(trimethylammonium) propane (DOTAP) have been described elsewhere [15–19]. Plasmid pRc/CMV HBS (5.6 kb) expressing the sequence coding for the S (small) region of HBsAg (subtype ayw) was supplied by Aldevron (Fargo, ND, USA) and pCMV.EGFP encoding enhanced green fluorescent protein was a gift from Dr Steven Hart. Both plasmids were radiolabelled with ³⁵S as described elsewhere [15,19]. Horseradish peroxidase-conjugated goat anti-mouse immunoglobulin IgG₁, IgG_{2a} and IgG_{2b}, and foetal calf serum were obtained from Sera-Lab (Crawley Down, Sussex, UK). Ninety-six-well flat-bottomed microtitre plates (Immunolon IB) were purchased from Dynatech Labs (Billingshurst, Sussex, UK). Recombinant hepatitis B surface antigen (HBsAg) (S region; ayw subtype) was supplied by Genzyme diagnostics (Kingshill, Kent, UK). All other reagents were of analytical grade.

2.2. Preparation of plasmid DNA-containing liposomes

pRc/CMV HBS was incorporated into liposomes by the dehydration–rehydration procedure [15–19,23]. In brief, 2 ml small unilamellar vesicles (SUV) prepared [23] by sonication and composed of 16 µmol PC or DSPC, 8 µmol DOPE, PE or cholesterol (molar ratio 1:0.5) and 1–16 µmol cationic lipid DOTAP were mixed with 100 µg plasmid DNA (and ³⁵S-labelled tracer of the same plasmid) to form lipoplexes, frozen at –20°C and freeze-dried overnight. Controlled [15–19] rehydration of the dry powders led to the formation of multilamellar [24] dehydration–rehydration vesicles (DRV liposomes) containing the DNA within their structure, presumably bound to the cationic charges of the inner bilayers [19]. DNA-containing DRV (DRV(DNA)) were then centrifuged twice at 25000g

for 40 min to remove non-entrapped DNA and resuspended in 0.01 M sodium phosphate containing 0.15 M NaCl (pH 7.4) (phosphate-buffered saline (PBS)) to the required volume. DNA entrapment into liposomes (Table 1) was estimated on the basis of ^{35}S radioactivity recovered in the suspended pellets. The same procedure as already described was used to entrap pCMV.EGFP (100 μg) into DRV composed of 16 μmol PC, 8 μmol DOPE and 4 μmol DOTAP.

2.3. Determination of vesicle size

The z -average diameter of DRV(DNA) liposomes was measured on a Autosizer 2c by photon correlation spectroscopy (PCS), at 20°C by diluting 20 μl dispersion to the appropriate volume with doubly filtered (0.22 μm pore size) distilled water [19].

2.4. Determination of vesicle zeta potential

The zeta potential, an indirect measurement of the vesicle surface charge, was measured in 0.001 M PBS at 25°C on a Malvern Zetasizer 3000 [19].

2.5. Electron microscopy

Cryo-electron microscopy of DRV(DNA) involved [24] forming a thin aqueous film on bare specimen grid (3–4 μm thick, with a fine 700 mesh honeycomb pattern of bars) by dipping the grid into the liposome suspension. After blotting the suspension-coated grid on filter paper, the thin film produced was rapidly (1 s) vitrified by plunging the grid into ethane and cooled to its melting point with liquid nitrogen. Preparation and blotting of thin films was carried out in a controlled environment using a fully automated system (PC-controlled, up to vitrification). The vitrified film was mounted in a cryo-holder (Gatan 626) and observed at –170°C in a transmission microscope (Philips CM12) operating at 120 kV. Micrographs were taken using low-dose conditions.

Table 1

Incorporation of plasmid DNA into liposomes: entrapment, zeta potential, and vesicle size^a

Liposomes	DNA incorporation (% of used)	Zeta potential (mV)	Size (nm \pm S.D. (PDI))
PC:DOPE:DOTAP (16 μmol :8 μmol :4 μmol)	94.0 \pm 2.8	32.1 \pm 0.3	979.3 \pm 95.9 (0.32)
PC:PE:DOTAP (16 μmol :8 μmol :4 μmol)	92.3 \pm 4.1	32.9 \pm 0.7	1093.6 \pm 81.3 (0.39)
DSPC:DOPE:DOTAP (16 μmol :8 μmol :4 μmol)	91.3 \pm 3.3	32.6 \pm 0.4	1024.6 \pm 152.6 (0.30)
PC:CHOL:DOTAP (16 μmol :8 μmol :4 μmol)	87.9 \pm 3.9	53.9 \pm 1.5	934.6 \pm 138.3 (0.35)
PC:DOTAP (16 μmol :8 μmol :4 μmol)	90.0 \pm 4.6	43.0 \pm 3.0	976.2 \pm 87.2 (0.31)

^a ^{35}S -labelled pRc/CMV HBS (100 μg) was incorporated into cationic DRV of various lipid compositions and lipid molar ratios as shown. Incorporation values were based on ^{35}S assay. The zeta potential of the DRV was measured in 0.001 M PBS at 25°C using a Zetasizer 3000. Vesicle z -average diameter was determined in an Autosizer 2c at 20°C. Results represent mean \pm S.D., $n = 3$ –5. Corresponding values for pCMV.EGFP (100 μg) entrapped in PC:DOPE:DOTAP (16:8:4 molar ratios) liposomes were 94 \pm 4.0 (% entrapped), 32.3 \pm 0.4 (mV) and 689 \pm 88 (nm \pm S.D.) ($n = 3$).

2.6. Immunisation protocol

Female Balb/c mice, 6–8 weeks old, were given two to four intramuscular (hind leg) injections of 10 μg (in 0.1 ml PBS) of either ‘naked’ or liposome-entrapped pRc/CMV HBS plasmid as shown in the captions to Figs. 2–6. Sera samples collected at time intervals were tested for anti-HBsAg (S region; ayw subtype) IgG₁, IgG_{2a} and IgG_{2b} by the enzyme-linked immunosorbent assay (ELISA) as previously described [15–17]. Endogenous levels of interferon- γ (IFN- γ) and interleukin-4 (IL-4) in whole spleens were determined by the method of Nakane et al. [25] as modified by de Souza et al. [26]. Individual spleens from mice injected intramuscularly twice with 10 μg naked or liposome-entrapped pRc/CMV HBS and with 1 μg HBsAg (in 0.2 ml of 0.9% NaCl) intravenously 24 h before death, were weighed, homogenised in ice-cold RPMI containing 1% 3-(cholamidopropyl-*o*-dimethylammonio)-1-propanesulphonate (CHAPS; Sigma) in a Dounce tissue homogeniser and 10% (w/v) homogenates were prepared. Homogenates were left on ice for 1 h and insoluble debris was removed by centrifugation at 2000g for 20 min. Standard capture ELISAs were used to determine IFN- γ and IL-4 levels. Maxisorb (NUNC, UK) plates were coated with primary monoclonal antibodies against IFN- γ and IL-4. Secondary biotinylated anti-mouse IL-4 and anti-mouse IFN- γ monoclonal antibodies (Pharmingen, USA) were used with streptavidin peroxidase (Dako, Denmark) and *o*-phenylenediamine (Sigma) as substrate. Recombinant IFN- γ and IL-4 standards were from Pharmingen. Spleens from a group of non-immunised (intact) mice treated as already described served as controls. Results (mean \pm S.D.) expressed as nanograms per spleen from at least four mice were analysed and compared using the Student’s *t*-test.

2.7. Expression of enhanced green fluorescent protein after intramuscular injection of the plasmid encoding the protein

Female Balb/c mice, 6–8 weeks old, in groups of three were injected intramuscularly into the right hind

Table 2
The effect of DOTAP content of DRV on DNA incorporation, vesicle size and zeta potential^a

DOTAP content (μmol)	Incorporation (% used)	Zeta potential (mV)	Vesicle size (nm)
1	95.9 ± 0.5	-1.1 ± 0.8	n/d
2	95.7 ± 2.8	13.2 ± 1.1	1095 ± 247
4	94.0 ± 2.8	32.1 ± 0.3	703 ± 109
8	96.3 ± 0.9	45.0 ± 2.3	645 ± 62
12	96.6 ± 2.8	48.3 ± 1.5	653 ± 84
16	95.5 ± 4.9	49.7 ± 0.2	607 ± 97

^a ^{35}S -labelled pRc/CMV HBS (100 μg) as in Table 1 was incorporated into DRV composed of 16 μmol PC, 8 μmol DOPE and various amounts of the cationic lipid DOTAP. Vesicle size (z average diameter) for DRV(DNA) containing 1 μmol DOTAP was too great to be measured by PCS and hence not determined (n/d). For other details, see footnote to Table 1. Results represent mean \pm S.D., $n = 3-5$.

leg with 10 μg (in 0.1 ml PBS) of naked or liposome-entrapped pCMV.EGFP. Forty-eight hours later, muscle tissue from the injected sites and the popliteal and inguinal lymph nodes were collected, adhered to cryostat chucks using Tissue-Teck (Miles Inc., USA) and frozen in liquid nitrogen, and sections (20 μm) cut in a Slee cryostat. Images were captured in a Nikon microphot-*fxa* microscope using incident fluorescence and Kodak Ektachrome 400 ASA film.

3. Results and discussion

3.1. DNA incorporation into liposomes

Values of pRc/CMV HBS entrapment (% of total used) in DRV of all compositions studied (Tables 1 and 2) were high (88–97%), even when the amount of cationic lipid (DOTAP) employed per preparation was as low as 1 μmol (Table 2). The use of DSPC (a high gel to liquid crystalline transition temperature (T_c) lipid) instead of PC or substitution of DOPE with PE had no influence on DNA entrapment values. Such ^{35}S -based entrapment values, also confirmed for pCMV.EGFP (footnote to Table 1), have been found

[15–19] to be reliable and to predominantly reflect actual DNA entrapment as opposed to vesicle surface complexation. The latter occurs when preformed 'empty' (water-containing) cationic liposomes are incubated with DNA [16,19] resulting in aggregates of 10–20 μm diameter. In contrast, DRV(DNA) are much smaller (around 1 μm diameter) (Table 1) and appear [19] to contain the DNA within the aqueous spaces in between the bilayers, presumably bound to the cationic charges. Indeed, cryo-electron microscopy (Fig. 1) of DRV(DNA) clearly showed that such constructs are multilamellar vesicles of similar appearance to that already seen by cryo-electron microscopy of neutral DRV-containing anions [24]. The multilamellar structure of DRV(DNA) was further confirmed (results not shown) by freeze fracture electron microscopy as previously applied for neutral DRV [27].

Table 1 (and footnote for pCMV.EGFP) also shows zeta potential values for DRV(DNA). Judging from results with pRc/CMV HBS, such values are influenced by the presence of DOPE or PE. Thus, values are lower (31–33 mV) when these lipids are present than when omitted (e.g. 43 mV for PC:DOTAP; Table 1) or substituted with cholesterol (e.g. 54 mV for PC:CHOL:DOTAP; Table 1). Interestingly, DOPE was

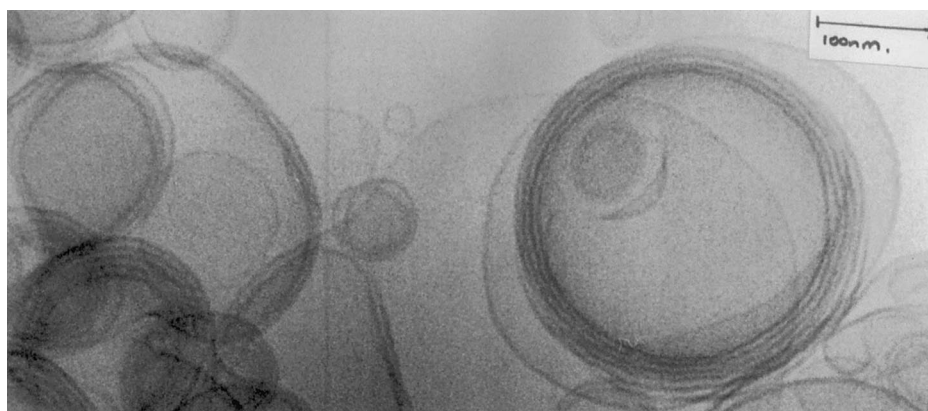


Fig. 1. Cryo-electron microscopy of DRV(DNA) composed of 16 μmol PC, 8 μmol DOPE and 4 μmol DOTAP, and prepared in the presence of 100 μg pRc/CMV HBS.

found [19] to also reduce the negative z -potential of similar phosphatidyl glycerol-incorporating anionic liposomes when compared with anionic vesicles where cholesterol replaces DOPE. It has been suggested [28] that such reductions of z -potential to less positive or less negative values in the presence of DOPE or PE result from the formation of salt bridges between the charge-bearing head groups of DOTAP or phosphatidyl glycerol and the zwitterionic head group of phosphatidylethanolamines. There was no significant difference in the zeta potential values between DSPC and PC DRV(DNA) (Table 1). As expected, and already found for a variety of polymer–DNA complexes [29], increasing the cationic lipid content of DRV(DNA) (incorporating a constant amount of DNA) led to an increase (from about 1 to 50 mV) in zeta potential values (Table 2).

The effect of DOTAP content on the size (diameter) of DRV prepared in the presence of 100 μg DNA is shown in Table 2. Results indicate that, as the DOTAP content increases to 2 μmol or higher, vesicle size is reduced to around 600–1100 nm (see also footnote to Table 1 for liposome-entrapped pCMV-EGFP). This effect of vesicle charge on the size reduction of DRV(DNA) has been attributed [19] to the charged surfaces repelling each other sufficiently during the dehydration rehydration steps of the DRV procedure so as to interfere with the progress of membrane adhesion and eventually fusion, thus leading to smaller DRV.

3.2. Immunisation with liposome-entrapped DNA

Having already established [14] that intramuscular injection of DRV(DNA) is more effective in inducing immune responses to the encoded antigen than injection of naked DNA or DNA complexed with preformed DRV, further related work was carried out to study the effect of varying the lipid composition of liposomes as well as their cationic charge on such responses. To allow the detection of liposome-mediated improvement (if any) of immune responses to the encoded antigen, doses of plasmid DNA were, as previously [3,15], low enough (10 μg) for naked DNA to fail to induce responses under the present conditions. In this respect, other workers [4] using the same (naked) pRc/CMV HBS plasmid employed multiple doses of 50–100 μg in order to obtain substantial levels of anti-HBsAg IgG.

Fig. 2 shows IgG₁ responses in mice 55, 66 and 91 days after the first of three injections of 10 μg pRc/CMV HBS, either naked or entrapped in liposomes composed of lipids as shown in Table 1. Results reveal that, at all time points measured, mice immunised with DRV(DNA) (Fig. 2, groups 1–4) elicited significantly higher ($P > 0.03$ –0.0001) immune responses than mice injected with naked DNA (Fig. 2, group 5). Moreover,

there was no significant difference in responses when DOPE in DRV(DNA) was replaced by PE (Fig. 2, compare groups 1 and 2). However, responses were significantly ($P < 0.01$ –0.001) reduced when the DOPE component in DRV(DNA) was omitted (Fig. 2, compare groups 1 and 4). A significant ($P < 0.01$ –0.001) reduction in IgG₁ response was also observed when DOPE in DRV(DNA) was replaced by cholesterol (Fig. 2, compare groups 1 and 3; 91 days). Incorporation of DOPE into the vesicle bilayer is known [29–32] to enhance the transfection activity in vitro of liposome–DNA complexes (lipoplexes), possibly [33,34] because

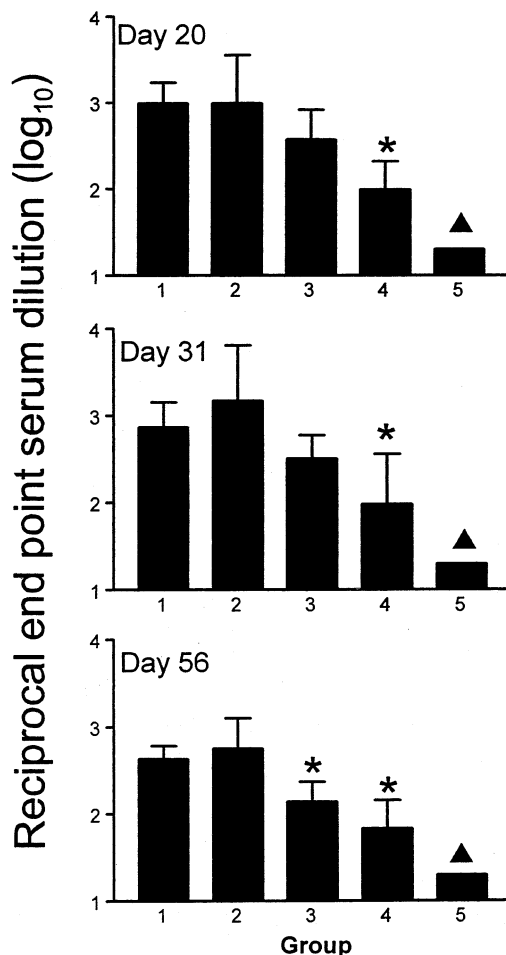


Fig. 2. Immunisation with liposome-entrapped pRc/CMV HBS: the effect of lipid composition. Balb/c mice in groups of five were injected intramuscularly on days 0, 14 and 35 either with 10 μg naked DNA (group 5) or with 10 μg pRc/CMV HBS entrapped in cationic liposomes composed of PC, DOPE and DOTAP (4:2:1 molar ratio, group 1); PC, PE and DOTAP (4:2:1 molar ratio, group 2); PC, CHOL and DOTAP (4:2:1 molar ratio, group 3); PC and DOTAP (4:1 molar ratio, group 4). Values at days 55, 66 and 91 after the first injection are means \pm S.D. ($n = 5$) of log₁₀ of the reciprocal end-point serial twofold serum dilutions required for OD readings to reach a value of about 0.200. Sera from untreated mice gave log₁₀ values of less than 2.0. * Values significantly ($P < 0.01$ –0.001) lower than those of the groups 1 and 2; \blacktriangle , values significantly ($P < 0.03$ –0.0001) lower than those of groups 1–4.

of the ability of DOPE to enter the H_{II} hexagonal phase. It is thought [35–37] that, by entering the H_{II} phase after the endocytosis of lipoplexes, DOPE promotes the disruption of the endosomal membrane and ensuing escape of plasmid DNA into the cytoplasm. The data of Fig. 2, indicating greater immune responses for DRV(DNA) incorporating DOPE (or PE), support this view: increased concentration of the plasmid in the cytoplasm as a result of the membrane disrupting activity of phosphatidylethanolamine should lead to a greater probability of plasmid entry into the nucleus and subsequent expression. In a separate experiment using pRc/CMV HBS (10 μ g), either as such (naked) or entrapped in DRV composed of PC, DOPE and DOTAP as described in Fig. 3, cell-mediated immunity was measured in terms of endogenous IFN- γ content of the spleens of mice immunised with pRc/CMV HBS and injected intravenously with 1 μ g encoded antigen 24 h before death. Results (Fig. 3) indicate much greater levels of the cytokine in the spleen of mice immunised with the liposome-entrapped plasmid. The failure to detect significant levels of IFN- γ in the present study in animals injected with naked plasmid could be attributed to the low amount used (10 μ g). Levels of IL-4 representing humoral immunity were also higher in the animals treated with liposomal plasmid, confirming data in Fig. 2.

In contrast to the transfection-promoting effect of DOPE [30,32], other studies [38] have reported that lipoplexes incorporating phospholipids with a high T_c inhibit transfection in vitro. This is in agreement with results in Fig. 4 showing that replacement of PC in DRV(DNA) with the high-melting DSPC leads to a significantly ($P < 0.03$) reduced IgG₁ response. The use of lipids with a high T_c (such as DSPC) in DRV(DNA) constructs renders bilayers more rigid and therefore [37,38] not as effective in interacting with the endosomal bilayer. Indeed, studies [39] in vitro with lipoplexes incorporating phospholipids of varying T_c have shown a direct correlation between bilayer fluidity and transfection activity. A similar effect of DSPC has been reported [40] in conjunction with the immunoadjuvant properties of DRV liposomes using tetanus toxoid as the antigen. As with DNA vaccination, it is likely that the putative interaction of endosomes with the liposomal membrane and ensuing release of antigen into the cytosol is inhibited by the 'solid' DPSC-incorporating liposomal membranes.

In another experiment, IgG responses were monitored in mice immunised with pRc/CMV HBS entrapped in DRV composed of PC and DOPE but incorporating a wide range of DOTAP content (1–16 μ mol). Results in Fig. 5 showing maximum IgG subclass (IgG₁, IgG_{2a} and IgG_{2b}) levels attained 69 days after the first injection suggest a trend of higher responses when 4 or 8 μ mol DOTAP are present in the

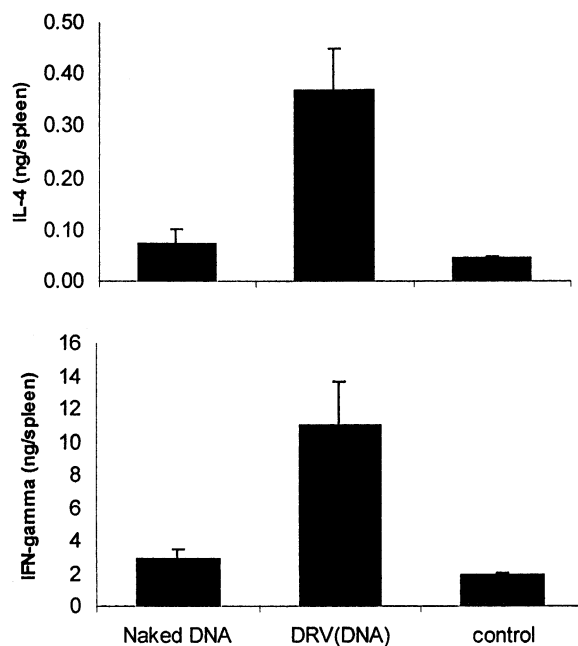


Fig. 3. Interferon- γ and interleukin IL-4 levels in the spleens of mice immunised with naked or liposome-entrapped pRc/CMV HBS. Balb/c mice in groups of four were injected on days 0 and 21 with 10 μ g pRc/CMV HBS either in the naked form or entrapped in liposomes composed of 16 μ mol PC, 8 μ mol DOPE and 4 μ mol DOTAP (DRV(DNA)). 'Control' represents cytokine levels in normal, non-immunised mice. Forty-one days after the first injection, mice were injected intravenously with 1 μ g HBsAg, killed 24 h later and their spleens subjected to cytokine analysis as described in Section 2. Each bar represents the mean \pm S.D. of a group of four mice.

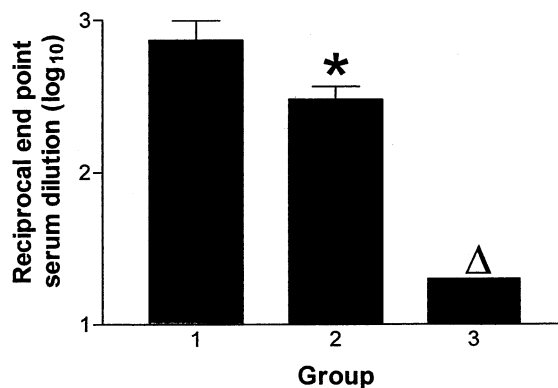


Fig. 4. Immunisation with liposome-entrapped pRc/CMV HBS: the effect of phospholipid T_c . Balb/c mice in groups of five were injected intramuscularly on days 0, 14 and 35 with 10 μ g pRc/CMV HBS entrapped in cationic liposomes containing PC, DOPE, DOTAP (4:2:1 molar ratio, group 1); DSPC, DOPE, DOTAP (4:2:1 molar ratio, group 2); naked DNA, (group 3). For other details see caption to Fig. 1. Values are from animals bled on day 66 after the first injection. * Values significantly lower ($P < 0.03$) than those of the PC:DOPE:DOTAP group; Δ , values significantly lower ($P < 0.0001$) than those of the DRV(DNA) groups.

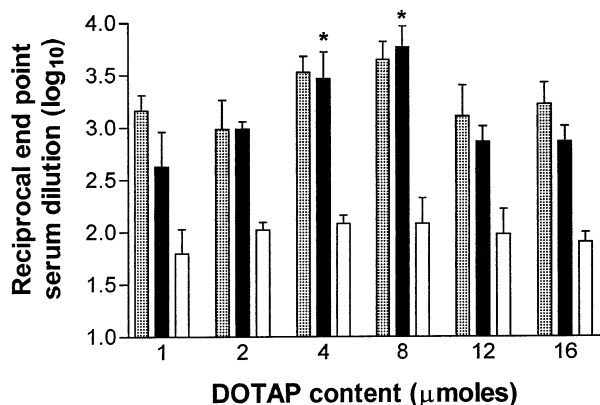


Fig. 5. Immunisation with liposome-entrapped pRc/CMV HBS: the effect of cationic charge. Balb/c mice in groups of five were injected intramuscularly on days 0,7,14 and 35 with 10 μ g pRc/CMV HBS entrapped in cationic liposomes composed of 16 μ mol PC, 8 μ mol DOPE and 1–16 μ mol DOTAP. Sera samples at 69 days after the first injection were tested by ELISA for IgG₁ (dotted bars), IgG_{2a} (black bars) and IgG_{2b} (open bars) responses against the encoded hepatitis B surface antigen. For other details see caption to Fig. 2. * IgG_{2a} responses significantly higher ($P < 0.02$ – 0.006) than those in mice immunised with other DOTAP formulations.

DRV(DNA). For instance, IgG_{2a} values from mice immunised with these formulations were significantly higher ($P < 0.02$ – 0.006) than those from mice immunised with other DRV(DNA) preparations incorporating lower or higher amounts of DOTAP. There was no significant difference in IgG_{2b} responses with any of the groups. These results suggest that the presence of 4 or 8 μ mol DOTAP in DRV (which relates to a theoretical +/– charge ratio of 3.2:1 to 6.4:1) may be an optimum cationic lipid to DNA ratio to employ in liposome-mediated vaccination. However, even with small amounts of cationic lipid (1 μ mol) present, significant immune responses can be obtained and this may be of importance if higher amounts of cationic lipid prove to be toxic. Inspection of the time course of IgG subclass values (Fig. 6) revealed no responses until week 6, after which values (especially for IgG₁ and IgG_{2a}) increased to peak at week 10 for all DOTAP contents. By week 13, all DOTAP groups showed a fall in IgG₁ responses whereas, in most cases, IgG_{2a} responses remained high. In this respect, it will be of interest in a future study to see whether the IgG₁:IgG_{2a} response ratios as observed here reflect similar ratios for specific IFN- γ (Th1) and IL-4 (Th2 response) production in the spleens of immunised mice. As anticipated from previous findings [3,15], there were no significant differences between IgG₁ responses in the two experiments of Fig. 2 and Figs. 5 and 6, where three and four injections of the plasmid were given, respectively, over 35 days.

It is generally accepted that efficient transfection with cationic liposomes relies on the cationic vesicle–DNA complexes (lipoplexes) possessing a slight excess of net positive charge that will allow binding of the complexes

with the anionic cell surface [41]. It is well known from our early studies [42] that the positive surface charge of cationic liposomes is masked by plasma proteins that impose a net negative charge on the surface of the vesicles. This potential problem was apparent even in the first reports using lipoplexes as transfection agents [43], with transfection being inhibited by serum-containing growth medium. More recently, it was reported [41] that interactions between liposome–DNA complexes and anionic molecules in the serum neutralised the positive charge of the complex and decreased transfection efficiency. Loss of transfection activity in the presence of serum may not, however, be solely due to neutralisation of the cationic surface charge. Observations [19,44] that anionic molecules can act competitively to release DNA from complexes with cationic vesicles or molecules raises the interesting possibility that, in vivo, negatively charged serum components (e.g. proteins) may interfere with transfection by competing with DNA for binding sites, thus bringing about its release. On the other hand, there is strong evidence to suggest [19,45,46] that, because DNA is entrapped within the aqueous phase of the multilamellar (Fig. 1) DRV vesicles, other anionic molecules fail to compete for binding sites. For instance, it has been shown in gel electrophoresis experiments [19] that, whereas the plasmid in lipoplexes (obtained by mixing preformed cationic DRV or SUV with DNA) is easily displaced by sodium dodecyl sulphate (SDS) through anionic competition, this occurs only to a minor extent with liposome-entrapped plasmid (generated as described in Section 2 by freeze-drying the lipoplexes and subsequent rehydration), presumably because, in the latter case, the plasmid is not as accessible to anions, especially to the much larger (than SDS) proteins. It is conceivable that the apparent failure [15] of lipoplexes to mount a substantial immune response to the encoded antigen results from the displacement of complexed pRc/CMV HBS by proteins in the interstitial fluid.

It can therefore be surmised that, in contrast to the events [1–5] associated with naked DNA immunisation by the intramuscular route, liposome-entrapped DNA given by the same route has a different fate. For instance, there is considerable degradation of naked DNA in situ [6], with some of the surviving material taken up by a minor fraction of myocytes [4] and, probably [6], a small number of APC thus requiring [1–5] relatively large doses of the vaccine to provoke substantial responses. In contrast, liposome-entrapped DNA is largely protected [18] from interstitial deoxyribonucleases by the bilayers surrounding the vaccine. Moreover, some of the liposomes (probably [20] those of larger size) are expected [21,22] to remain at the site of injection and slowly release their DNA content locally following their degradation by tissue phospholipases, with the surviving smaller vesicles delivering the

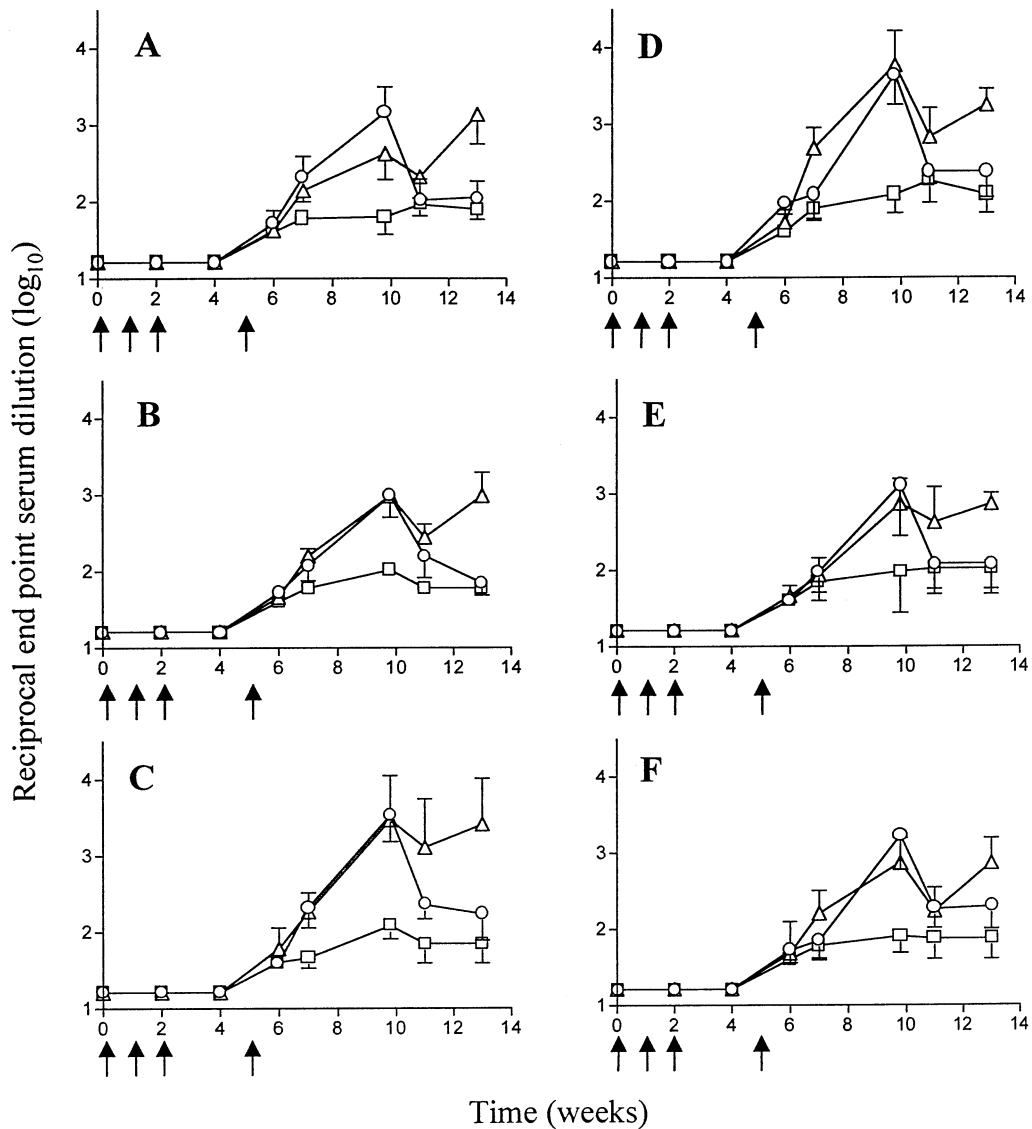


Fig. 6. Time course of immune responses in mice immunised with liposome-entrapped pRc/CMV HBS in the experiment of Fig. 5. Sera samples were collected at various time intervals and tested by ELISA for IgG₁ (circles), IgG_{2a} (triangles) and IgG_{2b} (squares) responses against the encoded hepatitis B surface antigen. (A) 1 μ mol DOTAP, (B) 2 μ mol DOTAP, (C) 4 μ mol DOTAP, (D) 8 μ mol DOTAP, (E) 12 μ mol DOTAP, and (F) 16 μ mol DOTAP. For other details, see caption to Fig. 5.

Fig. 7. Fluorescence images of muscle and lymph node sections from mice injected intramuscularly with 10 μ g liposome-entrapped or naked pCMV.EGFP and killed 48 h later. Sections from untreated animals were used as controls. For details, see Section 2.

remainder directly and efficiently to APC in the draining lymph nodes. Data (Fig. 7) obtained in an experiment where mice were injected intramuscularly with naked and liposome-entrapped pCMV.EGFP support this view. Results indicate much greater fluorescence intensity (presumably reflecting greater expression of enhanced green fluorescent protein) in both the injected muscle and the draining popliteal and inguinal lymph nodes of mice treated with the liposomal plasmid than in the animals treated with the same amount of naked plasmid. At the intracellular level, it is likely [32] that one of the steps in the pathway of liposome-mediated DNA immunisation, i.e. escape of DNA from endosomes following endocytosis of the DRV(DNA), is influenced by the composition of liposomes. Our results indicate that a phospholipid with a low T_c combined

with the fusogenic DOPE (or PE) and an appropriate surface charge (or zeta potential) contributes to optimal immune responses to the antigen encoded by the liposome-entrapped pRc/CMV HBS plasmid.

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