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A comparative study of cationic liposome and niosome-based adjuvant systems for protein subunit vaccines: characterisation, environmental scanning electron microscopy and immunisation studies in mice

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Abstract

Vesicular adjuvant systems composing dimethyldioctadecylammonium (DDA) can promote both cell-mediated and humoral immune responses to the tuberculosis vaccine fusion protein in mice. However, these DDA preparations were found to be physically unstable, forming aggregates under ambient storage conditions. Therefore there is a need to improve the stability of such systems without undermining their potent adjuvanticity. To this end, the effect of incorporating non-ionic surfactants, such as 1-monopalmitoyl glycerol (MP), in addition to cholesterol (Chol) and trehalose 6,6′-dibehenate (TDB), on the stability and efficacy of these vaccine delivery systems was investigated. Differential scanning calorimetry revealed a reduction in the phase transition temperature (T_c) of DDA-based vesicles by $\sim$12°C when MP and cholesterol (1:1 molar ratio) were incorporated into the DDA system. Transmission electron microscopy (TEM) revealed the addition of MP to DDA vesicles resulted in the formation of multi-lamellar vesicles. Environmental scanning electron microscopy (ESEM) of MP–Chol–DDA–TDB (16:16:4:0.5 μmol) indicated that incorporation of antigen led to increased stability of the vesicles, perhaps as a result of the antigen embedding within the vesicle bilayers. At 4°C DDA liposomes showed significant vesicle aggregation after 28 days, although addition of MP–Chol or TDB was shown to inhibit this instability. Alternatively, at 25°C only the MP-based systems retained their original size. The presence of MP within the vesicle formulation was also shown to promote a sustained release of antigen in-vitro. The adjuvant activity of various systems was tested in mice against three subunit antigens, including mycobacterial fusion protein Ag85B-ESAT-6, and two malarial antigens (Merozoite surface protein 1, MSP1, and the glutamate rich protein, GLURP). The MP- and DDA-based systems induced antibody responses at comparable levels whereas the DDA-based systems induced more powerful cell-mediated immune responses.

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Introduction

Several colloidal lipid- and surfactant-based particulate delivery systems, such as liposomes (Aramaki et al 1994), non-ionic surfactant vesicles (niosomes) (Brewer & Alexander 1992) as well as polymeric micro- and nano- particles (O'Hagan et al 1993; Florence et al 1995), have been employed in subunit vaccine delivery. Many of these delivery systems have been exploited not only for their inherent associated adjuvanticity, which augments immune response to poorly immunogenic yet otherwise promising vaccine candidates, but also for reasons such as increased safety in comparison with live vaccines, protecting the antigen from in-vivo degradation and thus allowing its administration by different routes, ability to sustain the antigen release over an extended period of time, intracellular delivery of antigen to achieve cell-mediated immune response and targeting the antigen-presenting cells (Gregoriadis 1990). Initial studies using niosomes as vaccine delivery systems (Brewer & Alexander 1992) suggested that merely mixing antigen with empty non-ionic surfactant vesicles was not sufficient for adjuvant activity; more recent studies have, however, demonstrated that mixing antigens such as Ag85B-ESAT-6 with

empty pre-formed liposomes composed of the cationic lipid dimethyldioctadecylammonium (DDA) or trehalose 6,6′-dibehenate (TDB) can produce effective immune responses (Davidsen et al 2005). This may be related to the ability of such vesicles to retain a high amount of the associated antigen (either via electrostatic adsorption or encapsulation), providing a greater capacity to target antigen to antigen-presenting cells or ability to form short-lived depots (Brewer & Alexander 1992). However, the adjuvanticity of these surfactant vesicles also appears to be dependent on structural characteristics such as vesicle size, surface charge, lipid-to-antigen ratio, lamellarity of bilayers and rigidity of the bilayer (Gregoriadis 1990; Gupta et al 1993).

Features of niosomes that make them desirable adjuvants are their apparent lack of toxicity (Rogerson et al 1988) and reported stability (Yoshioka et al 1994), although studies have also shown that addition of non-ionic surfactants to liposomes can greatly enhance the adjuvanticity of these preparations (Zigterman et al 1987, 1988). Similarly, cationic liposomes formulated using the quaternary ammonium surfactant DDA have also been reported as an effective adjuvant for eliciting both cell-mediated and humoral responses (Lindblad et al 1997; Holten-Andersen et al 2004).

In this paper, we investigate the stabilisation and adjuvant effect of various DDA formulations by supplementing the formulation with non-ionic surfactants, cholesterol and TDB. In addition to standard characterisation studies, morphological and structural analysis of these surfactant vesicles has been undertaken using environmental scanning electron microscopy (ESEM). Morphological investigations are usually performed using techniques such as scanning electron microscopy (SEM) (Lopez et al 2001), freeze fracture analysis (Egelhaaf et al 2003) or scanning tunnelling microscopy (STM) (Zareie et al 1997). However, most of these techniques require samples to be dried, fixed, frozen or coated with conducting materials before imaging, resulting in potential changes in the morphology. As a result these images can be poorly representative of these systems in real-time conditions. Alternatively transmission electron microscopy (TEM), and in particular ESEM, can image wet systems with no prior elaborate specimen preparation. Though TEM has been extensively used in imaging surfactant vesicles (e.g. Darwish & Uchegbu 1997; Arunothayanun et al 2000), ESEM is a new technique as far as imaging surfactant vesicles is concerned (Mohammed et al 2004). The underpinning advantage of ESEM over other microscopy methods is that it allows altering the sample environment dynamically through a range of pressures, temperatures and gas compositions, enabling hydration and dehydration processes to be followed as they happen in the sample chamber.

Materials and Methods

Materials

Dimethyldioctadecylammonium bromide (DDA) and α,α'-trehalose 6,6′-dibehenate (TDB) were obtained from Avanti Polar Lipids (Alabaster, AL). 1-Monopalmitoyl glycerol (C16:0) (MP) and cholesterol were purchased from Sigma Chemical Company (UK) Ltd. Methanol (extra pure), chloroform (extra pure) and 1 M hydrochloric acid, used to adjust pH in the Tris-buffer, were purchased from Merck (Darmstadt, Germany). Tris (Ultra Pure) was from ICN Biomedicals, Inc. (OH, USA) and Sephadex G-75 was obtained from Sigma-Aldrich (St Louis, MO, USA). Double-distilled water was used to prepare all samples.

The following antigens were used for immunisation of mice: non-His-tagged protein Ag85B-ESAT-6 was produced in *Escherichia coli* as described previously for the His-tagged version (Olsen et al 2001), purified by column chromatography and dissolved in 10 mM Tris-buffer, pH 7.4, at a concentration of 0.5 mg mL^{-1}; His-tagged MSP1 from *Plasmodium yoelii* and GLURP from *Plasmodium falciparum* were cloned, expressed and purified as previously described (Theisen et al 1995).

Alexa Fluor 350 dye kit for labelling Ag85B-ESAT-6 was obtained from Molecular Probes Ltd. For radiolabelling of the antigens, Iodo-gen pre-coated iodination tubes from Pierce Biotechnology (Rockford, IL, USA) were used and ^{125}I (NaI in NaOH solution) was purchased from Amersham Biosciences (Amersham, UK).

Splenocyte cultures were conducted using round-bottomed 96-well microtitre plates obtained from Nunc (Roskilde, Denmark). RPMI 1640 medium was obtained from Gibco (Invitrogen, Carlsbad, CA, USA), 2-mercaptoethanol from Sigma-Aldrich, glutamine (Gibco), pyrovate (Gibco), penicillin–streptomycin (Gibco), Hepes (Gibco), nonessential amino acids (MP Biochemicals, Ohio, USA) and 10% fetal calf serum from Biochrom AG (Berlin, Germany). Concanavalin A (ConA) was obtained from Sigma-Aldrich.

Plates for enzyme linked immunosorbent assay (ELISA) were from Nunc and HRP-conjugated secondary antibodies (rabbit anti-mouse IgG1 and IgG2b) were obtained from Zymed (San Francisco, CA, USA). TMB substrate was manufactured by Kem-En-Tec (Copenhagen, Denmark). Reagents for the IFN-γ ELISA (purified rat anti-mouse and biotin-labelled rat anti-mouse) were obtained from BD Pharmingen and the HRP-conjugated streptavidin was from Zymed.

Preparation of surfactant vesicles

Lipid hydration method

Multilamellar vesicles (MLV) were prepared using a technique based on the established lipid hydration method (Bangham et al 1965). Briefly, the chosen surfactants were dissolved in a 9:1 (v/v) solvent mixture of chloroform and methanol and the solvent was evaporated on a rotary evaporator to obtain a thin dry film. The film was flushed with nitrogen and hydrated with 2 mL of aqueous buffer or double-distilled water above the liquid-crystalline temperature (T_c) of the surfactants, and the suspension was allowed to stand for about 30 min above the T_c during which time

multilamellar vesicles (MLVs) were formed. Subsequently, Ag85B-ESAT-6, MSP1 or GLURP was added to these preformed MLVs at a fixed concentration of 0.01, 0.05 or 0.05 mg mL^{-1}, respectively.

Dehydration–rehydration method

The dehydration–rehydration procedure (Kirby & Gregoriadis 1984) was used for incorporation of antigen (Ag85B-ESAT-6, MSP1 and GLURP) into niosome-based vesicles. In brief, 2 mL MLVs, prepared as described above, were sonicated to produce small unilamellar vesicles (SUVs). These SUVs, composed of 16 μmol MP, 16 μmol cholesterol and 4 μmol DDA in the presence or absence of TDB (0.5 μmol), were mixed with 20 μg of Ag85B-ESAT-6 or 100 μg of MSP1 or GLURP, respectively, frozen at −70°C and freeze dried overnight. Controlled rehydration (Kirby & Gregoriadis 1984) of the dried powder led to the formation of antigen-containing multi-lamellar dehydration–rehydration vesicles (DRVs). DRV preparations were then ultra-centrifuged twice at 100 000 *g* for 20 min to remove non-entrapped or non-complexed antigen and resuspended in 0.01 M sodium phosphate buffer containing 0.15 M NaCl (pH 7.4; PBS) to the appropriate volume. Antigen adsorption or encapsulation in various vesicle preparations was determined based on ^{125}I radiation with, in the case of DRVs, ^{125}I radio-labelled Ag85B-ESAT-6, MSP1 or GLURP antigens being added to vesicles before freeze-drying. Adsorption/encapsulation of antigens was determined by measuring gamma radiation from the labelled antigen recovered in the resuspended pellets and the supernatants.

Labelling of Ag85B-ESAT-6 using Alexa Fluor 350 dye

Labelling of Ag85B-ESAT-6 was initially performed using Alexa Fluor 350 dye according to the manufacturer's protocol. Briefly, 1 mg of antigen (in a volume of ~ 500 μL of buffer) was added to the vial provided, which contained a pre-measured quantity of amine-reactive dye and a magnetic stir bar. Reaction was allowed to take place for 20 min. Separation of free dye from the labelled antigen was accomplished on a gravity-feed size-exclusion column, which was supplied with the kit. A sample of the eluted fluorescent band was collected and measured at absorbance and fluorescence emission maxima of 346 nm and 442 nm, respectively, and simultaneously measured for UV absorbance at 280 nm to confirm the presence of fluorescent labelled antigen and was stored at −20°C until required for further use.

^{125}I radiolabelling of antigens

Radiolabelling of the antigens was performed using Iodo-gen pre-coated iodination tubes (Pierce Biotechnology, Rockford, IL, USA). Briefly, antigen was diluted with Tris-buffer (25 mM, pH 8) and added to the pre-coated iodination tube. A pre-determined activity of ^{125}I (3.7 MBq) was then added to the iodination tube. This mixture was left for 15 min with intermittent shaking. Unlabelled antigen was removed using Sephadex G-75 gel column.

Antigen release and retention studies

The amount of subunit antigen (Ag85B-ESAT-6) released from the vesicles was determined by incubating antigen adsorbed/encapsulated vesicles in 15 mL 0.01 M Tris buffer or 0.01 M sodium phosphate buffer, pH 7.4 at 37°C in a shaking water bath. At regular time intervals, 1 mL of release medium was withdrawn and the same amount of fresh buffer was replaced. Withdrawn sample was ultra-centrifuged at 100 000 *g* for 1 h. Antigen release was then determined on the basis of radioactivity of ^{125}I-labelled antigen recovered in the re-suspended pellets. At regular time intervals, a similar procedure was adopted to determine antigen (Ag85B-ESAT-6) retention/encapsulation at 4°C and 25°C.

Determination of vesicle size and zeta-potential

The z-average vesicle diameter of the surfactant vesicles was measured by photon correlation spectroscopy on Zetaplus (Brookhaven Instruments) at 25°C by diluting 20 μL of the sample to an appropriate volume with doubly filtered (0.22 μm pore size) distilled water. The zeta-potential (an indirect measurement of the vesicle surface charge) was measured in 0.001 M sodium phosphate buffer, pH 7.4, at 25°C using Zetaplus (Brookhaven Instruments).

Differential scanning calorimetry (DSC)

Phase transitional behaviour of the formulations was studied using a differential scanning calorimeter (Perkin Elmer Diamond DSC). Briefly, 50 μL of vesicle dispersion was placed on the aluminium DSC pan and sealed. An empty sealed aluminium pan was used as a reference. The difference in the heat flow between both the pans was then measured as a function of temperature. Samples were scanned at a rate of 2.5°C min^{-1} from 10 to 60°C. Studies were conducted in triplicate.

Transmission electron microscopy (TEM)

Morphological analysis was carried out by TEM using JEOL 1200EX transmission electron microscope fitted with a LaB6 filament, with an operating voltage of 40–120 kV. A small drop of sample (10 μL) was placed on a polymer-filmed copper grid and allowed to stand for 2 min. Excess sample was removed using a filter paper, followed by addition of 10 μL of uranyl acetate. The grid was then allowed to stand for another 2 min, washed in distilled water and air dried, forming a thin film of stained vesicles, which was then viewed at an operating voltage of 70 kV.

Environmental scanning electron microscopy (ESEM)

Morphological characteristics and stability of MP-based non-ionic surfactant vesicle preparations were analysed using a Philips XL-30 field emission gun environmental scanning electron microscope. Briefly, 50 μL of the niosome formulation was loaded on the ESEM sample holder and examined under saturated water vapour conditions. Gradual reduction of pressure in the sample chamber resulted in controlled dehydration of the sample environment. A working temperature of 5°C was maintained in all experiments.

Immunisation of mice

Female C57BL/6j mice, 8–12 weeks old, were obtained from Harlan Scandinavia (Allerd, Denmark). The DDA-based vaccines were prepared by simply mixing preformed DDA liposome dispersions incorporating 0 or 11 mol% TDB, respectively, with the antigen of choice to a final Ag85B-ESAT-6, MSP1 and GLURP concentration of 0.01, 0.05 or 0.05 mg mL^{-1}, respectively (2, 10 or 10 μg, respectively, in each vaccine dose). The amount of DDA and TDB in each dose was 250 μg (0.4 μmol) and 0 or 50 μg (0 or 0.05 μmol), respectively. The MP-based vaccines were prepared by the dehydration–rehydration procedure as described above. Mice were immunised subcutaneously with the vaccines (0.2 mL/dose) at the base of the tails three times with a two-week interval between each immunisation. All animal testing and experiments were carried out subject to licensing from the Danish Animal Experiments Inspectorate.

Lymphocyte cultures

Splenocyte cultures from individual spleens of immunised mice (n = 3) were obtained 3 weeks after the last immunisation by passage of spleens through a metal mesh, followed by two washing procedures using RPMI. After recovery of the lymphocytes, cell cultures were established as previously described (Andersen et al 1991). Briefly, cultures were performed in triplicate in round-bottomed microtitre wells (Nunc, Denmark) containing 2×10^5 cells in a volume of 200 μL RPMI supplemented with 5×10^{-5} M 2-mercaptoethanol, 1% non-essential amino acids, 1% pyruvate, 1 mM glutamine, 1% penicillin–streptomycin, 1% Hepes and 10% fetal calf serum. Ag85B-ESAT-6, MSP1 and GLURP for re-stimulation were used in a concentration of 5 μg mL^{-1}. Wells containing medium only or 5 μg mL^{-1} of ConA were included in all experiments as negative and positive controls, respectively.

Culture supernatants were harvested from parallel cultures after 72 h of incubation in the presence of antigen, and the amounts of IFN-γ were determined by ELISA. In brief, microtitre plates were incubated with purified rat anti-mouse IFN-γ overnight, followed by blockage of the free-binding sites using 1% (w/v) bovine serum albumin (BSA) and 0.05% Tween in PBS. The culture supernatants were tested in triplicate and the IFN-γ detected by biotin-labelled rat anti-mouse IFN-γ followed by HRP-conjugated streptavidin.

Determination of antibody titres

Antibody titres were determined by ELISA as previously described (Rosenkrands et al 2005). Briefly, plates for enzyme linked immunosorbent assay (ELISA) were coated with Ag85B-ESAT-6, MSP1 or GLURP (0.05 μg/well) in PBS, and individual mouse sera (n = 3) were analysed in duplicate in five-fold dilutions. HRP-conjugated secondary antibodies (rabbit anti-mouse IgG1 and IgG2b) diluted 1/2000 in PBS containing 1% BSA were added, and Ag85B-ESAT-6, MSP1 and GLURP specific antibodies were detected by TMB substrate as described by the manufacturer. Antibody titres were then defined as the serum dilution that gave an absorbance value of 1.00 in the parallel portion of the curves, as previously described (Rosenkrands et al 2005).

Molecular modelling and statistical analysis

Molecular Modelling Pro software was used to calculate the operational critical packing parameter values of components in a system. Analysis of variance followed by Tukey's test was performed to compare the mean values of different groups. Kruskal–Wallis's non-parametric rank sum test followed by Dunn's post test was used for differences in humoral and cellular immune responses. Statistical significance was considered at $P < 0.05$ in all the studies.

Results and Discussion

Optimisation of vesicle composition

Surfactant vesicles were initially prepared by the lipid hydration method and were composed of DDA (1.25 mg mL^{-1}) with or without TDB (0.25 mg mL^{-1}). This vesicle composition was modified further by supplementing with several other non-ionic surfactants, including MP (C16:0), 1-monostearoyl glycerol (MS) or 1,2-dipalmitoyl glycerol (DP), all in combination with cholesterol (1:1 molar ratio, an optimum ratio for production of physically stable vesicles; Uchegbu & Florence 1995). These lipids and surfactants have been used in previous immunisation studies with success; TDB in DDA-based systems has been reported to improve the immune response to the tuberculosis vaccine fusion protein, Ag85B-ESAT-6, in mice and furthermore stabilise the formulation (Davidsen et al 2005). Similarly, MP-based niosomes have been shown to be effective adjuvants for BSA (Brewer & Alexander 1992).

Cationic vesicle formulations composed of MP or MS in the presence of cholesterol and DDA (molar ratio of

4:4:1) gave stable vesicles in aqueous buffer, pH 7.4, prepared by the dehydration–rehydration method. Interestingly, similarly formulated systems using the non-ionic surfactant DP in place of MP failed to form vesicles presumably due to either a chemical incompatibility between the surfactants used or as a result of critical packing parameter (CPP) of the system, which can be used to predict the vesicle-forming ability of surfactants (Israelachvili et al 1977). To support vesicle formation, a CPP value in the range 0.5–1.0 is required, whereas a CPP < 0.5 (indicating a large contribution from the hydrophilic head group area) is said to give spherical micelles and a CPP > 1.0 (indicating a large contribution from the hydrophobic group volume) would lead to precipitation. It is observed that the operational CPP value of all the components combined in DP-based formulation was 1.13, thus explaining the inability to form vesicles when using this combination of surfactants in the absence of other surfactants such as Solulan C-24, which can be used (Arunothayanun et al 2000). However, it is also evident that DDA played a key role in stabilizing the MP- and MS-based vesicles, as omission of DDA in the formulation caused precipitation. The vesicle size of both SUV and DRV systems was also significantly influenced by the choice of surfactant (results not shown), with MS-based systems being around 50% larger in measured diameter compared with their MP counterpart (100 nm vs 78 nm for SUV and 1432 nm vs 847 nm for DRV systems, respectively). This is presumably due to the longer alkyl chain length of monostearyl glycerol, which might be contributing to the expansion of the bilayer as the hydrophilic portion of the molecule is decreased relative to the hydrophobic portion (Israelachvili 1993; Uchegbu & Duncan 1997; Hao et al 2002).

Characterisation of formulations

Based on these initial characterisation studies and on previous reports of MP-based niosomes inducing high adjuvant activity compared with their MS-based counterpart (Brewer & Alexander 1992), MP-based systems were further investigated and compared with cationic liposome systems previously demonstrated to be effective adjuvants (Lindblad et al 1997; Holten-Andersen et al 2004; Davidsen et al 2005). Therefore, formulations composed of DDA only or DDA–TDB (4:0.5 μmol) with adsorbed antigen were compared with DRV systems composed of MP–Chol–DDA (16:16:4 μmol) and with or without inclusion of TDB (0.5 μmol) encapsulating three different antigens (i.e., Ag85B-ESAT-6, MSP1 and GLURP).

The physico-chemical characteristics of different preparations in the presence or absence of antigens were studied. Results shown in Table 1 demonstrate that the sizes of empty DDA vesicles were significantly smaller ($P < 0.05$) than their MP-based counterparts, with DDA liposomes being almost half the size of those composed of MP–Chol–DDA (421 nm vs 847 nm, respectively). However, no significant difference in vesicle size was observed when TDB was incorporated in DDA liposomes and MP–Chol–DDA systems. Zeta-potential analysis of these formulations showed that the supplementation of DDA vesicles with MP and cholesterol or TDB made no significant difference, with all vesicles having a net positive charge (50–60mV, Table 1), clearly resulting from the cationic nature of DDA, and suggesting that no marked

Table 1 Vesicle size, zeta-potential and antigen loading of vesicles with three different antigens (Ag85B-ESAT-6, MSP1 and GLURP)

Formulations	Vesicle size (nm)	Zeta potential (mV)	Antigen loading (% of amount used)
DDA	421.2 ± 26.1	52.4 ± 4.1	NA
DDA–TDB	416.3 ± 40.4	54.7 ± 4.7	NA
MP–Chol–DDA	847.1 ± 43.8	56.4 ± 1.6	NA
MP–Chol–DDA–TDB	877.0 ± 12.6	55.3 ± 2.2	NA
DDA–Ag85B-ESAT-6	488.1 ± 123.6	46.2 ± 4.6	69.9 ± 10.2
DDA–TDB–Ag85B-ESAT-6	485.8 ± 110.0	59.7 ± 4.7	67.0 ± 2.8
MP–Chol–DDA (Ag85B-ESAT-6)	1346.8 ± 140.0	57.1 ± 1.8	87.8 ± 0.5
MP–Chol–DDA–TDB (Ag85B-ESAT-6)	1031.6 ± 132.1	57.9 ± 3.4	89.3 ± 0.4
MP–Chol–DDA (MSP1)	958.6 ± 86.6	53.2 ± 1.2	60.4 ± 2.2
MP–Chol–DDA–TDB (MSP1)	1038.8 ± 89.2	52.7 ± 2.4	57.9 ± 0.8
MP–Chol–DDA (GLURP)	2547.9 ± 363.0	46.0 ± 2.9	58.5 ± 2.8
MP–Chol–DDA–TDB (GLURP)	2797.8 ± 530.6	45.7 ± 1.1	60.0 ± 3.7

Vesicles were formulated from dimethyldioctadecylammonium bromide (DDA) alone or in combination with 1-monopalmitoyl glycerol (C16:0) (MP) and cholesterol (Chol) (MP–Chol–DDA) and/or α,α'-trehalose 6,6′-dibehenate (DDA–TDB, 4:0.5 μmol; MP–Chol–DDA–TDB, 16:16:4:0.5 μmol). Antigens were either adsorbed (e.g. DDA–Ag85B-ESAT6) or entrapped via the DRV method (e.g. MP–Chol–DDA (Ag85B-ESAT-6)). Results represent mean ± s.d., n = 3. NA, not applicable.

electrostatic interactions between the DDA and the additional surfactants occurred at the surface of the formed vesicles.

Adsorption of Ag85B-ESAT-6 onto the surfaces of DDA and DDA–TDB liposomes resulted in no significant effect on vesicle size and zeta-potential systems in line with previously reported results (Davidsen et al 2005). In contrast, encapsulation of Ag85B-ESAT-6 in MP–Chol–DDA and MP–Chol–DDA–TDB DRV systems resulted in an increased vesicle size (Table 1) with no significant change in zeta-potential (Table 1), suggesting that the antigen may influence the formation of the DRV. A similar trend was observed with both MP–Chol–DDA and MP–Chol–DDA–TDB systems when the malaria antigen MSP1 was encapsulated (Table 1). In contrast, when another malaria antigen, GLURP, was encapsulated in these MP DRVs both in the presence and absence of TDB, the vesicle diameters were significantly increased to over 2.5 microns. This may be due to the high molecular weight of GLURP (~55 kDa), in addition to its high hydrophobic nature, causing it to associate with the hydrophobic regions of the vesicle bilayers and possibly encouraging a degree of vesicle fusion or influencing the packing arrangements of the surfactants. This interaction of the GLURP antigen with the vesicular bilayers is further reinforced by a small decrease in zeta-potential (Table 1).

Adsorption of Ag85B-ESAT-6 antigen to cationic DDA vesicles with or without TDB was almost 70% of initial amount used (0.01 mg mL^{-1}). Entrapment efficiency of MP-based systems was ~90% for Ag85B-ESAT-6 and about 60% for MSP1 and GLURP (Table 1). Similar to antigen adsorption, the presence of TDB did not affect the entrapment efficiency in any of the formulations compared with their counterparts in the absence of TDB with each of the antigens (Table 1).

The spatial location of the antigen (Ag85B-ESAT-6) on the MP-based vesicles was also investigated. After incubation of free fluorescently labelled antigen (Alexa Fluor 350) with empty MP-based preparations, only approximately 10% of the antigen was adsorbed onto the vesicle surface (results not shown), significantly lower than that seen after dehydration–rehydration (~90%; Table 1), suggesting that the majority of the antigen associated with the MP formulations is encapsulated within the vesicles.

Differential scanning calorimetry

DDA lipid bilayers undergo a main phase transition at a characteristic temperature (T_c) at which the lipid chains transfer from a lower temperature gel-phase dominated by ordered alkyl chain conformations to a high-temperature fluid-phase characterised by disordered alkyl chain conformations (Bloom et al 1991). The phase transition temperature (T_c) is detected as a peak in the heat capacity curve obtained by DSC.

The DSC scan (data not shown) of the MP molecule was characterised with a single well-defined endothermic peak possessing a T_c of 27.6 ± 1.2°C in agreement with a previous report (Gehlert et al 1995). DDA liposomes in aqueous buffer showed an endothermic peak with a T_c of 48.4 ± 0.6°C, characteristic of a highly cooperative gel–fluid phase transition in agreement with DSC results for DDA liposomes reported in the literature (Feitosa et al 2000). In addition, it has been reported previously that a shift in T_c to ~42.4°C was noted (Davidsen et al 2005) when TDB was introduced into DDA preparations. Here, introduction of MP and cholesterol into the DDA system further lowered the T_c of the system to 35.9 ± 0.9°C, presumably as a result of the addition of the combination of MP (with its much lower T_c, 26.4°C) and cholesterol, which is known to abolish the overall T_c of the systems (Horiuchi & Tajima 2000). Further, with incorporation of TDB in the MP DRVs, the endothermic peak slightly shifted increasing the T_c to 38.6 ± 0.6°C. This reduction in the T_c of DDA by ~12°C when MP and cholesterol were added enables these formulations to be made at lower temperatures compared with DDA and DDA–TDB liposomes, thus reducing the risk of antigen denaturation when higher temperatures are employed.

Stability of vesicles

Vesicle stability, in terms of vesicle size and zeta-potential, was studied at two different storage temperatures (4°C and 25°C). DDA vesicles in the presence or absence of TDB were initially about 500 nm (Figure 1A, C). At 4°C a steady rise in the vesicle size was observed for DDA liposomes, which led to eventual precipitation by day 28. Indeed, after only 7 days storage the measured vesicle diameter had almost doubled to 900 nm (Figure 1A). In line with previous studies (Davidsen et al 2005), the addition of TDB to DDA liposomes was shown to stabilise the liposome formulation, with these systems showing no significant increase in vesicle size during 28 days of storage at 4°C.

As already noted (Table 1), the addition of MP and cholesterol to DDA formulations resulted in the formation of vesicles of significantly larger diameter ($P < 0.05$) than their DDA counterparts (~1300 nm; Figure 1A). After 28 days storage, the mean vesicle size of these MP-based vesicles moderately increased to approximately 1900 nm (Figure 1A). Supplementation of these MP DRVs with TDB added further stability to the system with no significant change in vesicle size being recorded over the 28-day investigation (Figure 1A). Under these conditions the zeta-potential of the DDA and DDA–TDB remained constant (40–50 mV) with no significant changes measured over 28 days and both MP-based formulations displayed an initial small decrease in zeta-potential after 7 days, but no significant changes thereafter (Figure 1B).

At 25°C DDA liposomes (without the addition of TDB) displayed a steady increase in vesicle size with

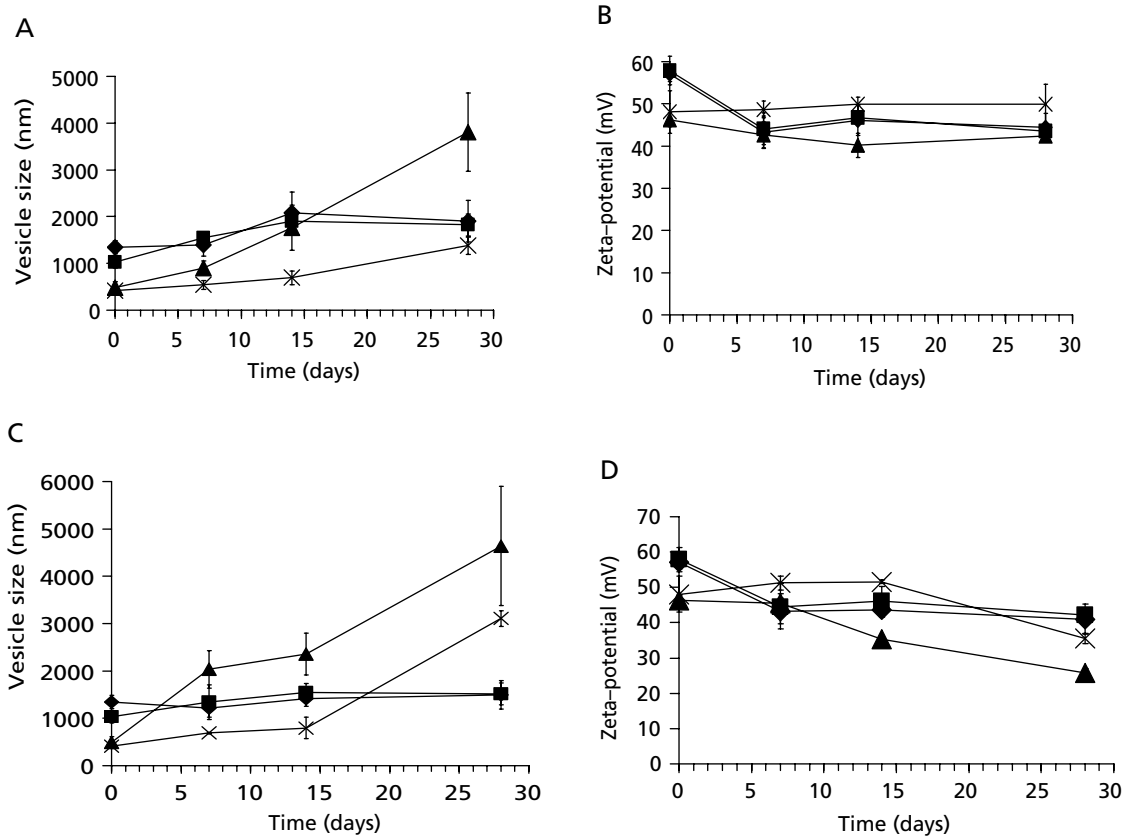

Figure 1 Vesicle size (A, C) and zeta-potential (B, D) of DDA (▲), DDA–TDB (×), MP–Chol–DDA (♦) and MP–Chol–DDA–TDB (■) preparations stored at 4°C (A, B) and 25°C (C, D). Results represent mean ± s.d., n = 3.

eventual precipitation noted by day 28, while those containing TDB maintained a submicron size until day 14 but increased to about 2900 nm by day 28 (Figure 1C). This increased vesicle size of DDA and DDA–TDB systems was in conjunction with a significant ($P < 0.05$) decrease in their zeta-potential (Figure 1D). In contrast, at 25°C neither of the MP-based formulations (with and without addition of TDB) showed significant change in vesicle size throughout the 28-day period and only an initial drop (day 7) in the zeta-potential was observed, similar to the MP-based vesicles stored at 4°C.

Taken together, the vesicle sizes of the MP DRVs (Figure 1A, C) in relation to their phase transition temperatures indicate that on storage above or nearer to their phase transition temperature, the vesicles remain relatively more stable presumably due to the surfactants existing in a liquid crystalline state (Arunothayanun et al 2000). However, storage at temperatures far below the T_c (i.e. at 4°C) led to an increase in vesicle size, presumably due to less thermal energy present around the vesicles. This is in accordance with thermodynamic theory; smaller vesicles require a higher input of energy to overcome the phenomenon of inherent instability of the system that might otherwise cause vesicles in the vicinity to fuse or aggregate (Uchegbu & Vyas 1998). A strong correlation between the T_c of the preparation and storage temperature also seems to exist, considering a 6°C fall in T_c from 48°C to 42°C when TDB was incorporated into the DDA system (Davidsen et al 2005) could also possibly be a contributing factor in the stabilisation phenomenon of TDB. For a future vaccine based on DDA liposomes, storage will most likely take place at 4°C, and both the DDA-based liposomes and MP-based formulations were stable at this temperature.

Antigen retention studies

After initial removal of non-adsorbed Ag85B-ESAT-6 via centrifugation, antigen retention to the liposomes after storage at both 4°C and 25°C was measured (Figure 2). After 7 days storage at 4°C (Figure 2A), antigen retention in both DDA and DDA–TDB formulations dropped to approximately 80% of the initial antigen adsorption. At time points thereafter, retention values for DDA–TDB preparations remained constant at 80% with no further losses of antigen being measured up to 28 days (Figure 2A). In contrast, further loss of antigen was associated with the DDA formulation with retention values being significantly ($P < 0.05$)

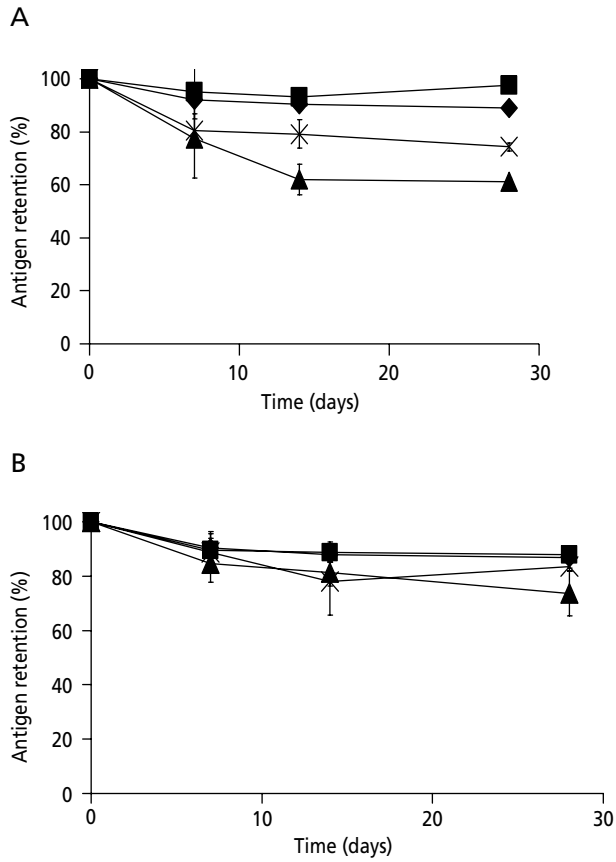

Figure 2 Antigen retention of DDA (▲), DDA–TDB (×), MP–Chol–DDA (♦) and MP–Chol–DDA–TDB (■) preparations in aqueous buffer, pH 7.4, stored at 4°C (A) and 25°C (B). ^{125}I-labelled Ag85B-ESAT-6 was adsorbed/encapsulated and antigen retention of Ag85B-ESAT-6 was determined on the basis of ^{125}I radioactivity recovered in the suspended pellets after ultracentrifugation. Results represent percentage retention of initially loaded antigen (Table 1), expressed as mean ± s.d., n = 3.

lower by day 14 (approximately 65% of initial adsorption values) compared with DDA–TDB formulations (Figure 2A). Storage of the formulations at 25°C reduced antigen loss from the DDA liposomes, with no significant differences in antigen retention between DDA and DDA–TDB formulations (Figure 2B). Both MP DRVs displayed a general enhanced stability, retaining about 90% of the encapsulated antigen over the study period.

Antigen release studies

Antigen release from the four different preparations was studied based on the activity of released ^{125}I-labelled Ag85B-ESAT-6 in physiological buffer, pH 7.4, as release medium at 37°C. DDA liposomes with and without added TDB showed an initial burst release in the first 2 h of up to 35% (of the total amount) followed by a steady state of ~ 40% being reached after 24 h (Figure 3).

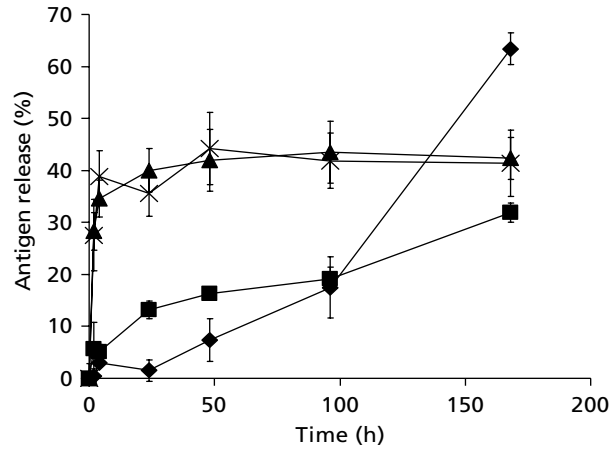

Figure 3 Antigen release under physiological conditions from DDA (▲), DDA–TDB (×), MP–Chol–DDA (♦) and MP–Chol–DDA–TDB (■) in aqueous buffer, pH 7.4, at 37 °C. Ag85B-ESAT-6 release was determined on the basis of radioactivity of ^{125}I-labelled Ag85B-ESAT-6 recovered in the suspended pellets after ultracentrifugation. Results represent percentage release of initially loaded antigen expressed as mean ± s.d., n = 3.

Both MP formulations displayed slower release characteristics: MP–Chol–DDA–TDB produced sustained release of antigen of approximately 30% (of the total amount) after 168 h, while MP–Chol–DDA initially had a significantly lower release rate (3% vs 12% after 24 h, respectively; $P < 0.05$) but rose to 65% after 168 h, the highest release shown among the four formulations (Figure 3).

Transmission electron microscopy (TEM)

The morphological characteristics of the vesicle preparations were investigated using TEM. In agreement with the particle size analysis (Table 1), DDA vesicles were smaller (~400 nm), with slightly angular structures and notable signs of vesicle aggregation (Figure 4A). Interestingly, incorporating TDB within the vesicle membranes produced more spherical and less aggregated vesicles (Figure 4B). Addition of antigen to both these vesicle formulations did not have a significant impact on their vesicle morphology (not shown). MP DRV systems were multilamellar in nature (Figure 4C) while the lamellarity of the DDA-based vesicles was not conspicuous (Figure 4A) using TEM, but these DDA vesicles have been shown to be unilamellar in nature using cryo-TEM (Davidsen et al 2005). Similar to the DDA liposomes (Figure 4A), MP–Chol–DDA vesicles showed partial aggregation of the vesicles (Figure 4D).

Environmental scanning electron microscopy (ESEM)

MP–Chol–DDA–TDB (16:16:4:0.5 μmol) vesicles were further assessed using ESEM. Micrographs (Figure 5)

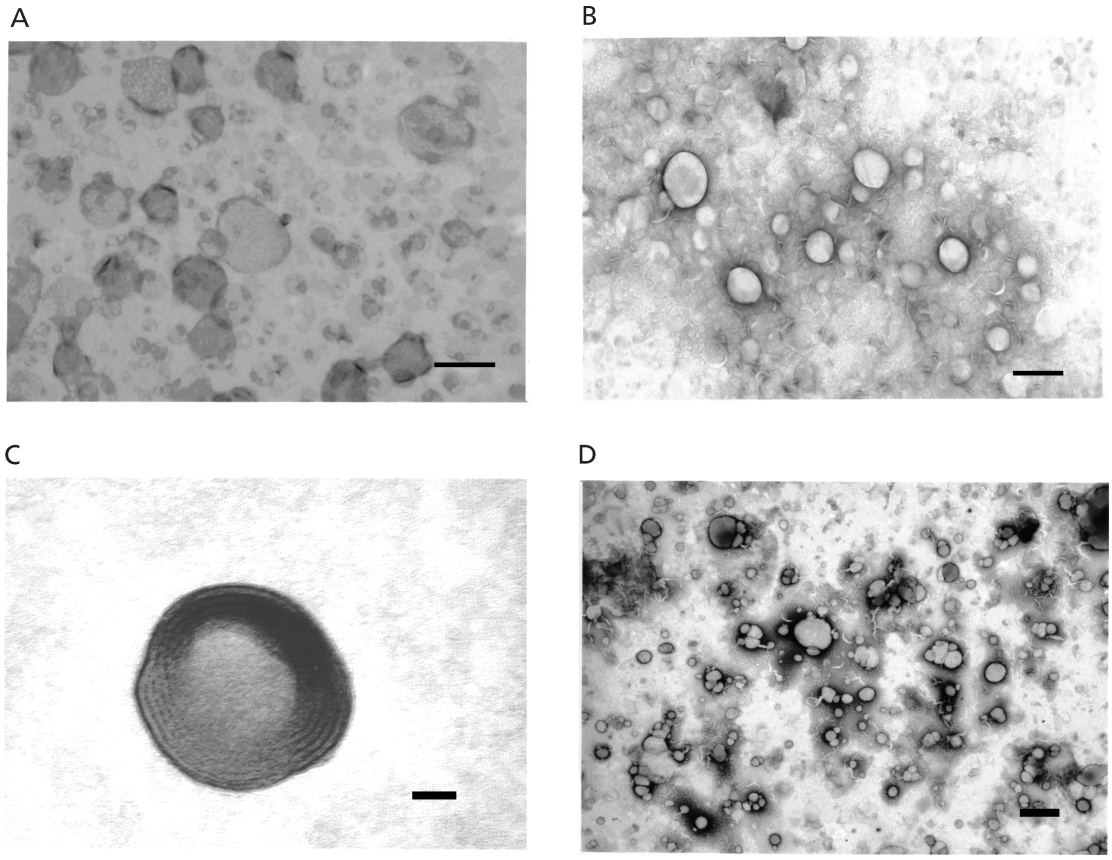

Figure 4 TEM micrographs of DDA- and MP-based preparations. A. DDA vesicles (preparation in the absence of TDB) revealed slightly angular structures, bar = 400 nm. B. DDA–TDB; the presence of TDB within the formulation made these structures more round in shape, bar = 300 nm. C. A single MP–Chol–DDA vesicle revealing multi-lamellar nature of the bilayers, bar = 200 nm. D. MP–Chol–DDA vesicles showed partial aggregation, bar = 1 μm.

revealed giant spherical vesicles of sizes between 10 and 30 μm compared with significantly smaller particle size measurements (850 nm), which may be a reflection of the overall population.

A comparative study was performed between the empty vesicles (Figure 5A, C, E) and antigen-loaded vesicles (Figure 5B, D, F). At a pressure of about 3.0 Torr, all the vesicles looked spherical and round in shape (Figure 5A, B). Vesicles loaded with antigen were observed to retain their structural and bilayer integrity more efficiently than the empty vesicles, as observed with reduction in sample chamber pressure. Gradual reduction of the pressure to 2.4 Torr resulted in the coalescence of empty vesicles (Figure 5C), with a few vesicles losing their spherical shape and flattening (indicated by arrows) to form lipid masses as the buffer medium evaporated, while antigen-incorporated vesicles resisted the destabilisation caused at a similar pressure (Figure 5D). Further reduction in operating pressure to 1.9 Torr resulted in both empty and antigen-incorporated MP–Chol–DDA–TDB vesicles coalescing completely as evaporation of the surrounding aqueous medium continued (Figure 5E, F). Salt crystallisation from the medium was visible amid the lipid–surfactant masses.

This ESEM analysis demonstrates that antigen incorporation could improve the stability of the MP DRVs, as revealed by enhanced resistance to coalescence during dehydration. Increased stability of antigen-incorporated vesicles may be due to the macromolecular antigen getting embedded in the spacing between adjacent lipid–surfactant chains of the vesicle bilayers and thereby increasing the rigidity of the vesicle bilayer; indeed, such type of increased stability was reported previously with entrapment of hydrophobic drugs (Uchegbu et al 1996) or macromolecular prodrugs (Gianasi et al 1997) in non-ionic surfactant vesicles and in liposome systems (Mohammed et al 2004).

Immunisation studies

The adjuvant effect of both MP- and DDA-based vesicle preparations was investigated by immunising C57Bl/6j mice with the Ag85B-ESAT-6 fusion protein. The

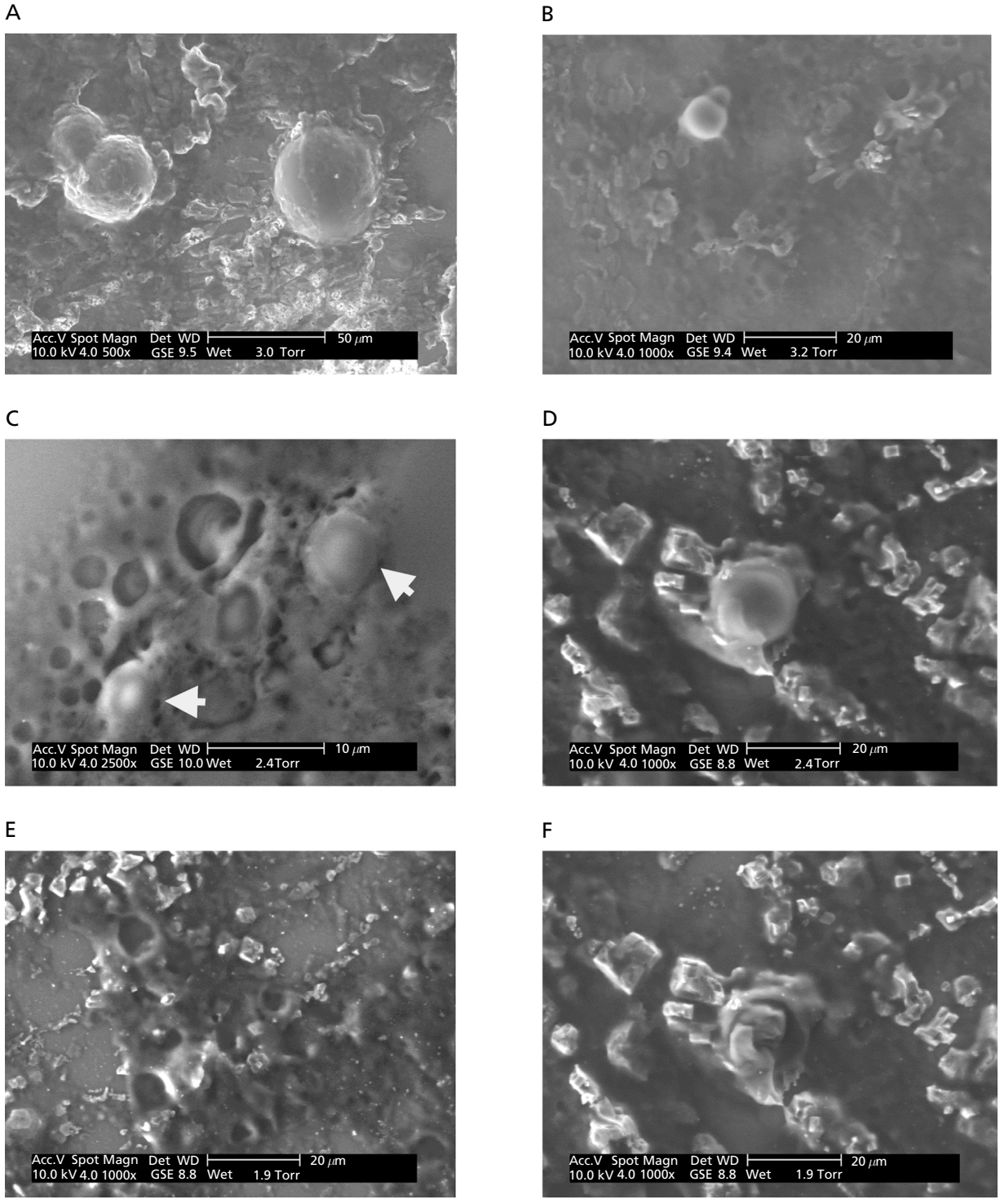

Figure 5 ESEM micrographs of empty (A, C, E) and Ag85B-ESAT-6 loaded (B, D, F) MP-based vesicles (MP–Chol–DDA–TDB; 16:16:4:0.5 μmol) suspended in aqueous buffer (pH. 7.4). Vesicles appear spherical and stable at an operating pressure of 3.0 Torr (A, bar = 50 μm) and 3.2 Torr (B, bar = 20 μm). Reduction of the ESEM operating pressure to 2.4 Torr resulted in the vesicles coalescing or collapsing with a couple of flattened vesicles observed (indicated by arrows) as the medium evaporates (C, bar = 10 μm), while at similar operating pressure vesicles in antigen-incorporated system retained their spherical nature (D, bar = 20 μm). Further reduction of operating pressure to 1.9 Torr resulted in vesicles completely collapsing with visible lipid/surfactant masses and salt crystallisation (E, F, bar = 20 μm).

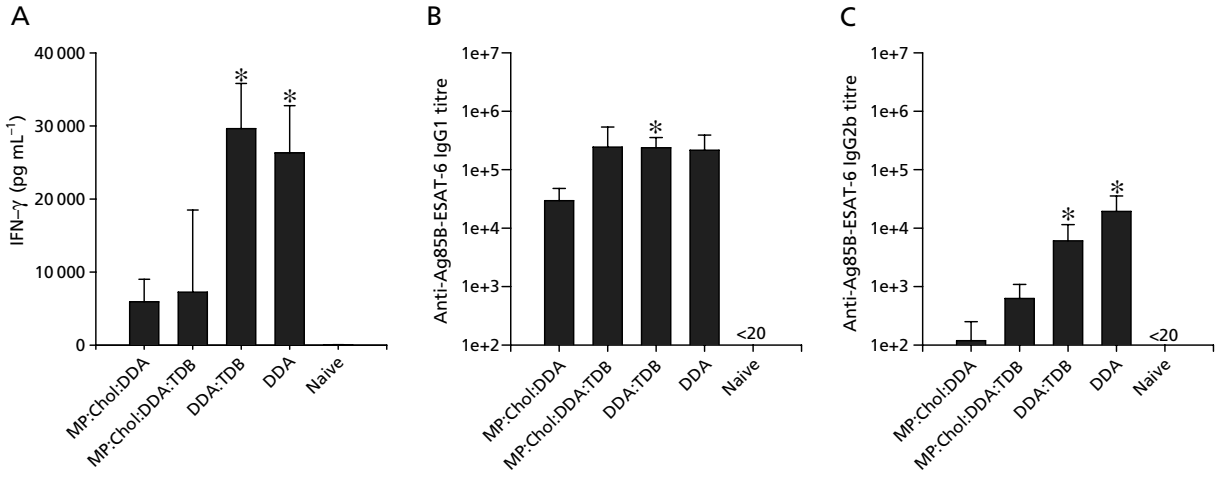

Figure 6 Immune responses against the *Mycobacterium tuberculosis* Ag85B-ESAT-6 fusion protein generated by MP- and DDA-based vesicle preparations. Release of IFN-γ was measured from spleen lymphocytes isolated from C57Bl/6j mice (n = 3) immunised three times with 2 μg of Ag85B-ESAT-6 encapsulated in MP–Chol–DDA and MP–Chol–DDA–TDB vesicles or adsorbed to DDA and DDA–TDB liposomes (A). Splenocytes were isolated 7 weeks after the first immunisation and re-stimulated in-vitro with the Ag85B-ESAT-6 (5 μg mL^{-1}). From the same mice, serum was also taken at this time point for determination of IgG1 (B) and IgG2b (C) Ag85B-ESAT-6-specific antibody titres. Asterisks denote values significantly higher than the naive controls.

specific immune response of the spleen cells was investigated by restimulation with Ag85B-ESAT-6 in-vitro (Figure 6A) and subsequently measuring IFN-γ as an indicator of a Th1 response. All formulations induced release of IFN-γ, with the DDA and DDA–TDB liposomes giving rise to the highest responses. The ability of the various vesicle preparations to induce antibody responses after immunisation was also investigated by measuring the antigen-specific titres of the IgG1 and IgG2b isotypes by ELISA. The IgG1 titres (Figure 6B) determined were at the same level for MP–Chol–DDA–TDB, DDA–TDB and DDA, whereas the highest IgG2b antibody titres (Figure 6C) were observed for the DDA-based liposomes.

In contrast to tuberculosis, for which a cell-mediated Th1 immune response is essential, protection against malaria caused by *Plasmodium falciparum* is primarily mediated by induction of antibodies (Cohen et al 1961). The adjuvant effect of MP- and DDA-based preparations was therefore investigated by immunising C57Bl/6j mice with the two malaria antigens MSP1 and GLURP. After immunisation with MSP1, high IgG1 titres were observed with all vesicle formulations (Figure 7A), whereas lower IgG1 titres were observed for GLURP (Figure 7C). The measured IgG2b titres induced after immunisation with MSP1 were highest for the two MP-based vesicle systems (Figure 7B), whereas comparable GLURP-specific IgG2b titres were observed with all four vesicle systems (Figure 7D).

Conclusions

Comparison of physico-chemical characteristics (zeta-potential and vesicle size) of liposome and MP-based systems suggests that despite the similar cationic surfactant content of all the four antigen free preparations, MP-based niosomes have measured z-average diameters approximately twice those of their DDA-based liposome counterparts. This trend is mainly attributed to the hydrophilicity of the lead surfactant. The relatively hydrophilic MP (HLB 7.2), with its single alkyl chain, gives rise to higher vesicle sizes compared with the DDA molecule, which possesses two 18 carbon alkyl chains and is relatively more hydrophobic in nature (HLB, 3.6).

In addition to these differences in physico-chemical characteristics, DDA-based systems were shown to effectively enhance cell-mediated Th1 immune responses against Ag85B-ESAT-6, with high IFN-γ levels compared with their MP-based counterparts, while induction of antibody responses, especially IgG1 titres, were comparable between both the systems. A strong Th2 humoral response in terms of IgG1 titres was obtained for malarial antigens (i.e., MSP1 and GLURP), which were comparable between the preparations, while MP-based vesicles showed high IgG2b titres. This can be attributed to the ability of vesicles to not only protect their protein content from protease attack in-vivo but also deliver it to antigen-presenting cells infiltrating the site of injection or in the lymphatics. Our results suggest that both DDA- and MP-based systems may be useful in enhancing the immunogenicity of the subunit vaccines, especially the former with the subunit antigen Ag85B-ESAT-6 against tuberculosis for which a high cell-mediated Th1 immune response is essential. The latter is of potential use with malarial antigens such as MSP1 and GLURP where induction of antibody titres is vital in combating the invading parasites.

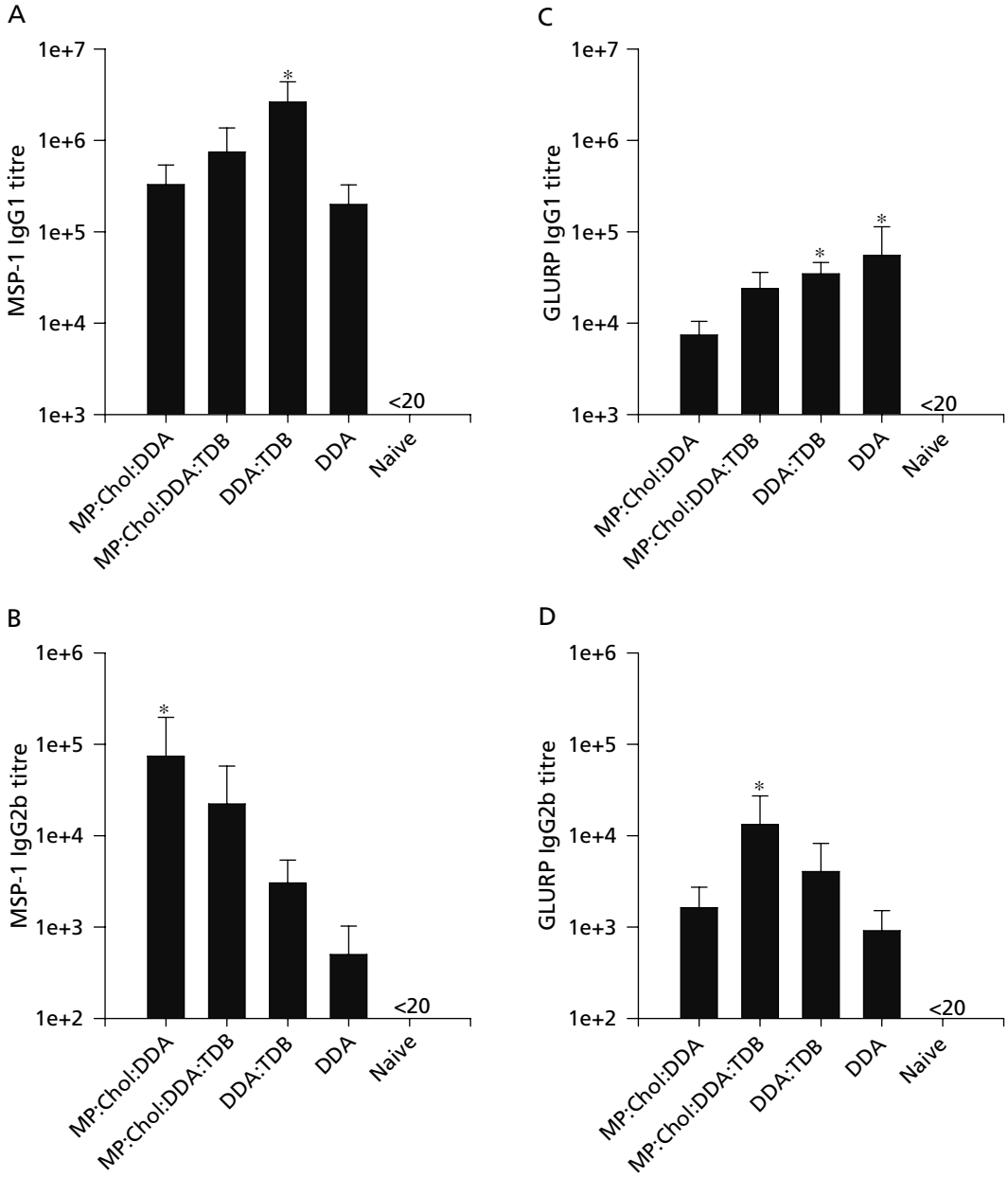

Figure 7 Antibody responses against the malaria proteins MSP1 and GLURP generated by MP- and DDA-based vesicle preparations. C57Bl/6j mice (n = 3) were immunised three times with 10 μg of MSP1 and GLURP, respectively, encapsulated in MP–Chol–DDA and MP–Chol–DDA–TDB vesicles or adsorbed to DDA and DDA–TDB liposomes. Serum was drawn 7 weeks after the first immunisation for determination of IgG1 (A, C) and IgG2b (B, D) MSP1- (A, B) and GLURP- (C, D) specific antibody titres. Asterisks denote values significantly higher than the naive controls.

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