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Biochimica et Biophysica Acta 1718 (2005) 22 – 31
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
http://www.elsevier.com/locate/bba

Characterization of cationic liposomes based on dimethyldioctadecylammonium and synthetic cord factor from *M. tuberculosis* (trehalose 6,6′-dibehenate)—A novel adjuvant inducing both strong CMI and antibody responses

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Received 3 May 2005; received in revised form 18 September 2005; accepted 25 October 2005
Available online 14 November 2005

Abstract

Incorporation of the glycolipid trehalose 6,6′-dibehenate (TDB) into cationic liposomes composed of the quaternary ammonium compound dimethyldioctadecylammonium (DDA) produce an adjuvant system which induces a powerful cell-mediated immune response and a strong antibody response, desirable for a high number of disease targets. We have used differential scanning calorimetry (DSC) to investigate the effect of TDB on the gel-fluid phase transition of DDA liposomes and to demonstrate that TDB is incorporated into DDA liposome bilayers. Transmission Electron Microscopy (TEM) and cryo-TEM confirmed that liposomes were formed when a lipid film of DDA containing small amounts of TDB was hydrated in an aqueous buffer solution at physiological pH. Furthermore, time development of particle size and zeta potential of DDA liposomes incorporating TDB during storage at 4 °C and 25 °C, indicates that TDB effectively stabilizes the DDA liposomes. Immunization of mice with the mycobacterial fusion protein Ag85B–ESAT-6 in DDA–TDB liposomes induced a strong, specific Th1 type immune response characterized by substantial production of the interferon-γ cytokine and high levels of IgG2b isotype antibodies. The lymphocyte subset releasing the interferon-γ was identified as CD4 T cells.

Keywords: Adjuvant; Immunomodulator; Liposome; Trehalose 6,6′-dibehenate; Dimethyldioctadecylammonium

1. Introduction

Subunit vaccines with improved safety profiles based on well-defined recombinant proteins or peptides are poorly immunogenic when administered alone. Adjuvants are therefore needed in the vaccine formulation to induce an adequate immune response. Selecting the appropriate adjuvant to specific antigenic components will not only enhance the level of immune response but will also determine the type of immune response induced [1]. In contrast to the substantial progress made in techniques to identify and produce antigens, the most widely used adjuvants are still aluminum based compounds (generically called alum); although a squalene based oil emulsion (MF59) and virosomes for use in influenza vaccines are approved for human use in Europe [1]. Alum generally promotes weak humoral Th2 type immune responses to protein subunits, and elicits insufficient cell-mediated Th1 immune responses [1,2]. In particular, the failure of alum to stimulate a cell mediated Th1 immune response, required for protective immunity against many cancers and infectious diseases has led to the search for more potent vaccine adjuvants that not only enhance the level of immune activation but also selectively induce cell-mediated immune responses.

The quaternary ammonium compound, dimethyldioctadecylammonium (DDA), has previously been reported as an effective adjuvant for eliciting cell-mediated and humoral responses. DDA was discovered as an adjuvant by Gall in

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0005-2736/$ - see front matter © 2005 Published by Elsevier B.V.
doi:10.1016/j.bbamem.2005.10.011

the mid 1960s [3] and has been tested in combination with a number of different viral and bacterial antigens in different animal species [4–9]. DDA is a synthetic amphiphilic lipid compound comprising a hydrophilic positively charged dimethylammonium head–group attached to two hydrophobic 18-carbon alkyl chains (tail). In an aqueous environment, DDA self-assemble into closed vesicular bilayers similar to liposomes made from natural phospholipids [10–13]. In general, vesicle size, polydispersity, thermotropic phase behavior, surface potential and physical stability of the DDA liposomes depend on the concentration of the amphiphile, preparation method and solvent conditions, e.g., buffer substance, pH and salt concentration [11,14–16]. Unfortunately, DDA liposomes are physically unstable. Small amounts of salt result in instantaneous aggregation, possibly due to reduced long range electrostatic repulsion between the positively charged DDA liposomes. Salt-induced aggregation is to some extent reversible as removing the salts restores the liposome dispersion [11,16–18]. However, even in pure water DDA vesicles aggregate over time, indicating that electrostatic repulsion forces between the cationic liposomes are insufficient to stabilize the vesicles [19].

In this paper, we demonstrate a unique way to increase both stability and adjuvant efficacy of DDA liposomes by incorporating trehalose 6,6′-dibehenate (TDB), a glycolipid comprising a 6,6′-diester of α,α′-trehalose with two long 22-carbon acyl chains (behenic acids), into the DDA liposome bilayers. TDB is a synthetic analogue of trehalose 6,6′-dimycolate (TDM) often referred to as cord factor, which is an immunostimulatory component of the mycobacterial cell wall [20,21]. However, compared to TDM, the TDB has shorter fatty acid chains which have been associated with lower toxicity [20,22]. As a model antigen, we selected the Ag85B–ESAT-6 fusion protein [8] which is a promising tuberculosis vaccine antigen. Administration of Ag85B–ESAT-6 in the DDA–TDB liposomes induces a very substantial CMI as well as humoral response with a strong Th1 bias, as demonstrated by the high titers of IgG2b and high levels of IFN-γ release from the T cells primed by the vaccine.

2. Materials and methods

2.1. Materials

Dimethyldioctadecylammonium (DDA) bromide and α,α′-trehalose 6,6′-dibehenate (TDB) were obtained from Avanti Polar Lipids (Alabaster, AL). The purity of the compounds was >99% by HPLC. 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 base (99%) and Sephadex G-75 were obtained from Sigma-Aldrich (St. Louis, MO). Purified water of Milli Q quality was used to prepare all samples. Non-his-tagged protein Ag85B–ESAT-6 was produced in Escherichia Coli as described previously for the His-tagged version [8], purified by column chromatography and dissolved in 10 mM Tris-buffer, pH 7.4, at a concentration of 0.5 mg/ml. Alhydrogel (Alum) was purchased at Brenntag Biosector (Frederikssund, Denmark). For radiolabelling of the antigen, Iodo-gen® pre-coated iodination tubes from Pierce Biotechnology (Rockford, IL) were used and ^{125}I (NaI in NaOH solution) was purchased from Amersham Biosciences (Amersham, UK).

Round-bottomed 96-well microtiter plates were obtained from Nunc (Roskilde, Denmark). RPMI 1640 medium was obtained from Gibco (Invitrogen, Carlsbad, CA), 2-mercaptoethanol from Sigma-Aldrich, glutamine (Gibco), penicillin–streptomycin (Gibco) HEPES (Gibco), and 10% fetal calf serum from Biochrom AG (Berlin, Germany). 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, was obtained from Zymed (San Francisco, CA). TMB substrate was manufactured by Kem-En-Tec (Copenhagen, Denmark).

2.2. Preparation of adjuvant liposomes

DDA liposomes in the presence or absence of TDB were prepared by two different methods:

2.2.1. Lipid film hydration method

Unilamellar DDA liposomes containing increasing concentrations of TDB were prepared by the film method. Weighed amounts of DDA and TDB were dissolved in chloroform/methanol (9:1, by volume) and the organic solvent was removed using a gentle stream of N_2 forming a thin lipid film at the bottom of the test tube. The lipid film was dried over night under low pressure to remove trace amounts of the organic solvent. Unilamellar vesicles were formed by simply hydrating the lipid film in 10 mM Tris-buffer at pH 7.4 [15,23,24]. The lipid film was hydrated for 20 min at a temperature 10 °C above the main phase transition of DDA (T_m ≈47 °C) [15,25] to ensure complete hydration.

The final DDA concentration was fixed at 1.25 mg/ml (2.0 mM) whereas the amount of TDB varied between 0 and 0.5 mg/ml (0 and 0.5 mM). Four different formulations were prepared containing the following amounts of TDB: 0, 0.125, 0.25, and 0.5 mg/ml (0, 0.125, 0.25, and 0.5 mM). The mole fraction of TDB corresponds to 0, 6.4, 11, and 20 mol%. DDA and TDB concentrations were determined by HPLC using a 5 μm diol column, a mobile phase composed of MeOH/$CHCl_3$/H_2O (75:20:5, v/v/v), and an evaporative light scattering detector. Ag85B–ESAT-6 was added to the preformed liposomes at a fixed concentration of 0.01 mg/ml.

2.2.2. Aqueous heat method

For comparison DDA adjuvant liposomes were also prepared as described previously [6]. Briefly, DDA was mixed into 10 mM Tris-buffer at pH 7.4 to a concentration of 1.25 mg/ml, heated to 80 °C for 20 min with intermittent shaking, and then cooled to room temperature. Where appropriate, 0.25 mg/ml of TDB (250 μl of a 1 mg/ml solution in 0.2% triethylamine) was then added, and the resulting solution was vortexed briefly. The Ag85B–ESAT-6 was finally added at a concentration of 0.01 mg/ml.

2.3. Dynamic light scattering: determination of liposome size and zeta potential

The z-average diameter of DDA liposomes containing increasing concentrations of TDB was determined by dynamic light scattering using the photon correlation spectroscopy (PCS) technique. The measurements were performed at 25 °C using either a ZetaSizer 4 (Malvern Instruments, Worcestershire, UK) equipped with a He-Ne gas laser (λ = 0.633 μm) or a ZetaPlus (Brookhaven Instrument Corporation, USA). Brookhaven or Malvern PCS software (v 1.52), depending on the system, was used for data acquisition and analysis. Polystyrene size standards 220±6 nm (Duke scientific corp., Duke, NC) was used to verify the performance of the instrument. For viscosity and refractive index, the values of pure water were used (1.0). The samples were diluted with 10 mM Tris-buffer at pH 7.4 to achieve the optimal vesicle concentration. Surface charge on the vesicles was measured indirectly via analysis of zeta potential at 25 °C using a ZetaPlus instrument (Brookhaven Instrument Corporation, USA) in a 1/10 solution of 10 mM Tris-buffer.

2.4. Transmission electron microscopy (TEM)

Morphological analysis was carried out by TEM using a JEOL 1200EX TEM fitted with a LaB6 filament, with an operating voltage from 40 to 120 kV. A small drop of sample (10 μl) was placed on a polymer filmed copper grid and allowed to stand for 2 min. The 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 which was viewed at 70 kV.

2.5. Cryo-TEM

Further morphological analysis was carried out by Cryo-TEM using a Philips CM120 BioTWIN transmission electron microscope. Samples for cryo-TEM were prepared under controlled temperature and humidity conditions within a controlled environmental verification system (CEVS). A small drop (5 μl) of sample was deposited on a Pelco Lacey carbon filmed grid. After carefully spreading of the drop, excess liquid was removed with a filter paper, forming thin (10–500 nm) sample films extending over the approx. 5 μm large holes in the polymer film. Then, the sample was immediately plunged into liquid ethane, held at −180 °C. The vitrified sample was then transferred in liquid nitrogen to an Oxford CT3500 cryo holder connected to the electron microscope. The sample temperature was kept below −180 °C. All observations were made in the bright field mode and an acceleration voltage of 120 kV. Digital pictures were taken with a Gatan Imaging Filter 100 CCD camera.

2.6. ^{125}I radiolabelling of Ag85B–ESAT-6

Radiolabelling of Ag85B–ESAT-6 was performed using the Iodo-gen® pre-coated iodination tubes (Pierce Biotechnology, Rockford, IL). Briefly, Ag85B–ESAT-6 was diluted with 50 μl 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 diluted up to 30 μl with 25 mM Tris-buffer and added to the iodination tube. This mixture was then left for 15 min, with intermittent shaking, to facilitate radiolabelling of Ag85B–ESAT-6.

Removal of the unlabelled Ag85B–ESAT-6 was performed by Sephadex G-75 gel column separation. In order to make the column, Sephadex G-75 (1%, w/v) was first soaked in double distilled water at 90 °C for 1 h, with stirring. The swollen gel was then packed into a 5 ml column and equilibrated with the 25 mM Tris-buffer.

Prior to separation, the reaction mixture from the iodination tube was further diluted with the Tris-buffer, and then passed through the column with 25 mM Tris-buffer as mobile phase. Aliquots of the eluted solution (0.5 ml) were collected and measured for gamma radiation using a Cobra™ CPM Auto-Gamma® counter (Packard Instruments Company inc., IL, USA) and also for UV absorbance at 280 nm, so as to confirm the presence of radiolabelled Ag85B–ESAT-6. The appropriate aliquots were then pooled and stored at −20 °C until required for further use.

2.7. Adsorption of antigen to adjuvant liposomes

The degree of adsorption of Ag85B–ESAT-6 to the liposomes was determined by ^{125}I radiation. Radiolabelled Ag85B–ESAT-6 was added to liposomes prepared as described above, mixed, and then allowed to stand for 10 min at ambient conditions. The formulation was then pelleted by ultracentrifugation (100,000×g for 1 h), resuspended in the original volume of 10 mM Tris-buffer, and then measured for gamma radiation. Adsorption of Ag85B–ESAT-6 was determined on the basis of ^{125}I radioactivity recovered in the suspended pellets. At regular time intervals, a similar procedure was adopted to determine Ag85B–ESAT-6 retention under different storage conditions (4 °C and 25 °C).

2.8. Immunization of mice

Female C57BL/6j mice, 8 to 12 weeks old, were obtained from Bomholtgaard (Ry, Denmark) and Harlan Scandinavia (Allerød, Denmark), respectively.

The vaccines were prepared by simply mixing preformed DDA liposome dispersions incorporating 0, 6.4, 11 or 20 mol% TDB, respectively, with the Ag85B–ESAT-6 stock solution to the final Ag85B–ESAT-6 concentration of 0.01 mg/ml (2 μg in each vaccine dose). The amount of DDA and TDB in each dose was 250 μg and 25, 50 or 100 μg, respectively. In vaccines with alum (500 μg in each dose), the antigen was added immediately before immunization.

Mice were immunized subcutaneously (s.c.) with the vaccines (0.2 ml/dose) at the base of the tails three times with a 2 weeks interval between each immunization.

2.9. Lymphocyte cultures

Blood samples were drawn from mice ($n=6$) 7 days after the last immunization by periorbital puncture and the blood lymphocytes were purified using Lympholyte®-Mammal (Cedarlane, Canada) according to the manufacturer's instructions. After recovery of the lymphocytes, the cells were washed twice in RPMI and cell cultures established as previously described [26]. In brief, cultures were performed in triplicate in round-bottomed microtiter wells (Nunc, Denmark) containing 2×10^5 cells in a volume of 200 μl RPMI supplemented with 5×10^{-5} M 2-mercaptoethanol, 1 mM glutamine, 1% penicillin–streptomycin, 1% HEPES and 10% fetal calf serum (all Gibco Invitrogen, Carlsbad, CA). Ag85B–ESAT-6 for re-stimulation was used in a concentration of 5 μg/ml. Wells containing medium only or 5 μg/ml of ConA were included in all experiments as negative and positive controls, respectively. Furthermore, 5 μg/ml of tetanus toxoid (SSI, Denmark) and 5 μg/ml of merozoite surface protein 1 (a generous gift by Dr. M. Theisen) was used as negative controls.

Splenocyte cultures ($n=3$) were obtained by passage of spleens through a metal mesh followed by two washing procedures using RPMI. To evaluate the responding subset of lymphocytes, the T cell receptor were blocked by adding monoclonal antibodies against CD4 and CD8 into the cultures 30 min before adding the splenocyte cultures. All dilutions of antibodies were based on previous titrations. Splenocyte cultures were grown as described for blood lymphocytes above.

Culture supernatants were harvested from parallel cultures after 72 h of incubation in the presence of antigen, and the amounts of IFN-γ and IL-5 were determined by ELISA as previously described [27,28].

2.10. Determination of antibody titres

Antibody titres were determined by ELISA as previously described [28]. Briefly, plates for enzyme linked immunosorbent assay (ELISA) were coated with Ag85B–ESAT-6 (0.05 μg /well) in PBS, and individual mouse sera from four mice per group were analyzed in duplicate in 5-fold dilutions. HRP-conjugated secondary antibodies (rabbit anti-mouse IgG1 and IgG2a, Zymed, USA) diluted 1/2000 in PBS containing 1% BSA were added, and Ag85B–ESAT-6 specific antibodies were detected by TMB substrate as described by the manufacturer. Antibody titres were then defined as the serum dilution which gives an absorbance value of 1.00 in the parallel portion of the curves as previously described [29].

3. Results and discussion

3.1. Dynamic light scattering: particle size and zeta potential

In an aqueous environment, DDA self-assemble into liposomes that have been used as an adjuvant for various vaccines [4,6–8]. The physico-chemical characteristics of DDA and DDA–TDB vesicles prepared by either the lipid film hydration method or the aqueous hydration method were studied. Results in Table 1 demonstrate that liposomes prepared by lipid hydration were significantly smaller than liposomes of the same composition prepared by the aqueous heat method (400–500nm vs. 800–1300nm, respectively; Table 1). Further, while supplementation of DDA liposomes with TDB resulted in no significant difference in vesicle size when prepared by the lipid hydration method, the opposite was apparent when liposomes were prepared by the aqueous heat method, with DDA–TDB vesicles being larger than their DDA counter-parts (Table 1), suggesting that potentially the TDB is less effectively incorporated/packaged within the lipid membranes when the latter method was adopted.

The zeta potential of liposomes can both directly influence liposome suspension stabilities and indirectly reflect vesicle

Table 1
Vesicle size and zeta potential of DDA liposomes prepared from DDA with and without TDB (11 mol%) by either the aqueous heat or lipid hydration method

Formulation	Aqueous heat method		Lipid hydration method	
	Size (nm)	ZP (mV)	Size (nm)	ZP (mV)
DDA	846±130	52±2.6	488±124	46±1.6
DDA+H1	929±113	47±2.6	421±26	52±3.1
DDA–TDB	1281±134	47±0.6	416±40	48±5.1
DDA–TDB+H1	1256±150	44±0.3	486±110	60±4.7

In both cases, the liposomes were dispersed in 10 mM Tris buffer with pH 7.4. The effect of Ag85B–ESAT-6 (H1) adsorption on vesicle size and zeta potential was measured by adding H1 (0.01 mg/ml) to the liposomes. Vesicle size and zeta potential (ZP) was measured using a Brookhaven ZetaPlus with zeta potential being measured in 1 mM Tris buffer pH 7.4. Results denote mean±S.D. from at least 3 batches.

surface net charge—a factor which can be used to evaluate the extent of charged lipid incorporation and head–group interaction. Zeta potential analysis of the DDA and DDA–TDB formulations show that all liposomes tested have a high net positive charge (44–52 mV; Table 1), clearly resulting from the cationic nature of DDA. Neither the method of preparation nor the inclusion of TDB within the liposome membranes was shown to make a significant difference to this positive surface charge, suggesting that no marked electrostatic interactions between the DDA and TDB lipids occurred at the surface of the vesicle. In addition, the adsorption of Ag85B–ESAT-6 antigen (0.01 mg/ml) to both DDA and DDA–TDB liposomes made no significant difference to the measured size or zeta potential of vesicles (Table 1), suggesting that at these concentrations adsorption of antigen on the vesicle surface made no significant impact on the liposomes physical attributes.

Unfortunately, dispersions of DDA liposomes are physically unstable and prolonged storage at 4 °C is not possible without the visible occurrence of aggregation and precipitates. This is supported by the particle size data shown in Fig. 1A. The average particle size of pure DDA liposomes increases rapidly during the first 10 days of storage at 4 °C due to aggregation or fusion of the liposomes, and after 45 days of storage it is impossible to determine the size of the liposomes using dynamic light scattering. In contrast, the liposomes containing small amounts of the glycolipid TDB showed little or no change in particle size, indicating that the presence of TDB in the DDA liposome bilayers leads to short- as well as long-term stabilization of the size of the liposomes. Similar results were also shown at room temperature (25 °C; Fig. 1B), with the particle size of DDA liposomes increasing approximately 4-fold after only 7 days, compared to DDA–TDB (11 mol%) which showed no significant increase in size after 14 days. However, by day 28, both formulations had shown a marked increase in vesicle size (Fig. 1B). Zeta potential analysis of these formulations reveals no major changes in the surface characteristics of liposomes stored at 4 °C for up to 28 days (Fig. 2A), however storage at room temperature conditions resulted in a marked reduction in the zeta potential of the DDA and DDA–TDB liposomes at day 14 and 28, respectively (Fig. 2B).

It has previously been shown that double chain glycolipids, such as TDB and TDM, inhibit fusion between phospholipid vesicles [30,31]. The stabilizing effect is presumably caused by the relatively large hydrophilic trehalose head–group increasing the overall hydration of the liposomal surface, preventing dehydration of the quaternary ammonium head–groups and aggregation caused by reduced charge repulsion. Alternatively, the trehalose moiety might act as a steric barrier that prevent close contact between opposing liposomes, which is a prerequisite for the formation of aggregation or fusion of the liposomes [30,31].

3.2. Differential scanning calorimetry (DSC)

Lipid bilayers formed of DDA undergo a main phase transition at a characteristic temperature (T_m) at which the liposomal bilayers go from a lower temperature gel-phase dominated by ordered alkyl chain conformations to a high-temperature fluid-phase characterized by disordered alkyl chain conformations [32]. The phase transition temperature (T_m) is detected as a peak in the heat capacity curve obtained by DSC. DSC thermograms for DDA dispersions containing increasing molar concentrations of the glycolipid TDB are shown in Fig. 3 and the parameters T_m, ΔH_m, and $\Delta T_{1/2}$ are summarized in Table 2.

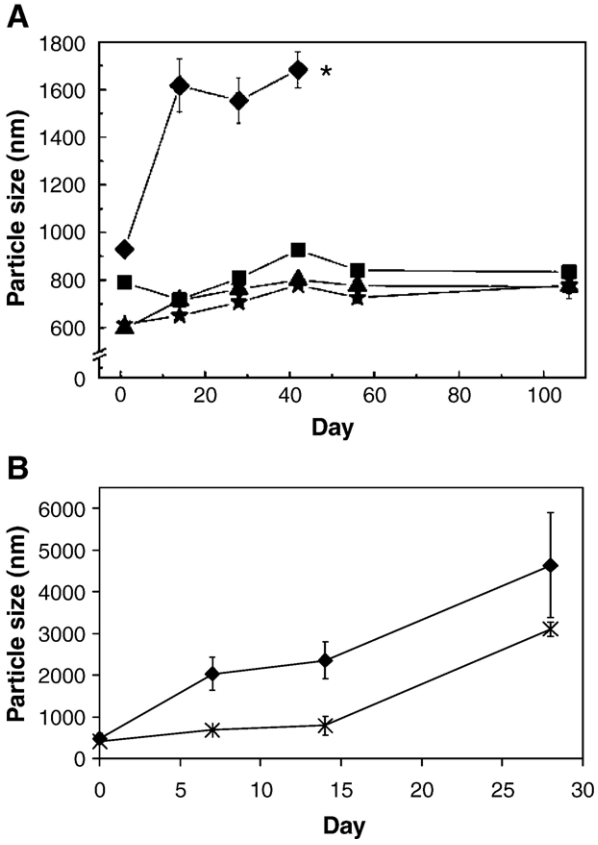

Fig. 1. (A) Time development of average particle size of DDA liposomes containing 0 mol% (–♦–), 6 mol% (–▲–), 11 mol% (–★–) and 20 mol% (–■–) TDB stored at 4 °C. The liposomes were dispersed in 10 mM Tris buffer adjusted to pH 7.4. A significant increase is observed for DDA liposomes without TDB after 14 days. (B) represents average particle of DDA (–♦–) and DDA/TDB (11 mol%) (–∗–) stored at 25 °C for up to 28 days.

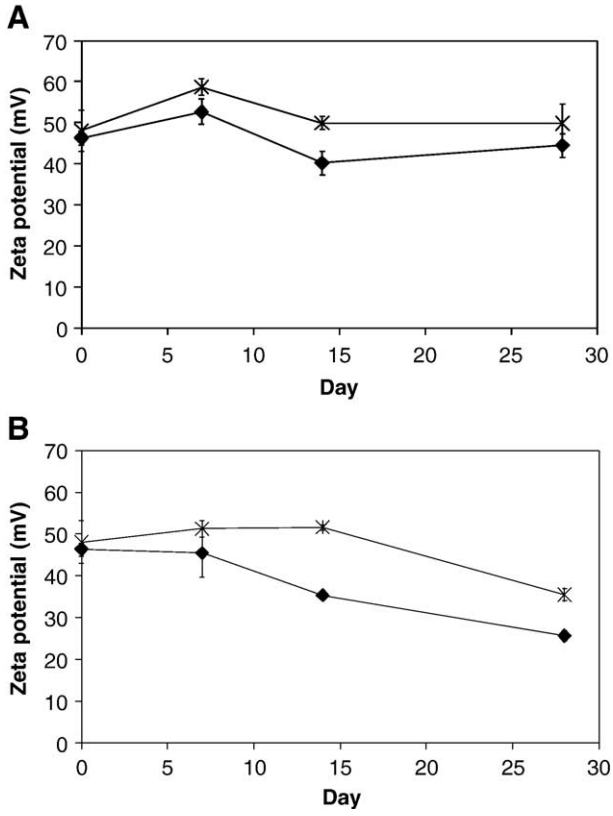

Fig. 2. Time development of average zeta potential of DDA (–♦–) and DDA/TDB (11 mol%) (–∗–) liposomes dispersed in 10 mM Tris-buffer (pH 7.4) and stored at (A) 4 °C and (B) 25 °C.

The DSC scan for pure DDA dispersed in 10 mM Tris at pH 7.4, is characterized by one sharp well defined endothermic peak with T_m ≈47.2 °C and $\Delta T_{½}$ ≈0.36 °C, characteristic of a highly cooperative gel-fluid phase transition in good agreement with DSC results for DDA liposomes reported in the literature [15,23,25,33,34]. In contrast, for DDA liposomes containing 6.4 mol% TDB a broadening of the gel-fluid phase transition peak ($\Delta T_{1/2}$=1.73 °C) was observed, and a shoulder was

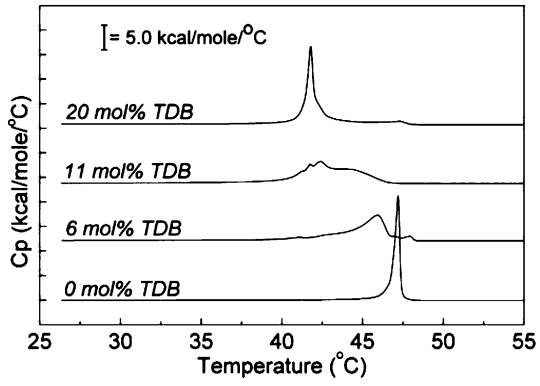

Fig. 3. Differential scanning heat capacity curves obtained at a scan rate of 30 °C/h for freshly prepared DDA liposomes incorporating 0, 6.4, 11 and 20 mol% TDB. The liposomes were dispersed in 10 mM Tris buffer adjusted to pH 7.4. The second of three DSC heating scans is shown. The curves have been normalized to molar content. A fall in phase transition temperature from 47.2 to 41.7 °C is observed. Notice that the scans have been displaced on the heat capacity axis for clarity.

Table 2
Thermodynamic parameters characterizing the gel to liquid phase transition in liposomes formed by DDA incorporating 0, 6.4, 11 and 20 mol% TDB obtained using differential scanning calorimetry at a scan rate of 30 °C/h

Mol% TDB	T_m (°C)	ΔH_m (kcal/mol)	$T_{½}$ (°C)
0	47.20	11.32	0.36
6	45.91	13.45	1.73
11	42.40 (47.4)	15.61	5.74
20	41.80	14.53	0.41

The liposomes were dispersed in 10 mM Tris buffer with pH 7.4.

detected at the high temperature side. In addition, introduction of TDB leads to a shift in T_m toward lower temperatures. The same kind of complex phase behavior was detected for DDA liposomes containing 11 mol% TDB. The broad gel-fluid phase transition revealed two components with an overall maximum at 42.4 °C, suggesting that more than one cooperative heat transition occurs, most likely due to changes in the local structure of the lipid bilayer, resulting in a lateral phase separation and formation of domains enriched in TDB [35]. This demonstrates that the acyl chains of the TDB glycolipids are in fact incorporated into the hydrophobic core of the DDA lipid bilayers causing a change in the lipid chain packing properties. This thermotropic phase behavior appeared to depend on the molar ratio of TDB, as the high temperature component of the heat transition peak practically disappears with increasing amounts of TDB, as indicated by the DSC thermograms for the dispersions containing 20 mol% TDB. Overall, the present DSC results demonstrate that TDB has a pronounced concentration dependent effect on the thermotropic phase behavior of DDA liposomes. It should be emphasized that despite the slight decrease in T_m observed when TDB is introduced into the bilayer, the liposomes do not break up. In fact, TDB significantly improves the colloidal stability of DDA dispersions as indicated by the development in particle size shown in Fig. 1A.

3.3. Adsorption of antigen

To characterize the antigen adsorption to DDA–TDB liposomes the mycobacterial Ag85B–ESAT-6 fusion protein was selected as model antigen. This protein has been identified as a promising vaccine antigen against tuberculosis in several studies [8,36,37] a disease for which a Th1 type immune response is required for protection.

The degree of adsorption of antigen (% of total used; 0.01 mg) to both DDA and DDA–TDB liposomes was determined using ^{125}I-labelled Ag85B–ESAT-6. The addition of TDB had no detrimental effect on protein adsorption, with both DDA and DDA–TDB formulations adsorbing approximately 70–80% of the total antigen initially added (Table 3). The adsorption of antigen to the formulation is most probably electrostatic in nature. The antigen is highly negatively charged and has a theoretical pI value of 4.80. In contrast, the zeta potential analysis demonstrates that all DDA–TDB formulations had a high net positive charge of 46 to 52 mM. The method of preparation was shown to impact on antigen

Table 3
Adsorption of antigen to adjuvant liposomes

Formulation	Aqueous heat method	Lipid hydration method
DDA	74.3±3.2%	69.9±10.2%
DDA–TDB	78.7±2.1%	67.0±2.8%

^{125}I-labelled Ag85B–ESAT-6 was adsorbed onto DDA or DDA–TDB (11 mol%) liposomes prepared by either the aqueous heat or lipid hydration method as in Table 1 and non-associated antigen removed by ultracentrifugation. Values denote mean±S.D. from at least 3 experiments.

adsorption, with slightly lower adsorption for liposomes prepared by the lipid hydration method; however, this could be related to the small vesicle size associated with this method, which could result in some small vesicle populations failing to pellet effectively during the centrifugation process.

After 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 (Fig. 4). After 7 days storage at 4 °C (Fig. 4A) antigen retention in both formulations dropped to approximately 80% of the initial antigen adsorption. Subsequently at time points thereafter, retention values for DDA–TDB liposomes remained constant at ~80% with no further losses of antigen being measured (Fig. 4A). In contrast, further loss of antigen was associated with the DDA formulation with retention values continuing to drop to approximately 65% (of initial adsorption values) at day 14 and subsequently levelling around this value thereafter (Fig. 4A). Storage of the formulations at 25 °C had no major impact on antigen retention compared to refrigerated samples over the period of analysis, however at this temperature, significant differences ($P<0.05$, Student's t-test) in antigen retention between liposomes with and without TDB were only apparent at day 28 (Fig. 4B).

3.4. Morphology studies

3.4.1. TEM

Initially, TEM was used to investigate the morphological characteristics of liposomes prepared by either the aqueous heat or lipid hydration method. Fig. 5A shows that DDA liposomes prepared by aqueous heat method are angular and relatively large in size (>1 μm) with notable signs of vesicle aggregates. The larger vesicle size of liposomes produced by aqueous heat compared to lipid hydration is also apparent when comparing the DDA–TDB (Fig. 5B vs. Fig. 5E) and DDA–TDB/protein (Fig. 5C vs. Fig. 5F) liposome formulations. The formulation of the liposomes had less of an impact on the morphological characteristics of the aqueous heat preparations, with no notable morphological changes seen upon addition of 11 mol% TDB (Fig. 5B) and protein (Fig. 5C) with vesicles remaining around 1 μm in size. However, the extent of aggregation appears reduced when TDB is incorporated within the liposomal membranes, again suggesting the stabilizing role TDB may play in the vesicle construct.

In agreement with the particle size analysis, when prepared by lipid hydration method, DDA liposomes (Fig. 5D) were smaller (~500 nm) and less angular than those prepared by aqueous heat (Fig. 5A), although some large vesicles and aggregates are present. Addition of 11 mol% TDB to the liposome formulation (Fig. 5E) gave more spherical vesicles which display less aggregation compared to their DDA counterparts, possibly again due to the bilayer stabilizing effect of the longer alkyl chain TDB. Similar to aqueous heat preparations, no obvious morphological change was apparent upon addition of protein to lipid hydration liposomes (Fig. 5F).

3.4.2. Cryo-TEM

The cryo-TEM micrograph shown in Fig. 6 confirms that liposomes are formed when a lipid film of DDA containing 11 mol% TDB is hydrated in Tris-buffer. The spontaneously formed DDA liposomes incorporating TDB displayed in Fig. 1 were rather polydisperse in size and shape, nevertheless most of the liposomes had a "lens-like" shape and diameters below 500 nm. However, as noted in the TEM investigations some giant vesicles with diameters in the micrometer range were also observed. As expected from the high content of positively charged DDA lipids the cryo-TEM micrograph shows that the vesicles were predominantly unilamellar. This general feature of bilayer systems containing charged lipids can be explained by inter-bilayer repulsive electrostatic forces, which prevent multiple bilayer stacking [38].

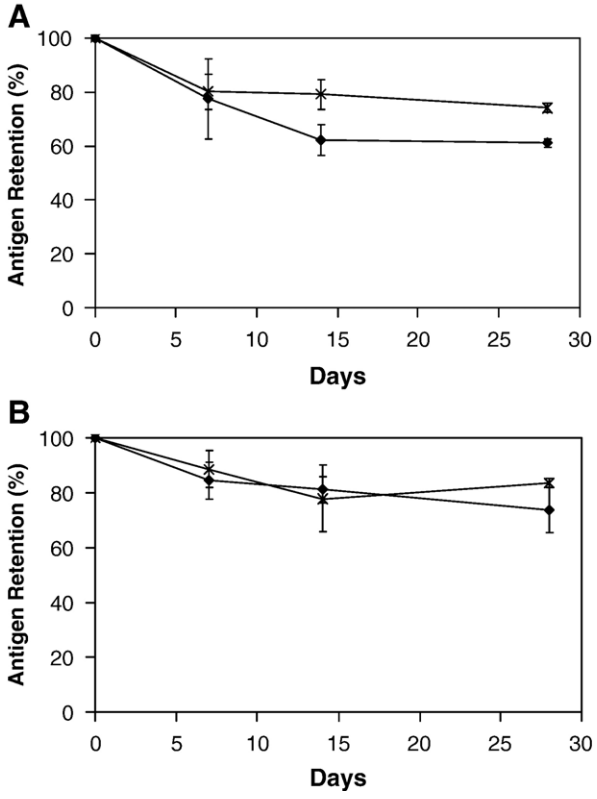

Fig. 4. Adsorbed antigen retention over time of DDA (–♦–) and DDA–TDB (11 mol%) (–∗–) liposomes dispersed in 10 mM Tris buffer (pH 7.4) and stored at 4 °C (A) and 25 °C (B). ^{125}I-labelled Ag85B–ESAT-6 was adsorbed to liposomes and antigen retention of Ag85B–ESAT-6 was determined on the basis of ^{125}I radioactivity recovered in the suspended pellets after ultracentrifugation. Results represent percentage retention of initial adsorbed antigen (Table 3) expressed as mean±S.D., $n=3$.

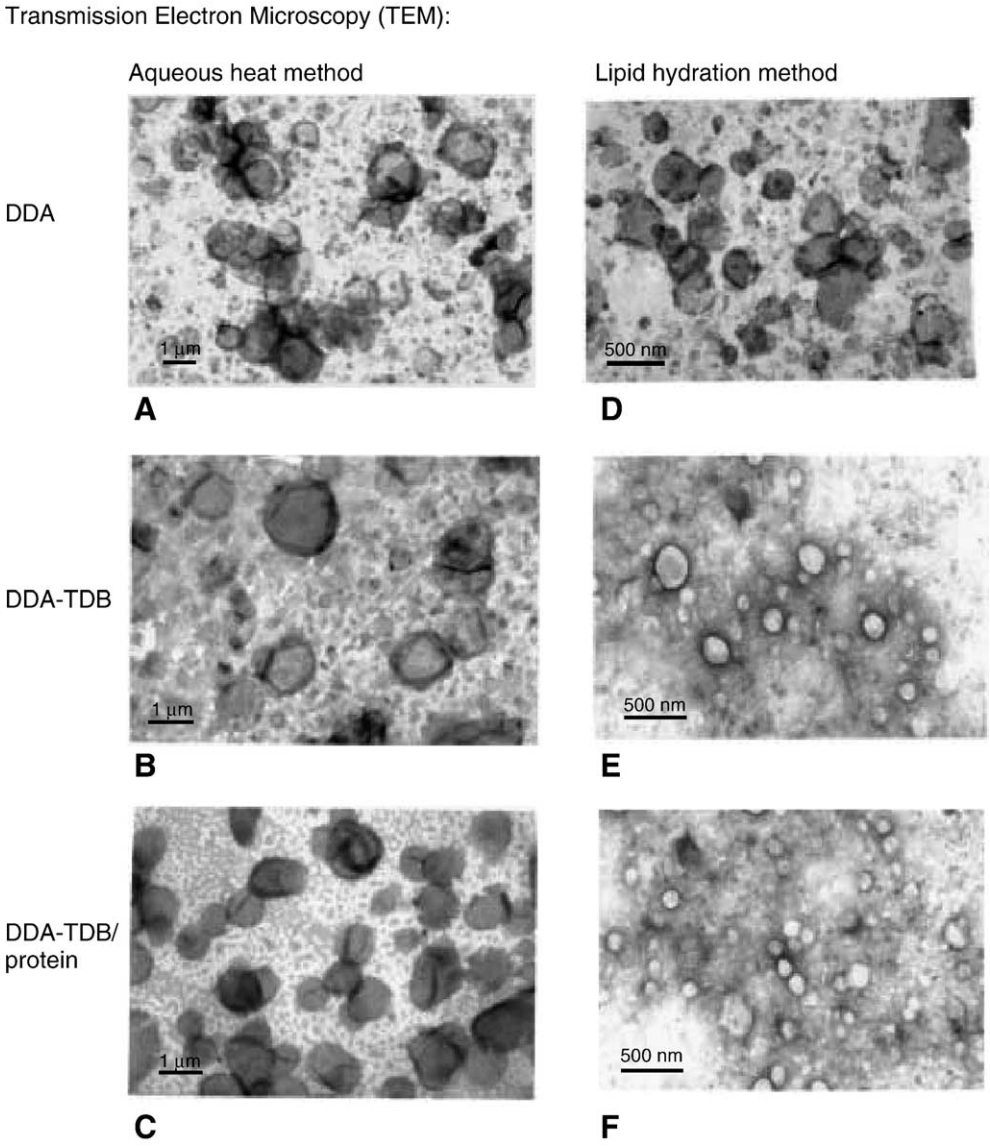

Fig. 5. DDA Liposomes in Tris-buffer prepared by aqueous heat method (A) were angular, and relatively large in size, and show significant vesicle aggregation. No morphological changes are seen upon addition of 11 mol% TDB (B) or protein (C). When prepared by lipid hydration method, DDA liposomes (D) were smaller and less angular than those prepared by aqueous heat (A), although some large vesicles are present. Addition of 11 mol% TDB to these DDA liposomes gave more spherical vesicles which display less aggregation (E). Similar to aqueous heat preparations, no obvious morphological change was apparent upon addition of protein to lipid hydration liposomes (F). In all formulations, liposomes prepared by the aqueous heat method are larger than the lipid hydration counterparts.

3.4.3. Immunological characterization of the DDA–TDB Ag85B–ESAT-6 vaccine

The adjuvant effect of the DDA–TDB formulation was subsequently tested in mice using the Ag85B–ESAT-6 fusion protein as a model antigen. Mice were immunized three times with the vaccine antigen mixed with DDA liposome dispersions incorporating 0, 6.4, 11 or 20 mol% TDB, respectively. One week after the third immunization, the specific immune response of the blood cells was investigated by restimulation with Ag85B–ESAT-6 in vitro (Fig. 7A) and subsequently measuring IFN-γ and IL-5 as indicators of a Th1 and Th2 response, respectively. All formulations with TDB were able to induce high levels of IFN-γ, with the formulation containing 11% TDB giving rise to the highest response (96.2 ± 1.6 ng/ml). Low levels of IL-5 (below 120 pg/ml) were seen with all formulations containing TDB (results not shown). No response was seen after re-stimulation with irrelevant antigens, i.e., tetanus toxoid or the merozoite surface protein from malaria (data not shown).

Furthermore, the ability of DDA–TDB to induce antibody responses after immunization was investigated by immunizing with Ag85B–ESAT-6 in the various formulations of DDA–TDB three times and measuring the levels of antigen-specific antibodies of the IgG1 and IgG2b isotypes by ELISA. The IgG2a titer which is widely used as an indicator of a Th1-type of immune response was not evaluated since the gene is deleted in C57Bl/6 mice [39]. As shown in Fig. 7B, the addition of TDB to DDA in general causes an elevation of the antibody levels with a level of IgG2b in the group receiving 11% TDB-DDA 9-fold higher compared to DDA alone. The groups receiving 11 and 6.4% TDB exhibited the highest ratio of IgG2b compared to

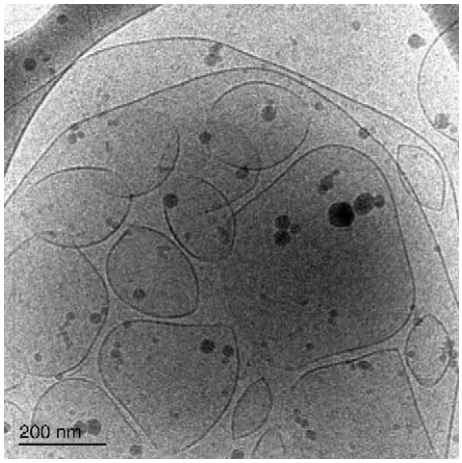

Fig. 6. Cryo-TEM micrograph of DDA vesicles containing 11 mol% TDB vitrified from 25 °C. The sample exists predominantly as unilamellar vesicle structures. Morphology and size varies between the vesicles. No bilayer fragments were observed. Scale bar=200 nm.

IgG1 (ratio 0.2) which is a characteristic seen with the induction of a Th1-immune response. Increasing the amount of TDB to 20% did not further enhance the immune response but rather had a negative influence on the levels of antibodies induced.

Based on these immunological data as well as the physico-chemical data on stability of DDA–TDB liposomes it was decided to use DDA liposomes with 11% TDB as the standard formulation. In order to evaluate the adjuvant activity of DDA–TDB compared to an adjuvant already approved for human use, mice were immunized with Ag85B–ESAT-6 in either DDA–TDB or Alum and the immune responses post-immunization evaluated. As shown in Fig. 8A, immunization with DDA/TDB leads to high levels of IFN-γ release and low levels of IL-5 whereas Alum-immunized mice exhibited the opposite pattern with a neglible IFN-γ secretion and higher levels of IL-5. DDA/TDB give rise to the same high levels of IgG1 antibody titers as seen after immunization with alum whereas the level of Ig2b antibodies were 8-fold higher after immunization with DDA–TDB compared to alum, indicating a dominant Th1-type of immune response obtained with DDA–TDB (Fig. 8B). To further evaluate the lymphocyte subset mediating the observed immune response, spleen cultures from immunized mice were blocked with monoclonal antibodies against the CD8 and CD4 receptors before restimulating with Ag85B–ESAT-6. Adding anti-CD4 to the cultures decreased the immune response considerably whereas minor effect was seen after blocking of the CD8 receptor (Fig. 9). The IFN-γ

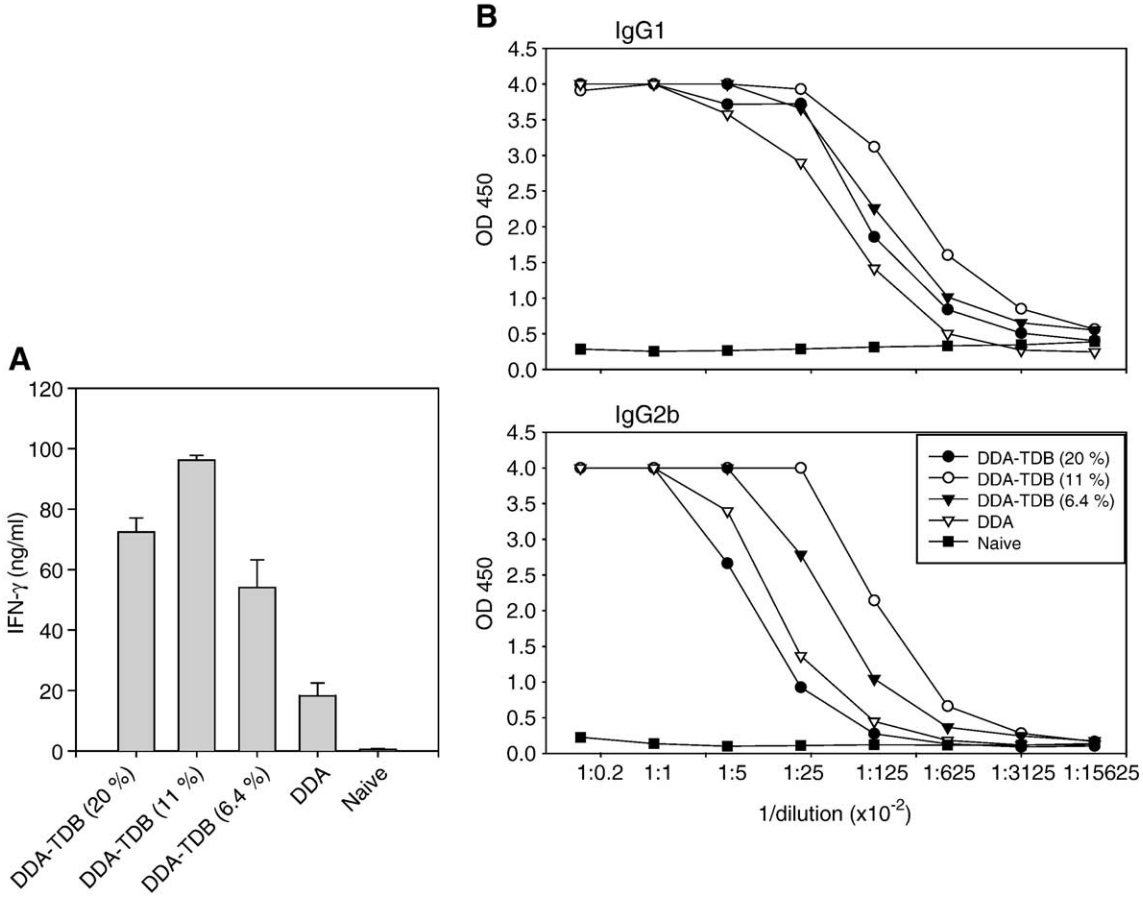

Fig. 7. Induction of immune response using DDA–TDB liposomes as adjuvant. Release of IFN-γ from blood lymphocytes isolated from C57Bl/6j mice immunized with 2 μg of Ag85B–ESAT-6 administered in DDA liposomes incorporating 0, 6.4, 11 or 20 mol% TDB, respectively (A). Blood lymphocytes were isolated 5 weeks after the first immunization and re-stimulated in vitro with the Ag85B–ESAT-6 (5 g/ml). IgG1 and IgG2b Ag85B–ESAT-6-specific antibody dilution curves (B).

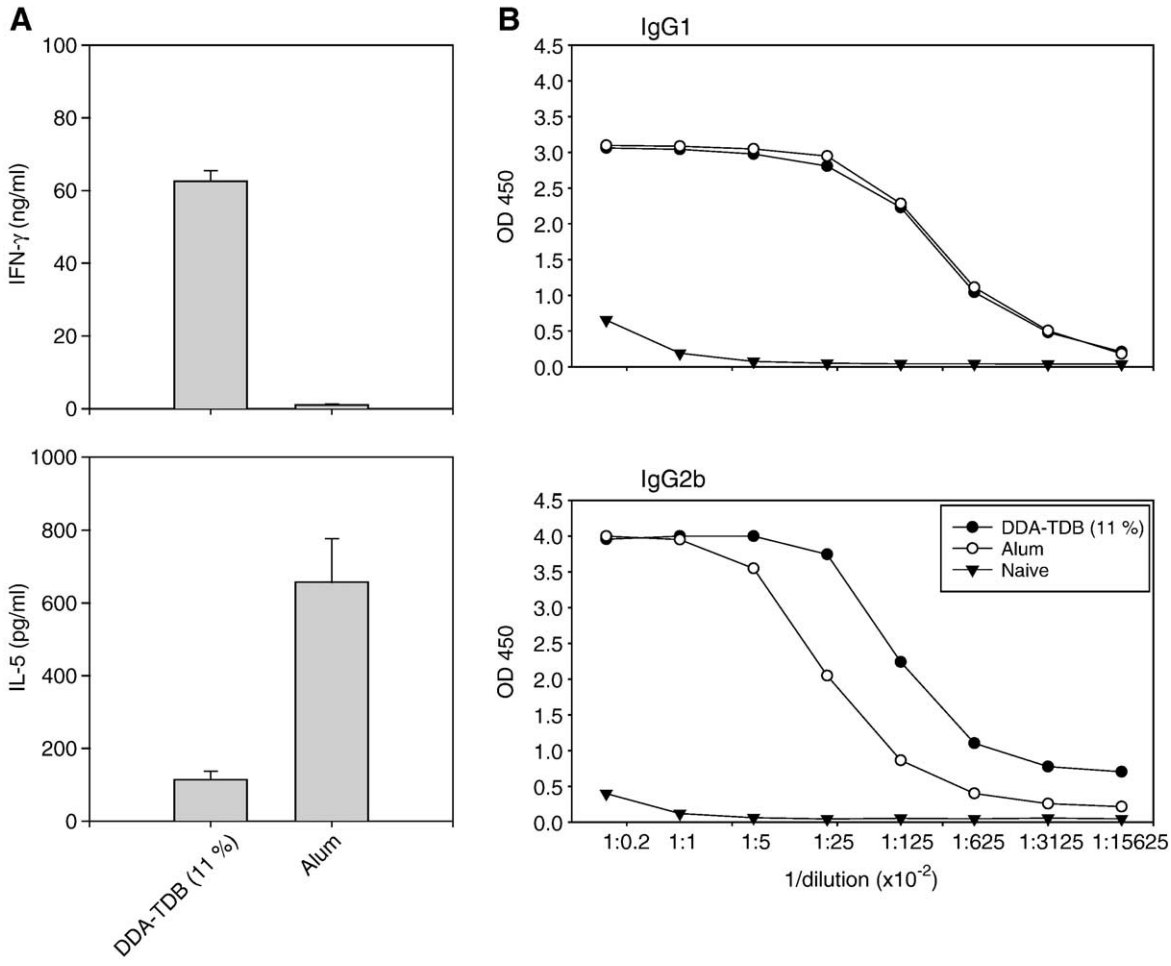

Fig. 8. Immune responses generated by DDA–TDB liposomes and alum. Release of IFN- and IL-5 from blood lymphocytes isolated from C57Bl/6j mice immunized with 2 μg of Ag85B–ESAT-6 administered in DDA–TDB with 11 mol% TDB incorporated or alum (A). IgG1 and IgG2b Ag85B–ESAT-6-specific antibody dilution curves (B).

release from the CD4 T cells was furthermore supported by flow cytometry analysis with intracellular staining of IFN-γ (results not shown).

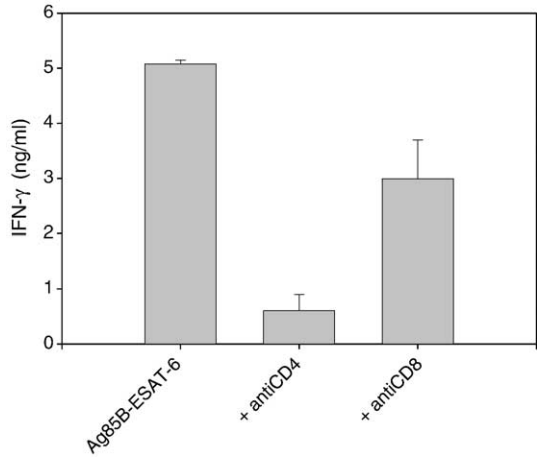

Fig. 9. Release of IFN-γ from spleen lymphocytes isolated from C57Bl/6 mice immunized with 2 μg of Ag85B–ESAT-6 emulsified in DDA/TDB. Splenocytes were isolated 1 week after the final immunization and blocked with anti-CD4 or anti-CD8 prior to re-stimulation with 5 μg/ml of Ag85B–ESAT-6.

The adjuvant liposomes presented here shows the commendable ability to stimulate both a cell mediated Th1 immune response and an antibody response. This broad stimulation of the immune response is a unique property of DDA–TDB and makes the adjuvant advantageous for a high number of disease targets including infectious diseases and cancers. The testing of DDA–TDB as an adjuvant for vaccines with different requirements is presently ongoing and would provide important information on the applicability of the adjuvant. Besides potentiating the immune response of DDA, incorporation of TDB was shown to effectively stabilize the DDA liposomes. This is, to our knowledge, the first time it has been demonstrated that cationic adjuvant liposomes are stabilized by incorporating synthetic glycolipid analogues of TDM into the liposome bilayers, thus overcoming the major obstacle for using DDA as an efficient adjuvant system, while simultaneously improving the immunogenicity of the adjuvant considerably.

Acknowledgements

This work was funded by the European Commission contract No. LSHP-CT-2003-503367.

We thank Birgitte Smedegaard, Lars Pedersen and Liselotte Skovsø Nielsen for excellent technical assistance.

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