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Research paper

Increased potential of a cationic liposome-based delivery system: Enhancing stability and sustained immunological activity in pre-clinical development

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ABSTRACT

The combination of dimethyl dioctadecyl ammonium bromide (DDA) and the synthetic cord factor trehalose dibehenate (TDB) with Ag85B-ESAT-6 (H1 fusion protein) has been found to promote strong protective immune responses against *Mycobacterium tuberculosis*. The development of a vaccine formulation that is able to facilitate the requirements of sterility, stability and generation of a vaccine product with acceptable composition, shelf-life and safety profile may necessitate selected alterations in vaccine formulation. This study describes the implementation of a sterilisation protocol and the use of selected lyoprotective agents in order to fulfil these requirements. Concomitantly, close analysis of any alteration in physico-chemical characteristics and parameters of immunogenicity have been examined for this promising DDA liposome-based tuberculosis vaccine. The study addresses the extensive guidelines on parameters for non-clinical assessment, suitable for liposomal vaccines and other vaccine delivery systems issued by the World Health Organisation (WHO) and the European Medicines Agency (EMEA). Physical and chemical stability was observed following alteration in formulations to include novel cryoprotectants and radiation sterilisation. Immunogenicity was maintained following these alterations and even improved by modification with lysine as the cryoprotective agent for sterilised formulations. Taken together, these results outline the successful alteration to a liposomal vaccine, representing improved formulations by rational modification, whilst maintaining biological activity.

1. Introduction

Subunit vaccines consisting of purified antigens represent a new era of safer and inactivated vaccines compared to killed or live attenuated microbes. They offer the potential for the reduction in side effects and avoidance of reversion to virulence, utilising an immunogenic part of the disease-causing agent, while avoiding the need to culture a hazardous pathogen [1]. The replacement of the whole organism with selected protein antigens not only reduces the amount of immunogen required for vaccination but also enables the immune response to be focused towards specific regions of the pathogen that are relevant to protection. Additionally, less dominant or more highly conserved epitopes can be presented more effectively than in the context of the whole organism [2]. Upon *in vivo* administration, the antigen requires protection against any extracellular degradation and needs to be taken up by the targeted immune cells [3]. In order to achieve this and to circumvent the weak immunogenicity of subunit antigens, a combination of an adjuvant and delivery system needs to be formulated [4,5]. The selection of an appropriate adjuvant and delivery system is vital to the generation of the desired immune response. With the advent of modern immunology, a plethora of natural as well as synthetic compounds have been investigated as vaccine adjuvants [6]. However, the selection of the right combination of delivery system and adjuvant may be key to the utilisation of particulate delivery systems for effective vaccines [7,8].

Delivery systems offer many options that include liposomes, polymer-based micro- and nanoparticles, virosomes, ISCOMS and many more. From these, liposomes were the first to be shown to act as immunological adjuvants [9]. Liposomes offer a flexible system for manipulation that can result in vesicles with varying lamellarity, physical characteristics, adsorption and encapsulation of antigens and pay load [10]. Despite the wealth of literature describing the potential of liposomes for vaccine delivery, the lack of availability of a purely liposome-based vaccine could potentially be attributed to their weak physical and chemical stability in solution. This may be avoided by the development of a stable, sterile, freeze-dried formulation capable of maintaining physical properties post hydration together with the ability to offer a chemically stable product when in the freeze-dried state [11]. However, the fabrication of a practical, immunogenic delivery system incorporating the adjuvant and the subunit protein antigen still remains an issue. Despite this, it is evident that the development of a stable freeze-dried vaccine has the potential for distribution independently of cold-chain infrastructure. In combination with a longer

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shelf-life, this can engender significant logistical, health and economic benefits [12].

The combination of dimethyl dioctadecyl ammonium bromide (DDA) and the synthetic cord factor trehalose dibehenate (TDB) has been found to promote strong protective immune responses against *Mycobacterium tuberculosis* infection without observable toxicity [13], and this system also offers significant potential for the improvement in immunogenicity of other subunit vaccines, such as hepatitis B [14]. Whilst this system was shown to be potent, formulation problems such as lack of stability and difficulties in the production of an economical sterile product, have led to extensive characterisation of this vaccine adjuvant system [15] and efforts to improve stability and generate a sterile product by freeze drying and γ-irradiation sterilisation, respectively [11].

Both the World Health Organisation (WHO) and the European Medicines Agency (EMEA) offer extensive guidelines on parameters for non-clinical assessment of vaccines [16,17]. Differences between these two useful sources are delineated only by definition as non-clinical or quality control measurements, respectively, and there is considerable overlap between parameters that are recommended as desirable for evaluation and characterisation of vaccine formulations. Despite the existence of these guidelines, investigations into candidate vaccines and vaccine delivery systems are rarely associated directly with the relevant regulatory criterion and recommendations. In a previous publication, we have outlined the implementation of lyophilisation and sterilisation protocols in the non-clinical and quality control assessment of vaccine formulations set out to directly support their transition from the bench to the clinic [11]. Within the development of a vaccine formulation that is able to facilitate the requirements of sterility, stability, and generation of a vaccine product with acceptable composition, shelf-life and safety profile, alterations in vaccine formulation may have to be made. Problems associated with obtaining a more stable lyophilised product have been circumvented by the use of selected cryo-/lyo-protectants and sterility ensured by the application of γ-irradiation sterilisation according to the dose recommended by the European Pharmacopoeia. However, the effect of these alterations on immunogenicity of the DDA/TDB vaccine formulations is unknown. The maintenance of immunogenicity is fundamental to vaccine efficacy, and confirmation of immunogenic properties is crucial to the fulfilment of WHO and EMEA guidelines. In the assessment presented here, the implication of these selected alterations to vaccine formulations, made in order to facilitate an enhanced vaccine product regarding sterility and stability, is assessed in terms of continuity of physico-chemical and biological characteristics, and thus, the potential for taking these optimised vaccine formulations towards the clinic is evaluated.

2. Materials and methods

2.1. Chemicals

Methanol (extra pure), chloroform (extra pure) and 1 M hydrochloric acid, used to adjust pH in the Tris buffer, were purchased from Fisher, UK. Tris base, carbohydrates (sucrose, trehalose and maltose), amino acid (lysine) and chloroform were obtained from Sigma–Aldrich Company Ltd., Poole, UK. All other chemicals used were of analytical grade.

2.2. Preparation of vaccines

The cryoprotectants tested for the study were chosen from two classes of compounds: the traditional sugar-based cryoprotectants, which included sucrose, maltose and trehalose, and amino acids, which included lysine. A concentration range of 2–10 mole/mole of lipid was tested, and size upon rehydration, dynamic viscosity and moisture content were chosen as indicators of effective lyophilisation. Ag85B-ESAT-6 (H1 fusion protein) was supplied by the Staten Serum Institut, Copenhagen, Denmark.

2.2.1. Formulation of freeze-dried liposomes

The organic phase consisting of the lipids was transferred into a 50-ml, spherical, round-bottomed quick-fit flask. The organic solvent was evaporated on a rotary evaporator (Buchi rotavapor-R) to obtain a dry film which was further flushed under a stream of nitrogen for 3 min to maximise the complete removal of solvents. The hydration step of the dried lipid film was carried out by the addition of 1 ml of Tris Buffer with different concentrations of the cryoprotectants, making up the final volume to 1 ml, and the flask was vortexed until all the lipid film had gone into solution. The liposomal suspension was then transferred into tubular type I clear glass injection vials/freeze drying vials, protein antigen (H1) added and covered with paraffin film which was ventilated (uniform punctures) to facilitate the removal of water during freeze drying. The freeze-drying protocol consisted of pre-freezing the liposomal formulation (dimethyldioctadecylammonium bromide (DDA, Avanti Polar Lipids (Alabaster, AL)): 1.25 mg/ml; α,α′-trehalose 6,6′-dibehenate (TDB, Avanti Polar Lipids (Alabaster, AL)): 250 μg/ml) at −70 °C for 30 min with or without the addition of cryoprotectant. This was followed by drying in two stages: −50 °C for 48 h and at −30 °C to a final temperature of 20 °C for 6 h, after which the vials were sealed and sterilisation was carried out as outlined below where appropriate.

2.2.2. Sterilisation of freeze-dried liposomes

The European Pharmacopeia specifies a dose of 25 KGy to produce sterile pharmaceuticals when the bioburden is not known. Briefly, the freeze-dried formulations were subjected to gamma sterilisation to a dose of 25 KGy using a Schering Healthcare IBL 437C irradiator. The radiation source was ^{137}Cs at a dose rate of 2.8 Gy/min. The sterilisation was carried out at ambient temperature.

These formulations were also subjected to stability trials where they were stored for up to 1 year at 25 °C/60% RH and for 6 months at 40 °C/75% RH to assess the long-term stability of these systems.

2.3. Vaccine characterisation

2.3.1. Size and zeta potential analysis

The liposomes were sized on a Zetaplus, Brookhaven Instruments, UK. A volume of 100 μl of the liposome suspension was diluted to 4 ml using ddH_2O, and the measurements were recorded at 25 °C. Each sample was the average of three readings, and each reading was a mean of measurements recorded for 3 min. Zeta potential was determined using a Zetaplus (Brookhaven Instruments) in ddH_2O at 25 °C using 50 μl of the dispersion diluted to 2 ml.

2.3.2. Viscosity and pH

Dynamic viscosity measurements were carried out using an Anton Paar AMVn automated microviscometer. The hydrated liposomal solutions were filled into the glass capillary tube, and the measurements were recorded at an angle tilt of 50° and −50°. The measurements were carried out at 20 °C. The pH measurements were recorded using a Mettler Toledo MP230 pH meter. The pH measurements were recorded after calibration with known standards.

2.3.3. Thermogravimetric analysis (TGA)

Thermogravimetric studies were carried out to measure the moisture content of the freeze-dried liposomes using a Perkin

Elmer Pyris 1 TGA. The samples were investigated between the temperature ranges of 50 °C and 140 °C at a scan rate of 10 °C/min. The values reported are the percentage loss in weight of the product.

2.4. Chemical characterisation of lipids

Chemical degradation of the irradiated liposomes was monitored using ^{1}H NMR to detect any degradation products that might have been formed as a result of exposure to high-energy radiation, and mass spectrometry was used as an additional technique to detect the mass of degraded moieties.

2.4.1. Nuclear magnetic resonance spectroscopy

NMR spectra were recorded on a Bruker AC-250 spectrometer at ^{1}H (250.1 MHz) referenced to tetramethyl silane (TMS). The freeze-dried formulation (10 mg) was dissolved in 2 ml of $CDCl_3$ and subjected to 64 scans, and the spectra were analysed. The analysis of gamma-sterilised DDA was carried out by dissolving 15 mg in 2 ml of $CDCl_3$.

2.4.2. Mass spectrometry

Mass spectra were recorded in electrospray ionisation mode with a Hewlett-Packard HP 5989B MS Engine apparatus using a HP 59987A API-electrospray LC/MS interface. The freeze-dried powder (5 mg) was dissolved in a solvent mixture (1 ml) consisting of $CHCl_3$ and CH_3OH, and the spectra were recorded.

2.5. Immunisation protocols

Experimentation strictly adhered to the 1986 Scientific Procedures Act (UK). All protocols have been subjected to stringent ethical review and were carried out in a designated establishment. Groups of five female BALB/c mice, approximately 6 weeks old, received doses of vaccine formulations containing DDA at 250 μg per dose, TDB at 50 μg per dose and 2 μg of Ag85B-ESAT-6 (H1 fusion protein) in 50 μl volume. Vaccine formulations were administered intramuscularly, and each mouse received three doses at intervals of two weeks. Serum samples were taken at 12 days after the first administration and at two week intervals thereafter.

2.5.1. Analysis of H1-specific antibody isotypes

Serum samples obtained at different time intervals after immunisation were analysed for the presence of anti-H1 IgG1, IgG2a and IgG antibodies by enzyme-linked immunosorbent assay (ELISA). ELISA plates (flat bottomed, high binding; Greiner Bio-One Ltd., Glos. UK) were coated with 60 μl of H1 per well (3 μg/ml) in PBS and incubated at 4 °C overnight. Unbound antigen was aspirated, and residual washings were removed by blotting firmly onto paper towel. Plates were blocked with 0.2 ml per well of 4% w/v Marvel (Premier Int. Foods Ltd., Lincs, UK) in PBS. Serially diluted serum samples (60 μl per well) were transferred to washed plates and incubated for 1 h at 37 °C. Anti-H1 antibodies were detected by the addition of horseradish peroxidase-conjugated anti-mouse isotype-specific immunoglobulin (goat anti-mouse IgG1, IgG2a or IgG; Oxford Biotechnology, Oxfordshire, UK), and subsequent addition of substrate solution, 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS; Sigma, Poole, Dorset, UK) in citrate buffer incorporating 5 μl of 30% H_2O_2/50 ml following repeated incubation and washing steps. Absorbance was measured at 405 nm (Bio-Rad, Herts, UK).

2.5.2. Spleen cell culture preparation

Upon termination of experiments, mice were humanely culled, and their spleens were aseptically removed and placed into ice-cold sterile PBS. Spleens were treated as follows: A crude suspension of spleen cells in 10 ml working media (RPMI 1640 cell culture medium supplemented with 10% (v/v) foetal bovine serum, 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml) (Gibco-Invitrogen, Paisley, UK) was prepared by gently grinding the spleen on a fine wire screen. After allowing the cell suspension to settle for approximately 5 min, the liquid was transferred to sterile 20-ml 'Falcon' tubes, without disturbing the cellular debris at the bottom. The cell suspension was centrifuged at 200*g* for 10 min. After centrifugation, the supernatant was removed, the cell pellet was resuspended in 10 ml fresh working media and the centrifugation procedure was repeated. These single-cell suspensions were used to assess antigen-specific cytokine production and antigen-specific recall responses.

2.5.3. Analysis of spleen cell proliferation

For the study of antigen-specific proliferative responses, aliquots of 150 μl volumes of sterile media or antigen in sterile media (at the concentrations stated) were seeded onto 96-well suspension culture plates (Greiner Bio-One Ltd., Glos., UK) and 150 μl volumes of viable splenocytes (approximately 1×10^7 cells/ml) added to each well. As a positive control, cells were co-cultured with concanavalin A (Sigma, Poole, Dorset, UK) at a concentration of 3 μg/ml. Covered plates were incubated in a humid, 5% CO_2 environment at 37 °C for 72 h. After 72-h incubation, half a microcurie of [^{3}H] thymidine (Amersham, UK) in 40 μl volumes of freshly prepared sterile working media was added to each well, and the incubation continued for a further 24 h. The well contents were harvested onto plain filter mats (Molecular Devices Ltd., Wokingham, UK) using a cell harvester (Titertek). After drying, the discs representing each well were punched from the filter mats into 5 ml volumes of scintillation fluid (Optiphase Hisafe III, Fisher Scientific UK Ltd., Loughborough), and the incorporation of [^{3}H] thymidine into the cultured cells was measured using standard counting procedures.

2.5.4. Analysis of cytokine production

Cytokines were detected by taking cell culture supernatants after 48 h of incubation with 2.5 μg/ml H1 fusion protein. The cell medium was separated by centrifugation, collected in eppendorfs and stored at −70 °C until analysed using DuoSet® capture ELISA kits (mouse IFN-γ, IL-2, IL-5, IL-6) purchased from R&D systems, Abingdon, UK, according to the manufacturer's instructions.

3. Results

3.1. Optimisation of cryoprotectant concentration

The characteristics of DDA/TDB liposomes have previously been described in detail [15], and the characteristics of the liposomes prepared for this study were in line with these previous studies with liposomes in the size range of 428 ± 124 nm and a zeta potential of ∼45 mV prior to freeze drying (data not shown). These liposomes were then freeze-dried in the absence of any lyoprotectant, and following freeze drying and subsequent hydration, the formulations showed a similar zeta potential but extensive vesicle aggregation with sizes over 1500 nm, suggesting that a lyoprotectant is necessary for product stabilisation (Fig. 1).

Given that previous studies have shown that sugars could be used as suitable protectants for liposomes during freeze drying [18,19], two sugars (trehalose and sucrose) and an amino acid (lysine) were tested as to their potential for stabilising the systems during freeze drying using a pre-optimised freeze-drying protocol. Initial pre-screening of each of these was undertaken at a range of protectant/lipid concentrations (2/1 to 10/1), and the concentration that promoted the best protection against increased vesicle

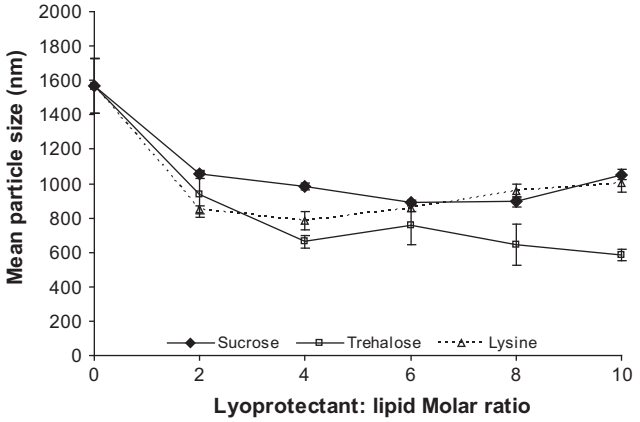

Fig. 1. The effect of lyoprotectant/lipid molar ratio on vesicle particle size after freeze drying. DDA/TDB MLV liposomes incorporating mycobacterial fusion protein Ag85B-ESAT-6 (H1; 10 μg) were prepared with various mol/mol ratios of sucrose, trehalose and lysine. Liposomes prior to freeze drying were 497 ± 113 nm. All values shown are the average values from three independent samples ± standard deviation.

size was identified and is summarised in Fig. 1. All protectants tested demonstrated a concentration-dependent effect on stabilising the DDA/TDB liposomes against aggregation with the trend in liposome stabilisation being related not only to the type of cryoprotectant employed but also to the concentration with ratios of 4/1 being the most effective for lysine, whilst high concentrations of 8/1 and 10/1 were required for sucrose and trehalose, respectively (Fig. 1). Therefore, whilst all the formulations after freeze drying were significantly ($P < 0.05$) larger in sizes compared to freshly prepared liposomes (~500 nm in size), given our previous studies [11] which had also shown the similarities in lyoprotection efficacy of sucrose and trehalose and the potential lower cost of using sucrose in production, sucrose at a 8/1 lyoprotectant/lipid molar ratio as a potential sugar-based lyoprotectant and lysine (at 4/1 molar ratio) as a possible novel amino acid-based protectant were taken forward for further analysis.

3.2. Gamma sterilisation

The potential of employing gamma irradiation to end-sterilise these liposomal systems was also investigated so as to produce a freeze-dried product that would have the potential to be applied in a clinical setting. DDA/TDB liposomes were formulated with Ag85B-ESAT-6 antigen (1.25 mg/ml DDA; 250 μg/ml and antigen 10 μg/ml) and freeze-dried either without a protectant or in the presence of sucrose or lysine at the ratios identified in Fig. 1. The various physico-chemical properties measured prior to and following gamma radiation at a dose of 25 KGy included particle size, zeta potential, dynamic viscosity, pH and moisture content, and these characteristics pre and post sterilisation are shown in Fig. 2. Results show that each of the parameters measured were reproducible and not significantly different ($P > 0.05$) following exposure to the recommended dose of gamma radiation (Fig. 2) with vesicles being around 700–800 nm in size (Fig. 2a), having zeta potentials of 40–45 mV (Fig. 2b) and dynamic viscosity values between 0.8 and 1.1 mPa s (Fig. 2c) when protectants were used in the formulations. Again, pH values were not influenced by sterilisation; however, the choice of protectant did make a difference, with formulations containing lysine having a slightly higher pH of ~7.8 compared to sucrose formulations (pH values of ~7.4; Fig. 2d). Analysis of residual moisture content using TGA showed that moisture content retained in the various formulations tested was in the range of 2–3.5% w/w (Fig. 2e), which is in line with the acceptable

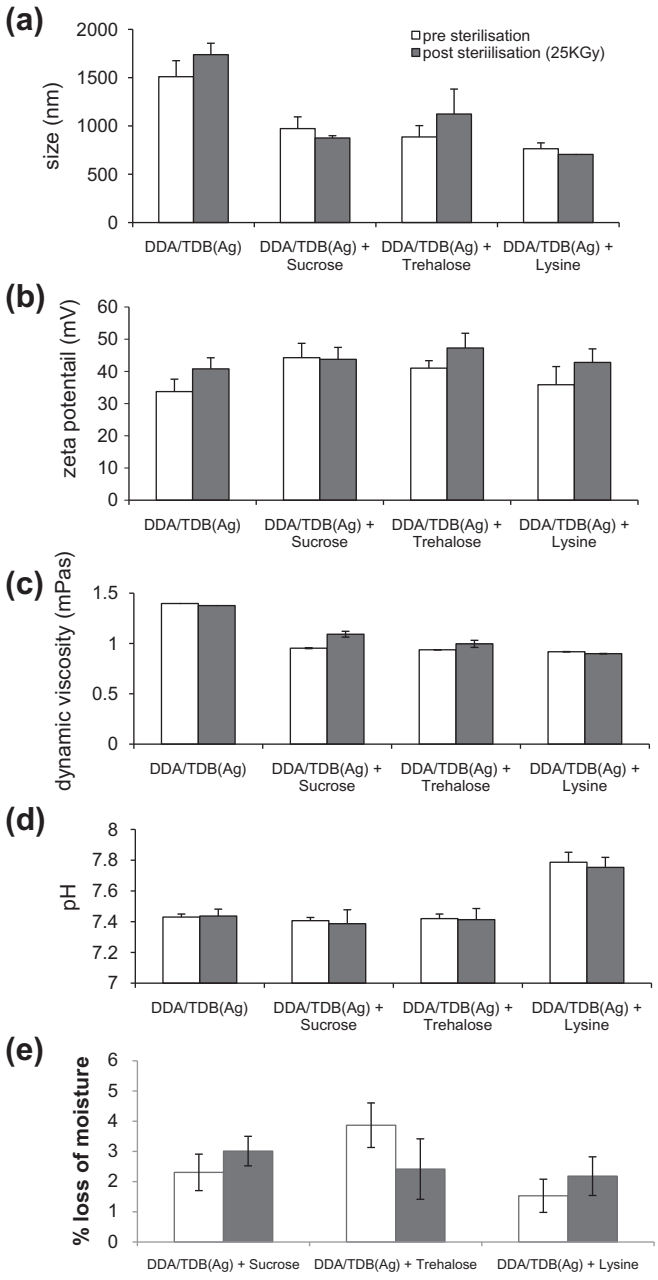

Fig. 2. Measurement of (a) mean volume diameter, (b) zeta potential, (c) dynamic viscosity, (d) pH and (e) residual moisture content of liposomal suspensions prior to and post sterilisation. DDA/TDB MLV liposomes incorporating mycobacterial fusion protein Ag85B-ESAT-6 (H1; 10 μg) were prepared in the absence and presence of optimised mol/mol ratios of sucrose (8/1), trehalose (10/1) and lysine (4/1). All values shown are the average values from three independent samples ± standard deviation.

limits recommended by the British Pharmacopeia and other regulatory authorities. The release of antigen from the liposome systems before and after sterilisation in the presence of lysine was also undertaken, and again no significant difference in release kinetics was shown after sterilisation compared to prior to the irradiation (Fig. 3).

Chemical analysis of the lipid components was also conducted, and the spectra from both the NMR and mass spectrometry pre and post gamma sterilisation and comparison of the scans suggested the absence of chemical degradation (Table 1).

These samples were also examined for stability on storage with the characteristics of freshly prepared formulations compared to

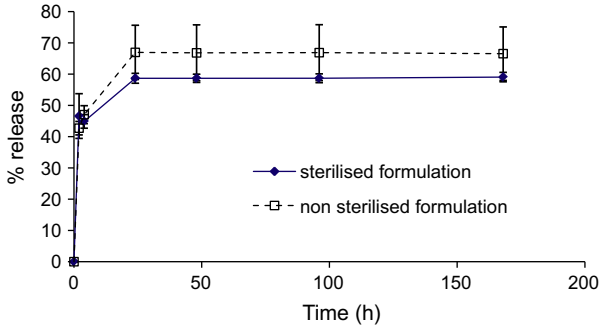

Fig. 3. Antigen release from liposomes pre and post sterilisation. Antigen release was monitored using radio-labelled H1 antigen.

Table 1
Summary of chemical analysis of lipids (mass spectrometry and NMR data values) before and after sterilisation.

	Before irradiation	After irradiation
m/z	551	551
Methyl group (non-shielded)	0.84 ppm	0.84 ppm
Methylene group	1.20 ppm	1.20 ppm
Methyl group (nitrogen shielded)	3.37 ppm	3.37 ppm

formulations, freeze-dried/sterilised and stored for 1 year at 25 °C ± 2 °C/60% RH ± 5% RH and under accelerated stressed conditions of 40 ± 2 °C/75 ± 5% RH. Results in Fig. 4 show that there was no significant difference in particle size, zeta potential or viscosity after 1-year storage in standard conditions and the equivalent of 2 years under accelerated conditions (Fig. 4a–c) respectively). In terms of moisture content, samples using trehalose as a lyoprotectant showed significant ($P < 0.05$) increases in measured moisture content when stored under accelerated conditions, whilst lysine also showed a trend of increased residual moisture upon storage compared to samples prior to storage (Fig. 4d). Previous studies have looked at the factors influencing changes in moisture content during the storage of freeze-dried vaccines [20], and they suggest a range of possible mechanisms for variations in water content including microleakage at the close seal and water vapour transfer through the closure. Given the lower initial moisture contents of the trehalose and lysine formulations (~1%; Fig. 4d), these systems would be more hygroscopic in nature and perhaps more prone to moisture uptake. Therefore, whilst all formulations were in sealed vials, subjecting these formulations to 60–75% RH (depending on standard or accelerated conditions) may have resulted in some moisture ingress and uptake by the low moisture content formulations and could be circumvented by further modification of the capping process, such as sealing the vials under vacuum, secondary crimping of the seals and possibly injecting an inert gas before sealing.

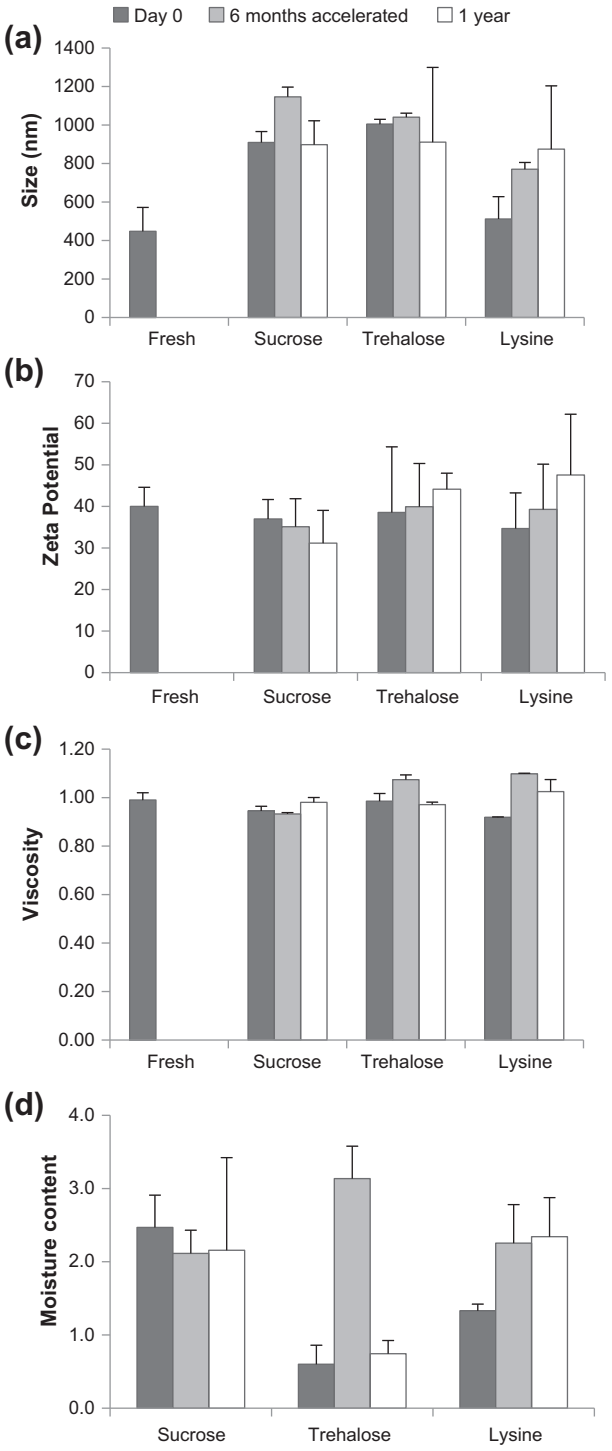

Fig. 4. Measurement of (a) mean volume diameter, (b) zeta potential, (c) dynamic viscosity and (d) residual moisture content of liposomal suspensions freshly prepared and subsequently freeze-dried and sterilised and stored for 1 year at 25 °C/60% RH and 6 months at 40 °C/75% RH. DDA/TDB MLV liposomes incorporating mycobacterial fusion protein Ag85B-ESAT-6 (H1; 10 μg) were prepared in the absence and presence of optimised mol/mol ratios of sucrose (8/1), trehalose (10/1) and lysine (4/1). All values shown are the average values from three independent samples ± standard deviation.

3.3. Maintenance of immunogenicity

To investigate the efficacy of these systems *in vivo*, immunisation studies were carried out where freshly prepared formulations (group 1) and formulations freeze-dried with lysine or sucrose (groups 2 and 3) were used. Mice were also immunised with DDA/TDB/H1 formulations which were freeze-dried with lysine and then gamma-sterilised (group 4). These groups were also compared to un-immunised naïve mice (group 5) or mice immunised with freshly prepared DDA/TDB/H1 containing lysine at isotonic concentrations but not subjected to freeze drying or gamma irradiation. For our analysis, we have looked at immunological parameters associated with the ability to rapidly respond to disease – antigen-specific antibody levels, the ability of spleen cells to respond to re-stimulation with antigen and more detailed analysis by antibody subtype investigation and scrutiny of the production of cytokines by proliferating spleen cells.

In terms of immunogenicity, from the antibody response data, for both IgG and IgG1 (Fig. 5A and B, respectively) at each of the time points measured, there were similar levels of responses between each of the DDA/TDB formulations, which were either freshly prepared (group 1), freeze-dried in the presence of lysine or sucrose (groups 2 and 3 respectively) or freeze-dried with lysine and subsequently gamma-irradiated (group 4). However, the freshly prepared DDA/TDB formulation (group 1) did give increased H1-specific IgG2a in comparison to the other formulations at the latest time point, 24 days after the last immunisation ($n = 5$, $P < 0.05$ in comparison with all other groups; Fig. 5C).

In contrast to this, there were more clearly observable differences between the groups when H1-specific spleen cell proliferation and cytokine production were analysed (Fig. 6). The group that received the freeze-dried/sterilised DDA/TDB formulated with lysine (group 4) showed higher levels of spleen cell proliferation in comparison with most other groups when stimulated with H1 at concentrations of 0.25 and 2.5 μg/ml (Fig. 6); however, the freeze-dried/sterilised DDA/TDB (group 4) was not significantly better than the corresponding non-sterilised formulation (i.e., freeze-dried DDA/TDB with lysine; group 2), yet group 4 did show H1-specific spleen cell proliferation levels higher than any of the other groups (Fig. 6, $n = 5$, $P < 0.05$). Apart from this, however, there were no differences between the vaccine formulations at either 0.25 or 2.5 μg/ml H1 concentrations.

Concomitant cytokine production revealed subtle differences between groups from the different cytokines assessed (Fig. 7). Only the groups receiving DDA/TDB with lysine or sucrose as the cryo/lyoprotectant (groups 2 and 3; Fig. 7A) showed significantly increased IL-5 production in comparison with the control group, and the only difference between immunised groups was that the freeze-dried DDA/TDB with lysine (group 2; Fig. 7A) gave enhanced IL-5 production in comparison with the freshly prepared DDA/TDB containing lysine in solution (group 6) (Fig. 7A; $n = 5$, $P < 0.05$). Analysis of H1-specific IL-6 production by splenocytes (Fig. 7B), on the other hand, revealed that all groups produced enhanced IL-6 relative to the naïve controls, but in a similar trend, all groups (except the naïve controls) showed increased IL-6 in comparison to the freshly prepared DDA/TDB (group 6) (Fig. 7B; $n = 5$, $P < 0.05$).

Interestingly, when H1-specific IL-2 was assessed, the vaccine formulation that had shown the highest cell proliferation (the freeze-dried sterilised DDA/TDB with lysine; group 4) also showed significantly increased IL-2 levels in comparison with all the other formulations ($n = 5$, $P < 0.05$) except the freshly prepared DDA/TDB (group 1; Fig. 8A). Also, there was a difference between the freshly prepared DDA/TDB formulation (group 1) with the formulation that was normalised for tonicity using lysine (group 6) with signif-

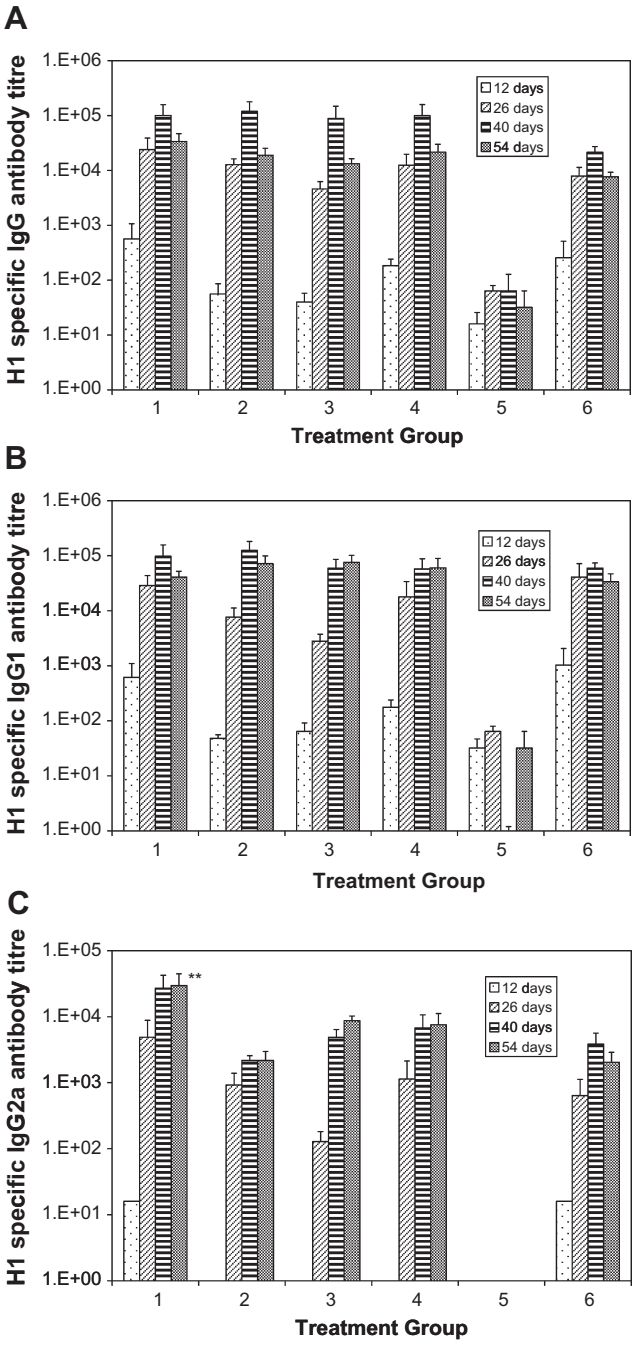

Fig. 5. H1-specific antibody titres. 1. DDA/TDB (freshly prepared); 2. DDA/TDB (freeze-dried – lysine); 3. DDA/TDB (freeze-dried – sucrose); 4. DDA/TDB sterilised (freeze-dried – lysine); 5. naïve control; 6. DDA/TDB (freshly prepared and containing isotonic concentrations of lysine). Vaccine doses for groups 1–4 were standardised regarding tonicity. Group 1 was given the vaccine dose initially identified as providing optimal immunogenicity [13,15]. (A) H1-specific IgG; (B) H1-specific IgG1; (C) H1-specific IgG2a. ** Denotes significantly increased H1-specific IgG2a in comparison with the other formulations at the latest time point ($n = 5$, $P < 0.05$).

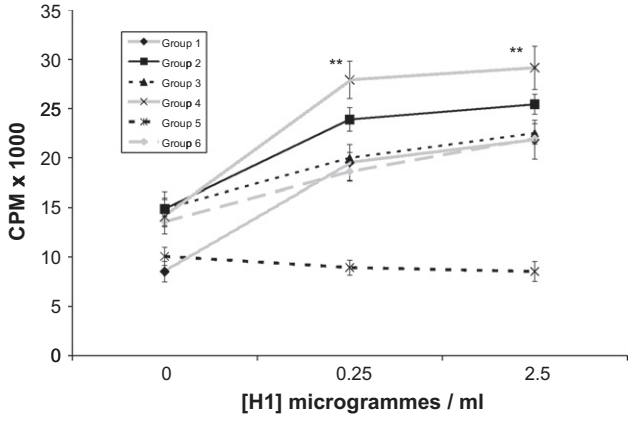

Fig. 6. Spleen cell proliferation in response to stimulation/re-stimulation with H1 antigen. 1. DDA/TDB (freshly prepared); 2. DDA/TDB (freeze-dried – lysine); 3. DDA/TDB (freeze-dried – sucrose); 4. DDA/TDB sterilised (freeze-dried – lysine); 5. naïve control; 6. DDA/TDB (freshly prepared with isotonic lysine). Vaccine doses for groups 1–4 were standardised regarding tonicity. Group 1 was given the vaccine dose initially identified as providing optimal immunogenicity [13,15]. ** Denotes significantly increased level of proliferation in comparison with all other groups except group 2 ($n = 5$, $P < 0.05$). All H1-stimulated cells from immunised groups had higher levels of cell proliferation in comparison with the naïve controls ($n = 5$, $P < 0.05$).

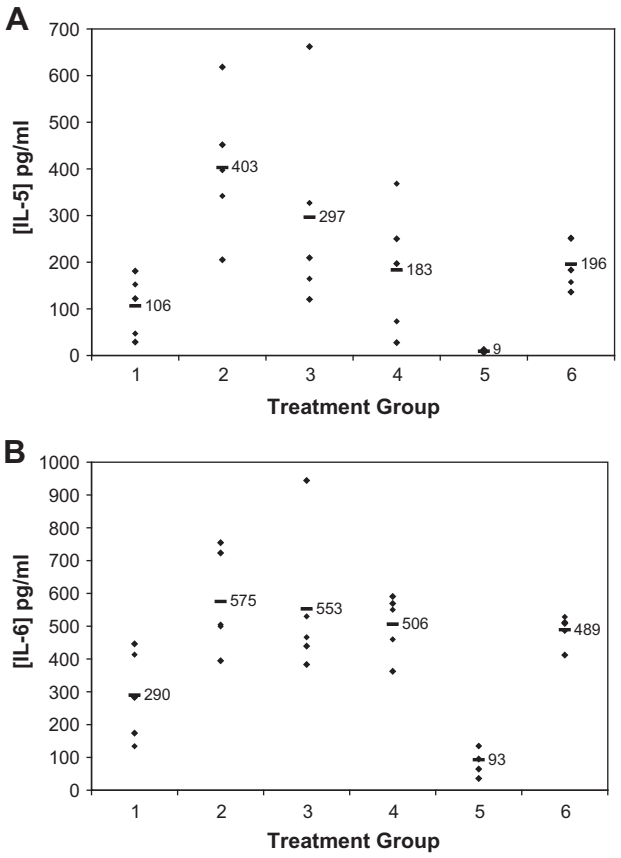

Fig. 7. H1-specific IL-5 and IL-6 production. (A) IL-5 from stimulated splenocytes; (B) IL-6 from stimulated splenocytes. Treatment groups: 1. DDA/TDB (freshly prepared); 2. DDA/TDB (freeze-dried – lysine); 3. DDA/TDB (freeze-dried – sucrose); 4. DDA/TDB sterilised (freeze-dried – lysine); 5. naïve control; 6. DDA/TDB (freshly prepared with isotonic lysine). Vaccine doses for groups 1–4 were standardised regarding tonicity. Group 1 was given the vaccine dose initially identified as providing optimal immunogenicity [13,15]. Filled diamonds represent the mean of three measurements for each individual spleen. Horizontal lines represent average group values ($n = 5$) and are shown numerically on the chart.

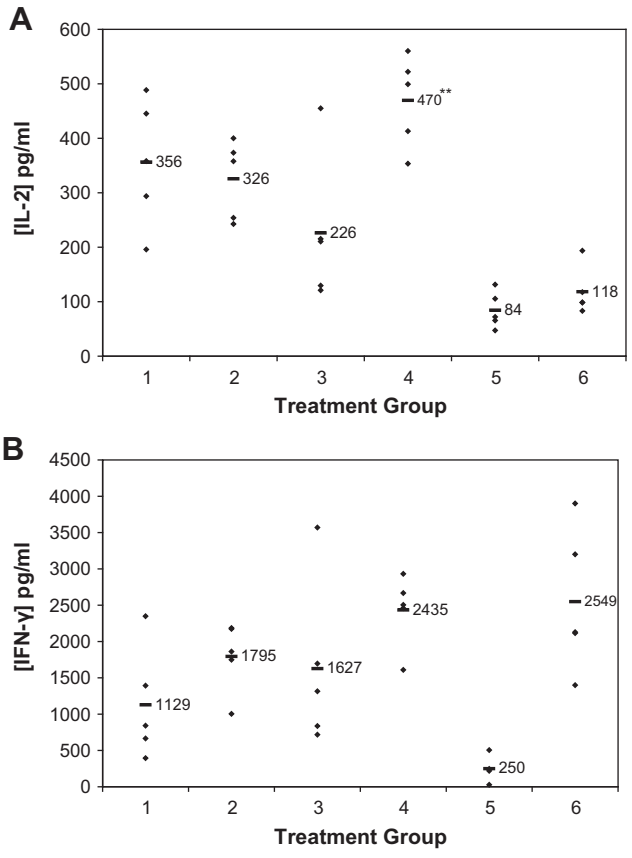

Fig. 8. H1-specific IL-2 and IFN-gamma production. (A) IL-2 from stimulated splenocytes. ** Denotes significantly increased IL-2 levels in comparison with all other formulations except treatment group 1 ($n = 5$, $P < 0.05$); (B) IFN-γ from stimulated splenocytes. Treatment groups: 1. DDA/TDB (freshly prepared); 2. DDA/TDB (freeze-dried – lysine); 3. DDA/TDB (freeze-dried – sucrose); 4. DDA/TDB sterilised (freeze-dried – lysine); 5. naïve control; 6. DDA/TDB (freshly prepared with isotonic lysine). Vaccine doses for groups 1–4 were standardised regarding tonicity. Group 1 was given the vaccine dose initially identified as providing optimal immunogenicity [13,15]. Filled diamonds represent the mean of three measurements for each individual spleen. Horizontal lines represent average group values ($n = 5$) and are shown numerically on the chart.

icantly higher H1-specific IL-2 being seen in group 1 ($n = 5$, $P < 0.05$).

Finally, comparing H1-specific IFN-γ (Fig. 8B), all groups receiving DDA/TDB/H1 formulations (groups 1–4 and 6) had significantly increased IFN-γ in comparison with controls ($P > 0.05$), and there were no other significant differences between the groups (Fig. 8B).

4. Discussion

Cryoprotectants have been widely used for the preservation of drug delivery systems (e.g., liposomes, microspheres) as well as biological molecules. The addition of a cryoprotectant during a freeze-drying regime helps in preventing the fusion of lipid membranes in the case of liposomes. The saccharides, which are the most extensively studied formulation protectors, essentially inhibit lipid fusion and phase separation and depress the transition temperature in the dry state without influencing membrane permeability [21]. The mechanism for formulation stabilisation is again an extensively discussed issue: Crowe et al. [22] have proposed that these cryoprotectants function by replacing the bound water of the lipid head group. They suggested that the polar region of the lipid head group interacts with the cryoprotectant, thus replacing the water around the bilayer (water replacement hypothesis). Alternatively, Koster et al. [23] proposed the formation of a vitreous layer of the cryoprotectant around the bilayer, which depresses the transition temperature of the phospholipids, thus preventing any drug leakage during gel-to-fluid-phase transformations. In terms of molecular interactions at the liposome, surface hydrogen bonding, van der Waals interactions and ion dipole interactions are the most often suggested mechanisms, and generally, hydrogen bonding along with the ability of the cryoprotectant to form a vitreous layer to suppress any translational motion of the molecules is a widely accepted mode of interactions for phospholipids. In terms of the effective cryoprotection of our DDA/TDB system, it can possibly be explained by three factors: (1) the cationic surface of the liposomes may interact with the dipole in the OH group of the various saccharides, (2) the ability to form a glass layer around the bilayer and concomitant lowering of the molecular mobility, together with the ability of the saccharides to form a spacing matrix between the liposomes [24] and (3) the possibility of interaction of the OH group in TDB with the OH of the saccharides by means of hydrogen bonding. Similarly, previous research has shown that the amino acids interact by means of formation of ion dipole bonds, which stabilises the freeze-dried protein [25], and given the basic nature of lysine, interactions between lysine and the anionic antigen used (Ag85B-ESAT-6 has an overall pI of 4.9 and is anionic at physiological pH ranges) may be involved in the stabilisation process.

The biphasic nature of the various cryoprotectants has been previously observed by other researchers. Suzuki et al. [26], studying the effect of concentration of glucose oligomers on freeze drying, have reported that when DPPC liposomes were lyophilised in the presence of maltotriose at either 7 mM or 15 mM, aggregation occurred; however, an intermediate concentration of 10 mM was found to be efficient in stabilising such vesicles. Similarly, Kundu et al. [27] reported that significant cryoprotection of goat sperm cells was offered by L-alinine, L-proline, glycine and L-glutamine. Protection was biphasic in nature, with an initial increase in the concentration of the cryoprotectants (up to 120 mM) supporting the recovery of the sperm cell mobility on rehydration, whereas further increases resulted in a loss of motility, suggesting cell damage upon rehydration. The decrease in cryoprotection efficiency beyond an optimum concentration of the stabilisers has been attributed to the increase in hydrophobicity around the bilayer which results in formulation destabilisation, thereby promoting fusion [28].

Gamma irradiation as a sterilisation technique has been widely investigated for pharmaceuticals. The application of this technique to develop sterile liposomes has, however, been limited. The damage caused by exposure to gamma radiation has been attributed to two factors: the direct influence of radiation on the bilayer and an indirect effect caused by the action of the reactive species that are generated by gamma radiation on the bilayer [29]. Additionally, chemical degradation of the lipids caused by the presence of unsaturated bonds due to peroxidation and formation of lysophospholipids, and free fatty acids with the eventual alteration of the hydrocarbon chain length has also been another factor [29,30]. The radiolysis of water results in the formation of reactive free radicals like OH, H and the hydrated electron [31]. The damage caused by the indirect pathway as a result of free radicals is far more deleterious when compared to the direct action on the lipid bilayers. The free radicals are very reactive and promote cascade of chain reactions with unlimited access to the lipid bilayer [31]. With the addition of radical scavengers e.g., tocopherol [32] sodium metabisulphite and more so, development of freeze-dried product [33] has shown promising results. However, the transition of formulation from a colloidal dispersion to a freeze-dried product has not been completely free of any chemical changes. ^{31}P NMR studies have shown that chemical changes have been observed when freeze-dried saturated phospholipid liposomes containing residual moisture were exposed to high-energy radiations, thought to be primarily due to indirect radical attack [33]. In interpretation of our results, it is possible that the residual moisture exists primarily as bound water interacting with the lipid head group and facilitates resistance to the generation of any reactive free radical species. However, the ^{1}H NMR protocol is concentration-limiting, and therefore, there is a possibility that some changes could escape this analysis. Previous investigations have also highlighted that the composition of the formulation with an emphasis on head group dictates the extent of degradation as seen in the case of DSPG liposomes, which were more sensitive when compared to DSPC liposomes [29,34,35]. There is limited published literature for the gamma sterilisation of non-phospholipids. The cationic lipid DDA consists of a quaternary nitrogen linked to the alkyl chain with the absence of any phosphate group. The chemical stability of the freeze-dried DDA liposomes can possibly be ascribed to two factors. Firstly, the presence of a cationic nitrogen at the head group could resistant radiation-induced damage unlike the negatively charged DSPG liposomes which showed a change in the phospholipid concentration post radiation [29,34,35]. Secondly, the development of a freeze-dried product with low levels of moisture content better resists any radiation-induced damage similar to previous reports [29,34,35].

Following sterilisation, the lack of any alterations in the liposome size can be due to the effective lyophilisation offered by the various protectants tested. Zuidam et al. [29] had highlighted that the presence of trehalose as a cryoprotectant in solution promotes radiation-induced damage. Similarly, Stensrud et al. [34] showed that despite the presence of saturated lipids in the formulations, radiation-induced damage was prevalent essentially due to the residual moisture content. The freeze-drying regime employed to generate the solid solutions with the DDA lipid was carefully optimised for the various stages ensuring the formation of a stable, amorphous and dry product with very low levels of moisture content well within the regulatory guidelines. Therefore, optimisation of the various stages of product development not only ensures the progress of formulation from one stage to another but can also have beneficial roles in other phases of development.

Defined correlates of protection against *M.* tuberculosis are still the subject of much debate. This facultative intracellular organism has evolved the ability to survive and multiply within human macrophages, and therefore, it is logical that a strong Th1 component may be effective against this highly important human pathogen. IFN-γ has long been associated with protection following initial convincing evidence [36,37]. More recently, however, it has become clear that this alone may not be sufficient and that other parameters may also need to be assessed [38]. Immune interaction with *M.* tuberculosis is highly complex [39], and it may be that some potential correlates of protection (such as IL-4δ2 [40]) may be difficult to assess or indeed may not even be present in some animal models [41]. Therefore, a broad analysis of immunogenicity provides a logical approach for the overall evaluation of the effects of formulation changes on performance in terms of relevant biological function of a vaccine against tuberculosis.

In terms of the maintenance of vaccine immunological characteristics, almost all of the antibody-mediated antigen-specific immune responses were comparable following the different modifications and treatments, with little apparent adverse effect on immunogenicity, with the exception that modified formulations were reduced in their ability to engender antigen-specific IgG2a in comparison to the freshly made preparation. However, cell proliferation results showed that the inclusion of lysine facilitated a superior level of antigen-specific spleen cell proliferation following radiation sterilisation. This is a surprising result as the inclusion of lysine has not previously been evaluated in this context. The analysis of IL-2 indicated that the increased cell proliferation observed following the use of lysine and sterilisation was probably due to T-cell expansion. However, this is not so for the non-freeze-dried, non-sterilised lysine formulation. This freshly made formulation showed lower levels of antigen-specific IL-2, whereas the inclusion of lysine plus sterilisation gave a higher level of antigen-specific spleen cell proliferation than any of the other groups, in conjunction with increased levels of IL-2. The mechanism of action of lysine certainly warrants further investigation. Delineation of the action of lysine in the context of both pharmaceutical and immunological roles would undoubtedly help to clarify a value and rationale for its use. Investigation into antigen-specific cytokine responses also failed to show any clear reduction in immunogenicity regarding the different modifications and treatments, and only the freshly made formulations showed the occasional reduced levels for some of the cytokines in terms of statistically significant results.

The extensive analysis outlined above has shown that the use of novel and optimised modifications to vaccine formulations can be successfully implemented to generate an improved vaccine product that meets essential criteria in terms of stability, sterility and immunogenicity. With new vaccines coming into clinical trials, including the Ag85B-ESAT-6 fusion protein [42], such comparative and broad non-clinical evaluation may help to underpin the

transition of vaccine formulations, such as those based on liposomes and delivery system technology, from the bench to the clinic.

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