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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