European Journal of Pharmaceutics and Biopharmaceutics 93 (2015) 231–241

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research Paper

Comparison of adjuvants for a spray freeze-dried whole inactivated virus influenza vaccine for pulmonary administration

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ARTICLE INFO

Article history:
Received 17 September 2014
Revised 25 March 2015
Accepted in revised form 9 April 2015
Available online 17 April 2015

Keywords:
Whole inactivated virus
Pulmonary vaccination
Spray freeze-drying
Adjuvants

ABSTRACT

Stable vaccines administered to the lungs by inhalation could circumvent many of the problems associated with current immunizations against respiratory infections. We earlier provided proof of concept in mice that pulmonary delivered whole inactivated virus (WIV) influenza vaccine formulated as a stable dry powder effectively elicits influenza-specific antibodies in lung and serum. Yet, mucosal IgA, considered particularly important for protection at the site of virus entry, was poorly induced. Here we investigate the suitability of various Toll-like receptor (TLR) ligands and the saponin-derived compound GPI-0100 to serve as adjuvant for influenza vaccine administered to the lungs as dry powder. The TLR ligands palmitoyl-3-cysteine-serine-lysine-4 (Pam_3CSK_4), monophosphoryl lipid A (MPLA) and CpG oligodeoxynucleotides (CpG ODN) as well as GPI-0100 tolerated the process of spray freeze-drying well. While Pam_3CSK_4 had no effect on systemic antibody titers, all the other adjuvants significantly increased influenza-specific serum and lung IgG titers. Yet, only GPI-0100 also enhanced mucosal IgA titers. Moreover, only GPI-0100-adjuvanted WIV provided partial protection against heterologous virus challenge. Pulmonary immunization with GPI-0100-adjuvanted vaccine did not induce an overt inflammatory response since influx of neutrophils and production of inflammatory cytokines were moderate and transient and lung histology was normal. Our results indicate that a GPI-0100-adjuvanted dry powder influenza vaccine is a safe and effective alternative to current parenteral vaccines.

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1. Introduction

Influenza virus is well known for its ability to cause seasonal epidemics and occasional pandemics. The virus is transmitted by aerosols and binds to and infects epithelial cells in the respiratory tract [1]. Pre-existing secretory IgA in the nose plays a vital role in preventing binding of influenza virus to its cellular receptor and further spreading of the virus in the respiratory tract [2,3]. Moreover, IgA is known for its ability to provide crossprotection against heterologous influenza virus [3,4]. However, currently available inactivated influenza vaccines are administered by the intramuscular, subcutaneous or intradermal route and do not induce mucosal IgA [5].

Induction of mucosal immunity requires vaccine administration to mucosal sites. Recently, the lung has appeared as a particularly attractive target site for mucosal vaccination since it has a large surface and harbors effective immune mechanisms [6,7]. Upon interaction with e.g. respiratory viruses, airway epithelial cells produce cytokines and chemokines promoting recruitment of inflammatory cells and directing the adaptive immune response according to stimuli received [8]. Antigen presenting cells like dendritic cells (DC), present beneath the epithelial layer, and alveolar macrophages (AM), present in the lumen, constantly patrol the large surface area of the lung [9,10]. Upon activation by pathogens, these epithelial cells, DC and AM effectively bypass the steady state anti-inflammatory T helper 2 (Th2) responses in the lung and initiate innate and adaptive immunity against the invading pathogen [11].

We previously showed that pulmonary delivery of spray freeze-dried (SFD) whole inactivated virus (WIV) influenza vaccine induces the production of systemic IgG antibody levels similar to those evoked by standard intramuscular (i.m.) immunization without causing inflammation in the lungs [12]. Pulmonary vaccination also resulted in induction of IgA antibodies in nose and lungs. However, the levels of IgA in the respiratory tract and the amount of systemic IgG2a antibodies, the protective antibody subclass in

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http://dx.doi.org/10.1016/j.ejpb.2015.04.004
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mice, were low [12]. An approach to overcome this drawback is addition of a potent adjuvant to the pulmonary vaccine.

An important class of adjuvants is Toll like receptor (TLR) ligands. TLR ligands are pathogen associated molecular patterns or substances mimicking such patterns that are recognized by TLRs present on the plasma membrane or the endosomal membranes of host cells. Binding of TLR ligands to TLRs induces engagement of the adaptor proteins MyD88 or TRIF, followed by further downstream signaling and eventually the activation of transcription factors. The most important transcription factors engaged upon TLR signaling are NfκB, driving the expression of pro-inflammatory cytokines, and interferon-regulatory factors (IRF) 3 and 7, driving expression of type I interferons [13,14]. Another class of adjuvants consists of saponins, glycosides usually derived from the bark of the Molina tree (*Quillaja saponaria*) which are composed of a triterpenoid backbone with complex oligosaccharide substituents. GPI-0100 is a semisynthetic derivative of the saponin Quil A with improved stability and strongly reduced toxicity [15,16]. GPI-0100 is not recognized by known pattern recognition receptors (PRRs) and its mechanism of action is so far poorly understood.

Our previous study showed that addition of a low amount (0.625 μg) of monophosphoryl lipid A (MPLA), a TLR4 agonist, to pulmonary delivered WIV improved IgA levels in the lung, but had little effect on IgA titers in the nose [17]. Therefore, to find a more effective pulmonary adjuvant we performed a head-to-head comparison of several candidate adjuvants. Among the TLR ligands we choose for Pam_3CSK_4 (recognized by TLR1 and TLR2), MPLA (TLR4) and CpG oligodeoxynucleotide (CpG-ODN-1826, TLR9). All these adjuvants have been described to improve humoral responses against parenterally administered split or subunit influenza vaccines [18,19] and have been successfully used as mucosal adjuvants [20,21]. Furthermore, we included GPI-0100 as adjuvant candidate. We earlier showed that pulmonary vaccination with GPI-0100-adjuvanted influenza subunit vaccine, administered as aerosol, induces potent mucosal and systemic antibody responses as well as efficient protection against virus challenge [7].

To find an optimal adjuvant that is compatible with formulation of the vaccine as dry powder and that effectively enhances systemic and mucosal immune responses to pulmonary administered vaccine, in this study, WIV was SFD along with the above mentioned adjuvants using inulin as stabilizer [12]. Inulin was chosen as stabilizer since it renders powders with excellent physiochemical and immunological stability during processing and during storage for 3 months at 30 °C [22,23]. For each of the adjuvants the amount used per vaccine dose was chosen on basis of the minimal amount required for effective adjuvantation according to the literature. We evaluated the compatibility of the adjuvants with the process of spray freeze-drying, and determined in mice the safety of pulmonary administration of the adjuvanted SFD vaccines, and the capacity of the vaccines to induce systemic and mucosal immune responses and protect from heterologous virus challenge.

2. Material and methods

2.1. Virus

A/PR/8. H1N1 (A/PR/8) was cultured in embryonated eggs as described previously [12].

2.2. Vaccine preparation

WIV derived from A/California/07/2009 H1N1 (A/Cal) was kindly provided by Solvay Biologicals (Weesp, The Netherlands). WIV was dialyzed overnight at 4 °C against HBS, pH 7.2, and protein content was determined by micro-Lowry assay. Purity of WIV was confirmed by SDS–PAGE followed by silver staining. The hemagglutinin (HA) content was assumed to be one third of the total protein for whole inactivated virus (based on the known protein composition of influenza virus and the molecular weight of the viral proteins). Adjuvanted vaccine solutions were prepared by adding adjuvant to WIV based on the ratios described in the literature for vaccine formulations and the experiments performed on RAW-Blue™ cells for NFkB activation (supp. Fig. 1). The amount of adjuvants used per vaccine dose (2.5 μg HA of WIV) was 2.5 μg Pam_3CSK_4 (EMC Microcollections GmbH, Tubingen, Germany) [20], 2.5 μg MPLA (Avanti Polar Lipids, Inc, Alabama, USA) [24], 10 μg CpG ODN 1826 (Eurogentec, Maastricht, The Netherlands) [25] or 12.5 μg GPI-0100 (Hawaii Biotech, Hawaii, USA).

2.3. Spray freeze-drying

Powder vaccine was prepared using inulin from dahlia tubers, MW ~5000, as stabilizer (Sigma–Aldrich, Chemie B.V. Zwijndrecht, The Netherlands). Spray freeze-drying was performed using previously optimized conditions [12,17]. Briefly, a mixture of WIV in HBS buffer containing 5% w/v inulin was prepared at an HA to inulin (w/w) ratio of 1:200. The mixture was then pumped at a flow rate of 5 ml/min through a two-fluid nozzle (diameter 0.5 mm) of a Büchi 190 Mini Spray Dryer (Büchi, Flawil, Switzerland) and sprayed in liquid nitrogen using an atomizing air flow of 600 l_n/h. After evaporation of the liquid nitrogen, the frozen droplets containing the active ingredients and inulin were placed on the shelf pre-cooled to a temperature of −55 °C of a Christ Epsilon 2–4 freeze dryer. Drying was performed at a pressure of 0.220 mBar with a condenser temperature of −85 °C. The shelf temperature was gradually increased from −55 °C to 4 °C during 32 h. Subsequently, the pressure was decreased to 0.055 mBar, and the shelf temperature was gradually increased to 20 °C during 11 h. The powder vaccine was then collected in a cabinet at a relative humidity of less than 10% and then stored at 4 °C in sealed injection vials until further use.

2.4. Physical characterization of powder vaccine

Geometric particle size distribution of powder vaccines was measured as described previously [17,23]. X10, X50 and X90 values of the geometric diameter were determined from cumulative undersize curves and the span was calculated as (X90–X10)/X50. The aerodynamic size distribution was calculated from the geometric particle size distribution by the following equation [26,27]:

$$d_{ae} = d_e\sqrt{\frac{\rho_p}{\rho_o\chi}}$$

where d_{ae} is the aerodynamic diameter, d_e the geometric particle size, ρ_p the density of the particles (g/cm^3), ρ_0 the unit density (1 g/cm^3) and, χ the dynamic shape factor. The density of the SFD particles was earlier determined to be 0.05 g/cm^3 and the shape was found to be spherical in scanning electron microscopy [23,26].

The specific surface area of powder vaccines was measured using a Tristar surface analyzer (Micrometrics Instrument Corp., Norcross, USA) as described previously [23].

Scanning electron microscope (SEM) images of powder vaccines were taken using a JEOL JSM 6301-F microscope (JEOL Ltd., Tokyo, Japan) as described previously [23]. Images were taken at magnification of 1000×.

2.5. Characterization of the cell-stimulating capacity of adjuvanted vaccines

The cell-stimulating capacity of adjuvants and adjuvanted vaccines before and after spray freeze-drying was measured using

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