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Research paper

In vivo investigation of twin-screw extruded lipid implants for vaccine delivery

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ABSTRACT

Sustained release systems have become the focus of attention in vaccine delivery as they may reduce or prevent the need for repeated dosing. In this work, lipid implants were prepared by twin-screw extrusion and investigated as vaccine delivery systems *in vivo*. The lipid implants consisted of cholesterol, soybean lecithin, and Dynasan 114. Ovalbumin (OVA) was employed as a model antigen and Quil-A (QA) as an adjuvant. In addition, OVA and QA loaded liposomes were prepared by the lipid-film hydration method, freeze-dried and then added to the lipid matrix prior to extrusion. Implants were administered subcutaneously and the kinetics of antigen release as well as the overall immune response stimulated were analysed by measuring $CD4^+$ and $CD8^+$ T cell proliferation, OVA-specific IgG production as well as cytokine (IFN-γ and IL4) secretion. Vaccine release from the implants was completed by 14 days. Inclusion of adjuvant into the implants was required for the generation of cellular and humoral immune responses. Inclusion of liposomes into the implant did not enhance the resulting immune responses generated.

1. Introduction

Modern vaccines often utilise subunit antigens such as peptides or proteins instead of the whole pathogen for safety and purity reasons [1]. However, subunit vaccines need to be administered multiple times in combination with immunostimulatory adjuvants in order to induce immunity [1]. This is because subunit vaccines are less immunogenic than whole-pathogen vaccines as they lack secondary signals required for the stimulation of immune responses [2].

The most commonly used and licensed adjuvant is alum. While the exact mode of action of alum is still point of discussion in the research community, a prolonged release from an antigen depot has been proposed to be important [3,4]. Thus, single-shot administration of a vaccine with sustained (7–10 days) and synchronised release of the antigen and adjuvant is a potential alternative to giving multiple vaccine doses and one which will potentially induce superior immune responses.

Different systems for the controlled release of vaccines have been evaluated, including controlled release particles, implants and depot systems. A variety of materials have been analysed, with early studies using non-degradable polymers, requiring surgical removal of the system after the drug has been released, and more recent studies utilising biodegradable systems [5]. Biodegradable PLGA nanoparticles have been investigated for the controlled release of protein antigens such as OVA and have shown great potential [6]. Systems can be developed to release antigen for a range of times from days up to months [6].

Implants as vaccine delivery systems have been investigated since the early 1970s. These implants consisted either of silicone, ethylene–vinyl–acetate copolymer, or collagen.

Later, lipid implant systems for sustained release of drug or antigen were investigated [7–9]. The majority of the implants were prepared by direct compression, a technique that can be applied in the laboratory setting, but is challenging for scale-up to larger batch sizes and the resulting disk-shaped implants are not feasible for human use. Using cholesterol and L-α-phosphatidylcholine in combination with an adjuvant, Myschik et al. [9] was able to show that lipid implants manufactured by direct compression were able to stimulate immune responses comparable to two immunisations

Abbreviations: OVA, ovalbumin; QA, Quil-A; D114, Dynasan 114; CHOL, cholesterol; FITC-OVA, ovalbumin, florescein CO; CFSE, 5,6-carboxy-fluoresceine diacetate succinimidyl ester; IFN-γ, interferon-γ; IL-4, interleukin-4.

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with an equivalent liquid vaccine [10]. Importantly, these implants stimulated CD8 immune responses – the type required for therapeutic cancer vaccines. Unfortunately, efficacy of cancer vaccines is low even with multiple doses of vaccine being given, demonstrating the need for new vaccine formulations [11].

The aim of the current work was to manufacture lipid implants for vaccine delivery by twin-screw extrusion and evaluate the efficacy of this implant system in stimulating immune responses *in vivo*. Twin-screw extrusion has been used successfully for the preparation of implants from triglycerides and mixtures of triglycerides for the sustained delivery of proteins [12], however, it has not been used to produce implants for vaccine delivery where the amount of active ingredient included is much lower. The extrusion process easily converts the raw materials into a product of uniform density and shape in a one-step production process by pushing it through a die under controlled conditions. Lipid implants consisting of cholesterol, soybean lecithin, and Dynasan 114 were prepared with a load of 20 μg antigen and 100 μg adjuvant. To combine the advantages of particulate delivery and sustained release, preformed liposomes were incorporated into the implants prior to extrusion.

After preparation, the implants were administered through a trocar into the subcutaneous tissue. For the analysis of the immune response, two sets of experiments were performed, one evaluating the kinetics of the release of the model antigen *in vivo*, a second one to evaluate the immune response *in vivo*. Ovalbumin in a liquid alum dispersion served as control.

2. Materials and methods

2.1. Materials

Ovalbumin from chicken egg white (OVA) grade V was purchased from Sigma–Aldrich. Ovalbumin, Fluorescein CO (FITC-OVA) from Life Technologies (Darmstadt, Germany). Cholesterol (CHOL), purity 95%, was purchased from AlfaAesar (Karlsruhe, Germany). Soybean Lecithin (approx. 90% phosphatidylcholine) was purchased from APPLICHEM LIFESCIENCE (Darmstadt, Germany). Purified Quil-A (QA) was sourced from Brenntag Biosector (Frederikssund, Denmark), as a lyophilised powder, and was used as supplied. Dynasan 114 (D114) was kindly provided by SASOL Germany GmbH (Witten, Germany). Chloroform (HPLC grade) was purchased from Fisher Scientific. Ultrapure deionised water having a conductivity of less than 0.055 mS (Milli-Q Water systems, Millipore, MA, USA) was used throughout the study. All other chemicals were of analytical grade.

The following agents were used for the immunological study: anti-CD16/CD32 antibody (2.4G2 Fc block), CD4-FITC, CD4-biotin, CD4 V500, CD8-APC, CD8-PE, CD8 PE-Cy 7, Vα2-PE, Vβ5.1-biotin, PI, CD122 FITC, CD44 APC, CD127 V450 and anti-CD3e (BD Biosciences). 5, 6-carboxy-fluoresceine diacetate succinimidyl ester was purchased from Molecular Probes. All single-cell suspensions were prepared in sterile complete Iscove's Modified Dulbecco's Medium [cIMDM; IMDM supplemented with 5% foetal bovine serum, 1% penicillin/streptomycin, 1% glutamax and 0.01% 2-mercaptoethanol all from Gibco Life Technologies (New York, USA)].

2.2. Methods

2.2.1. Preparation of lipid implants by twin-screw (tsc) extrusion

Implants were prepared from mixtures of soybean lecithin, CHOL, D114, with and without OVA and/or QA. Soybean lecithin and D114 were transferred into high grade stainless steel beakers for milling in a swing mill Retsch® CryoMill (Retsch Technology, Haan, Germany). After precooling the system with liquid nitrogen for 10 min at 5 Hz, soybean lecithin and D114 were ground for 1 min at 25 Hz. The obtained powder was mixed by hand, using a plastic mortar and pestle, with the remaining ingredients. The final mixture was then gradually blended with a mix of OVA and QA and subsequently fed into a twin-screw extruder (Haake MiniLab® Micro Rheology Compounder, Thermo Haake, Germany). The implants were extruded with closed bypass channel and a rotation speed of 40 rpm at an extrusion temperature of 45 °C. The resulting implants had a diameter of 2 mm and were subsequently cut into lengths of 0.5 cm, resulting in an implant mass of approximately 16 mg.

2.2.2. Preparation of implants by twin-screw extrusion containing preformed liposomes

Liposomes consisting of soybean lecithin and cholesterol were prepared using the lipid film hydration method as described previously [13]. Briefly, 0.9 mg of soybean lecithin and 0.23 mg of cholesterol were dissolved in 70 mL of chloroform [9]. The organic solvent was evaporated under reduced pressure in a water bath at 45 °C for approximately 1 h using a rotary evaporator (Laborota 4001, Heidolph, Germany). Residual chloroform was removed by flushing of the flasks with nitrogen. The thin lipid film was rehydrated by an aqueous solution (70 mL) containing OVA with or without QA. Glass beads were added to the flasks that were rotated for 1 h and an additional rehydration time of approximately 3 h was given for the samples to equilibrate. The liposome size and size distribution were determined using a Laser Scattering Particle Size Distribution Analyzer (LA-950, HORIBA Scientific). The obtained dispersion was then freeze-dried in a Christ Epsilon 2-6D freeze-drier (Christ, Germany) for a total duration of 44 h employing a conventional freeze-drying protocol.

Lyophilised powders were mixed to D114 and additional CHOL in a plastic mortar and subsequently fed into the twin-screw extruder. The extrusion was performed at 45 °C.

2.2.3. Applicability of tsc extrudates through trocar into cadaver pig skin

Extrudates of a length of 1 cm and a diameter of 2 mm were introduced into cadaver pig skin using a trocar with an inner diameter of 2 mm. After application into the pig skin, the skin was carefully cut open and the extrudates were retrieved. Pictures of the implants were taken before and after the application.

2.2.4. Texture analysis of implants

The mechanical stability, the hardness/softness of the lipid implants, was tested using a TA.XT *Plus* Texture Analyser (Stable Micro Systems). A stainless steel cylinder with a diameter of 5 mm was attached to the machine and used to compress the implants and thereby determine their hardness. For each measurement, $n = 3$ implants were analysed.

2.2.5. Differential scanning calorimetry (DSC)

All lipids were analysed by DSC (204 Phoenix, Netsch, Selb, Germany) prior to their use, to obtain a reference to identify eventual polymorph changes induced by processing or subsequent storage. Samples of approximately 4 mg were weighed into aluminium crucibles. Heating and cooling rates were set to 5 K/min between 20 °C and 160 °C. An empty crucible was used as the reference.

2.2.6. In vitro release of FITC-OVA from implants

FITC-OVA was incorporated into the implants to investigate the *in vitro* release of the model antigen. The extruded strand was cut into pieces of a length of 2.5 cm. The implants ($n = 3$) were placed into vials containing 1.8 ml PBS (pH 7.4, 0.01 M, 0.05% NaN_3) and incubated at 37 °C in a Heidolph Inkubator 1000. At defined time intervals samples were taken and the release medium was