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Immunogenicity of diphtheria toxoid and poly(I:C) loaded cationic liposomes after hollow microneedle-mediated intradermal injection in mice

Guangsheng Du[a], Mara Leone[a], Stefan Romeijn[a], Gideon Kersten[a,b], Wim Jiskoot[a], Joke A. Bouwstra[a,*]

[a] Division of BioTherapeutics, Leiden Academic Centre for Drug Research, Leiden University, Leiden 2300 RA, The Netherlands
[b] Department of Analytical Development and Formulation, Intravacc, Bilthoven 3720 AL, The Netherlands

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ABSTRACT

In this study, we aimed to investigate the immunogenicity of cationic liposomes loaded with diphtheria toxoid (DT) and poly(I:C) after hollow microneedle-mediated intradermal vaccination in mice. The following liposomal formulations were studied: DT loaded liposomes, a mixture of free DT and poly(I:C)-loaded liposomes, a mixture of DT-loaded liposomes and free poly(I:C), and liposomal formulations with DT and poly(I:C) either individually or co-encapsulated in the liposomes. Reference groups were DT solution adjuvanted with or without poly(I:C) (DT/poly(I:C)). The liposomal formulations were characterized in terms of particle size, zeta potential, loading and release of DT and poly(I:C). After intradermal injection of BALB/c mice with the formulations through a hollow microneedle, the immunogenicity was assessed by DT-specific ELISAs. All formulations induced similar total IgG and IgG1 titers. However, all the liposomal groups containing both DT and poly(I:C) showed enhanced IgG2a titers compared to DT/poly(I:C) solution, indicating that the immune response was skewed towards a Th1 direction. This enhancement was similar for all liposomal groups that contain both DT and poly(I:C) in the formulations. Our results reveal that a mixture of DT encapsulated liposomes and poly(I:C) encapsulated liposomes have a similar effect on the antibody responses as DT and poly(I:C) co-encapsulated liposomes. These findings may have implications for future design of liposomal vaccine delivery systems.

1. Introduction

Vaccination has become the most effective method for preventing infectious diseases, having led to the eradication of smallpox and severe restriction of other devastating diseases such as polio and measles (Jiang et al., 2017; Peek et al., 2008). However, there is still a need for new and better vaccines against emerging infectious diseases (Rappuoli et al., 2014). Nowadays, vaccination gains increasing attention also for therapeutic use against established diseases such as cancer and chronic auto-immune disorders (Melief et al., 2015). Most vaccines are delivered by intramuscular or subcutaneous injection. However, these injections need special training and can cause pain (Kim et al., 2012). To avoid the drawbacks of the hypodermic needles, microneedles have been developed. Microneedles are micro-sized needle structures with a length shorter than 1 mm and can be used to penetrate skin barrier in a non-invasive and pain-free way (Larraneta et al., 2016; Tuan-Mahmood et al., 2013; van der Maaden et al., 2012). Owing to the large number of antigen presenting cells in viable dermis and epidermis, dose-sparing may be achieved (Li et al., 2011).

Traditional vaccines are derived from attenuated organisms or inactivated pathogens and toxins. Attenuated vaccines have safety concerns as they may revert back to their virulent form (Reed et al., 2013). Inactivated vaccines like subunit antigens are safer but they are generally less immunogenic (Peek et al., 2008; Reed et al., 2013). To enhance and modify the immune response, immune modulators or nanoparticle delivery systems can be used (Zhao et al., 2014).

Ligands for toll-like receptors (TLRs) can be used as immune modulators to enhance the immune response against antigens by acting as a danger signal to the antigen-presenting cells. Among different types of TLR ligands, poly(I:C), which is a virus-associated double-stranded RNA, has been extensively investigated (Ammi et al., 2015). Poly(I:C) is a ligand for TLR3, which is located in the membrane of the endosomal compartments of dendritic cells. Previously, it was shown that compared to other TLR agonists, poly(I:C) induced a more effective IFN-γ secretion, which is an important linker of innate and adaptive immunity (Longhi et al., 2009). Furthermore, poly(I:C) has been shown to enhance anti-tumor immune responses and facilitate tumor elimination (Ammi et al., 2015).

* Corresponding author.
E-mail address: bouwstra@lacdr.leidenuniv.nl (J.A. Bouwstra).

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Nanoparticles have been shown to improve the immunogenicity of antigens by protecting the antigens from degradation, increasing their uptake by antigen-presenting cells and co-delivering antigens and immune modulators (Fan and Moon, 2015). Among different types of nanoparticle delivery systems, liposomes have been studied frequently because of their excellent biocompatibility and biodegradability (Giddam et al., 2012). Studies have shown that co-formulating antigen and TLR ligands in liposomes can enhance Th1 and $CD8^+$ T cell responses compared to mixture of antigen and adjuvant after intradermal vaccination. Some studies have shown that co-encapsulation of OVA with poly(I:C) or CpG in cationic liposomes significantly increased the IgG2a response (Th1 type) and the $CD8^+$ T cell response compared to OVA and adjuvant solutions (Bal et al., 2011; Du et al., 2017; Guo et al., 2013). Other studies have shown that peptide and poly(I:C) loaded cationic liposomes induced potent Th1 and $CD8^+$ T cell responses needed for tumor vaccination (Varypataki et al., 2017; Varypataki et al., 2015). These results are noteworthy, as nowadays there is an increasing need for potent cellular immune responses, e.g., for immunotherapy of cancer (Ammi et al., 2015; Fan and Moon, 2015; Hamdy et al., 2008) and intracellular pathogens (Chong et al., 2005; Zaric et al., 2013). However, it is not yet well understood whether the antigen and immune modulator need to be co-encapsulated in liposomes, or they can similarly modulate the immune response when encapsulated individually in liposomes. Therefore, the aim of this study was to examine whether co-encapsulation of antigen and adjuvant is required for enhancing and modulating the immune response.

In the present study, we chose diphtheria toxoid (DT) as a model antigen and studied the effect of encapsulation of DT and poly(I:C) in liposomes on immune responses in mice after hollow microneedle mediated intradermal immunization. DT and poly(I:C) were either individually encapsulated or co-encapsulated in 1,2-dioleoyl-3-trimethylammonium-propane chloride (DOTAP) based cationic liposomes. To investigate the modulation of immune responses by the liposomal formulations, IgG1 and IgG2a titers, which are indications of a Th2 and a Th1 type immune response, respectively (Maassen et al., 2003), were determined.

2. Materials and methods

2.1. Materials

DT (batch 04–44, 1 μg equal to 0.3 Lf) and diphtheria toxin were provided by Intravacc (Bilthoven, The Netherlands). Aluminum phosphate was purchased from Brenntag (Ballerup, Denmark). Egg phosphatidylcholine (EggPC), DOTAP and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) were ordered from Avanti Polar Lipids (Alabaster, AL). Polyinosinic-polycytidylic acid (poly(I:C)) (low molecular weight) and its rhodamine-labeled version were purchased from Invivogen (Toulouse, France). Foetal bovine serum (FBS), M199 medium (with Hanks' salts and L-glutamin), bovine serum albumin (BSA) and hydrofluoric acid ≥48% were ordered from Sigma-Aldrich (Zwijndrecht, The Netherlands). Glucose solution, L-Glutamine (200 mM), penicillin–streptomycin (10,000 U/ml) and 1-step™ ultra 3,3′,5,5′-tetramethylbenzidine (TMB) were obtained from ThermoFisher Scientific (Waltham, MA). HRP-conjugated goat anti-mouse total IgG, IgG1 and IgG2a were purchased from Southern Biotech (Birmingham, AL). Sulfuric acid (95–98%) was obtained from JT Baker (Deventer, The Netherlands). VivaSpin 2 and 500 centrifugal concentrators (PES membrane, MWCO 1000 kDa) were obtained from Sartorius Stedim (Nieuwegein, The Netherlands). Sterile phosphate buffered saline (PBS, 163.9 mM Na^+, 140.3 mM Cl^-, 8.7 mM HPO_4^{2-}, 1.8 mM H_2PO^{4-}, pH 7.4) was obtained from Braun (Oss, The Netherlands). 10 mM PB (7.7 mM Na_2HPO_4, 2.3 mM NaH_2PO_4, pH 7.4) was prepared in the laboratory. All the other chemicals used were of analytical grade and Milli-Q water (18 MΩ/cm, Millipore Co.) was used for the preparation of all solutions.

2.2. Preparation of liposomes

Liposomes were prepared by thin-film hydration followed by extrusion, as reported earlier (Varypataki et al., 2016). EggPC (25 mg/ml), DOPE (25 mg/ml) and DOTAP (25 mg/ml) in chloroform were mixed in a molar ratio of 9:1:2.5 in a round bottom flask. The organic solvent was evaporated by using a rotary evaporator (Buchi rotavapor R210, Flawil, Switzerland) for 1 h at 40 °C and 120 rpm. To prepare DT encapsulated liposomes (Lipo-DT), the lipid film was hydrated with 0.25 mg/ml DT dissolved in 10 mM PB (pH 7.4) by vortexing for 10 s, resulting in a 12.5 mg/ml lipid suspension. To prepare poly(I:C) encapsulated liposomes (Lipo-PIC), the lipid film was hydrated with 0.25 mg/ml poly(I:C) solution (containing 0.5% (w/w) rhodamine-labeled poly(I:C)). To prepare DT and poly(I:C) co-encapsulated liposomes (Lipo-DT-PIC), after lipid film hydration with DT solution, 0.25 mg/ml poly(I:C) (containing 0.5% (w/w) rhodamine-labeled poly (I:C)) dissolved in 10 mM PB (pH 7.4) was added slowly (2 μl/min) into the lipid suspension by using a syringe pump (NE-300, Prosense, Oosterhout, The Netherlands). Next, the lipid vesicles were extruded (LIPEX™ extruder, Northern Lipids, Burnaby, Canada) four times through a carbonate filter with a pore size of 400 nm and another four times through a filter with a pore size of 200 nm (Nucleopore Millipore, Amsterdam, The Netherlands). To remove the DT/poly(I:C) not associated with liposomes, the suspension was transferred into VivaSpin 2 centrifugal concentrators (1000 kDa MWCO) and centrifuged (Allegra X-12R, Beckman Coulter, Indianapolis, IN) for 6 h (350*g*, 22 °C). Finally, the liposomes were washed with 10 mM PB and kept at 4 °C in the refrigerator prior to use. The filtrates, containing the free DT/poly(I:C), were collected for determination of loading efficiency of DT and poly (I:C).

2.3. Characterization of liposomal formulations

2.3.1. Particle size and zeta potential measurements

The particle size of the liposomes was measured by dynamic light scattering by using a Nano ZS® zetasizer (Malvern Instruments, Worcestershire, U.K.). The zeta potential of liposomes was measured by the same instrument by using laser Doppler velocimetry. The liposomes were diluted with 10 mM PB (pH 7.4) to a concentration of 25 μg/ml for the measurements. The samples were measured 3 times with 10 runs for each measurement.

2.3.2. Determination of encapsulation efficiency (EE) and loading capacity (LC) of DT/poly(I:C) in liposomes

To determine the EE and LC of DT and poly(I:C), the intrinsic fluorescence intensity of DT (λ_{ex} 280 nm/λ_{em} 320 nm) and fluorescence intensity of rhodamine labeled poly(I:C) (λ_{ex} 545 nm/λ_{em} 576 nm) in the purification filtrates were measured by using a Tecan M1000 plate reader (Männedorf, Switzerland). The EE and LC were calculated by using Eqs. (1) and (2) as below:

$$EE = \frac{M_{loadedDT/poly(I:C)}}{M_{totalDT/poly(I:C)}} \times 100\% \quad (1)$$

$$LC = \frac{M_{loadedDT/poly(I:C)}}{M_{Liposomes+DT+poly(I:C)}} \times 100\% \quad (2)$$

where $M_{loaded\ DT/poly(I:C)}$ represents the mass of encapsulated DT or poly (I:C), $M_{total\ DT/poly(I:C)}$ is the total amount of DT or poly(I:C) added to the formulations and $M_{liposomes+DT+poly(I:C)}$ is the total weight of liposomes, DT and poly(I:C).

2.3.3. In vitro release of DT and poly(I:C) from liposomes

To study the *in vitro* release of DT and poly(I:C) from Lipo-DT, Lipo-PIC and Lipo-DT-PIC, the liposomes (containing about 80 μg/ml DT with or without 80 μg/ml poly(I:C)) were dispersed in PBS and shaken with a speed of 550 rpm at 37 °C by using an Eppendorf thermomixer