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Robust IgG responses to nanograms of antigen using a biomimetic lipid-coated particle vaccine

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ABSTRACT

New subunit vaccine formulations with increased potency are of interest to improve immune responses against poorly immunogenic antigens, to avoid vaccine shortages in pandemic situations, and to promote dose-sparing of potent adjuvant molecules that can cause unacceptable side effects in prophylactic vaccination. Here we report strong class-switched, high avidity humoral immune responses elicited by a vaccine system based on poly(lactide-*co*-glycolide) micro- or nano-particles enveloped by PEGylated phospholipid bilayers, with protein antigens covalently anchored to the lipid surface and lipophilic adjuvants inserted in the bilayer coating. Strikingly, these particles elicited high endpoint antigen-specific IgG titers ($>10^6$) sustained for over 100 days after two immunizations with as little as 2.5 ng of antigen. At such low doses, the conventional adjuvant alum or the molecular adjuvants monophosphoryl lipid A (MPLA) or α-galactosylceramide (αGC) failed to elicit responses. Co-delivery of antigen with MPLA or αGC incorporated into the particle bilayers in a pathogen-mimetic fashion further enhanced antibody titers by ~12-fold. MPLA provided the highest sustained IgG titers at these ultra-low antigen doses, while αGC promoted a rapid rise in serum IgG after one immunization, which may be valuable in emergencies such as disease pandemics. The dose of αGC required to boost the antibody response was also spared by particulate delivery. Lipid-enveloped biodegradable micro- and nano-particles thus provide a potent dose-sparing platform for vaccine delivery.

1. Introduction

The immune system has evolved to respond strongly to antigens encountered in micro- or nano-particulate form, likely reflecting the intrinsic particulate nature of foreign microbes. B-Lymphocytes are strongly activated by particles displaying repeat copies of antigens capable of crosslinking B-cell receptors [1–3], and particulate delivery also allows antigens to be processed and loaded onto class I MHC molecules, enhancing $CD8^+$ T-cell responses [4–6]. These findings, combined with the desire to control the duration of exposure to antigen via controlled release, have motivated extensive studies of biodegradable polymer micro- or nano-particles as potential vaccine delivery materials [7–10]. Such systems improve immune responses not only due to their ability to control the release rate of their components, but also due to the inherent potency of degradable particles as materials for vaccine delivery [11–13], particularly PLGA [14,15]. Additionally, particles can co-deliver immunostimulatory molecules on the same particle, targeting multiple classes of molecules to the same intracellular compartment [16–19].

However, these technologies have failed to move into the clinic in part due to the challenges of low antigen encapsulation efficiency and denaturation of protein antigen during the encapsulation process [8,10]. In order to avoid antigen denaturation, strategies based on the binding of antigens to the surfaces of particles post-synthesis have been pursued. This approach has the additional benefit of mimicking multivalent antigen display on natural pathogens. Examples of this approach include adsorption of antigens to charged PLGA particles [20,21] or covalent coupling of protein to reactive groups on particle surfaces [22,23].

We sought to combine this concept of antigen surface-display with a strategy for creating degradable particles whose surfaces could mimic microbial pathogens in their structure and surface chemistry.

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To this end, we began exploring lipid-enveloped micro- and nanoparticles composed of a biodegradable PLGA polymer core surrounded by a self-assembled phospholipid membrane. We recently reported on the nanoscale structure of lipid membrane assemblies formed when lipids are used as surfactants in emulsion/solvent evaporation syntheses of PLGA particles [24]. For a range of compositions, PLGA particles were prepared with a two-dimensionally fluid surface phospholipid bilayer surface that tightly envelopes the polymer core.

Here we report on *in vivo* testing of this lipid-coated particle system for delivery of protein antigens with or without co-delivered danger signals displayed in a native lipid context. The model protein antigen ovalbumin (ova) was conjugated to PEGylated lipids incorporated in the particle lipid shells. We also incorporated lipophilic molecular danger signals into the surface bilayers of these particles, focusing primarily on monophosphoryl lipid A (MPLA) and α-galactosylceramide (αGC). MPLA is a nontoxic lipopolysaccharide derivative that binds to Toll-like receptor 4, which is already in use in human vaccines including the papillomavirus vaccine Cervarix™ recently approved in the United States [25]. αGC is a synthetic glycolipid that can be loaded into non-classical MHC CD1d molecules by antigen presenting cells; αGC/CD1d complexes stimulate invariant natural killer T cells (NKT cells) through their conserved T-cell receptors [26]. αGC is in clinical development as a drug against cancer and autoimmunity, but has been recognized as a candidate vaccine adjuvant as well, in part due to the recently discovered role for NKT cells in promoting humoral immune responses [17]. Lipopolysaccharide and its derivatives, including MPLA, have been used as membrane-incorporated components in liposomal vaccines for many years [18,27,28], whereas this report provides one of the first applications of this concept to αGC.

In analyzing immune responses elicited by this lipid-coated particle system, we particularly explored immunizations using limiting (down to sub-nanogram) doses of both the antigen and molecular adjuvant molecules. Such an analysis is useful in several contexts. First, dose sparing of antigen is of significant interest in the setting of seasonal influenza vaccines, where production issues have in the past led to vaccine shortages, as well as in bioterrorism and pandemic vaccine development settings, where rapid deployment of limited vaccine stocks may be critical [29–34]. Second, dose sparing of molecular adjuvants such as MPLA and αGC lowers the likelihood of reactogenicity or systemic side effects that can block clinical translation of promising adjuvant candidates for prophylactic vaccines [35]. Lastly, dose titration is a powerful strategy for comparing potency of candidate vaccines in mice, allowing important differences in vaccine potency to be revealed that may be missed by immunizations with high antigen doses [36]. These quantitative features of vaccination are infrequently characterized in small-animal models but may be relevant for predicting the performance of candidate particle-based vaccines in non-human primates and humans.

2. Materials and methods

2.1. Materials

PLGA with a 50:50 lactide:glycolide ratio was purchased from Lakeshore Biomaterials (Birmingham, Alabama). The lipids 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (DOPG), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)2000] (mal-PEG2k-PE) were purchased from Avanti Polar Lipids (Alabaster, Alabama). Carboxyfluorescein succinimidyl ester (CFSE) was from Invitrogen (Carlsbad, CA). MPLA was purchased from Sigma Aldrich (St. Louis, Missouri), rhodamine-conjugated Pam3Cys was purchased from Invivogen (San Diego, California), and αGC was purchased from Toronto Research Chemicals Inc. (North York, Ontario, Canada). Aluminum hydroxide adjuvant (Imject) and *n*-succinimidyl s-acetyl (thiotetraethylene glycol) ($SAT(PEG)_4$) were purchased from Pierce Biotechnology. Solvents were from Sigma-Aldrich and used as received.

2.2. Lipid-enveloped particle synthesis

Lipid bilayer-enveloped microparticles were synthesized as previously reported [24]. Briefly, lipid (DOPC:DOPG:mal-PEG2k-PE 72:18:10 molar ratio) and polymer were co-dissolved in dichloromethane (DCM) and this organic phase was dispersed into distilled deionized ultrapure water (DDI water) by homogenization. After evaporation of DCM by stirring the emulsion for 12 h, solid PLGA particles with lipid bilayer coatings were recovered by centrifugation. Larger microspheres were separated from particles <1 μm by two sequential steps of centrifugation for 1 min at 1100 RCF. To prepare lipid-enveloped nanoparticles, we adapted a procedure published by Wassel et al. for the synthesis of poly(vinyl alcohol)-stabilized PLGA particles [37]. PLGA (30 mg) was co-dissolved in 1 mL DCM with 1.3 mg of DOPC, 0.34 mg of DOPG, and 0.62 mg of mal-PEG2k-PE to form the organic phase. An internal aqueous phase of 200 μL DDI water was dispersed in the organic phase by sonication for 1 min on ice using a Misonix XL2000 Probe Tip Sonicator (Farmingdale, NY) at 7 W output power. The resulting solution was immediately dispersed in 6 mL DDI water by sonication for 5 min on ice using the Misonix XL2000 at 12 W output power. DCM was evaporated overnight at ambient temperature and pressure while agitating the solution on an orbital shaker.

To purify polymer-core nanoparticles from free liposomes, particles were layered over a cushion of 30% sucrose in ultrapure water and centrifuged at 13,000 *g* for 5 min. The liposome-containing solution retained above the sucrose gradient was discarded, and the particles that formed a pellet below the sucrose gradient were retained. Self-assembly of lipids on particle surfaces was confirmed using electron microscopy. Particle size was determined using a Horiba Partica LA-950V2 Laser Diffraction Particle Size Analysis System, and confirmed using scanning electron microscopy and optical microscopy of microparticles.

2.3. Antigen conjugation to lipid-enveloped particles

To load lipid-enveloped particles with surface-displayed antigens, thiolated proteins were linked to the lipid surfaces via the maleimide terminus of mal-PEG2k-PE. As a model protein antigen, purified ovalbumin (ova, Worthington Biochemical, Lakewood, New Jersey) was passed through a Detoxi-Gel endotoxin removal affinity column (Pierce Biotechnology, Rockford, Illinois), and the resulting protein solution contained no endotoxin detectable by the Limulus Amebocyte Lysate assay (Lonza, Basel, Switzerland). Ova was modified with the heterobifunctional cross-linker $SAT(PEG)_4$ (Pierce Biotechnology, Rockford, Illinois) by adding a 10-fold molar excess of the crosslinker (2.2 mM) to ova solution (0.22 mM or 10 mg/mL) and incubating on a revolving rotator for 30 min at room temperature. To quench NHS groups on unreacted $SAT(PEG)_4$ molecules, 25 mM glycine was added, and protein was incubated for an additional 15 min rotating at room temperature. Quenched $SAT(PEG)_4$ was removed by buffer exchange with a 7000 MWCO desalting spin column (Pierce Biotechnology Rockford, Illinois) and stored for up to 16 h at 4 °C. Sulfhydryl groups on $SAT(PEG)_4$-modified ova were deprotected by adding 50 mM hydroxylamine and 2.5 mM EDTA (pH = 7.4) and rotating for 2 h at room temperature followed by a second buffer exchange into 10 mM EDTA (pH = 7.4). Particles (70 mg/mL) were then incubated with protein (5 mg/mL) for 4 h at 25 °C before washing with sterile saline to remove unbound antigen. Buffers and products of the synthesis contained no detectable endotoxin. An analogous procedure was used to couple green fluorescent protein (GFP) or fluorescein isothiocyanate (FITC)-labeled ova, and the resulting particles were visualized using a Zeiss LSM510 confocal fluorescence microscope. The