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Time course study of the antigen-specific immune response to a PLGA microparticle vaccine formulation

Qian Wang [a], Melody T. Tan [b], Brian P. Keegan [a], Meagan A. Barry [c], Michael J. Heffernan [a, d, e, *]

[a] Sabin Vaccine Institute and Texas Children's Hospital Center for Vaccine Development, Department of Pediatrics (Section of Pediatric Tropical Medicine), Baylor College of Medicine, 1102 Bates Street, Houston, TX 77030, USA
[b] Department of Bioengineering, Rice University, 6100 Main Street, Houston, TX 77005, USA
[c] Medical Scientist Training Program and Interdepartmental Program in Translational Biology and Molecular Medicine, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA
[d] Department of Molecular Virology & Microbiology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA
[e] National School of Tropical Medicine, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA

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ABSTRACT

Microparticle-based vaccine delivery systems are known to promote enhanced immune responses to protein antigens and can elicit T_H1-biased responses when used in combination with Toll-like receptor (TLR) agonists. It is important to understand the kinetics of the immune responses to microparticle-based protein vaccines in order to predict the duration of protective immunity and to optimize prime-boost vaccination regimens. We carried out a 10-week time course study to investigate the magnitude and kinetics of the antibody and cellular immune responses to poly(lactic-*co*-glycolic acid) (PLGA) microparticles containing 40 μg ovalbumin (OVA) protein and 16 μg CpG-ODN adjuvant (MP/OVA/CpG) in comparison to OVA-containing microparticles, soluble OVA plus CpG, or OVA formulated with Alhydrogel® aluminum adjuvant. Mice vaccinated with MP/OVA/CpG developed the highest T_H1-associated IgG2b and IgG2c antibody titers, while also eliciting T_H2-associated IgG1 antibody titers on par with Alhydrogel®-formulated OVA, with all IgG subtype titers peaking at day 56. The MP/OVA/CpG vaccine also induced the highest antigen-specific splenocyte IFN-γ responses, with high levels of IFN-γ responses persisting until day 42. Thus the MP/OVA/CpG formulation produced a sustained and heightened humoral and cellular immune response, with an overall T_H1 bias, while maintaining high levels of IgG1 antibody equivalent to that seen with Alhydrogel® adjuvant. The time course kinetics study provides a useful baseline for designing vaccination regimens for microparticle-based protein vaccines.

1. Introduction

The development of adjuvant systems for protein subunit vaccines is an important area of investigation in the field of vaccine biotechnology. Current aluminum-based adjuvants typically elicit antibody responses, whereas $CD8^+$ T cell effector responses are required for vaccines directed against intracellular pathogens and cancers [1,2]. The $CD8^+$ T cell effector response involves the polarization of $CD4^+$ T cells toward a T-helper (T_H)1 phenotype and the generation of antigen-specific $CD8^+$ effector T cells. A common approach to designing adjuvant systems aimed at T_H1-type immune responses is to incorporate an immunopotentiating agent, which directs T_H1 polarization, and a delivery vehicle, which provides a depot effect or targeting to antigen-presenting cells [1]. Several classes of immunopotentiating agents have been utilized, including cytokines, Toll-like receptor (TLR) agonists, and other pathogen-recognition receptor agonists [3,4]. A wide range of delivery vehicles have been developed for delivery of protein antigen and adjuvants, including polymer-based depot systems, microparticles, liposomes, and micelles [3–5].

CpG oligodeoxynucleotide (CpG-ODN, or CpG) sequences have been widely studied as immunomodulators, with certain CpG sequences established as inducers of strong T_H1-type immune responses [6,7]. In the United States, several Phase I or II clinical trials

* Corresponding author. Sabin Vaccine Institute and Texas Children's Hospital Center for Vaccine Development, Department of Pediatrics (Section of Pediatric Tropical Medicine), Baylor College of Medicine, 1102 Bates Street, Houston, TX 77030, USA. Tel.: +1 832 824 0512; fax: +1 832 825 0549.
E-mail address: michael.heffernan@bcm.edu (M.J. Heffernan).

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have been conducted using CpG 7909 as an adjuvant for cancer vaccines or vaccines against infectious diseases including influenza, malaria, and hepatitis B infection [8]. In preclinical studies, CpG 1826, an agonist for mouse TLR9, has been frequently employed as a vaccine adjuvant. When combined with *Leishmania major* whole-pathogen or recombinant protein vaccines, CpG 1826 improved protection against *L. major* challenge and enhanced T_H1-type immune responses in mice [9,10]. Similarly, the addition of CpG 1826 to recombinant *Trypanosoma cruzi* [11–14] and *Plasmodium yoelii* [15] protein vaccines resulted in increased protection in mouse models of Chagas disease and malaria, respectively, with evidence of T_H1-biased immune responses.

Microparticle delivery systems have been shown to increase the efficacy of protein-based vaccines in preclinical animal studies. A commonly used carrier material is poly(lactic-*co*-glycolic acid) (PLGA), a biodegradable polymer with properties amenable to the formation of drug- or protein-loaded microparticles by the solvent evaporation method [16]. Several studies have demonstrated that encapsulation of protein antigens and CpG in PLGA microparticles enhanced the immunogenicity in comparison to soluble formulations, including one study in which greater protection against *L. major* challenge was achieved [17–21]. This enhancement of immune responses may be partially attributed to improved delivery of antigen and CpG to the endosomes of antigen-presenting cells (APCs), where antigen is processed for presentation [22] and CpG engages TLR9 on the endolysosomal membrane [23–25].

It is important to understand the kinetics of immune responses to vaccines in order to predict the duration of protective immunity and to establish optimal immunization schedules for prime-boost administration. Several studies have demonstrated that the interval between prime and boost was critical for enhancing post-boosting protective immunity, in both humoral [26–28] and cellular [29–31] immune responses. Although the mechanisms have not been completely understood, it has been reported that the induction and maturation of antigen-specific memory B and T cells after the expansion phase contributes to such enhancement [28,32]. While several studies have reported time course kinetics of antibody and T cell immune responses to ovalbumin (OVA) model protein antigen delivered by PLGA microparticles or other delivery vehicles [33–37], there is a lack of information on T cell cytokine levels through the expansion and contraction phases, which is particularly needed for the development of T_H1-type vaccines. To address this need, we investigated the magnitude and kinetics of antibody and cellular immune responses to PLGA microparticles containing OVA protein and CpG, in comparison to OVA-containing microparticles, non-microparticle OVA plus CpG, or OVA formulated with Alhydrogel® aluminum hydroxide adjuvant. The immune response was characterized over a 10-week time course by measuring serum antibody titers and splenocyte cytokine expression from multiple cohorts of vaccinated mice, with a focus on markers of T_H1-type immune responses.

2. Materials and methods

2.1. Materials

Poly(lactic-*co*-glycolic acid) (PLGA, M.W. 7000–17,000, RESOMER® RG 502H), polyvinyl alcohol (PVA, M.W. 31,000–50,000, 98–99% hydrolyzed), albumin from chicken egg white (ovalbumin (OVA); A2512), D-mannitol (M9546), and 2-mercaptoethanol were purchased from Sigma–Aldrich (Milwaukee, WI). CpG-ODN 1826 sequence 5′-tccatgacgttcctgacgtt-3′, with phosphorothioate backbone, and IDTE (pH 8) buffer were obtained from Integrated DNA Technologies, Inc. (Coralville, IA). The cationic lipid 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP; 890890P) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Micro BCA™ Protein Assay was obtained from Thermo Fisher Scientific Inc. (Rockford, IL), and Alexa Fluor® 660 Protein Labeling Kit and Quant-iT™ OliGreen® ssDNA Assay were from Life Technologies Corporation (Carlsbad, CA). Alhydrogel® (aluminum hydroxide adjuvant) was purchased from Brenntag Biosector (Frederikssund, Denmark). PBS, RPMI-1640, fetal bovine serum (FBS), HEPES, non-essential amino acids, and antibiotic-antimycotic were purchased from Mediatech Inc. (Manassas, VA). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG1 and IgG2b were sourced from LifeSpan Biosciences, Inc. (Seattle, WA), and IgG2c from SouthernBiotech (Birmingham, AL). Bovine serum albumin (BSA) was purchased from KPL (Gaithersburg, MD). Tween 20 was purchased from Bio-Rad Laboratories, Inc. (Hercules, CA). Complete medium was prepared with RPMI-1640 medium plus 10% FBS, 10 mM HEPES, 1 mM sodium pyruvate, non-essential amino acids, 100 I.U. penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin B, and 50 μM 2-mercaptoethanol.

2.2. Animals

C57BL/6 and BALB/c female mice were obtained from Taconic Farms, Inc. (Hudson, NY). All mice were housed in the Texas Children's Hospital Feigin Center animal facility, operated by the Center for Comparative Medicine at Baylor College of Medicine (BCM). Experiments were conducted under an animal research protocol approved by the Institutional Animal Care and Use Committee at BCM and followed the BCM Guidelines for Animal Care and Use.

2.3. Encapsulation of OVA and CpG in PLGA microparticles

OVA was encapsulated in microparticles using a water-oil-water double emulsion method. Briefly, 5 mg of protein dissolved in 200 μL of PBS containing 0.5% PVA and 125 mg/mL mannitol, was homogenized with 200 mg of PLGA dissolved in 2 mL of dichloromethane using a PowerGen™ homogenizer (Fisher Scientific) at 24,000 rpm for 2 min. The primary emulsion was poured into 15 mL of 5% PVA solution (in 3× PBS) and homogenized at 24,000 rpm for another 2 min. The resulting emulsion was poured into 185 mL of 5% PVA solution and stirred for 4 h at room temperature to evaporate the dichloromethane. The microparticles were collected by centrifugation at 3000 g, washed twice using Milli-Q water, re-suspended, and lyophilized for 48 h. Parallel batches of microparticles were pooled during the washing step.

For imaging studies, OVA was first labeled with Alexa Fluor® 660 dye (AF660) per the manufacturer's protocol. The degree of labeling was determined to be 0.86 molecules of dye per protein molecule. AF660-labeled OVA was encapsulated in PLGA microparticles using the same method as above, except that the inner water phase contained 215 μg of AF660-OVA in 100 μL of PBS containing 0.5% PVA and the oil phase contained 100 mg of PLGA in 1.5 mL of dichloromethane.

To prepare CpG-loaded microparticles, CpG was first ion-paired with the cationic lipid DOTAP to create a complex that is soluble in dichloromethane [38], prior to encapsulation in PLGA microparticles by an oil-in-water single emulsion method. Briefly, 4 mg of CpG in 1 mL of IDTE buffer was mixed with 8.47 mg of DOTAP in 1 mL of dichloromethane, and then 2.1 mL of methanol was added into the mixture, which was pipetted until a single phase solution was obtained. Next, 1 mL of water and 1 mL of dichloromethane were added sequentially, resulting in phase separation, and the mixture was vortexed and centrifuged at 2000 g for 5 min to recover the organic phase containing CpG ion-paired to DOTAP. The ion-paired CpG was mixed with 200 mg of PLGA in a total oil phase volume of 2 mL, and then homogenized with 15 mL of 5% PVA solution (in 3× PBS) at 24,000 rpm for 2 min. The microparticles were collected by centrifugation at 6000 g, washed twice using Milli-Q water, re-suspended, and lyophilized for 48 h.

Microparticles were imaged by scanning electronic microscopy (SEM, JEOL JSM-6100). Protein encapsulation level was measured by Micro BCA™ Protein Assay after microparticles were digested in a mixture of DMSO, SDS, and NaOH [39]. To quantify CpG encapsulation, microparticles were digested in the same manner, followed by pH adjustment to neutral pH and measurement of CpG concentration by OliGreen® ssDNA Assay. The loading level (μg/mg) is defined as the mass of OVA protein (or CpG) divided by the mass of microparticles. The loading efficiency (%) is calculated as the actual loading level divided by the nominal loading level, which is defined as the starting OVA protein (or CpG) mass divided by the starting PLGA mass.

2.4. In vivo fluorescence imaging

An *in vivo* imaging study was carried out using female BALB/c mice aged 6–8 weeks at the beginning of the study. Mice ($n = 8$) were injected with 2 mg of PLGA [AF660-OVA] in PBS or an equivalent amount of AF660-OVA formulated with Alhydrogel® (prepared at a mass ratio of 8:1 (alum to OVA) based on our group's experience with other protein antigens). All injections were given subcutaneously (s.c.) in a volume of 100 μL in a shaved area on the right flank. Fluorescence imaging was performed with an IVIS® Lumina II *in vivo* imaging system (Caliper Life Sciences) in the Small Animal Imaging Facility at Texas Children's Hospital. Mice were anaesthetized using isoflurane gas prior to the injection and for all imaging sessions. The fluorescence imaging settings were as follows: excitation wavelength = 640 nm, emission wavelength = 704 nm, f-stop = 2 or 4, binning = 2, and exposure time = 1 or 2 s. The total radiant efficiency ([photons/sec]/[μW/cm^2]) was measured for uniform circular regions of interest (ROIs) using Living Image® software. The total radiant efficiency for each time point was plotted as the percentage of the initial reading (at time = 10 min).