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RESEARCH PAPER

Rapid Vaccination Using an Acetalated Dextran Microparticulate Subunit Vaccine Confers Protection Against Triplicate Challenge by *Bacillus Anthracis*

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ABSTRACT

Purpose A rapid immune response is required to prevent death from Anthrax, caused by *Bacillus anthracis*.

Method We formulated a vaccine carrier comprised of acetalated dextran microparticles encapsulating recombinant protective antigen (rPA) and resiquimod (a toll-like receptor 7/8 agonist).

Results We were able to protect against triplicate lethal challenge by vaccinating twice (Days 0, 7) and then aggressively challenging on Days 14, 21, 28. A significantly higher level of antibodies was generated by day 14 with the encapsulated group compared to the conventional rPA and alum group. Antibodies produced by the co-encapsulated group were only weakly-neutralizing in toxin neutralization; however, survival was not dependent on toxin neutralization, as all vaccine formulations survived all challenges except control groups. Post-mortem culture swabs taken from the hearts of vaccinated groups that did not produce significant neutralizing titers failed to grow *B. anthracis*.

Conclusions Results indicate that protective antibodies are not required for rapid protection; indeed, cytokine results indicate that T cell protection may play a role in protection from anthrax. We report the first instance of use of a particulate carrier to generate a rapid protective immunity against anthrax.

KEY WORDS anthrax · bacterial vaccine · inhalation anthrax · polymeric nanoparticle/microparticle · vaccine

ABBREVIATIONS

/MP	encapsulated in Ac-DEX microparticles
Ac-DEX	acetalated dextran
APCs	antigen presenting cell
AVA	anthrax vaccine absorbed
B. anthracis	Bacillus anthracis
CD*+	cluster designation 8 T cell (cyotoxic T cell)
CD4+	cluster designation 4 T cell (helper T cell)
CDC	Centers for Disease Control
CFUs	colony forming units
DC	dendritic cell
DMSO	dimethyl sulfoxide
ED50	effective dose where 50% inhibition is achieved
EE	encapsulation efficiency
EF	edema factor
i.t.	intratracheal
IFN	interferon

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IL	interlukein
LeTx	leathal anthrax toxin
LF	lethal factor
LPS	lipopolysaccharide
MHC	major histocompatibility complex
MP	micropartilces
PA	protective antigen
PBS	phosphate buffer solution
PLGA	poly(lactic-co-glycolic acid)
PVA	poly vinyl alcohol
Resiq	resiquimod
rLF	recombinent lethal factor
rPA	recombinent protective antigen
TEA	triethylamine
Th2	type two helper T cell
TLR	toll-like receptor
TNA	toxin neutralization assay
VLP	virus-like particle

INTRODUCTION

For a number of lethal infections, the time course from pathogen exposure to death is shorter than the duration of time required to create an effective humoral or cell-mediated immune response. An example of this is inhalational anthrax. For pulmonary *Bacillus anthracis* infection, death can result within 1 week. Comparatively, the current vaccine (BioThrax or Anthrax Vaccine Adsorbed (AVA)) requires up to 6 doses and 18 months to achieve protective antibody concentrations and protection (1). The Centers for Disease Control (CDC) classifies *B. anthracis* as a Category A agent due to its potential threat, ability to diffuse across large areas easily, and high lethality (~50% for inhaled anthrax) (2). Anthrax spores were used in the most recent example of domestic bioterrorism attacks in 2001 and have a history of use as a bioterrorism agent dating back to Scandinavia in 1916 (3). The lethality of anthrax is due to the toxemia. Bacteremia is also propagated, in part, due to the inhibition of macrophage clearance as a result of the negative charge on the bacterial coat. The diminished action by macrophages requires the activation of other immune cells to clear the pathogen.

The toxin component of anthrax is comprised of three separate proteins (protective antigen (PA), edema factor (EF), and lethal factor (LF)) that act in binary combinations during pathogenesis: PA + EF = edema, and PA + LF = cell death. PA has received much attention as an antigen, and is the primary protein component of the AVA vaccine due to the fact that PA's binding to the cell surface is critical for both edema and cell death due to *B. anthracis*. The potential threat of anthrax as a bioweapon, in conjunction with the inability of AVA to develop a rapid protective immunity, illustrates a clear need for additional technologies to accelerate the time for the formation of a protective immune response.

In addition to the need for an accelerated time to protective immunity, the history surrounding the use of AVA would indicate that new vaccine formulations are also needed. In 1970, human AVA was developed and this formulation was required for all service personnel in 1997 (4). The requirement of AVA for service personnel was suspended 2004 and was initiated again as a requirement in 2006 (4). Although the CDC reports vaccine incidents emerging from AVA to be in the same range as other vaccines (2), other scientists have reported an increased incidence in joint (5) and gastrointestinal (6) problems with the vaccine resulting in a negative perception by many Warfighters and the general public (7). Indeed, one study concludes: "The incidence of adverse reactions reported [in Vaccine Adverse Events Reporting System database] following anthrax vaccine was higher for every reaction analyzed in comparison to the adult vaccine control groups (6)." Clearly, additional safer formulations of the vaccine are needed.

AVA is a formulation of proteins from the *B. anthracis* culture media, absorbed on aluminum hydroxide ($AlOH_3$). $AlOH_3$ helps to stabilize the protein by absorbing them on the surface of the material, thus preventing them from absorbing on the glass vial the vaccine is stored. Once inside the body, proteins absorbed on $AlOH_3$ propagate a CD4+ helper T cell type two (Th2) response. This response primarily generates antibodies against PA, which can neutralize toxin. However, like several pathogens, *B. anthracis* grows and proliferates inside of a macrophage host, so activation of a CD8+ cytotoxic T cell response would also help to clear the pathogen (8). One method of generating a combined CD4+ and CD8+ response is to add an adjuvant in addition to, or in place of, alum. Novel vaccines for other pathogens have included the adjuvant CpG in formulations (9,10), fused antigen to toll-like receptor (TLR) agonists (11,12), and included the protein in a viral vector (13,14) all to enhance the response of subunit elements. Imidazoquinolines (e.g. imiquimod, resiquimod) are a group of FDA approved TLR 7/8 agonist (15,16) that are applied as topical creams, due to their extreme hydrophobicity. Our labs were the first to show the incorporation of the FDA approved TLR 7/8 agonist imiquimod into polymeric particles for immune applications (15). Macrophage and dendritic cell (DC) activation from Ac-DEX microparticles encapsulating imiquimod displayed a dose sparing response, in that less adjuvant was needed, compared to free adjuvant, for equal cellular activation (15). After the success of our encapsulation of imiquimod the logical next step was to also encapsulate a subunit protein to create a vaccine against a pathogen.

To this end, we have formed Ac-DEX microparticles encapsulating rPA and resiquimod, an imiquimod derivative that is a more potent TLR 7/8 agonist (17). By encapsulating resiquimod in Ac-DEX microparticles, we can deliver the potent immune activator intracellularly into the

phagosome of antigen presenting cells (APCs) by passively targeting these cells through size exclusion (18). rPA and resiquimod Ac-DEX particles were then evaluated for their efficacy in generating an accelerated immune response by vaccinating at 0 and 7 days, evaluating antibody concentration, cytokine production with antigen recall, and the toxin neutralizing capabilities of the serum antibodies. Additionally, A/J mice were challenged (i.t.) with *B. anthracis* Sterne strain on an accelerated and exhaustive schedule with three total challenges 7 days apart. Here, we report mouse survival as well as heart bacterial load.

MATERIALS AND METHODS

All chemicals were purchased from Sigma (St. Louis, MO) and used as received, unless otherwise indicated. Dextran $(C_6H_{10}O_5)_n$ 71 kDa, was used to synthesize acetalated dextran (Ac-DEX, 71 kDa, 59% cyclic acetal coverage) using the methods described in previous work (15,19,20). Resiquimod ($C_{17}H_{22}N_4O_2$; Mol. Wt. 314.4 g/mol) was purchased from Alexis Biochemicals, Enzo Life Sciences (Farmingdale, NY).

Ac-DEX Synthesis

Ac-DEX was reacted as outlined by Kauffman *et al.* (19). In brief, dextran was freeze dried and dissolved in anhydrous DMSO with pyridinium p-toluenesulfonate. Under anhydrous conditions the mixture was reacted with 2-methoxypropene for 4 hours, at which time the reaction was quenched with triethylamine (TEA). Basic water (Nanopure wate with TEA; pH9) was used to precipitate the product which was then filtered via vacuum, and freeze dried to yield a fluffy white solid. The product was then purified by dissolving it in ethanol, centrifuging (10 min, 10,000×*g*, Beckman RA-21, Los Angeles, CA, USA), basic water precipitation and freeze drying to yield purified Ac-DEX.

Ac-DEX NMR Analysis

Ac-DEX dissolved in deuterium oxide, was hydrolyzed with deuterium chloride and its spectrum was recorded with a 300 MHz ^{1}H-NMR (Bruker 300 Ultrashield). The cyclic: acyclic ratio of acetal substitution, which determines the degradation rate, was determined by evaluating the NMR spectra peaks as described in earlier publications (21). The Ac-DEX used for the encapsulation of recombinant protective antigen was 10 KMW dextran reacted for 4 h (cyclic coverage of 79.5%) and for encapsulation of resiquimod, 71 KMW dextran reacted for 5 min was used (cyclic coverage of 59.2%). These two different MW Ac-DEXs were formed because they have been shown to encapsulate their respective compound best.

Resiquimod Emulsion Particle Fabrication

Resiquimod and Ac-DEX were dissolved in dichloromethane (3 mg resiquimod/100 mg Ac-DEX), added to an aqueous solution containing 3% PVA, vortexed for 2 min and sonicated for 30 s (Misonix Ultrasonic Liquid Processor, 60 W, duty cycle 50%). The emulsion was then dispersed into the aqueous phase (0.3% PVA) that was spinning on a stir plate. After 3 hours, this solution was then centrifuged (12 min, 17500 rpm, 4°C), the supernatant discarded, and the particle sediment resuspended in basic water. The particles were washed two more times with basic water to remove excess drug and PVA. The microparticles were then suspended in basic water and freeze dried without additional compounds added (e.g. cryoprotectants). Blank microparticles were made following the same procedure but without adding adjuvant. Particle size ranging from 200 nm to 2 μm, as observed by SEM, was achieved.

Generation of Protective Antigen

To produce *B. anthracis* protective antigen, pQE30 (Qiagen) expressing wild type PA83 was transformed into *E. coli* M15. The resulting strain was inoculated into 1 L LB (Technova) containing 100 μg/mL ampicillin and 25 μg/mL kanamycin and grown overnight in 2.3 L shake flask at 30°C and 250 rpm. Ten liters of modified high cell density superbroth (AthenaES) was sterilized in a 10 L vessel and placed on a Bioflo 3000 fermenter (New Brunswick Scientific) and ampicillin and kanamycin were added to final concentration of 100 μg/mL and 25 μg/mL respectively. The fermentation was started by adding the overnight culture to the bioreactor to a final optical density (OD) of 0.2 at 600 nm and performed according to the following parameters: 37°C, pH7, airflow 1 vvm, and 40% DO. The culture was supplemented with 50% glucose (Technova) to maintain a final concentration of 5 g/L and was grown to an OD of 16.5 at 600 nm. The temperature reduced to 30°C and rPA expression was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; Sigma). Induction occurred for 4 hours after which the culture was harvested by centrifugation, the supernatants were discarded and the cell pellets frozen at −20°C. The cells were resuspended 1:5 with buffer containing 50 mM sodium phosphate, 1 M sodium chloride and 10 mM imidazole, pH7.5. The cells were lysed via microfluidization at 20,000 psi. The supernatant was clarified through centrifugation at 30,000×*g*. One liter of the clarified supernatant was then loaded onto four 5 ml HisTrap Crude FF columns (GE Healthcare) at 100 cm/h. The rPA was eluted with a linear 0–250 mM imidazole gradient over 15 CV. This was repeated three additional times with a total of 4 L of clarified supernatant being captured through affinity chromatography. The rPA was visualized through SDS-PAGE and the fractions containing rPA were pooled for 1%

triton X-114 (Sigma) phase separation to remove LPS. Triton X-114 was added to a final concentration of 1% and incubated on ice for 1 h, placed in a 37°C water bath for 15 min, centrifuged for 10 min at 40,000×*g* to separate the phases. The rPA pool was buffer exchanged into 50 mM HEPES, pH 7.5 using a 26/10 HiPrep desalt column. The buffer exchanged rPA was then loaded onto two 5 ml HiTrap CM FF columns and eluted in a 1 M NaCl step for 10 CV. The resulting rPA peak was loaded onto a 16/60 HiLoad superdex 200 gel filtration column in 5 ml volumes. The column was equilibrated with 50 mM HEPES, pH7.5 and run at a flow rate of 0.9 ml/min. Fractions containing full length rPA were pooled. The rPA was quantitated with bicinchoninic acid assay (BCA) kit (Peirce) and assayed for LPS using the Kinetic-QCL® Kinetic Chromogenic LAL Assay (Lonza) using the inhibition/enhancement program. The final concentration of rPA and LPS was determined to be 837.6 μg/ml and 3 EU/mg PA respectively. Protein was aliquoted into 1 ml samples and stored at −80°C.

Protective Antigen Emulsion Particle Fabrication

rPA was dissolved in phosphate buffer solution (PBS) and added to a solution containing Ac-DEX and dichloromethane to reach a final concentration of 1.5 mg PA/100 mg Ac-DEX. This mixture was homogenized for 30 s at 20 k rpm using Polytron PT 10–35 Homogenizer (Westbury, NY). A 3% polyvinyl alcohol (PVA) solution was added and homogenized again for 30 s to form a primary emulsion. The primary emulsion was then immediately added to second PVA solution (0.3% *w*/*w* in PBS) and stirred on a magnetic stir plate for 3 h to evaporate the organic solvent. The particles were isolated through centrifugation for 10 min at 4°C and 9160 rpm. The supernatant was decanted, the particles resuspended in basic water, washed two more times with basic water, freeze-dried without additional compounds added (e.g. cryoprotectants) and stored under at −20°C until use. Blank particles were prepared using similar method but without the addition of PA. As visualized via SEM, particle size ranging from 200 nm to 2 μm was achieved.

Encapsulation Efficiency

The encapsulation efficiency (EE) of resiquimod was calculated by reading the adjuvant's fluorescence (Ex: 260 nm/Em: 360 nm) using a Spectra Max Gemini XS microplate reader (Molecular Devices, Sunnyvale, CA). Particles containing resiquimod and blank particle samples were dissolved in DMSO to reach a concentration of 1 mg/mL in a 96 well plate. A standard curve of resiquimod in DMSO was also prepared. The fluorescence of the blank samples was measured and subtracted from the loaded samples. The concentration of resiquimod in the particles was determined from the calibration curve.

The encapsulation efficiency of rPA was determined using a fluorescamine (4-phenyl-spiro [furan-2(3H), 1′-phthalan] -3,3′ -dione) assay (22). rPA encapsulated Ac-DEX particles were degraded in PBS and 50-vol% formic acid nanopure water mixture (pH 3.0) at 37°C for 24 h. The pH was adjusted to 7.4 with a NaOH solution and the samples were aliquoted into a 96-well solvent resistance plate. A fluorescamine solution (3 mg/mL in acetone) was then added and the fluorescence of the resulting solution was read (Excitation: 400 nm/Emission: 460 nm) via FlexStation 3 Benchtop Multi-Mode Microplate Reader (Sunnyvale, CA). The amount of encapsulated protein was determined using rPA as a standard. The encapsulation efficiency of either formulation was determined by dividing the amount of protein or adjuvant encapsulated by the amount initially loaded into the particles, and then multiplied by 100%.

Particle Characterization

Samples were imaged using FEI NOVA NanoSEM 400. A 10 mg/mL solution of particles and basic water was made and a small amount (20 μl) was added to a silicon wafer (Ted Pella; Redding, CA). The samples were allowed to air dry, and then sputter coated with a layer of gold alloy for 120 s before analyzing with SEM. Particles size analysis was performed by dynamic light scattering (DLS) as outlined by Kauffman *et al.*(19)

Vaccination

The experiments reported herein were conducted in compliance with the Animal Welfare Act and in accordance with the principles set forth in the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animals Resources, National Research Council, National Academy Press, 1996. Lyophilized particles were weighed out and placed in microcentrifuge tubes for all experimental groups (Table I). Just prior to injection, the particles were suspended in 200 μl of PBS to reach a final protein and adjuvant concentration of 15 or 8 micrograms per mouse. For groups with alum, Imject (Thermo Scientific) was added to the suspension at a final concentration of 20 mg/ml (4 mg per a mouse) and the suspension was vortexed for 30 min. The appropriate suspension was then drawn into a 1 ml syringe and 200 μl was injected subcutaneously into the scruff of the mouse with a 25 gauge needle. Mice were vaccinated at day 0 and 7 in the same location.

The experimental groups, abbreviations, protein amount, resiquimod amount and alum concentrations are given.

Table I Experimental Groups for all *In Vivo* Studies

Name	Abbreviation	Protein (μg/mouse)	Resiquimod (μg/mouse)	Alum (mg)	Total Particles (mg/mouse)
Phosphate Buffer Solution (sham)	PBS	0	0	0	-
Blank microparticles	MP	0	0	0	9.2
Unencapsulated Protective Antigen	PA	15	0	0	-
Unencapsulated Protective Antigen with Alum (traditional formulation)	PA + Alum	15	0	4	-
Encapsulated Resiquimod	Resiq/MP	0	8	0	3.2
Free Protective Antigen and Encapsulated Resiquimod	PA + Resiq/MP	15	8	0	3.2
Encapsulated Protective Antigen	PA/MP	15	0	0	6.0
Encapsulated Protective Antigen and in separate microparticles, encapsulated Resiquimod	PA/MP + Resiq/MP	15	8	0	9.2
Encapsulated Resiquimod, Free Protective Antigen and Alum	Resiq/MP + PA + Alum	15	8	4	3.2

Anti-PA IgG Detection and Lethal Toxin Neutralization

Ten mice from each experimental group were vaccinated on day 0 and 7 as outlined above. On days −3, 14, 30, the lateral tail vein of each mouse was nicked and approximately 50 μl of blood was collected from each mouse. On day 42, mice were exsanguinated under deep anesthesia via intracardiac puncture. Whole blood was allowed to clot at 25°C for approximately 2 hours prior to separation by centrifugation at 10,000 rpm for 10 min. The recovered serum was then frozen and stored at −80°C.

Mouse anti-PA antibody concentrations were determined by ELISA as described by Albrecht *et al.* (23). Microtiter plates (Immulon-IV HBX,Thermo Labsystems, Franklin, MA) were coated with protective antigen overnight at 4°C in 0.2 M carbonate buffer (pH7.4) containing 1 μg/ml rPA (preparation described above). Following blocking and washing, quantitative standard curves composed of purified mouse anti-PA IgG antibodies, (produced in-house through established methods (24)) were serially diluted and added to the plate, as well as mouse serum samples which were serially diluted in duplicate. PA-specific antibodies which were bound to the capture antigen were detected using horseradish peroxidase-conjugated anti-mouse IgG antibodies (Kirkegaard and Perry Laboratories, Gaithersburg, MD). ELISA plates were developed with ABTS one-component substrate (KPL),stopped with the addition of 1% SDS and the absorbance at 405 nm was read with a standard ELISA plate reader. Readings in the linear range were compared to the quantitative standard curve to determine the anti-PA IgG concentrations (μg/ml). IgG antibody subclass (IgG1 or IgG2a) was determined from the day 42 terminal blood samples by ELISA as described above except that standard curves were generated with mouse IgG1 and IgG2a subclass controls (Invitrogen).

The antibody responses produced by each vaccine formulation were also evaluated for their ability to neutralize lethal anthrax toxin (LeTx), as measured by a toxin neutralization assay (TNA), detailed elsewhere (25,26). Prior to testing, rPA and recombinant LF (rLF) proteins were titrated for toxin potency on the J774A.1 mouse monocyte cell line (TIB-67; American Type Culture Collection, Manassas, VA) by subjecting cells (3×10^5/ml) to serial dilutions of LeTx (1 μg rPA : 0.8 μg rLF) and evaluating subsequent viability by quantifying the metabolic conversion of the tetrazolium dye XTT into its bright orange derivative, as measured by absorbance (480 nm). The effective concentrations of rPA and rLF that kill 99% of cells were determined to be 60.8 and 48.6 ng/ml, respectively, as calculated by sigmoidal dose–response (variable slope) non-linear regression analysis using GraphPad Prism v5.04 software. This dosage was combined with serial dilutions of vaccinated mouse serum and applied to J774A.1 cells, 3×10^5/ml, to calculate the effective dosage of serum that neutralizes LeTx activity by 50% (ED_{50}), as reported in terms of serum dilution factor. Serum samples from individual mice were tested in duplicate and compiled with the measurements from other group members ($n=10$ mice/group) to calculate group TNA titer, reported as ED50 dilution factors, or the reciprocal of the dilution that inhibits lethal toxin cytotoxicity by 50%.

Antigen-Specific Re-stimulation of Splenocytes

On day 42, mice were euthanized by exsanguination under deep anesthesia and the spleens were surgically removed. Each spleen was disrupted into a single cell suspension by pushing it through a 100 μm nylon cell strainer (Fisher) into a six-well tissue culture plate containing 5 ml of tissue culture medium [RPMI 1640+GlutaMax with penicillin-streptomycin (5 ml/500 ml), L-Glutamine (5 ml/500 ml), 10% Hyclone FBS heat inactivated (56° for 30+ min, 50 ml/500 ml), and 0.1% 2-Mercaptoethanol (0.5 ml/500 ml)] to create a single-cell suspension. The cells were centrifuged for 10 min at 1500 RPM, 4°C, the medium was removed and the pellet was

resuspended in the remaining drop. Two milliliters of ice cold ACK lysis buffer was added to lyse the red blood cells for precisely two minutes at which time 10 ml of tissue culture medium was added. The resulting suspension was again centrifuged for 10 min at 1500 rpm at 4°C. The supernatant was removed and the cells resuspended in 5 ml of complete RPMI. Viable cells (as determined by trypan blue exclusion) were counted in a hemocytometer and $1X10^6$ viable splenocytes from each spleen were added to a 96-well tissue culture plate. The splenocytes were then left unstimulated or stimulated in triplicate with either Concanavalin A (5 μg/ml) or rPA (12 μg/ml) in RPMI for 72 h at 37°C in 5% CO_2. At the end of this time period, cells were pelleted by centrifugation and the supernatants removed and stored at −20°C. Stimulated cytokine production was detected in the supernatants using a BioPlex Pro (BioRad) custom cytokine kit that included IL-2, IL-6, IL-17 and IFNγ; and were measured on a BioRad Luminex-200 according to the manufacturer's instructions.

Bacillus anthracis Challenge

Bacillus anthracis 34 F2 Sterne strain spores were prepared as previously described (27). The concentration of viable spores was determined by serial dilution and plating on Brain-Heart Infusion (BHI) agar plates. Mice were challenged three times at two different CFUs. Challenges occurred seven days after the last vaccination and continued each week thereafter (resulting in challenges on days 14, 21 and 28, with respect to the first vaccination) as described below. For the low-dose challenge approximately four LD_{50}s ($4.8x10^4$, $3.78x10^4$ and $4.35x10^4$ cfu) by intratracheal (i.t.) were given. Although typically higher LD_{50}s are used for anthrax challenges, the CFUs of the low dose bacterial challenge were determined by the funding source, Defense Advanced Research Projects Agency (DARPA), as a representative anthrax challenge as was the frequency of the challenge. To thoroughly evaluate our formulation, a high dose challenge was also performed. The high dose challenge, approximately 100 LD_{50}s (3.26×10^5, 5.1×10^5 and 6.6×10^5 CFU) were given, also by the i.t. route. For each challenge, instillation was as follows: Mice were lightly anesthetized with a mixture of Ketamine and Xylazine (80 mg/kg with mixed and 20 mg/kg respectively) administered intraperitoneally. The animal was then manually restrained in an upright position and padded forceps were used to gently open the mouth and hold the tongue down to the lower jaw to prevent swallowing. A second investigator then carefully administered 30 μl of spore suspension to the back of the mouth after which a gloved finger was used to cover the mouse nostrils to prevent obligate nasal breathing. The combination of holding the tongue to prevent swallowing and closing off the nostrils to prevent nasal breathing causes the mouse to aspirate the instilled fluid. The mouse is then returned to its cage and allowed to recover in the supine position to prevent loss of instilled agent.

Infected mice were monitored for a period of 14 days following challenge. The cause of death for those individuals which succumbed to the infection was verified by swabbing the heart for the presence of *B. anthracis*. Here, the hearts were aseptically removed from the chest cavity and a cross section was swiped across tryptic soy agar plates supplemented with 5% sheep blood. A similar procedure was followed for those individuals who survived to the study endpoint and were subsequently euthanized. The presence of *B. anthracis* colonies was considered verification that the cause of death was Anthrax. The absence of colonies was considered indicative of sterile immunity induced by the vaccine.

Statistics

All data except survival data was compared using a two-tailed student *T*-test, where a P-value of <0.05 was considered statistically significant.

RESULTS

For this study, we prepared rPA and resiquimod particles using Ac-DEX. Representative scanning electron micrographs of Ac-DEX microparticles are given in Fig. 1, with particles encapsulating rPA (a) and those encapsulating resiquimod (b). Particles were characterized for drug and protein loading, and the average results are presented in Table II. The average diameter and standard deviation of the particles, as measured through DLS, is given in Table II. The polydispersity of the particles is also listed in Table II. Particles are observed ranging from 200 nm to 2 μm, with particles >100 nm capable of only being endocytosed through phagocytosis(28,29). The release of rPA was observed to be similar to the macromolecule release observed by Bachelder *et al.*(30) Resiquimod release at pH7.4 and phagosomal pH (5.0) is given in Supplementary Material (Fig. S1).

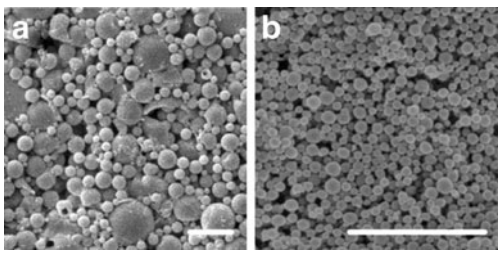

Fig. 1 Scanning electron micrograph of Ac-DEX microparticles encapsulating (**a**) protective antigen and (**b**) resiquimod. Scale bars represents 2 μm.

Table II Ac-DEX Microparticle Loadings for Resiquimod and Protective Antigen. Initial Resiquimod Loading was 3 mg drug/100 mg Ac-DEX and Recombinant Protective Antigen 1.5 mg Protein/100 mg Ac-DEX

	Encapsulation Efficiency (%)	Weight Loading (mg/100 mg Ac-DEX)	Diameter (nm)	Polydispersity Index
Resiquimod	8.3	0.25	194.1 ± 13.5	0.316
Protective Antigen	88.2	1.33	382.6 ± 12.8	0.366

Antibody Concentration and Toxin Neutralization

Blood samples were taken from each mouse on days −3, 14, 28 and 42. The result of anti-PA antibody generation is presented in Fig. 2a. Statistically higher antibody concentrations (P-value <0.05), compared to PA + alum control, were observed at days 14, 28 and 42 for the resiq/MP + PA/MP and the encapsulated resiq/MP + PA + alum groups. The antibody concentration was significantly higher for PA + resiq/MP group, compared to the PA + alum control, at days 28 and 42. Figure 2b reports the concentration of IgG subclass antibodies. Groups containing resiquimod (i.e. PA/MP + Resiq/MP and Resiq/MP + PA+ alum) produced a mixed TH1/TH2 response and generated a significantly higher concentration of IgG2a antibodies compared to those with just antigen (i.e. PA + alum and PA/MP).

The humoral response of the experimental conditions presented in Table I was evaluated at day −3, 14, 28 and 42, with respect to sub-Q vaccination that occurred at day 0 and 7. The conventional formulation of the anthrax vaccine (PA + Alum) generated significantly reduced concentrations compared to the PA/MP + Resiq/MP or Resiq/MP + PA + alum group (Fig. 2a). In addition, significantly higher levels of IgG2a were generated as well in these groups compared to PA + alum, whereas the levels of IgG1 were not significantly different compared to PA + alum at day 42 (Fig. 2b). The generation of IgG2a are expected for experimental conditions containing resiquimod, as it is a potent activator of B cells, resulting in antigen specific generation of IgG2a antibodies (16).

The ability of immunized mouse sera to neutralize anthrax lethal toxin was then subjected to *in vitro* analysis by a toxin neutralizing assay (TNA) (Fig. 3). The TNA titer is reported in terms of the effective serum dilution factor calculated to inhibit lethal toxin cytotoxicity by 50% (ED_{50}). The highest titers were produced by mice immunized with Resiq/MP + PA + alum, which produced significantly higher TNA titers than all other groups starting at day 14. PA + alum and PA + Resiq/MP vaccination also produced significant TNA titers but not until day 30. Figure 3 reports that neutralizing levels of antibodies are achieved only for the PA + alum, PA + Resiq/MP and Resiq/MP + PA + alum groups.

Antigen Recall

To help evaluate T cell specificity for protective antigen, splenocytes were isolated and cultured with antigen. The

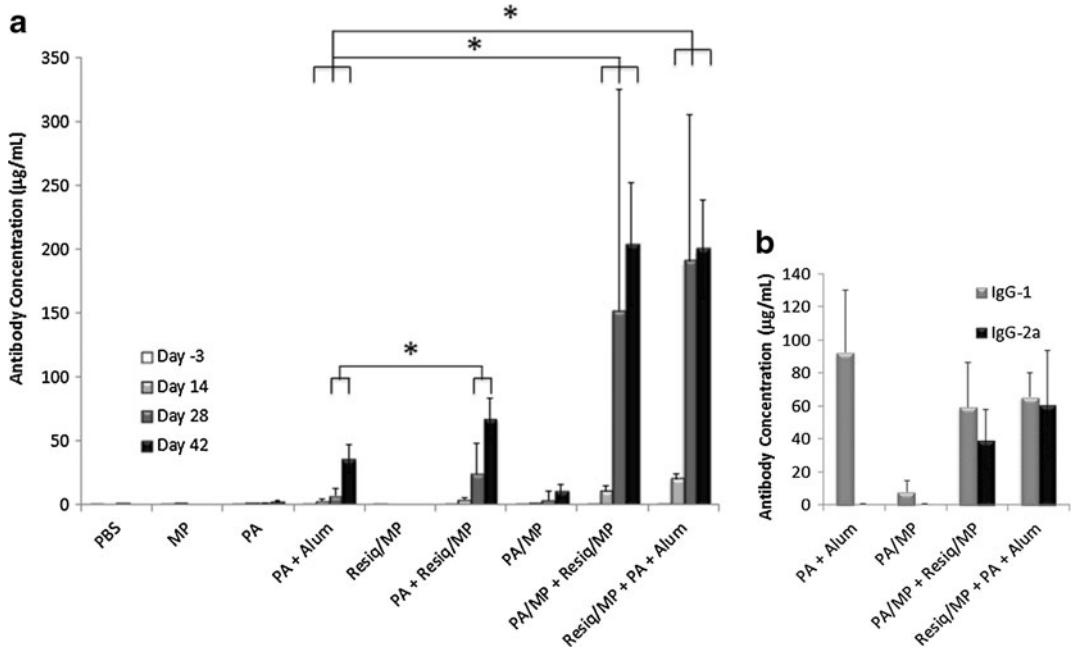

Fig. 2 Protective Antigen specific antibody concentration for blood samples obtained from mice vaccinated on day 0 and 7 with indicated experimental condition. (**a**) Presents total antigen specific IgG antibody concentration and (**b**) presents IgG subclass IgG1 and IgG2a concentration at day 42. Encapsulated condition is indicated with/MP. Resiq indicates 8 μg of resiquimod/mouse. PA is recombinant protective antigen at 15 μg/mouse. Data is presented as average ± standard deviation. $n = 10$. An * represents significance with respect to PA + Alum control at the respective time point.

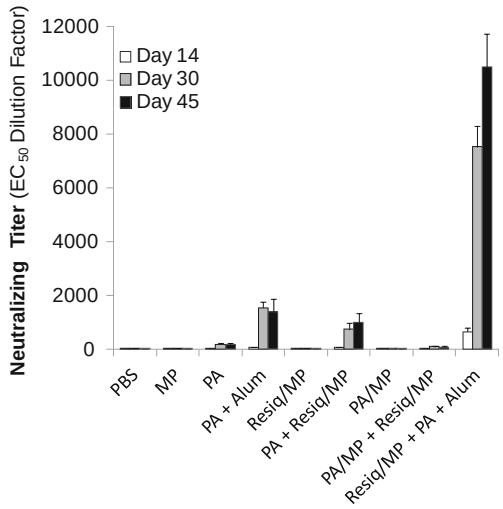

Fig. 3 Toxin neutralizing antibody (TNA) titers on day 14, 30 and 45 in mouse sera in response to immunization with various rPA formulations. TNA titer is reported as the effective serum dilution factor that inhibits lethal toxin cytotoxicity by 50% (ED_{50} – Table S2). Encapsulated condition is indicated with/MP. Resiq indicates 8 μg of resiquimod/mouse. PA is recombinant protective antigen at 15 μg/mouse. Data represent the average logistical regression ED_{50} values ± standard errors produced by groups of $n = 10$ mice.

supernatant from the culture was then analyzed for cytokine production via Luminex. The results of the most significant cytokines are presented in Fig. 4. Additional cytokines are presented in Supplementary Material Table SI. Figure 4a reports that significantly higher levels of IL-2 are produced by splenocytes isolated from mice vaccinated with encapsulated resiquimod and some formulation of antigen compared to PA + alum. Levels of IL-6 and IFN-γ are also significantly higher when comparing PA + alum and Resiq/MP + PA + alum (Fig. 4 b, d). For PA + Resiq/MP the levels of IFN-y are also significantly higher compared to PA + alum (Fig. 4d). Figure 2c shows that IL-17 production is on average higher for the PA/MP + Resiq/MP vaccinated splenocytes. Additional cytokines are presented in Supplementary Material Table S1.

Survival After Challenge

Based on the antibody concentration results (Fig. 2), experimental conditions were down-selected for the challenge experiments to include only the experimental conditions that had antibody concentrations at or above the rPA + alum group (i.e., PA + Alum, PA/MP, PA + Resiq/MP, Resiq/MP + PA + Alum, PA/MP + Alum). With the addition of PBS and blank particle controls, mice were vaccinated on day 0 and 7 as described above and subsequently challenged with *B. anthracis* on day 14, 21 and 28 (inset Fig. 5) at a low-dose. As Figure 5 demonstrates, the majority of the mice from both the PBS and blank microparticle (MP) control groups did not survive the low dose challenges resulting in a 10% survival rate by the end of the study. In contrast, all of the other experimental conditions displayed 100% survival, even after three low-dose *B. anthracis* challenges (Fig. 5). With the exception of the empty microparticles and PBS control groups, all vaccinated animals displayed 100% survival with the low dose challenges. In addition, when the hearts of the mice from the low dose challenge were swabbed and plated post mortem at day 42, PA/MP and PA/MP + Resiq/MP and Resiq/MPs + PA + alum all had sterile cultures, and PA + Resiq/MP had 9 sterile hearts out of 10 (Table III). The PA + alum group fared the worst out of the experimental groups with antigen, with only 7 out of 10 sterile hearts.

Given our success with three low dose challenges, we sought to determine the threshold of protection elicited by our vaccine. To this end, mice were also challenged at a higher dose (~50 LD_{50}) and significant survival was again observed in vaccinated groups (Fig. 6). Encapsulated PA produced modest survival (60% through three challenges) while 100% survival was observed with all other groups except the PBS controls (Fig. 6).

DISCUSSION

The need for an accelerated time to protection is necessary due to the frequency and diversity that new infectious pathogens are emerging as the rate of global surveillance and biomedical technologies for detection of diseases and infections increases. To this end, we have developed a subunit based microparticulate carrier system. Subunit vaccines are classified as the safest form of a vaccine by National Institutes of Allergy and Infectious Disease and formulation of a subunit vaccine using a particle based system has many advantages over traditional subunit formulations (e.g. protein absorbed on alum or adenoviral vectors with protein plasmid). A particulate platform facilitates needle-free applications of the vaccine (31), can provide CD8+ stimulation (21,30,32), and encapsulation of protein in has been shown to stabilize protein activity at temperatures outside the cold chain (33). For these reasons, particulate carriers are viable way to produce a safer subunit vaccine.

For our particulate formulation, we used Ac-DEX, which has several advantages over other commonly used systems (e.g. PLGA microparticles, liposomes, nanoemulsions, virus like particles (VLPs)). Ac-DEX is an acid sensitive polymer with benign pH neutral degradation products of dextran, an alcohol and acetone, a metabolic by-product (30). These degradation products do not shift the local pH, as can be observed with degradation of PLGA formulations (34,35). Furthermore, the range of degradation rates for Ac-DEX is more broad then most biopolymers, as it can range from hours to months by varying the reaction time and/or molecular

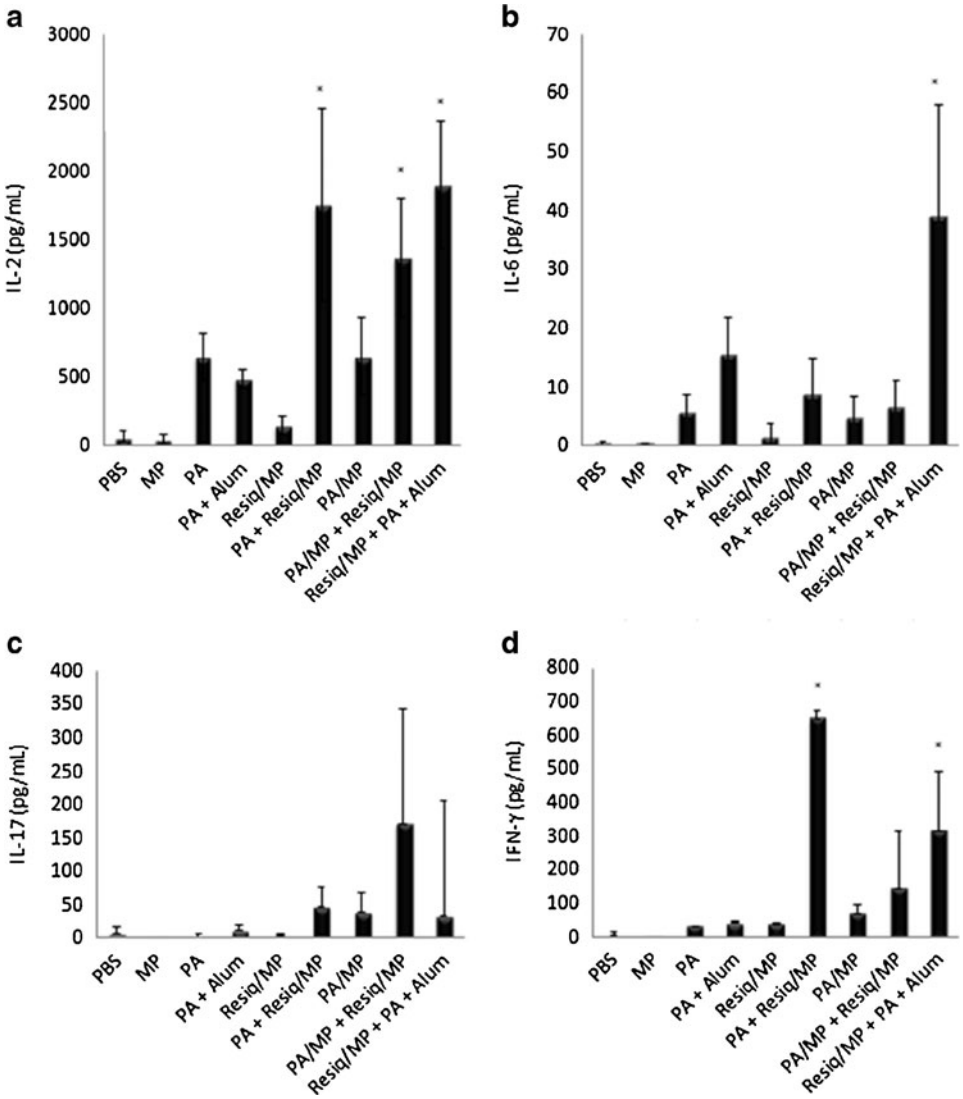

Fig. 4 Cytokine production for protective antigen recall assay with splenocytes isolated from mice vaccinated at day 0 and 7 with indicated experimental condition. Cytokines presented are (**a**) IL-2, (**b**) IL-6, (**c**) IL-17 and (**d**) IFN-γ. Additional cytokines are presented in Supplementary Material Table S1. Encapsulated condition is indicated with/MP. Resiq indicates 8 μg/mouse of resiquimod. PA is recombinant protective antigen at 15 μg/mouse. Data is presented as average ± standard deviation.

weight of the polymer (19,21). The degradation rate has shown to be critical in enhancing the presentation of protein antigen to both CD8+ and CD4+ T cells, as significantly greater presentation was observed with Ac-DEX compared to PLGA and iron oxide materials (21). Additionally, proteins encapsulated in Ac-DEX have displayed stable activity across a range of temperatures (−20 to 45°C) for up to 90 days, whereas PLGA microparticles fused together after 4 days at 45°C (33). Liposomes and nanoemulsions would also not be stable over this range of temperatures (36–38). For these reasons, Ac-DEX microparticles were chosen as a carrier system to accelerate the immune response to subunit antigens because of their many advantages compared to other biomaterials and vaccine formulations.

For this study, we prepared rPA and resiquimod particles using Ac-DEX (Fig. 1 and Table II). Since subunit vaccines are generally only weakly immunogenic, a danger signal or adjuvant is typically co-delivered to generate a protective immune response (39). The adjuvant we have chosen is resiquimod, which is an imidazoquinoline. Imidazoquinolines are usually applied topically in conjunction with alum or free protein (40), and they cannot be effectively applied in a parenteral fashion in their free form due to solubility issues. We present here a new method to vaccinate by using encapsulated resiquimod. With our resiquimod particle method, we can either mix our particles with other particles that have encapsulated protective antigen, or we could add the resiquimod particles with other adjuvants such as alum. rPA and resiquimod were not co-encapsulated into one particle because of the already low range of resiquimod encapsulation efficiency was further reduced during the double emulsion process required for encapsulation of protein encapsulation. We are currently exploring other methods of co-encapsulation, besides emulsion chemistry, to effectively encapsulate both compounds in one particle; however, by separately encapsulating the antigen and adjuvant, additional antigens can be co-administered without the need

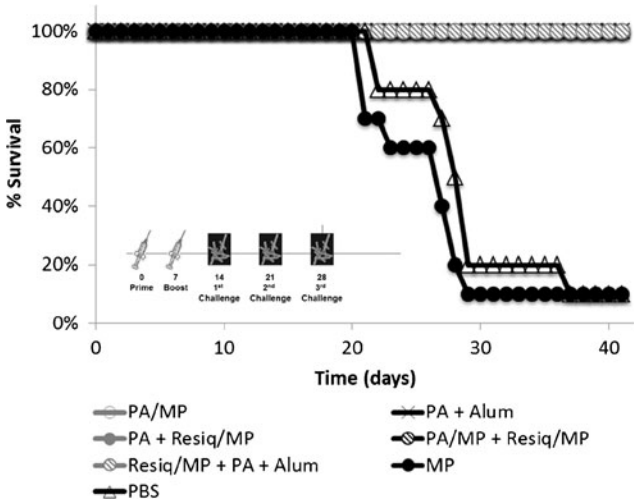

Fig. 5 Survival curves of mice vaccinated on day 0 and 7 and then challenged with an intra-tracheal instillation (average 4.3×10^4 CFUs) on day 14, 21 and 28, as indicated by the insert. A syringe represents injection of experimental conditions via sub-cutaneous injection. The numbers indicate the study day in which the indicated procedure was performed. All experimental groups except PBS and blank microparticle (MP) controls report 100% survival and are overlapping on the graph.

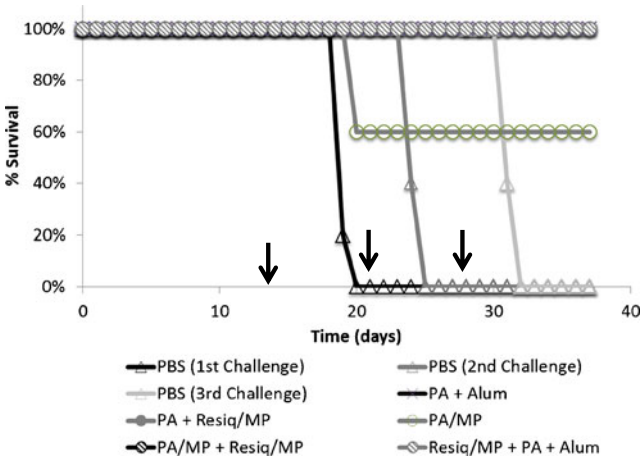

Fig. 6 Survival curves of mice vaccinated on day 0 and 7 and then challenged with an intra-tracheal instillation at a higher dose than Fig. 5. An average of $50xLD_{50}$ CFUs on day 14, 21 and 28, as indicated with arrows, were given. A PBS control group was used for each of the 3 challenges. The PA + Resiq/MP, PA/MP + Resiq/MP, PA + Alum, and Resiq/MP + PA + Alum groups all report a 100% survival and are overlapping on the graph.

for additional adjuvant. Once delivered to the subcutaneous compartment, the particles can then be taken by and processed inside the phagosome, where the natural pH shift from 7.4 to approximately 5 facilitates release of the particles' payload and activation of the phagosomal TLR 7/8 receptor (30,41).

In comparing the antibody concentration presented in Fig. 2 with other particulate formulations using rPA as an antigen, many of the variables become confounding because this study is the first of its kind to report an accelerated vaccination schedule using a particulate carrier. Commonly, vaccines, or more specifically anthrax vaccines, are applied in a mouse model on a time scale of three vaccinations 2–3 weeks apart. With a similar vaccination schedule (0, 3 and 6 week), a higher antibody concentration was observed with a PLGA-dendron DNA vaccine nanoparticle vaccine (42), however, in addition to a different vaccination schedule, plasmid not protein was used and BALB/c mice were used, which can generate a concentration approximately 5 times greater concentration than the mice used in this study (A/J mice) (43).

One other laboratory has developed a non-particulate vaccine that has shown antibody concentration with a shortened vaccination schedule and time to challenge. McConnell *et al.* vaccinated A/J mice (intramuscular) on day 0 and 4 with rPA expressing plasmid using an adenovirus vector. The anti-PA concentrations were higher at 3 and 4 weeks then what is reported here, but the concentrations were similar to those observed with AVA (44), which have been shown to have reduced concentrations compared to PA + alum formulations (45). Antibodies are considered protective against anthrax and here we report significantly higher antibody concentrations at day 14 in two of the 9 groups, compared to PA + alum. A major concern with the adenovirus vector (a type of VLP) used in McConnell *et al.*'s study, is that they cannot be broadly applied because individuals have a pre-existing immune response to the adenovirus, which results in the generation of antibodies that can neutralize transcriptions of the plasmid antigen, particularly with repeated use (46,47).

To evaluate the protection conferred by anti-PA antibodies, a toxin neutralization assay (TNA) is typically performed. It is well regarded that the results of a TNA correspond to survival after challenge with a traditional vaccine schedule (48). Other particulate formulations of vaccines have shown antibody concentrations that are ineffective in neutralizing toxin *in vitro* (42). It is thought that these antibodies are non-neutralizing because they are created against peptide sequences that do not interfere with the binding of PA to the cell. Neutralizing levels of antibodies

Table III Confirmation of *B. Anthracis* Infection from Heart Swabs of Mice Vaccinated and Challenged with Low Dose of CFUs. The Hearts of those Individuals which Succumbed to the Infection or Survived to the Study Endpoint and were Subsequently Euthanized were Swabbed for the Presence of *B. Anthracis*. The Number of Individuals from Each Group which were Sterile or Infected is Indicated. $n = 10$

	Infected	Sterile
PBS	10	-
MPs	9	1
PA + Alum	3	7
PA + Resiq/MPs	1	9
PA/MPs	-	10
PA/MPs + Resiq/MPs	-	10
Resiq/MPs + PA + Alum	-	10

are achieved only for the PA + alum, PA + Resiq/MP and Resiq/MP + PA + alum groups (Fig. 3) which corresponds to elevated levels of IL-6, which has been shown to mediate the protective role of the liver in acute response to anthrax (49) (Fig 4). However, all of the down selected experimental groups with antigen and/or adjuvant result in survival after all three low-dose challenges (Fig 5) and all but PA/MP had 100% survival with the high-dose challenges. It would appear that although TNA predicts survival when mice are vaccinated on a more traditional time scale, TNA does not predict survival on a shortened vaccination schedule. This conclusion is also supported by the bacterial load observed in the hearts (Table II). Sterile cultures were observed with experimental conditions where antibodies were only weakly toxin neutralizing (i.e. PA/MP and PA/MP + Resiq/MP). It would appear that a strong antibody response is not necessarily needed, but perhaps a protective CD8+ T cell mediated response is generated. The role of CD8+ response is reasonable, considering that the challenge occurs near the time where antibody generation begins (Fig. 2). Additionally, the role of CD8+ cells generated by a plasmid-based cancer vaccine with a shortened vaccination schedule was elucidated by Peng *et al.* They show that antigen specific CD8+ T cell activation was shown to generate a protective response that therapeutically reduced cancer volume (48). Furthermore, the use of a particulate system to generate a CD8 response from a protein antigen was first shown by the Rock Laboratory in 1993, wherein protein absorbed on iron oxide beads increased dramatically the presentation of the immunodominant peptide of the protein on major histocompatibility complex I (MHC I) (32). Later, work from the Fréchet group reported that protein encapsulated in particulate carriers comprised of acid-sensitive polymers resulted in significantly greater MHC I presentation over protein complexation with other biomaterials such as iron oxide beads and PLGA (21,30,50,51).

With our study, the levels of IFN-γ would indicate elevated levels of CD8+ and CD4+ T cell activity in the antigen with resiquimod groups, with the levels of IL-2 indicating increased T cell proliferation in these groups and several other experimental conditions (Fig 4) (41). For this study, CD8+ response per se is difficult to measure with protective antigen because the immunodominant peptide is not well characterized. Additional studies regarding proliferation of CD8+ T cells need to be performed to completely elucidate the role of CD8+ T cells in generating a protective immune response with a shorted vaccination schedule.

CONCLUSION

Here, we report the first instance of using a particulate carrier to confer protective immunity rapidly. A novel polymer that has benign degradation products, a broad range of degradation rates and shown enhanced CD8+ activation (Ac-DEX) was used in microparticulate form to generate this protective response. Ac-DEX microparticles encapsulated rPA and/or resiquimod and were administered by a subcutaneous vaccination twice, 1 week apart. By reducing the dosing frequency and time to challenge, we were able to protect animals against three challenges 1 week apart. Survival was not shown to be dependent on neutralization from antibodies and potentially could be from an effector CD8+ T cell response. This work can aid in the development of universal platform for delivery of subunit vaccines for rapid protection against intracellular pathogens. Future evaluation of this platform to generate a protective memory response can also lead to a safer vaccine or one with improved dosing, compared to commercially available anthrax vaccines (e.g. BioThrax).

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REFERENCES

1. Callahan M. DARPA-BAA-09-43: Broad Agency Announcement 7-Day Biodefense In: Agency DARP, editor. Arlington, VA Department of Defense; 2009.
2. Control CfD. Emergency Preparedness and Response: Anthrax. Atlanta: CDC; 2012 [cited 2012 3/8/12]; Available from: http://www.bt.cdc.gov/agent/anthrax/.
3. Bhargava D, Bhargava K, Sabri I, Siddharth M, Dave A, HG J, *et al.* Bioterrorism - "My Role as a Dentist". J Indian Acad Forensic Med. 2011;33(3):254.
4. Sternbach G. The history of anthrax. J Emerg Med. 2003;24 (4):463–7.
5. Vasudev M, Zacharisen MC. New-onset rheumatoid arthritis after anthrax vaccination. Ann Allergy Asthma Immunol. 2006;97 (1):110–2.
6. Geier MR, Geier DA. Gastrointestinal adverse reactions following anthrax vaccination: an analysis of the Vaccine Adverse Events Reporting System (VAERS) database. Hepatogastroenterology. 2004;51(57):762–7.
7. Mercola J. Is An Anthrax Vaccine Worth It? Redwood City: Care2.com, Inc.; 2012 [cited 2012 3/22/12]; Available from: http://www.care2.com/greenliving/is-an-anthrax-vaccine-worth-it.html.
8. Zhang Y, Qiu J, Zhou Y, Farhangfar F, Hester J, Lin AY, *et al.* Plasmid-based vaccination with candidate anthrax vaccine antigens induces durable type 1 and type 2 T-helper immune responses. Vaccine. 2008;26(5):614–22.
9. Rynkiewicz D, Rathkopf M, Sim I, Waytes AT, Hopkins RJ, Giri L, *et al.* Marked enhancement of the immune response to BioThrax (R) (Anthrax Vaccine Adsorbed) by the TLR9 agonist CPG 7909 in healthy volunteers. Vaccine. 2011;29(37):6313–20.

10. Klinman DM, Xie H, Ivins BE. CpG oligonucleotides improve the protective immune response induced by the licensed anthrax vaccine. Ann N Y Acad Sci. 2006;1082:137–50.
11. Lousada-Dietrich S, Jogdand PS, Jepsen S, Pinto VV, Ditlev SB, Christiansen M, *et al.* A synthetic TLR4 agonist formulated in an emulsion enhances humoral and type 1 cellular immune responses against GMZ2–a GLURP-MSP3 fusion protein malaria vaccine candidate. Vaccine. 2011;29(17):3284–92.
12. Bargieri DY, Rosa DS, Braga CJ, Carvalho BO, Costa FT, Espindola NM, *et al.* New malaria vaccine candidates based on the plasmodium vivax merozoite surface protein-1 and the TLR-5 agonist salmonella typhimurium FliC flagellin. Vaccine. 2008;26(48):6132–42.
13. van Oers MM. Vaccines for viral and parasitic diseases produced with baculovirus vectors. Adv Virus Res. 2006;68:193–253.
14. Almeida AP, Bruna-Romero O. Synergism/complementarity of recombinant adenoviral vectors and other vaccination platforms during induction of protective immunity against malaria. Mem Inst Oswaldo Cruz. 2011;106 Suppl 1:193–201.
15. Bachelder EM, Beaudette TT, Broaders KE, Frechet JM, Albrecht MT, Mateczun AJ, *et al. In vitro* analysis of acetalated dextran microparticles as a potent delivery platform for vaccine adjuvants. Mol Pharm. 2010;7(3):826–35.
16. Tomai MA, Imbertson LM, Stanczak TL, Tygrett LT, Waldschmidt TJ. The immune response modifiers imiquimod and R-848 are potent activators of B lymphocytes. Cell Immunol. 2000;203(1):55–65.
17. Dockrell DH, Kinghorn GR. Imiquimod and resiquimod as novel immunomodulators. J Antimicrob Chemother. 2001;48(6):751–5.
18. Manolova V, Flace A, Bauer M, Schwarz K, Saudan P, Bachmann MF. Nanoparticles target distinct dendritic cell populations according to their size. Eur J Immunol. 2008;38(5):1404–13.
19. Kauffman KJ, Kanthamneni N, Meenach SA, Pierson BC, Bachelder EM, Ainslie KM. Optimization of rapamycin-loaded acetalated dextran microparticles for immunosuppression. Int J Pharm. 2012;422(1–2):356–63.
20. Meenach SA, Kim YJ, Kauffman KJ, Kanthamneni N, Bachelder EM, Ainslie KM. Synthesis, optimization, and characterization of camptothecin-loaded acetalated dextran porous microparticles for pulmonary delivery. Mol Pharm. 2012;9(2):290–8.
21. Broaders KE, Cohen JA, Beaudette TT, Bachelder EM, Frechet JM. Acetalated dextran is a chemically and biologically tunable material for particulate immunotherapy. Proc Natl Acad Sci U S A. 2009;106(14):5497–502.
22. Udenfriend S, Stein S, Bohlen P, Dairman W, Leimgruber W, Weigele M. Fluorescamine: a reagent for assay of amino acids, peptides, proteins, and primary amines in the picomole range. Science. 1972;178(4063):871–2.
23. Albrecht MT, Li H, Williamson ED, LeButt CS, Flick-Smith HC, Quinn CP, *et al.* Human monoclonal antibodies against anthrax lethal factor and protective antigen act independently to protect against bacillus anthracis infection and enhance endogenous immunity to anthrax. Infect Immun. 2007;75(11):5425–33.
24. Little SF, Leppla SH, Cora E. Production and characterization of monoclonal antibodies to the protective antigen component of bacillus anthracis toxin. Infect Immun. 1988;56(7):1807–13.
25. Quinn CP, Dull PM, Semenova V, Li H, Crotty S, Taylor TH, *et al.* Immune responses to bacillus anthracis protective antigen in patients with bioterrorism-related cutaneous or inhalation anthrax. J Infect Dis. 2004;190(7):1228–36.
26. Li H, Soroka SD, Taylor Jr TH, Stamey KL, Stinson KW, Freeman AE, *et al.* Standardized, mathematical model-based and validated *in vitro* analysis of anthrax lethal toxin neutralization. J Immunol Methods. 2008;333(1–2):89–106.
27. Stojkovic B, Torres EM, Prouty AM, Patel HK, Zhuang L, Koehler TM, *et al.* High-throughput, single-cell analysis of macrophage interactions with fluorescently labeled bacillus anthracis spores. Appl Environ Microbiol. 2008;74(16):5201–10.
28. Foged C, Brodin B, Frokjaer S, Sundblad A. Particle size and surface charge affect particle uptake by human dendritic cells in an *in vitro* model. Int J Pharm. 2005;298(2):315–22.
29. Hirota K, Hasegawa T, Hinata H, Ito F, Inagawa H, Kochi C, *et al.* Optimum conditions for efficient phagocytosis of rifampicin-loaded PLGA microspheres by alveolar macrophages. J Control Release. 2007;119(1):69–76.
30. Bachelder EM, Beaudette TT, Broaders KE, Dashe J, Frechet JM. Acetal-derivatized dextran: an acid-responsive biodegradable material for therapeutic applications. J Am Chem Soc. 2008;130(32):10494–5.
31. Chadwick S, Kriegel C, Amiji M. Nanotechnology solutions for mucosal immunization. Adv Drug Deliv Rev. 2010;62(4–5):394–407.
32. Kovacsovics-Bankowski M, Clark K, Benacerraf B, Rock KL. Efficient major histocompatibility complex class I presentation of exogenous antigen upon phagocytosis by macrophages. Proc Natl Acad Sci U S A. 1993;90(11):4942–6.
33. Kanthamneni N, Sharma S, Meenach SA, Billet B, Zhao JC, Bachelder EM, *et al.* Enhanced stability of horseradish peroxidase encapsulated in acetalated dextran microparticles stored outside cold chain conditions. Int J Pharm. 2012;431(1–2):101–10.
34. Lu L, Peter SJ, Lyman MD, Lai HL, Leite SM, Tamada JA, *et al. In vitro* and *in vivo* degradation of porous poly(DL-lactic-co-glycolic acid) foams. Biomaterials. 2000;21(18):1837–45.
35. Liu Y. Schwendeman SP. Mol Pharm: Mapping microclimate pH distribution inside protein-encapsulated PLGA microspheres using confocal laser scanning microscopy; 2012.
36. Szoka Jr FC. The future of liposomal drug delivery. Biotechnol Appl Biochem. 1990;12(5):496–500.
37. Hamouda T, Chepurnov A, Mank N, Knowlton J, Chepurnova T, Myc A, *et al.* Efficacy, immunogenicity and stability of a novel intranasal nanoemulsion-adjuvanted influenza vaccine in a murine model. Hum Vaccin. 2010;6(7):585–94.
38. Andresen TL, Jensen SS, Jorgensen K. Advanced strategies in liposomal cancer therapy: problems and prospects of active and tumor specific drug release. Prog Lipid Res. 2005;44(1):68–97.
39. Janeway C, Travers P, Walport M, Shlomchik M. ImmunoBiology. 5th ed. New York: Garland Science; 2001.
40. Johnston D, Bystryn JC. Topical imiquimod is a potent adjuvant to a weakly-immunogenic protein prototype vaccine. Vaccine. 2006;24(11):1958–65.
41. Paul WE. Fundamental immunology. 4th ed. New York: Raven; 1994.
42. Ribeiro S, Rijpkema SG, Durrani Z, Florence AT. PLGA-dendron nanoparticles enhance immunogenicity but not lethal antibody production of a DNA vaccine against anthrax in mice. Int J Pharm. 2007;331(2):228–32.
43. Nemazee DA. Immune complexes can trigger specific, T cell-dependent, autoanti-IgG antibody production in mice. J Exp Med. 1985;161(1):242–56.
44. McConnell MJ, Hanna PC, Imperiale MJ. Adenovirus-based prime-boost immunization for rapid vaccination against anthrax. Mol Ther. 2007;15(1):203–10.
45. Williamson ED, Hodgson I, Walker NJ, Topping AW, Duchars MG, Mott JM, *et al.* Immunogenicity of recombinant protective antigen and efficacy against aerosol challenge with anthrax. Infect Immun. 2005;73(9):5978–87.
46. Tatsis N, Ertl HC. Adenoviruses as vaccine vectors. Mol Ther. 2004;10(4):616–29.
47. Bonnet MC, Tartaglia J, Verdier F, Kourilsky P, Lindberg A, Klein M, *et al.* Recombinant viruses as a tool for therapeutic vaccination against human cancers. Immunol Lett. 2000;74(1):11–25.

48. Peng S, Trimble C, Alvarez RD, Huh WK, Lin Z, Monie A, *et al*. Cluster intradermal DNA vaccination rapidly induces E7-specific CD8+ T-cell immune responses leading to therapeutic antitumor effects. Gene Ther. 2008;15(16):1156–66.
49. Popov SG, Popova TG, Grene E, Klotz F, Cardwell J, Bradburne C, *et al*. Systemic cytokine response in murine anthrax. Cell Microbiol. 2004;6(3):225–33.
50. Cohen JA, Beaudette TT, Tseng WW, Bachelder EM, Mende I, Engleman EG, *et al*. T-cell activation by antigen-loaded pH-sensitive hydrogel particles *in vivo*: the effect of particle size. Bioconjug Chem. 2009;20(1):111–9.
51. Kwon YJ, James E, Shastri N, Frechet JM. *In vivo* targeting of dendritic cells for activation of cellular immunity using vaccine carriers based on pH-responsive microparticles. Proc Natl Acad Sci U S A. 2005;102(51):18264–8.