AN ABSTRACT OF THE DISSERTATION OF

Brian R. Sloat for the degree of Doctor of Philosophy in Pharmacy presented on March 19, 2010.

Title: Rational Vaccine Development
1) Design of a Triantigen Nasal Anthrax Vaccine Cadidate
2) A Novel Lecithin Based Nanoparticle as a Vaccine Delivery System

Abstract approved:

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Currently, the only anthrax vaccine licensed for human use in the United States is the Anthrax-vaccine-absorbed (AVA or Biothrax®). AVA suffers from several drawbacks, including a complicated and lengthy dosing schedule that requires six initial injections administered over eighteen months, followed by annual boosters. Therefore, a new generation anthrax vaccine that can be easily administered for rapid mass immunization and induce strong immune responses not only against the anthrax protective antigen protein, but also against the other virulent factors of *Bacillus anthracis*. To address these needs, a prototypic triantigen nasal anthrax vaccine candidate that contains a truncated PA (rPA63), the anthrax lethal factor (LF), and the capsular poly-γ-D-glutamic acid (γDPGA) as the antigens and a synthetic double-stranded RNA, polyriboinosinic-polyribocytidylic (pI:C) acid as the adjuvant. This study identified the optimal dose of nasal pI:C in mice, as well as showed that pI:C enhanced the proportion of dendritic cells (DCs) in local draining lymph nodes (LNs)

and stimulated DC maturation. The γDPGA was shown to be immunogenic when conjugated to a carrier protein and dosed intranasally to mice. Further, the anti-PGA antibodies (Abs) were shown to be functional because they were able to activate complement and kill PGA-producing bacteria. Nasal immunization with LF alone and PA alone induced strong, functional anti-LF and anti-PA Abs. Nasal immunization of mice with the prototypic tri-antigen vaccine candidate induced strong immune responses against all three antigens. The immune responses protected macrophages against an anthrax lethal toxin challenge *in vitro* and enabled the immunized mice to survive a lethal dose of anthrax lethal toxin challenge *in vivo.*

When used as a nasal vaccine adjuvant, pI:C is generally considered to be safe. However, repeated high doses of pI:C tended to induce some side effects, including fever and abnormal liver functions. Therefore, new adjuvants are constantly being sought. Over the past several decades, an accumulation of research has demonstrated the usefulness of nanoparticles as antigen carriers with adjuvant activity. A novel lecithin-based nanoparticle was engineered from emulsions. Bovine serum albumin (BSA) and PA proteins were covalently conjugated onto the nanoparticles. Mice immunized with BSA conjugated nanoparticles developed strong anti-BSA Ab responses comparable to that induced by BSA adjuvanted with incomplete Freund's adjuvant and 6.5-fold stronger than that induced by BSA adsorbed onto aluminum hydroxide. Immunization of mice with the PA-conjugated nanoparticles elicited a quick, strong, and durable anti-PA Ab response that afforded protection of the mice against a lethal dose of anthrax lethal toxin challenge. The adjuvanticity of the

nanoparticles was likely due to their ability to move antigens into local draining LNs, to enhance the uptake of the antigens by antigen-presenting cells (APCs), and to activate APCs.

Most vaccines require cold-chain refrigeration for storage and distribution. A major challenge in the vaccine development field is to develop formulations that do not require refrigeration. The most commonly used process in the pharmaceutical field to convert vaccine suspensions into solids of sufficient stability for distribution and storage is lyophilization. Using 5% of mannitol plus 1% of polyvinylpyrrolidone, the immunogenicity of the lyophilized protein conjugated nanoparticles (BSA-NPs or PA-NPS) was found to be undamaged after a relatively extended period of storage at room temperature or under accelerated conditions (37^{o}C).

Rational Vaccine Development

1) Design of a Triantigen Nasal Anthrax Vaccine Candidate

2) A Novel Lecithin Based Nanoparticle as a Vaccine Delivery System

by

Brian R. Sloat

A DISSERTATION

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Presented March 19, 2010

Commencement June 2010

Doctor of Philosophy dissertation of Brian R. Sloat presented on March 19, 2010

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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ACKNOWLEDMEMENTS

I would like to express my sincere gratitude to my advisor, Dr. Zhengrong Cui, for allowing me to join his research team at Oregon State University, his scientific insights, encouragement, and financial support. I would like to thank Dr. J. Mark Christensen, Dr. Rosita Proteau, Dr. Virginia Lesser, and Dr. Mark Hoffman for their willingness to participate in my dissertation committee and for providing useful guidance.

I would also like to say thank you to Ms. Julie Oughton, Ms. Danielle King, and Dr. Nikki Marshall for their helpful assistance and interpretation in the flow cytometry experiments, Dr. Mike Nesson and Ms. Teresa Sawyer for their assistance in obtaining TEM images of the nanoparticles, Mr. Andrew Hau and Mr. Adam Campbell for their help in the fluorescent imaging of cells, and Ms. Stacey Vannorrison for her kind assistance on the DSC work.

I would like to thank the current students in Dr. Cui's lab, Ms. Nijaporn Yanasarn, Mr. Mike Sandoval, Ms. Letty Rodriguez, Ms. Xinran Li, and Mr. Amit Kumar, for their help and friendship both in and out of the lab. I would like to thank Dr. Woongye Chung, Dr. Zhen Yu, Dr. Qui Fu, and Dr. Dalia Shaker, former or current postdocs in Dr. Cui's lab, for their helpful collaborations and advice. I also highly appreciate Dr. Uyen Le, a former graduate student in Dr. Cui's lab, for her friendship, lab help and advice, and her amazing Vietnamese food throughout the years.

I am very grateful to OSU College of Pharmacy for its partial financial support in 2005 – 2009, the P. F. & Nellie Buck Yerex Graduate Fellowship for its partial financial support in 2007 – 2008, the Oregon Sports Lottery Scholarship for its partial financial support in 2008 – 2009, the James Ayres – OSU College of Pharmacy Travel Award and Sponenburgh Travel Award to attend a national conference in 2008 and 2009, respectively. I would also like to thank my current and former colleagues in the OSU College of Pharmacy, especially those in the Department of Pharmaceutical Sciences, for their friendship and guidance over the past several years.

Lastly, I would like to thank my family for their love, support, and encouragement over my many years of school, I am forever indebted to them! I would also like to thank Dr. Hirdesh Uppal and family for their help, guidance, and friendship.

CONTRIBUTION OF AUTHORS

Dr. Dalia S. Shaker (College of Pharmacy, Oregon State University) assisted with some animal experiments in chapter 4.

Dr. Uyen M. Le (College of Pharmacy, Oregon State University) assisted with some animal experiments in chapter 4.

Mr. Michael A. Sandoval (College of Pharmacy, Oregon State University; College of Pharmacy, University of Texas – Austin) assisted with experiments in chapters 5 and 6.

Mr. Andrew M. Hau (College of Pharmacy, Oregon State University) assisted with the fluorescent imagery in chapter 5.

Dr. Yongqun He (Unit for Laboratory Animal Medicine, University of Michigan Medical School) provided materials and assistance that made the publication of chapter 5 possible.

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2) A NOVEL LECITHIN BASED NANOPARTICLE AS A VACCINE DELIVERY SYSTEM

Chapter 1

GENERAL INTRODUCTION

1.1 Vaccines

A vaccine is a biological preparation that elicits an immune response against a particular disease. The concept of vaccination was first demonstrated by Edward Jenner more than 200 years ago. Jenner inoculated and protected an eight year old boy against smallpox using pus collected from the hand of a milkmaid infected with cowpox. Since then, the development and global use of vaccines against a variety of infectious agents is arguably one of the greatest medical achievements. Vaccination is often considered to be the most effective and economical method of preventing infectious diseases. Vaccines can be generalized into three categories: live or attenuated, inactivated (killed microorganisms, subunits, and toxoids), or nucleic acid (Ellis, 2001).

Live or attenuated vaccines consist of a microorganism that is able to replicate in a host or infect cells, and induce an immune response similar to that elicited by the natural infection. The microorganisms contained in such vaccines have their virulent properties disabled by biological or technical manipulations; however they remain infectious enough to function as a vaccine (Ellis, 2001). Vaccination with live

vaccines is successful due to their high immunogenicity, and their ability to stimulate both cell mediated immune responses and neutralizing antibodies (Ellis, 1999a; Ellis, 1999b). However, the potential safety concern associated with the use of live vaccines presents a limitation to their use and drives the development for safer vaccines.

Inactivated vaccines are a broad category consisting of inactivated whole bacteria or viruses, or isolated proteins, peptides, or protein toxins as antigens. Such vaccines contain immunogens that cannot replicate in the host. Inactivated whole bacteria vaccines are prepared from cultured bacterial cells that have been killed by heat or chemical agents (Ellis, 2001). Inactivated whole virus vaccines are prepared by collecting the supernatant from infected cell cultures when the virus is shed, or by simply lysing the cell pellet when the virus remains intracellular. The virus particles require purification and chemical inactivation before further use (Ellis, 2001). Biological and immunological approaches have helped identify proteins and peptides (isolated from microorganisms, produced by recombinant DNA technology or by direct chemical peptide synthesis, or expressed by DNA constructs) as candidate vaccine antigens, collectively referred to as subunit vaccines. While these inactivated vaccines overcome some of the safety concerns of live vaccines, they too have disadvantages. Inactivated vaccines, especially subunit vaccines, are poorly immunogenic, and often require an adjuvant to enhance immunogenicity. A priming dose followed by booster doses is often required to establish long-term protective immunity (Ellis, 2001). Another disadvantage common to most inactivated vaccines is their inability to elicit an effective cell-mediated immune response. The lack of a

cellular response may be the reason there are no effective vaccines against the infectious pathogens like human immunodeficiency virus or the pathogen causing malaria. To effectively protect a host against such pathogens, the induction cellular immune responses may be more critical than the production of neutralizing antibodies (Gurunathan et al., 2000). The need for vaccines that are both safe and effective at eliciting cellular and humoral immune responses simultaneously continues to drive research in this area.

Nucleic acid or DNA vaccines are taken up by cells and direct the synthesis of the vaccine's antigen, the antigen may be secreted by or associated with the cell in a way that would elicit a cellular or antibody response (Wolff et al., 1990). DNA vaccines are advantageous because of their ease or preparation, the expected amplification of both antigen synthesis and corresponding immune response, and the induction of both antibody and cellular immune responses (Ellis, 2001). Nevertheless, DNA vaccines tend to be poorly immunogenic, and while intramuscular injection of unformulated plasmid DNA into animals induced strong antibody and cellular immune responses, there is no evidence that similar results will occur in humans (Mumper and Ledebur, 2001). Therefore, improving the uptake of the DNA by using a vehicle, alternate routes of administration, and the use of adjuvants are all active areas of research in DNA vaccinology.

1.2 Immunological adjuvants

An adjuvant is a substance that stimulates an increased cellular and/or humoral immune response to a co-administered antigen (Ellis, 2001). Many vaccines, especially those consisting of purified proteins and peptides, are weakly immunogenic and require an appropriate adjuvant. Like vaccines, adjuvants can be generalized into three categories: particulates, non-particulates, and combined adjuvant compositions (Sinyakov et al., 2006). Particulate adjuvants are common in their physical structure and dimensions. They are particulate dispersions to which an antigen is bound or associated, and extend the duration of an antigen's presence at the injection site. This category of adjuvant includes the aluminum salts (Glenny, 1926), oil emulsions (Freund, 1937), and micro- and nanoparticles (Allison and Gregoriadis, 1974; Kreuter et al., 1976). The aluminum salts (generically called Alum) were identified in the 1920s, and are licensed for use in vaccines across the world (Glenny, 1926). Currently, aluminum hydroxide is the only adjuvant licensed for human use in the United States. However, Alum is a relatively weak adjuvant and has various limitations (Baylor et al., 2002). Examples of non-particulate adjuvants include saponins, cholera toxin, lipid A, and muramyl dipeptide derivatives. Cholera toxin is mostly used as a mucosal adjuvant, but has also been shown to be an effective skin adjuvant. Lipid A is a purified component of the bacterial lipopolysaccharide (LPS), and is capable of boosting the cellular immune responses. While no non-particulate adjuvants are approved for human use in the United States, monophosphoryl lipid A co-adjuvanted with Alum is licensed for use in an HBV vaccine in Europe (O'Hagan

and De Gregorio, 2009). Other non-particulate adjuvants, such as saponins, are currently in Phase I/II clinical trials (O'Hagan and De Gregorio, 2009). Examples of combined adjuvant compositions include Syntex adjuvant formulation, Ribi adjuvant system, as well as other immune stimulating complexes (Sinyakov et al., 2006). Presently, no combined adjuvant compositions are approved for human use, but several are in phase I and II clinical trials (O'Hagan and De Gregorio, 2009).

1.3 Immune responses, nasal immunization, Toll-like receptors, and polyriboinosinic-polyribocytidylic acid

The immune response consists of adaptive immunity and innate immunity. Innate immunity consists of antigen presenting cells (dendritic cells, macrophages, and leukocytes) that engulf and digest microorganisms and other foreign substances. Where adaptive immunity is mediated by clonally distributed T and B lymphocytes and is characterized by specificity and memory (Akira et al., 2001). The activation of the innate immune responses can be a requirement for the triggering of adaptive immunity.

The adaptive immune response is influenced by the generation of helper T cells (Th) and the resultant production of cytokines by these cells (Abbas et al., 1996). When stimulated by APCs with cognate antigen, the Th cells differentiate into helper T type 1 cells (Th1) or Th2. Th1 cells secrete interferon-γ (IFN- γ) and chiefly promote cellular immune responses, where Th2 cells secrete Interleukin-4 (IL-4), IL-5, IL-10, and IL-13, and promote antibody immune responses. The instructive cytokines IL-12 typically influences the generation of Th1 based immunity, where IL-

4 drives Th2 based immunity. Infection by intracellular pathogens induces primarily a Th1 dominant response, where intercellular infection typically induces a Th2 dominant response. APCs use costimulatory molecules, including CD80 and CD86 to signal T cells to induce clonal expansion of antigen-specific T cells. Antigen presentation in the absence of costimulation will lead to T cell anergy, whereas engagement by costimulatory molecules alone does not activate antigen-specific T cells. To induce effective immunogenicity, these stimuli must be provided simultaneously by APCs to T cells.

The resultant immune response is determined by how the antigen is presented to T and B cells by APCs. Endogenous antigens, those arising from intracellular infections, are processed into small peptides by the proteosomes, transferred to the endoplasmic reticulum, and bind to major histocompatibility complex (MHC) I molecules. The antigen loaded MHC I is then transported to the cell surface where it can be recognized by appropriate antigen specific $CD8^+$ T cells. This endogenous presentation results in a cellular immune response. However, if the antigen is taken up by APCs by endocytosis, the antigen will be processed inside the endosomes and lysosomes. The degraded antigen will be loaded into MHC II molecules and transported to the surface of the APC where it can be recognized by $CD4^+$ T cells. One of the outcomes of this presentation is the production of antibodies by B lymphocytes or the humoral immune response (Castellino et al., 1997). The naïve $CD4^+$ T cells can develop into either Th1 or Th2 helper cells. Th1 helper cells and

related cytokines help initiate a cellular immune response, whereas Th2 helper cells and related cytokines direct the antibody immune response.

Nearly all pathogens causing common infectious diseases of the respiratory, intestinal, and genital tract enter or infect through the large surface area of the mucosal membranes (Mestecky et al., 1997). The nasal mucosa is often the first point of contact for inhaled antigens, and has emerged as a promising route for vaccination to induce both peripheral and mucosal immunization (Davis, 2001; Renauld-Mongenie et al., 1996). The nose has been identified as an attractive route for immunization because it is easily accessible, and thus allows for the possibility of mass immunization while not requiring needles and syringes, or trained professionals. Additionally, the nose has a large absorption surface area, is highly vascularized, and after intranasal immunization, both mucosal and systemic immune responses can be induced. The dissemination of effector immune cells in the common mucosal immune system allow for immune responses to be induced at distant mucosal sites as well (Partidos, 2000).

The immune system of the respiratory tract can be divided into three parts: the surface epithelium and its underlying connective tissue that contains immunocompetent cells (macrophages, DCs, M-cells, and intraepithelial lymphocytes), the nose, larynx, and bronchus – associated lymphoid tissues, and the local lymph nodes that drain the respiratory system (Davis, 2001). The nose associated lymphoid tissue (NALT) is a well organized structure, consisting of B and T cell areas, which are covered by an epithelial layer containing M cells (Kuper et al.,

1992). Due to the lack of afferent lymphatics, exogenous antigens are sampled directly from the mucosal surface covering the NALT, primarily by the M cells (Davis, 2001). The DCs, which are rich in the epithelial tissues, may also pick up antigens present on the mucosal surface (Jahnsen et al., 2004).

Immunological studies have identified the Toll-like receptor (TLR) as a key regulator of both the innate and adaptive immune responses (Pandey and Agrawal, 2006). The TLR is a family of evolutionarily conserved transmembrane receptors that recognize distinct pathogen-associated molecular patterns (Akira et al., 2001). Upon activation, TLR signal transduction involves myeloid differentiation factor 88 (MyD88) mediated pathway that leads to the activation of nuclear factor (NF)-κB, and Toll IL-1 receptor (TIR) domain containing adaptor inducing IFN-β mediated pathway resulting in the activation of interferon regulatory factor 3 (Pandey and Agrawal, 2006). At least thirteen mammalian TLRs have been identified, although the expression of only 10 is known in humans (Takeda and Akira, 2003; Takeda et al., 2003). Further, several exogenous and endogenous TLR ligands have been identified, making TLRs a therapeutic target. One such ligand, double-stranded RNA (dsRNA), which is produced by many viruses during their replicative cycle (Honda et al., 2003; Okahira et al., 2005), was found to be an agonist of TLR3 (Alexopoulou et al., 2001). It was known in the 1960 -1970s that polyriboinosinic-polyribocytdylic (poly(I:C) or pI:C), a synthetic dsRNA, can boost immune responses (Giuntoli et al., 2002; Herman and Baron, 1971; Park and Baron, 1968; Stark et al., 1998; Yang et al., 1995). Poly(I:C) was shown to induce stable maturation of DCs (Verdijk et al., 1999),

activate natural killer (NK) cells (Schmidt et al., 2004; Sivori et al., 2004), promote the survival of activated $CD4^+$ T cells *in vitro* (Gelman et al., 2004), and to boost the number of antigen-specific $CD8^+$ T cells and their survival *in vivo* (Salem et al., 2005). Additionally, pI:C has proven to be effective at enhancing the immune responses against nasally dosed vaccines. For example, Ichinohe et al. reported that nasal immunization of mice with an inactivated hemaglutinin (HA) admixed with pI:C protected the mice against an influenza viral infection (Ichinohe et al., 2005). Similarly, Partidos et al. reported the induction of specific serum and mucosal antibodies, as well as cellular responses, to the HIV TAT protein when the molecular complex of TAT and pI:C was nasally administered into mice (Partidos et al., 2005).

1.4 Anthrax: clinical manifestations, virulence, and vaccination

The etiological agent of anthrax, *Bacillus anthracis* is a rod-shaped, non-motile, Gram-positive bacterium that forms highly resistant endospores under adverse conditions (Ascenzi et al., 2002). Three clinical forms of anthrax infection can occur depending on the site of inoculation: cutaneous, gastrointestinal, or inhalational. Ninety-five percent of all anthrax infections reported in the United States are cutaneous infections (Dixon et al., 1999; Taylor et al., 1993). Patients suffering from cutaneous anthrax often have a history of contact with animals or animal products. The primary skin lesion appears as a painless, pruritic papule 3 to 5 days after the introduction of endospores. The lesion forms a vesicle that undergoes necrosis and drying, leaving a black eschar surrounded by edema and several purplish vesicles

(Dixon et al., 1999). Cutaneous anthrax is rarely fatal, but antibiotic treatment is recommended. Gastrointestinal anthrax is rare and there have been no known cases in the United States, although the infection is serious and can be fatal. Symptoms appear 2 to 5 days after ingestion of undercooked, endospore-contaminated meat from a diseased animal (LaForce, 1994). Patients suffering from gastrointestinal anthrax often have intestinal ulcerations, fever, abdominal pain, blood-tinged diarrhea and vomit (Alizad et al., 1995; Dixon et al., 1999; Nalin et al., 1977). Symptoms in surviving patients generally subside within 10 to 14 days (Alizad et al., 1995). Inhalation anthrax has a fatality rate approaching 100%, even with aggressive antibacterial treatment (Friedlander, 1999). Anthrax spores are 1 – 2 μm in diameter, making them an optimal size for inhalation into the alveolar spaces (Brachman, 1980; Brachman et al., 1966; Dixon et al., 1999). Whereupon the spores are phagocytosed by macrophages and germinate into vegetative bacilli while being transported to the mediastinal and peribronchial lymph nodes, and are released into systemic circulation (Koehler et al., 1994). Bacilli reach as many as 10^7 to 10^8 organisms per milliliter of blood, causing massive septicemia (Dixon et al., 1999). Initial symptoms resemble that of an upper respiratory tract infection, and include a cough, fever, malaise, and muscle pain. Through the following several days, symptoms include dyspnea, chills, harsh cough, and eventual death of the host (Dixon et al., 1999).

Bacillus anthracis has two primary virulence factors, the secreted anthrax exotoxins and the capsule. The anthrax exotoxins have three components, the protective antigen (PA), the lethal factor (LF), and the edema factor (EF). While each

of these components are nontoxic individually, they can combine to form two binary toxins, the lethal toxin (LeTx, PA and LF) and the edema toxin (ET, PA and EF). The binding of PA onto receptors on the surface of host cells allows the entry of LF and EF into the cell cytosol, where they are toxic (Bradley et al., 2001; Scobie et al., 2003; Scobie et al., 2005). The LeTx is a zinc metalloprotease that has been shown to inactivate mitogen activated protein kinase kinase *in vitro* (Duesbery et al., 1998; Hammond and Hanna, 1998; Hanna et al., 1994; Klimpel et al., 1994; Vitale et al., 1998). Lethal toxin stimulates macrophages to release interleukin-1β and tumor necrosis factor α, which attribute to the sudden shock-like death of the host (Hanna et al., 1993; Hanna et al., 1992; Hanna et al., 1994). The ET is a calmodulin-dependent adenylate cyclase which is responsible for disrupting water homeostasis and causing massive edema (Dixon et al., 1999). Further, ET has been shown to inhibit neutrophil function *in vitro* (O'Brien et al., 1985). The poly-glutamic acid (PGA) capsule is another virulence factor, by which the *B. anthracis* is surrounded and protected from the host immune system (Welkos et al., 1993). *Bacillus anthracis* strains that are encapsulated within the PGA grow unhindered in infected hosts, whereas bacteria lacking the capsule are nearly avirulent (Welkos, 1991; Welkos et al., 1993). The exotoxins are responsible for inhibiting the immune response against the infection, where the capsule prevents phagocytosis of the vegetative anthrax bacilli.

Vaccination is considered the best option for the prophylaxis of anthrax. Currently, the only anthrax vaccine available in the United States is the anthrax-vaccine absorbed (AVA, now BioThrax®), an aluminum hydroxide (Alum) –

absorbed, formalin-treated culture supernatant of a toxigenic *B. anthracis* strain (Puziss et al., 1963). Previous studies demonstrated that the primary antigen component in the AVA was the PA protein. While effective, AVA has many limitations including occasional severe side effects, difficulty in maintaining consistency, and more importantly, a lengthy and complicated dosing schedule with six initial injections over 18 months, followed by annual booster injections (Turnbull, 2000; Wang and Roehrl, 2005).

A new generation anthrax vaccine is expected to target not only the anthrax protective antigen protein, but also other virulent factors of *B. anthracis*. A vaccine capable of inducing anticapsule Abs is expected to inhibit bacillus growth, thereby reducing anthrax toxin production, which is expected to be beneficial. The feasibility of inducing Abs against PGA and the efficacy of the anti-PGA Abs in protecting against anthrax infection have been confirmed in several studies (Chabot et al., 2004; Joyce et al., 2006; Kozel et al., 2004; Rhie et al., 2003; Schneerson et al., 2003). Further, Wang and Roehrl (2005) reported that after a naturally occurring human infection, 68-93% of patients developed anti-PA Abs, 43-55% developed anti-LF Abs, and 67-94% developed anti-PGA Abs (Wang and Roehrl, 2005). Further, there were data showing that vaccination of guinea pigs with PA and LF together afforded better protection against an aerosolized anthrax spore challenge than with PA alone (Ivins and Welkos, 1988). Taking these findings together, an anthrax vaccine that contains PA, LF, and PGA to target the vegetative bacilli and anthrax lethal toxin is expected to be more protective than the AVA.

1.5 Engineering of nanoparticles and nanoparticles as adjuvants

Solid lipid nanoparticles (SLN) were first described by Muller et al. more than a decade ago (Muller et al., 2000), since then they have attracted increasing attention not only as a drug carrier, but also as platform for vaccine design. Several methods exist to engineer SLN: high pressure homogenization using hot or cold dispersion techniques (Almeida, 1997), solvent emulsification and evaporation (Sjostrom et al., 1993), solvent emulsification and diffusion (Trotta et al., 2005), or from emulsion/ microemulsion precursors (Gasco, 1993). The latter technique involves the formation of an emulsion comprised of a hydrophobic oil phase and water phase that, upon heating and brief intermittent periods of sonication, forms a homogenous, opaque suspension of SLN with addition of a surfactant. This method of engineering nanoparticles has several advantages over the other techniques. The resulting nanoparticles are well-defined, uniform, and are reproducibly engineered without the use of high-torque mechanical mixing, homogenization, or microfluidization. Further, no harmful organic solvents are used during the manufacturing process. Lastly, the materials used in making the nanoparticles are potentially biocompatible.

The nanoparticles described herein are engineered using lecithin and glyceryl monostearate (GMS) as the matrix material and Tween 20 as an emulsifying agent. Lecithin is a complex mixture of phsphatides, consisting chiefly of phasphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, and other substances such as triglycerides and fatty acids. Further, lecithins are components of cell membranes and are regularly consumed as

part of a normal diet. They are extensively used in pharmaceutical applications as emulsifying, dispersing, and stabilizing agents and are included in intramuscular and intravenous injectables and parenteral nutrition formulations (Williams et al., 1984). It is generally regarded as safe (GRAS) and accepted in the FDA Inactive Ingredients Guide for parenterals (Weller, 1994). Tween 20 is a polyoxyethylene derivative of sorbitan monolaurate. It is also GRAS listed and included in the FDA Inactive Ingredients Guide for parenterals (Weller, 1994). Like lecithin, GMS is used in a variety of food, pharmaceutical, and cosmetic applications and is also GRAS listed. Taken together, this nanoparticle-based antigen delivery system is relatively safe, although more experiments need to be done to define its safety profile.

Recombinant protein antigens may help overcome the toxicities associated with the traditional vaccines prepared with live or attenuated microorganisms, but recombinant proteins are poorly immunogenic and a vaccine adjuvant is needed to enhance the immune responses. As previously mentioned, Alum remains to be the only adjuvant approved for human use in the U.S., and it is a relatively weak adjuvant with various limitations (Baylor et al., 2002; Lindblad, 2004a; Lindblad, 2004b). As the need for new alternative vaccine adjuvants to Alum becomes critical, micro- and nanoparticles have emerged as novel antigen carriers with adjuvant activity (Cui and Mumper, 2003; Peek et al., 2008; Singh et al., 2007; Wendorf et al., 2008). The adjuvanticity of micro- and nanoparticles is likely due to the easiness of APCs to take up antigens associated with the particles over soluble antigen. Data from recent studies suggests that nanoparticles have more potent adjuvant activity than

microparticles. For example, it was reported that particles with a diameter of 500 nm or less were optimal for uptake by DCs and macrophages (Foged et al., 2005; Kanchan and Panda, 2007). Additionally, Manolova et al. recently reported that small nanoparticles (20 - 200 nm) can freely drain into the LNs for antigen presentation, whereas DCs were required for the transport of large microparticles (0.5 – 2.0 microns) from the injection site to the LNs (Manolova et al., 2008). Therefore, it is likely that antigens associated with nanoparticles less than 200 nm can reach the draining LNs for antigen presentation not only by the trafficking of APCs, but also by direct draining, which is expected to lead to a strong immune response. Recent studies aimed at correlating the particle size of the antigen carrier to the resultant immune response are inconsistent. For example, Wendorf et al. reported that comparable immune responses were induced in mice by protein antigens (Env from HIV-1 and MenB from *Neisseria meningitides*) adsorbed onto anionic microparticles (~1 μm) and nanoparticles (110 nm) prepared with poly(lactic-co-glycolic acid) (PLGA) polymers (Wendorf et al., 2008). Contrarily, Gutierro et al. showed that when BSA was entrapped in PLGA particles of various sizes (1 μm, 500 nm, and 200 nm), the 1 μm particles generally induced a stronger serum anti-BSA IgG response than the smaller nanoparticles (Gutierro et al., 2002). Similarly, Kanchan and Panda reported that the HBsAg entrapped in PLGA particles of 200 – 600 nm induced a weaker anti-HBsAg antibody response than HBsAg entrapped in PLGA particles of 2 – 8 μm, when injected intramuscularly into rats (Kanchan and Panda, 2007). On the other hand, data from several other studies showed that the immune response induced by smaller

nanoparticles was more potent than that of the larger microparticles using protein antigens or plasmid DNA (Fifis et al., 2004; Kalkanidis et al., 2006; Minigo et al., 2007; Olbrich et al., 2002; Singh et al., 2000). For example, Fifs et al. reported that the optimal particle size was 40 – 50 nm when they compared the antibody and cellular immune responses induced by polystyrene particles ranging from 20 nm to 2 μm with antigens conjugated onto their surface (Fifis et al., 2004). Interestingly, the 40 – 50 nm was the size of the polystyrene particles before the protein antigen was conjugated onto their surface. When ovalbumin as an antigen was conjugated onto the particles, the resultant particle size was reported to be 223 nm (Kalkanidis et al., 2006), demonstrating that particles with a diameter of around 200 nm are ideal antigen carriers when the antigens are on the surface of the particles.

There is evidence to suggest that the particle's size may have different effects on the antigen-specific antibody or cellular immune responses (Caputo et al., 2009; Kanchan and Panda, 2007; Mann et al., 2009). In general, it seemed that microparticles promoted antibody responses, whereas nanoparticles promoted cellular responses (Kanchan and Panda, 2007). For example, Caputo et al. showed that HIV TAT protein adsorbed onto cationic polymeric nanoparticles (220 nm or 630 nm) tended to induce a stronger cellular immune response and a weaker antibody response than the same protein adsorbed onto large microparticles (~2 μm) (Caputo et al., 2009). Of great interest, Mann et al. reported that using oral biolosomes (bile salt in lipid vesicles) with influenza A antigens showed that the larger biolosomes (0.4 – 2.0

μm) generated an immune response that was significantly more biased towards Th1 than the smaller biolosomes (10 – 100 nm) (Mann et al., 2009).

1.6 Lyophilization

Cold chain refrigeration is often required for the distribution and storage of most vaccines. Developing a vaccine formulation that does not require refrigeration represents a major challenge in the vaccine development process. Usually, a dried solid state vaccine formulation is prepared using methods such as lyophilization to avoid or to slow down the degradation. Lyophilization, or freeze drying, is a dehydration process used to preserve or improve the stability of various pharmaceutical products including: proteins and nanoparticles. The freeze drying process is relatively slow and expensive. A lyophilization cycle typically requires three steps: freezing or solidification, ice sublimation during a primary drying phase, and the desorption of unfrozen water during a secondary drying phase. A typical pharmaceutical scale freeze dryer consists of a drying chamber with temperature controlled shelves, and a chamber housing a condenser. The condenser consists of a series of plates or coils capable of being maintained at or below -50°C. A series of vacuum pumps are connected to the condensing chamber to achieve a system wide pressure in the range of 4 to 40 Pa during operation (Abdelwahed et al., 2006b).

Solidification is the first step of lyophilization. The liquid containing the pharmaceutical product is cooled and ice crystals of pure water form. As solidification continues, more and more water contained in the liquid phase freezes

resulting in an increased concentration of the product in the liquid phase. As the liquid phase becomes more concentrated, the viscosity increases and inhibits further crystallization (Abdelwahed et al., 2006b). The highly concentrated, viscous liquid phase solidifies yielding an amorphic, crystalline, or amorphic-cystalline phase (Franks, 1992).

The primary-drying stage involves sublimation of ice from the frozen product. In this stage, (i) heat is transferred from the heating element of the shelf (if used) to the frozen product, and conducted to the sublimation front; (ii) water vapor from the sublimation process passes through the dried portion of the product to the surface of the sample, and lastly; (iii) the water vapor moves from the surface of the product and condenses onto the condensing element of the lyophilizer (Abdelwahed et al., 2006b). At the end of the primary-drying stage, pores in the dried product correspond to the spaces previously occupied by ice crystals (Williams and Polli, 1984). The secondary-drying stage involves the removal of bound water from the product, the water which did not solidify during the freezing stage, and did not sublimate off during the primary-drying stage (Abdelwahed et al., 2006b).

The Process of lyophilization introduces many stresses that could destabilize a colloidal suspension of nanoparticles. During the solidification of the nanoparticle suspension, there is a phase separation of the ice and the cryo-concentrated phase containing the nanoparticles. The freezing stress placed onto the particulate system can induce particle aggregation, irreversible fusion of the nanoparticles, or the crystallization of ice may induce a mechanical stress leading to the destabilization of

the nanoparticle system (Abdelwahed et al., 2006a). Therefore, excipients are added to the nanoparticles before freezing to protect the product from the freezing stresses (cryoprotectants) or drying stresses (lyoprotectants), and to increase the stability of the product for long term storage. The more popular cryoprotectants used for lyophilization of nanoparticles are the sugars: mannitol, trehalose, sucrose, and glucose (Abdelwahed et al., 2006b). These sugars are known to vitrify at a specific temperature (noted Tg′), thereby immobilizing the nanoparticles within a glassy matrix of cryoprotectant which can protect them from the freezing and drying stresses. Freezing of the nanoparticle suspension must occur below Tg′ of a frozen amorphous sample or below the eutectic crystallization temperature, the crystallization temperature of the soluble component as a mixture with ice, if it is in a crystalline state for complete solidification of the sample (Abdelwahed et al., 2006a; Tang and Pikal, 2004).

A lyophilized nanoparticle product should have several desirable characteristics. The physical and chemical characteristics of the product must be preserved including an elegant and solid cake, rapid reconstitution time, an acceptable suspension, unmodified particle size and size distribution, and unchanged activity of the active ingredient. Further, the desired product must have an acceptable relative humidity and remain stable over long-term storage.

Chapter 2

NASAL IMMUNIZATION WITH ANTHRAX PROTECTIVE ANTIGEN PROTEIN ADJUVANTED WITH POLYRIBOINOSINIC-POLYRIBOCYTIDYLIC ACID INDUCED STRONG MUCOSAL AND SYSTEMIC IMMUNITIES

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Pharmaceutical Research, 2006; 23(6): 1217-26

2.1 Abstract

The current anthrax-vaccine-adsorbed (AVA) was originally licensed for the prevention of cutaneous anthrax infection. It has many drawbacks, including the requirement for multiple injections and subsequent annual boosters. Thus, an easily administrable and efficacious anthrax vaccine is needed to prevent the most lethal form of anthrax infection, inhalation anthrax. We propose to develop a nasal anthrax vaccine using anthrax protective antigen (PA) protein as the antigen and synthetic double-stranded RNA in the form of polyriboinosinic-polyribocytidylic acid (pI:C) as an adjuvant. Mice were nasally immunized with recombinant PA admixed with pI:C. The resulting PA-specific antibody responses and the lethal toxin neutralization activity were measured. Moreover, the effect of pI:C on dendritic cells (DCs) was evaluated both in vivo and in vitro. Mice nasally immunized with rPA adjuvanted with pI:C developed strong systemic and mucosal anti-PA responses with lethal toxin neutralization activity. These immune responses compared favorably to that induced by nasal immunization with rPA adjuvanted with cholera toxin. Poly (I:C) enhanced the proportion of DCs in local draining lymph nodes and stimulated DC maturation. This pI:C-adjuvanted rPA vaccine has the potential to be developed into an efficacious nasal anthrax vaccine.

2.1. Introduction

Anthrax is an often fatal bacterial infection caused by *Bacillus anthracis* (Ascenzi et al., 2002). Although cutaneous and gastrointestinal anthrax infections have

produced documented fatalities, inhalation anthrax has a fatality rate close to 100%, with death occurring shortly after the onset of symptoms (Friedlander, 1999). Under adverse conditions, anthrax bacteria form endospores, which are sufficiently small enough for inhalation and deposition in the alveolar spaces of the host respiratory tract (Dixon et al., 1999). Anthrax is a toxin-mediated disease. Anthrax toxin contains three components, the protective antigen (PA), lethal factor (LF), and edema factor (EF). While no of these components alone is toxic, they can combine to form two binary toxins, the lethal toxin (LeTx, PA + LF) and the edema toxin (PA + EF). PA binds to the anthrax toxin receptor on cell surface and mediates the entry of LF and EF into host cell cytosol (Bradley et al., 2001), in where they are toxic (Ascenzi et al., 2002). Thus, anti-PA antibodies (Abs) that block the transport of LF and EF into cell cytosol have been shown to prevent the course of infection (Pitt et al., 2001).

Anthrax-vaccine adsorbed (AVA), an aluminum hydroxide (Alum)-adsorbed, formalin-treated culture supernatant of a toxigenic *B. anthracis* strain is the only licensed anthrax vaccine currently available in the U.S. (Puziss et al., 1963). Previous studies demonstrated that the only active antigen component in the AVA was the PA protein. AVA was originally licensed only for the prevention of cutaneous anthrax. Although it was shown to be effective against inhalational anthrax infection in many animal models, including non-human primates (Ivins et al., 1998), its efficacy against an inhalational anthrax infection in humans has yet been confirmed. Moreover, AVA suffers from a lengthy dosing schedule with 6 initial injections in the first year, followed by annual boosters (Turnbull, 2000; Wang and Roehrl, 2005). Thus, there

continues to be a need to develop alternative and improved anthrax vaccines to more effectively prevent against an anthrax infection.

A nasal anthrax vaccine would be advantageous not only because it will be easily administrable, but also because it is expected to induce systemic immune responses, as well as PA-specific mucosal immune responses in the respiratory tract, through which inhaled anthrax spores enter hosts (Dixon et al., 1999; Friedlander, 1999). Therefore, it is conceivable that the immune response induced by a nasal PA-based anthrax vaccine will be more effective in preventing an inhalational anthrax infection than a systemically injected rPA-based vaccine. Anti-PA Abs have been shown to recognize spore-associated proteins, to stimulate spore uptake by macrophages, and to interfere with the germination of spores in vitro (Cote et al., 2005; Welkos et al., 2002; Welkos et al., 2001), suggesting that anti-PA Abs in the respiratory mucosa will exert a certain extent of anti-spore activity. In support of this, it was recently reported that, in rabbits nasally immunized with PA protein adjuvanted with CpG oligos, serum LeTx neutralization Ab titers alone were not predictive of the survival of rabbits after a pulmonary anthrax spore challenge (Mikszta et al., 2005), which was in sharp contrast to the previous belief that serum LeTx neutralization Ab titers alone were predictive of the efficacy of an anthrax vaccine (Pitt et al., 2001). Similarly, using rPA-loaded PLGA (poly lactic-co-glycolic acid) microspheres, It was reported that mice that were immunized by a combination of intramuscular (i.m.) and nasal priming and boosting schedules were always fully protected against aerosol anthrax spore challenges, while i.m. injection of the PA-loaded microspheres alone

was not as effective (Flick-Smith et al., 2002). In fact, there had been a few more reports on evaluating the immune responses induced by nasally administered PA protein. While nasal PA protein alone failed to induce any anti-PA immune responses, nasal PA was immunogenic when it was incorporated into liposomes (Gaur et al., 2002) or a liposome-based system (Sloat and Cui, 2005), or when cholera toxin (CT) was used as a mucosal adjuvant (Boyaka et al., 2003).

Double-stranded RNA is produced by many viruses during their replicative cycle (Honda et al., 2003; Okahira et al., 2005). It was known in the 1960's-1970's that poly (I:C), a synthetic dsRNA, can boost immune responses (Giuntoli et al., 2002; Herman and Baron, 1971; Park and Baron, 1968; Stark et al., 1998; Yang et al., 1995). However, it was only recently found that dsRNA functioned through TLR3 (Alexopoulou et al., 2001), which stimulated more interest in studying the adjuvanticity of dsRNA. Poly (I:C) was recently shown to induce stable maturation of DCs (Verdijk et al., 1999), to activated natural killer (NK) cells (Schmidt et al., 2004; Sivori et al., 2004), to promote the survival of activated $CD4^+$ T cells in vitro (Gelman et al., 2004), and to boost the number of antigen-specific $CD8^+$ T cells and their survival in vivo (Salem et al., 2005). More recently, pI:C was demonstrated to enhance the immune responses against nasally dosed vaccines too. For example, Ichinohe et al. (2005) reported that nasal immunization of mice with an inactivated hemaglutinin (HA) admixed with pI:C protected the mice against an influenza viral infection (Ichinohe et al., 2005). Similarly, Partidos et al. (2005) reported the induction of specific serum and mucosal Abs, as well as cellular responses, to the HIV

TAT protein when the molecular complex of TAT and pI:C was nasally administered into mice (Partidos et al., 2005).

In this present study, we evaluated the anti-PA immune responses induced by nasal immunization of mice with rPA admixed with pI:C as an adjuvant, hypothesizing that pI:C is a strong mucosal adjuvant for PA protein. We reported that the nasally dosed rPA adjuvanted with pI:C induced strong mucosal and systemic anti-PA immune responses. Moreover, pI:C enhanced the proportion of DCs in local draining LNs and stimulated the maturation of DCs.

2.3 Materials and Methods

2.3.1 Nasal immunization

All animal studies were carried out following NIH guidelines for animal use and care. Poly (I:C) (10, 20, or 40 μg per mouse, GE Amersham Biosciences, Piscataway, NJ) and rPA (5 μg/mouse, List Biological Laboratories, Inc., Campbell, CA) were dissolved into phosphate buffered saline (PBS, 10 mM, pH 7.4, Sigma-Aldrich, St. Louis, MO). Mice (n = 5) were lightly anesthetized (i.p., pentobarbital, 6 mg/100 g, Abbott Laboratories, North Chicago, IL) and given a total volume of 20 μL of rPA/pI:C solution in two 10 μL doses, with 10-15 min between each dose, half in each nare. As controls, mice (n = 5) were subcutaneously (s.c.) injected with rPA (5 μg per mouse) admixed with aluminum hydroxide gel (Alum, 15 μg/mouse, USP grade, Spectrum Chemical and Laboratory Products, New Brunswick, NJ), nasally dosed with rPA admixed with cholera toxin (CT, 1 mg per mouse, List Biological

Laboratories) as a mucosal adjuvant, or left untreated. Mice were dosed on days 0, 7, and 14, bled via the tail vein on days 21 and 30, and euthanized on day 30 using CO_2. This experiment was repeated independently twice.

The pI:C was a duplex RNA polymer composed of a poly (I) strand and a poly (C) strand. According to GE Amersham product information, the two strands were synthesized independently, and then annealed together, which resulted in extensive heterogeneity in duplex lengths. The length of poly (I) and poly (C) was 152-539 and 319-1305 bases, respectively. The pI:C was believed to be primarily double-stranded in nature, although some single-stranded regions may exist. The endotoxin level in our pI:C solution was determined to be 2.4 ± 0.3 EU/mL using a Limulus Lysate Assay (Associates of Cape Cod, Inc., East Falmouth, MA).

2.3.2 Broncho-alveolar lavage (BAL) collection

Mice were euthanized 30 days after the first immunization. To collect the BALs, an incision was made in the trachea; care was taken to not cut the blood vessels close to the trachea. A 0.4 mL of sterile PBS was pipetted through the trachea towards the lungs, aspirated back into the pipette tip and re-injected once before the final withdrawal of the PBS solution. Lavage samples collected from each individual mouse were stored at -80ºC prior to further use.

2.3.3 ELISA for anti-PA Ab measurement

Enzyme-linked immunosorbent assay (ELISA) was used to determine anti-PA Ab levels. Briefly, EIA/RIA flat bottom, medium binding, polystyrene 96-well plates (Corning Costar, Corning, NY) were coated with 100 ng of rPA dissolved in 100 μL of carbonate buffer (0.1 M, pH 9.6) overnight at 4ºC. Plates were washed with PBS/Tween 20 (10 mM, pH 7.4, 0.05% Tween 20, Sigma) and blocked with 4% (w/v) bovine serum albumin (BSA, Sigma) in PBS/Tween 20 for 1 hour at 37ºC. Serum and BALs samples were diluted (2-fold serial except where mentioned) in 4% BSA/PBS/Tween 20, added to the plates following the removal of the blocking solution, and incubated for an additional 2.5 hours at 37ºC. The samples were removed, and the plates were washed 5 times with PBS/Tween 20. Horse radish peroxidase (HRP)-labeled goat-anti-mouse immunoglobulin (IgG, IgG1, IgG2a, IgM, or IgA, 5000-fold dilution, Southern Biotechnology Associates, Inc., Birmingham, AL) was added as the secondary Ab into the plates, followed by a 1 hour incubation at 37ºC. Plates were again washed 5 times with PBS/Tween 20. The presence of bound Ab was detected following a 30-min incubation at room temperature in the presence of 3,3',5,5'-Tetramethylbenzidine solution (TMB, Sigma), followed by the addition of sulfuric acid (0.2 M, Sigma) as the stop solution. The absorbance was read at 450 nm using a SpectraMax Plate reader (Molecular Devices Inc., Sunnyvale, CA).

2.3.4 Lethal toxin neutralization activity (TNA) assay

TNA was determined as described elsewhere with slight modifications (Boyaka et al., 2003; Sloat and Cui, 2005). Briefly, confluent J774A.1 cells were plated (5.0 x 10^4 cells/well) in sterile, 96-well clean-bottom plates (Corning Costar) and incubated at 37ºC, 5% CO_2 for 16 hours. A fresh solution (50 μL) containing rPA (400 ng/mL) and LF (40 ng/mL) was mixed with 50 μL of the diluted serum or BAL samples in triplicate and incubated for 1 hour at 37ºC. The cell culture medium was removed, and 100 μL of the serum/LeTx mixture was added to each well and incubated at 37ºC, 5% CO_2 for 3 hours. Cell viability was determined using an MTT assay kit (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide, Sigma-Aldrich) with untreated or LeTx alone treated cells as controls.

2.3.5 Splenocyte proliferation assay

Single cell suspensions from individual spleens were prepared as previously described (Cui and Mumper, 2002). Splenocytes were cultured at a density of 4 x 10^6 cells/mL with rPA (0 or 12.5 μg/mL) in complete RPMI medium (Invitrogen, Carlsbad, CA) for 5 days at 37°C, 5% CO_2 (Boyaka et al., 2003). Cell proliferation was determined using an MTT kit. Proliferation index was reported as the ratio of the final number of cells after stimulation with rPA (12.5 μg/mL) over that without stimulation (0 μg/mL).

2.3.6 The effect of nasal pI:C on local draining lymph nodes

Balb/C mice (n = 3) were lightly anesthetized, and then nasally dosed with 20 μL of sterile PBS, pI:C (20 μg, 1 mg/mL), or CT (1 μg) as described above. Twenty hours later, mice were euthanized, and their superficial and deep cervical LNs were removed. As controls, mice (n = 3) were s.c. injected in their hind leg footpads with PBS alone or lipopolysaccharide in PBS (LPS, 100 ng, List Biological Lab), and their draining popliteal LNs were removed 20 h later. The LNs from mice with same treatment were pooled and incubated with collagenase (1 mg/mL, Sigma) for 20 min at 37^{o}C, followed by a 10 more min incubation at room temperature after the incubation medium was supplemented with EDTA (10 mM, Sigma). Single LN cell suspension was prepared by forcing the LNs through a BD cell strainer (70 μm), stained with FITC-labeled anti-mouse CD11c Ab (1 μg/2 x10^{6} cells, BD Pharmingen, San Diego, CA) for 20 min at 4^{o}C, washed twice, and analyzed using a flow cytometer (FC500 Beckman Coulter EPICS V Dual Laser Flow Cytometer, Fullerton, CA).

2.3.7 CD80 and CD86 expression and TNF-α secretion by DCs

Bone morrow-derived DCs (BMDCs) were isolated as previously described (Cui et al., 2005). Briefly, bone marrow cells from the tibia and femur bones of C57BL/6 mice were depleted of lymphocytes (CD4, CD8, and NK cells) and cultured at a density of 5 x 10^{5} cells/mL in 10% FBS-containing RPMI 1640 with GM-CSF and IL-4 (10^{3} U/ml each, R & D Systems, Minneapolis, MN). Loosely adherent cells were collected on day 5. Over 90% of these cells were CD11c^{+} as confirmed by flow

cytometry. To determine the expression of CD80 and CD86 on the surface of DCs, BMDCs (2 x 10^6) were incubated with pI:C (50 μg/mL), LPS (100 ng/mL), or PBS for 16 hours. The cells were then stained with FITC-labeled anti-mouse CD80 or PE-labeled anti-mouse CD86 (Pharmingen) and analyzed by flow cytometry. Moreover, the TNF-α content in the BMDC culture supernatant was measured using a TNF-α ELISA kit from BD Pharmingen.

2.3.8 Statistics

Statistical analyses were completed using one-way analysis of variance (ANOVA) followed by the Fischer's protected least significant difference (PLSD) procedure. A p-value of ≤ 0.05 (two tail) was considered to be statistically significant. The normality of the data and equal variance between groups was checked prior to data analysis.

2.4 Results

2.4.1 Nasal immunization of mice with pI:C-adjuvanted rPA induced anti-PA Abs in their serum

As previously reported, nasal rPA alone (5 μg per mouse) failed to induce any detectable level of anti-PA IgG in mouse serum. However, nasal immunization of mice with rPA admixed with pI:C elicited a significant serum anti-PA IgG response when measured 30 days after the first immunization (Fig. 2.1). The serum IgG data shown were when the mice were dosed with 10 μg of pI:C. Increasing the dose of pI:C

to 20 and 40 μg per mouse did not lead to a significant increase in the serum anti-PA IgG titers induced (data not shown). Although the anti-PA IgG titer induced in mice nasally immunized with the pI:C-adjuvanted rPA was lower than that in mice s.c. injected with rPA adjuvanted with Alum, it was higher than that in mice nasally dosed with rPA adjuvanted with CT (Fig. 2.1). The anti-PA IgG titer induced by the nasal pI:C-adjuvanted rPA was more than 10,000 when measured 21 days after the first immunization. Moreover, although weak, PA-specific IgM was also detected in the serum of mice nasally immunized with rPA adjuvanted with pI:C (data not shown). No significant level of anti-PA IgA was detected in the serum of mice nasally dosed with rPA adjuvanted with pI:C. Judged from the levels of anti-PA IgG1 and IgG2a in mouse serum (Fig. 2.2), the anti-PA immune response induced by the nasal pI:C-adjuvanted rPA was more balanced, in contrast to the more IgG1-biased responses induced by s.c. injection of Alum-adjuvanted rPA or nasal immunization with CT-adjuvanted rPA (Fig. 2.2B). In fact, the anti-PA IgG2a was very weak or not detectable in mice immunized with CT- or Alum-adjuvanted rPA. All these findings demonstrated that nasal rPA adjuvanted with pI:C induced strong anti-PA Abs in mouse serum.

2.4.2 Nasal immunization of mice with pI:C-adjuvanted rPA induced anti-PA IgA in their lung mucosal secretion

Similar to nasal immunization of mice with rPA adjuvanted with CT, nasal rPA adjuvanted with pI:C also induced PA-specific IgA in mouse BALs (Fig. 2.3). The anti-PA IgA titer in mice nasally immunized with the pI:C-adjuvanted rPA

vaccine was, in fact, significantly higher than that induced by the nasal CT-adjuvanted rPA. As expected, s.c. injection of mice with rPA adjuvanted with Alum did not induce any anti-PA IgA in their BALs (Fig. 2.3). Also, nasal rPA alone failed to induce any anti-PA IgA in mouse BAL samples (Fig. 2.3).

2.4.3 The anti-PA antibody responses induced by nasal rPA adjuvanted with pI:C had anthrax lethal toxin neutralization activity

In order to evaluate the protective activity of the anti-PA Abs induced, the anthrax LeTx neutralization activity in the serum and BAL samples was measured using an in vitro macrophage (J774A.1) protection assay. As shown in Fig. 2.4, LeTx neutralization activity was induced in both the serum and the BALs of mice nasally immunized with rPA adjuvanted with pI:C, but only in the serum, not in the BALs, of mice s.c. injected with rPA admixed with Alum. Although not significant, the LeTx neutralization activity induced by the nasal rPA adjuvanted with pI:C tended to be stronger than that induced by the nasal rPA adjuvanted with CT (Fig. 2.4A). Moreover, the LeTx neutralization activity in the BALs of mice nasally immunized with the pI:C-adjuvanted rPA vaccine was significantly higher than that in mice nasally immunized with rPA adjuvanted with CT at the 5- and 10-fold BAL dilutions (Fig. 2.4B). As expected, no LeTx neutralization activity was detected in the BALs of mice s.c. injected with rPA admixed with Alum (Fig. 2.4B). Finally, LeTx-neutralization activity was not detectable in naïve mice and mice nasally dosed with rPA alone.

2.4.4 Splenocytes isolated from mice nasally immunized with pI:C-adjuvanted rPA proliferated after in vitro re-stimulation

To evaluate the proliferative immune response induced by nasal immunization of mice with the pI:C-adjuvanted rPA, the splenocytes isolated from the immunized mice were re-stimulated with rPA, and the final splenocyte number after the re-stimulation was determined. As shown in Fig. 2.5, nasal immunization with rPA adjuvanted with pI:C induced a significant splenocyte proliferation. Similarly, s.c. injection of rPA adjuvanted with Alum and nasal dosing of rPA admixed with CT both led to significant splenocyte proliferations (Fig. 2.5). Again, a proliferative response was not detected in mice nasally dosed with rPA alone without an adjuvant (Fig. 2.5).

2.4.5 Poly (I:C) enhanced the proportion of DCs in local draining lymph nodes and stimulated the maturation of DCs in vitro

To preliminarily elucidate the mechanism responsible for the strong anti-PA immune responses induced by nasal rPA when adjuvanted with pI:C, the effect of pI:C on DCs was evaluated. Nasal pI:C enhanced the proportion of $CD11c^+$ cells in local draining LNs, the superficial and deep cervical LNs (Fig. 2.6). In vitro, co-incubation of BMDCs with pI:C up-regulated the expression of DC maturation markers, CD80 and CD86, on the surface of DCs (Fig. 2.7A) and induced the secretion of TNF-α by the BMDCs into the cell culture supernatant (Fig. 2.7B).

2.5 Discussions

Due to the lengthy dosing schedule of AVA and the uncertainty of its efficacy against an inhalational anthrax infection, we proposed to develop a nasal rPA-based anthrax vaccine. However, in order to develop such a vaccine, an appropriate mucosal adjuvant is needed because data in published literature had clearly shown that nasal PA alone was unable to induce any detectable PA-specific immune responses (Boyaka et al., 2003; Sloat and Cui, 2005). Using CT and CpG oligos as adjuvants or incorporation of rPA into either liposomes or PLGA microspheres had been previously employed to address this issue (Boyaka et al., 2003; Flick-Smith et al., 2002; Gaur et al., 2002; Mikszta et al., 2005; Sloat and Cui, 2005). However, there continues to be a need for alternative mucosal adjuvants. Data shown in this present study clearly indicated that dsRNA, in the form of a synthetic pI:C, is a potential nasal anthrax vaccine adjuvant. When compared to nasal rPA adjuvanted with CT, which is considered by some as a "gold" standard for mucosal adjuvant, nasal rPA adjuvanted with pI:C induced higher anti-PA IgG titers in the serum, higher anti-PA IgA titers in BALs, and more importantly, stronger (or tending to be stronger) anthrax LeTx neutralization activity in the BAL and serum samples (Fig 2.1, 2.3, 2.4). All these findings pointed out the potential of this pI:C-adjuvanted nasal rPA vaccine. In future studies, we will evaluate the efficacy of the PA-specific immune responses induced by this nasal pI:C-adjuvanted rPA in protecting mice against an inhalational anthrax spore challenge. The fact that the anti-PA IgG titer in the serum of mice nasally immunized with rPA adjuvanted with pI:C was only slightly lower than that in mice s.c. injected

with rPA adjuvanted with Alum suggested that nasal rPA adjuvanted with pI:C will be effective in protecting mice against an inhalational anthrax spore challenge (Ivins et al., 1998; Mikszta et al., 2005). Moreover, this nasal rPA anthrax vaccine is expected to provide an appropriate model for us to further identify the advantage of the combination of mucosal and systemic anti-PA immune responses over systemic anti-PA immune responses alone in preventing against an inhalational anthrax infection.

As described earlier, dsRNA was established to be immuno-stimulatory in the 1960-1970's (Herman and Baron, 1971; Park and Baron, 1968). It was only until recently that TLR3 was identified as a receptor for dsRNA, which is a pathogen-associated molecular pattern molecule (Alexopoulou et al., 2001). Thus, it is expected that the strong anti-PA immune responses induced by this nasal pI:C-adjuvanted rPA vaccine was related to the dsRNA/TLR3 signaling. We have shown that, when nasally dosed with pI:C, the proportion of $CD11c^+$ cells in the local draining LNs of the dosed mice was significantly increased (Fig. 2.6). As a positive control, LPS, a ligand for TLR4, when s.c. injected into mouse hind leg footpads, also led to the increase of the proportion of $CD11c^+$ cells in the local draining popliteal LNs (Fig. 2.6). Although not very specific, CD11c is generally considered to be a DC-restricted marker in mice (Morelli et al., 2000). It is possible that the pI:C induced the migration of DCs into local draining LNs. Moreover, as previously reported (Alexopoulou et al., 2001), pI:C induced the maturation of DCs by stimulating the expression of secondary signal molecules, such as CD80 and CD86, on the surface of DCs (Fig. 2.7A) and the secretion of TNF-α cytokine by the DCs (Fig. 2.7B). These are important because it is

known that an antigen presentation without an appropriate secondary signal generally leads to an immune tolerance rather than an immune stimulation (Guermonprez et al., 2002). Taken together, all these findings suggested that the enhanced specific immune responses against the antigen of interest when the pI:C was used as an adjuvant may be partially attributed to the ability of pI:C to stimulate the migration and maturation of DCs.

Data presented in Fig. 2.3 indicated that, similar to previous reports (Ichinohe et al., 2005; Partidos et al., 2005), the Ab response induced by pI:C as an adjuvant was more IgG1/IgG2a balanced because both anti-PA IgG1 and IgG2a were induced, in contrast to the more IgG1-biased immune responses induced when Alum or CT were used as adjuvants. For example, the anti-PA IgG1/IgG2a ratio was changed from 0.8 in naïve mice to 6.6-25.6 in mice immunized by s.c injection of rPA admixed with Alum, and to 5.4-7.6 in mice nasally dosed with rPA admixed with CT (Fig. 2.3B). The more IgG1/IgG2a balanced anti-PA immune response induced by the nasal rPA adjuvanted with pI:C might be advantageous over an IgG1-biased immune response because it was thought that cellular immune responses, in addition to the PA-specific Ab response, might be beneficial for a full protection against an anthrax infection in different animal species (Wang and Roehrl, 2005).

Although further experiments have to be completed to precisely define the extent to which the inhalation of the pI:C-adjuvanted PA vaccine into the lungs was responsible for the induction of the anti-PA immune responses, we believe that the involvement of lung in the induction of anti-PA immune responses by our nasal pI:C-

adjuvanted PA vaccine was limited. It had been shown that the extent to which a nasally dosed vaccine travels to the lungs was largely determined by the volume of the vaccine dosed to mice (Davis, 2001). It was previously reported that when 50 or more μL of a particle suspension was dosed to anesthetized mice, the majority of it traveled into lungs. However, when the dose was decreased to 10 μL, they were mainly remained in the nare passages (Eyles et al., 1998). Our previous study also showed that, when a FITC-labeled liposome suspension was nasally administered to mice using the procedure we used in the present study (i.e., a total of 20 μL was dosed separately as two 10 μL with 10-15 min between each dosing), the majority of FITC-labeled liposomes were recovered in mouse nasal washes 4 hours later; while FITC-labeled liposomes were not detected in the lung washes (Cui et al., 2003). In future studies, FITC-labeled PA protein, admixed with pI:C, will be dosed nasally into mice to track PA proteins that reach the lungs.

In the present study, with a nasal pI:C dose as high as 40 μg, no gross inflammatory, allergic, or toxic effect was observed in mice. Ichinohe et al. (2005) also reported that, when pI:C was nasally given to mice daily for 9 days (25 μg/day), the body weight of the mice did not significantly change (Ichinohe et al., 2005). Histopathological examination also did not reveal any pathological change in the nasal areas of mice dosed with pI:C (Ichinohe et al., 2005). In our studies, an overall 40% fatality was observed when mice were nasally dosed with CT (1 μg/mouse once a week for 2 or 3 weeks); while no fatality was observed in other groups of mice who were not treated or were dosed with vaccines that did not contain CT. All these

findings suggested the pI:C as a strong and safe nasal mucosal adjuvant. Thus, more investigations on the nasal mucosal adjuvanticity of pI:C are warranted. Finally, it needs to be pointed out that in a previous clinical trial using as high as 75 mg of pI:C/m^2 on day 0 and then daily from day 7 to a maximum of 35 days, pI:C did induce some side-effects, including renal failure and hypersensitivity reactions in some patients (Robinson et al., 1976), which prompted the development of a much safer, modified form of pI:C, poly(I:C_{12}U), by introducing unpaired uracil and quinine bases in the pI:C (Strayer et al., 1994). Recently, it was shown that poly(I:C_{12}U) was as effective as pI:C in inducing the maturation of human monocyte derived DCs in vitro (Adams et al., 2003). In future studies, the poly(I:C_{12}U) may be used to replace pI:C, if needed.

In conclusion, we reported that nasal immunization of mice with anthrax PA protein with pI:C as an adjuvant induced strong PA-specific immune responses with anthrax lethal toxin neutralizing activity both in the systemic compartment and in the BALs. The strong immune response was likely to be due to the effect of pI:C on the immune system, such as the DCs, through the dsRNA/TLR3 signaling. This nasal pI:C-adjuvanted, rPA-based vaccine has the potential to be developed into a nasal anthrax vaccine to prevent against an inhalation anthrax infection.

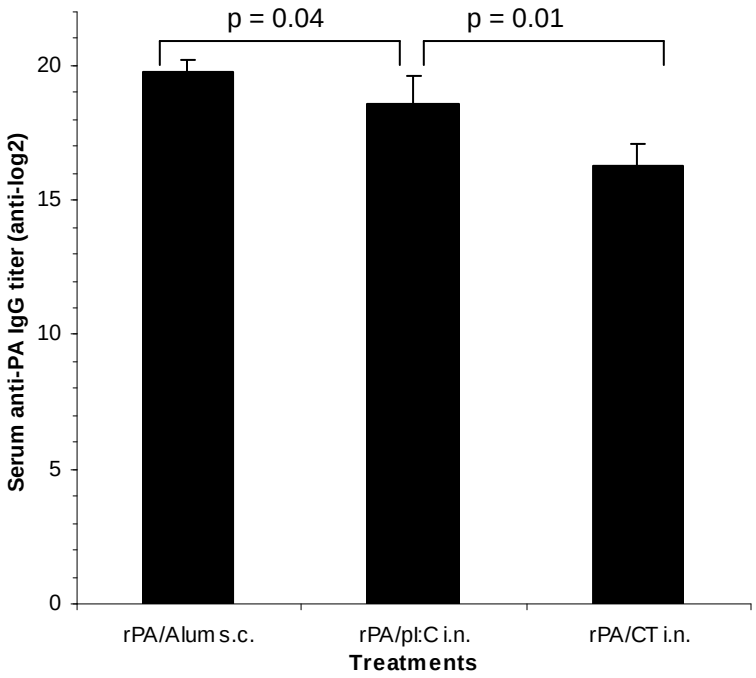
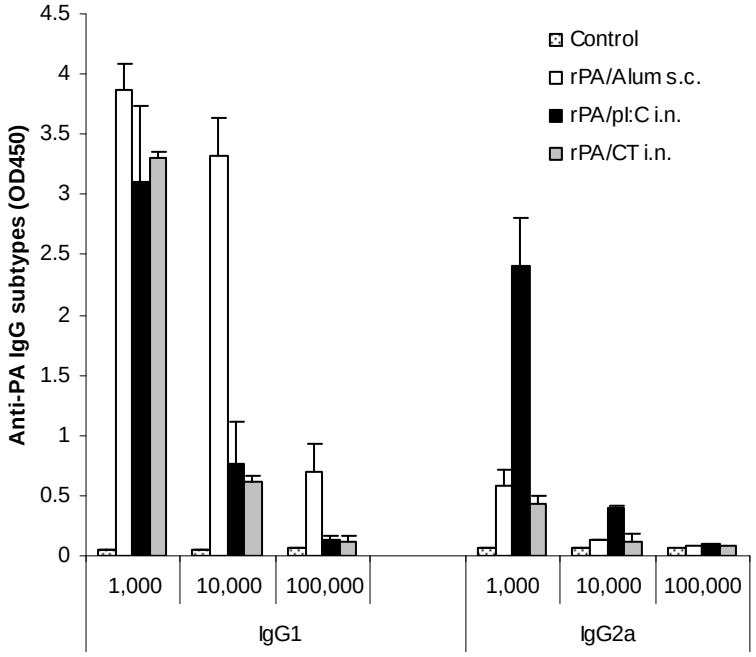


Figure 2.1 Serum anti-PA Ab responses. Balb/C mice (n = 5) were nasally dosed with rPA alone, rPA adjuvanted with pI:C (rPA/pI:C), or left untreated on days 0, 7, and 14. As controls, other two groups of mice (n = 5) were nasally dosed with rPA adjuvanted with CT (rPA/CT i.n., CT = 1 μg/mouse) or s.c. injected with rPA adjuvanted with Alum (rPA/Alum s.c., Alum = 15 μg/mouse). The dose of rPA and pI:C was 5 μg and 10 μg per mouse, respectively. On day 30, mice were bled, and total anti-PA IgG titer in their serum was determined using ELISA. The sera were diluted 2-fold serially. Nasal rPA alone failed to induce any detectable level of anti-PA IgG. ANOVA analysis showed that there were significant differences among those three treatments ($p << 0.05$). This experiment was repeated twice, and similar results were obtained. Data from one representative were shown (mean ± S.D., n = 5).

(A)



(B) **Ratio of IgG1 over IgG2a ($OD450_{IgG1}/OD450_{IgG2a}$)**

	1,000	10,000	100,000
Untreated	0.8	0.8	0.8
rPA/Alum s.c.	6.6	25.6	8.7
rPA/pI:C i.n.	1.3	1.9	n/a
rPA/CT i.n.	7.6	5.4	n/a

Figure 2.2

Figure 2.2 Serum anti-PA IgG subtypes. Balb/C mice (n = 5) were dosed with rPA alone, rPA/pI:C, or left untreated on days 0, 7, and 14. As controls, other groups of mice were injected (s.c.) with rPA adjuvanted with Alum or nasally dosed with rPA adjuvanted with CT. The IgG1 and IgG2a levels in the sera were determined on day 30 using ELISA after the serum samples were diluted 1,000-, 10,000-, and 100,000-fold. (A) Data shown were mean ± S.D. (n = 5). (B) The ratio of the OD450 for IgG1 and IgG2a ($OD450_{IgG1}/OD450_{IgG2a}$). N/A, for rPA/pI:C (i.n.) and rPA/CT (i.n.), the values in the 100,000-fold dilution were not reported because the IgG1 values of them were not significantly different from that of the untreated mice. Nasal rPA alone failed to induce any detectable level of anti-PA IgG subtypes.

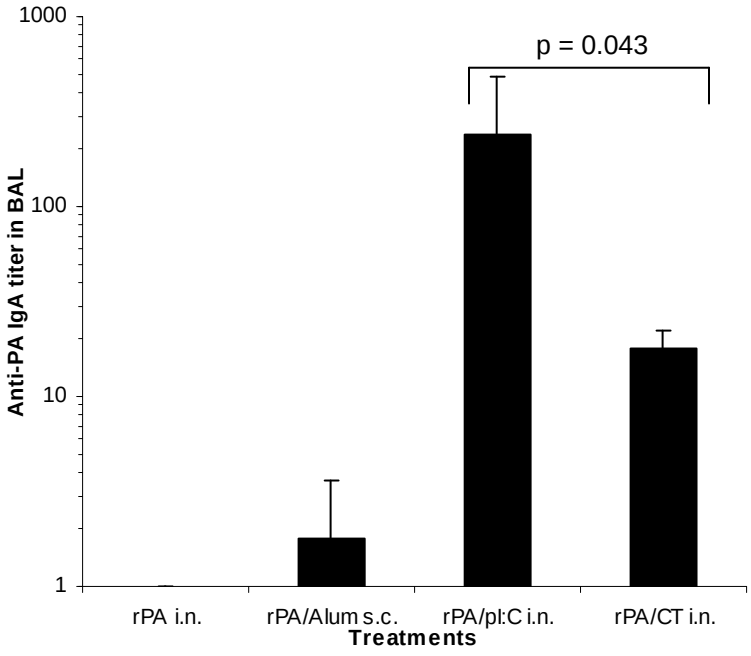


Figure 2.3 Nasal immunization with rPA admixed with pI:C induced anti-PA IgA in the BALs of immunized mice. Balb/C mice were dosed (i.n.) with rPA alone, rPA/pI:C, or left untreated on days 0, 7, and 14. As controls, mice were injected (s.c.) with rPA adjuvanted with Alum or nasally dosed with rPA adjuvanted with CT. On day 30, mice were euthanized, and their BALs were collected. Anti-PA IgA titer in the BAL samples from individual mouse was determined using ELISA. No anti-PA IgA was detected in the BALs of mice nasally dosed with rPA alone. Statistical analyses showed that the value of rPA/pI:C (i.n.) was significantly different from that of rPA/CT (i.n.) ($p = 0.043$, 2-tail t-test).

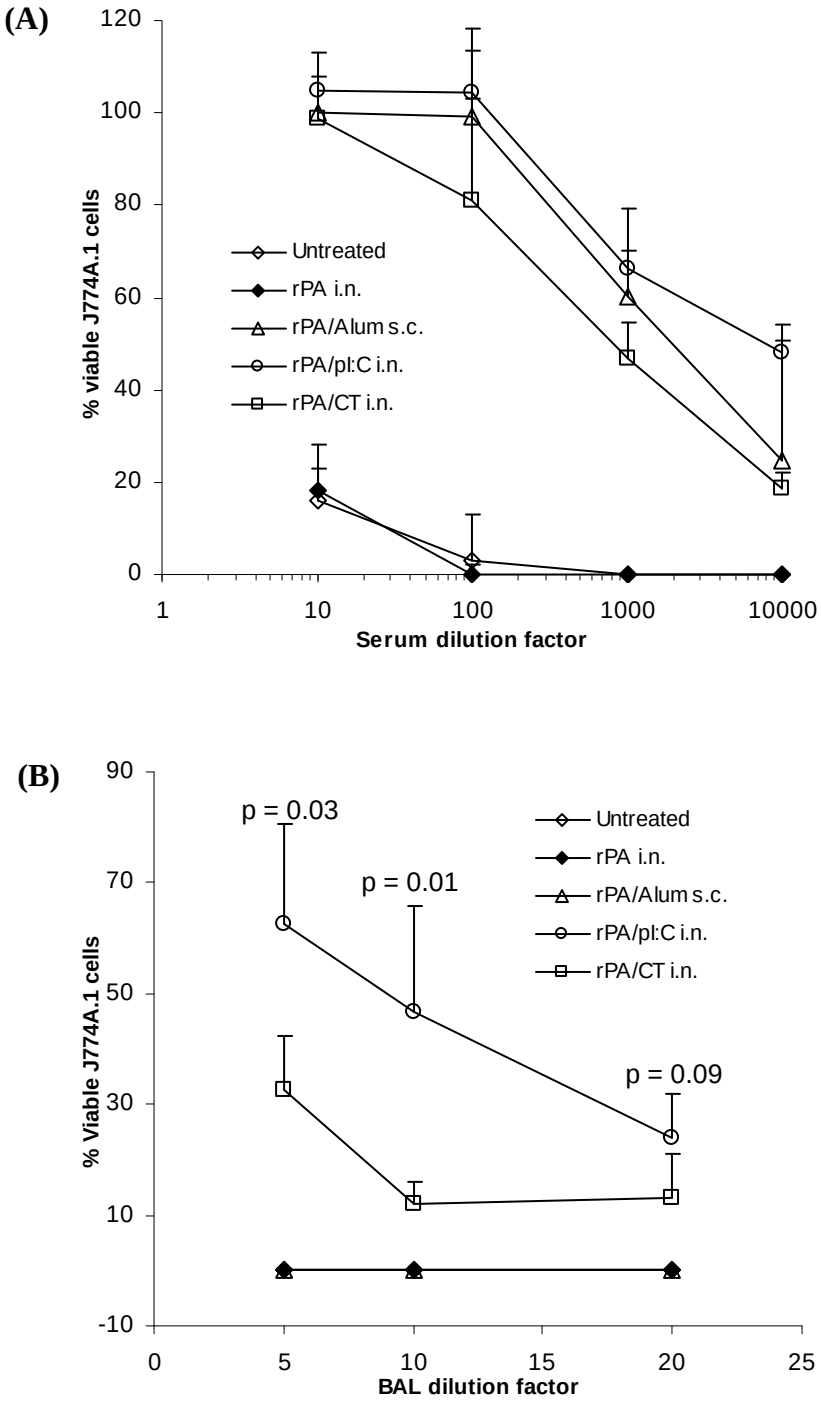

(A)
% viable J774A.1 cells
120
100
80
60
40
20
0
1
10
100
1000
10000
Serum dilution factor
Untreated
rPA i.n.
rPA/Alum s.c.
rPA/pI:C i.n.
rPA/CT i.n.
(B)
% Viable J774A.1 cells
90
70
50
30
10
-10
0
5
10
15
20
25
BAL dilution factor
p = 0.03
p = 0.01
p = 0.09
Untreated
rPA i.n.
rPA/Alum s.c.
rPA/pI:C i.n.
rPA/CT i.n.

Figure 2.4

Figure 2.4 Nasal immunization with rPA adjuvanted with pI:C induced strong LeTx neutralization activity (TNA) in the serum (A) and BAL (B) of immunized mice. Balb/C mice (n = 5) were dosed (i.n.) with rPA alone, rPA/pI:C, or left untreated on days 0, 7, and 14. As controls, mice were injected (s.c.) with rPA adjuvanted with Alum or nasally dosed with rPA adjuvanted with CT. On day 30, serum was collected. The 10-fold serial dilutions of each serum samples were incubated with J774A.1 cells (5×10^5/mL) in the presence of LeTx (400 ng/mL of PA, and 40 ng/mL of LF) for 3 h. The number of viable J774A.1 cells was determined using an MTT kit. BAL samples were diluted 5, 10, and 20-fold. In (A), the values from rPA/Alum (s.c.), rPA/pI:C (i.n.), and rPA/CT (i.n.) were comparable, although the neutralization activity in the serum of mice nasally immunized with rPA/CT tended to be weaker. In (B), TNA was not detectable in the BALs of untreated mice, mice nasally immunized with rPA alone, and mice injected (s.c.) with rPA adjuvanted with Alum. The LeTx neutralization activity in the BALs of mice nasally immunized with rPA/pI:C was significantly higher than that in mice immunized with rPA/CT (i.n.) in the 5- and 10-fold dilutions.

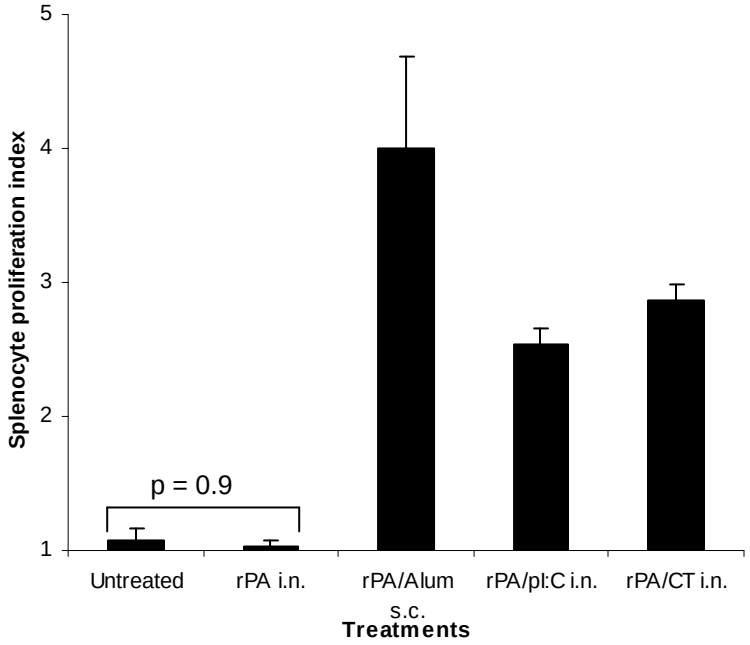


Figure 2.5 Splenocytes isolated from mice nasally immunized with rPA adjuvanted with pI:C proliferated after in vitro re-stimulation with rPA. Balb/C mice (n = 5) were dosed (i.n.) with rPA alone, rPA/pI:C, or left untreated on days 0, 7, and 14. As controls, mice were injected (s.c.) with rPA adjuvanted with Alum or dosed (i.n.) with rPA adjuvanted with CT. On day 30, mice were euthanized. Their spleens were harvested, and single splenocyte suspension from individual spleen was prepared. The splenocytes (4 x 10^6/mL) were incubated with rPA (0 or 12.5 μg/mL) for 5 days. The proliferation (final cell number) was measured using an MTT kit. ANONA analysis showed that there were significant differences among the values from different treatments ($p << 0.05$). Splenocytes isolated from mice nasally immunized with rPA alone did not proliferate ($p = 0.9$ vs. untreated). The values of rPA/Alum (s.c.), rPA/pI:C (i.n.), and rPA/CT (i.n.) were significantly different from each other [rPA/Alum (s.c.) vs. rPA/pI:C (i.n.), $p = 0.002$; rPA/Alum (s.c.) vs. rPA/CT (i.n.), $p = 0.03$; rPA/pI:C (i.n.) vs. rPA/CT (i.n.), $p = 0.007$], and were all higher than that of the untreated mice.

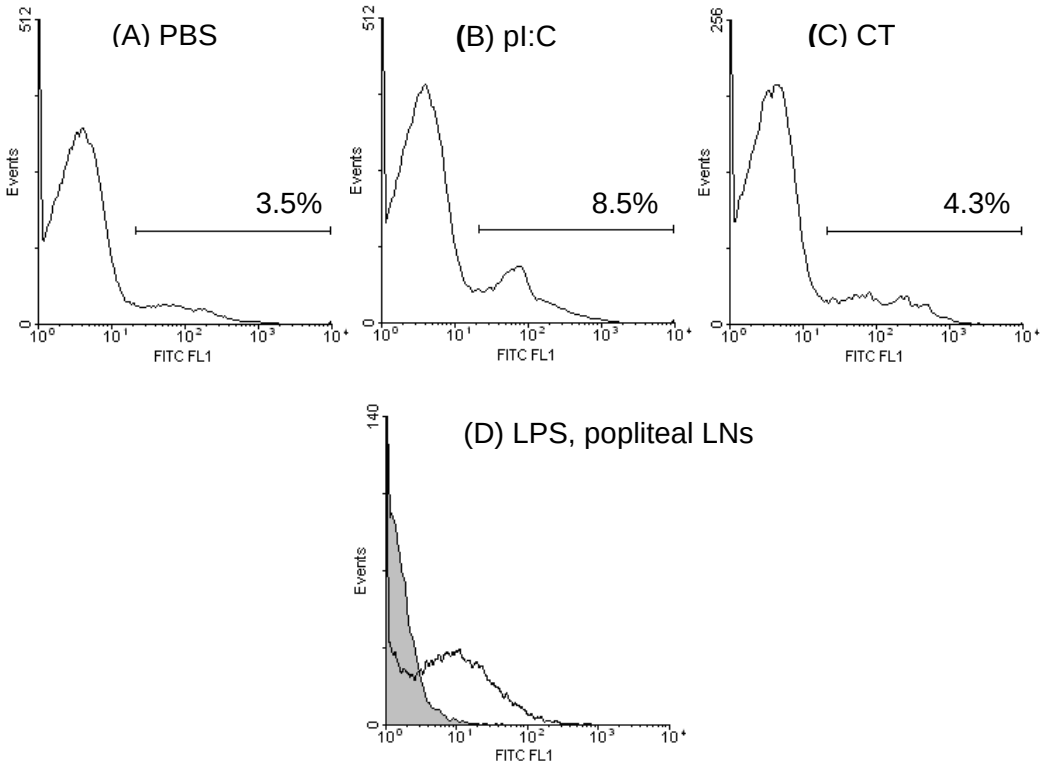


Figure 2.6 Nasal pI:C enhanced the proportion of $CD11c^+$ cells in the local draining LNs. Balb/C mice (n = 3) were lightly anesthetized, and nasally dosed with 20 μL of sterile PBS, pI:C (20 μg), or CT (1 μg). Twenty hours later, mice were euthanized, and their superficial and deep cervical LNs were collected. The LNs from mice with the same treatment were pooled, and single LN cell suspension was prepared. The LN cell samples (n = 3) were stained with FITC-labeled anti-mouse CD11c Ab, and analyzed by flow cytometry. Numbers shown above the bars in (A) – (C) were the mean percent of $CD11c^+$ cells from three independent flow cytometry measurements. In (D), as a positive control, mice (n = 3) were s.c. injected in their hind leg footpads with LPS (100 ng in PBS) or sterile PBS, and their draining popliteal LNs were removed 20 h later. The graph of the LPS injected mice (dark line) was overlaid on the graph of mice injected with sterile PBS (gray area).

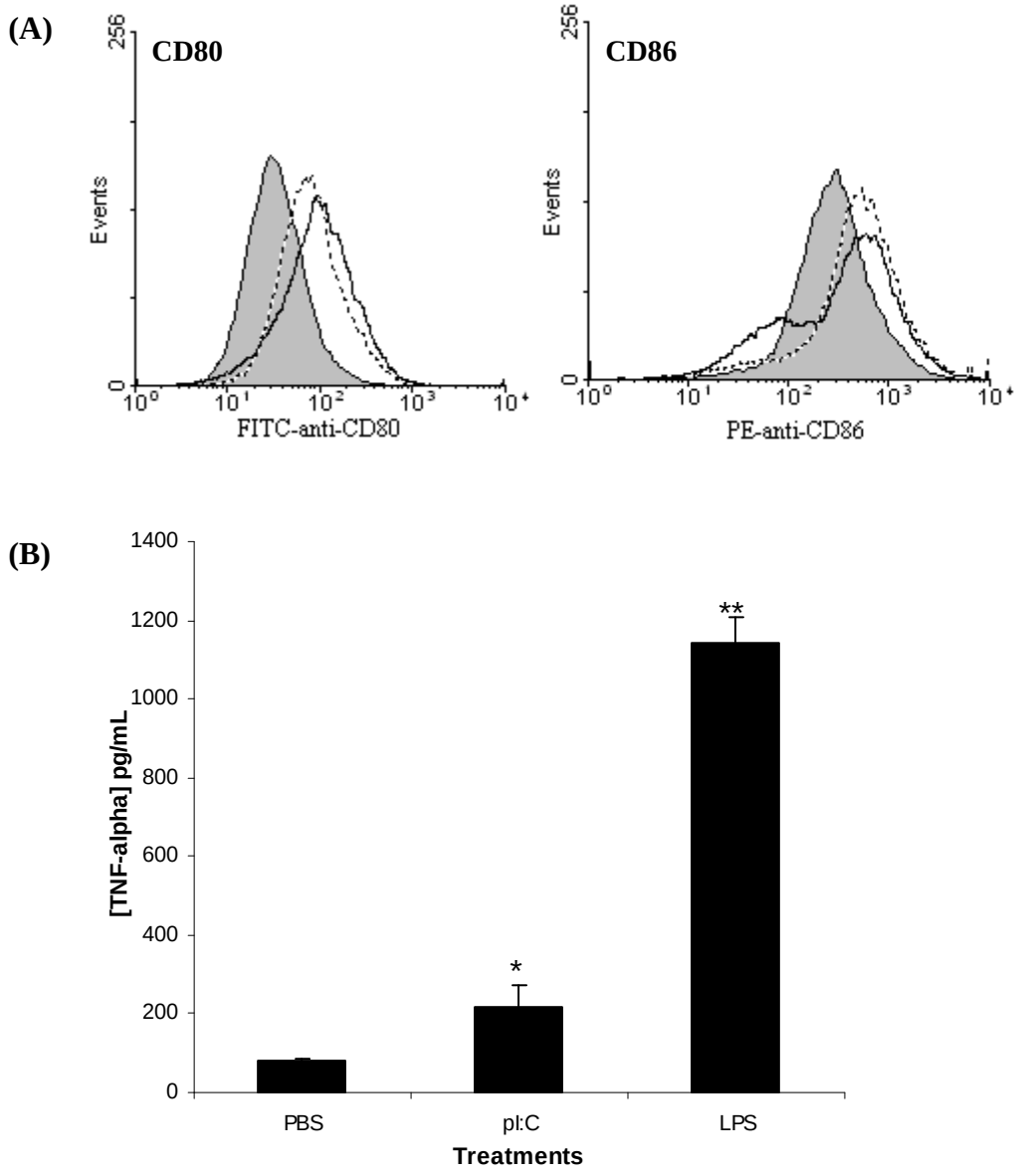

(A)
CD80
CD86
Events
256
0
10^0
10^1
10^2
10^3
10^4
FITC-anti-CD80
PE-anti-CD86
(B)
[TNF-alpha] pg/mL
1400
1200
1000
800
600
400
200
0
**
*
PBS
pI:C
LPS
Treatments

Figure 2.7

Figure 2.7 Poly (I:C) stimulated the expression of CD80 and CD86 on the surface of DCs and the secretion of TNF-α by DCs. (A) BMDCs (2×10^6) isolated from C57BL/6 mice were co-incubated with pI:C (50 μg/mL, dash line), LPS (0.1 μg/mL, solid line), or left untreated (gray area) for 16 h at 37°C. Cells were stained with FITC-anti-CD80 and PE-anti-CD86 and analyzed by flow cytometry. Data shown were one representative from three independent studies with similar results. (B) TNF-α content in the cell culture supernatant was measured using ELISA. Data were mean ± S.D. ($n = 3$). (*), value from pI:C was significantly higher than that from untreated mice ($p = 0.003$, t-test); (**), value from LPS was higher than that from the other two treatments ($p << 0.05$).

Chapter 3

NASAL IMMUNIZATION WITH A DUAL ANTIGEN ANTHRAX VACCINE INDUCED STRONG MUCOSAL AND SYSTEMIC IMMUNE RESPONSES AGAINST TOXINS AND BACILLI

Brian R. Sloat and Zhengrong Cui

Vaccine, 2006; 24: 6405 – 13

3.1 Abstract

Anthrax-vaccine-adsorbed (AVA), the only anthrax vaccine licensed in the U.S., suffers from many major drawbacks. Therefore, there is a need to develop new generation anthrax vaccines that can be easily administered and induce strong immune responses not only against the anthrax toxins, but also against the toxin-producing vegetative anthrax bacilli. In the present study, we evaluated the feasibility of inducing strong mucosal and systemic immune responses against both anthrax toxins and bacilli after nasal immunization using a synthetic double-stranded RNA (dsRNA), polyriboinosinic–polyribocytidylic acid (poly(I:C) or pI:C), as the adjuvant. We have shown that the capsular poly-γ-d-glutamic acid (PGA) from bacillus was immunogenic when conjugated to a carrier protein and dosed intranasally to mice. We further demonstrated that nasal immunization with the PGA–carrier protein conjugate in combination with the anthrax protective antigen (PA) protein induced both anti-PGA and anti-PA immune responses in mouse sera and lung mucosal secretions. The anti-PA antibody (Ab) response was shown to have anthrax lethal toxin neutralization activity; and the anti-PGA Abs induced were able to activate complement and kill PGA-producing bacteria. These findings demonstrated that it is feasible to develop a novel dual-action nasal anthrax vaccine.

3.2 Introduction

Inhalational anthrax, the most lethal form of anthrax, is caused by the inhalation and deposition of *Bacillus anthracis* spores into the respiratory tract of the

host. Wherein, they are ingested by macrophages and transported to regional lymph nodes (LNs). Once in the LNs, the spores germinate into vegetative bacilli and are released into the systemic circulation (Koehler et al., 1994). In this environment, genes responsible for capsule formation and anthrax toxin synthesis are activated (Koehler et al., 1994). The bacteria then replicate unimpeded, resulting in massive bacteremia, toxemia, and eventual death of the patients, leaving little opportunity for medical interventions (Dixon et al., 1999) and (Inglesby et al., 2002). Currently, the only licensed anthrax vaccine in the U.S. is the anthrax-vaccine-adsorbed (AVA), whose primary antigen component was identified to be the PA protein of anthrax toxins (Puziss et al., 1963). However, the AVA suffers from several major drawbacks, including a very complicated and lengthy dosing schedule with six initial injections during the first 18 months, followed by annual booster injections. Thus, there continues to be a need to develop alternative and efficacious anthrax vaccines.

B. anthracis has two primary virulence factors. The anthrax toxins, one of the virulence factors, are composed of the PA protein, the lethal factor (LF), and the edema factor (EF). While non-toxic individually, they can combine to form two binary toxins, the lethal toxin (LeTx, PA and LF) and the edema toxin (PA and EF). PA binds to anthrax toxin receptors on the surface of host cells and mediates the entry of LF and EF into the host cell cytosol (Bradley et al., 2001), where they are toxic (Ascenzi et al., 2002). Thus, it was expected that anti-PA Abs will block the transport of LF and EF inside cells, and thus, protect hosts against anthrax challenges. However, there was evidence indicating that anti-PA Abs alone may not provide the optimal protection

(Brossier et al., 2002) and (Wang and Roehrl, 2005). Another virulence factor is the capsular poly-γ-d-glutamic acid (PGA), by which *B. anthracis* is surrounded and protected from the host immune system (Welkos, 1991). Encapsulated *B. anthracis* strains grow unhindered in infected hosts, while bacteria lacking the capsule are nearly avirulent (Welkos, 1991) and (Welkos et al., 1993). Thus, a vaccine that induces Abs against PGA may inhibit bacillus growth, and thus, reduce anthrax toxin production. It has been shown that after natural infections, 67–94% of patients developed anti-PGA Abs (Welkos, 1991). Therefore, new generation anthrax vaccines are expected to target not only the PA, but also the capsular PGA to inhibit the growth of vegetative bacilli.

Although PGA alone was shown to be weakly or non-immunogenic, data from recent studies demonstrated that it was immunogenic when conjugated to carrier proteins (Rhie et al., 2003), (Schneerson et al., 2003) and (Joyce et al., 2006). Moreover, Kozel et al. (2004) reported that more than 90% of mice passively immunized with anti-PGA monoclonal Abs were protected from a lethal pulmonary anthrax spore challenge (Kozel et al., 2004). Chabot et al. (2004) reported that greater than 90% mice subcutaneously injected with a PGA–PA conjugate survived a lethal anthrax spore challenge (Chabot et al., 2004). Joyce et al. (2006) also showed that immunization of mice with a PGA-OMPC conjugate induced very high capsule-specific immune responses that protected mice against a lethal challenge from virulent *B. anthracis* (Joyce et al., 2006).

We propose to test the feasibility of dosing a vaccine containing both PA and PGA as antigens via the nasal route. A nasal anthrax vaccine is expected to be advantageous because it will be easily administrable, and thus, feasible for mass immunization in case of an emergency. Moreover, because inhaled anthrax spores enter hosts via their lung mucosal surface, it is conceivable that an optimal protection against it requires not only systemic, but also mucosal immune responses. In support of this, Mikszta et al. (2005) reported that in rabbits nasally immunized with PA protein adjuvanted with CpG oligos, the serum LeTx neutralization Ab titers alone were not correlated with the survival of the rabbits after a pulmonary anthrax spore challenge (Mikszta et al., 2005), which was contrary to the previously reported correlation between animal survival and the LeTx neutralization activity for AVA and other rPA-based vaccines when injected intramuscularly (Pitt et al., 2001). Similarly, Flick-Smith et al. (2002) reported that mice that were immunized by a combination of i.m. and nasal priming/boosting were always fully protected against an aerosol anthrax spore challenge, whereas i.m. immunization alone was not as effective (Flick-Smith et al., 2002).

In the present study, we reported that a PGA conjugate was immunogenic when administered nasally into mice. In addition, nasal immunization of mice with a vaccine containing a PGA conjugate and PA both as antigens induced strong anti-PGA and anti-PA immune responses in mouse serum and broncho-alveolar lavage (BAL) samples. A synthetic dsRNA, poly(I:C), was used as the adjuvant. Poly(I:C) was known to have adjuvant activity (Alexopoulou et al., 2001) (Verdijk et al., 1999),

(Schmidt et al., 2004), (Sivori et al., 2004), (Gelman et al., 2004), (Salem et al., 2005), (Schulz et al., 2005), (Ichinohe et al., 2005) and (Partidos et al., 2005). Recent studies (Ichinohe et al., 2005) and (Partidos et al., 2005), including ours (Sloat and Cui, 2006b), have proved it as a strong and safe nasal mucosal vaccine adjuvant.

3.3 Materials and Methods

3.3.1 Strain, inoculation, and culture methods

B. licheniformis was generously provided by the Bacillus Genetic Stock Center at the Ohio State University (Columbus, OH). Highly mucoid colonies were selected and grown aerobically in Erlenmeyer flasks with E-medium (Gelman et al., 2004). The formula of the E-medium in g/l was as follows: glycerol, 80.0; citric acid, 12.0; l-glutamic acid 20.0; NH_4Cl, 7.0; K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; $CaCl_2 \cdot 2H_2O$, 0.15; $MnSO_4 \cdot H_2O$, 0.104; $FeCl_2 \cdot 6H_2O$, 0.04. The aforementioned chemicals were all purchased from Sigma–Aldrich (St. Louis, MO). The E-medium was adjusted to a pH of 7.4 with 10 M NaOH prior to autoclaving. Cultures were incubated at 37 °C and shaken at 250 rpm for 36–48 h.

3.3.2 Purification of PGA

PGA was purified from *B. licheniformis* as previously described with modifications (Rhie et al., 2003) and (Perez-Camero et al., 1999). It was previously shown that the PGAs isolated from *B. anthracis* and *B. licheniformis* were chemically and immunologically identical (Mesnage et al., 1998) and (Makino et al., 1989). The

bacterial culture was centrifuged at 4 °C, 6500 × *g* for 30 min. The supernatant was decanted, and the PGA was precipitated with 3 vol. of ethanol at 4 °C overnight. The PGA precipitate was collected and dialyzed against deionized water. The water was replaced every 2 h. The PGA solution was acidified to a pH of 1.5 with 6 M HCl. The PGA was precipitated with 3 vol. of 1-propanol at −20 °C. The resulting precipitate was collected by centrifugation and washed twice with acetone and once with ethyl ether. The purified PGA was dissolved in water, dialyzed overnight, and lyophilized. The structure and purity of the PGA was verified by ^{1}H NMR spectroscopy (Bruker am 400 MHz FT NMR, Blue Lion Biotech, Snoqualmie, WA). Prior to further use, the PGA was sonicated for 1.5 h in an ice-cold water bath.

3.3.3 Synthesis of PGA-BSA conjugates

Five mg of bovine serum albumin (BSA, Sigma–Aldrich) and 15 mg of the PGA in 6 ml PBS (pH 7.4, 10 mM) was mixed with 50 mg 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC, Sigma–Aldrich) and stirred for 4 h at room temperature. The reaction product was desalted using a PD-10 column (GE Amersham Biosciences, Piscataway, NJ). The PGA–BSA conjugate was stored at −20 °C until further use.

3.3.4 Nasal immunization

All mouse studies were carried out following NIH guidelines for animal use and care. In an initial experiment to evaluate the immunogenicity of a nasally dosed

PGA conjugate, Balb/C mice (female, 6–8 weeks, *n* = 5, Simenson Labs, Gilroy, CA) were nasally dosed with PGA–BSA conjugate (20 μg/mouse) admixed with pI:C (10 μg/mouse, GE-Amersham Biosciences), PGA–BSA admixed with cholera toxin (CT, 1 μg/mouse, List Biological Laboratories Inc., Campbell, CA), or PGA–BSA alone in PBS. As controls, mice were either left untreated or subcutaneously (s.c.) injected with PGA–BSA adsorbed onto aluminum hydroxide gel (Alum, 15 μg/mouse, USP grade, Spectrum Chemical and Laboratory Products, New Brunswick, NJ). The CT-adjuvanted group and the Alum-adjuvanted group were included as two positive controls, not necessarily to compare their effectiveness with that of the pI:C. For nasal dosing, mice were lightly anesthetized (i.p., pentobarbital, 6 mg/100 g, Abbott Laboratories, North Chicago, IL) and given a total volume of 20 μl of the vaccine formulations in two 10 μl doses with 10–15 min between each dose, half in each nare. Mice were dosed on days 0, 7, and 14. They were euthanized on day 30 and bled by cardiac puncture. Their broncho-alveolar lavage (BAL) samples were also collected.

In another experiment, PGA–BSA (20 μg/mouse), rPA (5 μg/mouse), and pI:C (10 μg/mouse) were co-dissolved into 20 μl PBS (10 mM, pH 7.4) and nasally dosed to Balb/C mice (*n* = 6). As controls (*n* = 5), mice were s.c. injected with the mixture of rPA (5 μg/mouse) and PGA–BSA (20 μg/mouse) adsorbed onto Alum (15 μg/mouse), nasally dosed with rPA (5 μg/mouse) admixed with pI:C (10 μg/mouse), nasally dosed with PGA–BSA (20 μg/mouse) admixed with pI:C (10 μg/mouse), or left untreated. Mice were dosed on days 0, 7, and 14 as mentioned above. They were euthanized and bled on day 30. Their BAL samples and spleens were also collected.

Both experiments mentioned above were repeated twice. Similar results were obtained.

3.3.5 Broncho-alveolar lavage (BAL) collection

To collect the BALs, an incision was made in the trachea of euthanized mice. Care was applied to avoid wounding the blood vessels. A 0.4 ml of sterile PBS was injected through the trachea towards the lungs. Before the final withdrawal, the lavage was aspirated back into the pipette tip and re-injected back into the lungs. Lavage samples collected from individual mice were stored at −80 °C prior to further use.

3.3.6 Quantification of PA and PGA Abs

The levels of anti-PA and anti-PGA in the serum and BAL samples were determined using enzyme-linked immunosorbent assay (ELISA). Briefly, EIA/RIA flat bottom, medium binding, polystyrene, 96-well plate (Corning Costar, Corning, NY) were coated with 100 ng of rPA or purified PGA dissolved in 100 µl of carbonate buffer (0.1 M, pH 9.6) overnight at 4 °C. For anti-PA Ab measurements, plates were washed with PBS/Tween 20 (10 mM, pH 7.4, 0.05% Tween 20, Sigma–Aldrich) and blocked with 4% (w/v) BSA in PBS/Tween 20 for 1 h at 37 °C. Samples were diluted two-fold serially (or as where indicated) in 4% BSA/PBS/Tween 20, added to the plates following the removal of the blocking solution, and incubated for an additional 3 h at 37 °C. The serum or BAL samples were removed, and the plates were washed five times with PBS/Tween 20. Horse radish peroxidase (HRP)-labeled goat anti-

mouse immunoglobulin (IgG, IgG1, IgG2a, IgM, or IgA, 5000-fold dilution in 1% BSA/PBS/Tween 20, Southern Biotechnology Associates Inc., Birmingham, AL) was added into the plates, followed by another hour of incubation at 37 °C. Plates were again washed five times with PBS/Tween 20. The presence of bound Ab was detected following a 30 min incubation at room temperature in the presence of 3,3′,5,5′-tetramethylbenzidine solution (TMB, Sigma–Aldrich), followed by the addition of 0.2 M sulfuric acid as the stop solution. The absorbance was read at 450 nm using a SpectraMax Plate reader (Molecular Devices Inc., Sunnyvale, CA).

Anti-PGA Ab measurements were determined similarly; except that the 4% BSA in PBS/Tween 20 was replaced by 10% (v/v) horse serum (Sigma–Aldrich) in PBS/Tween 20 in the plate blocking and sample dilution steps. In addition, the secondary Abs were diluted in 2.5% (v/v) horse serum in PBS/Tween 20.

3.3.7 Lethal toxin neutralization activity (TNA) assay

TNA was determined as previously described (Boyaka et al., 2003) and (Sloat and Cui, 2006c). In brief, confluent J744A.1 cells were plated (5.0×10^4 cells/well) in sterile, 96-well, clean-bottom plates (Corning Costar) and incubated at 37 °C, 5% CO_2 for 24 h. A fresh solution (50 µl) containing rPA (400 ng/ml) and LF (40 ng/ml) was mixed with 50 µl of diluted serum or BAL samples and incubated for 2 h at 37 °C. The cell culture medium was removed, and 100 µl of the serum/LeTx mixture was added into each well and incubated for 3 h at 37 °C, 5% CO_2. Cell viability was determined

using an MTT kit (Sigma–Aldrich) with untreated and LeTx alone treated cells as controls.

3.3.8 Splenocyte proliferation assay

Single cell suspensions were prepared from individual mouse spleen as previously described (Cui and Mumper, 2002). Splenocytes were cultured at a density of 4×10^6 cells/ml with rPA (12.5 μg/ml) or without rPA in complete RPMI 1640 medium (Invitrogen, Carlsbad, CA) for 5 days at 37 °C, 5% CO_2 (Boyaka et al., 2003; Sloat and Cui, 2006c). The cell number was determined using an MTT kit. The proliferation index was reported as the ratio of the final number of cells with rPA re-stimulation over that without re-stimulation.

3.3.9 Complement-mediated bacteriolysis

To evaluate the functionality of the anti-PGA sera against bacilli, a complement-mediated bactericidal assay was completed (Rhie et al., 2003). Due to the unavailability of capsular *B. anthracis* and the biohazards associated with it, we used *B. licheniformis*. Briefly, pooled sera from groups of immunized mice were 10-fold serially diluted in PBS. Samples of 30 μl of *B. licheniformis* (~1000 CFU) in PBS were incubated with 50 μl of the diluted serum samples and 20 μl of human complement (diluted 1:20 in PBS, Sigma–Aldrich) at 37 °C for 1 h in a shaker–incubator. Samples from each incubation mixture (50 μl) were plated on LB agar plates, and the plates were incubated for 8 h at 37 °C. The number of colonies formed

was counted. As controls, bacteria were incubated with serum from untreated mice, human complement alone, or PBS alone before being plated onto LB agar plates. The percent of bacterial killing was calculated for each serum dilution by comparing with the number of colonies formed when the bacteria were incubated with serum from un-immunized mice.

3.3.10 Statistics

Statistical analyses were completed using one-way analysis of variance (ANOVA) followed by the Fischer's protected least significant difference (PLSD) procedure. A *p*-value of ≤ 0.05 (two-tail) was considered to be statistically significant. The normality of the data and equal variance between groups was checked prior to data analysis.

3.4 Results

3.4.1 Nasal immunization of mice with a PGA-BSA conjugate adjuvanted with pI:C induced anti-PGA Abs

Although PGA conjugated with a carrier protein was shown to be immunogenic when subcutaneously (s.c.) injected, it was still unknown whether a PGA conjugate can induce anti-PGA immune responses when dosed intranasally. As shown in Fig. 1A, nasal PGA–BSA conjugate alone induced a very weak anti-PGA IgG response. However, PGA–BSA conjugate admixed with pI:C induced a strong anti-PGA IgG Ab response in mouse serum. As expected, mice nasally immunized with PGA–BSA conjugate admixed with cholera toxin (CT) or s.c. injected with the

PGA–BSA conjugate adsorbed on Alum also induced anti-PGA IgG responses (Fig. 3.1A). The PGA-specific IgG response in mice s.c. injected with the PGA–BSA conjugate adsorbed on Alum and in mice nasally dosed with the PGA–BSA admixed with CT was predominately IgG1 (Fig. 3.1B). In contrast, strong IgG1 and IgG2a Abs were both elicited in mice nasally dosed with the PGA–BSA conjugate admixed with pI:C (Fig. 3.1B). Moreover, nasal PGA–BSA conjugate adjuvanted with pI:C also induced anti-PGA IgA in mouse BALs (Fig. 3.2). Anti-PGA IgA Ab was also detected in the BAL samples of mice nasally immunized with the PGA–BSA conjugate admixed with CT. However, as expected, mice s.c. injected with the PGA–BSA adsorbed on Alum or nasally dosed with the PGA–BSA conjugate alone did not induce any significant anti-PGA IgA responses in their BALs.

3.4.2 Nasal immunization of mice with the mixture of rPA and PGA-BSA adjuvanted with pI:C induced both anti-PGA and anti-PA Abs in their sera and broncho-alveolar lavages

In the next experiment, we evaluated the immune responses induced when mice were nasally dosed with the physical mixture of rPA and PGA–BSA, adjuvanted with pI:C. As shown in Table 3.1, strong anti-PGA Abs (IgG, IgM, and IgA) and anti-PA Abs (IgG and IgM) were induced in their sera. Mice nasally immunized with the PGA–BSA conjugate alone adjuvanted with pI:C had comparable anti-PGA Abs, but as expected, no anti-PA Abs. Similarly, nasal rPA alone adjuvanted with pI:C induced comparable levels of anti-PA Abs, but no anti-PGA Abs. Subcutaneous injection of the mixture of rPA and PGA–BSA conjugate adsorbed on Alum induced comparable

anti-PGA Abs, and the anti-PA Abs induced by it tended to be stronger, though not significant, than that in mice nasally dosed with the mixture of rPA and PGA–BSA conjugate. However, the s.c. injection failed to induce any detectable levels of anti-PGA IgA Abs in the serum. Mice injected (s.c.) with the mixture of rPA and PGA–BSA conjugate adsorbed on Alum were strongly IgG1-biased, while the subtypes of anti-PA and anti-PGA IgG in mice nasally immunized with antigens adjuvanted with pI:C were less IgG1-biased or more IgG1/IgG2a balanced (Table 3.2). Finally, as shown in Fig. 3.3, both anti-PGA IgA and anti-PA IgA were induced in the BAL samples of mice nasally dosed with the mixture of rPA and PGA–BSA conjugate adjuvanted with pI:C. Nasal PGA–BSA conjugate adjuvanted with pI:C induced only anti-PGA IgA in the BALs, while nasal rPA adjuvanted with pI:C induced only anti-PA IgA in the BAL samples. As expected, mice s.c. injected with the mixture of rPA and PGA–BSA conjugate adsorbed on Alum failed to induce any anti-PA or anti-PGA IgA in their BALs (Fig. 3.3).

3.4.3 Splenocytes isolated from mice nasally immunized with the physical mixture of rPA and PGA-BSA adjuvanted with pI:C proliferated after in vitro re-stimulation with rPA

To evaluate the proliferative immune response induced when mice were nasally immunized with the mixture of rPA and PGA–BSA adjuvanted with pI:C, splenocytes isolated from the mice were re-stimulated with rPA, and the final splenocyte number was determined. As shown in Fig. 3.4, nasal immunization with the mixture of rPA and PGA–BSA adjuvanted with pI:C induced a significant

splenocyte proliferation. Nasally dosed rPA alone adjuvanted with pI:C also induced a PA-specific proliferative response.

3.4.4 The anti-PA Ab response induced by the mixture of rPA and PGA-BSA conjugate adjuvanted with pI:C had lethal toxin neutralization activity

To evaluate the protective activity of the anti-PA Abs induced, the anthrax LeTx neutralization activity in the sera and the BALs of the immunized mice was measured using an in vitro macrophage (J774A.1) protection assay. As shown in Fig. 3.5, strong LeTx neutralization activity was induced in the serum of mice s.c. injected with the mixture of rPA and PGA–BSA conjugate adsorbed on Alum. Mice immunized with the mixture of rPA and PGA–BSA conjugate adjuvanted with pI:C and those immunized with rPA alone adjuvanted with pI:C had comparable LeTx neutralization activities, although relatively weaker than that in mice s.c. injected with the mixture of PGA–BSA and rPA adsorbed on Alum, possibly because the later tended to induce stronger anti-PA Abs as shown in Table 3.1 and Table 3.2. As expected, mice immunized with PGA–BSA alone adjuvanted with pI:C failed to induce any significant LeTx neutralization activity. Lethal toxin neutralization activity was also detected in the BAL samples of mice nasally immunized with rPA adjuvanted with pI:C or with the mixture of rPA and PGA–BSA adjuvanted with pI:C (titer $\geq$20, a 400 μl BAL sample was collected).

3.4.5 The anti-PGA immune response induced by the mixture of rPA and PGA-BSA conjugate adjuvanted with pI:C can activate complement-mediated bacterial killing

To evaluate the functionality of the anti-PGA sera against bacilli, a complement-mediated bactericidal assay was completed. As shown in Fig. 3.6, the serum samples from mice nasally immunized with the mixture of rPA and PGA–BSA adjuvanted with pI:C can activate complement and had a bacillus-killing activity that was comparable to that from mice s.c. injected with the mixture of rPA and PGA–BSA adsorbed on Alum.

3.5 Discussions

In the present study, we first proved that the capsular PGA purified from bacillus was immunogenic when conjugated to a carrier protein and dosed nasally to mice, although an appropriate adjuvant was required. Moreover, when the mixture of a PGA conjugate and rPA were nasally dosed into mice, both anti-PGA and anti-PA immune responses were induced, although it appeared that the antigen mixture did not produce a synergistic effect. The anti-PA Ab responses induced were able to neutralize PA and thus protect macrophages against a lethal dose of anthrax LeTx challenge. Similarly, the anti-PGA Ab responses induced were able to activate complement-mediated bacillus killing, when *B. licheniformis* was used. Although the PGAs from *B. anthracis* and *B. licheniformis* are chemically and immunologically identical (Mesnage et al., 1998) and (Makino et al., 1989), the capsular PGA adheres to *B. anthracis*, but *B. licheniformis* sheds significant amounts of its capsule (Welkos, 1991), (Perez-Camero et al., 1999) and (Mock and Fouet, 2001). In future studies, we will measure the bacillus-killing activity using a capsular *B. anthracis* strain.

As reasoned earlier, a growing body of evidence is supporting the idea that an efficacious anthrax vaccine should be able to induce immune responses not only against the anthrax toxin, but also against the vegetative bacilli (Wang and Roehrl, 2005). AVA and many other anthrax vaccines currently under development are all primarily based on the anthrax PA protein alone, which can only induce immune responses to neutralize the toxins. However, anthrax toxemia starts only after the bacteremia has already started (Dixon et al., 1999) and (Inglesby et al., 2002), and the level of anti-PA Abs required to confer immunity to *B. anthracis* is expected to be high and may be difficult to induce and maintain (Schneerson et al., 2003). Thus, the addition of anti-PGA Ab-induced opsonophagocytic killing of vegetative bacilli is expected to prevent or inhibit bacteremia, and thus, augment the protection mediated by the anti-PA Abs against high levels of anthrax spores that might occur during a bioterrorist attack (Schneerson et al., 2003). In fact, there had been a few recent publications that attempted to design such a dual-action anti-PA and anti-PGA anthrax vaccine (Rhie et al., 2003), (Schneerson et al., 2003), (Chabot et al., 2004) and(Wang et al., 2004). For example, Rhie et al. (2003) reported that s.c. injected PGA–PA conjugate adsorbed on Alum induced both anti-PGA and anti-PA immune responses (Rhie et al., 2003). Similarly, Schneerson et al. (2003) and Wang et al. (2004) reported that s.c. injection of PA conjugated with PGA polypeptides also induced anti-PA and anti-PGA immune responses (Schneerson et al., 2003) and (Wang et al., 2004).

However, in all those studies, the PGA–PA conjugates were adsorbed on Alum and dosed to mice by injection (Rhie et al., 2003), (Schneerson et al., 2003), (Chabot

et al., 2004) and (Wang et al., 2004), which still did not address one of the major drawbacks of the current AVA for injection, its complicated and lengthy dosing schedule. This prompted us to evaluate the feasibility of dosing the PGA–PA dual antigen vaccine via the nasal route. As demonstrated in the present study, such a nasal anthrax vaccine can induce both anti-PGA and anti-PA immune responses (Table 3.1). Moreover, it is expected to be cost-effective, patient-friendly, and suitable for mass immunization (Davis, 2001). Finally, it is expected that the induction of immune responses in the site of anthrax spore entrance, the lung mucosa, will lead to an improved protection against an inhalational anthrax spore challenge, when compared to a vaccine that can only induce immune responses systemically. Although anthrax bacteria do not produce anthrax toxin until the inhaled and phagocytized anthrax spores germinate into vegetative bacilli, which does not happen in the lung surface, recent data have shown that rabbits that were induced to produce anti-PA IgA in their lung mucosal were better protected from an inhalational anthrax spore challenge (Mikszta et al., 2005) and (Flick-Smith et al., 2002). It is possible that the anti-PA Abs in the lung mucosal secretion might have reacted with some PA-binding protein(s) on the spore surface, and thus, inhibited the germination of the spores. Spore-associated proteins recognized by anti-PA Abs were shown to be present on the surface of spores (Welkos et al., 2001) and(Welkos et al., 2002). Moreover, anti-PA Abs were also shown to stimulate the phagocytosis of spores by macrophages and inhibit the germination of spores in vitro (Welkos et al., 2001) and (Welkos et al., 2002)Because purified protein antigens, such as the PGA–BSA conjugate and rPA, were not or only

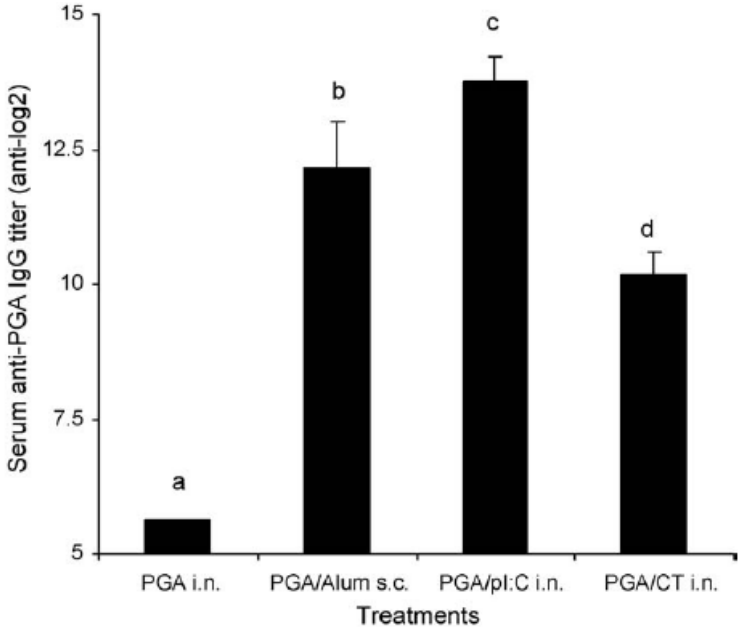
weakly immunogenic when given alone, we used the synthetic dsRNA, poly(I:C), as a nasal mucosal vaccine adjuvant. An agonist to Toll-like receptor 3 (TLR3) (Alexopoulou et al., 2001), poly(I:C) was known to have adjuvant activity. More recently, pI:C was successfully used intranasally in a couple of studies to induce anti-HIV Tat and anti-hemagglutinin immune responses (Ichinohe et al., 2005) and (Partidos et al., 2005). In a previous study, we have shown that nasal dosing of mice with anthrax PA protein admixed with pI:C induced strong anthrax lethal toxin neutralizing anti-PA Ab responses systemically as well as in the lung mucosa (Sloat and Cui, 2006b). The adjuvant activity of pI:C was likely from its interaction with TLR3. Nasal pI:C was shown to up-regulate the expression of TLR3 and alpha/beta interferons as well as Th1- and Th2-related cytokines (Ichinohe et al., 2005). We have also shown that nasal pI:C induced the accumulation of dendritic cells into local draining LNs, the superficial and deep cervical LNs (Sloat and Cui, 2006b). At a dose of 25 μg per mouse daily for 9 days, pI:C did not induce any significant side-effects in mice. In addition, 25 μg of pI:C, when intracerebrally injected into mice, did not lead to any significant toxicity in the mouse brain (Ichinohe et al., 2005). In the present study, the nasal dose of pI:C was 10 μg per mouse because we have shown in a previous pI:C dose response study that 10 μg of pI:C per mouse induced the strongest anti-PA Ab responses (Sloat and Cui, unpublished data). Further increasing the dose of pI:C to 20 or 40 μg per mouse did not improve the resulted specific immune responses. Moreover, it also needs to be pointed out that the IgG Ab responses induced by the nasal pI:C included both IgG1 and IgG2, in contrast to the

predominately IgG1-biased responses induced when cholera toxin or Alum was used as the adjuvant. The more balanced IgG subtype (Fig. 3.1 and Table 3.2) and the proliferative splenocyte responses suggested that both Ab and cellular immune responses had been induced when the pI:C was used as an adjuvant. In fact, it was known that pI:C was a strong cellular immune response inducer (Salem et al., 2005) and(Cui and Qiu, 2006). Thus, using the pI:C as an adjuvant might be beneficial for the induction and maintenance of a long-lasting antibody response, which are lacking for the current AVA. Finally, pI:C as an adjuvant has some advantages over the previously well-characterized bacterial CpG motifs. CpG motifs have been proven to be an excellent vaccine adjuvant in small animal models. However, it is often difficult to transfer the success in murine models to humans, probably due to the species-specific differences between the cell type distribution of TLR9, the receptor for CpG motifs, in mice and humans. In humans, TLR9 is only present on plasmacytoid DCs (pDCs) and B cells (Kadowaki et al., 2001). Thus, only pDCs and B cells respond to TLR9 ligands. All other effects of TLR9 ligands on human immune cells seem to be indirect and dependent on factors produced by pDCs and B cells. The situation in mice is significantly different because, not only pDCs and B cells, but also most other DCs, as well as macrophages, express TLR9, and thus respond directly to TLR9 ligation (Kadowaki et al., 2001). Unlike TLR9, TLR3 is expressed in myeloid DCs (mDCs) and T cells as well as fibroblast and other non-immune cells in humans (Ulevitch, 2004). Thus, pI:C and CpG oligos together are expected to be more potent than each of them alone in humans by complementing each other.

Finally, when dosed intranasally, it is possible that the vaccine formulation may travel to the lung or even the gastrointestinal tract, especially when the volume of the vaccine dosed to mice is large (Davis, 2001). In the present study, the volume of the vaccine solution was 20 μl per mouse, and the 20 μl was divided into two 10 μl doses with 10–15 min between each dose, half in each nare. Data from our previous study have shown that, when mice were dosed using this method, the extent to which the vaccine traveled to the lungs and the gastrointestinal tract was very limited (Sloat and Cui, 2006a).

In conclusion, we have confirmed that nasal PGA was immunogenic when the PGA was conjugated to a carrier protein and dosed with an appropriate adjuvant. A nasal vaccine containing PA and PGA as antigens induced both anti-PA and anti-PGA immune responses. In future studies, we will directly conjugate PGA and PA, nasally dose the conjugate to mice, and validate the efficacy of the induced immune responses in an inhalational anthrax spore challenge study.

(A)


15
12.5
10
7.5
5
Serum anti-PGA IgG titer (anti-log2)
a
b
c
d
PGA i.n.
PGA/Alum s.c.
PGA/pI:C i.n.
PGA/CT i.n.
Treatments

(B)

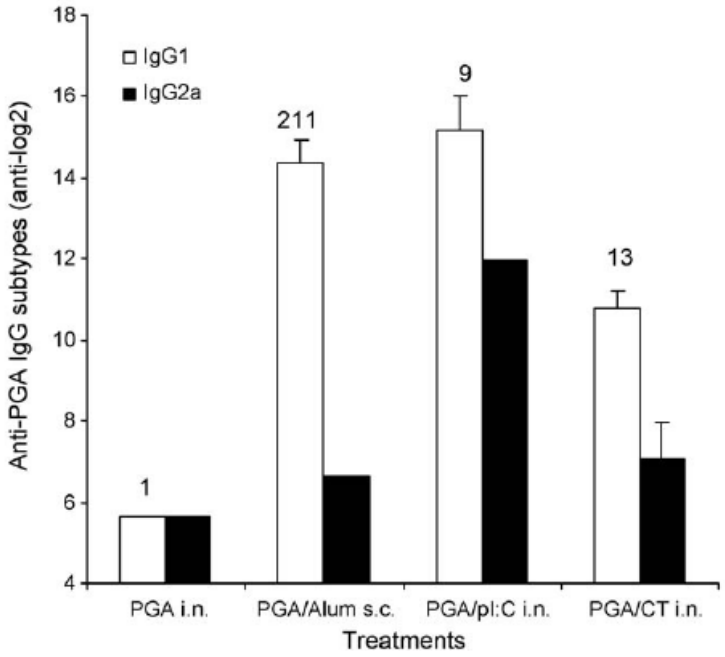

18
16
14
12
10
8
6
4
Anti-PGA IgG subtypes (anti-log2)
IgG1
IgG2a
1
211
9
13
PGA i.n.
PGA/Alum s.c.
PGA/pI:C i.n.
PGA/CT i.n.
Treatments

Figure 3.1

Figure 3.1 Serum anti-PGA IgG and its subtypes. Balb/C mice (n = 5) were nasally dosed with PGA–BSA admixed with CT (PGA/CT, i.n.) or pI:C (PGA/pI:C, i.n.). As controls, mice were either left untreated, nasally dosed with PGA–BSA alone (PGA, i.n.), or s.c. injected with PGA–BSA adsorbed on Alum (PGA/Alum, s.c.). (A) The total anti-PGA IgG titer in mouse serum samples. The (a)–(d) indicate that the values from all treatments are significantly different from one another ($p \ll 0.05$). (B) The anti-PGA IgG1 and IgG2a titers in sera. The numbers above the bars represent the ratio of the IgG1 titer and the IgG2a titer (IgG1/IgG2a). All three adjuvanted PGA formulations induced significant anti-PGA IgG1. The anti-PGA IgG1 values from PGA/Alum (s.c.) and PGA/pI:C (i.n.) are not different from each other ($p = 0.11$), but are both significantly higher than that from the PGA/CT (i.n.). Only the PGA/pI:C (i.n.) induced a significant anti-PGA IgG2a response. Data reported are mean ± S.D.

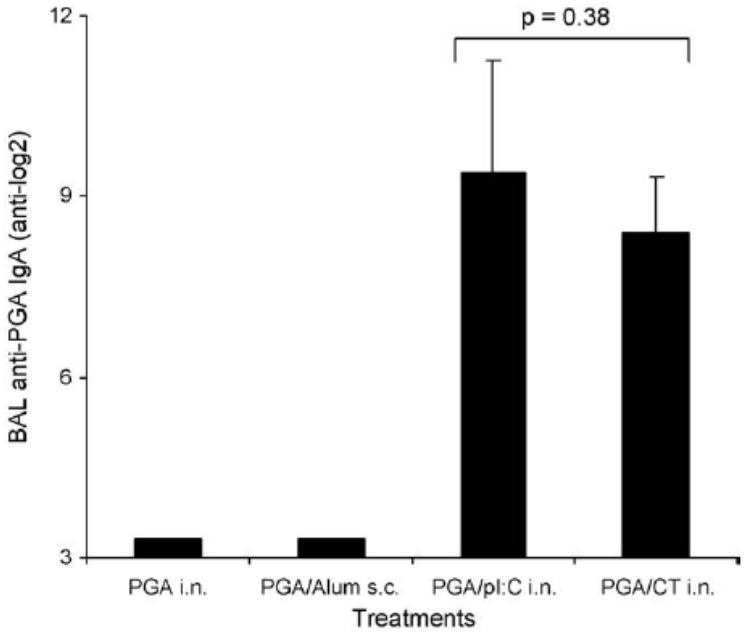


Figure 3.2 Anti-PGA IgA in mouse BALs. Balb/C mice (n = 5) were nasally dosed with PGA–BSA adjuvanted with CT or PGA–BSA adjuvanted with pI:C. As controls, mice were either left untreated, nasally dosed with PGA–BSA alone, or s.c. injected with PGA–BSA adsorbed on Alum. The anti-PGA IgA Ab titer in the BAL samples from individual mouse was determined. The titers for PGA/pI:C (i.n.) and PGA/CT (i.n.) were comparable (p = 0.38). Data reported are mean ± S.D.

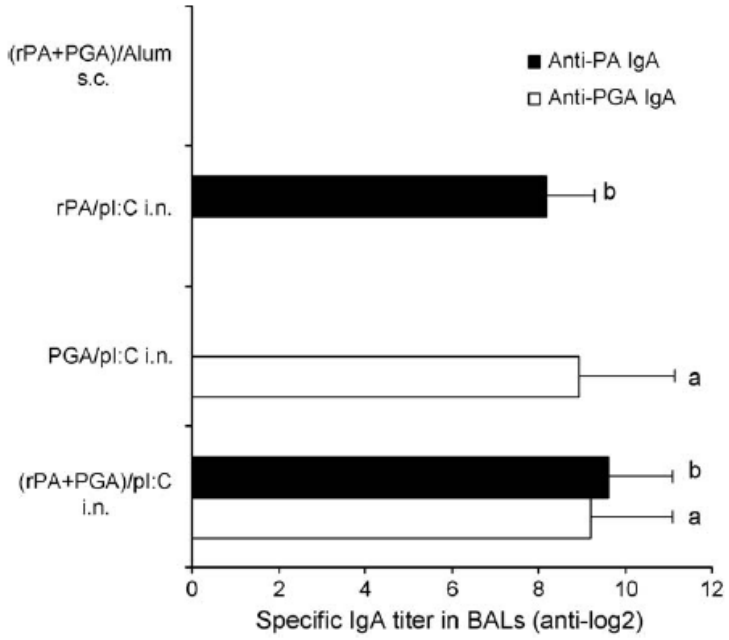


Figure 3.3 Anti-PGA and anti-PA IgA Abs in mouse BALs. Balb/C mice (n = 5–6) were nasally dosed with rPA/pI:C, PGA–BSA/pI:C, or the mixture of rPA and PGA–BSA adjuvanted with pI:C. As controls, mice were either left untreated or s.c. injected with a mixture of rPA and PGA adsorbed on Alum. The anti-PGA and anti-PA IgA titers in the BAL samples from individual mouse were determined: (a) indicates that the anti-PGA IgA values from PGA/pI:C (i.n.) and (rPA + PGA)/pI:C (i.n.) are not different from each other (p = 0.85); similarly, (b) indicates that the anti-PA IgA values from rPA/pI:C (i.n.) and (rPA + PGA)/pI:C (i.n.) are not different from each other (p = 0.13). Data reported are mean ± S.D.

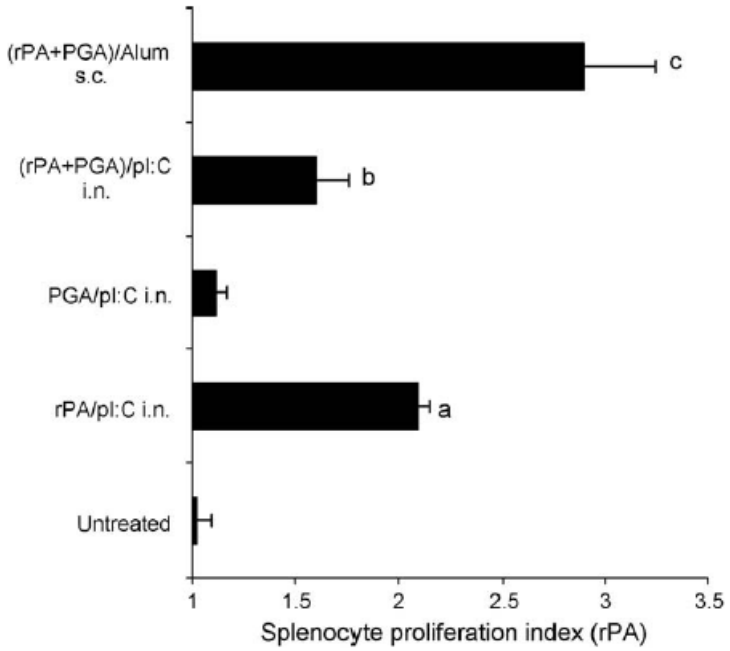


Figure 3.4 Splenocyte proliferation after in vitro stimulation with rPA. Balb/C mice were nasally dosed with rPA/pI:C, PGA/pI:C, or (rPA + PGA)/pI:C. As controls, mice were either left untreated or s.c. injected with (rPA + PGA)/Alum. The proliferation of the splenocytes after in vitro re-stimulation with PA was evaluated. The (a–c) indicate that the values from the three different treatments were significantly different from one another. Data reported are mean ± S.D. (n = 5–6).

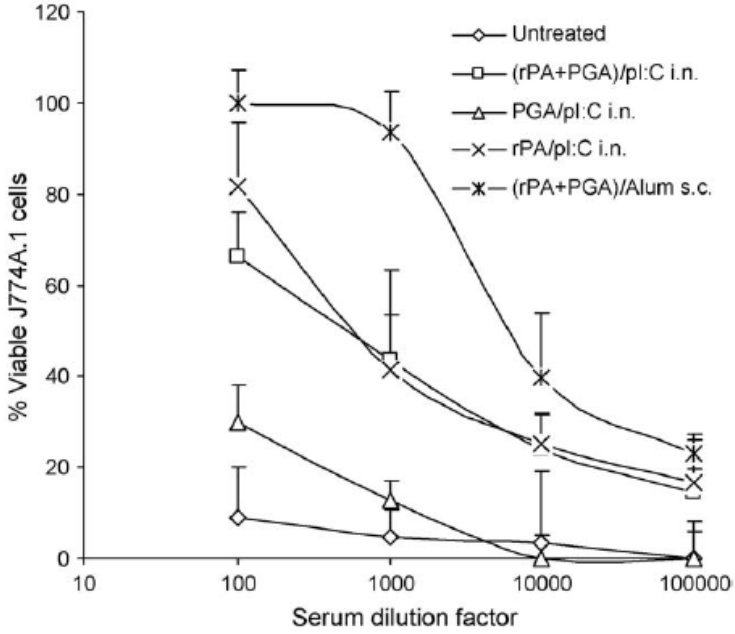


Figure 3.5 Lethal toxin neutralization activity. Balb/C mice were nasally dosed with rPA/pI:C, PGA/pI:C, or (rPA + PGA)/pI:C. As controls, mice were either left untreated or s.c. injected with (rPA + PGA)/Alum. The serum samples were diluted 10-fold serially and incubated with J774A.1 cells in the presence of anthrax LeTx. The percentages of the viable J774A.1 cells were estimated and reported. Data reported are mean ± S.D. (n = 5–6).

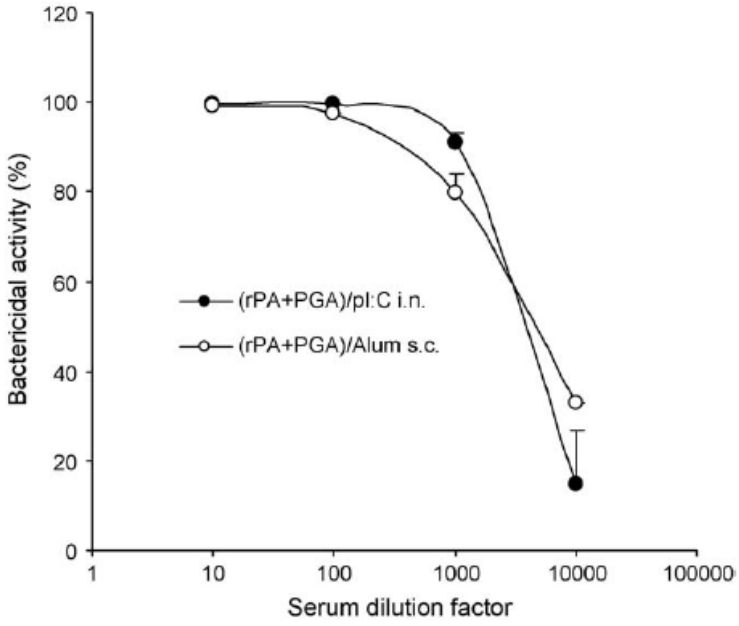


Figure 3.6 Complement-mediated bactericidal activity. Balb/C mice (n = 5–6) were nasally dosed with (rPA + PGA)/pI:C, s.c. injected with (rPA + PGA)/Alum, or left untreated. The ability for the anti-PGA Abs induced to activate complement and kill *B. licheniformis* bacilli was measured and reported. Serum samples from each treatment were pooled together. Reported are mean ± S.D. from three independent measurements.

Table 1
Serum Ab responses to PGA and rPA

	Treatment and route (anti-log_2)				*p*-Value
	(rPA + PGA)/pI:C, i.n.	PGA/pI:C, i.n.	rPA/pI:C, i.n.	(rPA + PGA)/Alum, s.c.	
Anti-PGA IgG	13.5 ± 0.8	13.2 ± 0.6	N/D	11.3 ± 1.9	0.03
Anti-PGA IgM	10.8 ± 0.4	10.9 ± 0.5	N/D	7.4 ± 2.1	0.004
Anti-PGA IgA	8.1 ± 0.9	8.7 ± 0.8	N/D	N/D	0.32
Anti-PA IgG	17.1 ± 0.7	N/D	17.5 ± 0.8	17.7 ± 0.5	0.44
Anti-PA IgM	12.2 ± 0.9	N/D	10.6 ± 5.3	12.8 ± 1.3	0.52

Table 3.1 Balb/C mice (n = 5–6) were i.n. dosed with rPA alone, PGA–BSA alone, or the mixture of rPA and PGA–BSA, all adjuvanted with pI:C. As controls, mice were either left untreated or s.c. injected with the mixture of rPA and PGA–BSA adsorbed on Alum. PA- and PGA-specific Abs in their sera were measured. Values shown are the anti-log_2 of the Ab titers (mean ± S.D.). N/D = not detectable. The anti-PGA IgG and IgM values for (rPA + PGA)/Alum (s.c.) are significantly different from those of the other two treatments. The p-values were from ANOVA analyses.

Table 2
Serum IgG subtypes specific to PGA and rPA

	Treatment and route (anti-log_2)				*p*-Value
	(rPA + PGA)/pI:C, i.n.	PGA/pI:C, i.n.	rPA/pI:C, i.n.	(rPA + PGA)/Alum, s.c.	
Anti-PGA IgG1	14.5 ± 0.4	14.8 ± 0.6	N/D	13.3 ± 0	≪0.05
Anti-PGA IgG2a	12.3 ± 0.8	10.5 ± 0.6	N/D	7.0 ± 0	≪0.05
IgG1/IgG2a	4.4	19.7		78.8	
Anti-PA IgG1	16.6 ± 1.3	N/D	17.4 ± 0.4	17.6 ± 1.0	0.24
Anti-PA IgG2a	16.4 ± 0.9	N/D	16.8 ± 0.8	13.4 ± 0	≪0.05
IgG1/IgG2a	1.1		1.5	18.4	

Table 3.2 Balb/C mice were i.n. dosed with rPA, PGA, or the mixture of rPA and PGA–BSA, all adjuvanted with pI:C. As controls, mice were either left untreated or s.c. injected with the mixture of rPA and PGA–BSA adjuvanted with Alum. PA- and PGA-specific IgG subtypes (IgG1 and IgG2a) in their sera were measured. Values shown are the anti-log_2 of the Ab titers (mean ± S.D.). For anti-PGA IgG1, the value from (rPA + PGA)/Alum (s.c.) was different from those of the other two treatments. For anti-PGA IgG2a, the values from the three treatments were all different from one another. For anti-PA IgG2a, the value from (rPA + PGA)/Alum (s.c.) was different from those of the other two treatments. The IgG1/IgG2a indicates the ratio of the IgG1 titer over that of the IgG2a titer.

Chapter 4

NASAL IMMUNIZATION WITH THE MIXTURE OF PA63, LF, AND A PGA CONJUGATE INDUCED STRONG ANTIBODY RESPONSES AGAINST ALL THREE ANTIGENS

Brian R. Sloat, Dalia S. Shaker, Uyen M. Le, and Zhengrong Cui

FEMS Immunology and Medical Microbiology, 2008; 52: 169-79

4.1 Abstract

A new generation anthrax vaccine is expected to target not only the anthrax protective antigen (PA) protein, but other virulent factors of *Bacillus anthracis*. It is also expected to be amenable for rapid mass immunization of a large number of people. We proposed to address these needs by designing a prototypic tri-antigen nasal anthrax vaccine candidate that contained a truncated PA (rPA63), the anthrax lethal factor (LF), and the capsular poly-γ-D-glutamic acid (γDPGA) as the antigens and a synthetic double-stranded RNA (dsRNA), polyriboinosinic-polyribocytodylic acid (poly(I:C)) as the adjuvant. We identified the optimal dose of nasal poly(I:C) in mice, demonstrated that nasal immunization of mice with the LF was capable of inducing functional anti-LF antibodies (Abs), and showed that nasal immunization of mice with our prototypic tri-antigen vaccine candidate induced strong immune responses against all three antigens. The immune responses protected macrophages against an anthrax lethal toxin challenge *in vitro* and enabled the immunized mice to survive a lethal dose of anthrax lethal toxin challenge *in vivo*. The anti-PGA Abs were shown to have complement-mediated bacteriolytic activity. After further optimization, this tri-antigen nasal vaccine candidate is expected to become one of the newer generation anthrax vaccines.

4.2 Introduction

Bacillus anthracis, the etiological agent of anthrax, is a rod-shaped, non-motile, gram-positive bacterium that forms highly resistant endospores under adverse

conditions (Ascenzi et al., 2002). Depending on the site of inoculation, anthrax infection can occur in three clinical forms: cutaneous, gastrointestinal, or inhalational. Cutaneous and gastrointestinal anthrax are rare and highly treatable, but inhalation anthrax has a fatality rate approaching 100%, with death occurring shortly after the onset of symptoms (Friedlander, 1999). Upon inhalation, anthrax spores are deposited into the alveolar spaces of the respiratory tract of the host and ingested as inert particles by local macrophages (Dixon et al., 1999). Wherein they are transported to the mediastinal and peribronchial lymph nodes, germinate into vegetative bacilli, and are released into the systemic circulation (Koehler et al., 1994). Released bacilli begin unimpeded extracellular multiplication while secreting fatal levels of anthrax toxins (Wang and Roehrl, 2005).

Vaccination is considered the best option for the prophylaxis of anthrax. Currently, the only anthrax vaccine available in the U.S. is the anthrax-vaccine adsorbed (AVA, now BioThrax®), an aluminum hydroxide (Alum)-adsorbed, formalin-treated culture supernatant of a toxigenic *B. anthracis* strain (Puziss et al., 1963). However, there still is room to improve the AVA. For example, the AVA has a lengthy and complicated dosing schedule with 6 initial injections in the first 18 months and subsequent yearly booster injections. Moreover, the real composition of the AVA is unknown, although the primary antigen component was identified to be the PA protein of the anthrax toxins (Puziss et al., 1963), which prompted the development of new generation anthrax vaccines based on recombinant PA. Finally, the AVA is administered by subcutaneous needle-syringe injection, which may not be amenable

for self-administration or for administration by untrained personnel, and thus, not suitable for rapid mass immunization in the case of an emergency. A newer generation anthrax vaccine is expected to address all these issues.

B. anthracis has two primary virulence factors, anthrax toxins and the capsule. Anthrax toxins have three components, the PA, the LF, and the edema factor (EF). Individually, these components are nontoxic. However, they can combine to form two binary toxins, the lethal toxin (LeTx, PA and LF) and the edema toxin (PA and EF). The binding of the PA to receptors on the surface of host cells allows the entry of the LF and the EF into the cell cytosol, where they are toxic (Bradley et al., 2001; Scobie et al., 2003; Scobie and Young, 2005). Thus, anti-PA Abs were shown to block the transport of LF and EF into cells and aid in the progression of the infection. The PGA capsule is another virulence factor, by which the *B. anthracis* is surrounded and protected from the host immune system (Welkos et al., 1993). *B. anthracis* strains that are encapsulated within the PGA grow unhindered in infected hosts, whereas bacteria lacking the capsule are nearly avirulent (Welkos, 1991; Welkos et al., 1993). Thus, a vaccine capable of inducing anti-capsule Abs is expected to inhibit the bacillus growth, and thus, reduce the anthrax toxin production, which is expected to be beneficial. The feasibility of inducing Abs against PGA and the efficacy of the anti-PGA Abs in protecting against anthrax infection have been confirmed in several recent studies (Chabot et al., 2004; Joyce et al., 2006; Kozel et al., 2004; Rhie et al., 2003; Schneerson et al., 2003). Moreover, it was reported that after naturally occurring human infections, 68-93% of the patients developed anti-PA Abs, 43-55% developed

anti-LF Abs, and 67-94% developed anti-PGA Abs (Wang and Roehrl, 2005). Furthermore, there were data showing that vaccination of guinea pigs with PA and LF together afforded a better protection against an aerosolized anthrax spore challenge than with PA alone (Ivins and Welkos, 1988). Taken all these findings together, we propose to develop an improved prototypic anthrax vaccine candidate that contains purified PA(63), LF, and PGA to target all three components.

To address the need for an immunization approach suitable for rapid mass immunization, we propose to dose this vaccine candidate intranasally. Intranasal dosing with specialized devices is expected to be amenable for self-administration or for administration by non-professionals.

In the present study, we evaluated the immune responses induced by a nasal vaccine candidate containing PA (PA83 or PA63), LF, and a PGA conjugate. The PA63 was used to reduce the potential toxicity when PA and LF were dosed together. It was previously shown that the PA63 fragment generated by trypsinizing PA83 was less toxic than the PA83 (Novak et al., 1992). There also were data showing that Abs against a PA63 preparation whose toxicity was up to 100-fold less than that of the PA83 protect rabbits and non-human primates against a lethal spore challenge (Hepler et al., 2006). The PGA was conjugated to a carrier protein to improve its immunogenicity because the PGA alone is weakly or non-immunogenic (Chabot et al., 2004; Rhie et al., 2003; Schneerson et al., 2003). A synthetic double-stranded RNA (dsRNA), poly(I:C), was used as a vaccine adjuvant. Data from recent studies have shown that the poly(I:C) was a safe and potent nasal vaccine adjuvant (Asahi-Ozaki et

al., 2006; Ichinohe et al., 2005; Partidos et al., 2005; Sloat and Cui, 2006a; Sloat and Cui, 2006b). We have shown that strong and functional immune responses against all three antigens were induced after only 2 or 3 intranasal doses with this prototypic tri-antigen vaccine candidate, and that the anti-PA Abs induced were as strong as that induced by s.c. injection of rPA83 adsorbed onto aluminum hydroxide gel (Alum).

4.3 Materials and Methods

4.3.1 Bacterial strain, inoculation, and culture methods

Bacillus licheniformis was obtained from the Bacillus Genetic Stock Center (BGSC) at the Ohio State University (Columbus, OH). Highly mucoid colonies were selected and grown aerobically in Erlenmeyer flasks with E-medium (Perez-Camero et al., 1999). The formula of the E-medium in g L^{-1} was as follows: glycerol, 80.0; citric acid, 12.0; L-glutamic acid, 20.0; NH_4Cl, 7.0; K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; $CaCl_2 \cdot 2H_2O$, 0.15; $MnSO_4 \cdot H_2O$, 0.104; $FeCl_2 \cdot 6H_2O$, 0.04. The aforementioned chemicals were all purchased from Sigma-Aldrich (St. Louis, MO). The E-medium was brought to boiling temperature, followed by shaking to dissolve all components, allowed to cool to room temperature, and adjusted to a pH of 7.4 with NaOH prior to autoclaving. Cultures were maintained in a shaker-incubator at 37˚C for 36–48 h.

4.3.2 Purification of γDPGA

The γDPGA was purified from *B. licheniformis* as previously described (Sloat and Cui, 2006a). The bacterial culture was centrifuged at 4˚C, 6,500 x g for 30 min.

The supernatant was collected, and the γDPGA was precipitated with 3 volumes of ethanol at 4˚C overnight. The γDPGA precipitate was collected and dialyzed against de-ionized water, which was replaced every 2 h over 6-8 h. The γDPGA solution was acidified to a pH of 1.5 with HCl, followed by precipitation with 3 volumes of 1-propanol at -20˚C. The resultant precipitate was collected by centrifugation and washed twice with acetone and once with ethyl ether. The purified γDPGA was dissolved in water, dialyzed extensively, and lyophilized. The structure and purity of the γDPGA was verified by ^{1}H NMR spectroscopy (Bruker am 400 MHz FT NMR, Blue Lion Biotech, Snoqualmie, WA) and UV-Vis scanning from 190 to 300 nm (DU-640 Spectrophotometer, Beckman Coulter, Fullerton, CA), and the purity was estimated to be greater than 96%. The percent of the D-isomer glutamic acids can be increased to 85%, depending on the concentration of the Mn in the culture medium. Prior to further use, the γDPGA was sonicated for 1.5 h in an ice-cold water bath sonicator (Ultrasonic Cleaner Model 150T, VWR International, West Chester, PA.).

4.3.3 Synthesis of PGA-BSA conjugates

The γDPGA-BSA conjugate was prepared as previously described (Rhie et al., 2003; Sloat and Cui, 2006a). Five mg of bovine serum albumin (BSA, Sigma-Aldrich, catalog # A8303), 15 mg of γDPGA, and 50 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC, Sigma-Aldrich) were dissolved in PBS (pH 7.4, 10 mM) and stirred for 4 h at room temperature. The reaction product was de-salted

using a PD-10 column (GE Amersham Biosciences, Piscataway, NJ). The γDPGA-BSA conjugate was lyophilized and stored at -20˚C until further use.

4.3.4 Nasal immunization

All mouse studies were carried out following NIH guidelines for animal use and care. For nasal dosing, BALB/c mice (female, 6-8 weeks, Simonsen Laboratories, Inc., Gilroy, CA) were lightly anesthetized intraperitoneally with pentobarbital (6 mg per 100 g, Abbott Laboratories, North Chicago, IL) before dosing. To identify the optimal dose of poly(I:C), mice were nasally dosed with rPA83 (5 μg per mouse, List Biological Laboratories, Inc., Campbell, CA) admixed with various concentrations of poly(I:C) (0.1, 1.0, 10.0, 20.0, or 40.0 μg per mouse, GE Healthcare Biosciences, Piscataway, NJ). As a negative control, mice were left untreated. Mice were dosed on days 0 and 14, euthanized on day 28, and bled by cardiac puncture.

To evaluate the feasibility of inducing functional anti-LF Abs by nasal LF, BALB/c mice were nasally dosed with various amounts of LF (5, 10, 20, or 40 μg per mouse, BEI Resources, Manassas, VA) admixed with poly(I:C) (10 μg per mouse). As controls, mice were either left untreated or nasally dosed with LF (5 μg per mouse) admixed with CT (1 μg per mouse). Mice were dosed on days 0 and 14, euthanized on day 28, and bled by cardiac puncture.

In another animal experiment, mice were nasally dosed with the mixture of rPA83 (5 μg per mouse), LF (5 μg per mouse), and the γDPGA-BSA conjugate (20 μg per mouse) admixed with poly(I:C) (10 μg per mouse). Each dose was administered in

two 10 μL doses with 10-15 min between each dose, half in each nare. As a positive control, mice were also nasally dosed with the mixture of rPA83, LF, and γDPGA-BSA, adjuvanted with cholera toxin (CT, 1 μg per mouse, List biological Labs). Negative control mice were left untreated. Mice were dosed on days 0 and 14, euthanized on day 55, and bled by cardiac puncture. Their spleens were also harvested for splenocyte proliferation assay.

Finally, BALB/c mice were nasally dosed with 20 μL of the mixture of rPA63 (15 μg per mouse, List Biological), LF (20 μg per mouse), and the γDPGA-BSA conjugate (20 μg per mouse), admixed with poly(I:C) (10 μg per mouse). As controls, mice were left untreated, s.c. injected with the mixture of rPA63, LF, and γDPGA-BSA adsorbed onto Alum (50 μg per mouse, USP grade, Spectrum), or nasally dosed with rPA63 alone (15 μg or 24 nmoles) admixed with poly(I:C), rPA83 alone (20 μg or 24 nmoles) admixed with poly(I:C), or the LF alone (20 μg) admixed with poly(I:C). Mice were dosed on days 0, 14, 28, and bled via the tail vein on day 42.

4.3.5 Quantification of Abs against PA, γDPGA, LF, and poly(I:C)

The levels of anti-PA83, anti-LF, and anti-γDPGA in the serum samples were determined using ELISA. Briefly, EIA/RIA flat bottom, medium binding, polystyrene, 96-well plates (Costar) were coated with 100 ng of rPA83, LF, or the pure PGA dissolved in 100 μL carbonate buffer (0.1 M, pH 9.6) at 4°C overnight. For anti-PA83 and anti-LF Ab measurements, the plates were washed with PBS/Tween 20 (10 mM,

pH 7.4, 0.05% Tween 20) and blocked with 4% (w/v) BSA in PBS/Tween 20 for 1 h at 37°C. Samples were diluted 2-fold serially (or as indicated) in 4% BSA/PBS/Tween 20, added to the plates following the removal of the blocking solution, and incubated for an additional 3 h at 37°C. The serum samples were removed, and the plates were washed 5 times with PBS/Tween 20. HRP-labeled goat anti-mouse immunoglobulin (IgG, IgG1, IgG2a, IgM, or IgA, 5,000-fold dilution in 1% BSA/PBS/Tween 20, Southern Biotechnology Associates, Inc. Birmingham, AL) was added into the plates, followed by another hour of incubation at 37°C. Plates were again washed 5 times with PBS/Tween 20. The presence of bound Ab was detected following a 30 min incubation at room temperature in the presence of 3,3',5,5'-tetramethylbenzidine solution (TMB, Sigma-Aldrich), followed by the addition of 0.2 M sulfuric acid as the stop solution. The absorbance was read at 450 nm using a SpectraMax Plate reader (Molecular Devices Inc. Sunnyvale, CA). Ab titers were derived by comparing the OD450 values of the samples with the OD450 plus 2 x S.D. of the negative control mice.

The anti-γDPGA Abs were determined similarly, except that the 4% BSA in PBS/Tween 20 was replaced by 10% (v/v) horse serum (Sigma-Aldrich) in PBS/Tween 20 in the plate blocking and sample dilution steps. In addition, the secondary Ab was diluted in 2.5% (v/v) horse serum in PBS/Tween 20.

The anti-dsRNA Abs were determined similarly. The plates were coated with the poly(I:C) (100 ng per well). As a negative control, the plates were coated with carbonate buffer only. As a positive control, the serum sample was replaced by a

primary anti-dsRNA monoclonal antibody (J2 IgG2a, English and Scientific Consulting Bt., Szivak, Hungary). The secondary Ab used was the HRP-labeled goat anti-mouse IgG2a. We have confirmed that the amount of poly(I:C) bound to the plates was correlated to the concentration of the poly(I:C) in solution.

4.3.6 Splenocyte proliferation assay

Proliferative immune response was evaluated by measuring splenocyte proliferation after *in vitro* re-stimulation. Spleens from individual mice were pooled, and single cell suspensions were prepared as previously described (Cui and Mumper, 2002). Splenocytes were cultured at a density of 4 x 10^6 cells per mL and re-stimulated with rPA83 (12.5 μg mL^{-1}), LF (10 μg mL^{-1}), or pure PGA (10, 50, or 100 μg mL^{-1}) in complete RPMI 1640 medium (Invitrogen, Carlsbad, CA) for 5 d at 37°C, 5% CO_2 (Boyaka et al., 2003; Sloat and Cui, 2006c). As a control, splenocytes were cultured without re-stimulation. The cell numbers were determined using an MTT kit (Sigma-Aldrich). The proliferation index was reported as the ratio of the final OD570 value of cells with re-stimulation over that without re-stimulation.

4.3.7 Lethal toxin neutralization activity (TNA) assay

To evaluate the functionality of the anti-PA and / or anti-LF Abs against anthrax lethal toxin, a TNA assay was performed as previously described (Sloat and Cui, 2006a). In brief, confluent J744A.1 cells (10,000) were plated in sterile, 96-well, clean-bottom plates and incubated at 37°C, 5% CO_2 for 24 h. A solution (50 μL)

containing rPA83 (400 ng mL^{-1}) and LF (100 ng mL^{-1}) was mixed with 50 µL of diluted serum samples and incubated for 2 h at 37°C. The cell culture medium was removed, and 100 µL of the serum/LeTx mixture was added into each well. After 6 more h of incubation at 37°C, 5% CO_2, the cell viability was determined using an MTT kit, with untreated and LeTx alone treated cells as controls.

4.3.8 Preparation of *B. licheniformis* spore suspension

The spore suspension of *B. licheniformis* was prepared as described elsewhere (Feijo et al., 1997). Briefly, *B. licheniformis* cultures were shaken at 37˚C, 250 rpm for 36–48 h and inoculated on LB agar plates. The plates were incubated for 5 d at 37°C to encourage sporulation. Spores from each plate were collected after the addition of 5 mL of sterile, ice-cold de-ionized water to the plate surface, followed by removal with a sterile scraper. The spore suspensions were washed 10 times successively with ice-cold de-ionized water, followed by centrifugation at 10,000 x g for 20 min. After the final wash, the spores were re-suspended in PBS (pH 7.0, 10 mM) and heated for 12 min at 80°C to kill any remaining vegetative bacteria. The spore suspension was immediately cooled in an ice-water bath and then stored at 4°C until further use.

4.3.9 Complement-mediated bacteriolysis assay

To evaluate the functionality of the anti-γDPGA Abs induced, a complement-mediated bactericidal assay was completed as described elsewhere with modifications (Chabot et al., 2004). Due to the biohazards associated with *B. anthracis*, *B.*

licheniformis was used. It has been shown that the PGAs from *B. anthracis* and *B. licheniformis* were immunologically cross-reactive (Makino et al., 1989; Mesnage et al., 1998). Also, Abs raised against the PGA from *B. licheniformis* were shown to bind to the PGA from *B. anthracis* with high affinity, and passive immunization with the Abs against *B. licheniformis* PGA protected mice against a lethal dose of anthrax spore challenge (Kozel et al., 2004). Briefly, *B. licheniformis* spores were re-suspended in LB broth and incubated at 37°C for 90 min without shaking. These freshly germinated vegetative bacterial cells were centrifuged for 5 min at 14,000 rpm, and re-suspended in Gey's buffer (PBS (pH 7.4, 10 mM), 1 mM $CaCl_2 \cdot 2H_2O$, 0.5 mM $MgCl_2 \cdot 6H_2O$, 2% BSA, and 0.1% glucose) to a concentration of ~100 CFU in 60 μL. Serum samples from individual mice were pooled, heat-inactivated (56°C, 30 min), and diluted 10-fold serially in PBS. The assay condition was consisted of 60 μL of bacillus cell suspension, 20 μL of heat-inactivated serum, and 20 μL of human complement (Sigma, diluted 1:4 in PBS). The mixture was incubated at 37°C for 1 h without shaking. Samples from each incubation mixture (50 μL) were plated on LB agar plates, and the plates were incubated for 8 h at 37°C. The number of colonies formed was determined. As controls, bacteria were incubated with human complement alone or with PBS alone before being plated onto the LB agar plates. The percent of killed bacterial cells was calculated by comparing with the number of colonies formed when the bacteria were incubated with complement alone.

4.3.10 *In vivo* LeTx challenge study

To preliminarily evaluate the extent to which nasal immunization with the mixture of rPA63, LF, and PGA-BSA conjugate admixed with poly(I:C) can protect the mice against an anthrax LeTx challenge, an *in vivo* LeTx challenge study was completed. Two weeks after the third immunization, mice were injected via the tail vein with the mixture of rPA83 (90 μg) and LF (45 μg) (i.e., 7.5 x LD_{50}) (Aulinger et al., 2005). Mice were monitored several times daily for two weeks. As controls, un-immunized mice and mice nasally immunized with rPA63 alone admixed with poly(I:C) or with LF alone admixed with poly(I:C) were also challenged similarly. At the conclusion of the two-week observational period, all surviving mice were euthanized and bled by cardiac puncture to collect serum samples. The anti-PA83 IgG titer, the anti-LF IgG titer, and the lethal toxin neutralization activity in the serum samples were determined.

4.3.11 Statistics

Statistical analyses were completed using ANOVA followed by the Fischer's protected least significant difference procedure. A p-value of ≤ 0.05 (two tail) was considered to be statistically significant. The normality of the data and equal variance between groups was checked prior to data analysis.

4.4 Results

4.4.1 The optimal nasal dose of poly(I:C) was determined to be 10 μg in mice.

To identify the optimal dose of nasal poly(I:C), mice were nasally dosed with the rPA83 admixed with various amounts of poly(I:C). As shown in Fig. 4.1A, mice nasally dosed with the rPA83 admixed with 0.1 or 1.0 μg of poly(I:C) failed to induce adequate anti-PA83 IgG Abs. However, mice nasally dosed with the rPA83 admixed with 10 μg of poly(I:C) induced a significantly higher level of anti-PA IgG Abs, when compared to untreated mice. Further increasing the dose of the poly(I:C) from 10 to 20 or 40 μg did not lead to any further increase in the resultant anti-PA83 IgG level (Fig. 4.1A). Therefore, all following experiments were completed using 10 μg of poly(I:C) per mouse. Data in Fig. 4.1B also showed that the nasal poly(I:C) did not induce any detectable level of anti-dsRNA Abs in mouse serum samples.

4.4.2 Nasal immunization of mice with the LF induced functional anti-LF Abs.

To evaluate the feasibility of inducing anti-LF Abs by nasal LF, mice were nasally dosed with various amounts of LF adjuvanted with poly(I:C). As shown in Fig. 4.2A, nasal LF induced anti-LF IgG in the serum samples. Increasing the dose of the LF tended to increase the resultant anti-LF IgG levels (LF dose *vs*. anti-LF IgG titer, $R^2 = 0.89$). Moreover, data in Fig. 4.2B showed that the anti-LF Abs have anthrax lethal toxin neutralization activity.

4.4.3 Nasal immunization with the mixture of rPA83, LF, and a γDPGA conjugate induced anti-PA, anti-LF, and anti-γDPGA Ab responses

Shown in Table 4.1 are the anti-PA83, anti-LF, and anti-γDPGA Ab titers in the serum samples when the mice were nasally immunized with the physical mixture of PA83, LF, and the γDPGA conjugate using the poly(I:C) or the CT as an adjuvant. Apparently, nasal immunization with the mixture of those three antigens induced anti-PA, anti-LF, and anti-γDPGA Abs, and the Ab responses were biased toward IgG1 (Table 4.1). The anti-LF IgG titer was relatively weaker, probably due to the low dose of the LF administered. In addition, the splenocytes isolated from the immunized mice also proliferated significantly after *in vitro* re-stimulation with rPA83 or LF (Fig. 4.3A, B), although proliferation was not detectable when re-stimulated with the γDPGA. Finally, the Abs (both anti-PA and anti-LF) induced were able to neutralize anthrax lethal toxin, and thus, protect mouse macrophages (J774A.1) against a lethal toxin challenge *in vitro* (Fig. 4.3C). Similarly, the anti-γDPGA Abs induced were also able to inhibit the growth of the γDPGA-producing freshly germinated vegetative *B. licheniformis* bacterial cells by complement-mediated bacteriolysis (Fig. 4.3D).

4.4.4 Nasal immunization with the mixture of rPA63, LF, and the γDPGA conjugate induced Ab responses against all three antigens

In this experiment, the feasibility of using the PA63, instead of the PA83, in the antigen mixture was evaluated. Moreover, to enhance the anti-LF Abs induced, the dose of the LF in the antigen mixture was increased from 5 to 20 μg per mouse. Data in Fig. 4.4A showed that nasal immunization of mice with the mixture of rPA63, LF,

and γDPGA-BSA induced anti-PA, anti-LF, and anti-PGA Abs. The specific Abs induced were comparable to that in mice s.c. injected with the antigen mixture adjuvanted with Alum (Fig. 4.4A). In addition, the levels of the serum anti-PA83 Abs induced by the nasal rPA83 and nasal rPA63 were comparable ($p = 0.07$) (Fig. 4.4A).

The Abs induced by this tri-antigen nasal vaccine candidate were able to protect all mice ($n = 4$) against a lethal dose ($7.5 \times LD_{50}$) of anthrax LeTx challenge (Table 4.2). Nasal rPA63 alone adjuvanted with poly(I:C) or LF alone adjuvanted with poly(I:C) both provided protection too (Table 4.2). However, one mouse in each of those two groups died 5 days after the challenge, and another mouse in each group showed signs of LeTx intoxication, although they slowly recovered by the end of the study. Also, one mouse that was immunized with the LF alone adjuvanted with poly(I:C) failed to regain consciousness after the injection of the LeTx. As expected, all un-immunized mice ($n = 5$) died within 36 hours after the LeTx challenge.

Finally, the levels of anti-PA83 and anti-LF Abs in the post-challenge serum samples from mice nasally immunized with the tri-antigen vaccine candidate (PA63, LF, and the γDPGA conjugate) were comparable to that in mice nasally immunized with the rPA63 alone or with the LF alone, respectively (data not shown). However, when the serum samples from the mice that survived the challenge were pooled to test their ability to neutralize anthrax lethal toxin using the *in vitro* macrophage protection assay, the serum samples from mice nasally immunized with the tri-antigen vaccine candidate increased the NC_{50} (neutralizing concentration) of the anthrax LeTx by 1.7-fold when compared to that from mice immunized with the rPA63 alone as the antigen

($p = 0.02$), and by 9-fold when compared to that from mice immunized with the LF alone as the antigen ($p = 0.001$) (Fig. 4.4B).

4.5 Discussions

The present study has 3 principal findings. (i) We identified the optimal dose of poly(I:C) as a nasal vaccine adjuvant to be 10 μg per mouse, and the poly(I:C) did not induce any detectable anti-dsRNA Abs. (ii) We showed that nasal immunization of mice with LF induced functional anti-LF Abs. (iii) We demonstrated that a prototypic tri-antigen vaccine candidate consisted of rPA63, LF, and a γDPGA conjugate induced Abs against all three antigens and protected the mice against a lethal dose of anthrax LeTx challenge.

Data from several studies have shown that anti-PA Abs alone did not provide the optimal protection against inhalation anthrax (Brossier et al., 2002; Ivins et al., 1998; Little and Knudson, 1986; Turnbull et al., 1986). It was thus thought that an efficacious anthrax vaccine should be able to rapidly induce immune responses against both virulence factors, the capsule, by which vegetative bacillus cells are encapsulated, and the anthrax toxins (Wang and Roehrl, 2005). The AVA and the new generation anthrax vaccines currently under development are primarily based on the anthrax PA protein alone. However, the level of anthrax toxin neutralizing Abs required to confer immunity to *B. anthracis* is expected to be high and may be difficult to induce and maintain if a large number of spores are inhaled (Dixon et al., 1999; Inglesby et al., 2002). Thus, the addition of anti-γDPGA Ab-mediated

opsonophagocytic killing of vegetative bacillus cells is expected to prevent or inhibit bacterial replication and augment the protection mediated by anthrax toxin neutralizing Abs. This is expected to be particularly true in the case of a post-exposure combination therapy of anthrax with a vaccine and an antibiotic. In addition, the PA serves as a carrier to facilitate the entry of LF and EF into the host cell cytosol (Bradley et al., 2001). Thus, a vaccine that can induce both anti-PA and anti-LF Abs is expected to provide a better protection than vaccines consisting of PA alone, and there were previous data that were supportive of this (Ivins and Welkos, 1988).

There have been many attempts to design an anthrax vaccine to target both the anthrax toxins and the vegetative bacilli. For example, it was reported that s.c. injection of rPA83 conjugated with a PGA peptide or protein induced both anti-PA and anti-PGA immune responses (Schneerson et al., 2003; Wang et al., 2004). Similarly, Wimer-Mackin et al. (2006) reported that nasal immunization of rabbits with a rPA83 protein-PGA peptide conjugate generated both anti-PA and anti-PGA Abs (Wimer-Mackin et al., 2006). However, no efforts were directed toward evaluating the extent to which the induction of anti-PGA Abs would further improve the resultant protective activity against anthrax spore challenges. Likewise, there have been several recent studies where both PA and LF were dosed together as antigens in an anthrax vaccine (Galloway et al., 2004; Hermanson et al., 2004; Ivins and Welkos, 1988; Price et al., 2001), and a possible synergistic effect was suggested for the resultant LeTx neutralization activity. For example, it was reported that when guinea pigs were vaccinated with PA in combination with LF, 81% of them survived an

anthrax spore challenge, whereas only 69% of guinea pigs vaccinated with PA alone as the antigen survived, indicating that vaccination with PA and LF together afforded a greater protection than with the PA alone (Ivins and Welkos, 1988). Our *in vitro* anthrax lethal toxin neutralization data shown in Fig. 4.4B were also supportive of this. Therefore, it is expected that a tri-antigen anthrax vaccine, such as the one we proposed, will provide a more robust protection against an anthrax infection, particularly when used for post-exposure prophylaxis. More experiments have to be completed in future studies to confirm this.

In the present study, we used the poly(I:C), a synthetic dsRNA, as the nasal mucosal vaccine adjuvant. Poly(I:C) was known to have adjuvant activity (Herman and Baron, 1971; Park and Baron, 1968). In the present study, the anti-PA and anti-LF Abs induced were able to protect macrophages in an *in vitro* neutralization assay (Fig. 4.2C). Additionally, the anti-γDPGA Abs induced were able to inhibit the growth of freshly germinated *B. licheniformis* bacterial cells (Fig. 4.2D). It is likely that the complement-mediated bacteriolysis assay used in the present study more accurately reflected that of a natural infection than a similar assay we previously used (Sloat and Cui, 2006a), in which the bacillus cells in their exponential proliferative growth stage were used. In the exponential proliferative stage, the bacteria also shed their PGA. It is worthy to point out that the anti-γDPGA Abs induced in this study may or may not be protective against *B. anthracis* (Chabot et al., 2004; Joyce et al., 2006; Kozel et al., 2004), and future experiments using *B. anthracis* strain need to be completed to confirm this. In a couple of recent studies, poly(I:C) was dosed intranasally to induce

anti-HIV Tat and anti-hemaglutinin immune responses (Ichinohe et al., 2005; Partidos et al., 2005). Poly(I:C) is an agonist of Toll-like receptor 3 (TLR3) and was shown to have multiple mechanisms to promote the induction of immune responses (Alexopoulou et al., 2001; Le Bon et al., 2003; Schulz et al., 2005). Nasal poly(I:C) was shown to up-regulate the expression of the TLR3 and alpha/beta interferons, as well as T helper type I (Th1)- and Th2-related cytokines (Ichinohe et al., 2005). In a preliminary pre-clinical safety study, poly(I:C) did not generate any detectable side-effects when repeatedly dosed nasally to mice at 10 μg daily for 9 consecutive days (Ichinohe et al., 2005). Additionally, intracerebral injection of poly(I:C) at a dose as high as 25 μg per mouse did not lead to any detectable toxicity in mouse brain tissues (Ichinohe et al., 2005). Finally, we have also demonstrated in the present study that poly(I:C) did not induce any detectable levels of anti-dsRNA Abs in mouse serum samples (Fig. 4.1B). All these pre-clinical data suggested that the poly(I:C) is safe when used as a nasal vaccine adjuvant. In fact, the safety profile of poly(I:C) dosed by other routes had been previously investigated in several clinical trials (Bever et al., 1986; Cornell et al., 1976; Giantonio et al., 2001; Guggenheim and Baron, 1977; Levine et al., 1979; Salazar et al., 1996). It was shown to be generally safe, although repeated high doses (e.g, i.p. injection) tended to induce some side effects, including fever and abnormal liver function (Bever et al., 1986; Cornell et al., 1976; Giantonio et al., 2001; Guggenheim and Baron, 1977; Levine et al., 1979; Robinson et al., 1976; Salazar et al., 1996). However, we speculate that when used as a vaccine adjuvant and at a relatively low dose for 2 or 3 times only, nasal poly(I:C) is unlikely to generate

severe side effects. More experiments will be carried out to evaluate the safety of nasal poly(I:C) as an adjuvant in future pre-clinical and clinical trials.

In conclusion, we have shown that a prototypic anthrax vaccine candidate that contained PA (PA83 or PA63), LF, and a γDPGA-carrier protein conjugate was able to induce strong Ab immune responses against all three antigens after only two or three intranasal doses. When fully optimized, a tri-antigen nasal vaccine similar to the present vaccine candidate is expected to represent one of the newer generation anthrax vaccines. In future studies, we will directly conjugate the γDPGA to PA and use a detoxified mutant LF to validate the efficacy of the resultant immune responses in protecting mice against an inhalational anthrax spore challenge.

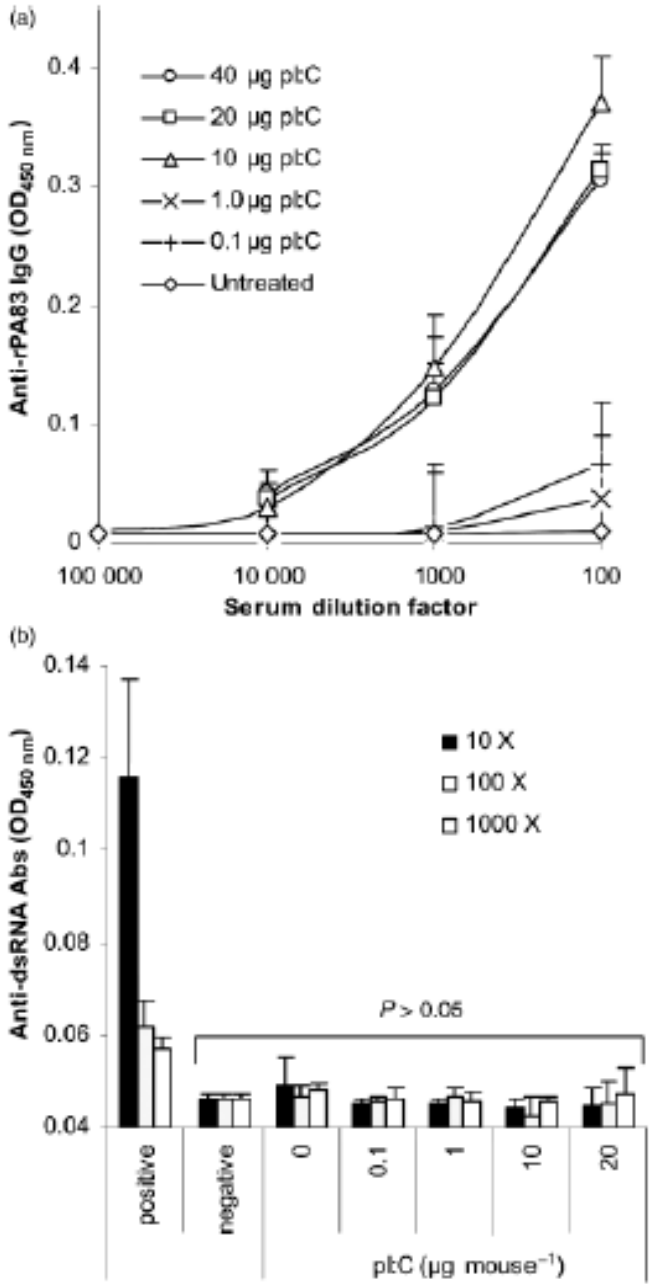

(a)
Anti-rPA83 IgG ($OD_{450\ nm}$)
0.4
0.3
0.2
0.1
0
40 µg ptC
20 µg ptC
10 µg ptC
1.0 µg ptC
0.1 µg ptC
Untreated
100 000
10 000
1000
100
Serum dilution factor
(b)
Anti-dsRNA Abs ($OD_{450\ nm}$)
0.14
0.12
0.1
0.08
0.06
0.04
10 X
100 X
1000 X
P > 0.05
positive
negative
0
0.1
1
10
20
ptC (µg mouse^{-1})

Figure 4.1

Figure 4.1 (A) The optimal dose of nasal poly(I:C) was identified to be 10 μg. Mice (n = 5) were dosed (i.n.) with rPA83 admixed with poly(I:C). Control mice were left untreated. The values were not different from one another when the poly(I:C) doses were 10, 20, and 40 μg/mouse. Similarly, the values were not different from that of the untreated control when the poly(I:C) doses were at 1.0 and 0.1 μg/mouse of poly(I:C). Nasal rPA83 alone failed to induce any detectable anti-PA Abs. (B) Nasal poly(I:C) did not induce any detectable anti-dsRNA Abs in mouse serum samples. The bracket indicated that at all three dilutions, the values for the negative control (i.e., wells not coated with dsRNA) was not different from that of serum samples from mice dosed with poly(I:C). In the positive control wells, the primary antibody used was the purified anti-dsRNA IgG2a.

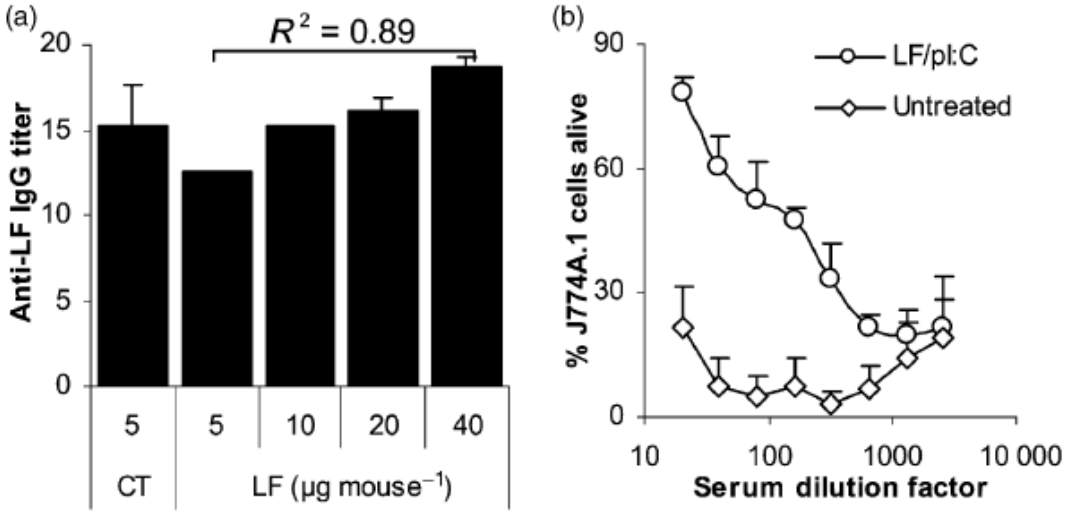


Figure 4.2 Nasal LF induced functional anti-LF Ab responses. (A) Anti-LF IgG titers (anti-$\log_2$). Linear regression analysis indicated that increasing the dose of the LF tended to increase the resultant anti-LF IgG titer ($R^2 = 0.89$). An ANOVA test resulted in a p value of 3.05×10^{-5} when the anti-LF IgG titers of the 4 difference LF doses were compared. (B) The anti-LF Abs protected macrophages (J774A.1) from a lethal toxin challenge. Data reported are mean ± S.D. (n = 5).

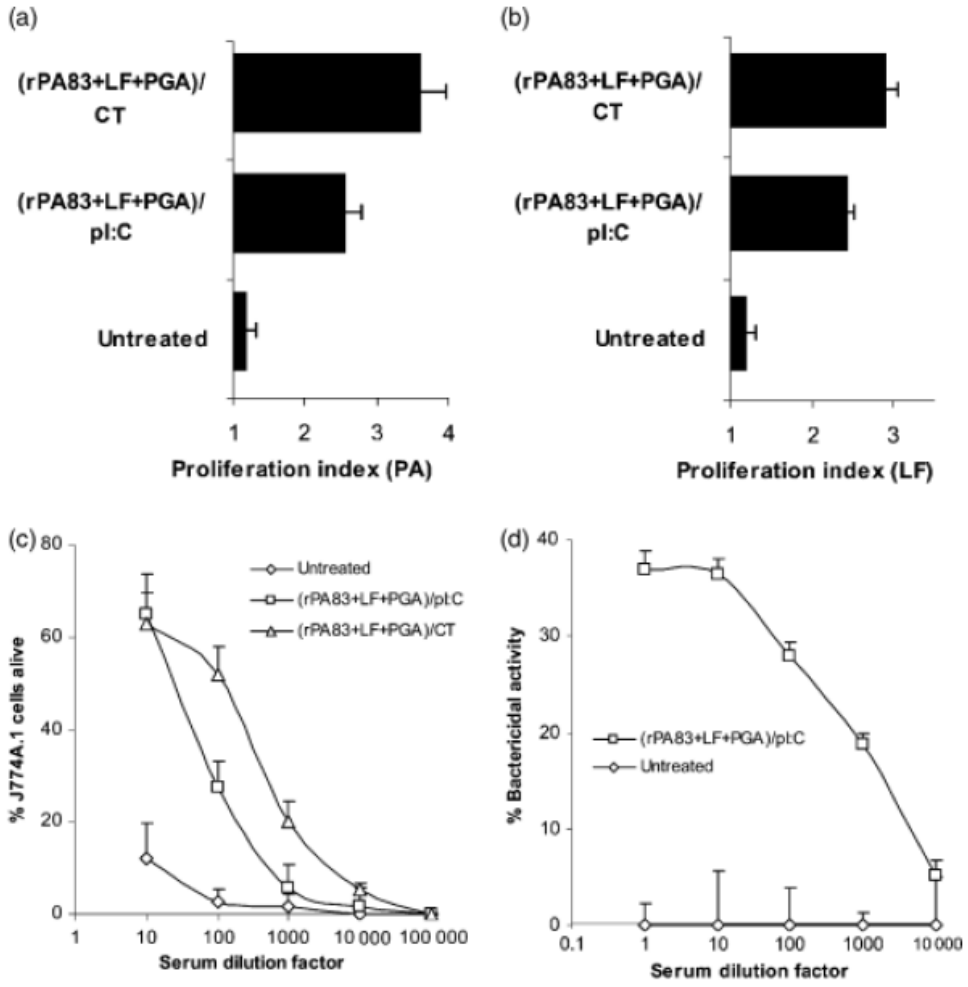

(a)
(rPA83+LF+PGA)/ CT
(rPA83+LF+PGA)/ pI:C
Untreated
1
2
3
4
Proliferation index (PA)
(b)
(rPA83+LF+PGA)/ CT
(rPA83+LF+PGA)/ pI:C
Untreated
1
2
3
Proliferation index (LF)
(c)
% J774A.1 cells alive
0
20
40
60
80
Untreated
(rPA83+LF+PGA)/pI:C
(rPA83+LF+PGA)/CT
1
10
100
1000
10 000
100 000
Serum dilution factor
(d)
% Bactericidal activity
0
10
20
30
40
(rPA83+LF+PGA)/pI:C
Untreated
0.1
1
10
100
1000
10 000
Serum dilution factor

Figure 4.3

Figure 4.3 Nasal immunization of mice with the mixture of rPA83, LF, and γDPGA-BSA adjuvanted with poly(I:C) induced anti-PA, anti-LF, and anti-γDPGA immune responses. (A) Splenocyte proliferation after *in vitro* re-stimulation with rPA. The proliferation indices of the two treated groups were significantly different from each other ($p = 0.003$) and greater than that of the untreated mice ($p = 0.001$). (B) Splenocyte proliferation after *in vitro* re-stimulation with LF. The proliferation indices of the two treated groups were significantly different from each other ($p = 0.001$) and greater than that of the untreated mice ($p = 0.001$). (C) The Abs (anti-PA and anti-LF) protected macrophage cells against anthrax lethal toxin *in vitro*. (D) The anti-γDPGA Abs had complement-mediated bacteriolytic activity against *B. licheniformis*. The activity of the untreated control was set to zero. All data reported are mean ± S.D. ($n = 3$).

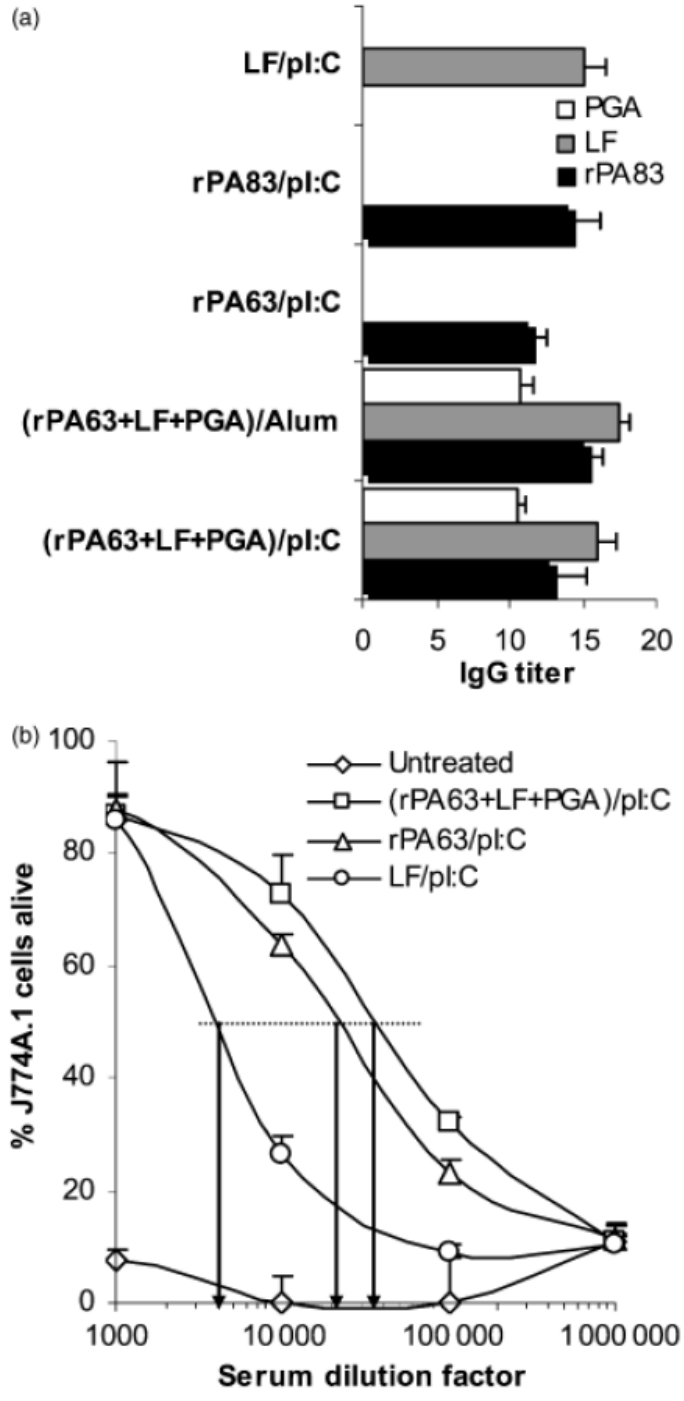

(a)
LF/pI:C
rPA83/pI:C
rPA63/pI:C
(rPA63+LF+PGA)/Alum
(rPA63+LF+PGA)/pI:C
PGA
LF
rPA83
0
5
10
15
20
IgG titer
(b)
100
80
60
40
20
0
% J774A.1 cells alive
Untreated
(rPA63+LF+PGA)/pI:C
rPA63/pI:C
LF/pI:C
1000
10 000
100 000
1 000 000
Serum dilution factor

Figure 4.4

Figure 4.4 Nasal immunization with the physical mixture of rPA63, LF, and γDPGA-BSA adjuvanted with poly(I:C) induced functional anti-PA83, anti-LF, and anti-γDPGA Abs in mouse serum samples. (A) The anti-PA83, anti-LF, and anti-γDPGA Ab titers did not differ among groups ($p = 0.11$, 0.25, and 0.56 for PA, LF, and PGA, respectively). Data reported are mean ± S.D. ($n = 5$). (B) The serum samples from mice immunized with the tri-antigen vaccine candidate more effectively protected macrophages against an anthrax lethal toxin challenge. The serum samples from mice that survived the anthrax lethal toxin challenge were collected and pooled. Arrows indicate the NC_{50} for each treatment. Data reported are the mean ± S.D. ($n = 3$).

Table 4.1 Anti-PA83, anti-LF, and anti-PGA Ab titers in mouse serum samples. Titers are in anti-$\log_2$ (mean ± S.D., n = 5).

	Vaccine formulations	
	(rPA83+LF+PGA)/pI:C, i.n.	**(rPA83+LF+PGA)/CT, i.n.**
Anti-PA IgG	17.3 ± 1.2	16.7 ± 1.5
Anti-PA IgG1	17.5 ± 1.8	17.7 ± 1.3
Anti-PA IgG2a	14.0 ± 2.3	15.1 ± 1.3
IgG1/IgG2a	11	6
Anti-PA IgA	9.4 ± 0.8	8.6 ± 3.0
Anti-PA IgM	4.6 ± 3.0	7.7 ± 4.1
Anti-LF IgG	10.2 ± 0.6	15.2 ± 3.8
Anti-LF IgG1	10.4 ± 1.1	13.7 ± 4.0
Anti-LF IgG2a	4.6 ± 2.8	11.4 ± 0.5
IgG1/IgG2a	56	5
Anti-PGA IgG	13.4 ± 1.1	12.4 ± 0.6
Anti-PGA IgG1	13.8 ± 1.6	12.4 ± 0.9
Anti-PGA IgG2a	9.9 ± 1.9	8.6 ± 1.8
IgG1/IgG2a	13	15
Anti-PGA IgA	7.2 ± 2.2	4.8 ± 2.1
Anti-PGA IgM	11.1 ± 2.5	8.2 ± 2.1

Table 4.2 *In vivo* anthrax lethal toxin challenge study.

	Untreated	**(rPA63+LF+PGA)/pI:C i.n.**	**rPA63/pI:C i.n.**	**LF/pI:C i.n.**
# of mice died	5/5	0/4	1/4	2/4 [a]
# of mice with signs of LeTx intoxication [b]	5/5	0/4	2/4	2/3

a. One mouse in this group failed to regain consciousness after the injection of the lethal toxin.
b. Severe signs of intoxication included body and facial swelling, un-responsiveness, inactiveness, and refusal of food and water.

Chapter 5

STRONG ANTIBODY RESPONSES INDUCED BY PROTEIN ANTIGENS CONJUGATED ONTO THE SURFACE OF LECITIN-BASED NANOPARTICLES

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and Zhengrong Cui

Journal of Controlled Release, 2010; 141: 93 - 100

5.1 Abstract

An accumulation of research over the years has demonstrated the utility of nanoparticles as antigen carriers with adjuvant activity. Herein we defined the adjuvanticity of a novel lecithin-based nanoparticle engineered from emulsions. The nanoparticles were spheres of around 200 nm. Model protein antigens, bovine serum albumin (BSA) or *Bacillus anthracis* protective antigen (PA) protein, were covalently conjugated onto the nanoparticles. Mice immunized with the BSA-conjugated nanoparticles developed strong anti-BSA antibody responses comparable to that induced by BSA adjuvanted with incomplete Freund's adjuvant and 6.5-fold stronger than that induced by BSA adsorbed onto aluminum hydroxide. Immunization of mice with the PA-conjugated nanoparticles elicited a quick, strong, and durable anti-PA antibody response that afforded protection of the mice against a lethal dose of anthrax lethal toxin challenge. The potent adjuvanticity of the nanoparticles was likely due to their ability to move the antigens into local draining lymph nodes, to enhance the uptake of the antigens by antigen-presenting cells (APCs), and to activate APCs. This novel nanoparticle system has the potential to serve as a universal protein-based vaccine carrier capable of inducing strong immune responses.

5.1 Introduction

Recombinant protein antigens may help overcome the toxicity concerns associated with many of the traditional vaccines prepared with live, attenuated or killed pathogens. However, recombinant proteins are often weakly immunogenic or

non-immunogenic on their own, and a vaccine adjuvant is usually needed to enhance the resultant immune responses. Aluminum adjuvant, such as the aluminum hydroxide (Alum), remains to be the only adjuvant approved for human use in the U.S. It forms a precipitate when combined with soluble antigen, and the slow release of the antigen from the precipitate at the injection site causes prolonged, strong antibody responses (Baylor et al., 2002; Lindblad, 2004). However, Alum is a relatively weak adjuvant and has various limitations (Baylor et al., 2002). Therefore, there is a critical need to search for or to devise alternative vaccine adjuvants to improve the immune responses induced by recombinant protein antigens.

In recent years, particles of nanometer or micrometer scales are increasingly used as antigen carriers, and it is generally accepted that microparticles and nanoparticles have adjuvant activity (Cui and Mumper, 2003; Peek et al., 2008; Singh et al., 2007), which is likely due to the easiness for antigen-presenting cells (APCs) to take up antigens associated with the particles, as compared to free antigens in solution. In theory, one expects that nanoparticles have a more potent adjuvant activity than microparticles. It was reported that particles with a diameter of 500 nm or less were optimal for uptake by APCs such as dendritic cells (DCs) and macrophages (Foged et al., 2005; Kanchan and Panda, 2007). In addition, data from a recent study showed that small nanoparticles (20-200 nm) can freely drain to the lymph nodes (LNs) for antigen presentation, whereas DCs were required for the transport of large microparticles (0.5-2 μm) from the injection site to the LNs (Manolova et al., 2008). Therefore, it is likely that antigens associated with nanoparticles can reach the

draining LNs for antigen presentation not only by the trafficking of APCs, but also by direct draining, which is expected to lead to a strong immune response. However, recent data from studies aimed at correlating the particle size of the antigen carriers and the resultant immune responses are rather controversial. For example, Wendorf et al. (2008) reported that comparable immune responses were induced in mice by protein antigens (Env from HIV-1 and MenB from *Neisseria meningitides*) adsorbed onto anionic microparticles (~1 μm) and nanoparticles (110 nm) prepared with poly (lactic-co-glycolic acid) (PLGA) polymers (Wendorf et al., 2008). However, data from a study by Gutierro et al. (2002) showed that when BSA was entrapped in PLGA particles of different sizes (200 nm, 500 nm, and 1 μm), the 1 μm microparticles generally induced a stronger serum anti-BSA IgG response than the 200 nm or 500 nm nanoparticles (Gutierro et al., 2002). Similarly, Kanchan and Panda (2007) reported that the HBsAg entrapped in PLGA particles of 200-600 nm induced a weaker anti-HBsAg antibody response than HBsAg entrapped in PLGA particles of 2-8 μm, when injected intramuscularly into rats. On the contrary, data from many other studies showed that the adjuvanticity of smaller nanoparticles was more potent than that of the larger microparticles using protein antigens or plasmid DNA (Fifis et al., 2004; Kalkanidis et al., 2006; Minigo et al., 2007; Olbrich et al., 2002; Singh et al., 2000). More interestingly, Fifis et al. (2004) reported that the optimal carrier particle size was in the range of 40-50 nm when they compared the immune responses, both antibody and cellular, induced by polystyrene particles ranging from 20 nm to 2 μm with antigens conjugated on their surface (Fifis et al., 2004). However, the 40-50 nm was

the size of the polystyrene particles before the protein antigen was conjugated onto their surface. When the OVA as an antigen was conjugated onto the 49 nm particles, the size of the resultant particles was reported to be 232 nm (Kalkanidis et al., 2006), which was more relevant. It is unclear why the conjugation of the OVA onto the 49 nm polystyrene particles quadrupled the diameter of the resultant particles. Nevertheless, it demonstrated that particles with a diameter of around 200 nm are ideal antigen carriers when the antigens are on the surface of the particles. Finally, it is becoming evident that the size of the particles may have different effects on the antigen-specific antibody or cellular immune responses(Caputo et al., 2009; Kanchan and Panda, 2007; Mann et al., 2009). It seemed that microparticles promote humoral response where as nanoparticles (< 1 μm) promote cellular response (Kanchan and Panda, 2007). For example, Caputo et al. (2009) showed that HIV TAT protein adsorbed on cationic polymeric nanoparticles (220 nm or 630 nm) tended to induce a stronger cellular immune response and a weaker antibody response than the same protein adsorbed on large microparticles (~ 2 μm) made of the same materials (Caputo et al., 2009). To make it more interesting, data from Mann et al.'s recent study using oral bilosomes (bile salt in lipid vesicles) with influenza A antigens showed that the larger bilosomes (400-2000 nm) generated an immune response that had a significantly greater Th1 bias than the smaller bilosomes (10-100 nm) (Mann et al., 2009).

Previously, we reported the engineering of 100-200 nm nanoparticles using lecithin as the matrix material (Cui et al., 2006). Lecithins are components of cell

membranes and are regularly consumed as part of a normal diet. They are used extensively in pharmaceutical applications as emulsifying, dispersing, and stabilizing agents and are included in intramuscular and intravenous injectables and parenteral nutrition formulations (Williams et al., 1984). In the present study, we modified the lecithin nanoparticles by including glyceryl monostearate (GMS) in the matrix and replacing the previous cationic surfactant with a non-ionic surfactant. We then evaluated the adjuvanticity of the modified nanoparticles in a mouse model using two model antigens, BSA or the *Bacillus anthracis* protective antigen protein. The antigens were chemically conjugated onto the surface of the nanoparticles because it was shown that when an antigen was covalently coupled to nanoparticles, it induced a stronger immune response than when it was simply adsorbed to the nanoparticles (Fifis et al., 2004). The anthrax PA protein was the functional antigen used in this study. Anthrax is a toxin-caused disease. There is no evidence that a cellular immune response is needed to control the anthrax toxins (Wang and Roehrl, 2005). Therefore, we only focused on the evaluation of the antibody responses.

5.3 Materials and Methods

5.3.1 Engineering of nanoparticles from emulsions

Nanoparticles were prepared from emulsions. Briefly, soy lecithin (3.5 mg, Alfa Aesar, Ward Hill, MA) and GMS (0.5 mg, Gattefosse Corp., Paramus, NJ) were weighed into a 7-ml glass scintillation vial. One ml of de-ionized and filtered (0.2 μm) water was added into the vial, followed by heating on a hot plate to 70-75ºC with

stirring and brief intermittent periods of sonication (Ultrasonic Cleaner Model 150T, VWR International, West Chester, PA). Upon formation of a homogenous milky slurry, Tween 20 was added in a step-wise manner to a final concentration of 0-1.2% (v/v). The resultant emulsions were cooled to room temperature while stirring to form nanoparticles. The particle size was determined using a Coulter N4 Plus Submicron Particle Sizer (Beckman Coulter Inc., Fullerton, CA).

The conjugation of proteins (BSA from Sigma-Aldrich or PA from BEI resources, Manassas, VA) to the nanoparticles was completed as previously described (Cui et al., 2005b). A reactive maleimide group was incorporated into the nanoparticles by adding 5% (w/w) of 1, 2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-[4-(p-maleimidophyl)butyramide] (Avanti Polar Lipids, Alabaster, AL) to form m-nanoparticles (m-NPs). The proteins were thiolated using 2-iminothiolane (Traut's reagent, Sigma-Aldrich). Briefly, the proteins were diluted into PBS (0.1 M with 3.0 mM EDTA, pH 8), followed by the addition of Traut's reagent (20 x molar excess). After 60 min of incubation at room temperature, the proteins were purified using a PD10 column (Amersham Biosciences, Bellefonte, PA). To react the thiolated proteins with the m-NP, 1 ml of freshly prepared m-NPs were mixed with the thiolated proteins (1 mg BSA or 0.25 mg PA) in PBS (0.1 M, pH 7.4). The pH was adjusted to 6.5, and the reaction mixture was stirred under N_2 gas for 12-14 h at room temperature. Unconjugated proteins were separated from the nanoparticles by gel permeation chromatography (GPC, Sepharose 4B, 6 x 150 mm) or ultracentrifugation (1.0×10^5 *g*). In the GPC, the eluted fractions were analyzed for

nanoparticles by measuring the absorption at 269 nm, and the protein content in every fraction was determined using a Bradford's reagent (BioRad, Hercules, CA). As an additional method to confirm the conjugation of the proteins to the nanoparticles, the fraction containing the BSA-conjugated nanoparticles (BSA-NPs) was diluted 100-fold in carbonate buffer and used as the capture antigen to coat the plates in an enzyme-linked immunosorbent assay (ELISA). The primary antibody was mouse anti-BSA anti-serum.

5.3.2 Transmission electron micrography (TEM)

The size and morphology of the nanoparticles were examined using transmission electron microscopy (TEM, Philips CM12 TEM/STEM) in the Oregon State University Electron Microscope Facility (Cui and Mumper, 2002).

5.3.3 Stability of nanoparticles in simulated biological media

To evaluate the stability of the nanoparticles in simulated biological media, the nanoparticle suspension was diluted in normal saline (0.9% NaCl) or 10% FBS (v/v) in normal saline and incubated at 37ºC for 30 min. The particle sizes at 0 and 30 min were measured.

5.3.4. In vitro uptake of nanoparticles by DC2.4 cells

Nanoparticles were conjugated with BSA that was pre-labeled with fluorescein-5(6)-isothiocyanate (FITC, Sigma-Aldrich). Thiolated BSA was labeled

with FITC following the manufacturer's instruction (Promega). DC2.4 cells (1.0 x 10^6 cells/well), a mouse dendritic cell line (Shen et al., 1997), were seeded into 24-well plates and allowed to grow overnight. The cells were then incubated with 50 µl of the FITC-BSA-NPs for 6 h at 37ºC under 5% CO_2 or 4ºC. The cells were then washed three times with PBS (10 mM, pH 7.4), lysed with 0.5% SDS (w/v), and analyzed for fluorescence intensity using a BioTek Synergy HT Multi-Detection Microplate Reader (BioTek Instruments, Inc. Winooski, VT). Fluorescence intensity was normalized using FITC-BSA as a control. Data were presented as the fold of fluorescence intensity increase (FITC-BSA-NP over FITC-BSA). Alternatively, the nanoparticles were labeled with FITC directly by incorporating 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-carboxyfluorescein (Avanti Polar Lipids) (5% w/w) into the nanoparticles prior to the conjugation of the BSA to generate BSA-NPs-FITC, which were used in the following fluorescence microscopic study and *in vivo* distribution experiments.

To microscopically evaluate the uptake of the nanoparticles, DC2.4 cells (2 x 10^4) were plated on poly-D-lysine-coated glass coverslips for 24 h. Cells were incubated with BSA-NPs-FITC and maintained at 4°C or 37°C for 6 h. Cells were then washed with PBS (10 mM, pH 7.4), fixed in 3% paraformaldehyde for 20 min, and washed three additional times prior to mounting on slides with Fluoromount G® (SouthernBiotech, Birmingham, AL). Bright-field and fluorescent images were obtained using a Zeiss AutoImager Z1 microscope (Carl Zeiss, Thornwood, NY) with a Zeiss 20× objective.

5.3.5 In vivo distribution and uptake of the nanoparticles in LNs

All mouse studies were carried out following NIH guidelines for animal use and care. BALB/c mice (female, 6 -8 weeks) were from Simonsen Laboratories Inc. (Gilroy, CA). Twenty-five µl of BSA-NPs-FITC or FITC-labeled nanoparticles without BSA (FITC-NPs) in suspension were subcutaneously (s.c.) injected into the footpads of the hind legs of the mice (n = 3). The lipopolysaccharide (LPS) from *E. coli* (Sigma-Aldrich, 100 ng per footpad) was used as a positive control. Mice in the negative control group were injected with sterile PBS (10 mM, pH 7.4) or left untreated. Mice were euthanized 23-24 h later, and their popliteal (and inguinal) LNs were removed and pooled to prepare single cell suspension (Cui et al., 2005a). To examine the activation of DCs, the cells from the popliteal LNs were stained with antibodies against CD11c and CD86 (BD Pharmingen, San Diego, CA) (Cui et al., 2005a) and analyzed using a flow cytometer (FC500 Beckman Coulter EPICS V Dual Laser Flow Cytometer, Fullerton, CA).

5.3.6 Immunization Studies

The vaccine formulations were administered to mice by s.c. injection. To quantify the amount of antigen proteins on the nanoparticles, the antigens (BSA or PA) were labeled with FITC before being conjugated onto the nanoparticles, and the fluorescence intensity was measured using a fluorescence spectrometer. We have repeated this method multiple times to make sure that the same amount of protein antigens were consistently conjugated to a fixed amount of nanoparticles. In the

control groups, the dose of the aluminum hydroxide (Alum) was 50 µg/mouse, and the dose of the incomplete Freund's adjuvant (IFA) was 100 µl/mouse (mixed with BSA in PBS at a ratio of 1:2). Except where mentioned, mice were dosed on days 0, 14, and 28 and euthanized on day 42. To measure the durability of the antibody response, mice were bled via the craniofacial vein. ELISA, splenocyte proliferation assay, and the *in vitro* and *in vivo* lethal toxin neutralization assays were all completed as previously described (Sloat and Cui, 2006). For the ELISA, EIA/RIA flat bottom, medium binding, polystyrene, 96-well plate (Corning Costar, Corning, NY) were coated with 100 ng of PA in 100 µl of carbonate buffer (0.1 M, pH 9.6) or BSA in PBS (10 mM, pH 7.4) overnight at 4 ◦C. For anti-PA Ab measurement, plates were washed with PBS/Tween 20 (10 mM, pH 7.4, 0.05% Tween 20, Sigma–Aldrich) and blocked with 4% (w/v) BSA in PBS/Tween 20 for 1 h at 37 ◦C. Samples were diluted two-fold serially in 4% BSA/PBS/Tween 20, added to the plates following the removal of the blocking solution, and incubated for an additional 3 h at 37 ◦C. The serum samples were removed, and the plates were washed five times with PBS/Tween 20. Horse radish peroxidase (HRP)-labeled goat anti-mouse immunoglobulin (IgG, 5000-fold dilution in 1% BSA/PBS/Tween 20, Southern Biotechnology Associates Inc., Birmingham, AL) was added into the plates, followed by another hour of incubation at 37 ◦C. Plates were again washed five times with PBS/Tween 20. The presence of bound Ab was detected following a 30 min incubation at room temperature in the presence of 3,3',5,5'-tetramethylbenzidine solution (TMB, Sigma–Aldrich), followed by the addition of 0.2 M sulfuric acid as the stop solution. The

absorbance was read at 450 nm. Anti-BSA Abs were determined similarly; except that the 4% BSA in PBS/Tween 20 was replaced by 5% (v/v) horse serum (Sigma–Aldrich) in PBS/Tween 20 in the plate blocking and sample dilution steps. In addition, the secondary Abs were diluted in 1.25% (v/v) horse serum in PBS/Tween 20. The antibody titers were determined by considering any OD value higher than the mean plus two times of the standard deviation (mean + 2 x S.D.) of the negative control group as positive. In the splenocyte proliferation assay, the splenocytes were stimulated with 12.5 μg/ml of PA protein(Sloat and Cui, 2006). In the *in vivo* lethal toxin neutralization assay, the dose of the anthrax lethal toxin was 90 μg of PA83 and 45 μg of LF per mouse (i.e., 7.5 x LD_{50}). Finally, mice used in the *in vivo* challenge experiment were different from those used to collect serum samples for the *in vitro* neutralization assay.

5.3.7 Statistics

Statistical analyses were completed using ANOVA followed by the Fischer's protected least significant difference procedure. Standard linear regression analysis was used to analyze the PA dose response data. A p-value of ≤ 0.05 (two-tail) was considered statistically significant.

5.4 Results

5.4.1 The lecithin-based nanoparticles were spherical and stable in simulated biological media

The nanoparticles were engineered from emulsions, consisting of 3.5 mg/ml lecithin and 0.5 mg/ml GMS. As shown in Fig. 5.1A, at least 0.4% of Tween 20 was required to emulsify the lecithin and GMS. Increasing the concentration of the Tween 20 resulted in smaller nanoparticles and a decrease in the turbidity of the nanoparticle suspensions. When the Tween 20 was increased to 1%, nanoparticles of 178 ± 20 nm were generated, and further increasing the concentration of the Tween 20 did not lead to a further decrease in the resultant particle size and the turbidity of the particle suspension. Thus, nanoparticles prepared with a final Tween 20 concentration of 1% were used for further studies. Shown in Fig. 5.1B is a typical TEM micrograph of the unpurified nanoparticles. The nanoparticles were spherical (Fig. 1B and inset). Finally, the nanoparticles did not aggregate during a 30 min incubation period at 37°C in normal saline or 10% FBS in normal saline (Fig. 5.1C).

5.4.2 BSA was conjugated onto the surface of the nanoparticles.

Two approaches were used to confirm that the BSA was conjugated onto the surface of the nanoparticles, GPC and ELISA. Data in Fig. 5.2A showed that the GPC column can be used to separate the nanoparticles from free BSA protein. Data in Figs. 5.2B and C demonstrated the conjugation of BSA onto the nanoparticles. After the BSA was reacted with the nanoparticles, the particle peak and the BSA peak overlapped at fraction 7. However, when the nanoparticles and BSA were simply physically mixed for the same amount of time as the conjugation reaction, the particle peak and the BSA peak did not overlap. In the ELISA, BSA was only detected when

the fraction 7 from the reaction product of the nanoparticles and BSA, not when the fraction 7 from the nanoparticles alone or from the physical mixture of the nanoparticles and BSA was used to coat the plates (Fig. 5.2D), demonstrating the conjugation of the BSA onto the nanoparticles. The size of the resultant BSA-conjugated nanoparticles was 201 ± 5 nm.

5.4.3 The nanoparticles facilitated the uptake of the antigen by APCs

To identify the extent to which the nanoparticles can facilitate the uptake of antigen by APCs, FITC-BSA-NPs were incubated with DC2.4 cells. As shown in Fig. 5.3A, the DC2.4 cells took up significantly more BSA when the BSA was conjugated onto the nanoparticles than free BSA ($p < 0.001$). Moreover, the fluorescence intensity ratio was significantly stronger at 37°C than at 4°C (Fig. 5.3A), indicating that the nanoparticles were internalized by the DC2.4 cells, not simply bound to their surface, which was further confirmed by the micrographs shown in Fig. 5.3B. Strong fluorescence signal was detected only in DC2.4 cells incubated with the FITC-labeled nanoparticles at 37^{o}C, but not in the cells incubated at 4^{o}C.

5.4.4 The trafficking of BSA-NPs into the LNs and the activation of APCs

Twenty-four h after the injection of the BSA-NPs-FITC in the hind footpads, the FITC was detectable in the popliteal and inguinal LNs (Fig. 5.4A), more in the local popliteal LNs than in the distal inguinal LNs (~3.7% vs. 1.9%). Close to 15% of the $CD11c^{+}$ cells in the popliteal LNs were found to be FITC positive or have taken up the FITC-labeled BSA-NPs (Fig. 4B, 100 x 0.43/(0.43+2.52)). Similarly, roughly 15%

of the popliteal LN cells that took up the BSA-NPs ($FITC^+$) were $CD11c^+$ (Fig. 5.4B, 100 x 0.43/(0.43+2.43)). Finally, the nanoparticles increased the percent of CD11c and CD86 double positive cells in the popliteal LNs. Over 1.67% of the popliteal LN cells were $CD11c^+$ and $CD86^+$ 23 h after the injection of the nanoparticles, as compared to 2.04% in mice injected with the *E. coli* LPS (100 ng/footpad) (Fig. 5.4C).

5.4.5 Immunization of mice with the BSA-NPs induced strong anti-BSA Ab responses

Data in Table 5.1 showed that the BSA-specific IgG and IgM titers induced by the BSA-NPs were comparable to that induced when the IFA was used as an adjuvant ($p = 0.88$), but significantly higher than when the Alum was used as an adjuvant ($p < 0.001$). The BSA-NPs, BSA/IFA, and BSA/Alum induced comparable levels of anti-BSA IgG1, but the anti-BSA IgG2a and IgG3 induced by the BSA/Alum was significantly weaker than that induced by the BSA-NPs and the BSA/IFA (Table 5.1).

5.4.6 Immunization of mice with PA conjugated onto the nanoparticles induced functional anti-PA antibody responses

The diameter of the PA-conjugated nanoparticles (PA-NPs) was 197 ± 6 nm. Shown in Fig. 5.5A is the effect of the dose of the PA protein on the resultant anti-PA IgG titers in the serum samples of mice immunized with the PA-NPs. Within the dose range of 1 to 10 μg/mouse, increasing the dose of the PA led to increased anti-PA IgG ($p < 0.001$ from linear regression) (Fig. 5.5A). Moreover, the anti-PA IgG titers induced by the PA-NPs at the dose of 5 μg PA were comparable to that induced by 5

μg PA adjuvanted with Alum. Seven days after the first dose at 5 μg of PA per mouse, anti-PA IgG was detectable in mice immunized with the PA-NPs, but not in mice dosed with the PA adjuvanted with Alum (Fig. 5.5B) ($p < 0.001$). However, after the second dose, the anti-PA antibody titers in both groups became comparable (Fig. 5.5B). The splenocytes isolated from mice immunized with the PA-NPs proliferated when re-stimulated with the PA protein (Fig. 5.5C). Finally, the anti-PA antibodies induced by the PA-NPs were able to neutralize anthrax lethal toxin and protect mouse macrophages (J774A.1) from a lethal toxin challenge *in vitro* (Fig. 5.5D). Mice immunized with the PA-NPs survived a lethal dose (7.5 x LD_{50}) of anthrax LeTx challenge, and the lethal toxin challenge itself significantly enhanced the anti-PA IgG titers in the surviving mice (Table 5.2).

5.5 Discussions

Our data in the present study showed that protein antigens conjugated onto the surface of nanoparticles prepared with lecithin and GMS induced quick, strong and long-lasting antibody immune responses when subcutaneously injected into mice. Depending on the antigens used, the adjuvanticity of the nanoparticles was stronger than that of the Alum and was comparable to that of the IFA. Moreover, the antibody responses developed more quickly when the antigen was adjuvanted using the nanoparticles than using Alum. The potent adjuvanticity of the nanoparticles was likely due to the nanoparticles' ability to enhance the uptake of the antigens by APCs, to move the antigens into local draining LNs, and to activate APCs.

BSA (~66 KDa) is a serum protein with normal immunogenicity. At the beginning, we used it as an antigen to confirm the conjugation of the protein antigens onto the surface of the nanoparticles and to evaluate the adjuvanticity of the nanoparticles. Our data in Fig. 5.2 clearly showed that successful conjugation of the BSA onto the nanoparticles and that unconjugated BSA can be separated from the nanoparticles by GPC. Importantly, the BSA conjugated onto the nanoparticles generated anti-BSA antibody titers 5-6.5-fold higher than those induced when the BSA was adjuvanted using the Alum (Table 5.1, IgG and IgM). In fact, data in Table 5.1 showed that the nanoparticles have similar adjuvanticity as the IFA, a well known strong adjuvant (Chang et al., 1998). The nanoparticles appeared to have adjuvant properties that favor humoral immunity, although less biased than the Alum. If a more balanced antibody response or an increased cell-mediated immune response is needed, one may have the option of including a co-adjuvant such as the low toxicity LPS (Goldstein et al., 1992) or the CpG oligos into the nanoparticle formulation.

In order to evaluate the functionality of the immune response induced by antigens delivered using the nanoparticles, we used the *B. anthracis* PA protein. The toxicity of the *B. anthracis* toxin is mainly from the lethal factor and the edema factor. PA is needed for the natural entrance of the LF and EF into the host cells (Bradley et al., 2001). Thus, antibodies that can neutralize the PA in serum are expected to block the transport of LF and EF into the cytosol and prevent the course of *B. anthracis* infection. Similar to the BSA-NPs, the PA-NPs were able to induce a strong anti-PA IgG response, and increasing the dose of the PA tended to increase the resultant anti-

PA IgG titer (Fig. 5.5A). Slightly different from the BSA-NPs, at the dose of 5 μg/mouse, the PA-NPs and the PA admixed with Alum induced comparable anti-PA IgG titers (Fig. 5.5A). This may be due to that unlike the BSA, the PA protein is strongly immunogenic in inducing anti-PA antibody responses (Kenney et al., 2004) and extremely foreign to the mice used in this study. In our splenocyte proliferation assay, we found a proliferation index of 2.5. The relatively small proliferation index may be due to reasons that the mice were dosed with only 1 μg of the PA protein and that the index was derived from the whole splenocytes, not the purified T cells. Shown in Fig. 5.5B are the kinetics of the anti-PA IgG responses induced by PA conjugated onto the nanoparticles. Interestingly, mice immunized with the PA-NPs developed detectable anti-PA IgG following a single immunization (Fig. 5.5B). However, mice immunized with the PA admixed with Alum failed to respond to the priming, and anti-PA IgG was detectable in those mice only after a boost immunization (Fig. 5.5B). The ability of an anthrax vaccine to induce an immune response immediately after the first immunization is likely critical in the case of post-exposure combination therapy of anthrax with a vaccine and an antibiotic. Finally, the anti-PA antibodies induced by PA-NPs were found to be functional. Serum from mice immunized with the PA-NPs was able to neutralize anthrax lethal toxin and protect mouse macrophages in culture (Fig. 5.5D). Moreover, mice immunized with the PA-NPs also survived a lethal dose of anthrax lethal toxin challenge (Table 5.2).

The ability of the nanoparticles to enhance the immune responses induced by antigens conjugated on their surface was likely due to their ability to enhance the

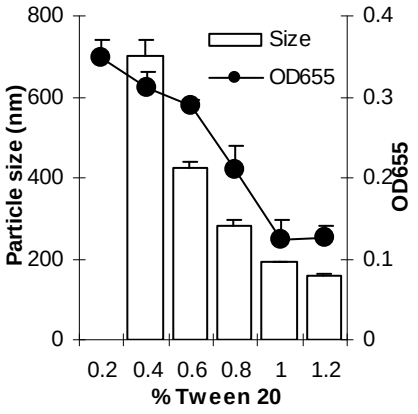
trafficking of the antigens into local draining LNs, to improve the uptake of antigens by APCs, and to activate the APCs. For example, our data in Fig. 5.3 clearly showed that nanoparticles facilitated the uptake of the BSA antigen conjugated on their surface, likely because the DCs were more effectively taking up particulates than the BSA protein in solution. Data from previous studies showed that particles with a diameter of 500 nm or below were optimal for uptake by APCs such as DCs and macrophages (Foged et al., 2005; Kanchan and Panda, 2007). Our BSA-NPs was around 200 nm, which is within the optimal size range for the uptake by APCs. Moreover, our data in Fig. 4A showed that close to 4% of the cells in local draining popliteal LNs became $FITC^+$ at 24 h after the BSA-NPs labeled with FITC were injected into the footpad of the hind legs, suggesting that the FITC-labeled nanoparticles were present in ~4% of the LN cells. It is unclear to what extent those particles moved into the LNs by direct draining or by the trafficking of the DCs. However, only about 15% of the $FITC^+$ cells in the popliteal LNs were $CD11c^+$, suggesting that the direct draining of the nanoparticles into the local draining LNs may have been extensive. Nevertheless, Manolova et al. (2008) reported that particles of 20-200 nm drained freely to LNs (Manolova et al., 2008). Finally, our data indicated that the nanoparticles also activated APCs in the draining LNs because an increased percent of the cells in popliteal LNs became $CD11c^+$ and $CD86^+$ after the subcutaneous injection of the nanoparticles (Fig. 5.3C).

Finally, our nanoparticles were engineered with lecithin and GMS as the matrix materials and Tween 20 as an emulsifying agent. Lecithin is a complex mixture

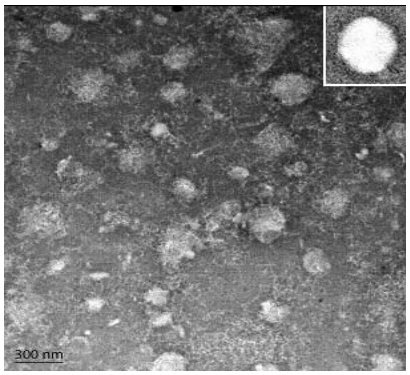
of phosphatides consisting chiefly of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol and other substances such as triglycerides and fatty acids. It is GRAS (generally regarded as safe) listed and accepted in the FDA Inactive Ingredients Guide for parenterals (e.g., 0.3-2.3% for intramuscular injection)(Wade A, 1994). Tween 20 is a polyoxyethylene derivative of sorbitan monolaurate. It is also GRAS listed and included in the FDA Inactive Ingredients Guide for parenterals (Wade A, 1994). GMS is used in a variety of food, pharmaceutical, and cosmetic applications and is also GRAS listed. Its LD_{50} in mouse was reported to be 0.2 g/kg by the intraperitoneal route (Wade A, 1994). Therefore, although more experiments will have to be carried out in the future to define the safety profile of the nanoparticles, we consider this nanoparticle-based antigen delivery system relatively safe.

We engineered a lecithin-based nanoparticle as a promising antigen delivery system. Immunization of mice with antigens conjugated on the surface of the nanoparticles induced a quick, strong, durable, and functional immune response. The adjuvant activity of the nanoparticles was likely due to their ability to facilitate the uptake of the antigens by APCs, to improve the trafficking of the antigens into local draining LNs, and to activate APCs.

(A)



(B)



(C)

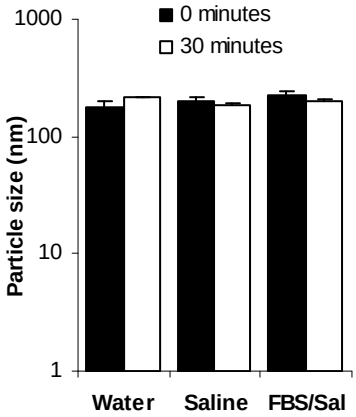


Figure 5.1

Figure 5.1 Lecithin-based nanoparticles were spherical and stable in simulated biological media. (A) The effect of the concentration of the Tween 20 on the size of the resultant nanoparticles (white bars) and the turbidity (OD655) of nanoparticle suspension (filled circles). (B) TEM of the nanoparticles. Inset is to show the spherical nature of the nanoparticles. (C) The nanoparticles did not aggregate in normal saline or 10% FBS in normal saline. Particle sizes were measured immediately and following a 30-min incubation period at 37ºC. Data reported are mean ± S.D. (n = 3).

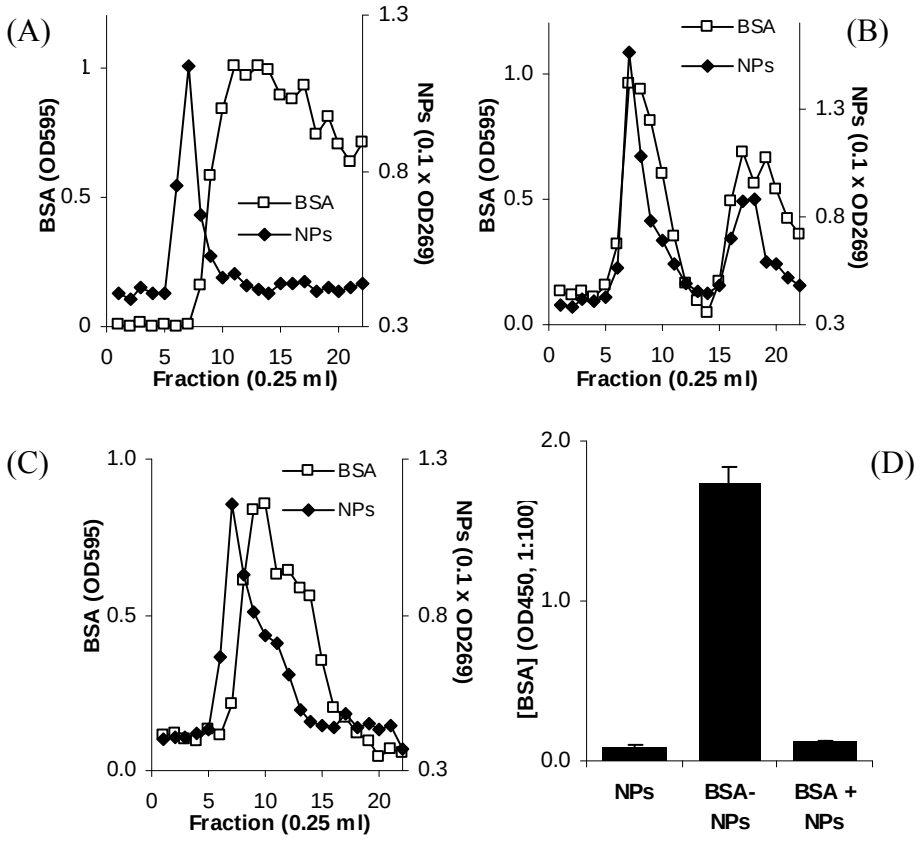
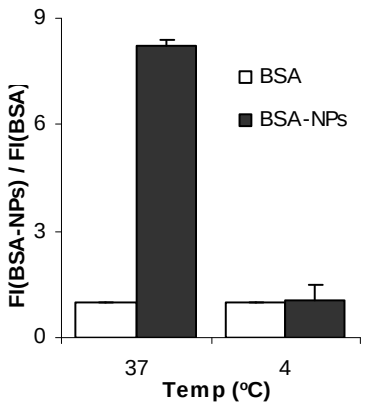


Figure 5.2 BSA was conjugated onto the nanoparticles. (A) The Sepharose 4B column can separate the nanoparticles from free BSA protein. Nanoparticles and the BSA were applied to the column separately. Nanoparticles were detected by measuring the absorption at 269 nm. BSA was detected at 595 nm after staining with Bradford's reagent. (B) and (C) The chromatogram of the reaction product of BSA and nanoparticles or the physical mixture of BSA and nanoparticles. In A, B, and C, data reported were the mean values from three independent determinations. The S.D. was not shown for clarity. In all cases, fraction 7 contained nanoparticles that could be reasonably separated from free BSA. (D) ELISA confirmed the conjugation of the BSA onto the nanoparticles. Fraction 7 from the nanoparticles alone (A), the reaction product of the BSA and nanoparticles (B), and the physical mixture of the BSA and nanoparticles (C) were used to coat the ELISA plate. The primary Ab was anti-BSA anti-serum. * The value of the BSA-NPs was significantly different from that of the other two ($p < 0.05$). Data presented are the mean ± S.D. ($n = 3$).

(A)


9
6
3
0
FI(BSA-NPs) / FI(BSA)
BSA
BSA-NPs
37
4
Temp (ºC)

(B)

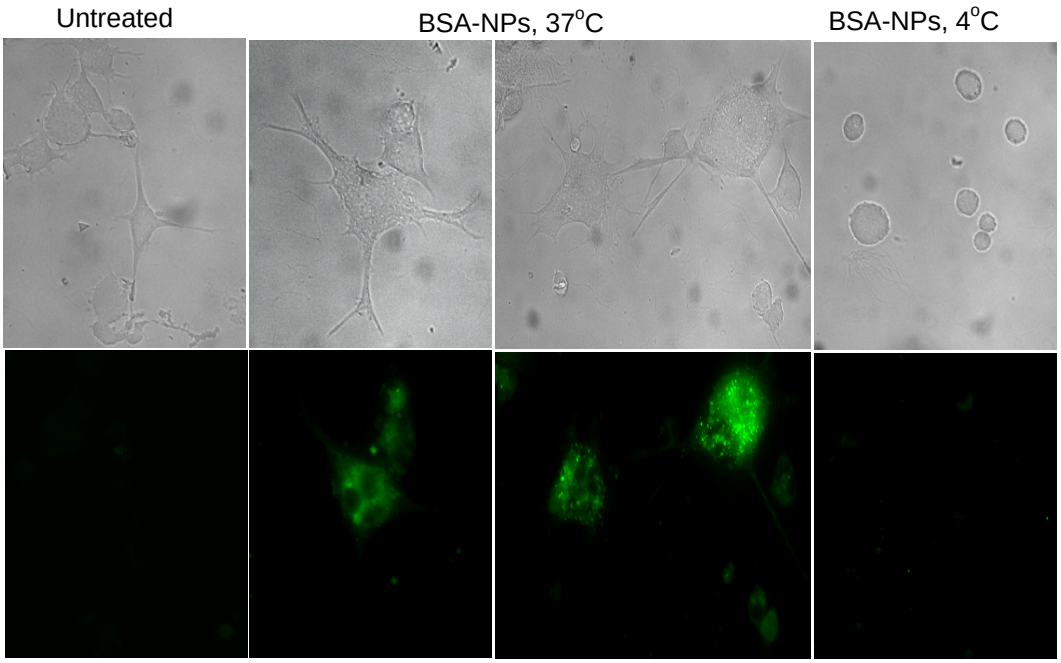

Untreated
BSA-NPs, 37°C
BSA-NPs, 4°C

Figure 5.3

Figure 5.3 The uptake of BSA-NPs by DC2.4 cells in culture. (A) The uptake of FITC-labeled BSA conjugated onto the nanoparticles by DC2.4 cells. Cells (1.0×10^6) were incubated with FITC-BSA-NPs or FITC-BSA for 6 h at 37°C or 4°C. Data reported are the ratio of the fluorescence intensity of cells treated with FITC-BSA-NPs over that with FITC-BSA. (B) Microscopic graphs showing the uptake of the FITC-labeled, BSA-conjugated nanoparticles. Cells were incubated with BSA-NPs-FITC for 6 h at 37^oC or 4^oC and observed under a bright-field microscope (top panel) or a fluorescence microscope (bottom panel).

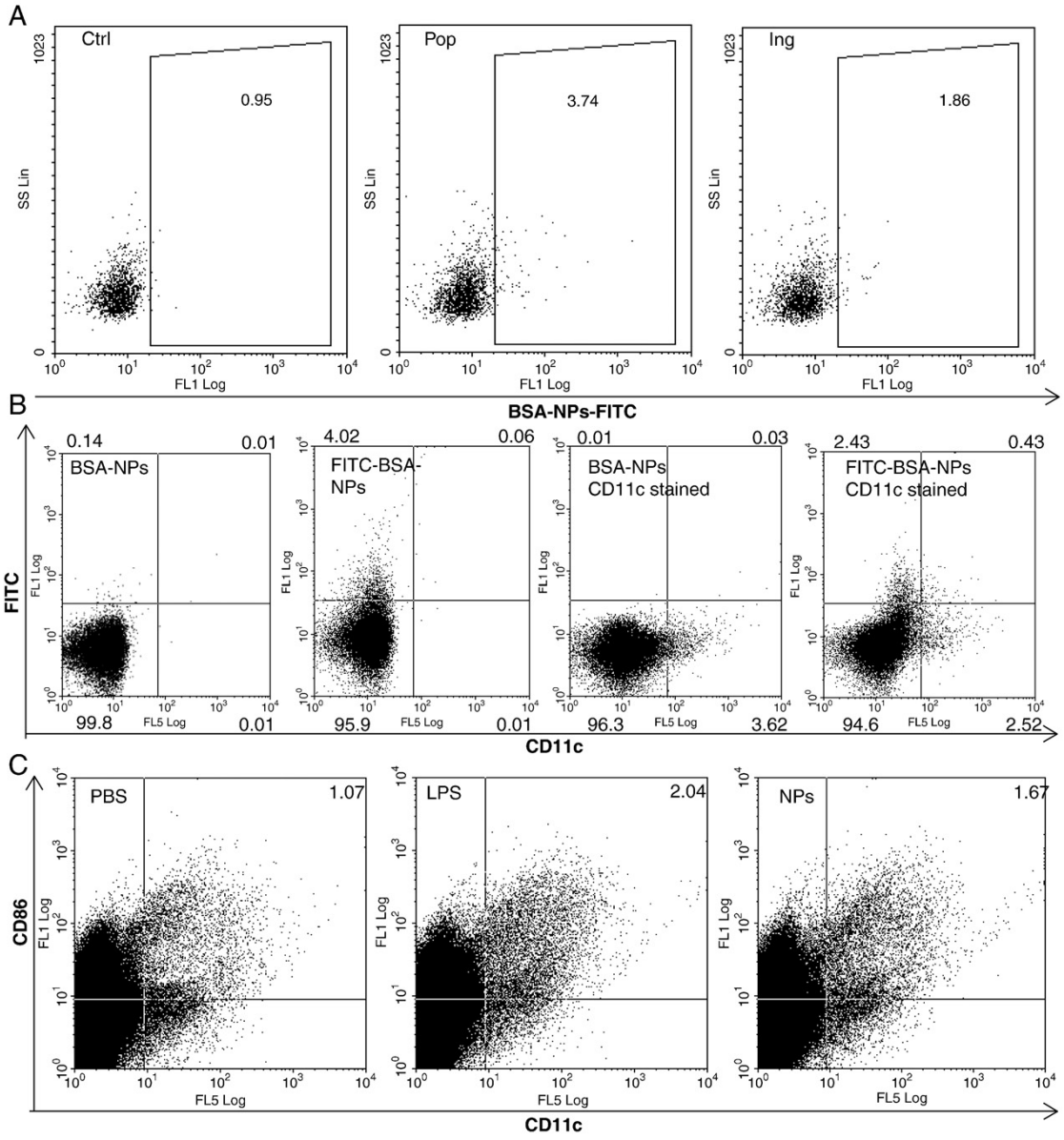

A
Ctrl
0.95
Pop
3.74
Ing
1.86
SS Lin
FL1 Log
BSA-NPs-FITC
B
0.14
0.01
BSA-NPs
4.02
0.06
FITC-BSA-NPs
0.01
0.03
BSA-NPs CD11c stained
2.43
0.43
FITC-BSA-NPs CD11c stained
FITC
99.8
0.01
95.9
0.01
96.3
3.62
94.6
2.52
FL5 Log
CD11c
C
PBS
1.07
LPS
2.04
NPs
1.67
CD86
FL1 Log
FL5 Log
CD11c

Figure 5.4

Figure 5.4 Trafficking of BSA-NPs into the LNs. (A) BSA-NPs were detectable in local and distal draining LNs. Mice (n = 3) were injected in the hind footpads with BSA-NPs-FITC. Popliteal and inguinal LN cells were collected 24 h after the injection. Ctrl indicates cells from the popliteal LNs of untreated mice. Pop, popliteal LNs; Ing, Inguinal LNs. Numbers are the % of cells that were FITC positive. (B) The uptake of BSA-NPs by DCs in the popliteal LNs. Mice were injected in the hind footpads with BSA-NPs or BSA-NPs-FITC. Popliteal LNs were collected 24 h later. Cells were stained with PE-Cy7-labeled anti-mouse CD11c. (C) Nanoparticles increased the percent of CD86 and CD11c double positive cells in the popliteal LNs. Mice (n = 3) were injected in the hind footpads with PBS, LPS from *E. coli*, or the NPs without BSA. Popliteal LNs were collected 23 h later and pooled. Cells were stained with PE-Cy7-anti-CD11c and FITC-anti-CD86.

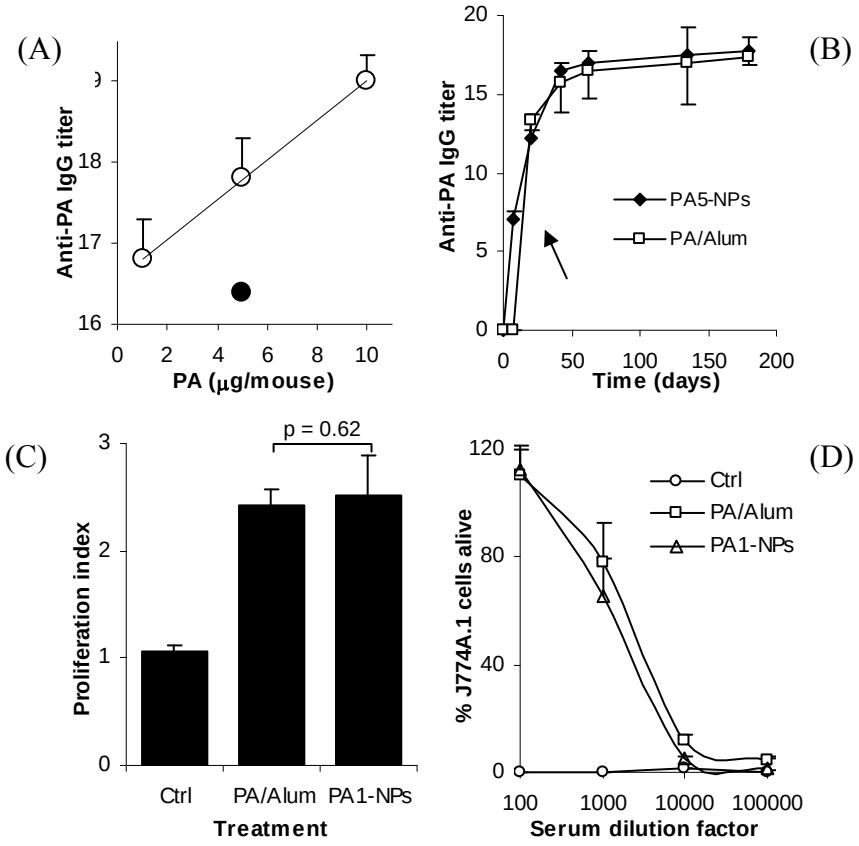


Figure 5.5 Immunization of mice with PA-NPs induced functional anti-PA Ab responses. (A) The effect of the dose of the PA on the resultant anti-PA IgG titer. Mice (n = 5) were dosed with PA-NPs (○) with 10, 5, or 1 μg of PA. Control mice were dosed with PA/Alum (5 μg PA) (●) or left untreated on days 0, 14, and 28 and bled on day 42. Linear regression showed that the slope of the regression line is not zero ($p < 0.001$). The values of the PA/Alum and the PA-NPs with 5 μg of PA were comparable ($p = 0.32$). Data shown are mean ± S.E.M. (B) The kinetics of the anti-PA IgG response. Mice (n = 5) were immunized on days 0, 14, and 28 with PA-NPs or PA/Alum (5 μg PA/mouse). The arrow indicates that the values of the PA5-NPs and the PA/Alum were different on day 7 ($p < 0.001$). (C) Splenocytes proliferated after *in vitro* re-stimulation with PA. Ctrl is the proliferation index from untreated mice. The values of PA1-NP (1 μg PA/mouse) and PA/Alum (5 μg PA/mouse) were comparable ($p = 0.62$). (D) The anti-PA antibodies protected J744A.1 cells from a lethal toxin challenge in culture. Ctrl indicates serum from untreated mice. Mice in the PA1-NP group were dosed with 1 μg PA/mouse, and those in the PA/Alum group were dosed with 5 μg PA/mouse. Data reported are mean ± S.D.

Table 5.1 Anti-BSA antibody titers in mouse serum samples.

	Treatment (titers in anti-log_2)		
	BSA-NPs	**BSA/IFA**	**BSA/Alum**
Anti-BSA IgG	13.5 ± 1.5	14.3 ± 0.5	10.8 ± 1.0*
Anti-BSA IgG1	16.0 ± 1.9	17.5 ± 1.7	16.0 ± 0
Anti-BSA IgG2a	9.9 ± 1.1	11.1 ± 0.6	6.6 ± 0*
Anti-BSA IgG3	8.4 ± 1.1	8.4 ± 1.5	6.6 ± 0*
Anti-BSA IgM	8.5 ± 0.9	7.9 ± 1.0	6.1 ± 0.6*

Mice (n = 5) were dosed with BSA-NPs, BSA/Alum, BSA/IFA, or left untreated on days 0 and 14 (5 μg BSA/mouse) and bled on day 28. Data shown are mean ± S.D. The data of the BSA-NPs were the average from two independent experiments. * The anti-BSA Ab titers induced by the BSA/Alum were lower than the other treatments ($p < 0.01$).

Table 5.2 Mice immunized with the PA-NPs survived a lethal dose of anthrax lethal toxin challenge, and the lethal toxin challenge significantly boosted the anti-PA IgG titer in the surviving mice.

	Control	**PA-NPs**	**PA/Alum**
# of mice died / # challenged [a]	4/4	0/3	0/3
Anti-PA IgG titer before challenge [b]	NA	17.8 ± 1.1	16.4 ± 1.9
Anti-PA IgG titer after challenge [c]	NA	19.6 ± 1.1	20.0 ± 0

[a] Mice were immunized on days 0, 14, and 28 (5 μg PA/mouse), challenged (i.v.) with anthrax lethal toxin two weeks after the last immunization, and monitored for 14 days after the challenge.
[b] IgG titer 4 days before the challenge (n = 5).
[c] IgG titer 14 days after the challenge (n = 3).
The values before and after the challenge were different from each other (PA-NPs, $p = 0.02$, PA/Alum, $p = 0.03$, dose of the PA was 5 μg/mouse). Titers are in anti-$\log_2$. Data shown are mean ± S.D.

Chapter 6

TOWARDS PRESERVING THE IMMUNOGENICITY OF PROTEIN ANTIGENS CARRIED BY NANOPARTICLES WHILE AVOIDING THE CHOLD CHAIN

Brian R. Sloat, Michael A. Sandoval, and Zhengrong Cui

International Journal of Pharmaceutics, submitted 2010

6.1 Abstract

Nanoparticles are an attractive vaccine carrier with potent adjuvant activity. Data from our previous studies showed that immunization of mice with lecithin-based nanoparticles with protein antigens conjugated onto their surface induced a strong, quick, and long-lasting antigen-specific immune response. In the present study, using these antigen-conjugated nanoparticles as a model, we evaluated the feasibility of preserving the immunogenicity of protein antigens carried by nanoparticles without refrigeration. The nanoparticles were lyophilized, and the immunogenicity of the antigens was evaluated in a mouse model using bovine serum albumin or the *Bacillus anthracis* protective antigen protein as model antigens. With proper excipients, the nanoparticles can be lyophilized while maintaining the immunogenicity of the antigens. Moreover, the immunogenicity of the bovine serum albumin model antigen conjugated onto the nanoparticles was undamaged after a relatively extended period of storage at room temperature or under accelerated conditions (37^{o}C) when the nanoparticles were lyophilized with 5% mannitol plus 1% polyvinylpyrrolidone. To our knowledge, the present study represents an early attempt to preserve the immunogenicity of the protein antigens carried by nanoparticles without refrigeration.

6.2 Introduction

Cold chain refrigeration is required for the storage and distribution of most vaccines. One major challenge in vaccine development is to develop formulations that do not require refrigeration. Proteins are increasingly used as antigens to prepare

vaccines. However, proteins alone are generally only weakly immunogenic, and a vaccine adjuvant is needed to improve the immunogenicity of the proteins (Clark and Cassidy-Hanley, 2005; Perrie et al., 2008). A growing body of evidence demonstrated the feasibility and advantages of using nanoparticles as an adjuvant or vaccine delivery system for protein antigens (Singh et al., 2007). It will be beneficial if future nanoparticle-based vaccines can be stored without refrigeration, preferably at ambient temperature, while preserving the immunogenicity of the antigens. However, it is well known that many protein pharmaceuticals in liquid formulations are rarely stable enough for long-term storage. Usually, a dried solid state formulation of the proteins is prepared using methods such as lyophilization to avoid or to slow down the degradation of the proteins, making it possible to store the protein pharmaceuticals for months or even years at ambient temperature (Carpenter et al., 1997). To assure the long-term stability of proteins in a dried solid state, the glass transition temperature (Tg) of the protein formulations must be higher than the planned storage temperature so that molecular motion and any associated degradation will be minimized (Carpenter et al., 1997; Chang et al., 1996; Roy et al., 1992).

Previously, we reported the preparation of nanoparticles from lecithin-in-water emulsions (Cui et al., 2006; Sloat et al., 2010; Yanasarn et al., 2009), which have the potential to serve as a universal protein-based antigen carrier capable of inducing strong immune responses (Sloat et al., 2010). Using two model antigens, bovine serum albumin (BSA) and the *B. anthracis* protective antigen (PA) protein, we showed that mice immunized with the BSA-conjugated nanoparticles (~200 nm in diameter)

developed strong anti-BSA antibody responses comparable to that induced by BSA adjuvanted with incomplete Freund's adjuvant, and stronger than that induced by BSA adsorbed onto aluminum hydroxide (Sloat et al., 2010). Mice immunized with the PA-conjugated nanoparticles elicited a quick, strong, and durable anti-PA antibody response that afforded protection of the mice against a lethal dose of anthrax lethal toxin challenge (Sloat et al., 2010). We attributed the potent adjuvanticity of the nanoparticles to their ability to move the antigens into local draining lymph nodes, to enhance the uptake of the antigens by antigen-presenting cells, and to activate the antigen-presenting cells (Sloat et al., 2010).

In a preliminary study, we found that the immunogenicity of a protein antigen (BSA) conjugated onto the surface of the nanoparticles decreased significantly after only one month of storage at room temperature in an aqueous suspension. Therefore, we decided to use these antigen-conjugated nanoparticles as a model to evaluate the feasibility of developing a nanoparticle-based vaccine formulation that can be stored at a temperature above refrigeration temperature while maintaining the immunogenicity of the antigens. We provided data showing that the antigen-conjugated nanoparticles can be lyophilized if proper excipients were used. We were able to lyophilize the antigen-conjugated nanoparticles into the blend of mannitol and polyvinylpyrrolidone (PVP), resulting in a formulation with a glass transition temperature (T_g) exceeding room temperature. In a 2.5-month storage study, we showed that the lyophilized protein antigen-conjugated nanoparticles can be stored at room temperature or even at 37^{o}C without any detectable damage to the immunogenicity of the antigen. We

conclude that storage of the nanoparticle-based vaccines in a dried form may represent a viable approach to preserve the immunogenicity of the antigens while avoiding refrigeration.

6.3 Materials and Methods

6.3.1 Preparation of nanoparticles

Nanoparticles were prepared as previously described (Sloat et al., 2010). Briefly, soy lecithin (3.5 mg, Alfa Aesar, Ward Hill, MA) and glyceryl monostearate (GMS, 0.5 mg, Gattefosse Corp., Paramus, NJ) were weighed into a 7-ml glass scintillation vial. One ml of de-ionized and filtered (0.2 μm) water was added into the vial, followed by heating on a hot plate to 70-75ºC with stirring and brief intermittent periods of sonication (Ultrasonic Cleaner Model 150T, VWR International, West Chester, PA). Upon formation of homogenous milky slurry, Tween 20 (Sigma-Aldrich, St. Louis, MO) was added in a step-wise manner to a final concentration of 1% (v/v). The resultant emulsions were allowed to stay at room temperature while stirring to form nanoparticles. The size of the nanoparticles was determined to be 178 ± 20 nm using a Coulter N4 Plus Submicron Particle Sizer (Beckman Coulter Inc., Fullerton, CA). To prepare the maleimide containing nanoparticles (m-NPs), 1,2-dipalmitoyl-s*n*-glycero-3-phosphoethanolamine-N-[4-(p-maleimideophyl)butyramide] (Avanti Polar Lipids, Alabaster, AL), which has a reactive maleimide group, was included in the lipid mixture (5%, w/w).

6.3.2 Conjugation of protein antigens onto the nanoparticles

The conjugation of the protein antigens onto the nanoparticles was completed as previously described (Cui et al., 2005; Sloat et al., 2010). We used two antigens, BSA (69 KDa, isoelectric point (pI) = 4.7) from Sigma-Aldrich and the protective antigen (PA) protein (83 KDa, PI = 5.6) from Biodefense and Emerging Infections (BEI) Research Resources Repository, Manassas, VA). Prior to the conjugation, the proteins were thiolated using 2-iminothiolane (Traut's Reagent, Sigma-Aldrich). The proteins were diluted into phosphate buffered saline (PBS, 0.1 M with 3.0 mM EDTA, pH 8.0), followed by the addition of Traut's reagent (20 x molar excess) and a 60 min incubation at room temperature. Thiolated proteins were purified using a PD10 column (Amersham Biosciences, Bellefonte, PA). To react the thiolated proteins with the m-NPs, 1 ml of freshly prepared m-NPs were mixed with the thiolated proteins (1 mg BSA or 0.25 mg PA) in PBS (0.1 M, pH 7.4) and stirred under N_2 gas for 12-14 h at room temperature. Unconjugated proteins were removed by ultracentrifugation (1.0 x 10^5 *g*). The amount of proteins conjugated onto the nanoparticles was estimated as previously described using fluorescein-labeled proteins (Sloat et al., 2010). The size of the BSA-conjugated nanoparticles was 201 ± 5 nm, and that of the PA-conjugated nanoparticles was 197 ± 6 nm. It was estimated that about 65-70 μg of the antigen proteins (BSA or PA) were conjugated onto 1 mL of the nanoparticles.

6.3.3 Lyophilization and storage of the nanoparticles

Thirty percent (w/v) stock solutions of potential lyoprotectants (sucrose, dextrose, mannose, PVP (average molecular weight, 40,000), mannitol, lactose, and trehalose, all from Sigma-Aldrich) were prepared with de-ionized and filtered (0.2 μm) water. To the nanoparticles in suspension, an equal volume of twice the desired concentration of the lyoprotectant was added. Upon complete mixing, the nanoparticles were frozen in liquid nitrogen overnight followed by lyophilization using a FreeZone 6 Liter Freeze Dry System (Labconco, Kansas City, MO). Water was added to reconstitute the nanoparticles. The number of inversions required for the complete re-suspension of the nanoparticles was recorded.

In the storage study, the lyophilized, protein-conjugated nanoparticles were sealed into 2 ml amber vials with standard rubber stoppers and aluminum caps (Wheaton Science Products, Millvile, NJ). They were then stored in dark at -80^{o}C, 4^{o}C, room temperature (22^{o}C), or 37^{o}C for 2.5 months. The size of the nanoparticles did not change significantly after the storage.

The moisture content of the lyophilized cake was determined using a gravimetric method (Shackell, 1909). The weight of the dried samples (n = 3) before and after the storage was measured and compared. Further, each sample was heated at an elevated temperature (> 100^{o}C) for 2 h, and the final weight was used to determine the moisture accumulation during the storage period. The weight gain (likely from water) was 0.18 ± 0.04%, 0.45 ± 0.11%, 0.47 ± 0.14%, and 0.30 ± 0.11% for the -80^{o}C, 4^{o}C, room temperature, and 37^{o}C samples, respectively.

The BSA-conjugated nanoparticles were used in the lyophilization and storage studies, while the PA-conjugated nanoparticles were lyophilized using the optimal excipients found in the lyophilization of the BSA-nanoparticles.

6.3.4 Differential scanning calorimetry (DSC)

DSC measurements were carried out using a TA Instruments Differential Scanning Calorimeter (New Castle, DE) to measure the glass transition temperature of the samples. Samples weighing between 7 and 10 mg were sealed into the aluminum DSC pans, which were placed in sample cells under nitrogen. Samples were scanned from -20 to 200°C at 10°C per min. Mannitol, PVP, and the physical blend of mannitol and PVP were scanned once and allowed to cool before final measurements were taken. The T_g of the pure PVP was found to be about 168^{o}C.

6.3.5 Immunization Studies

All mouse studies were carried out following NIH guidelines for animal use and care. BALB/c mice (female, 6-8 weeks) were from Simonsen Laboratories, Inc. (Gilroy, CA). The vaccine formulations were administered by subcutaneous injection. Except where mentioned, group of mice (n = 5) were dosed with 5 μg of antigens on days 0, 14, and 28, and euthanized and bled on day 42. As controls, mice were either left untreated or vaccinated with 5 μg of antigens adsorbed onto 50 μg aluminum hydroxide gel (Alum) (Spectrum Chemicals and Laboratory Product, New Brunswick, NJ).

6.3.6 Enzyme linked immunosorbent assay (ELISA) and toxin neutralization assay

The levels of anti-BSA and anti-PA IgG in serum samples were determined using ELISA (Sloat et al., 2010). EIA/RIA flat bottom, medium binding, polystyrene, 96-well plates (Corning Costar, Corning, NY) were coated with 100 ng of PA in 100 µl of carbonate buffer (0.1 M, pH 9.6) or BSA in PBS (10 mM, pH 7.4) overnight at 4^{o}C. For anti-BSA Ab measurement, plates were washed with PBS/Tween 20 (10 mM, pH 7.4, 0.05% Tween 20, Sigma–Aldrich) and blocked with 5% (v/v) horse serum in PBS/Tween 20 for 1 h at 37^{o}C. Samples were diluted two-fold serially in 5% horse serum/PBS/Tween 20, added to the plates following the removal of the blocking solution, and incubated for an additional 3 h at 37 ^{o}C. The serum samples were removed, and the plates were washed five times with PBS/Tween 20. Horse radish peroxidase (HRP)-labeled goat anti-mouse immunoglobulin (IgG, 5,000-fold dilution in 1.25% horse serum/PBS/Tween 20, Southern Biotechnology Associates Inc., Birmingham, AL) was added into the plates, followed by another hour of incubation at 37◦C. Plates were again washed five times with PBS/Tween 20. The presence of bound antibody was detected following a 30 min incubation at room temperature in the presence of 3,3’,5,5’-tetramethylbenzidine solution (TMB, Sigma–Aldrich), followed by the addition of 0.2 M sulfuric acid (Sigma-Aldrich) as the stop solution. The absorbance was read at 450 nm. Anti-PA IgG was determined similarly; except that the 5% horse serum in PBS/Tween 20 was replaced by 4% (w/v) BSA in PBS/Tween 20 in the plate blocking and the sample dilution steps. In addition, the secondary antibody was diluted in 1% (w/v) BSA in PBS/Tween 20. The antibody

titers were determined by considering an OD value higher than the mean plus two times of the standard deviation (mean + 2 x S.D.) of the negative control group as positive. Antibody titers were reported as the anti-log2 values.

The lethal toxin neutralization assay was also completed as previously described (Sloat and Cui, 2006). In brief, confluent J774A.1 cells were plated (5.0 x 10^4 cells/well) in sterile, 96-well, clean-bottom plates (Corning Costar) and incubated at 37^{o}C, 5% CO2 for 24 h. A fresh solution (50 μL) containing rPA (400 ng/mL) and lethal factor (100 ng/mL, BEI Resources) was mixed with 50 μL of diluted serum samples and incubated for 2 h at 37^{o}C. The cell culture medium was removed, and 100 μL of the serum/lethal toxin mixture was added into each well and incubated for 3 h at 37^{o}C, 5% CO2. Cell viability was determined using an MTT (3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide) kit (Sigma–Aldrich) with untreated and lethal toxin alone treated cells as controls.

6.3.7 Statistics

Statistical analyses were completed using ANOVA followed by the Fischer's protected least significant difference procedure. A p-value of ≤ 0.05 (two tail) was considered significant. The normality of the data and equal variance between groups was checked prior to data analysis.

6.4 Results and Discussions

6.4.1 Significant loss of immunogenicity when the antigen-conjugated nanoparticles were stored as an aqueous suspension

We aimed at using the protein antigen-conjugated nanoparticles previously developed in our laboratory as a model to evaluate the feasibility of storing protein antigens carried by nanoparticles at a temperature above refrigeration while maintaining the immunogenicity of the antigens. It was expected that the immunogenicity of the protein antigens conjugated onto the nanoparticles would be damaged significantly when they were stored as an aqueous suspension. To confirm it, the BSA-conjugated nanoparticles suspended in PBS were left at room temperature for up to a month and used to immunize mice. As shown in Fig. 6.1, the anti-BSA IgG titer induced by the BSA-nanoparticles after the storage was significantly lower than that induced by the freshly prepared BSA-nanoparticles ($p = 2 \times 10^{-5}$), demonstrating a significant loss of immunogenicity. Possible reasons for the loss of the immunogenicity may include the aggregation of the BSA-nanoparticles or the chemical degradation of the BSA proteins. In the following studies, we used this nanoparticle system as a model to test the feasibility to preserve the immunogenicity of the protein antigens carried by nanoparticles by storing the antigen-conjugated nanoparticles in a lyophilized dried formulation.

6.4.2 Lyophilization of the antigen-conjugated nanoparticles

Based on previous data on the storage of protein pharmaceutics, we hypothesized that the immunogenicity of the protein antigens conjugated onto

nanoparticles can be preserved at a temperature above refrigeration if the nanoparticles are to be stored in a dried solid formulation. Therefore, we first evaluated the feasibility of lyophilizing the protein antigen-conjugated nanoparticles. BSA-conjugated nanoparticles were frozen and lyophilized using several protectants. As shown in Fig. 6.2A, mannitol and sucrose at 5% adequately preserved the size of the BSA-nanoparticles. The 5% was chosen because it was found to be sufficient to protect the antigen-free nanoparticles during the freezing step (Sloat and Cui, unpublished data). Although dextrose was found to preserve the size of the BSA-nanoparticles (data not shown), it was not further considered because it is a reducing sugar and should be avoided in the lyophilization of proteins (Wang, 2000). Interestingly, the size of the BSA-conjugated nanoparticles was also preserved even without any lyoprotectant (Fig. 6.2A). It is likely that the polymeric BSA molecules conjugated on the surface of the nanoparticles helped prevent the aggregation of the BSA-nanoparticles. However, an aesthetically appealing lyophilized cake was not formed when a lyoprotectant was not used (Fig. 6.2B). Only when mannitol was used, an elegant, un-collapsed cake was formed (Fig. 6.2B), and the cake required the fewest number of inversions to completely reconstitute (Fig. 6.2C).

6.4.3 Lyophilization of antigen-conjugated nanoparticles using mannitol (5%) and PVP (1%) as excipients did not affect the immunogenicity of the antigen

Sugars such as trehalose and sucrose with a relatively high glass transition temperature (Tg) are typically used as the primary excipient to formulate dried vaccines (Chen and Kristensen, 2009), but our data in Fig. 6.2 clearly showed that

mannitol at 5% was optimal to lyophilize the BSA-nanoparticles. However, mannitol has a very low glass transition temperature of 11-14^{o}C (Kim et al., 1998), making it unlikely to store the dried antigen-conjugated nanoparticles at a temperature above room temperature (22^{o}C) while preserving the immunogenicity of the antigen proteins. Therefore, we tested the feasibility of including the polymeric PVP as a co-protectant to lyophilize the BSA-nanoparticles. PVP (MW 40,000) has a T_g of 160-168^{o}C. It also has a favorable safety profile, even when injected subcutaneously or intramuscularly (La-Chapelle, 1966; Wade A, 1994). Blending it into the mannitol can potentially increase the T_g of the lyophilized powder of the BSA-nanoparticles. Moreover, in a preliminary study, we found that the inclusion of the PVP (1%) slightly, but significantly, increased the antibody response induced by the antigen-conjugated nanoparticles lyophilized with 5% mannitol (Sloat and Cui, unpublished data). Shown in Fig. 6.3A was the effect of PVP at various concentrations, with and without mannitol (5%), on the size of the lyophilized BSA-nanoparticles. Reconstituted BSA-nanoparticles lyophilized with 5% mannitol and 1% PVP had a particle size comparable to that of the freshly prepared BSA-nanoparticles (Fig. 6.3A), and thus, were used for further studies.

To test whether the lyophilization step had damaged the immunogenicity of the antigen, the BSA-nanoparticles lyophilized using 5% mannitol plus 1% PVP were immediately reconstituted and used to immunize mice. As shown in Fig. 6.3B, the anti-BSA IgG titer induced by the lyophilized BSA-nanoparticles was not different from that induced by the freshly prepared BSA-nanoparticles.

In order to test whether the antisera induced by the lyophilized antigen-conjugated nanoparticles were still functional, we replaced the BSA antigen with the protective antigen (PA) protein of *B. anthracis*. When the PA protein was used as an antigen, the neutralizing activity of the anti-PA antibodies induced can be easily measured using an *in vitro* anthrax lethal toxin neutralization assay (Sloat and Cui, 2006). Again, the anti-PA IgG titer induced by the lyophilized PA-nanoparticles was not different from that induced by the freshly prepared PA-nanoparticles (Fig. 6.3B). Importantly, the anti-PA antisera induced by the lyophilized PA-nanoparticles had a similar anthrax lethal toxin neutralization activity as those induced by the freshly prepared PA-nanoparticles, as illustrated by their ability to protect the mouse J774A.1 macrophages from the anthrax lethal toxin (Fig. 6.3C). Anthrax lethal toxin is comprised of the PA protein and a lethal factor protein. The PA protein is non-toxic itself, but it is required to transport the lethal factor into cells such as macrophages, and the lethal toxin is toxic only when inside cells (Young and Collier, 2007). Therefore, neutralizing anti-PA antibodies can prevent lethal factors from entering the J774A.1 macrophages and protect them from the toxicity of the lethal toxin. Apparently, the lyophilization step did not cause any detectable damage in the immunogenicity of the antigens conjugated onto the nanoparticles.

6.4.4 The immunogenicity of the antigens conjugated onto the nanoparticles can be potentially preserved without refrigeration

To test whether the immunogenicity of the antigens conjugated onto the surface of the nanoparticles can be potentially preserved by storing the nanoparticles

in a lyophilized solid form at a temperature above refrigeration temperature, the BSA-nanoparticles lyophilized with 5% mannitol plus 1% PVP were stored at room temperature or 37^{o}C in sealed amber vials for 2.5 months and used to immunize mice. The anti-BSA IgG titers induced by the stored nanoparticles were then compared to that induced by the freshly prepared BSA-nanoparticles. As controls, the lyophilized BSA-nanoparticles were also stored at -80°C or 4°C for 2.5 months. As shown in Fig. 6.4, the anti-BSA IgG titer induced by the lyophilized BSA-nanoparticles stored for 2.5 months at room temperature was not different from that induced by the freshly prepared BSA-nanoparticles. In fact, storage of the lyophilized BSA-nanoparticles at 37^{o}C for 2.5 months also did not cause any detectable change in the immunogenicity of the BSA-nanoparticles (Fig. 6.4). This was probably due to the vitrification of the BSA-nanoparticles with the PVP and mannitol (Crowe et al., 1998), which resulted in a lyophilized powder with a relatively high T_g of 58-59^{o}C, as compared to 13-14^{o}C for the BSA-nanoparticles lyophilized in mannitol alone or 36^{o}C for the BSA-nanoparticles lyophilized in PVP alone. A 2.5-month of storage at 37^{o}C could equal to about 20 months at room temperature (22^{o}C), assuming an activation energy (Ea) value of 24.5 kcal/mol (Connors KA, 1985). Clearly, although a longer term of storage study has to be completed to confirm it, our data suggested that refrigeration can be potentially avoided while preserving the immunogenicity of the antigens conjugated onto the nanoparticles by storing the nanoparticles in a lyophilized formulation.

Finally, new technologies that allow the storage and distribution of vaccines while avoiding cold chain are actively sought. So far, the most effective approach

remains to be the formulation of the vaccines into a dried solid form (Amorij et al., 2008; Chen and Kristensen, 2009). There are many methods of drying, such as freeze drying, spray drying, spray-freeze drying, vaccine drying, and supercritical fluid drying (Amorij et al., 2008). Because the freezing and/or drying processes can significantly damage the (protein) antigens in the vaccine formulation, excipients, often sugars, are used as a stabilizer during the freezing and/or drying steps. In the present study, we used freeze drying, which may be expensive to generate a dried solid, but the damage to proteins during the freeze drying is minimal, and there are plenty of industrial experience (Amorij et al., 2008). Mechanistically, it is believed that the stabilizers allow the formation of a matrix of the vaccine embedded in an amorphous sugar glass. The sugar glass provides a physical barrier between particles and molecules and reduce the diffusion and mobility of molecules, and thus, prevent the aggregation and degradation of the proteins (Amorij et al., 2008). Moreover, during the drying process, the sugar replaces the water molecules in the hydrogen-bonding interaction with the protein molecules and thus help preserve the integrity of the proteins (Amorij et al., 2008). Although the lyophilization of nanoparticles is not new, our present study represented a successful early effort toward the development of a nanoparticle-based vaccine formulation that can be stored and distributed while avoiding the cold-chain.

In conclusion, we showed that protein antigen-conjugated nanoparticles can be lyophilized without damaging the immunogenicity of the antigens, and that the immunogenicity of the antigens can potentially be preserved by storing the

nanoparticles in a dried powder form, while avoiding refrigeration. This approach is likely applicable to protein antigens in other nanoparticle formulations. We are planning to test the feasibility of developing an antigen-conjugated nanoparticle formulation with a shelf-life of up to 2 years at ambient temperature.

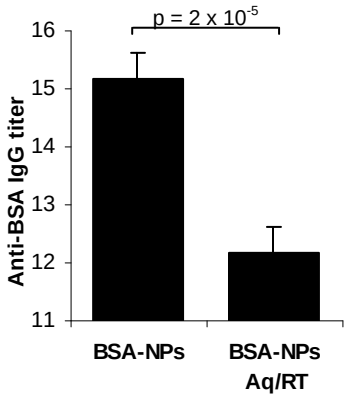


Figure 6.1 Significant decrease of immunogenicity when the BSA-conjugated nanoparticles were stored in an aqueous suspension. BSA-conjugated nanoparticles (BSA-NPs) were stored at room temperature in an aqueous suspension (BSA-NPs, Aq/RT) for a month, and the anti-BSA IgG titer induced by them was compared to that of freshly prepared BSA-NPs. Data shown are mean ± S.D. from group of 5 mice (anti-log2 values).

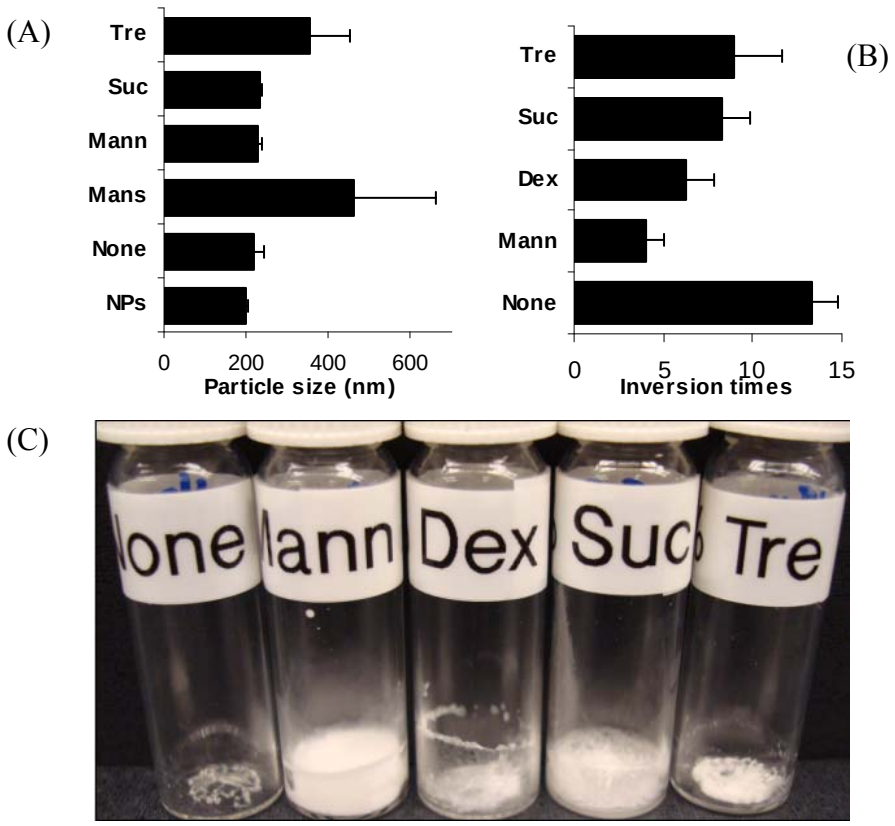


Figure 6.2 The lyophilization of the BSA-conjugated nanoparticles. (A) The size of the BSA-nanoparticles lyophilized with different excipients and immediately after reconstitution. The concentration of the excipient was 5% (w/v). (Suc: sucrose, Dex: dextrose, Mans: mannose, Mann: mannitol, Tre: trehalose). "None" indicates that no excipient was used. " NPs" means the freshly prepared BSA-nanoparticles. (B) The number of inversions needed to reconstitute the lyophilized BSA-nanoparticles. Data are mean ± S.D. (n = 3). (C) Photos of the lyophilized BSA-nanoparticles.

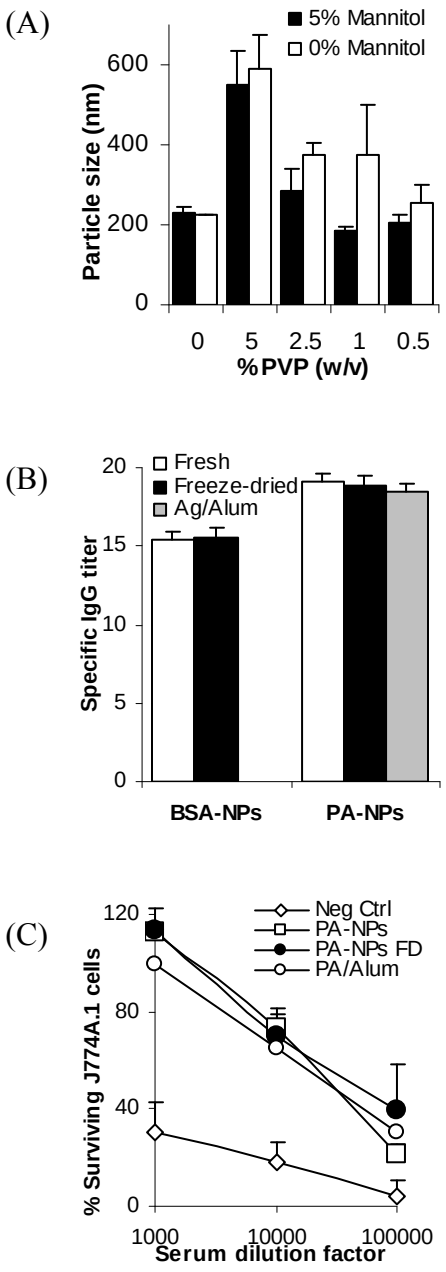

(A)
5% Mannitol
0% Mannitol
Particle size (nm)
600
400
200
0
0
5
2.5
1
0.5
%PVP (w/v)
(B)
Fresh
Freeze-dried
Ag/Alum
Specific IgG titer
20
15
10
5
0
BSA-NPs
PA-NPs
(C)
Neg Ctrl
PA-NPs
PA-NPs FD
PA/Alum
% Surviving J774A.1 cells
120
80
40
0
1000
10000
100000
Serum dilution factor

Figure 6.3

Figure 3. Lyophilization did not damage the immunogenicity of the antigens conjugated onto the surface of the nanoparticles. (A) Lyophilization of the BSA-nanoparticles using both mannitol and PVP. (B) The lyophilized antigen-conjugated nanoparticles (Freeze-dried) were as immunogenic as freshly prepared ones (Fresh). BSA and protective antigen (PA) protein were used as model antigens. For the PA, a PA adsorbed onto Alum (Ag/Alum) group was included as a control. Data are mean ± S.D. from 5 mice per group. (C) The anthrax lethal toxin neutralization activity of the serum samples from mice immunized with lyophilized/freeze-dried PA-NPs (PA-NPs FD) or freshly prepared PA-NPs. Serum samples from mice in individual group were pooled, and the data reported were mean ± S.D. from 4 measurements.

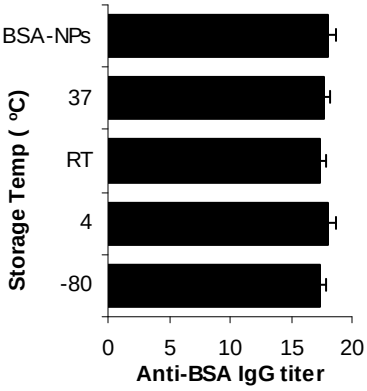


Figure 4. After 2.5 months of storage at 37°C, room temperature, 4°C, or -80°C, the lyophilized BSA-conjugated nanoparticles were as immunogenic as the freshly prepared BSA-conjugated nanoparticles. Group of mice (n = 5) were dosed 3 times every two weeks with 5 μg of BSA and bled one week after the last dosing. Data are mean ± S.D.

Chapter 7

GENERAL CONCLUSIONS

We confirmed that PGA conjugated to a carrier protein is immunogenic when administered intranasally with an appropriate adjuvant. The data presented in our studies showed that a prototypic anthrax vaccine candidate that contains PA, LF, and γDPGA-carrier protein conjugate was able to induce strong Ab immune responses against all three antigens when dosed intranasally using pI:C as an adjuvat. The anti-PA and anti-LF Ab responses protected macrophages against an anthrax lethal toxin challenge *in vitro* and enabled the immunized mice to survive a lethal toxin challenge *in vivo*. Further, the anti-PGA Abs were shown to have complement-mediated bacteriolytic activity. The strong immune responses were likely due to the effect of pI:C on the immune system, such as the DCs, through dsRNA/TLR3 signaling. When fully optimized, a tri-antigen nasal vaccine similar to the presented here is expected to represent of the newer generation anthrax vaccines. In future studies, the γDPGA will be directly conjugated to PA and a detoxified mutant LF will replace the wildtype LF. The efficacy of the resultant immune responses in protecting mice against an inhalational anthrax spore challenge will be determined.

In the studies using lecithin-based nanoparticles as antigen carriers with adjuvant activity, we found that mice immunized with BSA conjugated nanoparticles developed strong anti-BSA immune responses. In fact, the resulting immune response was comparable to that induced by BSA adjuvanted with incomplete Freund's adjuvant, and statistically stronger than that induced by BSA adsorbed onto Alum.

Further, mice were immunized with PA conjugated nanoparticles rapidly developed a strong and robust anti-PA antibody response that afforded protection against a lethal dose of anthrax lethal toxin. The adjuvanticity of the nanoparicles was determined to be the result of their ability to move antigens into local draining LNs, to enhance the uptake of the antigens by APCs, and to activate APCs. This novel nanoparticle system has the potential to serve as a universal protein-based vaccine carrier capable of inducing strong immune responses. However, the physical and chemical instability of the protein conjugated nanoparticles presents a major challenge for long-term storage. Lyophilization studies determined that freeze-drying the protein conjugated nanoparticle suspension with 5% of mannitol and 1% of PVP can potentially preserve the immunogenicity of the antigens in a dried powder form, while avoiding refrigeration. Future studies will be aimed at determining the efficacy of a peptide conjugated nanoparticle vaccine. Further, we are planning to test the feasibility of developing an antigen-conjugated nanoparticle formulation with a shelf-life of up to 2 years at ambient temperature.

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Appendix A

STRONG MUCOSAL AND SYSTEMIC IMMUNITIES AGAINST ANTHRAX FOLLOWING NASAL IMMUNIZATION WITH A PROTECTIVE ANTIGEN PROTEIN INCORPORATED IN LIPOSOME PROTAMINE-DNA PARTICLES

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Pharmaceutical Research, 2006; 23(2): 262-9

A.1 Abstract

The very lengthy and complicated dosing schedule for the current anthrax vaccine adsorbed (AVA), which was licensed in the U.S. for the prevention of cutaneous anthrax infection, calls for the development of an efficacious and easily administrable vaccine to prevent against the most lethal form of anthrax infection, inhalation anthrax. We propose to develop a nasal anthrax vaccine using anthrax protective antigen (PA) protein carried by LPD particles. PA was incorporated in LPD particles and nasally dosed to mice. The resulting PA-specific immune response and lethal toxin neutralization activity were measured. Mice nasally immunized with PA incorporated into LPD particles developed both systemic and mucosal anti-PA responses. The anti-PA immunities induced included the production of anti-PA antibodies (IgG and IgM in the serum and IgA in nasal and lung mucosal secretions) and the proliferation of splenocytes after in vitro stimulation. The anti-PA IgG subtype induced was mainly IgG1. Finally, anthrax lethal toxin neutralization activity was detected both in the serum and in the mucosal secretions. The anti-PA immune response induced by nasal PA incorporated in LPD was comparable to that induced by nasal PA adjuvanted with cholera toxin or subcutaneously injected PA adjuvanted with aluminum hydroxide.

A.2 Introduction

Anthrax is an often fatal bacterial infection caused by a rod-shaped, nonmotile, gram-positive bacterium, *Bacillus anthracis* (Ascenzi et al., 2002a; Dixon et al.,

1999). Inhalation anthrax infection is the most lethal anthrax, with a death rate of close to 100% (Friedlander, 1999). It involves massive bacteremia and toxemia with unnoticed initial symptoms until the onset of hypotension, shock, and sudden death (Chaudry et al., 2002; Dixon et al., 1999; Mourez et al., 2002). Although *B. anthracis* strains are sensitive to many antibiotics, such as ciprofloxacin, the lack of early symptoms and the rapid course of the disease make antibiotic therapy practically ineffective for an inhalation anthrax infection. Thus, there is a need for an efficacious vaccine against inhalation anthrax. The only anthrax vaccine currently licensed in the U.S. is the AVA, an aluminum hydroxide (Alum)-adsorbed, formalin-treated culture supernatant of a toxigenic *B. anthracis* strain (Puziss et al., 1963). AVA has many limitations, including the difficulty in maintaining consistency, the relatively high rate of local and systemic side effects, and most importantly, a very complicated and lengthy dosing schedule. The vaccination begins with a series of subcutaneous injections occurring over 18 months with subsequent annual boosters (Leppla et al., 2002; Puziss et al., 1963). Moreover, AVA was originally licensed only for the prevention of cutaneous anthrax, and its efficacy for inhalation anthrax prevention in humans has yet to be confirmed.

Anthrax is a toxin-mediated disease. Anthrax toxin contains 3 components, the protective antigen, the lethal factor (LF), and the edema factor (EF). Individually, these components are not toxic. However, they can combine to form two binary toxins, the lethal toxin (LeTx, i.e., LF + PA) and the edema toxin (ET, i.e., EF + PA). PA binds to a cell-surface anthrax toxin receptor (ATR) and mediates the entry of LF

and EF into the cytosol of host cells (Bradley et al., 2001). LF and EF are only toxic when they are inside cells (Ascenzi et al., 2002a; Ascenzi et al., 2002b). Thus, Abs that neutralize PA block the transport of both LF and EF into the cytosol, and in doing so, block the course of infection. The only demonstratable protective component in the AVA is the PA. Anti-PA IgG, actively induced or passively administered, had been shown to confer protection to animals challenged with *B. anthracis* either by aerosol or by intravenous injection, demonstrating that anti-PA Abs play an important role in the immunity against *B. anthracis* (Pitt et al., 2001; Turnbull et al., 1986). Moreover, anti-PA Abs have been shown to recognize spore-associated proteins, to stimulate spore uptake by macrophages, and to interfere with the germination of spores in vitro (Welkos et al., 2002; Welkos et al., 2001). Thus, many recent anthrax vaccine developments have focused on the production of a subunit vaccine based on the PA protein.

In order to protect against inhaled anthrax spore infection, such as what happened in the Fall of 2001, an efficacious anthrax vaccine that can be readily used for mass immunization in the event of an emergency is needed. A nasal anthrax vaccine could be a good candidate because it will be simpler to administer than the AVA injectable. In addition, because inhaled anthrax spores enter hosts through the respiratory mucosal surface, it is conceivable that a nasal anthrax vaccine that can induce PA-specific immune responses both in the systemic compartment and in the mucosa of the respiratory tract will be more effective. Finally, because of their anti-spore activity (Welkos et al., 2002; Welkos et al., 2001), anti-PA Abs generated in the

mucosal secretions of the respiratory tract should theoretically be able to bind to inhaled spores and prevent them from entering into hosts, and thus provide improved immunity against inhalation anthrax. In support of this, recent data showed that, in rabbits and mice nasally immunized with PA, serum toxin neutralization activity (TNA) alone was not predictive of the survival of animals after an inhalation anthrax challenge (Flick-Smith et al., 2002; Mikszta et al., 2005), which was in contrast to the previous belief that serum TNA was predictive of the effectiveness of anthrax vaccine (Little et al., 2004; Pitt et al., 2001).

The nasal mucosa is an important arm of the mucosal system. It has (i) an epithelial compartment on the surface of the epithelium and the underlying connective tissue that contains immuno-competent cells, such as macrophages, dendritic cells (DCs), and intraepithelial lymphocytes; (ii) the organized lymphoid structure of nose-associated lymphoid tissues (NALTs); (iii) the lymph nodes draining the respiratory system (Kiyono and Fukuyama, 2004). The Waldeyer's ring, which includes the nasopharyngeal tonsil or adenoid, the pair of palatine tonsils, the pair of tubal tonsils, and the inguinal tonsils, is present in the human pharynx (Hellings et al., 2000). NALT is a well organized structure, consisting of B and T cells areas, which are covered by an epithelial layer containing M cells (Kuper et al., 1992). Due to the lack of afferent lymphatics, exogenous antigens are sampled directly from the mucosal surface covering the NALT, to a large extent by M cells (Davis, 2001). The DCs, which are rich in the epithelial tissues, may also pick up antigens present on the mucosal surface (Jahnsen et al., 2004). Thus, the nasal mucosa is expected to be an excellent site to

administer vaccine, which was clearly demonstrated by the launch of FluMist® into the market.

The feasibility of inducing anti-PA immune response by nasal PA protein has been previously confirmed (Boyaka et al., 2003; Flick-Smith et al., 2002; Gaur et al., 2002; Mikszta et al., 2005). Although nasal administration of PA alone cannot induce any detectable anti-PA immune response, entrapment of PA in PLGA microspheres or liposomes (soy PC), or co-administration of PA with an adjuvant, such as cholera toxin (CT) and CpG motif-containing oligos, enabled the nasally administrated PA to induce anti-PA immune responses in murine models (Boyaka et al., 2003; Flick-Smith et al., 2002; Gaur et al., 2002; Mikszta et al., 2005). Boyaka et al. (2003) detected anthrax lethal TNA in mucosal secretions, when mice were nasally dosed with PA adjuvanted with CT (Boyaka et al., 2003). Although CT is a powerful mucosal adjuvant (Lycke, 2004), its severe toxicity precludes its potential use in humans (Fujihashi et al., 2002). Currently, the feasibility of replacing the wild type CT with its detoxified form is being tested. Mikszta et al. (2005) also detected specific anti-PA Abs in the lung lavages of mice nasally immunized with PA adjuvanted with CpG motif-containing oligos (Mikszta et al., 2005). However, it is known that the adjuvanticity data of CpG oligos obtained in murine are difficult to be transferred to humans due to the significant difference that exists in their DC biology (Kadowaki et al., 2001). Nevertheless, there continues to be a need for the development of an efficacious PA-based nasal vaccine.

Previously, we have reported a liposome-based particle, named LPD (liposome-Polycation-DNA), as a vaccine carrier for subcutaneous administration of protein-based vaccines (Cui and Huang, 2005), although the LPD particles *per se* were not an effective delivery system for DNA vaccine (Tan et al., 1999). LPD was engineered by combining cationic liposomes with protamine-condensed DNA. Upon mixing, the components spontaneously rearrange to form virus-like particles with the condensed DNA inside the liposomes. An antigen of interest may be incorporated into these LPD particles simply by adding the antigen into the preparation prior to the spontaneous rearrangement (Cui and Huang, 2005). We hypothesized that nasal dosing of PA protein incorporated into LPD particles will induce strong anti-PA immune responses both in the peripheral blood and in mucosal secretions, especially the respiratory tracts, of immunized mice.

A.3 Materials and Methods

A.3.1 Preparation of PA protein incorporated LPD particles

Liposomes and LPD were prepared as previously described (Cui and Huang, 2005). Briefly, small unilamellar liposomes composed of 1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP, Avanti Polar Lipids, Inc., Alabaster, AL): cholesterol (Sigma-Aldrich, St. Louis, MO, molar ratio 1:1) were prepared by a thin film hydration method followed by membrane extrusion (0.1 μm). The DOTAP concentration was fixed at 10 mg/ml. The LPD was composed of DOTAP/cholesterol liposomes, protamine (Sigma), and an empty plasmid DNA (pNGVL from the

National Gene Vector Laboratory, Ann Arbor, MI) in a ratio of 9.0:0.6:1.0 (w/w/w). Plasmid pNGVL is a DNA vector engineered for the construction of DNA vaccine. It has CpG motifs with appropriate flanking sequences. The plasmid was amplified in *E. coli* and purified with an Endo-Free plasmid purification kit from Qiagen (Valencia, CA). To prepare LPD, liposomes (21.5 μL, 10 mg/ml) and protamine (7.5 μL, 2 mg/ml) were dispersed in 75 μL of aqueous solution containing 10% dextrose (Sigma). Seventy-five μL of aqueous solution containing pNGVL (50 μg) and rPA (30 μg) was added drop wise into the mixture of liposomes and protamine while gently shaking. The mixture was allowed to stay at room temperature for at least 15 min prior to further use. The incorporation efficiency for the rPA in LPD was estimated to be ~80% using SDS-PAGE gel electrophoresis. In SDS-PAGE gel, unincorporated rPA moved into the gel, while those incorporated remained in the loading well. A comparison of the intensity of the rPA band from rPA/LPD preparation with that when same amount of pure rPA was loaded in a different well in the same gel allowed us to roughly estimate the incorporation efficiency. The size of the rPA-incorporated LPD particles was determined using photon correlation spectroscopy to be around 200 nm.

A.3.2 Nasal immunization

Female Balb/C mice (6-8 weeks, 18-20 g, Charles River Laboratories, Inc., Wilmington, MA) were nasally immunized once a week for three consecutive weeks with 5 μg of rPA (List Biological Laboratories, Inc., Campbell, CA) in 25 μl of LPD suspension. For nasal immunization, mice were lightly anesthetized with pentobarbital

(7 mg/100 g, Abbott Laboratories, North Chicago, IL) and given a total volume of 25 μL of rPA/LPD, in two doses of 12.5 μL with 10-15 min between each dose. As controls, mice (n = 5) were either left untreated, nasally (i.n.) dosed with rPA alone in phosphate buffered saline (PBS, 10 mM, pH 7.4), nasally dosed with rPA adjuvanted with cholera toxin (CT, 1 μg/mouse, List Biological Laboratories), or subcutaneously (s.c.) injected with rPA (5 μg/mouse) admixed with Alum (aluminum hydroxide gel, USP, Spectrum Chemical and Laboratory Products, New Brunswick, NJ, 15 μg/mouse). Mouse blood and lung and nasal washes were collected 28 days after the first immunization. This immunization experiment was repeated two times.

To collect nasal and lung washes, an incision was made in the trachea of euthanized mice. The nasal wash was collected by pipeting 0.2 ml of sterile PBS (10 mM, pH 7.4) through the trachea towards the nose. Fluid was collected in a microfuge tube as it exited the nares. The lung wash was obtained by pipeting 0.4 ml of PBS into the trachea towards the lungs, aspirating back into the pipet tip, and re-injecting once before the final withdrawal of the PBS fluid from the lung. The samples were stored at -80 ^{o}C prior to further use.

A.3.3 ELISA for anti-PA Ab measurement

The levels of anti-PA Abs were determined using enzyme-linked immunosorbent assay (ELISA). Briefly, EIA/RIA flat bottom, medium binding, polystyrene 96-well plates from Corning Costar (Corning, NY) were coated at 4°C overnight with 100 ng of rPA (List Biological Laboratories) dissolved in 0.1 M

carbonate buffer (pH 9.6). Plates were washed with PBS/Tween 20 (10 mM, pH 7.4, 0.05% Tween 20) and blocked with 4% (w/v) BSA (Sigma) in PBS/Tween 20 for 1 hr at 37°C. Serum samples were diluted in 4% BSA/PBS/Tween 20, added to the plates, and incubated for 2.5 hr at 37°C. Goat-anti-mouse immunoglobulin (IgG, IgA, IgM, IgG1, or IgG2a) conjugated to horseradish peroxidase (HRP, Southern Biotechnology Laboratories, Birmingham, AL) were added as secondary Abs. The presence of bound Ab was detected following a 30 min incubation at room temperature in the presence of TMB substrate (3,3',5,5'-Tetramethylbenzidine, Sigma). The reaction was stopped by adding sulfuric acid (Sigma, 0.2 M). The absorbance was read at 450 nm using a SpectraMax plate reader (Molecular Devices Inc., Sunnyvale, CA). The levels of anti-PA IgA in the lung and nasal washes were also determined similarly.

The sample dilution was completed by 2- or 10-fold serial dilutions. In cases of serial 2-fold dilutions, specific Ab level was reported as titer (anti-$\log_2$). Otherwise, it was reported as the OD450 after the TMB solution was incubated at room temperature for 30 min.

A.3.4 Lethal toxin-neutralization activity (TNA) assay

TNA was determined as described elsewhere with modifications (Boyaka et al., 2003). Briefly, confluent J774A.1 cells were seeded (5×10^4 cells/well) in sterile, 96-well, clean-bottom plates (Corning Costar) and incubated at 37°C, 5% CO_2 for 12 hr. A fresh solution containing 40 ng/ml LF (List Biological Laboratories) and 400 ng/ml rPA was mixed with an equal volume of diluted serum or mucosal wash

samples in triplicate and incubated for 1 hr at 37°C. The mixture was then added to J774A.1 cells and incubated at 37°C, 5% CO_2 for 3 hr. Cell viability was determined using an MTT kit (Sigma), with untreated cells and LeTx alone treated cells as controls.

A.3.5 Splenocyte proliferation assay

Single splenocyte suspension was prepared as previously described (Cui and Mumper, 2002). Splenocytes from individual spleens were cultured at a density of 4 x 10^6 cells/ml and stimulated with various concentrations of rPA (0 or) in complete RPMI medium (Invitrogen, Carlsbad, CA) for 5 days at 37°C, 5% CO_2 (Boyaka et al., 2003). Boyaka et al. (2003) reported the 12.5 μg/ml of rPA as the optimal concentration to induce splenocyte proliferation (Boyaka et al., 2003). Cell proliferation was determined using an MTT kit.

A.3.6 Statistics

Statistical analyses were completed using one-way analysis of variance (ANOVA) followed by Fischer's protected least significant difference (PSLD) procedure. A p-value of ≤ 0.05 (two-tail) was considered to be statistically significant.

A.4 Results

A.4.1 Nasal immunization of mice with rPA-incorporated LPD particles (rPA/LPD) induced strong anti-PA Ab responses in the serum.

As previously reported, nasal rPA alone did not induce any significant anti-PA IgG production (data not shown). However, nasal immunization of mice with rPA/LPD induced high level of anti-PA IgG in their sera, which was comparable to that induced by nasal rPA adjuvanted with CT or s.c. rPA adjuvanted with aluminum hydroxide (Fig. A.1). Serum anti-PA IgG was detectable 15 days after the first immunization (data not shown). Nasal rPA/LPD and nasal rPA adjuvanted with CT also induced anti-PA IgM in mouse sera, while IgM was not detected in the serum of mice injected (s.c.) with rPA adjuvanted with Alum (data not shown).

A.4.2 The anti-PA response was Th2-biased.

To understand the T helper (Th) cell response, the level of IgG subtypes, IgG1 and IgG2a, in the serum was measured using ELISA. Similar to injection (s.c.) of rPA adjuvanted with Alum, nasal rPA/LPD induced a high level of anti-PA IgG1, but not IgG2a, suggesting that the immune response induced was biased towards Th2 (Fig. A.2). As reported by Boyaka et al. (2003), Ab response induced by nasal rPA adjuvanted with CT was also biased towards Th2.

A.4.3 Specific anti-PA IgA was induced in both the nasal and lung washes.

To investigate the mucosal immune response induced, the levels of anti-PA IgA in the nasal and lung washes were measured. As shown in Figure A.3, nasal rPA/LPD induced high level of anti-PA IgA in both nasal and lung washes. In fact, the level of mucosal anti-PA IgA induced by nasal rPA/LPD was comparable to that induced by nasal rPA adjuvanted with CT. In contrast, no anti-PA IgA was detected in the nasal and lung washes of mice s.c. injected with rPA adjuvanted with Alum.

A.4.4 Splenocytes isolated from mice nasally immunized with rPA/LPD proliferated after in vitro stimulation with rPA protein

To investigate whether a proliferative immune response was induced by our nasal rPA/LPD vaccine, splenocytes isolated from immunized mice were stimulated with rPA for 5 days, and the cell proliferation was measured. As shown in Figure A.4, although not as strong as the proliferation of splenoctyes isolated from mice nasally immunized with rPA adjuvanted with CT, significant splenocyte proliferation was observed in mice nasally immunized with rPA/LPD. As expected, splenocytes isolated from mice injected (s.c.) with rPA adjuvanted with Alum also proliferated significantly.

A.4.5 Nasal rPA/LPD vaccine induced protective lethal toxin-neutralization activity

To evaluate the protective activity of the anti-PA Abs induced, the anthrax LeTx neutralization activity in the serum and mucosal wash samples was measured

using an in vitro macrophage (J774A.1) protection assay. As shown in Figure A.5A, strong LeTx neutralization activity was induced in the serum of mice nasally immunized with rPA/LPD. In fact, the LeTx neutralization activities in the sera of mice nasally immunized with rPA/LPD, nasally immunized with rPA adjuvanted with CT, and s.c. injected with rPA adjuvanted with Alum were comparable. LeTx neutralization activity was also detected in the nasal and lung washes of mice nasally immunized with rPA/LPD or rPA adjuvanted with CT (Figure A.5B and data not shown). In contrast, no neutralization activity was detected in the nasal and lung washes of mice nasally dosed with rPA alone or s.c. injected with rPA adjuvanted with Alum.

A.5 Discussions

Although cutaneous and gastrointestinal anthrax infections have produced documented fatalities, inhalation anthrax is the most lethal form of anthrax, with a fatality rate close to 100%, if proper antibiotic intervention is not provided (Friedlander, 1999). Upon inhalation of anthrax spores by a susceptible host, the spores are taken up locally by phagocytic cells. The phagocytized spores are either cleared or carried to regional LNs, where surviving spores germinate and outgrow (Guidi-Rontani et al., 1999; Hanna and Ireland, 1999; Welkos et al., 2001). The vegetative bacilli proliferate, spread systemically, and secrete anthrax toxins (Welkos et al., 2001). An anti-spore immune response that interferes early in this sequence of events, before the onset of bacteremia and toxemia, could conceivably provide more

effective protection against a lethal anthrax infection (Welkos et al., 2001). Anti-PA Abs were shown to have anti-spore activities (Welkos et al., 2002; Welkos et al., 2001). For example, spore-associated proteins recognized by anti-PA Abs were shown to be present on the surface of spores (Welkos et al., 2001). Moreover, anti-PA Abs were also shown to stimulate the phagocytosis of spores by macrophages and inhibit the germination of spores in vitro (Welkos et al., 2001). Thus, it is conceivable that a nasal PA protein-based vaccine can be an excellent candidate to induce anti-PA Ab responses both in the mucosa of respiratory tract to block anthrax spore uptake and germination and in the serum to neutralize anthrax toxins resulted from bacteremia. Moreover, a nasal anthrax vaccine is expected to be more patient-friendly to administer and potentially more cost-effective than a sterile injectable.

Results in this study clearly demonstrated that protective anti-PA immunities were induced in the mucosal secretions of mice when they were nasally immunized with rPA protein incorporated in our LPD particles. We showed that anti-PA IgA was induced in the nasal and lung washes of mice nasally immunized with rPA/LPD (Figure A.3). More importantly, the mucosal anti-PA immune response was shown to have LeTx neutralization activity in a J774A.1 macrophage protection assay (Figure A.5B). Based on some recently published data, we speculate that this strong LeTx-neutralization anti-PA immunity, which was comparable in strength to that induced by nasal rPA adjuvanted with CT, will provide a certain extent of anti-spore activity, and thus, interfere with the early stage of inhalation anthrax infection. We will confirm this activity in future studies. Mikszta et al. (2005) reported that in rabbits immunized

nasal with rPA adjuvanted with CpG oligos, serum toxin neutralization Ab titers alone were not predictive of the survival of rabbits after an aerosol anthrax spore challenge, which was in contrast to the previously reported correlation between the effectiveness of an anthrax vaccine and the serum TNA induced by it when AVA or other rPA-based vaccine was injected intramuscularly (Little et al., 2004; Pitt et al., 2001). The authors suggested that it was possible that the nasal route of vaccine dosing may provide stronger local responses at mucosal surfaces, thus enabling better protection from an aerosol challenge, despite the lower serum TNA titers. Similarly, using rPA-loaded PLGA microspheres, Flick-Smith et al. (2002) reported that mice immunized with a combination of i.m. and nasal priming and boosting schedules were always fully protected against aerosol anthrax spore challenges, while i.m. injection of the PA-loaded microsphere alone was not as effective, emphasizing the importance of stimulating both mucosal and systemic immune systems to obtain a full protection (Flick-Smith et al., 2002).

Strong anti-PA Abs were also induced in the systemic compartment of mice nasally immunized with our rPA/LPD vaccine (Fig. A.1, A.4, and A.5A). Anti-PA IgG titers higher than 1,000 was detected in mouse serum 15 days after the first dose (data not shown). On day 28, the serum anti-PA IgG titer was comparable to that induced by nasal rPA adjuvanted with CT and s.c. rPA adjuvanted with Alum (Fig. A.1). The splenocytes isolated from mice nasally dosed with rPA/LPD also proliferated significantly after in vitro stimulation with rPA protein (Fig. A.4). Although the types of the splenocytes that proliferated still need to be identified in

future studies, based on the Th2-biased Ab response as suggested by the high ratio of IgG1/IgG2a (13.0 ± 6.5), it was likely that $CD4^+$ T cells proliferated. The LeTx neutralization activity in the serum of mice nasally immunized with rPA/LPD was comparable to that in mice s.c. injected with rPA/Alum or nasally immunized with rPA adjuvanted with CT (Fig. A.5A). Thus, we expect our nasal rPA/LPD vaccine to be at least as effective as rPA adjuvanted with the other two standard vaccine adjuvants (CT and aluminum hydroxide) in protecting against anthrax challenge. Meanwhile, our nasal rPA/LPD is advantageous in that, in contract to CT, which has severe toxicity, LPD has an established good safety profile in a recent clinical trial (Leone et al., 2000). In our studies, 40% of mice nasally dosed with CT (1 μg/mouse) died during the experiments, while no mortality was observed in any other mice dosed similarly without CT. In addition, nasal rPA/LPD is more convenient and patient-friendly to administer than the s.c. injection of rPA adjuvanted with Alum. In future studies, we will evaluate the protective activity of nasal rPA/LPD in pulmonary anthrax spore challenging studies and further elucidate the relationship between the presence of anti-PA Abs in the mucosal secretion of the respiratory tract and the effectiveness of an vaccine against inhalation anthrax spore challenge in mice.

In the current study, the rPA dose per mouse was only 5 μg. Boyaka et al. (2003) previously reported that rPA alone was not immunogenic when dosed nasally (Boyaka et al., 2003). However, when CT was used as an adjuvant, there was a correlation between the dose of nasal rPA and the resulting anti-PA Ab response, with 40 μg/mouse being the optimal dose (Boyaka et al., 2003). Further increase of the

dose of rPA to above 40 μg/mouse did not lead to higher anti-PA Ab response. The anti-PA IgG titer in mice nasally dosed with 40 μg rPA was more than 100-fold higher than that in mice nasally dosed with 10 μg of rPA (Boyaka et al., 2003). If this holds true for our nasal rPA/LPD, we expect that the anti-PA IgG titer induced by our nasal rPA/LPD with 40 μg of rPA will be at least comparable with that induced by 40 μg of nasal rPA adjuvanted with CT. We will evaluate the effect of rPA dose in our nasal rPA/LPD on the resulting anti-PA immune response in future studies.

We have also evaluated the anti-PA IgG subtypes induced by our nasal rPA/LPD vaccine, which suggested that a Th2-biased antibody response was induced (Fig. 2), because the IgG1/IgG2a ratio obtained was 13.0 ± 6.6, in contract to the 0.6 ± 0.1 observed in the naïve mice. The high level of anti-PA IgG1 induced is important because it was shown that the anti-PA IgG1 subtype has a high affinity to PA protein (Little et al., 1988). A Th2-biased immune response is also beneficial for the development of an anthrax vaccine because anthrax is a toxin-mediated disease, and a Th2 response aids in the production of Abs. However, it is counterintuitive to observe that the anti-PA Ab response was biased towards Th2 because there were plasmids in our LPD particles, and the unmethylated CpG motifs in bacterial plasmid DNA usually screw the resulting immune response towards Th1 when the DNA is intramuscularly injected into mice (Cui, 2005; Gurunathan et al., 2000). In fact, in our previous studies, we found that the pNGVL and the cationic liposomes in the LPD both had adjuvant activity and that the induced immune response was Th1-biased when the LPD was subcutaneously injected into mice (Cui et al., 2005; Cui and

Huang, 2005). The nasal route of administration may be responsible for the Th2-biased response observed in the current study. It is known the immune response from DNA vaccine usually biases towards Th1 after i.m. injection (Gurunathan et al., 2000). However, the response is usually biased towards Th2 when DNA vaccine is intradermally (i.d.) injected using gene-gun (Gurunathan et al., 2000). In addition, the low dose of DNA applied to mice using gene-gun was thought by some to be responsible for the Th2-biased response (Cui, 2005). The dose of pNGVL applied nasally was about 4 μg/mouse, which was a relatively low dose and may explain the observed Th2-biased response. Finally, the Th2-biased Ab response may also suggest that the adjuvanticity from the plasmid DNA in our rPA/LPD vaccine may not be required for it to induce the anti-PA immune response, which is not surprising because our previous studies showed that the plasmid DNA in the LPD was not absolutely required for LPD to be immunostimulatory (Cui et al., 2005). Replacement of the CpG motif-containing plasmid with CpG motif-free oligos did not significantly change the immunostimulatory activity of the LPD (unpublished data). We will exploit this possibility in future studies by using rPA/LPD prepared with CpG motif-free oligos. The dispensability of the plasmid DNA in the rPA/LPD can be advantageous because it will eliminate the uncertainty for transferring the success of rPA/LPD in mice to humans.

Mechanistically, the immunogenicity of rPA/LPD could be mainly due to its particulate nature, which can be readily taken up by M cells in the NALTs when compared to rPA protein in solution. In nasal immunization, it is known that the

nasally dosed vaccine has a great potential to be inhaled into the lung. Lung involvement usually led to a stronger specific immune response, but can potentially cause serious proinflammatory response in the lung, and thus limit it use in humans (Bracci et al., 2005; Davis, 2001; Janakova et al., 2002). With our dosing technique and FITC-labeled rPA/LPD, we have confirmed that nasally dosed FITC-labeled rPA/LPD mainly remained in the nare passages 4 hours after dosing, while was not detectable in the lung washes (data not shown), suggesting that the contribution from lung immunization to the induced PA-specific immune response was limited. As to the potential involvement of gut-associated lymphoid tissue in inducing anti-PA immune response, it is difficult to speculate to what extent our rPA/LPD had traveled to the gastrointestinal tract after nasal dosing. However, we tend to believe that GALT involvement was insignificant because it is known that a very large dose is generally required for an orally dosed vaccine to induce any significant immune response.

As mentioned early, a few recent publications have described nasal dosing of rPA protein as an antigen in animal models (Boyaka et al., 2003; Flick-Smith et al., 2002; Gaur et al., 2002; Mikszta et al., 2005). However, our nasal rPA/LPD is advantageous not only because of the strong mucosal and systemic anti-PA immune responses induced by it, but also because of the established good safety profile of LPD. When systemically injected via the i.v. route, LPD was shown to be transiently inflammatory by producing cytokines such as (Tan et al., 1999). LPD had been intraventricularly administered to children with Canavan disease for gene therapy in a previous clinical trial and was shown to be well tolerated (Leone et al., 2000). In the

present studies, we did not observe any gross inflammatory, allergic, or toxic effect when mice were nasally dosed with our rPA/LPD. In fact, the high anti-PA IgG1/IgG2a ratio suggested that Th1-driving proinflammatory cytokines (e.g., IFN-γ and IL-12) observed after i.v. injection of LPD were not induced after nasal dosing with rPA/LPD. Finally, as mentioned earlier, LPD was prepared via a spontaneous rearrangement process after cationic liposomes and protamine/DNA complex were mixture together. Also, LPD particles can be lyophilized and stored at room temperature for months (Li et al., 2000). Thus, we believe this nasal rPA/LPD vaccine holds a great potential to be developed into a human anthrax nasal vaccine.

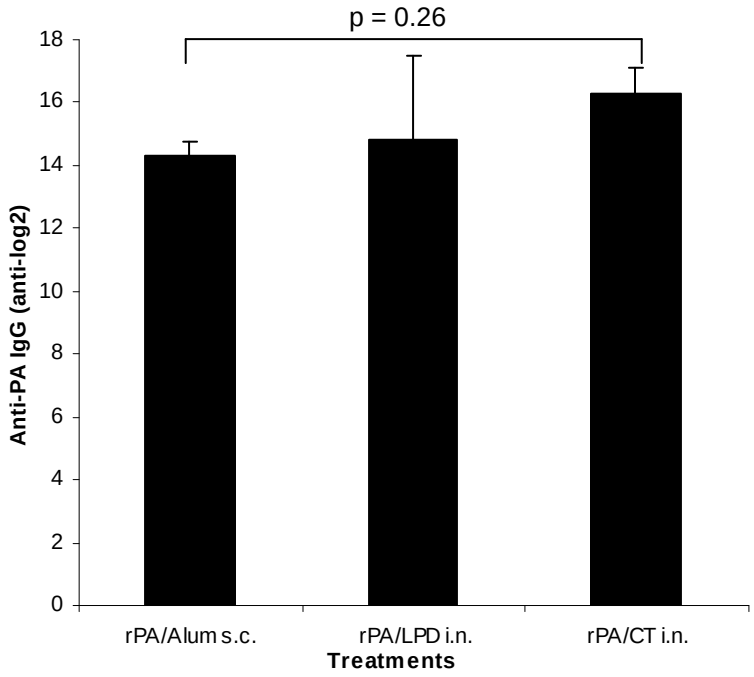

Figure A.1 Serum anti-PA Ab responses. Balb/C mice (n = 5) were nasally dosed with rPA alone, rPA-incorporated LPD (rPA/LPD), or left untreated on days 0, 7, and 14. As controls, other two groups of mice (n = 5) were nasally dosed with rPA adjuvanted with CT (rPA/CT i.n., CT = 1 μg/mouse) or s.c. injected with rPA adjuvanted with Alum (rPA/Alum s.c., Alum = 15 μg/mouse). The dose of rPA was 5 μg/mouse. On day 28, mice were bled, and total anti-PA IgG titer in the serum was determined using ELISA with the serum being diluted 2-fold serially. Nasal rPA alone failed to induce any detectable level of anti-PA IgG. ANOVA analysis showed that there was not any significant difference among those three treatments ($p = 0.26$). This experiment was repeated twice, and similar results were obtained. Data from one representative are shown.

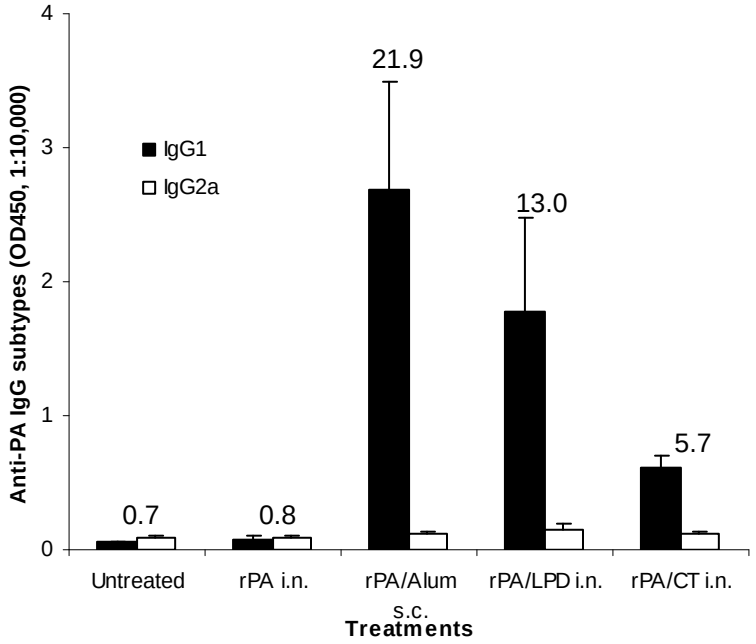

Figure A.2 Serum anti-PA IgG subtypes. Balb/C mice (n = 5) were dosed with rPA alone, rPA/LPD, or left untreated on days 0, 7, and 14. As controls, other groups of mice were injected (s.c.) with rPA adjuvanted with Alum or nasally dosed with rPA adjuvanted with CT. The IgG1 and IgG2a levels in the serum were determined on day 28 using ELISA after the sera were diluted 1,000-fold. The ratio of the OD450 for IgG1 and IgG2a ($OD450_{IgG1}/OD450_{IgG2a}$) was reported as numbers above the bars. Statistical analysis did not reveal any significant difference among all the values for IgG2a ($p = 0.13$, ANOVA). The IgG1 values from rPA/Alum (s.c.), rPA/LPD (i.n.), and rPA/CT (i.n.) were all significantly higher than that from untreated mice.

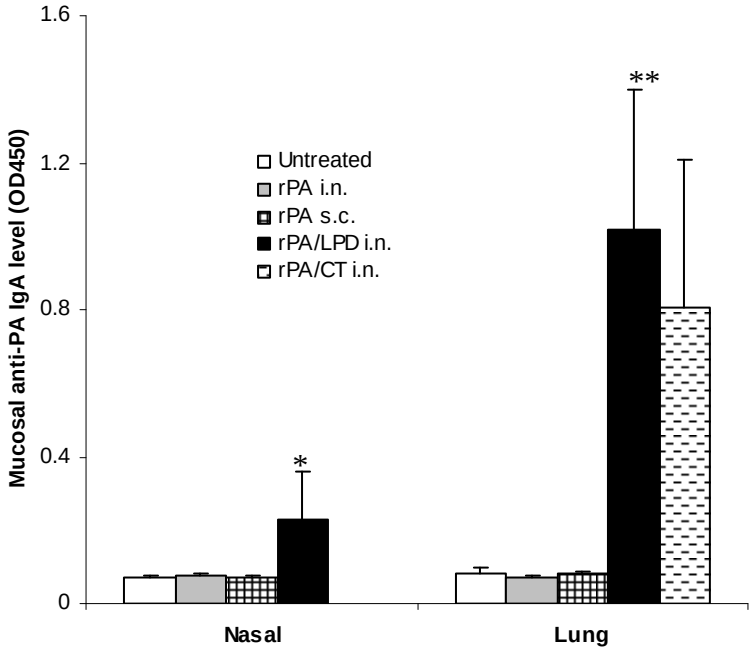

Figure A.3 Anti-PA IgA in nasal and lung washes. Balb/C mice were dosed (i.n.) with rPA alone, rPA/LPD, or left untreated on days 0, 7, and 14. As controls, other groups of mice were injected (s.c.) with rPA adjuvanted with Alum or nasally dosed with rPA adjuvanted with CT. On day 28, mice were sacrificed. Both nasal and lung washes were collected. Anti-PA IgA level in samples from individual mouse was determined using ELISA after the washes were diluted 10-fold. * indicates that, for nasal washes, the value for rPA/LPD (i.n.) was significantly higher than that of the others. Nasal washes were not collected from mice nasally dosed with rPA/CT. ** indicates that, for lung washes, the values from rPA/LPD (i.n.) and rPA/CT (i.n.) were not different from each other, but significantly higher than that from the others.

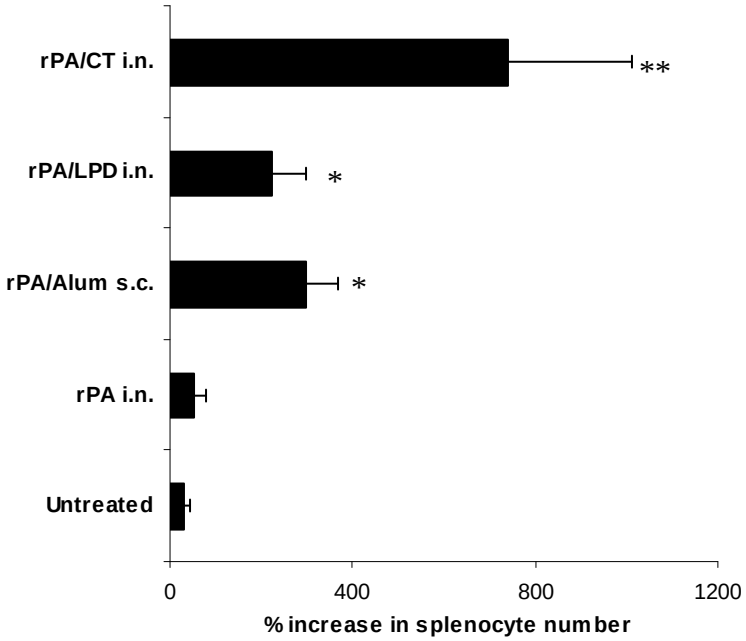

Figure A.4 In vitro proliferation of splenocytes after stimulation with rPA. Balb/C mice were dosed (i.n.) with rPA alone, rPA/LPD, or left untreated on days 0, 7, and 14. As controls, other groups of mice were injected (s.c.) with rPA adjuvanted with Alum or nasally dosed with rPA adjuvanted with CT. On day 28, mice were sacrificed. Their spleens were harvested, and single splenocyte suspension from individual spleen was prepared. The splenocytes (4×10^6/ml) were incubated with rPA (0 or 12.5 μg/ml) for 5 days. The proliferation was measured using an MTT test kit. ANONA analysis showed that there was significant difference among the values from different treatments ($p << 0.05$). * indicates that the value for rPA/Alum (s.c.) was not different from that for rPA/LPD (i.n.), but higher than that from the untreated mice and mice nasally dosed with rPA alone. ** indicates that the value for rPA/CT (i.n.) was significantly higher than that of the others.

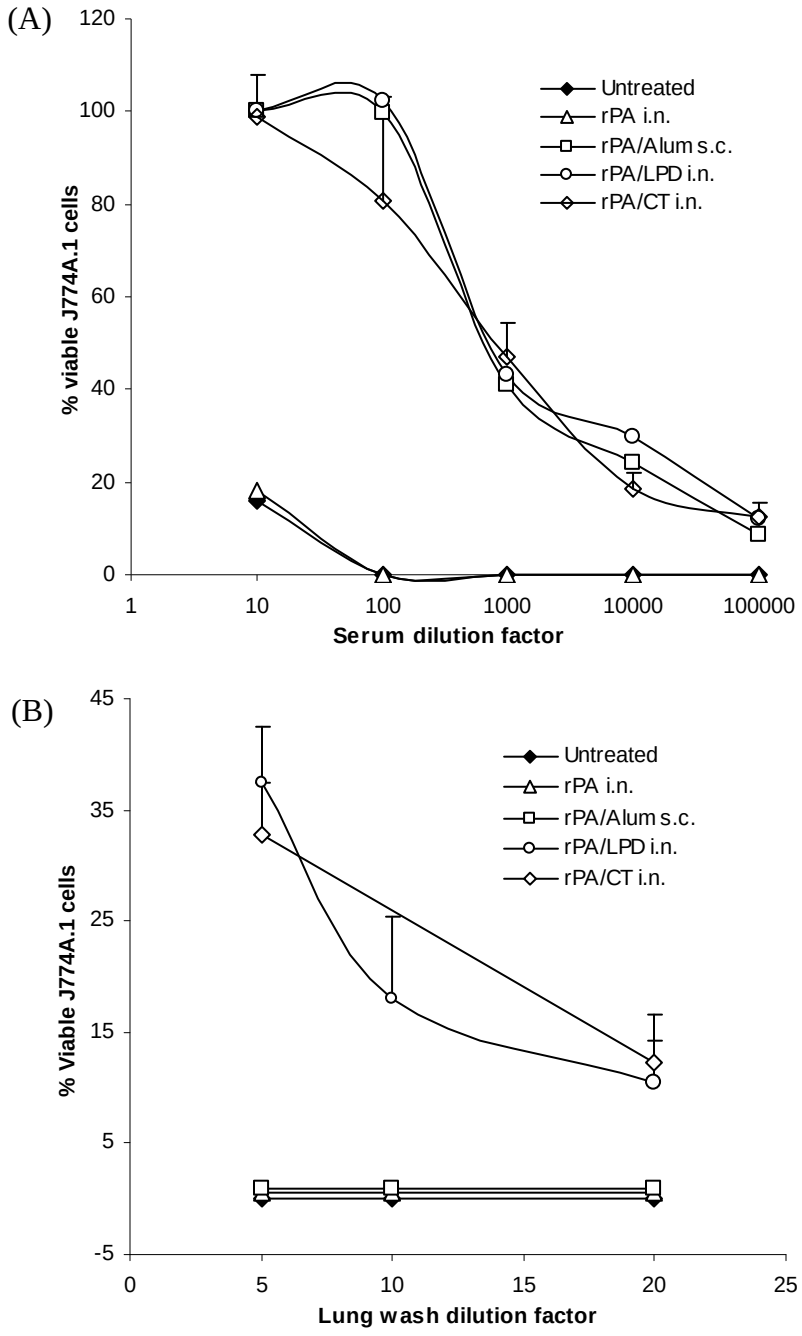
(A)
120
100
80
60
40
20
0
% viable J774A.1 cells
1
10
100
1000
10000
100000
Serum dilution factor
Untreated
rPA i.n.
rPA/Alum s.c.
rPA/LPD i.n.
rPA/CT i.n.
(B)
45
35
25
15
5
-5
% Viable J774A.1 cells
0
5
10
15
20
25
Lung wash dilution factor
Untreated
rPA i.n.
rPA/Alum s.c.
rPA/LPD i.n.
rPA/CT i.n.

Figure A.5

Figure A.5 Nasal rPA/LPD induced strong LeTx neutralization activity in serum (A) and lung washes (B). Balb/C mice (n = 5) were dosed as mentioned in Materials and Methods. On day 28, sera were collected. The 10-fold serial dilutions of each serum samples were incubated with J774A.1 cells (5×10^5/ml) in the presence of anthrax lethal toxin (LeTx, 400 ng/mL of PA and 40 ng/mL of LF) for 3 h. The percent of viable J774A.1 cells was determined using an MTT test. In (A), only the standard deviations from rPA/CT (i.n.) were included to show that the LeTx neutralization activity induced by rPA/LPD (i.n.), rPA/Alum (s.c.), and rPA/CT (i.n.) were not significantly different from one another. In (B), TNA was not detected in the lung washes of untreated mice and mice nasally immunized with rPA alone or s.c. injected with rPA adjuvanted with Alum. The data for rPA/CT (i.n.) after 10-fold dilution were not collected.

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Appendix B

EVALUATION OF THE IMMUNE RESPONSE INDUCED BY A NASAL ANTHRAX VACCINE BASED ON THE PROTECTIVE ANTIGEN PROTEIN IN ANESTHETIZED AND NON-ANESTHETIZED MICE

Brian R. Sloat and Zhengrong Cui

Journal of Pharmacy and Pharmacology, 2006; 58(4): 439-47

B.1 Abstract

To better protect against an inhalational anthrax infection, a nasal anthrax vaccine based on the protective antigen (PA) protein of *Bacillus anthracis* could be an attractive alternative to the current Anthrax-Vaccine-Adsorbed (AVA), which was licensed for cutaneous anthrax prevention. Previously, we have demonstrated that anti-PA immune responses comparable to that in mice subcutaneously immunized with PA protein adjuvanted with aluminum hydroxide were induced in both the systemic compartment and the mucosal secretions of the nose and lung of anesthetized mice when they were nasally immunized with PA protein incorporated into previously reported LPD (Liposome-Protamine-DNA) particles. In this present study, we evaluated the anti-PA immune response induced by the nasal PA/LPD particles in non-anesthetized mice and compared it to that in anesthetized mice. Our data showed that the anti-PA antibody (Ab) response and the anthrax lethal toxin-neutralization activity induced by the nasal PA/LPD in non-anesthetized mice was relatively weaker than that in anesthetized mice. However, the splenocytes isolated from the nasally immunized mice, anesthetized and non-anesthetized, proliferated comparably after in vitro re-stimulation. By evaluating the uptake of fluorescence-labeled LPD particles by phagocytes in the nasal and broncho-alveolar lavages of mice after the nasal administration, we concluded that the relatively weaker anti-PA immune response in the non-anesthetized mice might be partially attributed to the reduced retention of the PA/LPD particles in the nasal lavages of the non-anesthetized mice. Data collected in

this study are expected to be useful for future anthrax nasal vaccine studies when mice are used as a model.

B.2 Introduction

Bacillus anthracis is the etiologic agent of anthrax. There are three clinical forms of anthrax infections, cutaneous, gastrointestinal, and inhalational (Ascenzi et al., 2002). Without a proper antibiotic intervention, all three forms of infections may lead to massive bacteremia and toxemia followed by hypotension, shock, and sudden death (Chaudry et al., 2002; Dixon et al., 1999; Mourez et al., 2002). Being the most lethal, inhalational anthrax infects the host through the respiratory tract. Anthrax is a toxin-mediated disease. Anthrax toxin contains three components, the protective antigen, the lethal factor (LF), and the edema factor (EF). While each of these components is not toxic alone, they combine to form two binary toxins, the lethal toxin (LeTx, i.e., LF + PA) and the edema toxin (i.e., EF + PA). PA binds to the anthrax toxin receptor on the cell surface and mediates the entry of LF and EF into the cytosol of host cells (Bradley et al., 2001). LF and EF are only toxic when inside cells (Ascenzi et al., 2002). Thus, Abs that neutralize PA block the transport of both LF and EF into the cytosol and prevent the course of infection.

The only licensed anthrax vaccine currently available in the U.S. is the AVA, an aluminum hydroxide (Alum)-adsorbed, formalin-treated culture supernatant of a toxigenic *B. anthracis* strain (Puziss et al., 1963). While efficacious against cutaneous anthrax as licensed, AVA has many limitations including occasional severe side-

effects, difficulty maintaining consistency, and more importantly, a lengthy and complicated dosing schedule. Moreover, its efficacy against inhalational anthrax infection in humans has yet to be confirmed. Numerous animal studies have shown that the primary immunogen in the AVA is the PA component of the anthrax toxins (Pitt et al., 1999; Welkos et al., 2001), and anti-PA IgG had been shown to confer protections to animals challenged with *B. anthracis* (Pitt et al., 2001; Turnbull et al., 1986), demonstrating that anti-PA Abs play an essential role in the immunity against *B. anthracis*. Thus, many recent anthrax vaccine developments have focused on the production of a subunit vaccine based on the PA protein.

A nasal PA-based anthrax vaccine could be an ideal alternative to the current AVA. It is advantageous in that a nasal anthrax vaccine will be self-applicable, making it possible to vaccinate a large population of people in case of an emergency. In addition, because anthrax spores enter hosts through the mucosal surface of the respiratory tract, it is conceivable that an optimal protection against an inhalational anthrax infection will require not only systemic, but mucosal immune responses, particularly in the respiratory mucosa. Finally, because anti-PA Abs have been shown to recognize spore-associated proteins, to stimulate spore uptake by macrophages, and to interfere with the germination of spores in vitro (Welkos et al., 2002; Welkos et al., 2001), anti-PA Abs induced in the respiratory mucosa should theoretically be able to neutralize spores, and thus, prevent the entrance of spores into hosts. Thus, it was no surprise that, in rabbits nasally immunized with PA in a powder form, serum toxin neutralization Ab titers alone were not predictive of the survival of rabbits after an

inhalational anthrax spore challenge (Mikszta et al., 2005), which was in sharp contrast to the previous belief that serum toxin neutralization Ab titers alone were predictive of the efficacy of an anthrax vaccine (Pitt et al., 2001).

The nasal mucosa is an important component of the mucosal immune system, and thus, an ideal site to administer vaccines, especially when a mucosal immune response in the respiratory tract is desired (Brandtzaeg et al., 1999; Davis, 2001; Kiyono and Fukuyama, 2004; Kuper et al., 1992). The launch of the Nasal Flu® and the FluMist® into the market clearly demonstrated the feasibility of nasal immunization (Glezen, 2004). Although the Nasal Flu® was pulled off the market due to its strong association with the Bell's Palsy disease (Mutsch et al., 2004), a recent post-approval review of the FluMist® did not reveal any unexpected serious adversary effects (Izurieta et al., 2005). In fact, there also had been a few attempts to administer PA-based anthrax vaccine formulations via the nasal route in murine models (Boyaka et al., 2003; Flick-Smith et al., 2002; Gaur et al., 2002; Mikszta et al., 2005).

Previously, we have demonstrated that anti-PA immune responses with strong anthrax LeTx-neutralization activity were induced in both the broncho-alveolar lavages (BALs) and the systemic compartment of mice nasally dosed with rPA incorporated into LPD (Liposome-Protamine-DNA) particles (Sloat and Cui, 2005). The LPD particles were engineered by combining cationic liposomes and protamine-condensed DNA. Upon mixing, the components spontaneously rearrange to form a virus-like structure with the condensed DNA inside the lipid membrane (Li et al., 1998). In a previous clinical trial, LPD was shown to be well-tolerated (Leone et al.,

2000). The antigen of interest may be incorporated into the LPD particles by simply adding it into the LPD preparation prior to the spontaneous rearrangement (Cui and Huang, 2005; Dileo et al., 2003). Similar to what has been carried out in most other studies, the rPA/LPD particles were dosed to mice under anesthesia (Sloat and Cui, 2005). However, there is evidence in the literature showing that the immune response induced in mice could vary significantly, depending on whether the mice were under anesthesia or not (Bracci et al., 2005; Davis, 2001; de Haan et al., 1995; Janakova et al., 2002; Ryan et al., 1999; Yetter et al., 1980). The involvement of lung has proven to be responsible for the variations in the immune responses induced (Janakova et al., 2002).

In the present study, we evaluated the anti-PA immune response induced by our nasal rPA/LPD vaccine in mice dosed with or without prior anesthesia. We hypothesized that the anti-PA immune responses induced will be significantly different. The information collected in this study should be relevant for future anthrax nasal vaccine developments when mice are used as a model.

B.3 Materials and Methods

B.3.1 Materials

Female Balb/C mice (6-8 week-old, 18-20 g) were purchased from Charles River Laboratories (Wilmington, MA). LF and rPA were purchased from List Biological Laboratories, Inc. (Campbell, CA). 1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP) and FITC-labeled 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine

(FITC-DOPE) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Cholesterol, protamine, dextrose, albumin from bovine serum (BSA), 3,3',5,5'-tetramethylbenzidine (TMB), sulfuric acid, and the MTT kit were purchased from Sigma-Aldrich (St. Louis, MO). Plasmid DNA (pNGVL), which does not encode any antigen, was from the National Gene Vector Laboratory (Indianapolis, IN). It was amplified and purified using an endo-free plasmid purification kit from Qiagen (Valencia, CA). Aluminum hydroxide (Alum) gel (USP grade) was purchased from Spectrum Chemical and Laboratory Products (New Brunswick, NJ). Polystyrene 96-well plates were purchased from Corning Costar (Corning, NY). Horseradish peroxidase (HRP)-labeled anti-mouse immunoglobulins (IgG, IgG1, IgG2a, and IgA) were purchased from Southern Biotechnology Laboratories (Birmingham, AL). RPMI 1640 medium was purchased from Invitrogen (Carlsbad, CA).

B.3.2 Preparation of Liposome and LPD

LPD and liposomes were prepared as previously described (Cui and Huang, 2005). Briefly, 100-nm size small unilamellar liposomes composed of DOTAP (10 mg/mL) and cholesterol (molar ratio 1:1) were prepared by the thin film hydration method followed by membrane extrusions. The LPD was composed of DOTAP/cholesterol liposomes, protamine, and pNGVL in a ratio of 9.0:0.6:1.0 (w/w/w), respectively. To prepare the LPD, 21.5 μL of liposome (10 mg/mL) and 7.5 μL of protamine (2 mg/mL) were dispersed in 75 μL of aqueous solution containing 10% dextrose. Another 75 μL of aqueous solution containing 50 μg of pNGVL with

30 µg of rPA was added drop wise into the mixture of liposomes and protamine while gently shaking. The incorporation efficiency of the rPA in the LPD particles was estimated to be ~80% using SDS-PAGE gel electrophoresis (Sloat and Cui, 2005). In the SDS-PAGE gel, unincorporated rPA moved into the gel, while those incorporated remained in the loading well. A comparison of the intensity of the rPA band from rPA/LPD preparation with that when the same amount of pure rPA was loaded in a different well in the same gel allowed us to roughly estimate the incorporation efficiency.

B.3.3 Intranasal Immunization

All animal studies were carried out following National Institutes of Health guidelines for animal use and care. Anesthetized mice (n = 5, i.p., pentobarbital, 6-7 mg/100 g) were given a total volume of 25 µL (5 µg rPA/mouse) of the rPA/LPD in suspension, which was divided into two-12.5 µL and dosed 10-15 min apart. Non-anesthetized mice (n = 5) were similarly given a total of 25 µL of rPA/LPD while fully awake, half in each nare. Additionally, as controls, mice (n = 5) were intranasally dosed with rPA alone in phosphate buffered saline (PBS, 10 mM, pH 7.4), subcutaneously (s.c.) injected with rPA (5 µg/mouse) admixed with Alum (15 µg/mouse) as an adjuvant, or left untreated. Mice were dosed on days 0, 7, and 14. They were euthanized and bled by cardiac puncture on day 28. The experiment was repeated independently twice.

B.3.4 Collection of Nasal Lavage (NL) and Broncho-Alveolar Lavage (BAL)

Twenty-eight days after the first immunization, mice were euthanized, and NLs and BALs were collected. The trachea was exposed. To obtain NLs, 0.2 mL of sterile PBS was passed through the trachea toward the nose and collected in a microfuge tube as it exited the nares. BALs were obtained by pipetting 0.4 mL of PBS towards the lungs via the trachea. The lavage was aspirated back into the pipet tip and re-injected once before the final withdrawal of the PBS fluid. Lavage samples were stored at -80°C prior to further use.

B.3.5 ELISA for Anti-PA Ab Measurement

Enzyme-linked immunosorbent assay (ELISA) was used to determine the anti-PA Ab levels. Briefly, EIA/RIA flat bottom, medium binding, polystyrene 96-well plates from Corning Costar were coated with 100 ng of rPA protein dissolved in 100 µL of 0.1 M carbonate buffer (pH 9.6) overnight at 4°C. Plates were washed once with PBS/Tween 20 (10 mM, pH 7.4, 0.05% Tween 20) and blocked with 4% (w/v) BSA in PBS/Tween 20 for 1 hour at 37°C. Serum samples were diluted in 4% BSA/PBS/Tween 20, added to the plates following the removal of the blocking solution, and incubated for an additional 2.5 hours at 37°C. The serum samples were removed, and the plates were washed five times with PBS/Tween 20. HRP-labeled goat-anti-mouse immunoglobulin (IgG, IgA, IgG1, or IgG2a; 5000-fold dilution) was added as the secondary Ab into the plates, and the plates were incubated for one hour at 37°C. The plates were again washed five times with PBS/Tween 20. The presence

of bound Ab was detected following a 30-minute incubation of the plates at room temperature in the presence of TMB substrate followed by the addition of sulfuric acid (0.2 M) as the stop solution. The absorbance was read at 450 nm using a SpectraMax Plate reader (Molecular Devices Inc., Sunnyvale, CA). Anti-PA Ab levels in the BALs and NLs were also determined similarly.

B.3.6 Anthrax lethal Toxin-Neutralization activity (TNA) Assay

TNA was determined as described elsewhere with modifications (Boyaka et al., 2003). Confluent J774A.1 cells were plated (5.0×10^4 cells/well) in sterile, 96-well, clean-bottom plates and incubated at 37°C, 5% CO_2 for overnight. A fresh solution (50 μL) containing LF (40 ng/mL) and rPA (400 ng/mL) was mixed with 50 μL diluted serum sample in triplicate and incubated for 2 hour at 37°C. The cell culture medium was removed, and 100 μL of the serum/LeTx mixture was added to each well and incubated at 37°C, 5% CO_2 for 3 hours. Cell viability was determined using an MTT test kit with untreated cells and LeTx alone treated cells as controls.

B.3.6 Splenocyte Proliferation Assay

Single splenocyte suspension from individual spleens was prepared as previously described (Cui and Mumper, 2002). Splenocytes were cultured at a density of 4×10^6 cells/mL and stimulated with either 0 or 12.5 μg/mL of rPA in complete RPMI medium for 5 days at 37°C, 5% CO_2 (Boyaka et al., 2003). The final cell numbers were determined using an MTT kit. Cell proliferation ratio was reported as

the ratio of the final splenocyte number after the cells were cultured in the presence of rPA (12.5 μg/mL) for 5 days over the final number of the same splenocyte preparation after they were cultured in the absence of rPA (0 μg/mL) for 5 days.

B.3.8 Uptake of FITC-labeled LPD particles by phagocytes in BAL and NL

Anesthetized and non-anesthetized mice (n = 3) were dosed as described above with FITC-labeled LPD particles. Four hours later, mice were euthanized, and their BAL and NL were collected. The uptake of FITC-LPD particles by phagocytes in the lavages was analyzed using a flow cytometer (BD LSR II laser benchtop, San Jose, CA). FITC-labeled LPD particles were prepared as stated above with DOTAP liposomes containing FITC-labeled DOPE (0.5 %, w/w). The % of FITC-positive cells was determined using the un-dosed mice as a reference.

B.3.9 Statistics

Statistical analyses were completed using one-way analysis of variance (ANOVA) followed by Fischer's protected least significant difference (PSLD) procedure. When two groups were compared, the t-test was used. A p-value of ≤ 0.05 (two tail) was considered to be statistically significant.

B.4 Results

B.4.1After intranasal immunization with rPA incorporated into LPD particles (rPA/LPD), the level of anti-PA IgG induced in anesthetized mice was greater than that in non-anesthetized mice.

As shown in Fig. B.1A, anesthetized mice intranasally immunized with the rPA/LPD had levels of anti-PA IgG similar to that in mice s.c. immunized with rPA adjuvanted with Alum in their sera 28 days after the first immunization. The anti-PA IgG titers in the anesthetized mice immunized with the rPA/LPD and in mice injected (s.c.) with rPA adjuvanted with Alum were comparable (Fig. B.1B). Anti-PA IgG was also induced in non-anesthetized mice nasally immunized with the rPA/LPD, although the level was lower than that in anesthetized mice (Fig. B.1A). The end-point titer of the anti-PA IgG in the non-anesthetized mice was above 100.

B.4.2 The anti-PA response was Th2-biased.

To understand the T helper (Th) cell response induced, the levels of anti-PA IgG subtypes, IgG1 and IgG2a, in the serum were also measured using ELISA. As shown in Fig. B.2, similarly to s.c. injection of rPA adjuvanted with Alum, anesthetized mice intranasally immunized with rPA/LPD produced a high level of anti-PA IgG1, but not IgG2a, indicating that the immune response induced was Th2-biased. Although relatively weaker, the immune response induced by the nasal rPA/LPD in non-anesthetized mice was also Th2-biased (Fig. B.2A).

B.4.3 Nasal rPA/LPD induced specific anti-PA IgA in the BALs and NLs of anesthetized mice.

Specific anti-PA IgA levels in the BALs and NLs were also measured using ELISA to evaluate the mucosal immune response induced. As shown in Fig. B.3, anti-PA IgA was detected in both the NLs and the BALs of anesthetized mice nasally

immunized with the rPA/LPD. In fact, after 100-fold dilution, the OD450 in the BAL samples of the anesthetized mice was still more than 2-fold higher than that of the untreated control. However, anti-PA IgA was not detected in the NLs and BALs of non-anesthetized mice when the samples were diluted 10-fold. It is possible that anti-PA IgA could be detectable at a lower dilution. As expected, no anti-PA IgA was detected in the BALs and NLs of mice s.c. injected with rPA adjuvanted with Alum.

B.4.4 Splenocytes isolated from both anesthetized and non-anesthetized mice immunized with nasal rPA/LPD proliferated after in vitro stimulation with rPA.

To investigate whether a proliferative immune response was induced by the nasal rPA/LPD vaccine, splenocytes isolated from immunized mice were cultured in the presence of rPA for 5 days, and the cell proliferation was measured. As shown in Fig. B.4, splenocytes isolated from both anesthetized and non-anesthetized mice nasally immunized with the rPA/LPD proliferated significantly. The proliferation of the splenocytes isolated from the non-anesthetized mice tended to be weaker than that from anesthetized mice, but not significant due to the relatively large standard deviations observed.

B.4.5 Nasal immunization with rPA/LPD induced anthrax lethal toxin neutralization activity in both anesthetized and non-anesthetized mice.

An in vitro macrophage (J774A.1) protection assay was carried out to evaluate the protective activity of the anti-PA Abs induced. Fig. B.5 shows that the LeTx neutralization activity induced in the sera of anesthetized mice nasally dosed with the rPA/LPD was comparable to that in mice s.c. injected with rPA adjuvanted with

Alum. The LeTx-neutralization activity detected in the serum of non-anesthetized mice nasally dosed with rPA/LPD was weaker than that in the anesthetized mice (Fig. 5). In addition, a weak LeTx neutralization activity was also detected in the NLs and BALs of some of the mice. After a 20-fold dilution, LeTx neutralization activity was detectable in the BALs of 80% (4 out of 5) anesthetized mice (i.e., 26 ± 8% of the J774A.1 cells were still alive when challenged with LeTx) and 3 out of 5 non-anesthetized mice (18 ± 5% of the J774A.1 cells were still alive). Similarly, after a 5-fold dilution, LeTx neutralization activity was detectable in the NLs of all of the anesthetized mice (25 ± 3% of the J774A.1 cells were still alive).

B.4.6 Deposition rPA-LPD particles in the nose and lung of anesthetized and non-anesthetized mice.

To define the deposition of the nasally dosed rPA/LPD particles in the nose and lung of the mice, the uptake of rPA/LPD by phagocytes in the mucus layer of the nose and the lung was measured. A FITC-labeled rPA/LPD suspension was intranasally administered to anesthetized and non-anesthetized mice. Four hours later, the BALs and NLs were collected and analyzed using flow cytometry. More phagocytes in the NLs of anesthetized mice were FITC positive (24.9 ± 12.8%) than in the non-anesthetized mice (3.19 ± 0.96%). Surprisingly, FITC-labeled rPA/LPD particles were rarely found in the BALs of both anesthetized and non-anesthetized mice, suggesting a very limited deposition of the rPA/LPD particles into the lung after the nasal administration.

B.5 Discussion

Previously, we have demonstrated that protective anti-PA immune responses were induced in the BALs and NLs, as well as the systemic compartment of anesthetized mice nasally immunized with rPA incorporated into LPD particles. In the present study, we hypothesized that significant differences will exist between the anti-PA immune responses induced in anesthetized and non-anesthetized mice when the mice are nasally dosed with rPA/LPD. While using general anesthesia makes it easier to intranasally dose mice, the immune responses induced in anesthetized mice could be very different from that in the non-anesthetized mice.

In agreement with our previous data (Sloat and Cui, 2005), anesthetized mice were found to have significantly stronger anti-PA Ab responses and LeTx neutralization activity in both the mucosal and systemic compartments (Fig. B.1 and B.5); however, the anti-PA Ab response and LeTx neutralization activity in non-anesthetized mice were relatively weaker (Fig B.1 and B.5). This is in agreement with others showing that anesthetics, regardless of the method of delivery (e.g., i.p., i.v., inhalational), generally led to stronger specific immune responses in anesthetized mice (Bracci et al., 2005; Janakova et al., 2002). For example, Janakova et al. (2002) reported that when lightly anesthetized mice were immunized with a meningococcal outer membrane vesicle vaccine, the median Ab concentrations were close to 10 times higher than that in non-anesthetized mice (Janakova et al., 2002). Similarly, Braccci et al. (2005) reported that a single intranasal immunization of anesthetized mice with hemagglutinin mixed with type I interferon (IFN) as an adjuvant induced a significant

increase in serum IgG and IgA, while that induced in non-anesthetized mice was relatively weaker (Bracci et al., 2005). It has been shown that in fully awake mice, fluid applied to the nares was not easily inhaled, but a volume of 20 μL or more was inhaled rapidly while the mice were under anesthesia (Yetter et al., 1980). Moreover, it was demonstrated that particles administered as drops of liquid suspension into the nares of anesthetized mice filled the lower respiratory tract 1,000-fold more than in non-anesthetized mice (Janakova et al., 2002). Antigens taken up by the bronchus-associated lymphoid tissues (BALTs) in the lung was thought to contribute to the stronger immune response induced in anesthetized mice (Eyles et al., 1998; Neutra et al., 1996). It was previously considered to be advantageous to have a vaccine reach the lung to achieve a total respiratory immunization in humans (de Haan et al., 1995; el Guink et al., 1989; Yetter et al., 1980). However, deeply inhaled vaccine may lead to severe pulmonary inflammations (Saunders et al., 1999; Simecka et al., 2000; Szarka et al., 1997).

The anesthetized mice had a strong anti-PA IgA response in both the BALs and NLs (Fig. B.3). Similarly, LeTx neutralization activity was detected in 5 out of 5 of the NLs (5-fold dilution) and 4 out of 5 of the BALs (20-fold dilution) of the anesthetized mice. However, no anti-PA IgA was detected in the BALs and NLs of non-anesthetized mice when the lavages were diluted 10 times (Fig. B.3). Weaker LeTx neutralization activity was detected in the BALs of some of the non-anesthetized mice, suggesting the existence of a weak anti-PA immunity in them. Our results were consistent with a previous study using a different vaccine, where measurable levels of

secretory IgA were detected in the respiratory tract of anesthetized mice, but not in non-anesthetized mice (Bracci et al., 2005). Interestingly, Janakova et al. reported that anesthesia led to a higher level of specific Abs in serum but not in the mucosal secretions (Janakova et al., 2002). Janakova et al. (2002) lightly anesthetized mice by i.v. injection of propofol. The mice recovered completely 1 to 2 min after the anesthesia administration (Janakova et al., 2002). However, it is unlikely that this light anesthesia *per se* was responsible for the difference observed. For example, de Hann et al. (1995) previously showed that the mucosal immune responses induced by a nasal vaccine in heavily anesthetized mice (i.e., pentobarbital) were significantly stronger than that in non-anesthetized mice (de Haan et al., 1995). Similarly, Ryan et al. (1999) have also shown that the specific IgA titers induced by a nasal vaccine in lightly anesthetized mice (i.e., isofluorane) were also significantly stronger than that in non-anesthetized mice (Ryan et al., 1999).

To preliminarily elucidate the mechanism behind the weaker anti-PA response induced in non-anesthetized mice by our rPA/LPD particles, we identified the location of the rPA/LPD particles by measuring the uptake of nasally administered FITC-labeled LPD particles by phagocytes in the NLs and BALs. Surprisingly, unlike what has been reported by others, no significant difference was revealed in the uptake of FITC-labeled LPD particles by the phagocytes in the BALs from anesthetized and non-anesthetized mice. In fact, the uptake of FITC-labeled LPD particles by phagocytes in the BALs in both anesthetized and non-anesthetized mice was very limited (i.e., < 1% of the phagocytes was FITC positive). Eyles et al. (1998)

demonstrated that a volume of 50 µL of nasal solution could result in as high as 40% of it entering the lung; however, when a smaller volume of 10 µL was dosed, it was primarily retained in the nasal airways in mice (Eyles et al., 1998). In our present study, a volume of about 25 µL was divided into two-12.5 µL, which were then dosed 10-15 min apart, suggesting that 25 µL was still a relatively small volume. We suspect that the greater retention of FITC-LPD particles in the nasal airways of the anesthetized mice than in the non-anesthetized mice might contribute to the increased anti-PA IgG response and LeTx neutralization activity induced in the anesthetized mice (Fig B.1-B.3, B.5). Finally, although it is difficult to predict to what extent the nasally administered rPA/LPD particles had traveled into the gastrointestinal tract, we believe that the contribution of the gut-associated lymphoid tissues (GALTs) in the induction of anti-PA immune responses by the rPA/LPD particles was very limited. A previous study had shown that, when influenza surface antigen hemaglutinin (HA)-entrapped liposomes were orally dosed to mice, they failed to induce any anti-HA immune responses; while the same amount of the HA-entrapped liposomes, when dosed intranasally, were able to induce strong anti-HA immune responses, both systemic and mucosal (de Haan et al., 1995).

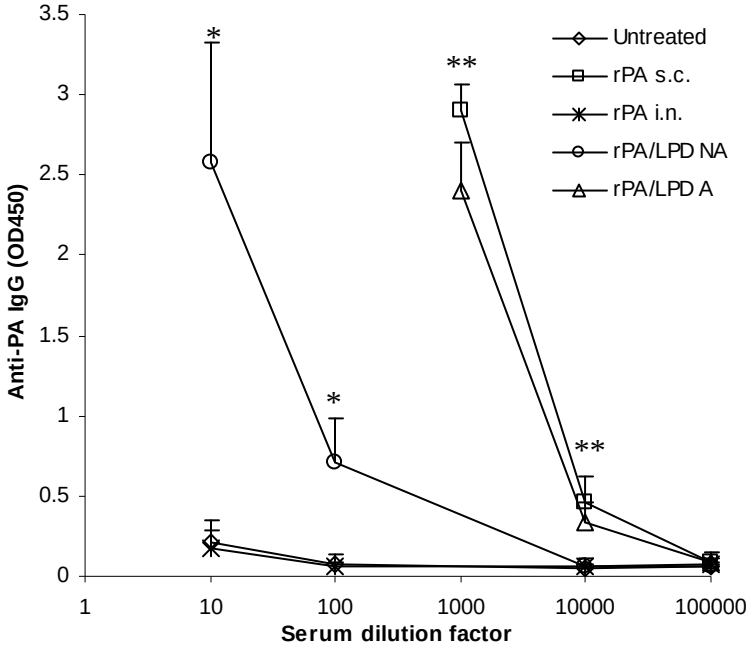
Although the anti-PA IgG level in non-anesthetized mice was much lower than in anesthetized mice, it was interesting to observe that splenocytes isolated from anesthetized and non-anesthetized mice proliferated comparably after a five-day in vitro re-stimulation (Fig B.4). The re-stimulation step might have made the otherwise different proliferation rates comparable (Lu et al., 2004). Moreover, it is important to

point out that, although the anti-PA Ab response induced by the rPA/LPD in the anesthetized mice was significantly stronger than that in the non-anesthetized mice, the immune response remained to be Th2 biased. Thus, anesthesia did not change the type of the anti-PA immune response induced (Fig. B.2A).

Finally, from this study we can clearly conclude that the immune response to rPA in anesthetized mice nasally dosed with rPA/LPD was stronger than that in non-anesthetized mice. Despite this, nasal rPA/LPD still holds great potentials in preventing inhalational anthrax infection. It has been suggested that unlike in mice, vaccines administered intranasally as drops may lead to strong systemic Ab responses in humans (Janakova et al., 2002). Although the Nasal Flu® had been pulled off the market due to its strong association with the Bell's Palsy disease (Mutsch et al., 2004), the Flumist® is currently available for human use, and no unexpected serious post-approval adversary effects have been identified with this vaccine (Izurieta et al., 2005). The Flumist® is administered intranasally as one 0.5 mL dose with annual boosters to account for antigenic variations in the influenza strains. A sprayer is inserted into the nose, and half of the dose is administered in each nostril. We suspect that the rPA/LPD vaccine will be more effective in fully awake humans if it is dosed using a device similar to that for the administration of the Flumist®. Moreover, even though the nasal rPA/LPD induced higher levels of anti-PA Abs in anesthetized mice than in non-anesthetized mice, it remains to be seen whether the anesthetics would make a difference in protection against an inhalational anthrax spore challenge. Nevertheless, our future studies will be focused on improving the immune response

induced by the rPA/LPD particles in non-anesthetized mice, which might be accomplished by either increasing the dose of the rPA/LPD or by increasing the retention of the rPA/LPD particles in the nasal cavity. We are currently preparing a mucoadhesive rPA/LPD formulation, hypothesizing that it will help retain more nasally dosed rPA/LPD particles in the nasal cavity of non-anesthetized mice, and thus, will lead to enhanced anti-PA immune responses.

(A)


3.5
3
2.5
2
1.5
1
0.5
0
Anti-PA IgG (OD450)
1
10
100
1000
10000
100000
Serum dilution factor
Untreated
rPA s.c.
rPA i.n.
rPA/LPD NA
rPA/LPD A
*
*
**
**

(B)

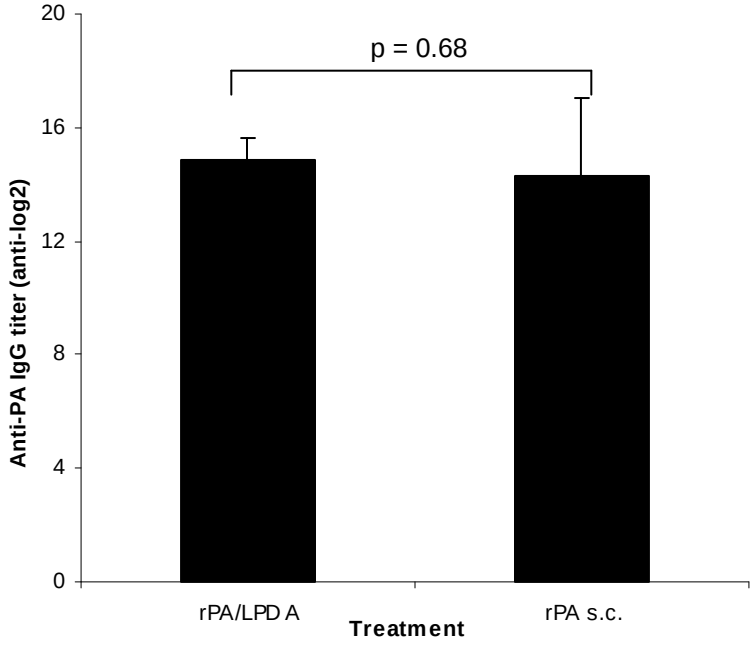
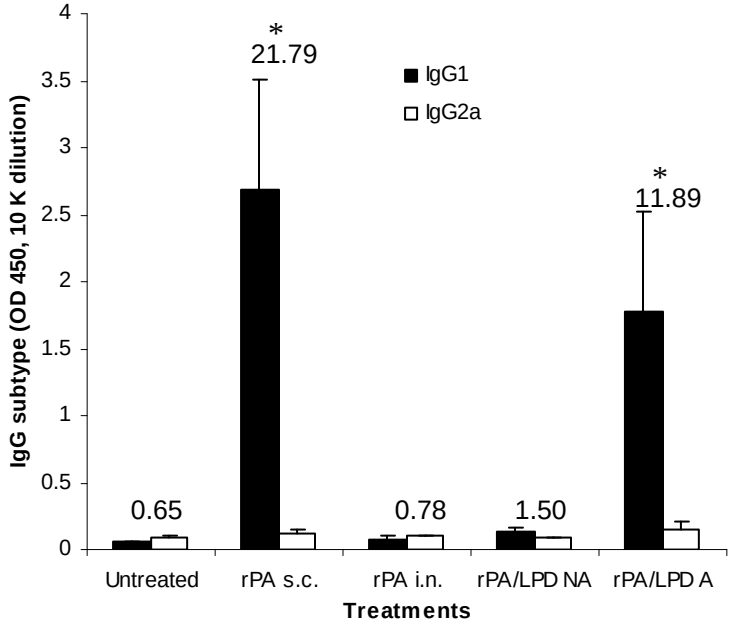

20
16
12
8
4
0
Anti-PA IgG titer (anti-log2)
p = 0.68
rPA/LPD A
rPA s.c.
Treatment

Figure B.1

Figure B.1 Serum anti-PA IgG Ab response. Anesthetized (rPA/LPD A) and non-anesthetized (rPA/LPD NA) Balb/C mice (n = 5) were nasally dosed with rPA (5 μg) incorporated in the LPD on days 0, 7, and 14. Other groups (n = 5) were nasally dosed (i.n.) with rPA (5 μg) alone, subcutaneously (s.c.) injected with rPA adjuvanted with Alum (15 μg/mouse), or left untreated. On day 28, mice were euthanized, and their serum samples were collected. (a) The total anti-PA IgG levels in mouse sera were determined after the sera were diluted 10-fold serially. * indicates the values for rPA/LPD (NA) were significantly different from that of the untreated and the rPA (i.n.) in the 10- and 100-fold dilutions. ** indicates that, at the 1,000- and 10,000-fold dilutions, the values for rPA (s.c.) and rPA/LPD (A) were not significantly different from each other, but higher than that of the others. At the 100,000-fold dilution, there was no significant difference among the OD450 values from all five treatments. (b) The anti-PA IgG titers in mice s.c. injected with rPA adjuvanted with Alum or in anesthetized mice nasally dosed with the rPA/LPD. Serum samples were diluted 2-fold serially. The two values were not different from each other ($p = 0.68$). Data shown were mean ± S.E.M. (n = 5). The experiment was repeated independently twice, and similar results were obtained. Data from one representative were shown.

(A)


IgG subtype (OD 450, 10 K dilution)
4
3.5
3
2.5
2
1.5
1
0.5
0
*
21.79
*
11.89
IgG1
IgG2a
0.65
0.78
1.50
Untreated
rPA s.c.
rPA i.n.
rPA/LPD NA
rPA/LPD A
Treatments

(B)

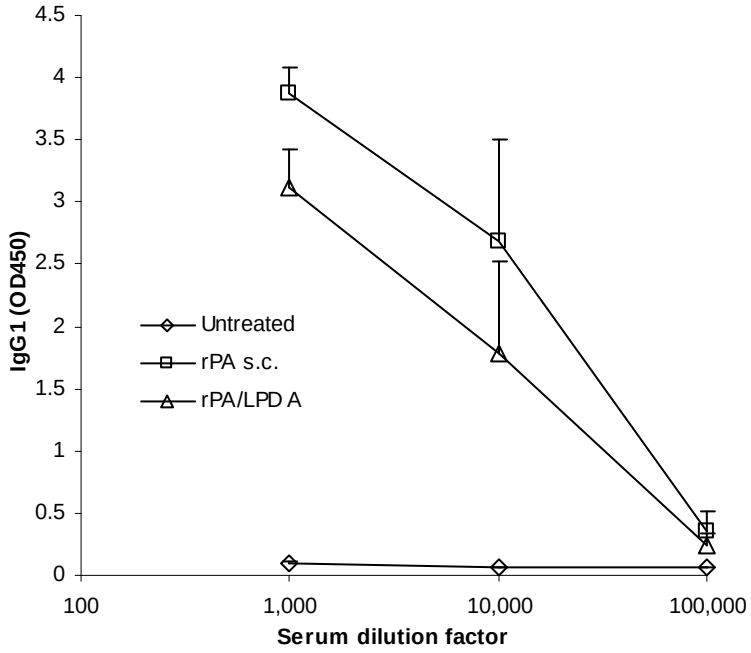

IgG1 (OD450)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Untreated
rPA s.c.
rPA/LPD A
100
1,000
10,000
100,000
Serum dilution factor

Figure B.2

Figure B.2 Serum anti-PA IgG subtypes. (a) Anesthetized (rPA/LPD A) and non-anesthetized (rPA/LPD NA) Balb/C mice (n = 5) were intranasally dosed with rPA/LPD on days 0, 7, and 14. Other groups (n = 5) were treated (i.n.) with rPA alone, s.c. injected with rPA adjuvanted with Alum, or left untreated. The IgG1 and IgG2a levels in the sera on day 28 were determined using ELISA after the sera were diluted 10^4-fold. * indicates that the IgG1 values for rPA (s.c.) and rPA/LPD (A) were not different from each other. Numbers above the bars were the values of the $OD450_{IgG1}/OD450_{IgG2a}$. (b) The OD450 values of the anti-PA IgG1 levels in the sera of mice s.c. injected with rPA adjuvanted with Alum and in anesthetized mice nasally immunized with rPA/LPD particles after the sera were diluted 1,000-, 10,000-, and 100,000-fold. Data shown were mean ± S.D. (n = 5).

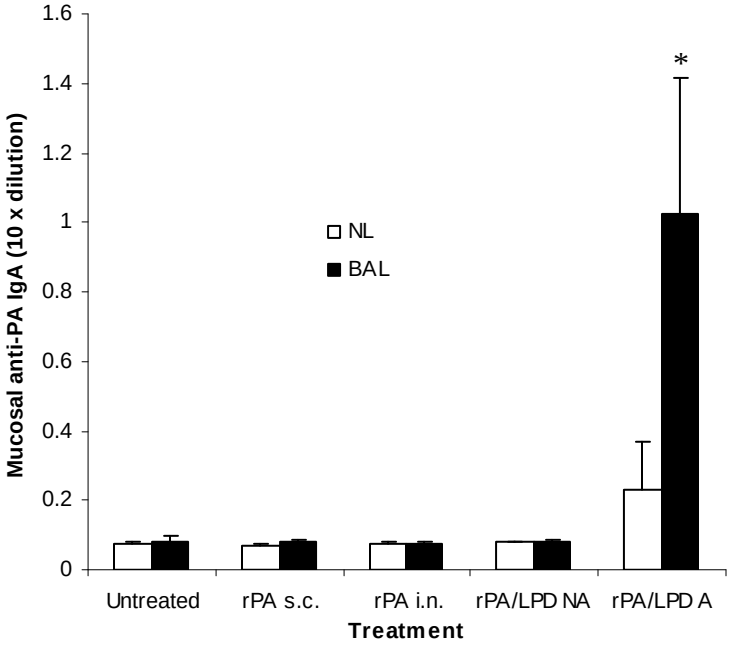


Figure B.3 Anti-PA IgA in the nasal and broncho-alveolar lavages. Anesthetized (rPA/LPD A) and non-anesthetized (rPA/LPD NA) Balb/C mice (n = 5) were intranasally dosed with rPA/LPD on days 0, 7, and 14. Other groups (n = 5) were treated with rPA alone, s.c. injected with rPA adjuvanted with Alum, or left untreated. On day 28, mice were euthanized, and their NL and BAL samples were collected. The IgA level was determined using ELISA after the lavages were diluted 10-fold. * indicates that the OD450 values in the NLs and BALs of the anesthetized mice rPA/LPD (A) were significantly different from that of the others.

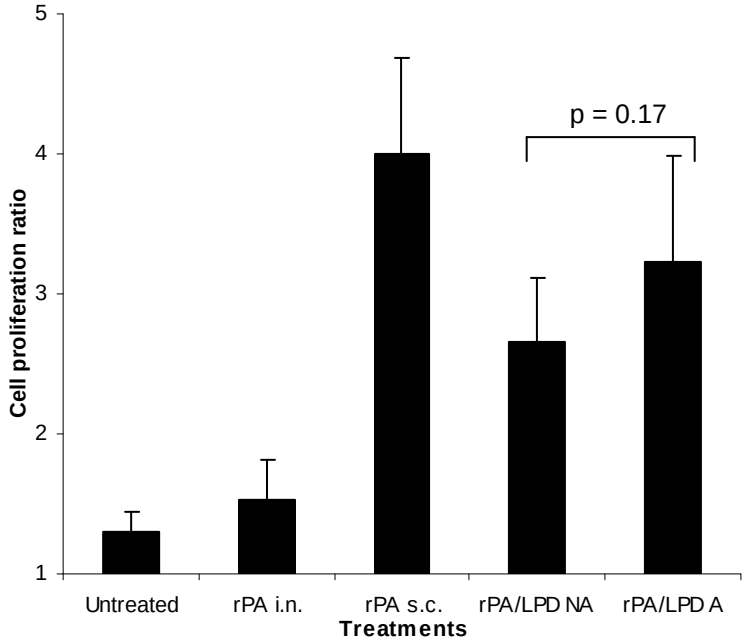


Figure B.4 Proliferation of splenocytes after in vitro re-stimulation with rPA. Anesthetized (rPA/LPD A) and non-anesthetized (rPA/LPD NA) Balb/C mice (n = 5) were intranasally dosed with rPA/LPD on days 0, 7, and 14. Other groups (n = 5) were treated with rPA alone, s.c. injected with rPA adjuvanted with Alum, or left untreated. On day 28, mice were euthanized, and their spleens were harvested. Single splenocyte suspension from individual spleens was prepared. Splenocytes (4 x 10^6/mL) were incubated with rPA (12.5 μg/mL) for 5 days. The final splenocyte number was determined using an MTT kit. The proliferation ratio was calculated by dividing the final splenocyte number after the splenocytes were cultured in the presence of rPA for 5 days by the final splenocyte number after the splenocytes were cultured in the absence of rPA for 5 days. The p value of 0.17 indicates that the values for rPA/LPD (NA) and rPA/LPD (A) were comparable to each other. The value for the rPA (s.c.) was not different from that of the rPA/LPD (NA) (p = 0.13), but was higher than that of the others.

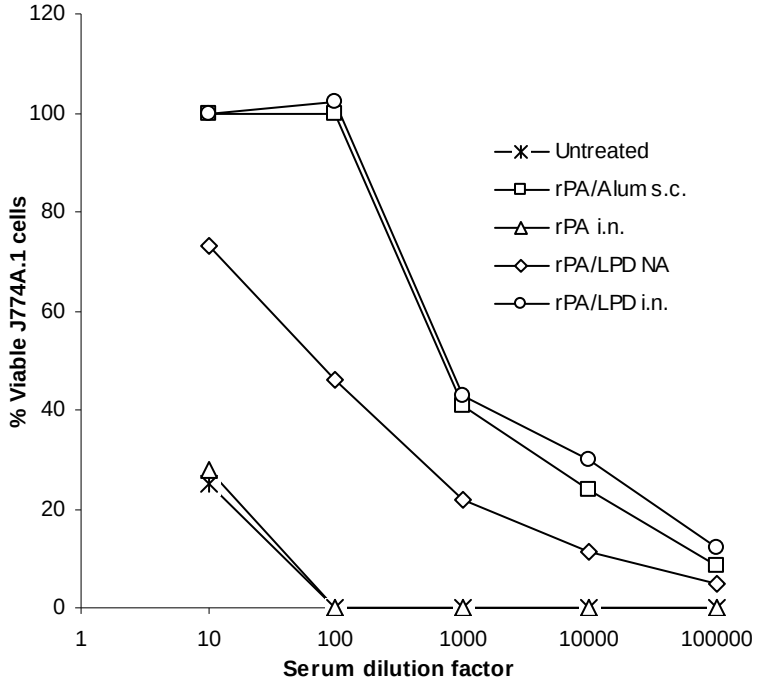


Figure B.5 Intranasal rPA/LPD induced LeTx neutralization activity in both anesthetized and non-anesthetized mice. Balb/C mice (n = 5) were dosed as mentioned in Materials and Methods. On day 28, sera were collected. Each sample was diluted 10-fold serially and incubated with J774A.1 cells (5 x 10^5/mL) in the presence of anthrax LeTx (400 ng/mL of PA and 40 ng/mL of LF) for 3 h. The percent of viable cells was calculated in comparison to the number of cells remained after they were treated with either LeTx alone or were left untreated. Data shown were the means from five mice. Standard deviations were not shown for a better view of the trend.

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