1. Literature Review

1.1 *Bacillus anthracis*

1.1.1 History

Anthrax was the first disease for which a causative bacterium, *Bacillus anthracis*, was positively identified (35). The disease derives its name from the Greek word for coal, *anthrakis*, due to the coal-like black lesions found on the skin in cutaneous anthrax (50). Casimir Davaine and Pierre Rayer first observed rod-like organisms present in the blood of anthrax-infected animals and humans in 1850. By 1863, Davaine showed that those rods were most likely the cause of anthrax since unexposed sheep did not develop the disease (25). Robert Koch developed a method for culturing pure *B. anthracis* in 1876. This method allowed him to be the first to elucidate the complete life cycle of anthrax from spore to vegetative bacterium and back to spore again (10,25). Koch also used *B. anthracis* to develop and prove his postulates regarding the germ theory of disease (25). Louis Pasteur created the first major vaccine against anthrax in livestock in 1881 (10,25). However, despite the existence of anthrax vaccines since around 1870, the disease remains a threat to livestock and even humans particularly in developing continents such as Asia, Africa and South America (35). In addition, *B. anthracis* was documented to have been part of the biological arsenals of many nations, including the U.S. at one time. With the Biological Weapons Convention of 1972, production of these weapons was outlawed (47). Even so, *B. anthracis* is still believed to be part of the biological arsenals of a least 17 nations (50). Taking into consideration the current world environment and the unpredictable nature of terrorism, developing a highly effective

vaccine with the ability to fully protect against all forms of the disease would be an important component to add to our national biological defense arsenal (21,25).

1.1.2 Biology of *Bacillus anthracis*

B. anthracis is an aerobic, gram-positive, non-motile rod (62). The bacterium measures 1-1.5 m by 3-10 m (49). Spore formation occurs centrally or paracentrally and causes no bacterial swelling (31,50). Spore formation occurs when nutrients are depleted as happens after host death and exposure to air (8). *B. anthracis* spores are highly resistant to various environmental changes and can survive indefinitely in soil, air, water and vegetation despite extreme heat or cold, dessication, chemical treatment or ultraviolet exposure (33,35,49). The highly resistant nature of the spore aids in the persistence of the disease in an area (33). The bacteria grow readily on all conventional microbiology media at 37°C including sheep blood agar and produce non-hemolytic colonies (50). Colony appearance on agar is typically 4-5mm rough, white colonies with a characteristic comma shape or tail often referred to as "curly-hair" or “medusa head” colonies (49,50). *B. anthracis* occurs singly or in pairs in tissue and forms long chains in culture giving a classic "boxcar" appearance (35).

B. anthracis is part of the *B. cereus* group of bacilli which includes *B. cereus*, *B. thuringiensis*, and *B. mycoides* (31). Anthrax can be differentiated from other members of the group by several methods. All members of the *B. cereus* group, except *B. anthracis*, are resistant to penicillin because of a chromosomally encoded betalactamase (31). Other characteristics, which differentiate *B. anthracis* from other *Bacillus* species, are the absence of hemolysis, lack of motility and the presence of an antiphagocytic capsule consisting of D-glutamic acid (49).

1.1.3 Pathogenesis of *Bacillus anthracis*

The disease manifests itself in one of three forms: cutaneous, inhalational or gastrointestinal depending upon the route of spore entry (33,78). The two latter forms, inhalational and gastrointestinal anthrax, are the most fatal and rare. Cutaneous anthrax accounts for up to 95% of all anthrax infections throughout the world and is mainly due to occupational exposure (8,31). The most common areas of exposure with cutaneous anthrax are the head, neck and limbs (31). Spores are often introduced subcutaneously via a cut or skin abrasion, although skin trauma may not be required (8,31). Incubation periods after spore exposure generally range from 1-10 days (8). Initial symptoms often present as a painless, pruritic papule that resembles an insect bite at the site of infection (8). The papule becomes vesiculated in 1-2 days with occasional hemorrhage (8). These vesicles rupture to form depressed ulcers with focal edema that develop the characteristic dry necrotic black center (8,31). Generally the disease will remain localized, however, patients may develop systemic symptoms including fever, malaise and headache (8). Antibiotic treatment does not halt the progression of the papule to ulceration (31,50). Differential diagnosis includes brown recluse spider bite, cellulitis, ulceroglandular tularemia, accidental vaccinia, ecthyma gangrenosum, and cat scratch disease (8). Gram stain and culture of any lesions are recommended for diagnosis before antibiotic treatment is initiated (8,31). The mortality rate for cutaneous anthrax without antibiotic treatment is reported as 20%, while with antibiotic treatment, death is rare (50).

Inhalational anthrax occurs as the result of spore deposition in the alveolar spaces of the lung (50). Historically, inhalational anthrax is a rare occupational disease of people who worked with raw wool, hence the name “wool-sorters disease” (8). However,

as evidenced by the 1979 Sverdlovsk incident in the former USSR and the intentional release of spores in the United States in 2001, inhalational anthrax would be the form most often seen in a biowarfare or bioterrorism event (8). Once inside the lung, alveolar macrophages engulf the spores and transport them to the mediastinal and hilar lymph nodes where they germinate to vegetative bacteria (8,31). Upon germination, the bacteria begin multiplication and production of toxin (31). Once germination occurs, symptoms of disease onset appear rapidly (50). Typically onset occurs 1-10 days after exposure (31). Inhalational anthrax is a fulminant disease that most often occurs in 2 stages (8). Initial symptoms are often nondescript or "flu-like" and similar to those of atypical pneumonia (8,31). Stage 1 symptoms include fever, chills, drenching sweats, headache, non-productive cough, chest pain, nausea and vomiting (8,50). This stage can last from a few hours to a few days and may be followed by a brief apparent recovery (50). Stage 2 soon follows and is characterized by dyspnea, fever, diaphoresis and shock (50). Chest radiographs often show a widened mediastinum, which is consistent with lymphadenopathy (50). Pleural effusions are highly characteristic of this disease and usually contain bloody fluid (8). Use of computerized tomography (CT) scans of the chest show characteristic features of hyperdense (hemorrhagic) mediastinal and hilar lymph nodes, mediastinal edema and pleural effusions (8). Differential diagnosis includes influenza, tuberculosis, tularemia, sarcoidosis, histoplasmosis, lymphoma, silicosis, tumor, aneurysm and alveolar proteinosis (8). Blood culture and *B. anthracis* polymerase chain reaction (PCR) of sterile fluids are important in the diagnosis of inhalational anthrax (8). Treatment with antibiotics is required but initiation generally should begin before stage 2 begins in order to ensure survival of the patient (8,49).

Mortality rates for inhalational anthrax generally range between 89-99% but could be considerably lower if treatment is begun before stage 2 occurs (8,49,50).

Gastrointestinal anthrax, the most rare form of anthrax, can manifest itself in one of two ways depending upon where the spores deposit themselves along the gastrointestinal tract. Spores, which settle in the upper gastrointestinal tract, lead to the development of oropharyngeal anthrax. While spores settling in the lower gastrointestinal tract, including the terminal ileum and cecum, develop gastrointestinal anthrax (49,50). Gastrointestinal anthrax develops as the result of ingesting spore contaminated meat (31). Incubation generally ranges from 2-5 days (31). Pathologic examination of infected mesentary shows the presence of bacilli in the mucosal and submucosal lymphatic tissue as well as mesenteric lymphadenitis (31). Ulceration of the mesentary is a characteristic symptom of gastrointestinal anthrax (31,49). Other common symptoms seen in this form of anthrax include regional lymphadenopathy, edema, nausea, vomiting, malaise, bloody diarrhea, acute abdomen, fever, dysphagia and sepsis (31,49). Morbidity is due to blood loss, fluid and electrolyte imbalance and shock (49). Death results from intestinal perforations or anthrax toxemia (31). Diagnosis is often the result of gram staining of peritoneal fluid to reveal large bacilli or the culturing of ascites fluid (31,49). Gastrointestinal anthrax has an extremely high rate of mortality given the difficulty of early diagnosis (50). Antibiotic treatment may save the patient from sepsis and death, however, like cutaneous anthrax, it cannot halt the progression of the ulcers (50).

Infection by *B. anthracis* begins when spores are introduced through the skin or mucosa (39). Spores are then phagocytosed by local macrophages and transported to the

regional lymph nodes that drain the site of introduction (79). *B. anthracis* is an extracellular pathogen that requires an intracellular step to initiate infection (79). Those spores, which survive phagocytosis, germinate inside the macrophage (31). The specific trigger for spore germination is unknown. However, generally spores begin germinating upon entering an environment rich in amino acids, nucleosides and glucose such as is found in an animal or human host (50). It is theorized that spore germination may be triggered inside the macrophage by host-specific signals such as elevated temperature (>37°C) and CO_2 concentrations (>5%) and presence of serum components (31). Virulence plasmid pXO1 encodes a germination operon *ger*X whose deletion affects germination of spores in macrophages. This operon consists of 3 predicted proteins GerXA, GerXB and GerXC, which may form a receptor specifically detecting germinants within a host (79). The vegetative bacilli are then released by the macrophage and continue to multiply in the lymphatic system (31). The infection extends to successive nodes until the lymphatic system is overwhelmed and the bacilli enter the bloodstream (31,79). Massive septicemia occurs when bacilli count reaches up to 10^7 to 10^8 organisms per milliliter of blood (31).

Fully virulent strains of *B. anthracis* express two known virulence factors, both of which are plasmid-encoded (62). Regulation of expression of the genes encoded on pXO1 and pXO2 is mediated by transcriptional activation of *atx*A encoded on pXO1, whose activity is regulated by the previously mentioned host-specific factors (29,31). pX01 is a 184.5 kilobase pair (kb) plasmid encoding the proteins comprising the anthrax toxins. pXO2 is a 95.3 kb plasmid encoding the genes which make up the poly-D-glutamic acid capsule (31). The genes encoded on pXO2 include *cap*B, *cap*C, *cap*A and

dep for capsule synthesis and degradation (31). In addition, *acp*A, another minor virulence regulatory gene positively affected by *atx*A is also present on the plasmid (12,79). Function of *acp*A appears to be restricted to positive control of capsule gene expression (63).

pXO1 carries the structural toxin genes *pag*A (PA), *lef* (LF), *cya* (EF); regulatory elements; a resolvase and transposase; and the *ger*X operon (79). The 44.8kb region of the plasmid harboring these genes has been termed a pathogenicity island (PI) because it is flanked by inverted IS1627 regions (79). This 44.8kb region or PI is the source of the second known virulence factor: the 3 component exotoxin consisting of the protective antigen (PA), lethal factor (LF), and edema factor (EF) (117). The anthrax toxin works on the common A-B model of bacterial exotoxin activity. This model requires a B or binding moiety and an A or enzymatic moiety for toxic activity to occur. Many common intracellularly acting toxins such as cholera, diphtheria, pertussis and botulinum toxin are explainable using the A-B model (88,122). However, the anthrax toxin is unique in that it consists of one B component and two A components (Figure 1.1) (9,88,118,122). Edema toxin (EdTx) consists of EF, which is a calcium and calmodulin dependent adenyl-cyclase and PA (31). EdTx causes a rise in the intracellular cAMP levels to non-physiological concentrations (60). These high cAMP levels upset water homeostasis and induce massive edema (31). In addition, EdTx inhibits phagocytic and oxidative burst abilities and stimulates chemotaxis of human neutrophil function (31,79). Lethal toxin (LeTx) consists of LF, which is a zinc metalloprotease and PA (41). LeTx inactivates mitogen-activated protein kinase 1 and 2 causing death of macrophages (31,41). The LeTx toxin is principally responsible for anthrax toxemia (60). In addition, LeTx

stimulates macrophages to secrete TNF-α and IL-1β, which mediate damaging inflammatory cascades leading to host shock and death in systemic anthrax (31,49). Both toxins render the host more susceptible to infection (49). Individually, PA, LF, and EF are biologically inactive as toxins (78,111). For toxic activity to occur, PA must be present. PA initially binds to the receptor on the membrane of the target cell and is cleaved by furin from inactive to active form (60). The activated PA then binds with 6 other cleaved PA molecules forming a heptameric pore that serves as the delivery vehicle of LF or EF (41,60). EF and LF bind to this heptamer competitively (9). Once bound the heptamer and factor are endocytosed (60). The endocytic vacuole fuses to an endosome that triggers an acidic pH. The change in pH results in a conformational change in the PA heptamer forming a transmembrane pore through which the associated factor is delivered to the cell cytoplasm (41,60).

1.1.4 Vaccination Strategies

PA is a very important component of the anthrax toxin for this reason: this protein plays a major role in anthrax immunity after both immunization and infection (99). A number of antigens of *B. anthracis* have been studied for their ability to induce protective immunity against the disease. Of the known antigens including the capsule, S-layer, surface polysaccharides and other proteins, only those proteins, which together make up the anthrax toxin, cause detectable production of antibodies (78,102,110). Of the three proteins, EF, LF and PA, only PA elicits antibodies that are protective against the disease (75,100,117). This immunity is thought to occur as a result of neutralizing the activity of

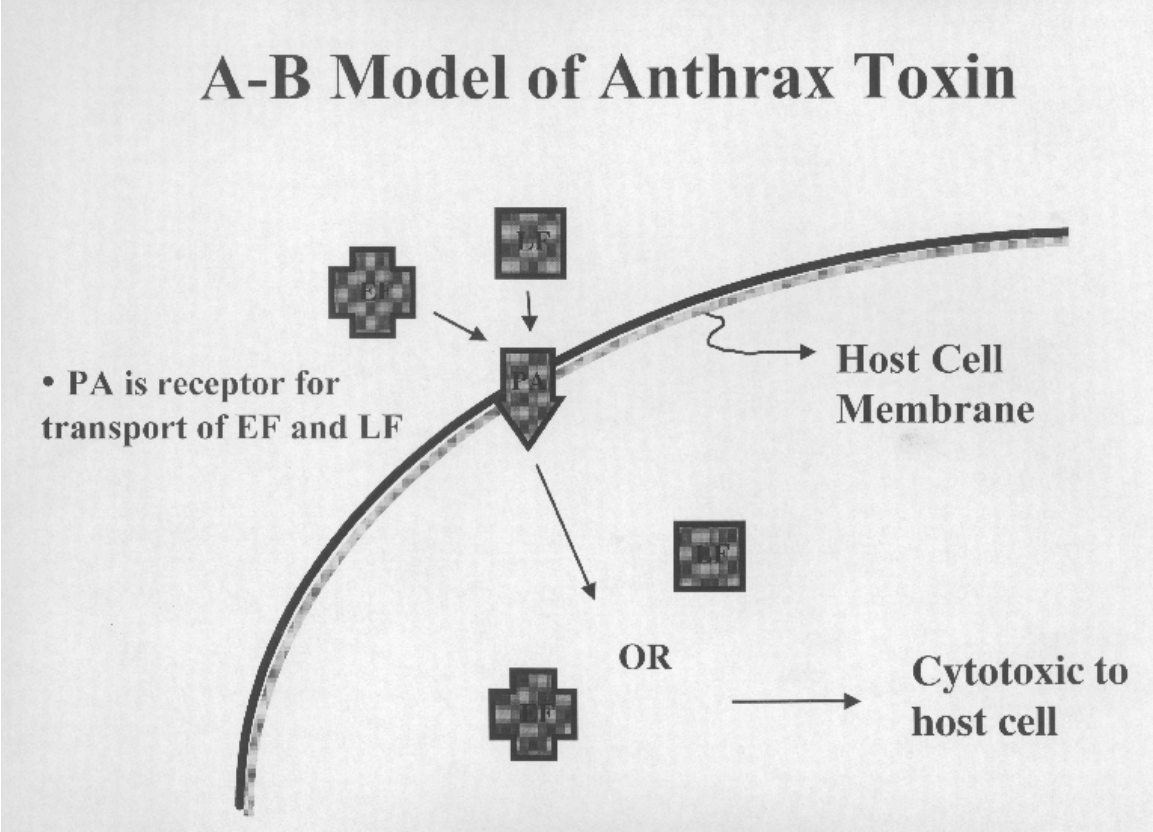

Figure 1.1: The A-B model of the anthrax toxin. The A-B model of anthrax consists of a B moiety and 2 A moieties. Seven PA molecules bind to one cell receptor then act as the effector for the binding and internalization of EF and LF. Since there are two A moieties,EF and LF bind competitively to PA. (A.S. Prince, J Clin. Invest. 112: 656-658, 2003 (89) shows an excellent description of the process.)

the anthrax toxin (36). Antibodies to PA will either block the protein from binding to host cell receptors or once bound will block the action of furin cleavage. Either situation renders PA biologically inactive. Without active PA bound to the cell, EF and LF cannot enter the cell. Thus, the anthrax toxin's influence on the host is halted (102).

Therefore, since PA is the only antigen known to induce protective antibodies against anthrax, the protein has become the main focus of anthrax vaccine research (52,110). PA, when produced in the absence of LF and EF, has been shown to be capable of producing effective protection both as a purified protein and when used in a recombinant or attenuated vaccine (110).

However, protection studies have shown that high antibody titers to PA do not correlate with level of protection (36,108). In fact, the veterinary live spore vaccine produced from the Sterne strain of *B. anthracis*, gives better and more prolonged protection against infection by the bacterium than merely adjuvanted PA even though antibody levels induced are much lower (36,99,104,106,108,110). The knowledge that spore vaccines confer stronger, more reliable immunity to the disease seems to point to a role for cell-mediated immunity (CMI) in protection of the host (28,78,85,99,109,110).

The anthrax vaccine licensed for human use in the United States was developed by the Michigan Department of Public Health (MDPH) and is prepared by BioPort Corporation (75). The AVA (anthrax vaccine adsorbed) is a subunit vaccine in that it is a cell-free extract. The vaccine is an aluminum hydroxide-adsorbed sterile culture filtrate containing mostly PA (55). The filtrate is derived from a fermentor culture of a non-encapsulated, toxigenic strain of *B. anthracis* called V77-NPI-R (55,53). The vaccine strain is cultured in a synthetic medium that promotes synthesis and secretion of PA preferentially over other proteins during the growth phase (55).

The human anthrax vaccine has several negative characteristics. For full immunity, a course of six immunizations over eighteen months followed by annual boosters is required (53,75). Local reactions have been noted in those persons receiving this vaccine in numbers as high as 35%; this local reaction can take the form of local pain, redness and inflammation (53,55,102). Another drawback of this vaccine is the apparent inability of the vaccine to fully protect guinea pigs from aerosol challenge with highly virulent strains of *B. anthracis*, even after a full course of immunizations (28,52,53,55). This last problem could be due to the assumption that only a antibody-

mediated response, mainly to the PA, is enough to confer protection as opposed to a CMI or antibody-mediated response to other anthrax proteins required for full protection (28,53).

The licensed anthrax vaccine for veterinary use, in the United States, is a live spore preparation produced by the Colorado Serum Company (24). The strain of anthrax used in this vaccine was developed by Sterne in the 1930's (105,106,110). The *B. anthracis* Sterne strain is non-encapsulated and attenuated (52). The Sterne strain lacks the pXO2 plasmid encoding the capsule but retains the pXO1 plasmid encoding the exotoxin. Various studies have shown this vaccine to be superior to cell-free vaccines in affording protection even against highly virulent strains of anthrax. This protection is possibly due to the induction of CMI response in the animal (53,78,85,110). The live spore vaccine requires only one initial immunization (two in areas where the disease is endemic) followed by yearly boosters for full immunity (24). However, this anthrax vaccine has two negative characteristics. The strain used in the veterinary vaccine retains the ability to cause local necrosis at the site of injection and disease in some animal species such as goats and llamas (53,78).

It is this possible disease induction, albeit a rare occurrence, which keeps Western nations from using a live vaccine to immunize humans against anthrax (78). However, the former USSR developed a live spore vaccine for human use derived from a Sterne-like strain known as STI. The STI vaccine was licensed for safe administration by scarification and subcutaneous inoculation initially (98,99). Later, after clinical trials, the vaccine was also judged to be safe and effective if given by aerosol route (78,99).

Adverse effects of this vaccine seem to be limited to a transient elevation in temperature and, in the case of subcutaneous injection, a slight swelling at site of inoculation (98).

The efficacy of the STI vaccine is judged by the anthraxin test. Anthraxin is a heat-stable polysaccharide-protein-DNA complex derived from a non-encapsulated strain of *B. anthracis* (99). This complex does not contain capsular or toxigenic material produced by *B. anthracis* (99). The anthraxin skin test works on the principle of the tuberculin skin test and is based on cell-mediated immunity (96,97,98). The anthraxin complex is injected intradermally and read 24 hours later. Positive reactors exhibit local erythema, with a diameter of at least 8 mm, and induration, which lasts for 48 hours (97). This test reliably identifies vaccine-induced immunity in guinea pigs, sheep, and humans, as well as human patients with histories of anthrax 20-30 years in the past, well after antibodies against *B. anthracis* proteins have disappeared (99).

While knowledge of the role of CMI in anthrax immunity is scarce, recent studies have demonstrated that live vaccines (not necessarily live spores) afford better protection than the chemical component vaccines (99). However, patients and health care workers are reluctant to use a live spore anthrax vaccine, even if the strain is avirulent, for fear of its conversion to the virulent form. Therefore, studies of subunit PA vaccines adjuvanted with substances that elicit nonspecific CMI responses are being pursued.

PA alone, with no adjuvant, is unable to completely protect against a spore challenge. This is especially true if the protein becomes degraded. Proteolytic digestion of PA into fragments smaller than the biologically active 63kDa size, yield protein products that are incapable of inducing antibodies able to provide protection (83). In order for PA to induce protective antibodies, the protein must be of the 63-83kDa size.

In studies comparing injection of PA alone or PA combined with some adjuvant, either chemical or bacterial in origin, PA alone was less efficacious that any combination by a factor of about 4 (54). The least efficacious adjuvant was saponin; the same used in the AVA vaccine. Chemical and bacterial product adjuvants, that stimulate CMI, confer higher levels of protection than those that only elicit a antibody-mediated response (56). In fact, PA combinations using bacterial products as adjuvants conferred superior protection over those combined with chemical adjuvants (54). These induced CMI responses are non-specific in nature and do not involve response to PA. Due to this observation, PA has been expressed in several different bacterial and viral species such as *Escherichia coli, Salmonella typhimurium*, *Bacillus subtilis*, and vaccinia virus. These constructs have been tested for vaccination efficacy against virulent anthrax spore challenge (6).

It is hoped that these new live recombinant bacterial strains expressing PA could be used as potential live vaccines against anthrax. The hypothesis is that these live attenuated bacterial strains will be able to induce a CMI response that will enhance the protective abilities of PA against spore challenge. Previous studies have suggested a need for both antibody-mediated and CMI activation to achieve superior immunity against *B. anthracis* (54,56,57).

The first recombinant bacterial strain to express PA was *E. coli*. Leppla and Vodkin cloned the *pag* gene into a plasmid vector, transformed *E. coli* and checked for recombinants using Western blot and ELISA (117). Several colonies producing PA were identified, however, the level of protein expression was extremely low and the PA synthesized was degraded (96). Until recently, one was able to isolate PA from *E. coli,*

but it was badly degraded and functionally inactive. In 1999, researchers in India using *E. coli* were able to purify recombinant PA of correct size and functionally active (44). This recombinant protein will undergo vaccine trials which will be the first such trials using *E. coli*.

PA has been expressed in *S. typhimurium* and the recombinant protein seems to be more stable than that produced by *E. coli* as well as functionally and immunologically active. *S. typhimurium* expressing PA was used in a vaccine trial comparing its efficacy as a live recombinant vaccine against PA protein combined with adjuvants. In this trial, the live vaccine had an efficacy rate of 33% when given orally. This is comparable to the efficacy rate of adjuvanted PA, which conferred 37% protection (28). Further studies into the usefulness of this recombinant strain are being pursued.

In addition to expressing PA in bacteria, the protein has also been expressed in both vaccinia virus and baculovirus. PA was expressed in the WR and Connaught strains of vaccinia virus (48). Vaccine trials of these two recombinant strains in mice showed that WR-PA conferred 60% protection, while the Connaught-PA failed to protect at all (48). The baculovirus-PA strain had a 50% efficacy rate. These results show that PA expressed in a virus is intact, functional and protective. The new constructs could be useful in future vaccine development (48).

Perhaps one of the best characterized recombinant bacterial strains expressing PA is *B. subtilis*. *B. subtilis* clones have been shown to produce PA in levels equal to or greater than those seen in *B. anthracis* (53,100). Expression of PA in this strain seems to be very stable and functionally active (8,51). Vaccination trials utilizing live *B. subtilis* also appear to be very promising (8,51,53). Clones expressing PA have been compared

to both the AVA and live spore vaccines in efficacy studies. Results have shown that the *B. subtilis* clones have efficacy equal to the live spore vaccine and better than the AVA vaccine (51,53).

1.2 Brucellosis

1.2.1 The Genus *Brucella*

Brucellosis is one of numerous zoonotic diseases that can occur in both humans and animals. In animals, the most obvious sign of disease is abortion (38). Human brucellosis is characterized mainly by undulant fever and malaise (103).

Brucella infected animals and humans often present with widespread granulomas in areas such as the lymph nodes, bone marrow, liver and spleen. Abscesses have also been observed in bone, liver, spleen, kidney and the brain (26). Placentitis is often seen in pregnant animals, with resulting abortion. Due to the frequent involvement of the mammary glands, *Brucella* is usually shed in milk. The organism is also present in aborted fetuses, fetal membranes and uterine discharge (38).

Natural transmission of *Brucella* is thought to occur by ingestion. This is due to the large numbers of organisms present in aborted tissue (38). Transmission occurs when animals ingest contaminated food and water or lick a recently aborted fetus (26). Infection may also result in humans by ingesting infected raw milk or other non-pasteurized dairy product. Also, *Brucella* can enter the host through abraded skin or following contact with mucous membranes (20).

Due to the extensive economic losses *Brucella* infection can bring, eradication of the disease worldwide is very important. Vaccination is an effective means of protecting animals that have not been exposed to the disease (38). However, since treatment of

infected livestock with antibiotics is not economically feasible, the U.S. has adopted the test-and-slaughter policy for cattle. Cattle that give a positive reaction in the serum agglutination and other tests are separated from the herd and slaughtered. The remaining cattle in the herd are quarantined and calf-hood vaccination performed. A herd is considered brucellosis free if it tests negative 2 or 3 successive times in the serum agglutination test (38).

The causative agent of brucellosis is a bacterial strain from the genus *Brucella*. The genus consists of six recognized species: *B. abortus, B. melitensis, B. suis, B. ovis, B. canis,* and *B. neotomae* (22). There are a number of reports demonstrating the occurrence of novel strains of *Brucella* in marine animals and corresponding nomen of *B. cetaceae* and *B. pinnipediae* (23,69). Classification is based upon differences in pathogenicity and natural host. The major agents of brucellosis, in terms of zoonotic potential are the *Brucella* species: *B. abortus, B. melitensis,* and *B. suis* (38).

Brucellae are gram-negative, non-motile, facultative intracellular bacteria (22). These bacteria are able to survive and even multiply inside the macrophage (38,84). *Brucellae* do not have a protective capsule and do not produce spores. The various species of the genus *Brucella* share a close taxonomic relationship, which extends to the genetic level (73,74,84). Studies underway at Virginia Tech suggest that as few as 100 genes allow the three species to be differentiated (84). All genetic information for *Brucella* organisms is chromosomally encoded and share at least a 90% homology across the genus (20,74,103). Unlike other bacteria, *Brucellae* do not appear to harbor plasmids (103).

Several virulence factors aid *Brucella* in their survival inside macrophages and other cells (84). The first and probably most important factor is the presence of the O-side chain, a linear polymer of mannose residues, on the lipopolysaccharide (LPS) of smooth strains. The O-side chain is the most exposed antigen structure in *Brucella* (22,26). The O-side chain of *Brucella* LPS induces a antibody-mediated response that is somewhat protective in mice but not in cattle (26). In fact, it appears that production of antibodies of certain subisotypes against *Brucella* O-side chain interferes with complement activation. Interference in this process could then allow *Brucella* to survive longer in cattle and set up a persistent infection (103).

Several species of *Brucella* are naturally of the smooth morphology. These species include *B. abortus, B. melitensis* and *B. suis*, although these species can also exhibit a rough phenotype as well (22). *B. ovis* and *B. canis* occur naturally as rough species. Rough colony morphology in *Brucella* is the result of the lack of the O-side chain on the LPS (26). Therefore, these strains do not induce antibodies against the immunodominant O-side chain, which interfere with differentiating field infections from vaccinated animals.

1.2.2 Overview of Brucellosis Vaccines

Elucidation of the factors, especially those that induce highly protective immune responses, is important in the development of effective vaccines. Also, the development of a highly efficacious vaccine means that it possesses several characteristics, including induction of long-term immunity, minimal interference with diagnostic tests, easy production and storage, posing no danger to the recipient, low cost and maintains a high level of quality (78). Vaccination of animals with live *Brucella* induces both antibody-

mediated and CMI responses. The strength and duration of these responses depends highly upon the vaccine used to induce the reaction and other factors such as dose and route of vaccination (78).

Several vaccines against *Brucella* that have been developed for use in humans and animals will be discussed here. As is the case with immunization against anthrax, the worldwide eradication campaign against brucellosis involves the use of both live and killed/subunit vaccines. However, none of these human vaccines are being used today.

Immunization studies in laboratory animals using the subunit or killed vaccines against *Brucella* have not been promising. Examples of these killed vaccines are *B. abortus* strain 45/20 and *B. melitensis* H38.

B. abortus strain 45/20 is an adjuvanted vaccine of whole cells exhibiting the rough phenotype. No O-side chain is present in the preparation and therefore, the vaccine does not cause interference in serum agglutination tests. Strain 45/20 requires 2 initial doses, 6-12 weeks apart followed by annual boosters (38). Local reaction at site of injection may occur but killed strain 45/20 has not been shown to induce abortion (78).

B. melitensis H38 is an adjuvanted vaccine first developed for use in sheep and goats. This vaccine is composed of formol-killed whole cells of the smooth phenotype. Strain H38 induced immunity has not been well characterized. The vaccine is also shown to cause local reaction at site of inoculation and due to the presence of O-side chain in the preparation, O-side chain antibodies interfere with serum diagnosis of infection (38,78).

Several live vaccines for use in animals have been developed worldwide with varying degrees of success in protecting against brucellosis. *B. abortus* strain 104-M isolated from a cow was developed in the former USSR. Virulence, immunogenicity and

antigenic structure are reported to be stable (78). *B. suis* strain 2, developed in China, consists of an attenuated smooth strain of biovar 1 of *B. suis.* This vaccine has been used in several animal species; immunization with strain 2 does not seem to induce abortion in pregnant animals (78). Serologic interference by the vaccine seems to be low and short-lived.

The three most widely used live vaccines against brucellosis are *B. melitensis* Rev 1, *B. abortus* strain 19, and *B. abortus* RB51. *B. melitensis* Rev 1 was developed for use in sheep and goats and was derived from a virulent smooth strain of *B. melitensis* (26,38). The vaccine strain exhibits reduced virulence and induces effective immune responses in vaccinated animals. Vaccination is performed in young sheep and goats subcutaneously; a lower dose can be used to immunize adult animals. The vaccine induces serum antibodies that are persistent; strain Rev 1 may induce abortion in pregnant animals (78).

B. abortus strain 19 is a viable smooth strain used in cattle since the 1930's. The positive and negative characteristics of this vaccine are well known. Strain 19 is primarily used for calf-hood immunizations but vaccination of adults is also possible (78). Normally, this vaccine is given in one dose and it is believed to provide about a 70% protection rate over the lifetime of the animal (38,87). However, studies have shown that administering a booster shot to calves may afford added protection (78). Strain 19 elicits a mainly CMI response which is very important in brucellosis immunity. One drawback of the vaccine, however, is the smooth phenotype of the strain. A smooth strain expresses O-side chain on the cell surface and induces corresponding antibody responses against the carbohydrate side chain. Antibodies produced against the O-side chain of the LPS interfere with standard serologic tests. Vaccinated positive reactors

cannot be distinguished from infected positive reactors (70,87). Vaccination with strain 19 can induce abortion in pregnant cattle (67). In addition to induction of abortion and interference with serologic tests, strain 19 is also pathogenic to humans (70,81).

While strain 19 apparently provides long-term efficacious immunity against infection by *Brucella*, the adverse characteristics associated with the vaccine and its ability to only protect against *B. abortus* species signals a need for an improved vaccine (27). An improved brucellosis vaccine would have the following characteristics: inability to induce O-side chain antibodies that interfere with serologic tests, induction of long-term effective immunity with one dose, and inability to cause abortion or induce infection in vaccinates and humans. The vaccine should also be a stable strain that does not revert to virulence *in vivo* (95).

The vaccine strain *B. abortus* RB51 meets these criteria; it is a stable rough mutant of *B. abortus* derived from parental strain 2308 (94). The mutant was obtained after passage of strain 2308 on media containing the antibiotic rifampin (87,95). Following serial passages, a highly attenuated mutant, rifampin resistant and essentially devoid of the O-side chain of LPS, was obtained (94). Strain RB51 passaged through and isolated from mice retains its highly attenuated, avirulent characteristics (27,94,95). Due to the lack of O-side chain in the LPS of strain RB51, vaccination with this strain does not induce antibodies that interfere with serologic testing of animals. Therefore, it is much easier to distinguish those animals that have been immunized from those which are infected (19,94,95).

Immunity induced by strain RB51 consists of both a antibody-mediated and CMI type (95). The CMI response, extremely important to immunity against *Brucella*, seems

to be highly induced with this vaccine. One injection confers protection from challenge with strain 2308 and field strains and with an efficacy at least equal to strain 19 (107,123). In addition to providing protection against *B. abortus* strains, a study using a mouse model indicated strain RB51 may be efficacious against *B. melitensis* and *B. suis* as well (27,123).

In addition to conferring protection against various species of *Brucella*, strain RB51 has other positive characteristics. One very important feature is the apparent inability or very low ability to induce abortion in pregnant animals (19,67,70,95). Also, accidental exposure to the strain during vaccination of animals or other situations has not caused full-fledged disease in humans. This suggests that strain RB51 may be avirulent in humans (27). Numerous studies using strain RB51 as a vaccine have shown that this strain has most, if not all, of the characteristics desired in the ideal *Brucella* vaccine. Due to this, strain RB51 was approved for use against bovine brucellosis in the U.S.A. by the USDA in 1996 (26,27,87).

Prevention of brucellosis in humans is not mediated by vaccination, as no effective human vaccines are available; but the elimination of the animal reservoir decreases the incidence (26,78). In the past, vaccination in humans against brucellosis was used in an attempt to prevent the disease. Varied successes and adverse effects accompanied use of these vaccines.

Strain *B. melitensis* Rev 1, the live vaccine strain used to immunize sheep and goats, has been studied for efficacy in humans and primates. Results showed that finding a dose, which was protective but did not cause disease, was too difficult to justify using strain Rev 1 in humans (78). *B. abortus* 19BA was used in the former USSR to

immunize humans against *B. melitensis* and protection lasts for up to one year. The theory behind the use of this vaccine was the idea the *B. abortus* is less pathogenic in humans and can confer cross-immunity to *B. melitensis* (26,78). Due to the presence of O-side chain on the LPS of strain 19BA, many immunized humans test positive for O-antibodies in serologic testing (78). In China, humans are vaccinated with live attenuated strain *B. abortus* 104M; protection is observed for one year (26,78). Immunization may result in erythema at site of inoculation; other side effects include headache and weakness.

Two non-living vaccines have been used to immunize humans against brucellosis. In the former USSR, a protein-polysaccharide complex derived from the cell wall of smooth *Brucella* strains has been used as an alternative to strain 19BA (26,78). This vaccine is reported as safe and of low reactogenicity when compared to strain 19BA known to cause severe adverse reactions (78). An immunogenic, phenol-insoluble fraction of *B. abortus* and *B. melitensis* has been used to immunize humans in France (26,78). The efficacy of this and other vaccines used in humans have not been well established, but seem to be of limited efficacy (26,78). It is important to note that the use of these vaccines has been discontinued due to low levels of protection and side effects.

1.3 *Brucella abortus* RB51 as a Delivery Platform

Various heterologous proteins (derived from species other than *Brucella*) have been expressed in strain RB51 in an attempt to create a dual vaccine. Studies have clearly demonstrated that strain RB51 can express a variety of heterologous antigens and induce a strong Th1 mediated CMI response as well as an antibody mediated (Th2) immune response. The expression of a foreign reporter protein, β-galactosidase of *E.*

coli, and the 65-kDa heat-shock protein (HSP65) of *M. bovis* in strain RB51 has been studied. The genes for β-galactosidase (*lacZ*) or HSP65 were cloned into plasmids and used to transform strain RB51. Mice vaccinated with either of the β-galactosidase expressing recombinant RB51 strains developed specific antibodies of predominantly IgG2a isotype, and *in vitro* stimulation of their splenocytes with β-galactosidase induced the secretion of IFN-γ but not IL-4. A Th1 type of immune response to HSP65, as indicated by the presence of specific serum IgG2a but not IgG1 antibodies, and IFN-γ but not IL-4 secretion by the specific antigen stimulated splenocytes, was also detected in mice vaccinated with strain RB51 containing pBBgroE::hsp65. Studies in mice indicated that expression of β-galactosidase or HSP65 did not alter either the attenuation characteristics of strain RB51 or its vaccine efficacy against *B. abortus* 2308 challenge (108).

A second study examined 2 other recombinant RB51 strains, RB51/SOD/85A which over expresses *B. abortus* Cu-Zn SOD with simultaneous expression of 85A and RB51/ESAT which over-expresses ESAT-6. Both 85A and ESAT-6 are protective antigens of *M. bovis*. Antibodies specific to 85A were not detected in mice vaccinated with strain RB51/SOD/85A. However, upon stimulation with 85A, splenocytes from these mice secreted high levels of IFN-γ but not IL-4. Mice vaccinated with strain RB51/ESAT developed ESAT-6-specific antibodies predominantly of the IgG2a subisotype and upon stimulation with ESAT-6, splenocytes from these mice secreted moderate levels of IFN-γ but not IL-4. Vaccination of mice with these recombinant strains significantly enhanced protection against *Brucella* challenge compared to the mice immunized with strain RB51 alone (109).

2. Dissertation Overview

2.1 Rationale of Dissertation

Currently, increased research activity in the field of anthrax vaccine development is aimed at a vaccine whose main component is the *B. anthracis* PA protein adjuvanted with some sort of strong CMI inducer. An ideal vaccine will require fewer immunizations, elicit fewer side effects and induce a stronger and more prolonged immunity against highly virulent strains of *B. anthracis* even under aerosol exposure. If the stigma of using a live vaccine for immunization can be overcome, an attenuated recombinant bacterial strain would be a good choice as a vaccine delivery platform. Since heterologous gene expression is possible in strain RB51 and mice immunized with the recombinants respond by producing specific antibodies, the presence of PA antibodies is expected in mice immunized with strain RB51/PA. The induction of antibodies against PA and the CMI response induced by strain RB51/PA should be sufficient to confer protection against two corresponding bacterial zoonotic/bioterror disease threats. The creation and use of this dual vaccine could be economically and militarily important.

The overall goal of this dissertation is to develop a protective, modified live vaccine candidate against both anthrax and brucellosis using *Brucella abortus* RB51 as the delivery platform. This candidate vaccine will use *B. abortus* RB51 expressing partial PA to stimulate a antibody-mediated response against anthrax. Strain RB51 will express only the domain 4 of the PA protein, the receptor binding site, since work by Flick-Smith *et al*, has shown that antibodies to this domain are protective against anthrax spore challenge (37). In addition, the CMI response induced by strain RB51 may aid in providing non-specific and perhaps specific protection against anthrax.

Therefore, I hypothesize that a live attenuated *B. abortus* RB51 bacterial strain will be able to induce a specific or nonspecific CMI response that will enhance the protective abilities of PA against spore challenge. Previous studies have suggested a need for both antibody-mediated and CMI activation to achieve superior immunity against *B. anthracis* (51,83,85). Literature searches have yielded many references to papers stating that live vaccines, whether they be natural or recombinant in nature, tend to confer better immunity against anthrax than subunit/adjuvanted vaccines do. For this reason, I have decided to express the *B. anthracis* PA gene in *B. abortus* vaccine strain RB51. Successful expression of this protein in strain RB51 would possibly enable the development of a vaccine that protects against two economically and militarily important bacterial diseases: anthrax and brucellosis. In addition, both pathogens have been adapted as components of bioweapons programs in a number of countries and there are no effective human vaccines to protect against aerosol infections.

We previously attempted to develop a dual vaccine for livestock against both brucellosis and anthrax by expressing the PA protein of *B. anthracis* in *B. abortus* strain RB51 and assessed the immune response in A/J mice (Chapter 2). This strain RB51/PA did express full size PA protein, however, it was in limited quantities. A/J mice immunized with the strain mounted antibody-mediated immune responses to the vaccine that were partially protective against challenge with *B. anthracis* Sterne spores at $50xLD_{50}$. In addition, expression of PA by strain RB51 did not interfere with the protective capabilities of the vaccine against *Brucella* challenge. While the results of this study were encouraging, the efficacy of the vaccine was less than desirable.

In order to improve the efficacy of the previous vaccine candidate, several new strains of RB51/PA were generated by fusing different *Brucella* signal sequences to the expressed PA gene in order to determine if localization of the protein plays a role in induction of a protective immune response. The signal sequences for superoxide dismutase (SOD) and 18kDa outer membrane protein were used. The PA sequence was fused to each of these signal sequences or to no signal sequence in order to localize the protein into three areas of the *Brucella* cell: the periplasmic space, outer membrane and cytosol respectively. In addition, the codon usage of the PA protein was converted from that of *B. anthracis* to that of *Brucella*. The G/C content of these organisms differs greatly with *Brucella* possessing the higher G/C content (59,91). Since *B. anthracis* PA protein codes for use of several rare tRNA populations in *Brucella*, this could be a possible explanation for the low expression of PA, i.e., all or some of the cognate tRNA pools were depleted. By changing the PA sequence to code for *Brucella* preferred codons, the synthesis of the protein is predicted to be increased.

2.2 Overview of My Previous Work

A/J mice immunized with live *Brucella abortus* RB51 expressing *Bacillus anthracis* protective antigen (PA)

Abstract

Bacillus anthracis is a facultative intracellular bacterial pathogen causing cutaneous, gastrointestinal or respiratory disease in many vertebrates, including humans. Commercially available anthrax vaccines for immunization of humans are of limited duration and do not protect against the respiratory form of the disease; those available for use in animals have caused disease in some susceptible species. *Brucella abortus* is a facultative intracellular bacterium that causes chronic infection in animals and humans. As with other intracellular pathogens, cell mediated immune responses (CMI) are crucial in affording protection against brucellosis. *B. abortus* strain RB51 can elicit antibody-mediated responses and protective cell mediated immunity against *Brucella* in cattle and other animal species. Since the protective antigen (PA) of *B. anthracis* is known to induce protective antibodies, it was decided to test whether the *pag* gene encoding PA could be expressed in *Brucella* producing a dual vaccine to protect against both brucellosis and anthrax. The *pag* gene was transcriptionally fused to the promoter of the *Brucella groE* gene encoding heat shock protein, subcloned into a broad host range plasmid (pBBR1MCS) and shown by immunoblotting to express in *B. abortus* RB51. Immunization and challenge studies were performed using the A/J mouse, an immunocompromised vertebrate model. As determined by ELISA, antibody titers against PA were induced by strain RB51/PA. Preliminary results demonstrate that the dual vaccine is capable of producing protection against a live challenge with *B. abortus* and some low level of protection against live spores of *B. anthracis* Sterne.

Introduction

Brucellosis is one of several zoonotic diseases that can occur in both human and animals. In animals, the most obvious sign of disease is abortion (38). Human brucellosis is characterized mainly by undulant fever and malaise (103). Natural transmission of *Brucella* is thought to occur by contact with mucous membranes or abrasions in the skin (20). Transmission occurs when animals ingest contaminated food and water or lick a recently aborted fetus (26,38). Infection may also occur in humans ingesting infected milk or other dairy products.

Due to the extensive economic losses *Brucella* infection in animals and humans can induce, eradication of the disease worldwide is very important. Vaccination is an effective means of protecting animals that have not already been exposed to the disease (38). However, since treatment of infected livestock with antibiotics is not economically feasible, the U.S. has adopted the test-and-slaughter policy (112). There are no known effective vaccines for the prevention of brucellosis in humans.

Several virulence factors aid *Brucella* in their survival inside macrophages and other cells (22). The first and probably most important factor is the presence of the O-side chain associated with the lipopolysaccharide (LPS) of smooth strains. The O-side chain is the most exposed antigen structure in intact *Brucella* and induces specific antibodies that are the diagnostic hallmark of a *Brucella* infection (22).

Vaccination of animals with live *Brucella* vaccine strain induces both antibody-mediated and cell-mediated immune (CMI) responses (95). The strength and duration of these immune responses depends highly upon the vaccine strain used and other factors such as dose and route of vaccination (78).

The vaccine strain *B. abortus* RB51, used for cattle, is a stable rough mutant of *B. abortus* derived from parental strain 2308 (94). Due to the lack of O-side chain in the LPS of strain RB51, vaccination with this strain does not induce antibodies that interfere with serologic testing of animals (19,94,95). The CMI response, extremely important to immunity against *Brucella*, seems to be highly induced with this vaccine. Immunization of cattle with strain RB51 confers protection from challenge with strain 2308 and field strains and with an efficacy at least equal to strain 19 (107,123).

Bacillus anthracis is a spore-forming bacterium whose spores can survive in dry form for indefinite periods of time and when airborne cause disease if inhaled (21). *B. anthracis* spores are highly resistant to environmental changes such as temperature extremes, ultraviolet exposure, dessication, and chemical treatment (33,35). It is the highly resistant nature of the spores of *B. anthracis* that aids in the persistence of the bacterial disease in an area (33). The disease can take three forms: cutaneous, respiratory or gastrointestinal, depending upon the route of spore entry (33,78). The latter two forms of the anthrax are the most fatal and most rare.

B. anthracis expresses two known virulence factors, both of which are plasmid-encoded (62). The first virulence factor is the poly-D-glutamic acid capsule. The capsule has anti-phagocytic properties that enable the bacterium to resist a host's defenses (99). The genes encoding the capsule are located on the 90 kilobase (kb) pXO2 plasmid (7).

The second known virulence factor is the tripartite exotoxin consisting of the protective antigen (PA), lethal factor (LF), and edema factor (EF) (117). All of the proteins, which collectively make up the anthrax toxin, are encoded by a 175kb plasmid called pXO1 (120). LF is thought to destroy host cells by disrupting the mitogen-activated protein kinase pathway (34,90). EF is a calcium and calmodulin dependent adenylyl cyclase, which causes cellular edema in the host by increasing cAMP levels (90,118). PA is a protein that binds to host cell surface receptors (118). Once seven PA molecules have bound to a receptor, a channel is formed in the cell wall that facilitates entry of LF or EF into the cell's cytoplasm (90,117). Alone PA, LF, and EF are biologically inactive as toxins (78,111).

PA is a very important component of the anthrax toxin for another reason: this protein plays a major role in anthrax immunity after both immunization and infection (99). This immunity occurs due to the neutralization of the activity of the anthrax toxin (36). PA, when produced in the absence of LF and EF, has been shown to be capable of producing effective protection both as a purified protein and when used in a recombinant or attenuated vaccine (110).

Previous studies have suggested a need for both antibody-mediated and CMI responses to achieve superior immunity against *B. anthracis* (54,56,57). We decided to express the *B. anthracis* PA gene in *B. abortus* vaccine strain RB51 to create a vaccine capable of affording protection against two diseases: anthrax and brucellosis.

The results of this study show the *pag* gene encoding the PA antigen of *B. anthracis* can be expressed in *B. abortus* RB51, the A/J mouse model immunized with the dual live vaccine produces specific antibodies against PA, and upon immunization A/J mice were protected against a challenge by virulent *B. abortus* 2308 but only barely protected against an avirulent Sterne spore challenge.

Materials and Methods

Bacterial strains, media and growth conditions:

All *Brucella* culture work was performed in a BSL-3 laboratory. *B. abortus* RB51 was grown on Trypticase Soy Broth (TSB) or SOC-B (6% trypticase soy both, 10mM NaCl, 2.5mM KCL, 10mM $MgCl_2$, 10mM $MgSO_4$ and 20mM glucose) (72). *B. abortus* 2308 was grown on TSB. All cultures were grown at 37° at 200 rpm. Strains transformed with plasmids were grown on solid or liquid media containing one of the

following antibiotics: ampicillin (Amp) 100ug/mL, chloramphenicol (Cm) 30ug/mL. Bacto-Agar was purchased from Fisher Biotech (Norcross, GA).

PCR amplification of *pag* gene:

Plasmid pUTE41, obtained from Dr. T. Koehler (University of Texas) contains the complete *pag* gene encoding the protein PA (62). Primers were designed to amplify only the open reading frame (ORF) for the mature protein. The forward primer: GGA TCC ACA AAA AGG AGA ACG TAT ATG AAA AAA CGA AAA GTG added a recognition site for the restriction enzyme *BamHI*. The reverse primer: TCT AGA CAC CTA GAA TTA CCT TAT CCT ATC TCA TAG CCT TTT added a recognition site for the restriction enzyme *XbaI*. The amplified *pag* was cloned into pCR2.1 (Invitrogen) digested with *BamHI* and *XbaI*.

Construction of the pBBSOD-PA and pBBGroE-PA plasmids:

pCR2.1 containing the 2.32kb *pag* gene was digested according to manufacturer's instructions (Invitrogen) with *BamHI* and *XbaI*. The plasmids pBBSOD (115) and pBBGroE (115) were also digested 16-18 hours with *BamHI* and *XbaI*. The DNA fragments were purified by Qiaquick Gel Extraction kit (Qiagen) as per the manufacturer's protocol. Ligation reactions were set up using the purified fragments and incubated at 4ºC overnight. *E. coli* DH5 Top10 cells (GibcoBRL) were transformed with these ligation reactions by heat shock method as per manufacturer's instruction. After transformation, the cells were spread on TSB-Cm plates and incubated overnight at 37ºC. Several clones were picked from those that grew overnight and checked for recombinant plasmid using Clonechecker, a rapid screen protocol (Invitrogen). These

clones containing the plasmids were designated pBBSOD-PA and pBBGroE-PA (Figures 2.1,2.2).

Transformation of *B. abortus* RB51:

Competent *B. abortus* RB51 cells were made and transformed with the plasmid constructs via electroporation (72). Transformed cells were plated onto TSB-Cm plates and incubated at 37ºC for 4 days.

Protein Analysis:

Extracts of *B. abortus* RB51 (20 ml culture) transformed with pBBSOD, pBBGroE, pBBSOD-PA, or pBBGroE-PA were separated on SDS-PAGE gels to check for expression of PA. Sample aliquots from both cell pellets and culture media were examined on gels. The procedure as described by Laemmli was followed with some modifications (65). A 12.5% acrylamide gel was used following standard protocol using the Mini-Protean® II gel apparatus (Bio-Rad, Rockville, NY) (4). Gels were run 90 minutes at 25mA/gel in SDS-Page electrophoresis buffer (25mM Tris, 0.19M Glycine, 0.1% SDS, pH 8.3).

Western blot:

Gels were run for 90 minutes at 25mA per gel. Proteins were transferred from the gel to a nitrocellulose membrane (0.45µ, Osmonics, Inc.) by electro-transfer in preparation for immunoblotting. Nitrocellulose membranes with attached proteins were blocked in 1% bovine serum albumin for 3 hours to prevent non-specific binding of the primary (1º) antibody (rabbit anti-PA) (S. Leppla, NIH) to the membrane. The blocked membranes were then exposed overnight on a shaker at 4ºC to the 1º antibody diluted 1:1000. The membranes were washed three times with TBS-Tween20 and secondary (2º)

antibody (anti-rabbit IgG HRP-labeled) added at a 1:2000 dilution for 2-3 hours at room temperature. The membranes were washed again three times and the substrate, 4-chloro-1-napthol, was added to the blot and observed for formation of protein bands.

Immunization of mice with *B. abortus* RB51/pBBGroE±PA:

A/J mice (Jackson Laboratories, Bar Harbor, ME) were divided into 4 groups. Five mice were designated as controls and injected intraperitoneally (IP) with 0.2mL of sterile saline. Ten mice were designated the GroE group and were injected IP with $3.6x10^8$ colony forming units (CFU) of *B. abortus* RB51 transformed with pBBGroE plasmid. Eleven mice were designated the pBBGroE-PA group. These mice were injected IP with $4.3x10^8$ cfu of *B. abortus* RB51 transformed with the pBBGroE-PA plasmid. Eleven mice were designated the PA group and were injected IP with 3 μg of pure PA protein.

Challenge of mice:

The mice were bled at 6 and 8 weeks post immunization. At 8 weeks post immunization, the mice were challenged. Three naïve mice and 5 mice from each of the pBBGroE and pBBGroE-PA groups were injected IP with $2.4x10^4$ cfu of *B. abortus* 2308. At 2 weeks post challenge the mice were sacrificed and the spleens were harvested, homogenized and aliquots plated on TSB plates to determine the clearance of the strain 2308.

The 5 mice receiving saline, 5 pBBGroE mice, 6 pBBGroE-PA and 6 PA immunized mice were then injected IP with $5.6x10^4$ spores of *B. anthracis* Sterne strain, the live veterinary vaccine (Colorado Serum Company, Denver, CO) (24).

Western blots using mouse serum:

Pure PA protein (~3ug) was loaded into each well of a 12.5% SDS-PAGE gel, electrophoresed and transferred to nitrocellulose membranes as mentioned above. Membranes were blocked with 1% BSA and cut into strips; each strip represents one lane of PA. Each strip was placed into a sealed bag and 1mL of mouse sera diluted 1:25 in 1% BSA was added. These strips were incubated at 4ºC for 3 days. The strips were then washed 3 times with 10mL of TBS and exposed to 2º antibody: anti-mouse IgG (HRP-labeled) at a 1:1000 dilution for 3 hours. Addition, of the 4-chloro-1-napthol substrate after washing, revealed which mice were producing antibodies to PA.

ELISA:

96-well plates were coated at 4°C overnight using 1□g of PA per well in bicarbonate buffer. Plates were then blocked with 2% BSA in phosphate buffered saline (PBS) overnight at 4°C. Plates were washed 3 times with wash buffer (PBS/0.05%Tween-20). Serum samples, from immunized mice, diluted 1:100 were added to each well and incubated at room temperature for 3 hours. Plates were washed 4 times with wash buffer and secondary antibody, goat anti-mouse IgG (HRP-labeled) were added to the wells at a dilution of 1:5000 and incubated at room temperature for 30 minutes. Plates were washed 5 times with wash buffer. 100□l of TMB substrate (KPL, Gaithersburg, Maryland) was added to each well and incubated at room temperature for 10-30 minutes. Stop solution (0.18M sulfuric acid) was added to each well and the plate was read at 450nm.

Statistical Analysis:

Counts of bacterial CFU in the spleens of mice were analyzed by the paired *t* test using Sigma Plot software. P values equal to or less than 0.01 were considered significant.

Results

Construction of pBBSOD-PA and pBBGroE-PA plasmids.

Restriction digestion of both pBBSOD and pBBGroE with *BamHI* and *XbaI* allowed subsequent ligation of the 3.23kb insert into each plasmid (Figures 2.1,2.2). Transformation of *E. coli* DH5 cells yielded Cm resistant clones. Restriction digests of the plasmid purified from these clones showed the *pag* insert in proper orientation. Single and double digests were performed on each plasmid with *BamH1* and *Xba1* in order to determine the presence of the *pag* gene.

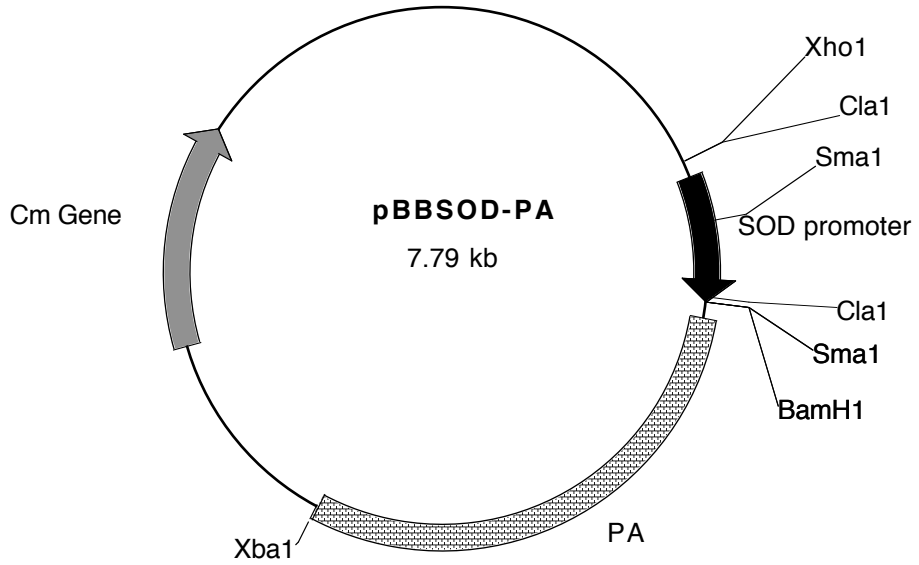

Figure 2.1: Plasmid pBBSOD-PA. Derived by ligation of 3.23kb *pag* gene encoding PA into *BamHI* and *XbaI* sites of pBBSOD (R. Vemulapalli, VPI&SU).

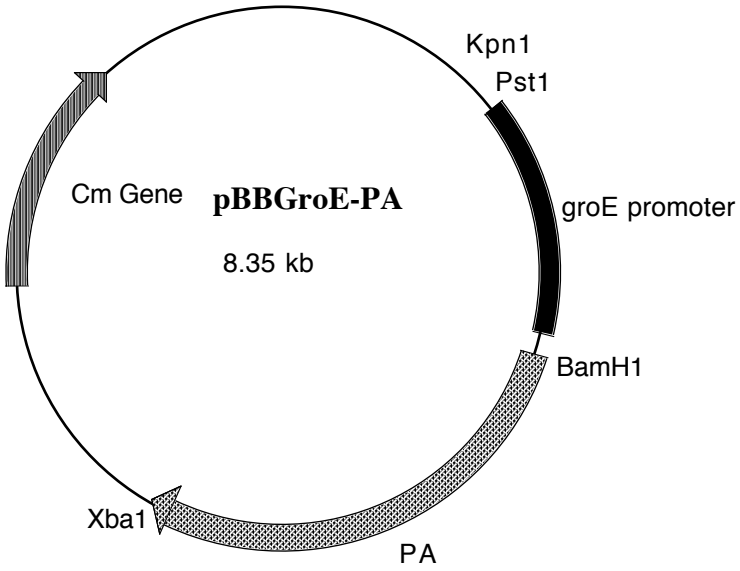

Figure 2.2: Plasmid pBBGroE-PA. Derived by ligation of 3.23kb *pag* gene encoding PA into *BamHI* and *XbaI* sites of pBBGroE (R. Vemulapalli, VPI&SU).

Expression & Stabilization of PA in *B. abortus* RB51:

Competent *B. abortus* RB51 cells were transformed with either pBBSOD-PA or pBBGroE-PA by electroporation and plated onto TSB-Cm plates (72). Cm resistant clones were grown individually and analyzed by Western blot to check for expression of PA. Because PA is a thermolabile protein (3), the cultures were placed on ice while being prepared for SDS-PAGE electrophoresis. Cell pellets and culture media were collected, solubilized in loading buffer and analyzed by immunoblotting (65). Full size PA (83kDa) was not observed on these gels. However, the degraded PA bands observed in the extracts of transformed *E. coli* (data not shown) were also present in extracts *Brucella* as well. This degradation of PA in *Brucella* could have resulted during the heat killing of the strain RB51 prior to loading on the SDS-PAGE gel.

Subsequent *Brucella* cultures were kept on ice for every step of sample preparation, except for the 1 hour heat inactivation step. When the PA expressed in

cultures were visualized by immunoblotting, a dark band could be seen corresponding to the GroE-PA construct (Figure 2.3), but only a faint band corresponding to the full size protein (63-83 kDa) was seen in the SOD-PA protein which could not be photographed. Thus it is possible that the GroE promoter is more efficient at expressing PA in *Brucella* than the SOD promoter (data not shown). No bands were seen in any of the lanes containing the *Brucella* culture media samples, however degraded PA was seen in the unconcentrated culture media of *E. coli* (data not shown).

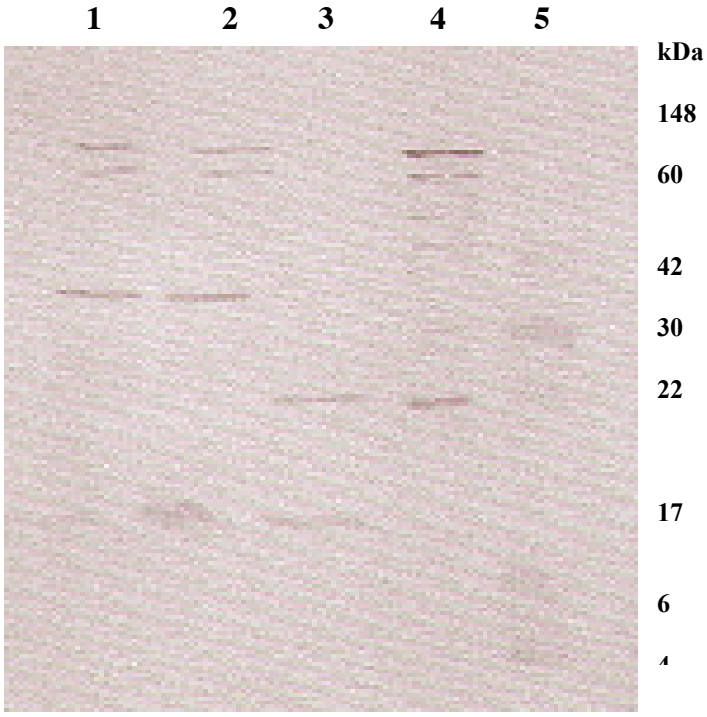

Figure 2.3: Western blot of *Brucella*/pBBGroE-PA clones. Cells were harvested under cold conditions and cell extracts prepared by adding Laemmli sample buffer. Lanes 1-2 are extracts from strain pBBGroE-PA, lane 3 is an extract from strain pBBGroE, lane 4 is purifired (3 □g) PA and lane 5 contains protein molecular mass markers. Anti-PA serum was used to visualize PA.

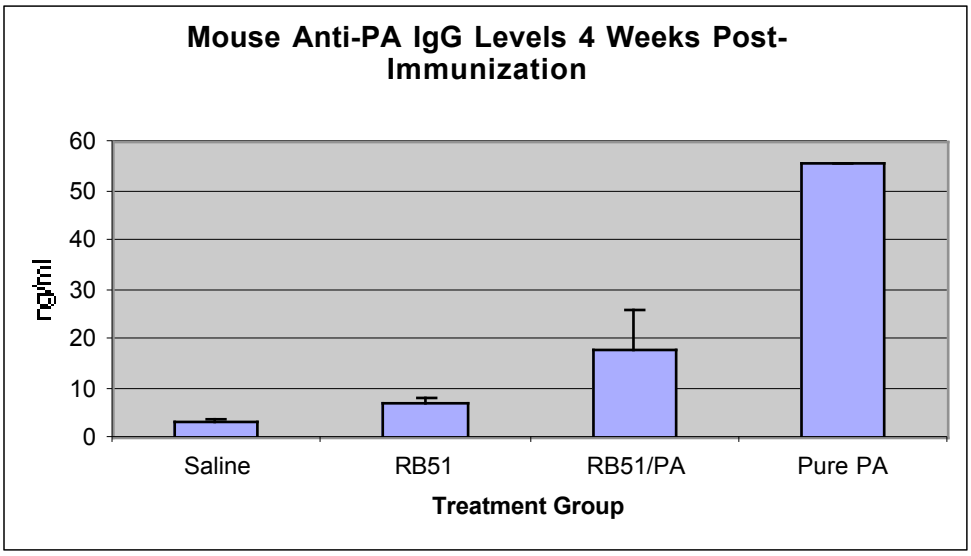

Figure 2.4: ELISA of serum from mice immunized with PA at 4 weeks post-immunization using a serum dilution of 1:50. Mice immunized with either strain RB51/PA or purified PA developed detectable IgG antibodies specific for PA.

Protection assessment of strain RB51/pBBGroE-PA:

Since GroE is a heat shock protein, it is known to be up- regulated during times of stress such as when *Brucella* is replicating in macrophages (115). Twenty-six female A/J mice were divided into 3 groups. Five mice were injected with sterile saline and 10 mice were injected with *B. abortus* RB51/pBBGroE. The remaining 11 mice received *B. abortus* RB51/pBBGroE-PA. The mice were bled at weeks 6 and 8 after immunization and immunoblot analysis and ELISA revealed the presence of antibodies against PA in the serum at week 6 (Figure 2.4); antibodies persisted through to week 8 in mice immunized with strain RB51/pBBGroE-PA and PA. Of the eleven mice injected with strain RB51/pBBGroE-PA, 10 were positive PA reactors (Figure 2.5). Each of the mice immunized with PA reacted positively to PA.

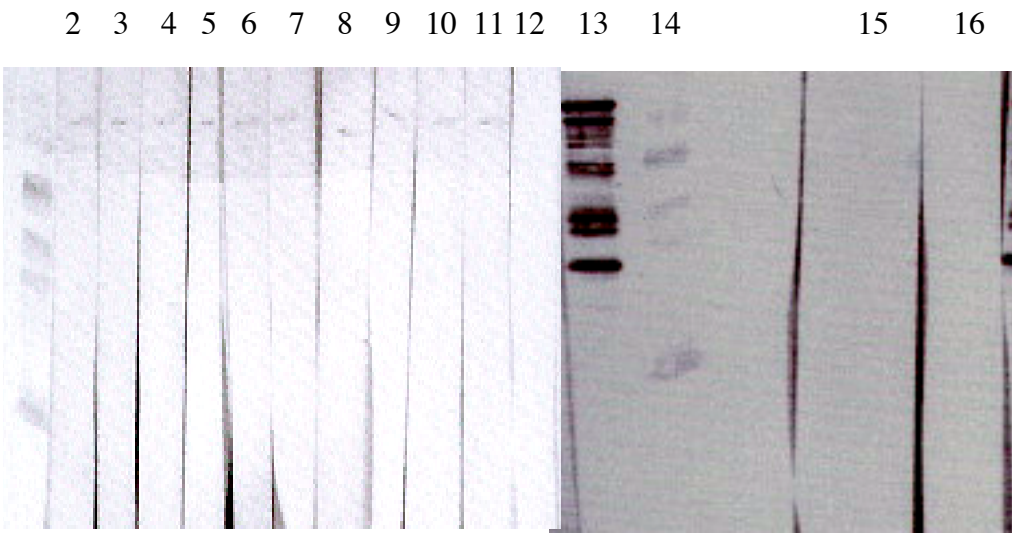

Figure 2.5. Western blots of pure PA protein exposed to sera from immunized mice. Lanes 1 and 13 are protein molecular mass markers. Lanes 2-12 are pure PA (~3ug) exposed to sera from mice immunized with *Brucella* expressing PA (pBBGroE-PA). Lane 14 is pure PA protein (~3ug) exposed to rabbit anti-PA serum. Lane 15 is pure PA (~3ug) exposed to serum from a mouse immunized with sterile saline, lane 16 is PA incubated in serum from a mouse immunized with *Brucella* not expressing PA (pBBGroE).

Since strain RB51 is able to confer protection against *Brucella* after a single immunization, and antibodies against PA were detectable, these animals were challenged. At 7 weeks post-immunization, the 5 mice receiving saline, the 5 mice receiving strain RB51/pBBGroE and the six mice receiving strain RB51/pBBGroE-PA were challenged with $5.6x10^4$ spores of the Sterne strain. The remaining mice in each group were challenged with $2.4x10^4$ cfu of *B. abortus* 2308.

The endpoint for those mice challenged with Sterne strain was death; any survival indicates protection. The mice immunized with saline survived for 4 days. The mice immunized with strain RB51/pBBGroE survived longer than the saline group but all mice in the group eventually died by day 7. The mice receiving strain RB51/pBBGroE-PA survived even longer (an average of 5.5-6 days); one mouse survived the challenge dose. This extended survival rate in the immunized mice suggests that the immune responses

were somewhat protective (Figure 2.6). On days 1-3 post challenge, mice in all groups were observed for signs of illness. On day 2, all mice presented with scruffy coats and reduced activity and appetite. On day 3 all mice were inactive and not eating. On day 4, all mice in the saline control group died as well as 3 mice in the pBBGroE group and 2 in the pBBGroE-PA group. On day 5, one mouse from each of the 2 remaining groups died. By day 7, all mice from the saline and pBBGroE groups were dead and 1 mouse from the pBBGroE-PA group survived. By day 8, this surviving mouse's coat returned to a normal appearance and its activity and appetite increased.

The endpoint for those mice challenged with *B. abortus* strain 2308 was determination of CFU/spleen at 2 weeks post-challenge. Mice were sacrificed and spleens harvested and cultured to observe clearance of the *Brucella*. In order for a *Brucella* vaccine to be considered protective, it must confer at least 1 log of protection over that achieved by saline alone (114). The clearance data suggests that the additional expression of the PA protein in *B. abortus* strain RB51 does not affect its ability to protect against a *Brucella* challenge. Comparison of groups immunized with either strain RB51/pBBGroE or RB51/pBBGroE-PA showed no difference in the level of protection against *Brucella* as measured by splenic clearance (Figure 2.7).

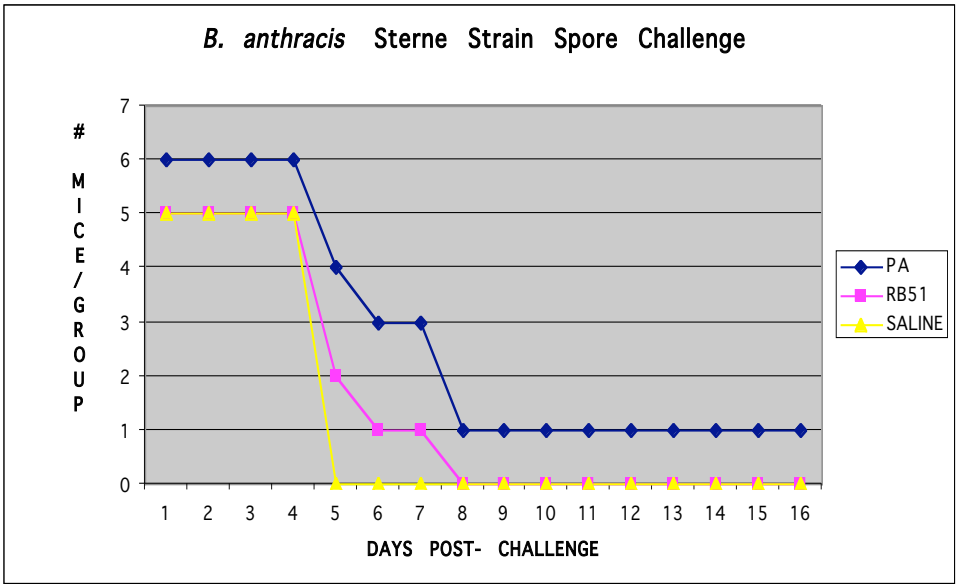

Figure 2.6: Survival of mice challenged with Sterne vaccine spores. Each mouse at day 0 received $5.6x10^4$ spores IP. The PA group corresponds to A/J mice immunized with the strain RB51/pBBGroE-PA. The RB51 group corresponds to A/J mice immunized with the strain RB51/pBBGroE. The saline group corresponds to A/J mice immunized with sterile saline.

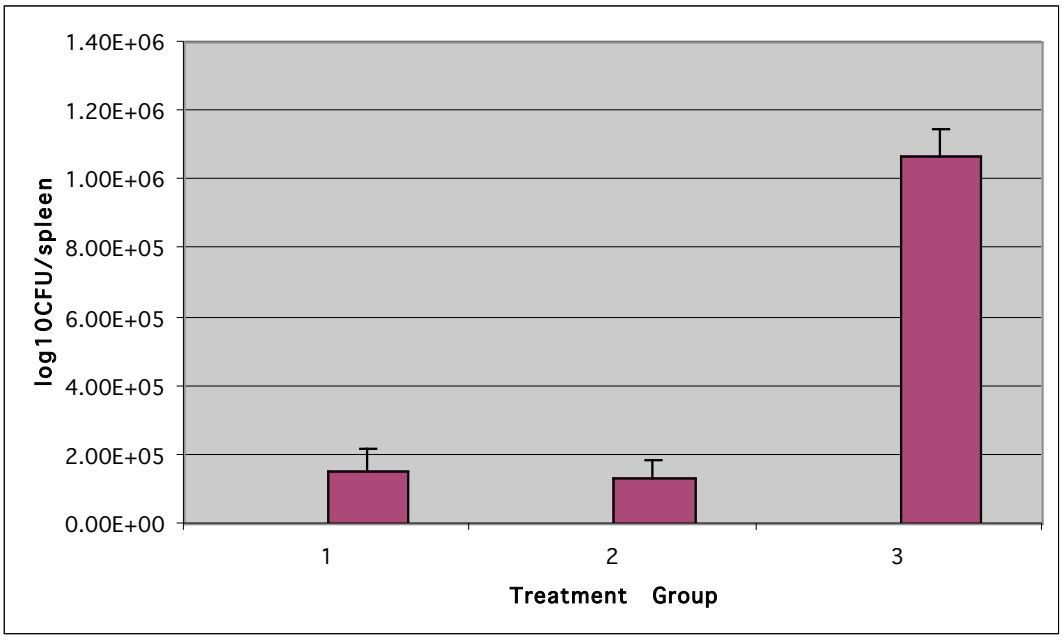

Figure 2.7: Clearance of *B. abortus* 2308 from A/J mice. Time of challenge was at 56 days post-immunization. Group 1 was immunized with the strain RB51/pBBGroE, group 2 with the strain RB51/pBBGroE-PA, and group 3 with sterile saline. The P value (by the paired t-test) for the t-test of group 1 vs. group 3=0.000251; group 2 vs. group 3=0.00905. The P value for the t-test of group 1 vs. group 2=0.403.

Discussion

While we know that strain RB51 is protective and able to synthesize homologous or heterologous antigens, those proteins need to be protective antigens in order to induce immunity (115). In this study, the PA protein of *B. anthracis* was chosen, since numerous studies have shown PA induces protective antibodies in immunized humans and animals; it is also the most common component of any anthrax vaccine (52,99,110).

The dual vaccine tested here used strain RB51 to synthesize and deliver PA to mice. One very important aspect of this dual vaccine's characteristic is the ability of the synthesized PA to offer detectable but limited protection against challenge by *B. anthracis* spores. Since the induced antibodies to PA were not fully protective, the vaccine's level of PA expression needs to be further refined. Studies currently underway include improving expression of PA by increasing plasmid copy number and examining whether an added signal sequence to the PA affects the level of specific immune responses. In a pilot study using BALB/c mice (Charles River Laboratories, Wilmington, MA), three mice were placed in each group and injected IP with a dose of $5x10^8$ cfu *B. abortus* RB51 containing either the pBBSOD or pBBSOD-PA. Western blots showed no reaction of the mouse sera to pure PA even after each mouse was given a second immunization of ~$2.5x10^8$ cfu *B. abortus* strain RB51/SOD-PA. Because other studies showed the SOD gene is down regulated in *Brucella* following uptake into macrophages (115), it was decided to use the pBBGroE-PA construct in the vaccine trial reported here. It may also be possible to identify a promoter other than GroE that is more strongly expressed during a *Brucella* infection; PA fused to this promoter would be a candidate vaccine for providing more protection against an anthrax challenge.

In addition to the antibodies induced, the ability of these recombinant strains to protect against infection by *B. anthracis* spores could be due to the nonspecific induction of cell mediated immunity (CMI); this arm of the immune system has been theorized to be an important component of anthrax immunity (6,54,56,57). Strain RB51 is a strong inducer of CMI (94,107,123) and may play some type of protective role. Further experiments are necessary to demonstrate that CMI is indeed contributing to protection induced by strain RB51/PA.

Western blots of extracts from strain RB51/pBBGroE-PA revealed its ability to produce full size PA. This is essential in the development of PA antibodies, as PA degradation products are unable to induce antibodies since they are biologically inactive both as antigens and toxin components (83). To further support our contention that the PA synthesized by strain RB51 is immunogenic and stimulates antibodies that recognize full length PA, we observed that 10 of the 11 female A/J mice immunized with *B. abortus* strain RB51/ pBBGroE-PA, developed antibodies recognizing full length PA (Figure 2.5).

The first and most common way to test the efficacy of an anthrax vaccine is to challenge immunized mice with spores from a virulent strain of *B. anthracis*. The second and somewhat safer way is to use a strain of mouse that is susceptible to the veterinary vaccine, Anthrax Spore Vaccine, produced by the Colorado Serum Company (Denver, CO). The live spore vaccine is derived from an avirulent strain of *B. anthracis*, known as Sterne strain, and is toxigenic but non-encapsulated. The A/J mice, a strain susceptible to avirulent anthrax spores (119,121), was used for this protection study. Injection of a lethal dose of Sterne spores into A/J mice results in a course of infection resembling the

pathogenesis of disease of fully virulent spores (119,121). Injection of ~10^4 spores results in 80-90% mortality in about 6 days (121). This is comparable to injection of mice with 6 spores from virulent *B. anthracis* Vollum 1B spores resulting in death in about 3 days (119). Therefore, since injection of susceptible mice with an avirulent strain of *B. anthracis* gives similar pathogenesis and endpoint, it is considered a useful and safer challenge model when testing the efficacy of a new anthrax vaccine (121).

The anthrax protection study in this research yielded some tantalizing results. Challenge with $2.4x10^4$ cfu of *B. abortus* strain 2308 and subsequent splenic clearance studies revealed that the strain RB51 immunized mice (either strain RB51/pBBGroE or pBBGroE-PA) had a 1 log greater rate of clearance than the saline immunized group. The strain RB51 vaccine is considered successful if it confers protection in terms of splenic clearance at the level of 1-2 logs greater than saline control mice (107). In addition, the mice challenged with *B. anthracis* Sterne strain provided some indications of protection. These mice were given a challenge dose of $5.6x10^4$ spores and observed for signs of illness and subsequent death. For unimmunized A/J mice receiving this dose of spores, the expected mortality rate is ~95% with an average time to death of ~4.5 days (121).

Overall, the trends of the survival curves of each challenged group are encouraging. The saline group did not survive beyond the expected period of time before succumbing to the challenge dose (121). The mice immunized with strain RB51/pBBGroE died more slowly than those in the saline group and possibly indicates a role of CMI in some non-specific protection. However, this non-specific protection was not enough to protect these mice as they also died from the challenge. The mice

immunized with strain RB51/pBBGroE-PA survived the longest before succumbing to the challenge dose; 5 of the 6 mice died.

The overall protection rate induced with this particular strain of RB51 is not as impressive as the protection rates observed with other recombinant bacterial systems producing PA (5,28,48,51,53). However, the level of protection and increased time to death post challenge indicates that the strain RB51 dual vaccine could be further refined to provide protection vaccination against anthrax and brucellosis.

In summary, the results of this research have demonstrated that *B. abortus* RB51 is able to express the full size PA encoded by the *pag* gene of *B. anthracis*. In addition, the immunization of A/J mice and subsequent challenge with *B. abortus* strain 2308 shows no interference in the vaccine strain's ability to confer protection while expressing PA. The challenge of A/J mice with *B. anthracis* Sterne strain spores indicates that the PA produced by strain RB51/pBBGroE-PA is able to confer a low level of protection.

3. Generation of *Brucella abortus* RB51 Vaccine Expressing *Bacillus anthracis* Protective Antigen

3.1 Abstract

Bacillus anthracis is a facultative intracellular bacterial pathogen that can cause cutaneous, gastrointestinal or respiratory disease in many vertebrates, including humans. Commercially available anthrax vaccines for immunization of humans are of limited duration and do not protect against the respiratory form of the disease. *Brucella abortus* is a facultative intracellular bacterium that causes chronic infection in animals and humans. As with other intracellular pathogens, cell mediated immune responses (CMI) are crucial in affording protection against brucellosis. *B. abortus* strain RB51 has been shown to be useful in eliciting protective CMI and antibody-mediated responses against *Brucella* in cattle and other animal species. Since the protective antigen (PA) of *B. anthracis* is known to induce antibodies, the *pag* gene encoding PA was expressed in *Brucella abortus* RB51, producing a dual vaccine to protect against both brucellosis and anthrax. In a previous study, the entire *pag* gene was expressed in strain RB51 and following immunization it induced antibodies against PA in A/J mice. However, PA stability and protective efficacy were less than desirable. The current study involved using a synthetic gene corresponding to domain 4 (PA4) of the *pag* gene utilizing the native codon usage of *Brucella*. The synthetic PA4 was ligated to *Brucella* signal sequences of *Brucella* 18kDa protein, superoxide dismutase or no signal sequence in an attempt to localize the PA4 to the outside cell envelope, periplasmic space or cytosol respectively. Comparisons of the expression level and stability of the native and synthetic PA4 in *Brucella* were assessed by immunoblot.

3.2 Introduction

Three proteins secreted by *B. anthracis* collectively constitute the anthrax toxin (101). These proteins are PA, EF and LF, which act in binary combinations to produce 2 toxic activities (101). PA is a required component of each of the 2 toxin combinations. This protein appears to have at least 3 functions in relation to the anthrax toxin: receptor binding, LF or EF binding and translocation of the toxic complex to the host cell cytosol (101). The crystal structure of PA has been mapped and shows that the protein consists of 4 distinct and functionally independent domains (37).

Full size PA protein is 735 amino acids long and 83 kilodalton (kDa) in size. Each domain is specifically required for a particular step in the anthrax toxin activity (17). Domain 1 is divided into domain 1a (residues 1-167) and 1b (residues 168-258) (37). Cleavage of mature PA by furin releases domain 1a, the N-terminis of the protein (17). Domain 1b remains part of the active protein (63kDa) and contains, along with domain 3, receptor sites for EF and LF (37). Domain 2 (residues 259-487) is a □-barrel containing a large flexible loop, which seems important in pore formation (17). Domain 3 (residues 488-595) is the smallest domain with a hydrophobic stretch thought to be involved in protein-protein interactions (17). Domains 2 and 3 are believed to form part of the heptameric pore of PA on the cell surface (37). Domain 4 (residues 596-735), the carboxyl-terminal end, is required for binding to the host cell receptor (82,113). This domain has limited contact with the other 3 domains (Figure 3.1) (17).

Antibodies to PA are essential in providing immunity to anthrax infection and studies indicate that it is domain 4 that contains the protective epitope of PA as antibodies to other domains do not convey protection without PA4 (37,66). *B. anthracis* vaccine strains expressing mutant forms of PA without domain 4 were unable to protect mice (15,16). The crystal structure of PA shows a 19 amino acid loop in domain 4, which is much more exposed than the other 3 domains that are in very close contact with each other. It is this structural arrangement of the protein, which may make the epitopes of domain 4 the most prominent for recognition by immune cells (37).

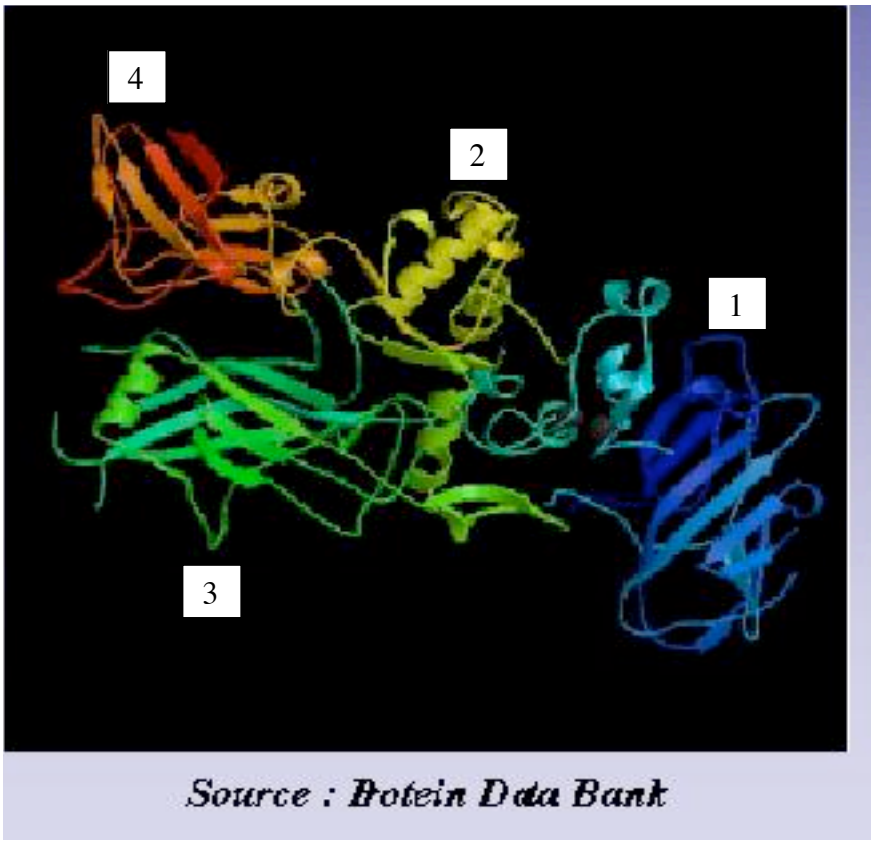

Figure 3.1: Crystal structure of *B. anthracis* protective antigen (http://www.rcsb.org/pdb/). Blue area is domain 1: 1a is light blue in color, 1b is darker. Domain 2 is yellow. Domain 3 is green. Domain 4 is red. Domains are also numerically labeled.

For gene expression, steps up to transcription are independent of the protein coding sequence and are able to be adjusted by manipulating vector sequences. However, control of gene expression at the translational level is mostly governed by the coding sequence within the gene (61). Organisms that have a highly skewed base composition, have been observed to follow compositional constraints as the main factor in determining the codon usage variation among the genes (45,58). It has been suggested that translational selection plays a part in manifesting the codon usage bias of highly expressed genes and the preferred codons in these genes are recognized by the most

abundant tRNAs (45). Therefore, another way to increase the yield of a protein is to modify the coding sequence of an individual gene without altering the amino acid sequence of the gene product (61,68). This strategy has been previously employed to improve expression of heterologous proteins in *E. coli* and mammalian cells (61,68).

Therefore, any recombinant vaccine expressing PA should contain domain 4 as part of the expressed protein. In a previous study (Chapter 2), the entire *pag* gene was expressed in *B. abortus* strain RB51. While PA expression was observed and subsequent PA antibodies found in mice, the PA expression levels were very low and truncated proteins were often observed. This observation may be due in part to the difference in G/C content between *Brucella* and *B. anthracis* (*Brucella* = 57%, *B. anthracis* = 36%) causing depletion of certain aminoacyl tRNA pools and thus truncated PA production (84,91). To overcome the problem of differences in codon usage between *Brucella* and *B. anthracis*, the codons within domain 4 of PA were converted to those of *Brucella* in order to obtain better expression of the protein. The synthetic PA4 was then fused to 2 different *Brucella* signal sequences and expressed in strain RB51. This study is an attempt to express PA4 in different locations within the *Brucella* cell (cytosol, periplasmic space, and outer membrane) to determine which location would result in the most efficient presentation to the immune system, i.e. as judged by antibody titer and/or protection.

3.3 Materials and Methods

Bacterial strains, media and growth conditions:

The *Brucella* and anthrax vaccine strains used in this study are listed in Table 3.1. *E. coli* TOP10 DH5 (Invitrogen, Carlsbad, CA) were grown in Luria-Bertani media

(Fisher Biotech, Norcross, GA) or Trypticase Soy Broth (Difco Laboratories, Detroit, MI) (30). *B. abortus* RB51 was grown on Trypticase Soy Broth and SOC-B (6% trypticase soy both, 10mM NaCl, 2.5mM KCL, 10mM $MgCl_2$, 10mM $MgSO_4$ and 20mM glucose) (72). All cultures were grown at 200 rpm at 37ºC at normal atmosphere. Cultures transformed with plasmids (listed in Figure 3.9) were grown on selective media containing one of the following antibiotics: ampicillin (Amp) 100ug/mL, chloramphenicol (Cm) 30ug/mL. Bacto-Agar was purchased from Fisher Biotech (Norcross, GA).

Bacterial Species and Strain	**Attributes**
Brucella abortus RB51	Lacks O-side chain, rifampin resistant, reduced virulence
Bacillus anthracis Sterne	Non-encapsulated, toxin producer, reduced virulence

Table 3.1: List of bacterial strains used in this study.

Reagents and enzymes:

Restriction endonucleases and T4 DNA ligase were purchased from Invitrogen (Carlsbad, CA). Ready-to-Go PCR beads were purchased from Pharmacia Biotech (Piscataway, NJ). DNA miniprep kit, PCR cleanup kit, and gel extraction kits were purchased from Qiagen (Valencia, CA). Agarose and restiction enzymes were purchased from Fisher Scientific (Norcross, GA). All other reagents were purchased from Sigma Chemical Corporation (St. Louis, MO).

Construction of PA Domain 4 (PA4) using *Brucella* codon usage:

PA4 was converted from the codon usage of *B. anthracis* to that of *Brucella* using bacterial species codon usage tables found on the National Center for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov). For this, the most common of the

amino acid codons for *Brucella* replaced the corresponding ones in PA4 gene of *B. anthracis*. In order to express this new PA4, the gene was commercially synthesized. Three 104base oligos and 2 linker oligos were synthesized by Sigma Genosys (The Woodlands, TX). These 5 oligos were linked together using DNA ligation techniques previously described (Figure 3.2) (Chapter 2). Briefly, the oligos were added to a ligation reaction at a 1:4 ratio (oligo:linker) using 5x concentration T4 ligase (Invitrogen, Carlsbad, CA) at 16°C overnight. The ligation reaction was then added directly to a PCR reaction using Ready-to-Go PCR beads (Amhersham Pharmacia, Piscataway, NJ). The PCR reaction mixture was in a final volume of 25uL and contained primers at a concentration of 1pmol/uL, DNA at a concentration of 50-100ng/uL, and water. The mixture was over-laid by mineral oil to prevent evaporation. The PCR protocol required 35 cycles for completion: denaturation at 65ºC for 1minute, annealing at 58ºC for 90 seconds, and elongation at 72º for 1 minutes, with a final elongation step of 7 minutes using primers PA4 Forward and PA4 Reverse for PCR reactions (Table 3.2).

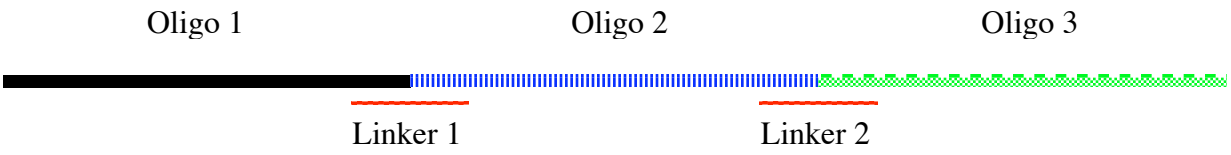

Figure 3.2: Schematic of construction of synthetic PA4. Three 104base oligos were ligated together using overlapping linkers to yield a single-stranded oligo 312b in length that was then amplified by PCR.

Other PCR protocols:

In order to place the proper *Brucella* signal sequence in front of PA4, additional PCR steps were performed using various primers to amplify the 18kDa and *sod*C signal sequences and PA4 (Table 3.2). These PCR products were then cleaned up using a

Qiagen PCR clean up kit (Valencia, CA) and ligated together and into plasmid pBBGroE using the protocol previously described (Chapter 2).

Cloning Protocol:

The broad host range plasmid pBBR1MCS was used to create the pBBSOD and pBBGroE plasmids. A partial sequence of the *Brucella* gene including the promoter region of either superoxide dismutase (SOD) or heat shock protein (GroE) was added to pBBR1MCS to create plasmids pBBSOD or pBBGroE (R. Vemulapalli, VPI&SU) (115). PCR products were ligated into the pBBGroE plasmid. Ligation reactions had a final volume of 10uL and contained a 1:1 to 1:2 ratio of vector to insert, 1ul of 10x ligation buffer, 1U of T4 DNA ligase and water. Ligation reactions were incubated overnight at 16ºC and then used to transform *E. coli* DH5µ cells by a heat shock method (32). Transformed cells were plated onto TSA-Cm plates and grown overnight at 37ºC. Colonies were picked from the plate and used to inoculate 10mL cultures of TSB broth and grown at 37ºC overnight on a shaker. These cultures were then centrifuged at 2060 x g at 4ºC for 10 minutes to form a cell pellet. Extraction of plasmid DNA was done using the Qiaprep Spin Miniprep kit (Qiagen, Valencia, CA).

Oligo #1 **5'** A GAC ATC CGC AAG ATC CTG TCG GGC TAT ATC GTC GAA ATC GAA GAC ACC GAA GGC CTG AAG GAA GTC ATC AAC GAC CGC TAT GAC ATG CTG AAC ATC TCG **3'**
Oligo #2 **5'** TCG CTG CGC CAG GAC GGC AAG ACC TTT ATC GAC TTT AAG AAG TAT AAC GAC AAG CTG CCG CTG TAT ATC TCG AAC CCG AAC TAT AAG GTC AAC GTC TAC GCC GTC **3'**
Oligo #3 **5'** ACC AAG GAG AAC ACC ATC ATC AAC CCG TCG GAA AAC GGC GAC ACC TCG ACC AAC GGC ATC AAG AAG ATC CTG ATC TTT TCG AAG AAG GGC TAC GAA ATC GGC TGA **3'**

Linker #1 **5'** CTG GCG CAG CGA CGA GAT GTT CAG **3'**
Linker #2 **5'** GGT GTT CTC CTT GGT GAC GGC GTA GAC **3'**
PA4 Forward: 5' **CCCGGGGGATCCAGACATCCGCAAG** 3'
PA4 Reverse: **5'** GACGTCCCGCGGTCAGCCGATTTCGTAGCC **3'**
nPA4 Forward: **5'** CCCGGGGGATCCAGATATAAGAAAA **3'**
nPA4 Reverse: **5'** GAGCTCCCGCGGTTATCCTATCTC **3'**
SOD signal sequence Forward: **5'** TCTAGACAGGGGGAATGGCCTTACGGT **3'**
18kDa signal sequence Forward: **5'** GAATTCCGACCCGCGCGC **3'**
18kDa signal sequence Reverse: **5'** GATATCGCACCCGGCCAGGACAATGCC **3'**
18kDa/sPA4 linker **5'** GGGCCCCTGTAGGCGTTCTAG **3'**

Table 3.2**:** PCR primers and linkers used in generating plasmids. Synthesized oligos and linkers for PA4.

Generation of plasmid pBBGroE/PA has been previously described (Chapter 2). Plasmid pBBGroE/PA4 was generated by directly ligating PA4 into pBBGroE using restriction endonucleases *Xba*I and *Sac*II. pBBGroE/SOD'::PA4 was first subcloned into pBBSOD using restriction endonucleases *Sma*I and *Sac*II. This allowed PA4 to be fused to the signal sequence and N-terminis of SOD in the correct reading frame for expression. The cassette containing the promoter/signal sequence/N-terminis/PA4 was then PCR amplified from the pBBSOD/PA4 plasmid and cloned into pBBGroE using restriction endonucleases *Xba*I and *Sac*II. Plasmid pBBGroE/SOD'::nPA4 was created in the same way. PBBGroE/SOD'::PA4/SOD was created using pBBGroE/SOD'::PA4 as the backbone plasmid. A cassette containing the promoter GroE::*Brucella* Cu-Zn SOD

fusion was cloned into the vector using restriction endonuclease *Kpn*I. Plasmid pBBGroE/18::PA4 was created by PCR amplifying the 18kDa signal sequence out of genomic DNA extracted from *B. abortus* 2308. This PCR product was then cleaned up using Qiagen PCR Cleanup kit (Valencia, CA) and ligated to PA4 using the ligation protocol described above. The ligation reaction was then amplified using primers PA4 Reverse and 18kDa signal sequence Forward. The PCR reaction was then ligated into pBBGroE using restriction endonucleases *EcoR*V and *Xba*I.

Once all plasmids were generated, restriction enzyme digestion with the appropriate endonucleases followed by gel electrophoresis, PCR analysis and DNA sequencing (Virginia Bioinformatics Institute, Blacksburg, VA) confirmed the correct orientation of the gene and signal sequences within the plasmid.

Heat shock transformation:

Transformation of *E. coli* TOP10 DH5 cells was accomplished using the heat shock method as described by Invitrogen (Carlsbad, CA) (32).

Transformation of *B. abortus* RB51:

Competent *B. abortus* RB51 cells were made and transformed with the plasmid constructs via electroporation (72). Transformed cells were plated onto TSB-Cm plates and incubated at 37ºC for 4 days. Cm resistant colonies were tested for transformation by performing Western blot analysis on specific extracts using specific PA antiserum.

SDS-PAGE of recombinant RB51/PA strains:

Forty microliters of cell pellet extracts from a 20 ml culture of *B. abortus* RB51 transformed with the plasmids pBBGroE, pBBGroE/PA, pBBGroE/PA4, pBBGroE/18::PA4, pBBGroE/SOD'::PA4, or pBBGroE/SOD'::PA4/SOD were prepared

with equal volumes of 2x Laemmli buffer and separated on SDS-PAGE gels to check for expression of PA using the procedure of Laemmli with some modifications (65). A 12.5% acrylamide gel was used following standard protocol using the Mini-Protean® II gel apparatus (Bio-Rad, Rockville, NY) (4). Gels were run 90 minutes at 25mA/gel in SDS-Page electrophoresis buffer (25mM Tris, 0.19M Glycine, 0.1% SDS, pH 8.3).

Analysis of PA4 proteins by western blot:

Gels were run for 90 minutes at 25mA per gel. Proteins were then transferred from the gel to nitrocellulose membrane (7.5cm x 10cm) in preparation for immunoblotting by electro-transfer. Nitrocellulose membranes with attached proteins were blocked in 1% bovine serum albumin overnight to prevent non-specific binding of the 1º antibody (rabbit anti-PA) (S. Leppla, NIH) to the membrane. The blocked membranes were then exposed to the 1º PA antibody at a 1:1000 overnight on a shaker at 4ºC. The membranes were then washed three times and 2º antibody (goat anti-rabbit IgG) (Sigma Chemical Co., St. Louis MO) at a 1:2000 dilution added to the blot for 3 hours at room temperature. The membranes were washed again three times and the substrate, 4-chloro-1-napthol, was added to the blot and reaction halted by rinsing blot with dH_2O. Purple color developed where the primary antibody recognized protein bands.

3.4 Results

Amino Acid	**Codon**	**% in *Brucella Sp.***	**% in *B. anthracis***
Alanine (A)	GCA	15	44
	GCC	39	8
	GCG	24	17
	GCU	22	31
Arginine (R)	AGA	2	40
	AGG	4	10

	CGA	4	15
	CGC	56	6
	CGG	9	6
	CGU	25	23
Asparagine (N)	AAC	52	25
	AAU	**48**	**75**
Aspartic Acid (D)	GAC	50	17
	GAU	50	83
Cysteine (C)	UGC	77	30
	UGU	23	70
Glutamine (Q)	CAA	13	77
	CAG	87	23
Glutamic Acid ((E)	**GAA**	**69**	**75**
	GAG	31	25
Glycine (G)	GGA	6	38
	GGC	60	12
	GGG	8	19
	GGU	26	31
Histidine (H)	CAC	35	19
	CAU	**65**	**81**
Isoleucine (I)	AUA	5	30
	AUC	69	15
	AUU	26	55
Leucine (L)	CUA	1	12
	CUC	26	4
	CUG	38	5
	CUU	24	14
	UUA	1	50
	UUG	10	15
Lysine (K)	AAA	17	72
	AAG	83	28
Methionine (M)	**AUG**	**100**	**100**
Phenylalanine (F)	UUC	73	26
	UUU	27	74
Proline (P)	CCA	7	38
	CCC	20	8
	CCG	59	18
	CCU	13	36
Serine (S)	AGC	22	9
	AGU	4	29
	UCA	7	22
	UCC	28	6
	UCG	32	8
	UCU	7	25
Stop (.)	**UAA**	**44**	**52**
	UAG	12	28

	UGA	44	20
Threonine (T)	ACA	9	47
	ACC	46	8
	ACG	36	18
	ACU	9	27
Tryptophan (W)	**UGG**	**100**	**100**
Tyrosine (Y)	UAC	36	25
	UAU	**64**	**75**
Valine (V)	GUA	6	41
	GUC	33	7
	GUG	29	21
	GUU	**32**	**31**

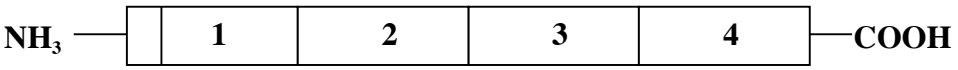

Table 3.3: Codon Usage in *Brucella* and *Bacillus anthracis.* Bold indicates codon remaining unchanged. The most common codon for each amino acid used by *Brucella* replaced that used by *B. anthracis.* A schematic of protective antigen protein domains 1-4. Domain 1 consists of 1a and 1b. Domain 1b remains part of the active protein (63kDa) and contains along with domain 3, receptor sites for EF and LF. Domain 2 is a □-barrel containing a large flexible loop possibly important in pore formation. Domain 4 is the host cell receptor-binding site.

Generation of Synthetic PA4 and plasmids:

The domain 4 of PA was converted from a codon usage that is considered A/T rich (36%) to the codon usage of Brucella that is G/C rich (57%) in an effort to increase its expression level and stability in *B. abortus* strain RB51. The first step in the process was to examine the DNA sequence of PA4 and choose the codons favored by *Brucella* over that of *B. anthracis* while keeping the encoded amino acid the same (Table 3.3). Once the new sequence was deciphered, the synthetic PA4 was designed in 3 oligos each of 104base in length (Figure 3.2). These three oligos were joined in proper sequence using overlapping linkers in an overnight ligation reaction. This ligation reaction was then PCR amplified using primers PA4 Forward and PA4 Reverse and run on a 1% agarose gel to observe the size of the amplified product. A product of the expected size,

~312bp, was observed. Products of the PCR reactions were sequenced and plasmid sequences were then aligned with either the native PA4 or synthetic PA4 using Sequencher software (Gene Codes Corp., Ann Arbor, MI). Each sequenced plasmid aligned correctly with the appropriate DNA sequence of the PA4 gene in one direction. Generally the reverse PA4 primer was used for sequencing reactions. To scale drawings of the sequenced plasmids can be seen in Figures 3.3 – 3.7.

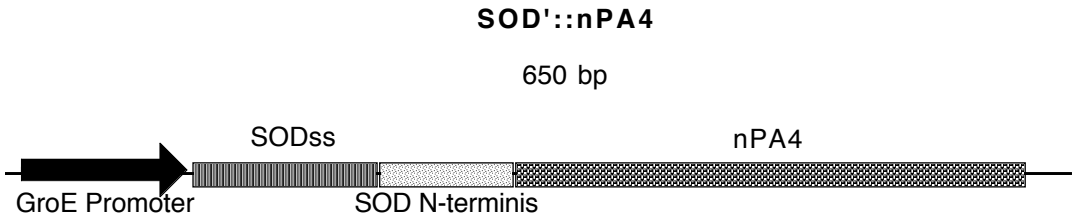

Figure 3.3: Linear representation of plasmid SOD'::nPA4. PA4 (in *B. anthracis* codon usage) is fused to the *Brucella* Cu-Zn SOD signal sequence and N-terminis. The whole gene is under control of the GroE promoter.

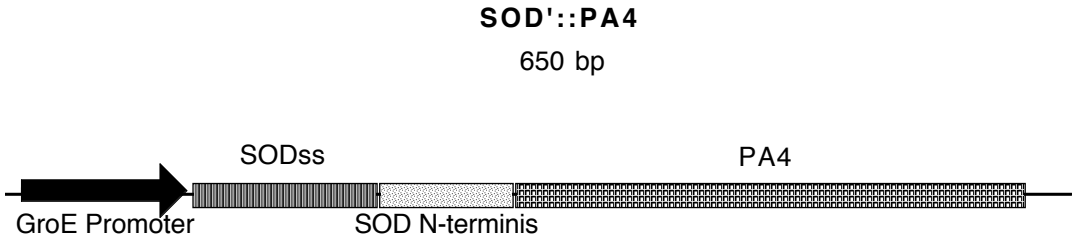

Figure 3.4: Linear representation of plasmid SOD'::PA4. PA4 (in *Brucella* codon usage) is fused to the *Brucella* Cu-Zn SOD signal sequence and N-terminis. The whole gene is under control of the GroE promoter.

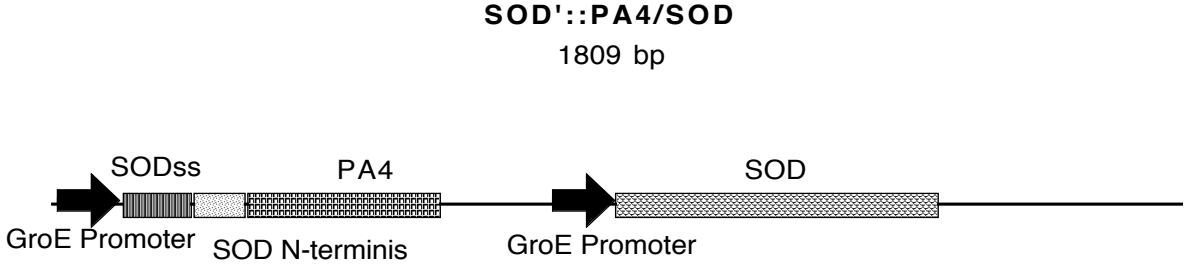

Figure 3.5: Linear representation of plasmid SOD'::PA4/SOD. PA4 (in *Brucella* codon usage) is fused to the *Brucella* Cu-Zn SOD signal sequence and N-terminis. The whole gene is under control of the GroE promoter. In addition, the *Brucella* Cu-Zn SOD under the control of the GroE promoter was placed on the plasmid for SOD overexpression.

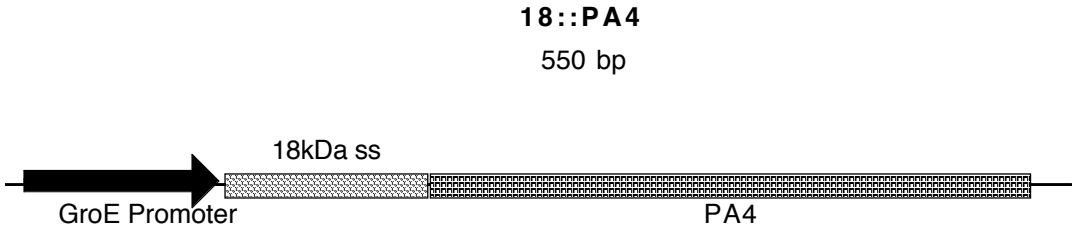

Figure 3.6: Linear representation of plasmid 18::PA4. PA4 (in *Brucella* codon usage) is fused to the *Brucella* 18kDa outer membrane protein signal sequence. The whole gene is under control of the GroE promoter.

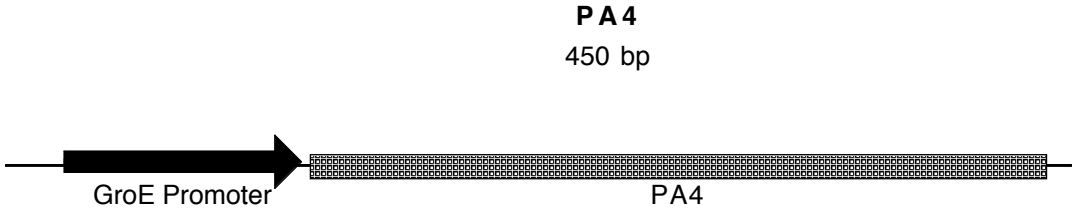

Figure 3.7: Linear representation of plasmid PA4. PA4 (in *Brucella* codon usage) is not fused to any signal sequence. The gene is under control of the GroE promoter.

Transformation of *B. abortus* strain RB51 with plasmids:

The plasmids generated were used to transform strain RB51 by electroporation. Each plasmid was created to localize the PA4 protein to a different area of the bacterial cell: outer cell envelope, periplasmic space or cytosol. In addition,

pBBGroE/SOD'::PA4/SOD was created to over-express Cu-Zn SOD as well as PA4 in the periplasmic space (Table 3.4 and Figure 3.8).

RB51/PA
Brucella abortus strain RB51 transformed with pBBGroE/PA. Contains complete *pag* gene and expresses full size PA. CmR.

RB51/SOD'::PA4
Brucella abortus strain RB51 transformed with pBBGroE/SOD'::PA4. Contains domain 4 of *pag* gene (*Brucella* codon usage) and expresses PA4 fused to SOD signal sequence and N-terminis. CmR.

RB51/SOD'::nPA4
Brucella abortus strain RB51 transformed with pBBGroE/SOD'::nPA4. Contains domain 4 of *pag* gene (native *Bacillus* codon usage) and expresses PA4 fused to SOD signal sequence and N-terminis. CmR.

RB51/18::PA4
Brucella abortus strain RB51 transformed with pBBGroE/18::PA4. Contains domain 4 of *pag* gene (*Brucella* codon usage) and expresses PA4 fused to 18kDa signal sequence. CmR.

RB51/PA4
Brucella abortus strain RB51 transformed with pBBGroE/PA4. Contains domain 4 of *pag* gene (*Brucella* codon usage) and expresses PA4 with no signal sequence. CmR.

RB51/SOD'::PA4/SOD
Brucella abortus strain RB51 transformed with pBBGroE/SOD'::PA4. Contains domain 4 of *pag* gene (*Brucella* codon usage) and expresses PA4 fused to SOD signal sequence and N-terminis. CmR. Overexpresses Cu-Zn SOD.

Table 3.4: Plasmids Used In Study and Description. Localization was checked by western blot of culture media.

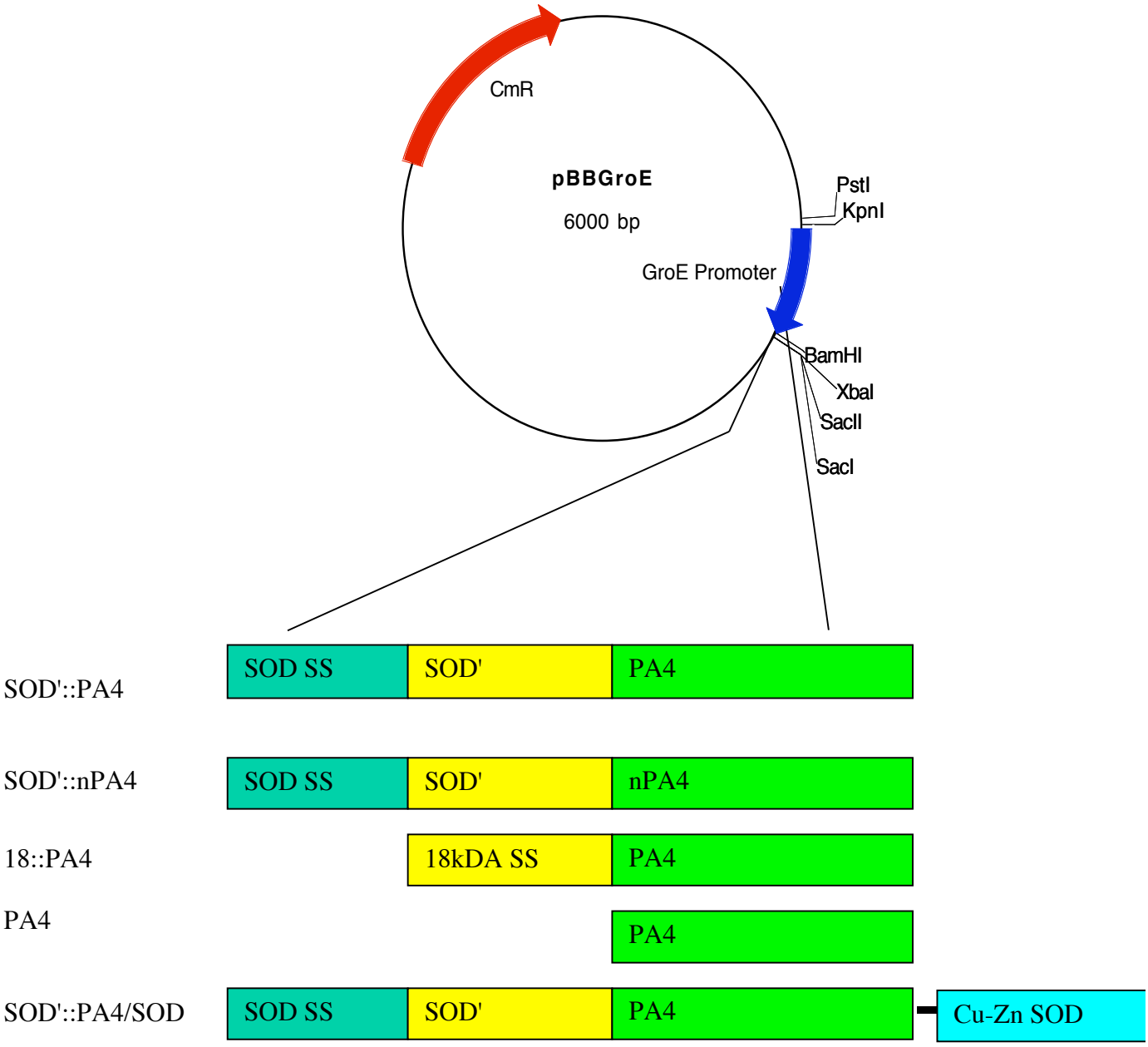

Figure 3.8: Schematic of pBBGroE/PA plasmids. Domain 4 of *B. anthracis* protective antigen was converted to the codon usage of *Brucella* and then fused to *Brucella* signal sequences for expression in various locations of the *B. abortus* strain RB51 cell.

Western Blot analysis of PA4 production:

Cell extracts from strain RB51 cultures expressing PA4 were probed with rabbit PA antibodies (S. Leppla, NIH) to check for protein expression. Each RB51/PA strain produced PA4 protein that was recognizable by these antibodies. Strains RB51 and RB51 expressing full size PA were not recognized by these PA antibodies (Figure 3.9).

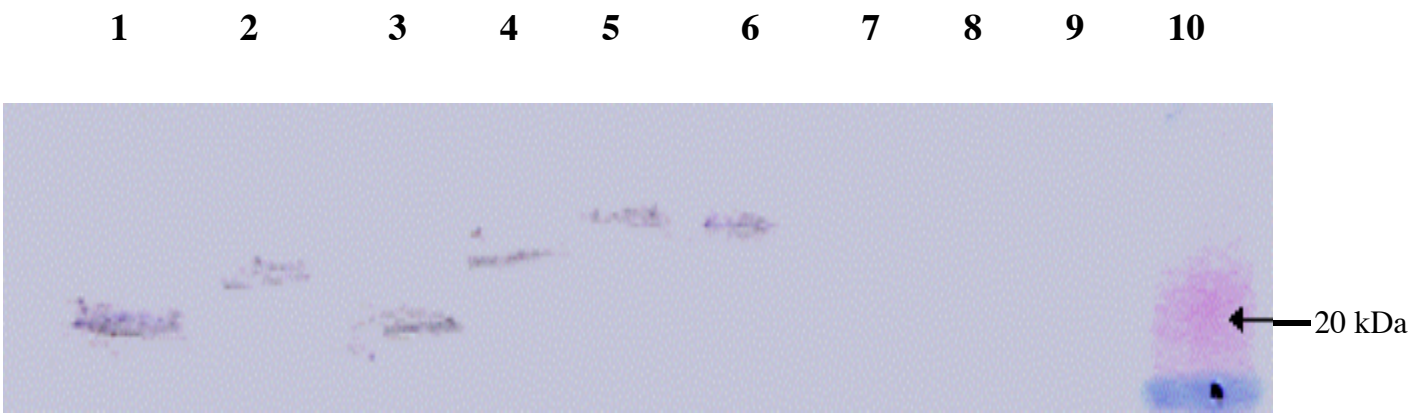

Figure 3.9: Western Blot of *Brucella* extracts expressing PA4. Developed using rabbit PA antibodies. Lane 1-purified PA4 from inclusion bodies of pRSET/PA4, lane 2-RB51/18::PA4, lane 3-RB51/PA4, lane 4-RB51/SOD'::nPA4, lane 5-RB51/SOD'::PA4/SOD, lane 6-RB51/SOD'::PA4, lane 7-blank, lane 8-RB51, lane 9-RB51/PA and lane 10-molecular weight marker. PA4 is about 19kDa.

3.5 Discussion

Control of gene expression at the translational level can be governed by the sequences within the gene (61). Organisms with a highly skewed base composition (G/C or A/T rich) have been noted to follow constraints which serve as the main factor in determining the codon usage variation of the genes (45,58). Almost every protein encoding sequence that has been analyzed seems to display a bias in codon usage for that organism (80). Most often the codon bias reflects the genome composition bias, however, there is evidence that selection discriminates between synonymous codons in some organisms (80,93). In each synonymous group there is usually a major codon per

amino acid, which is complimentary to the most abundant tRNA (80,93). Highly expressed genes show a higher degree of this bias and a clear correlation between synonymous codon usage bias and protein amount has been reported (93). This kind of bias, found in highly expressed genes is said to be the consequence of selection for translation efficiency (58). Therefore, one way to increase the efficiency and yield of a protein is to modify the coding sequence of the gene so that it calls for the most abundant codon without altering the amino acid sequence of the product, a strategy previously employed for heterologous gene expression in *E. coli* (61,68).

PA could be considered a highly expressed gene of *B. anthracis* since the protein is required for the bacterium to exert effects on the host (101). Without PA, the anthrax toxins are unable to enter the host cell (101). The PA protein of *B. anthracis* has been expressed in *B. abortus* strain RB51 in a previous study. While strain RB51 was indeed able to express PA, the stability and amount of protein production was less than desirable. One possible explanation for this could be the great difference in the G/C content between *Brucella* and *B. anthracis*, i.e. 57 vs. 38% respectively (84,91). In its native state the gene sequence of PA required some codons that are not considered abundant or preferable in *Brucella*. The production of native PA could have been halted due to the depletion of those tRNAs leading to truncated proteins or "degraded" proteins observed in the previous study. While strain RB51 expressing native PA was able to induce antibodies to PA, these antibodies or their levels were not very protective possibly due to a lack of sufficient PA presented to the host's immune system after the *Brucella* vaccine was processed by antigen presenting cells.

This study was an attempt to produce a higher level of PA production by changing the codon usage of the gene to that of *Brucella* so as not to deplete the tRNA supply before the protein was fully processed. In addition, only the most antigenic/protective epitope domain of the PA protein was expressed. This allowed the fusion of the domain to different *Brucella* signal sequences in order to localize PA4 in a location other than the cytosol of strain RB51. PA is secreted by *B. anthracis*, thus, it is more readily seen by the host's immune system since it is outside of the bacterium. By localizing the PA4 to the periplasmic space and the outer membrane, it was reasoned that these locations would facilitate better presentation of the protein to the immune system thereby eliciting a more protective immune response.

Western blot showed that the synthetic PA4 was indeed produced by *B. abortus* strain RB51. Each of the possible vaccine candidates produced PA4 that was recognizable by PA antibodies (Figure 3.9). This indicates that *Brucella* is producing the protein using abundant *Brucella* codons. PA bands observed appear to be of slightly differing sizes possibly due to signal sequences fused to PA4 causing it to migrate at different sizes. Each signal sequence fused to PA domain 4 added from ~100bp to ~200bp depending on the sequence added. This difference in sequence size would significantly change the size of the protein but may be the cause of the observed differences in band migration on the Western blot. The lower weight of the band representing SOD'::nPA4 appears to be slightly lower than the corresponding band for SOD'::PA4. This observation could be due to that presence of truncated PA4 in SOD'::nPA4 since the codon usage of the PA4 gene in this construct is native *B. anthracis*; possibly leading to depletion of highly used tRNAs. Each band was seen near

the expected molecular weight. In addition, it appears, based on PA band intensity that there may be some differences in the amount of synthetic PA4 produced by the different vaccine constructs. These constructs will be used to immunize A/J mice and the immune responses observed for efficacy against live challenge by *B. anthracis* Sterne spores and *B. abortus* 2308.

4. Evaluation of Mice Immune Responses to *Brucella abortus* RB51 Vaccines Expressing Protective Antigen Domain 4 of *Bacillus anthracis*

4.1 Abstract

Bacillus anthracis is a facultative intracellular bacterial pathogen that can cause cutaneous, gastrointestinal or respiratory disease in many vertebrates, including humans. Commercially available anthrax vaccines for immunization of humans are of limited duration and are not proven to protect against the respiratory form of the disease and commercially available live vaccines for animals have been shown to cause disease in certain species. *Brucella abortus* is a facultative intracellular bacterium that causes chronic infection in animals and humans. As with other intracellular pathogens, cell mediated immune responses (CMI) are crucial in affording protection against brucellosis. *B. abortus* strain RB51 has been shown to be useful in eliciting protective CMI and antibody-mediated responses against *Brucella* in cattle and other animal species. Since the protective antigen (PA) of *B. anthracis* is known to induce antibodies, the domain 4 (PA4) of the *pag* gene encoding PA fused to various *Brucella* protein signal sequences was expressed in *B. abortus* strain RB51. A synthetic PA4 was constructed using *Brucella* native codon usage in an attempt to boost protein production and stability. Comparisons of the expression level and stability of the native and synthetic PA4 in *Brucella* was assessed by protection studies in A/J mice in order to determine the protective capabilities of the various vaccine candidates against a live challenge with either *B. abortus* or spores of *B. anthracis* Sterne. Live challenge revealed partial protection of mice against Sterne spores and full protection against *B. abortus* 2308. In order to improve anthrax protection levels, mice were immunized with the RB51/PA4 strains and boosted with purified PA. Challenge results indicate an improvement in protection in mice boosted with PA.

4.2 Introduction

The *pag* gene of *Bacillus anthracis* codes for the PA protein, which is one component of the 2 toxins produced by the bacterium, the other two components are LF and EF (66). These toxin components when used as antigens have been shown to confer protective immunity against anthrax (102). However, numerous studies have shown PA to be the component that is essential in an anthrax vaccine (64). The primary immunogenic component of the human vaccine is PA and the Sterne strain used for vaccination of animals produces PA as well. However, each of these vaccines has a history of undesirable side effects (102).

Currently, increased research activity in the field of anthrax vaccine development is aimed at a vaccine whose main component is the *B. anthracis* PA protein adjuvanted with some sort of strong CMI inducer. An ideal vaccine will require fewer immunizations, elicit fewer side effects and induce a stronger and more prolonged immunity against highly virulent strains of *B. anthracis* even under aerosol exposure. Once the stigma of using a live vaccine for immunization can be overcome, an attenuated recombinant bacterial strain would be a good choice as a vaccine delivery vector.

For toxin activity, PA is required and seems to have at least 3 different functions: receptor binding, LF or EF binding and translocation of the toxic complex to the cytosol (101). The PA protein is composed of 4 distinct domains which each play a role in the various functions of the protein. The C- terminis of PA is involved in receptor binding to host cells delivering the toxic complex to the cell (82).

This C- terminis is referred to as domain 4 (16). A previous study has shown that 2 monoclonal antibodies, 3B6 and 14B7, are able to recognize the receptor binding region of domain 4 and block the ability of PA to bind to its receptor (66). The antibodies are able to protect murine macrophage J774A.1 cells from PA-LF (LeTx) challenge (113). Additionally, truncation of PA in the C- terminis reduces the toxicity of LeTx due to a decrease in the ability of PA to bind to the host cell receptor (37,113).

A previous study by Flick-Smith *et al.*, (37) showed that while antibodies against PA are required for protection against anthrax challenge, those antibodies need not recognize the entire protein. In their study, A/J mice were immunized with varying combinations of domains of PA and antibody responses measured following immunization and survival after challenge with *B. anthracis* Sterne spores. All 4

domains of PA were able to induce antibodies in the mice, however, not all domains could be considered protective. All mice in groups immunized with fusion proteins containing domain 4 were fully protected against challenge (37). This study and previous studies employing truncated PA to immunize mice indicate that domain 4 carries the protective epitope of PA (15,16,37).

Vaccination of animals against brucellosis with live *Brucella abortus* strain RB51 induces response from both arms of the immune system (95). The strength and duration of the response depends on factors such as dose and route of vaccination (78). Strain RB51 is a strong inducer of CMI with an ability to express heterologous proteins (derived from other species) that in turn induces an immune response dependent upon the antigen (115).

Since heterologous gene expression is possible in strain RB51 and mice immunized with the recombinants respond by producing specific antibody, the presence of PA antibodies is expected in mice immunized with strain RB51/PA. The presence of antibodies against PA and the CMI response induced by strain RB51/PA should be sufficient to confer protection against two corresponding bacterial disease threats. The creation and use of this dual vaccine could potentially be economically and militarily important.

Therefore, in this study, we used various *B. abortus* strain RB51 vaccine candidates expressing PA4 under different *Brucella* signal sequences to immunize A/J mice and assessed the ability of these vaccine candidates to protect mice against *B. abortus* 2308 and *B. anthracis* Sterne spore challenge.

4.3 Materials and Methods

Immunization Protocol Study 1:

A/J mice (Jackson Laboratories, Bar Harbor, ME) were divided into 9 groups of 14 mice each. One group of mice was designated as controls and injected intraperitoneally (IP) with 0.2mL of sterile saline. Another group was designated the RB51 group and injected IP with $3.6x10^8$ cfu of *B. abortus* RB51. Fourteen mice were designated for each of the following groups: RB51/PA, RB51/SOD'::PA4, RB51/SOD'::nPA4, RB51/18::PA4, RB51/PA4, RB51/SOD'::PA4/SOD and pure PA protein dissolved in sterile saline. These mice were injected IP with $3.5x10^8$ cfu of *B. abortus* RB51 transformed with the appropriate PA plasmid. One final group of 14 mice were each immunized with ~3.2□g of pure PA dissolved in sterile saline as a positive control. Eleven mice from each group were used in challenge studies 8 weeks post-immunization and the remaining 3 mice in each group were used for CMI studies.

Immunization Protocol Study 2:

A/J mice (Jackson Laboratories, Bar Harbor, ME) were divided into 7 groups of 6 mice each. One group of mice were designated as controls and injected intraperitoneally (IP) with 0.2mL of sterile saline. Another 2 groups were designated the RB51 group and were injected IP with $3.6x10^8$ cfu of *B. abortus* RB51. Six mice were designated for each of the following groups: RB51/SOD'::PA4, RB51/18::PA4, RB51/PA4, and pure PA protein dissolved in sterile saline. These mice were injected IP with $3.5x10^8$ cfu of *B. abortus* RB51 transformed with the appropriate PA plasmid. One final group of 6 mice were each immunized with ~3.2□g of pure PA suspended in sterile saline. All groups except the saline and one GroE group were boosted with an IP injection of 3.2□g PA on

week 4. The remaining 2 groups received an IP injection of sterile saline. All mice from each group were used in challenge studies 10 weeks post-immunization using *B. anthracis* Sterne spores.

Western Blot:

Pure PA protein (3μg) (List Biologicals Laboratories Inc., Campbell, CA) was loaded onto SDS-PAGE gels and electrophoresed as previously described. Gels were run for 90 minutes at 25mA per gel. Proteins were then transferred from the gel to nitrocellulose membrane (7.5cm x 10cm) in preparation for immunoblotting by electro-transfer. Nitrocellulose membranes with attached proteins were blocked in 1% bovine serum albumin overnight to prevent non-specific binding of the 1° antibody (rabbit anti-PA) (provided by S. Leppla, NIH) to the membrane. The blocked membranes were then exposed to the 1° antibody diluted 1:100 overnight on a shaker at 4°C. The membranes were then washed 3 times and 2° antibody, goat anti-rabbit IgG (HRP labeled) (Sigma, St. Louis, MO), at a 1:1000 dilution added to the blot for 3 hours at room temperature. The membranes were washed again three times and the substrate, 4-chloro-1-napthol, was added to the blot. Color reaction was stopped by washing blot in dH_2O. Purple color developed where the primary antibody recognized protein bands.

ELISA:

96-well plates (MaxiSorp™, Nalgene-Nunc International, Rochester, NY) were coated at 4°C overnight using 1μg of PA per well in bicarbonate buffer. Plates were then blocked with 2% BSA in PBS overnight at 4°C. Plates were washed 3 times with wash buffer (PBS/0.05%Tween-20). Serum samples, from immunized mice, diluted 1:100 in 2% BSA were added (in triplicate) to each well and incubated at room temperature for 3

hours. Plates were washed 4 times with wash buffer and secondary antibody, anti-mouse isotype specific (IgG, IgG1, IgG2, IgM) goat (HRP-labeled) 2° antibodies (Sigma, St. Louis, MO) were added to the wells at a dilution of 1:5000 and incubated at room temperature for 30 minutes. Plates were washed 5 times with wash buffer. 100 l of TMB Microwell, 1-component substrate (KPL, Gaithersburg, Maryland) was added to each well and incubated at room temperature for 10-30 minutes. Stop solution (0.18M sulfuric acid) was added to each well and the plate was read at 450nm. The quantity of antibody was determined according to the linear regression equation of the standard curve for each antibody isotype.

Neutralizing Antibodies (NtAb) Assay:

Briefly, neutralizing PA antibodies are determined by their ability to protect the J774A.1 mouse macrophage cell line from cell death due to exposure to lethal toxin. A modification of the NtAb assay proposed by Halperin was followed (46). J774A.1 cells were used at a concentration of 6-8x10^5/ml in DMEM with 2mM L-glutamine (Cambrex, Walkersville, MD) supplemented with 5% fetal bovine serum (FBS) (Sigma, Atlanta, GA), 50U/ml penicillin, and 50 g/ml streptomycin (Sigma, Atlanta, GA). 0.2ml aliquots of the cell suspension were added to 96-well plates (Nunc round bottom plates) and incubated 24 hours at 37°C with 5% CO_2. Immune sera were serially diluted by two-fold serial dilutions (1:50-1:400) in PBS/0.05%Tween-20 and incubated at room temperature for 1 hour with PA and LF at a final concentration of 5 g/ml and 2 g/ml respectively. After the incubation period, 10 l of each dilution of sera were added to the J774A.1 cells. Plates were incubated at 37°C in 5% CO_2 for 5 hours. After this incubation, 10 l of saline solution containing 0.75% MTT (3-[4,5-dimethylthiazol-2yl]-2,5-

diphenyltetrazolium bromide) (Sigma, St. Louis, MO) were added to each well. The plates were incubated again under same conditions for 30 minutes, and 100µl of acidified isopropanol (0.8% HCl) were added to each well. Absorbance at 540nm was determined after an additional 1 hour incubation at room temperature (46).

CMI Response Analysis

Mice were sacrificed by CO_2 asphyxiation and their spleens were removed under aseptic conditions. Single cell suspensions were prepared from the spleens. Briefly, red blood cells were lysed using ACK solution (0.1M NH_4Cl, 1mM $KHCO_3$, 0.1mM EDTA [pH7.3]) and the splenocytes washed with RPMI 1640 media (Invitrogen, Carlsbad, CA). The splenocytes were cultured in 96-well U bottom plates (Corning, Acton, MD) at a concentration of $5x10^5$ viable cells/well. RPMI 1640 medium supplemented with 2mM L-glutamine, 10% heat- inactivated fetal bovine serum (FBS) (Fisher, Norcross, GA) and 1ml of penicillin/streptomycin (Penicillin 100U/ml; Streptomycin 100µg/ml) was used for culturing splenocytes. Splenocytes were stimulated with the following antigens: 1µg of concanavalin A (ConA); heat inactivated $1x10^6$ of *B. abortus* RB51, $1x10^3$ of *B. anthracis* Sterne, 1µg of pure PA, 1µg of *E. coli* SOD (Sigma Chemical Co., St. Louis, MO) and media/no additives. The splenocytes were incubated in 5% CO_2, at 37°C for 5 days for cytokine ELISA and 2 days for lymphocyte proliferation assay.

Lymphocyte Proliferation Assay

Splenocytes were cultured from mice as described above. Twenty-four hours after incubation at 37°C in a 5% CO_2 incubator, 20µl Alamar Blue dye was added to each well and the plate was incubated an additional 24 hours. The plate was read on a Cyto-Fluor II Fluorescence Multi- Well Microplate Reader (Perceptive Biosystems, Inc.,

Framingham, MA). Alamar Blue dye fluoresces only when it is reduced. The wells of the plate were excited at 530 nm with emission measured at 590 nm (2,42).

Cytokine ELISA:

Splenocytes were cultured from mice as described above and incubated in U bottom 96-well plates (Corning, Acton, MD) for 5 days in an incubator at 5% CO_2 and 37°C. The culture supernatants were transferred to a new 96- well plate and stored at -70°C until a sandwich ELISA was performed to quantify cytokine levels in the supernatants. Cytokine ELISA was performed using Ready-Set-Go Mouse TH1/TH2 ELISA kit (eBioscience, San Diego, CA) Briefly, 96- well MaxiSorp™ immunoplates (Nalge-Nunc International, Rochester, NY) were coated overnight at 4°C with 0.1 g/well of purified anti-mouse cytokine coating antibody diluted in Coating Buffer. The coating buffer was discarded and the plate was washed 3 times with Wash Buffer and blotted dry. The plate was blocked at room temperature for 1 hour using 1X Assay Diluent. The diluent was discarded and the wells washed as above. Cytokine standards were serially diluted (1:2) with assay diluent to make the standard curves. Cytokine standards and samples were added to the wells in triplicate and incubated at room temperature for 2 hours. The samples were discarded and the plate was washed five times as above. Biotin-conjugated detection antibody was added to the plate and incubated for 1 hour at room temperature and the plate was washed 5 times as above. Avidin-HRP was added to the wells and incubated for 30 minutes at room temperature. The plates were washed a total of seven times as described above. TMB substrate solution is then added to the wells and incubated for 15 minutes. Stop solution is added top the wells and the plate is

read at 450nm. The quantity of cytokines was determined according to the linear regression equation of the standard curve.

Analysis of Proteins "Secreted" into Culture Media:

Strains RB51, RB51/SOD'::PA4, RB51/SOD'::PA4/SOD, RB51/18::PA4, RB51/PA4, RB51/SOD'::PA4, and purified PA4 were grown in 20 ml of TSB medium to a cell density of $5x10^8$ cells/ml. Culture supernatants were prepared by removing the cells by centrifugation at 5000 x g and the media acidified with trichloroacetic acid (TCA) at a final concentration of 5% and incubated overnight at 4°C. The acidified media were centrifuged at 8,000 x g for 10 minutes and the precipitated proteins were washed with 1 volume of acetone, dissolved in 100 µl 2x Laemmli buffer and boiled for 10 minutes. The samples (35 µl) were loaded onto 12.5% SDS-PAGE gels and transferred to membranes for Western Blot as described earlier. Membranes were blocked overnight at 4°C in 1% BSA. Rabbit PA 1° antibody (S. Leppla, NIH) was added to the membrane at a 1:1000 dilution and incubated at room temperature overnight. After washing 3 times, goat to rabbit IgG 2° antibody (Sigma, St. Louis, MO) was added at a 1:1000 dilution and incubated at room temperature for 3 hours. The membrane was again washed 3 times and the substrate was added for color development.

Challenge of mice:

The mice were bled at 4 and 6 weeks post immunization. At 8 weeks post immunization, the mice were challenged. Five mice from each of the groups were injected IP with $2.4x10^4$ cfu of *B. abortus* 2308. At 2 weeks post challenge the mice were sacrificed by CO_2 asphyxiation and the spleens were harvested aseptically and placed in sterile Corning glass tubes containing sand (0.75g/tube) and 1ml of TSB.

Spleens were homogenized using a sterile pestle and 10- fold serial dilutions made in TSB. Five, 10 l drops from each dilution were plated on TSA plates and incubated at 37°C for 5 days to determine the CFU per spleen, i.e., the clearance of strain 2308. The number of colonies in the lowest dilution countable was used to quantify the bacteria per spleen in CFUs/spleen using the following formula:

CFU/spleen = Total CFU/dilution x 20 x dilution factor

Counts of bacterial CFU in the spleens of mice were analyzed by the paired *t* test using Microsoft Excel. Groups were compared to the negative control group (saline) and to each other. P values equal to or less than 0.01 were considered significant.

The 6 remaining mice in each group were then injected IP with $2.4x10^4$ spores of *B. anthracis* Sterne strain and protection was assessed by survival over 14 days after which any surviving mice were euthanized.

4.4 Results

Immunization and antibody-mediated immune response analysis of study 1.

A/J mice were immunized by IP route with the various RB51/PA strains at a dose of ~$3.5x10^8$ cfu. The negative control group was immunized with sterile saline. Mice in the positive control group each received ~3.2 g of pure PA. The immunization and challenge protocol is described in Table 4.1 and Figure 4.1.

At 4 weeks post-immunization, mice were bled retro-orbitally and the sera were used to probe western blot membranes loaded with full size PA (83kDa) and PA4 (Figure 4.2). The serum from each group of mice immunized with an RB51/PA strain contained antibodies that recognized the PA protein. Sera from the saline and RB51 immunized mice did not recognize the protein.

Mouse Group	Vaccine Used for Immunization
1	Saline
2	RB51
3	RB51/SOD'::nPA4
4	RB51/SOD'::PA4
5	RB51/PA4
6	RB51/18::PA4
7	RB51/SOD'::PA4/SOD
8	Pure PA
9	RB51/PA

Table 4.1: Mice were assigned to these groups for the immunization and protection studies.

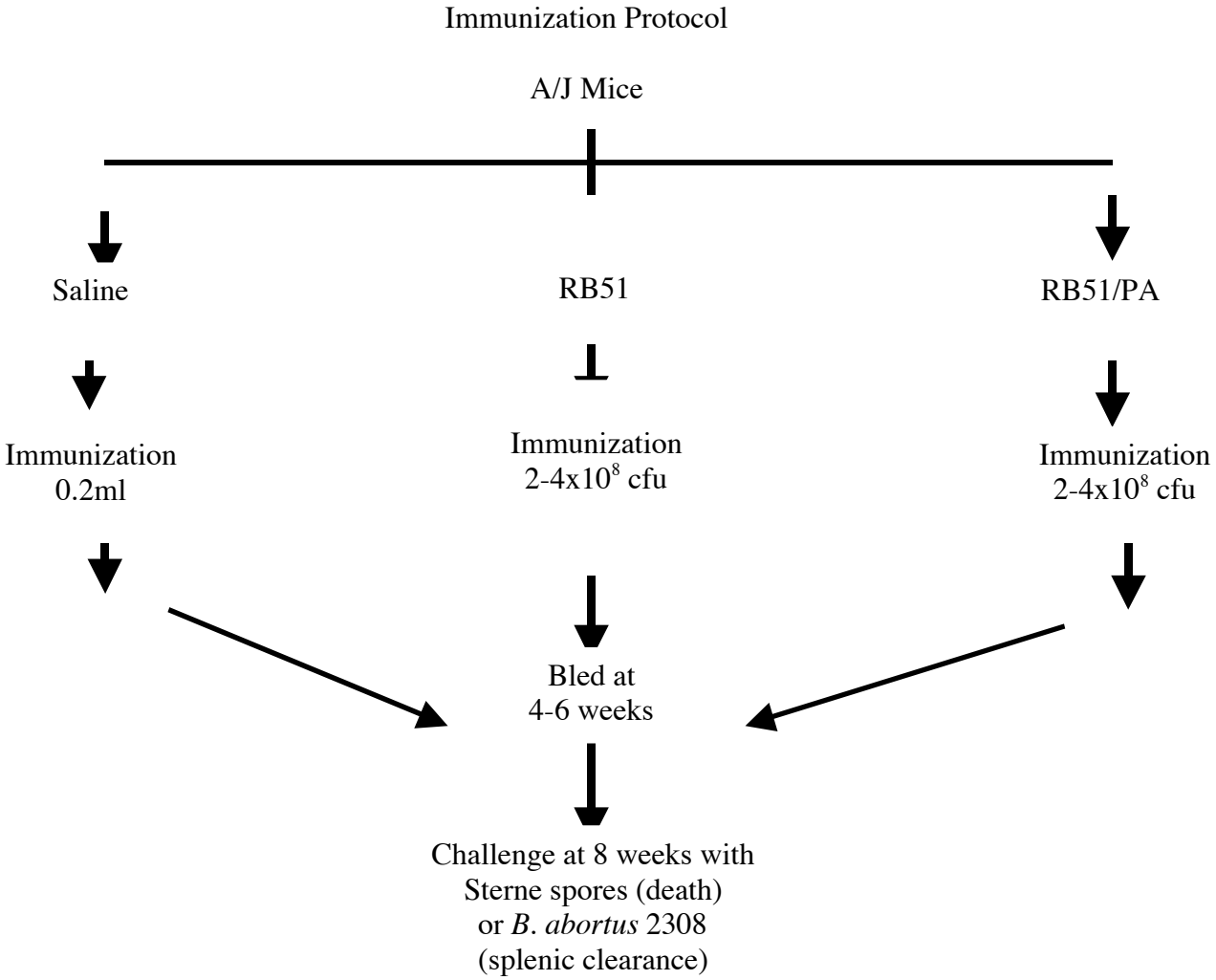

Figure 4.1: Schematic of mouse immunization and challenge schedule.

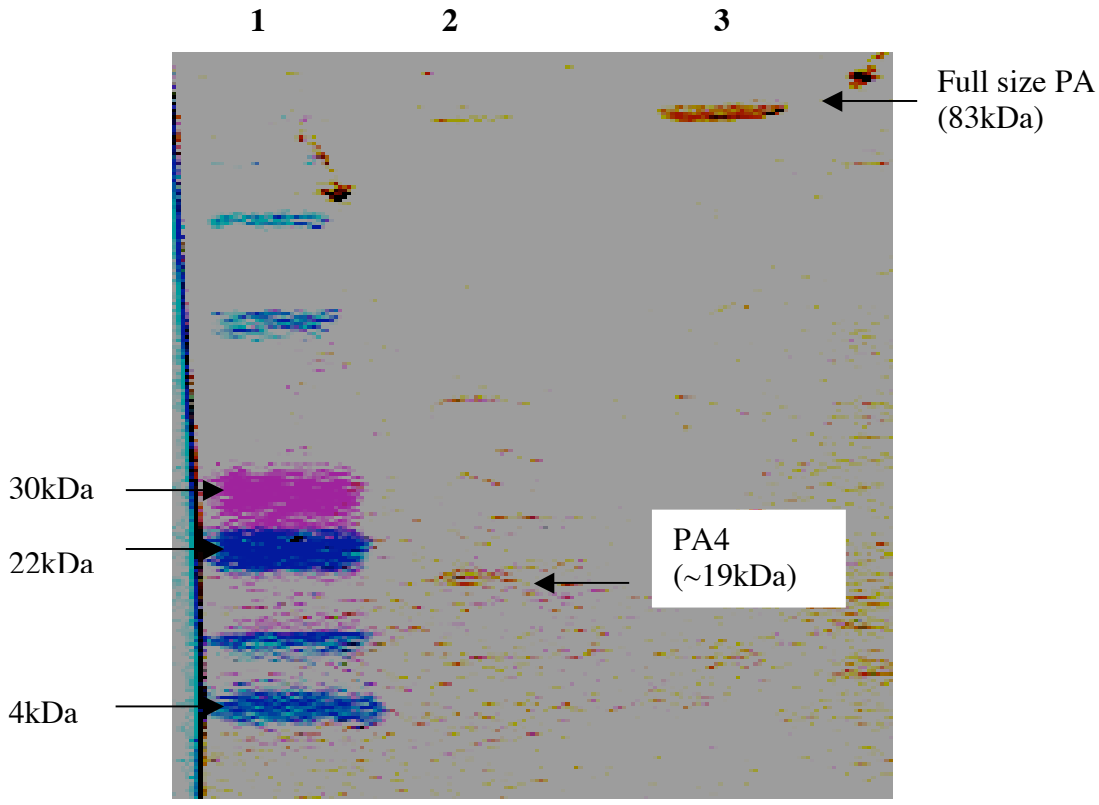

Figure 4.2: Representative western blot of mouse sera (1:50 dilution) exposed to extracts containing either full size PA or PA4. Each mouse group immunized with a RB51/PA strain produced antibodies that were able to recognize the proteins. Sera from mice immunized with saline or strain RB51 did not recognize the proteins.

Subisotype ELISAs were performed to determine the serum antibody levels against PA for IgG, IgG1, IgG2a and IgM for each immunization group. IgG titers against PA were higher in each immunization group exposed to PA relative to the saline and strain RB51 immunized groups (Figure 4.3). This indicates that an immune response to PA has been induced by the RB51/PA vaccines. IgG1 titers were very low for most of the immunization groups except for the group injected with pure PA and RB51/SOD'::PA4 (Figure 4.4). A high level of IgG1 antibodies was expected for the mice immunized with pure PA since the protein is known to induce an antibody-mediated response. Compared to IgG1 anti-PA levels, the IgG2a anti-PA levels were much higher in all immunization groups (Figure 4.5). This result is indicative of a Th1 response to PA

in these A/J mice, which is correlated to a CMI response. All groups mounted an IgM response to PA (Figure 4.6).

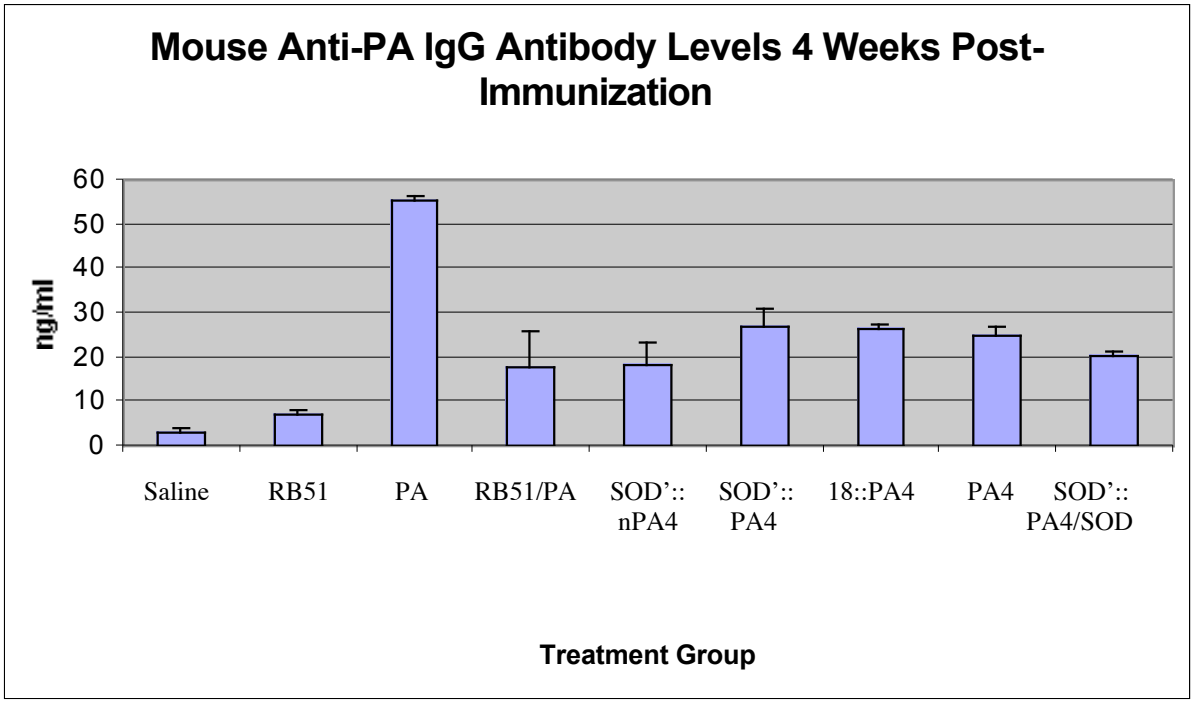

Figure 4.3: IgG specific PA antibodies induced by immunization with strain RB51/PA vaccine candidates.

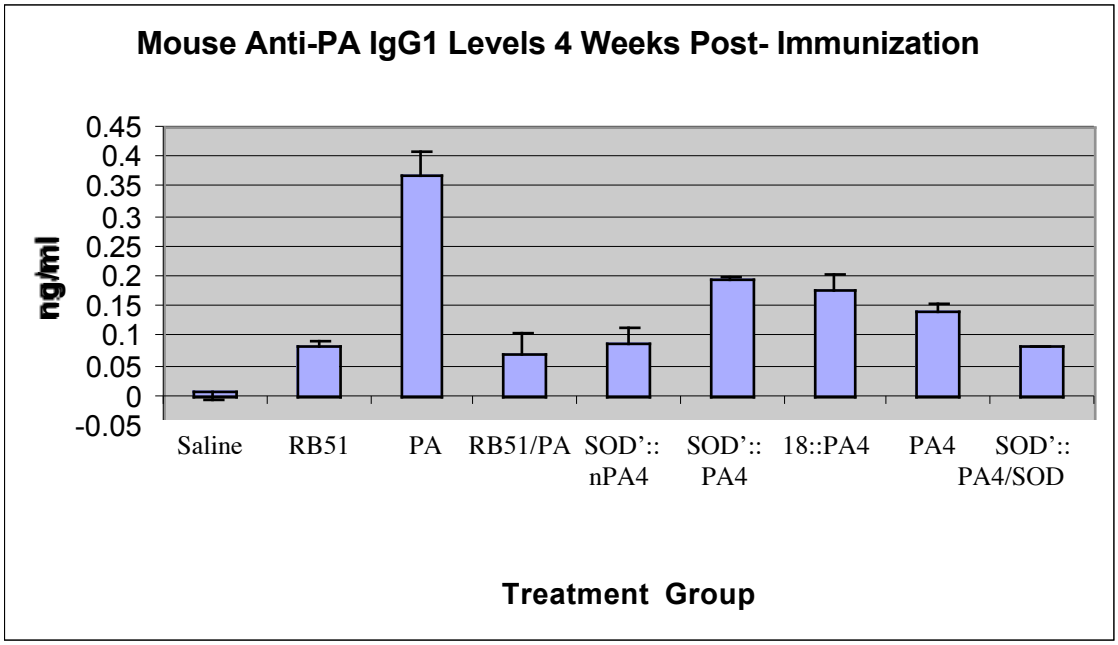

Figure 4.4: IgG1 specific PA antibodies induced by immunization with *Brucella* vaccine candidates.

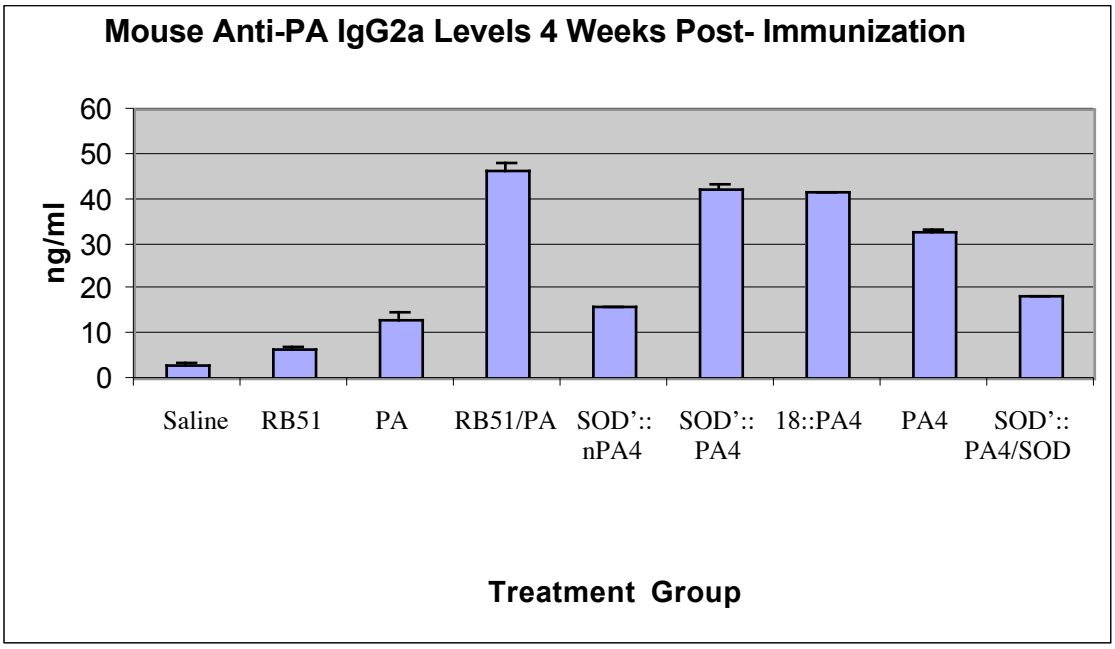

Figure4.5: IgG2a specific PA antibodies induced by immunization with *Brucella* vaccine candidates.

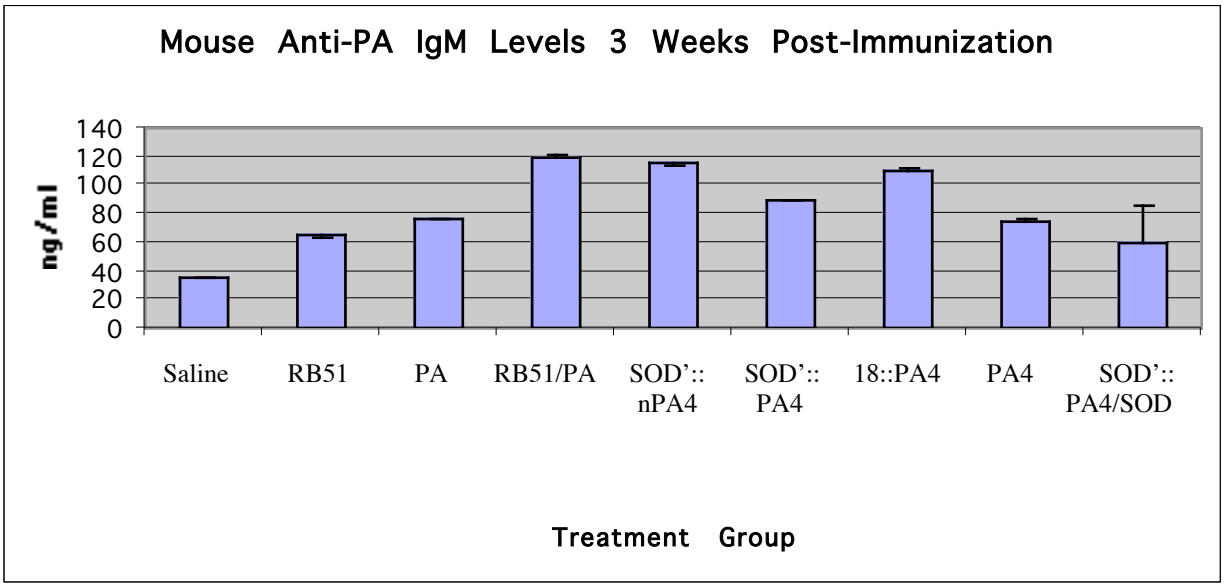

Figure 4.6: IgGM specific PA antibodies induced by immunization with *Brucella* vaccine candidates.

Neutralizing antibody assay

The ability of the immunized mouse serum to protect mouse macrophage J774A.1 cells against LeTx was tested (Figure 4.7). Dilutions of 1:50, 1:100, 1:200 and 1:400 mouse sera as well as 5 μg of PA and 2 μg of LF (which comprises LeTX) were added to these cells and incubated in 5% CO_2 at 37°C for 1 hour. Incubation of the dye MTT with live cells results in a dark blue formazan product whose absorbance is read at 540 nm. Cells protected by the immunized mouse serum produced the blue formazan product while cells unprotected (i.e. killed) formed no blue product. Some partial protection was observed in the macrophages incubated with serum from mice immunized with strain RB51. Overall, the sera from mice immunized with one of the vaccines (strain RB51/PA) or PA were more protective (OD values ranging from 0.5952 to 0.8482) than strain RB51 alone. However, no sera fully protected the macrophage cells from exposure to this concentration of LeTx. Exposure to LeTx at lower doses may have yielded more protection.

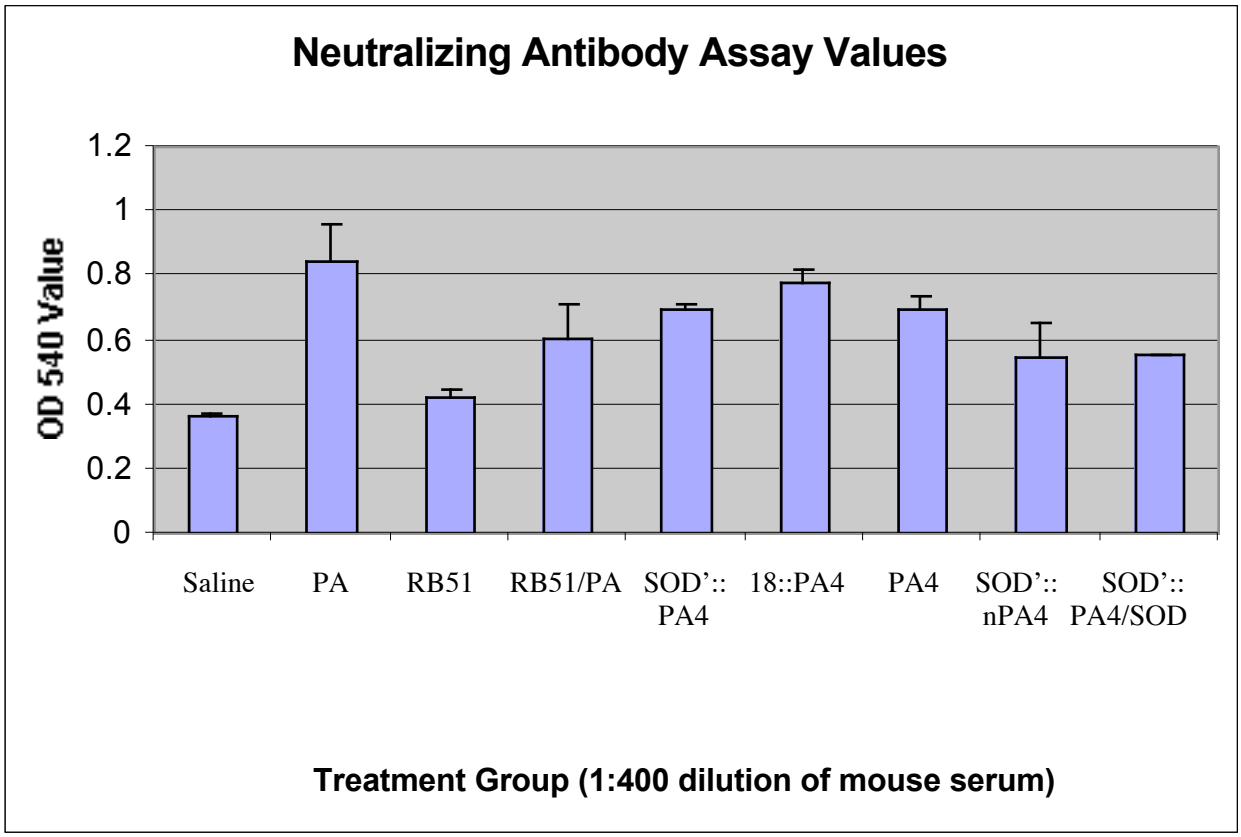

Figure 4.7: Neutralizing antibody assay to determine protection of mouse macrophage J774A.1 cells against challenge with LeTx (5□g of PA and 2□g of LF). Value of negative control (cells not exposed to LeTX) was 0.9842. Value of positive control (cells exposed to LeTx) was 0.1537.

Cell mediated immune response.

Lymphocyte proliferation assay (LPA) and cytokine ELISAs were performed to determine INF-□and IL-4 titers against antigens including: ConA (1□g), PA (1□g), SOD (1□g), heat inactivated *B. abortus* ($1x10^6$ cfu), heat inactivated *B. anthracis* ($1x10^3$ cfu), or media (no additives). Mouse splenocytes were incubated in 96-well plates in 5% CO_2 at 37°C for 5 days for cytokine ELISA and 2 days for LPA. All samples were run in triplicate. Il-4 values for each mouse immunization group were very low for each antigen indicating the immune response was not primarily a antibody-mediated one (Figure 4.8). In support of these results, the INF-□ELISA indicated a strong CMI response to strain RB51, ConA and pure PA in 3 mouse groups exposed to PA (Figure 4.9).

LPA assay results revealed that splenocytes from all groups were stimulated by ConA (1 □g) (Table 4.2). Splenocytes from groups receiving immunizations of strains of RB51 were stimulated by the heat killed strain RB51 antigen ($1x10^6$ cfu). In addition, PA (1 □g) stimulated splenocyte proliferation in 3 groups: PA, SOD'::PA4, 18::/PA4 and PA4. Values were determined by subtracting the following value (average background + 2 S.D.) from the average value of each group to a specific antigen. Any positive value was termed lymphocyte proliferation.

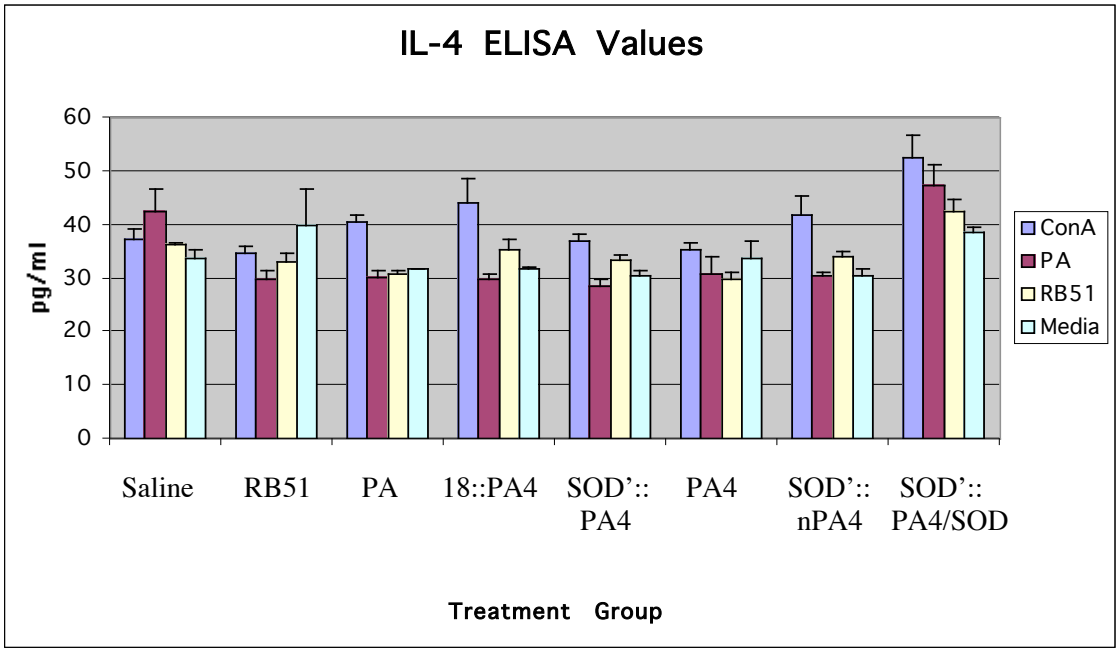

Figure 4.8: IL-4 ELISA of splenocytes of mice immunized with RB51/PA strains. Splenocytes were exposed to ConA, PA, heat inactivated RB51 and media.

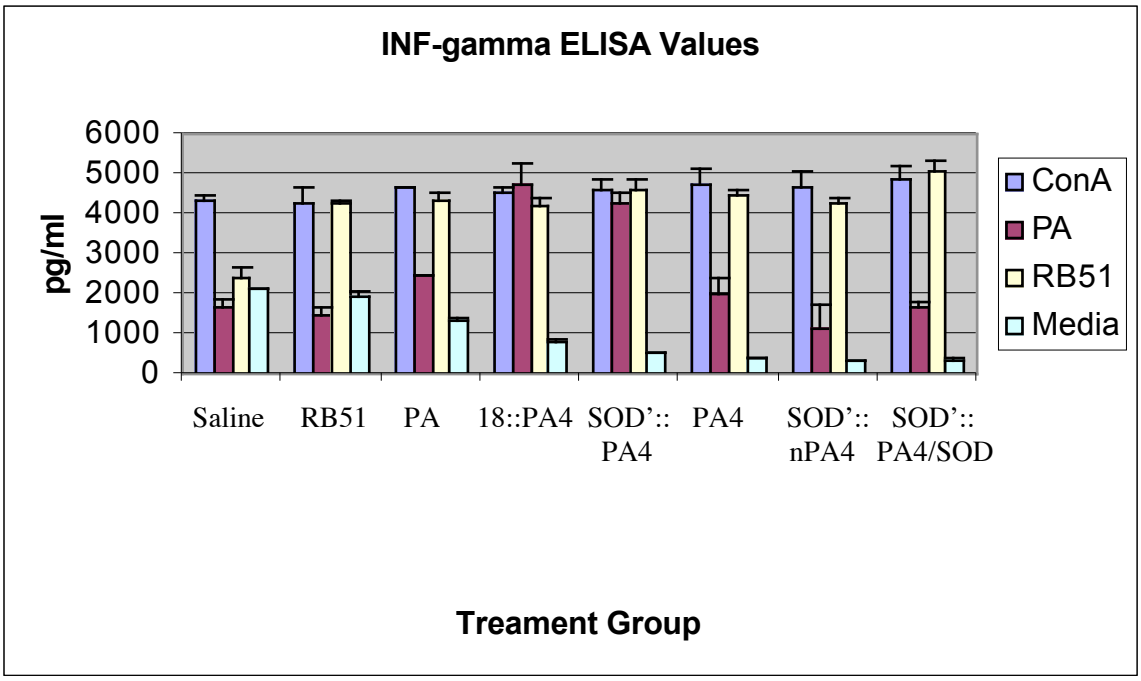

Figure 4.9: INF-□ ELISA of splenocytes of mice immunized with RB51/PA strains. Splenocytes were exposed to ConA, PA, heat inactivated RB51 and media.

Mice Immunization Groups

	Saline	RB51	PA	RB51/PA	PA4	SOD`::PA4	SOD`::PA4/SOD	18::PA4	SOD`::nPA4
ConA	575	380	624	304	806	381	418	703	657
RB51	0	100	0	20	100	110	97	103	115
SOD	0	0	0	0	0	0	0	0	0
Sterne	0	0	0	0	0	0	0	0	0
PA	0	0	45	0	20	190	0	21	0
Media	0	0	0	0	0	0	0	0	0

Table 4.2: Lymphocyte proliferation assay of splenocytes from immunized mice. Stimulating antigens were 1□g of ConA, 1□g of SOD, $1x10^6$ CFU heat inactivated RB51, $1x10^3$ CFU heat inactivated Sterne, 1□g pure PA, and media. Values are adjusted such that background is subtracted from average.

Challenge with *B. abortus*2308 and *B. anthracis* Sterne spores.

A/J mice were challenged with either $2.4x10^4$ cfu of *B. abortus* 2308 or $2.4x10^4$ spores of *B. anthracis* Sterne strain. Mice challenged with *Brucella* were sacrificed by CO_2 asphyxiation 2 weeks post-challenge. Their spleens were aseptically removed, ground up, serially diluted in TSB, and plated onto TSA plates. The plates were then incubated at 37°C for 5 days and the cfu determined to measure the rate of bacterial clearance from the spleens of the strain RB51/PA immunized mice as compared to that of saline and strain RB51 immunized mice.

Splenic clearance study reveals that there is no statistical difference in the abilities of the RB51/PA strains or strain RB51 to protect a mouse against *Brucella* challenge. As expected, the mice immunized with saline or PA were not protected against *Brucella* challenge (Figure 4.10).

Following a challenge by 20xLD_{50} Sterne spores, some encouraging results were observed. The endpoint of this study was death or survival over a period of 14 days. As expected, no saline or strain RB51 immunized mice survived the spore challenge. However, 3 groups exposed to some form of PA did contain survivors. Full protection against challenge was not seen in any group. A/J mice immunized with PA were 60% protected (4/6), the group designated as RB51/18::PA4 was 50% protected (3/6) and the group designated as RB51/PA4 was 17% protected (1/6) (Table 4.3).

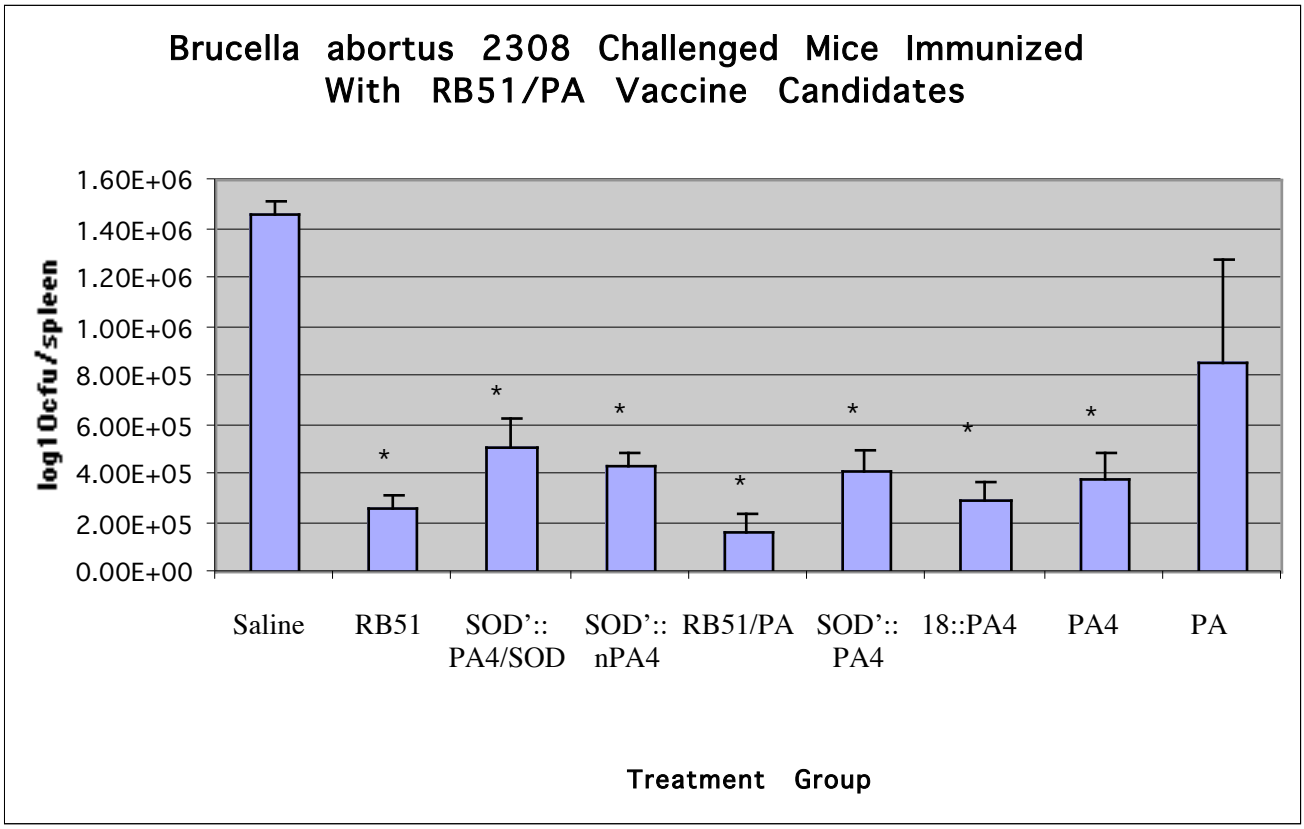

Figure 4.10: Splenic Clearance of *B. abortus* 2308 by A/J mice immunized with various *Brucella* vaccine candidates. Time of challenge was at 56 days post- immunization. Group 1 was immunized with sterile saline, group 2 with strain RB51, group 3 with strain RB51/SOD'::PA4/SOD, group 4 with strain RB51/SOD'::nPA4, group 5 with strain RB51/PA, group 6 with strain RB51/SOD'::PA4, group 7 with strain RB51/18::PA4, group 8 with strain RB51/PA4 and group 9 with pure PA. (* indicates protection significant to saline and PA.) ($p \leq 0.01$)

Treatment Group	# Mice Surviving (% Protection)	Mean Days to Death
Saline	0/6 (0%)	4
RB51	0/6 (0%)	4
RB51/PA	0/6 (0%)	4
RB51/SOD'::nPA4	0/6 (0%)	3
RB51/SOD'::PA4/SOD	0/6 (0%)	3
RB51/SOD'::PA4	0/6 (0%)	5
RB51/PA4	1/6 (17%)*	5
RB51/18::PA4	3/6 (50%)*	6
Pure PA	4/6 (67%)*	7

Table 4.3: Challenge with $2.5x10^4$ *B. anthracis* Sterne spores. Survival over 14 days was observed. No group was fully protected against challenge. However, 3 groups (*) displayed partial protection.

Analysis of "secreted" proteins by western blot.

While challenge results were encouraging, one puzzling result was observed. The strain RB51/SOD'::PA4/SOD was constructed as a vaccine to over-express Cu-Zn SOD along with PA4. Previous work has demonstrated that over-expression of this form of SOD in strain RB51 enhances protection afforded by the vaccine (114). It was predicted that this vaccine construct would be significantly more protective against both challenge organisms due to this SOD expression. However, there was no enhanced protection observed against *Brucella* relative to strain RB51. In addition, mice immunized with

RB51/SOD'::PA4/SOD died faster on average than mice immunized with saline or strain RB51.

One theory to explain this observation is that since SOD and PA4 are about the same size (20kDa and 19kDa respectively), they are competing to be "secreted" out of the *Brucella* cell when either is located in the periplasmic space. Unpublished observations (H. Misra, Virginia Tech) found this SOD in the culture media when over-expressed as a result of being plasmid encoded in strain RB51. The SOD is thought to be "secreted" out of the cell when the periplasmic space becomes over-loaded with SOD. This observation is significant because *Brucella* as a species is not known to secrete any proteins despite the presence of secretory pathway genes in the bacterial genome (1,11,18,92). In order to observe if strain RB51/PA4 expressed PA4 and "secreted" it into the culture media in the same amount as strain RB51/SOD'::PA4, the proteins in the culture media grown with the strains were precipitated using TCA. These precipitated proteins were then analyzed (5□g each sample) by western blot using PA antibodies generated in rabbit (S. Leppla, NIH). Western blot revealed that strain RB51/SOD'::PA4/SOD (lane 2) did not deposit as much PA4 in the culture media as RB51/SOD'::PA4 (lane 3) since the former PA band was much lighter (Figure 4.11). This indicates that PA4 and SOD are competing for "secretion" into the culture media, which may explain why this RB51 strain did not protect as well as was predicted. However, neither strain produced sufficient PA outside of the cell to induce a protective immune response. In addition, strain RB51/18::PA4 (lane 4) deposited PA into the culture media indicating that the PA expressed by this strain was at or near the cell's outer membrane as was intended by the signal sequence (18kDa). No PA band was

observed in the media of strain RB51/PA4 (lane 5) as expected since the PA would be located in the cytosol of the bacterial cell due to lack of signal sequence. No bands are seen in lanes 6 and 7 because strain RB51 does not produce PA naturally and strain RB51/PA would produce a band of PA much higher in the gel.

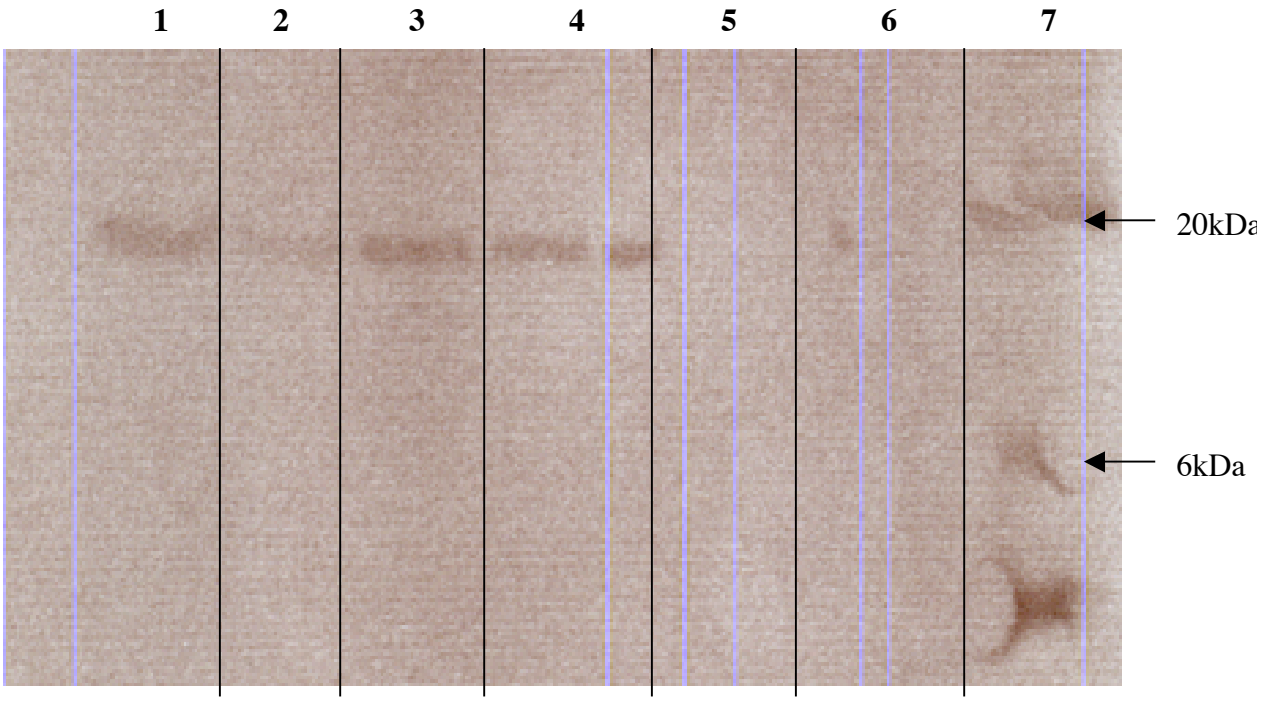

Figure 4.11: Western Blot of "secreted" PA4 in media of *B. abortus* strain RB51 cultures developed using Rabbit PA antibody. Lane 1 is purified PA4 from inclusion bodies of pRSET/PA4, lane 2 is RB51/SOD'::PA4/SOD, lane 3 is RB51/SOD'::PA4, lane 4 is RB51/18::PA4, lane 5 is RB51/PA4, lane 6 is RB51 and lane 7 contains molecular weight markers.

Immunization and antibody-mediated immune response analysis of study 2.

Previously in this study, *B. abortus* strain RB51 expressing PA4 fused to the signal sequence of *Brucella* 18kDa protein was able to afford 50% protection, while strain RB51 expressing PA4 with no signal sequence gave 17% protection against *B. anthracis* Stern spore challenge. In an attempt to increase protection, mice were boosted

with an IP injection of 3.2μg of pure PA protein after an initial immunization with *Brucella* vaccine candidates RB51, RB51/PA4, RB51/18::PA4, RB51/SOD'::PA4, sterile saline or pure PA. Protection was assessed by live challenge with *B. anthracis* Sterne spores.

A/J mice were immunized by IP route with the various RB51/PA strains at a dose of ~$3.5x10^8$ cfu. The negative control group was immunized with sterile saline. Each mouse in the positive control group received ~3.2 μg of pure PA; immunization vaccine is described in Table 4.4.

Mouse Group	Vaccine Used For Immunization
1	Saline
2	RB51
3	RB51 boosted with PA
4	RB51/SOD'::PA4 + PA
5	RB51/PA4 + PA
6	RB51/18::PA4 + PA
7	PA + PA

Table 4.4: Mice were designated into one of these groups for this study.

At 4 and 7 weeks post-immunization, mice were bled retro-orbitally and the sera were assayed by ELISA to determine PA titers (Figure 4.12-4.14). Subisotype ELISAs were performed to determine the serum levels against PA for IgG, IgG1, IgG2a and IgM for each immunization group. IgG titers against PA were higher in each immunization group after receiving the PA boost (Figure 4.12). Indicating that a stronger immune response to PA has been induced. IgG1 titers were higher after the PA boost indicating

that the protein was inducing a stronger antibody-mediated response (Figure 4.13). A high level of IgG1 antibodies was expected for the mice immunized with pure PA since the protein is known to induce a antibody-mediated response. IgG2a titers were lower after the PA boost compared to mice only immunized with an RB51/PA strain indicating a balancing of the CMI and antibody-mediated response (Figure 4.14).

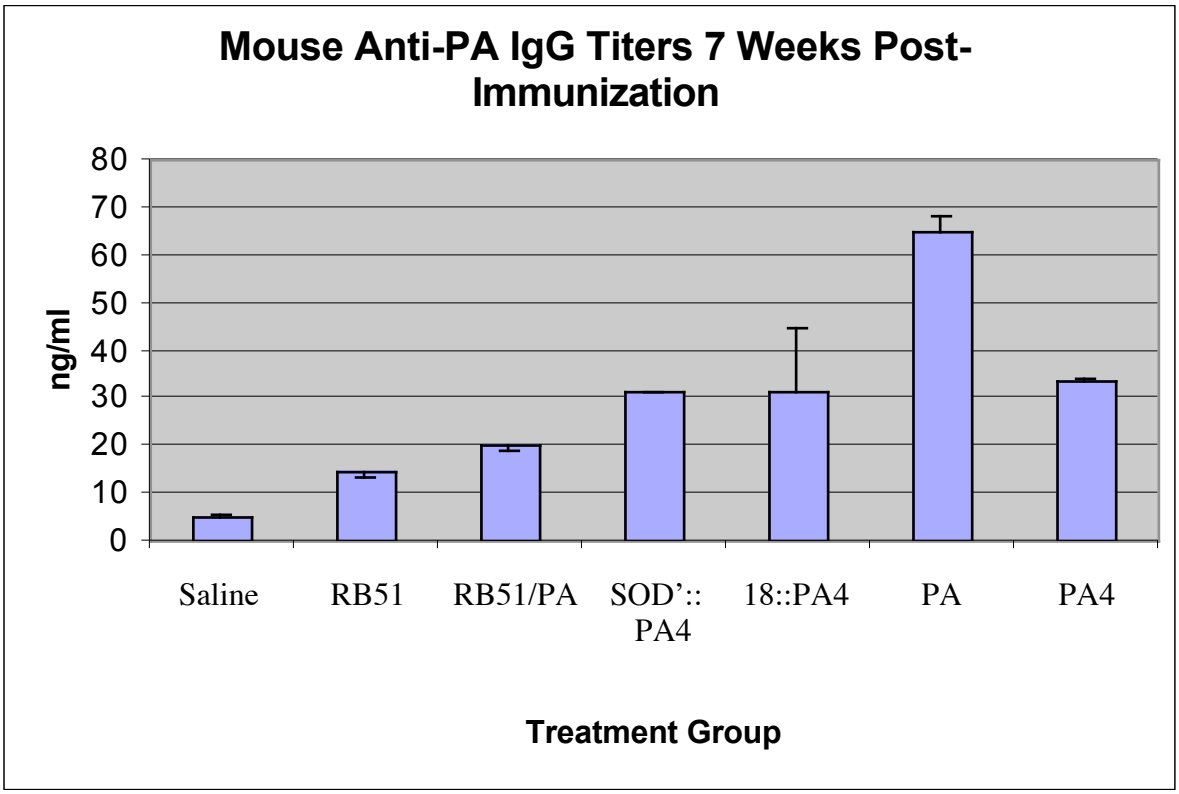

Figure 4.12: IgG specific PA antibodies induced by immunization with *Brucella* vaccine candidates with a pure PA boost at week 7.

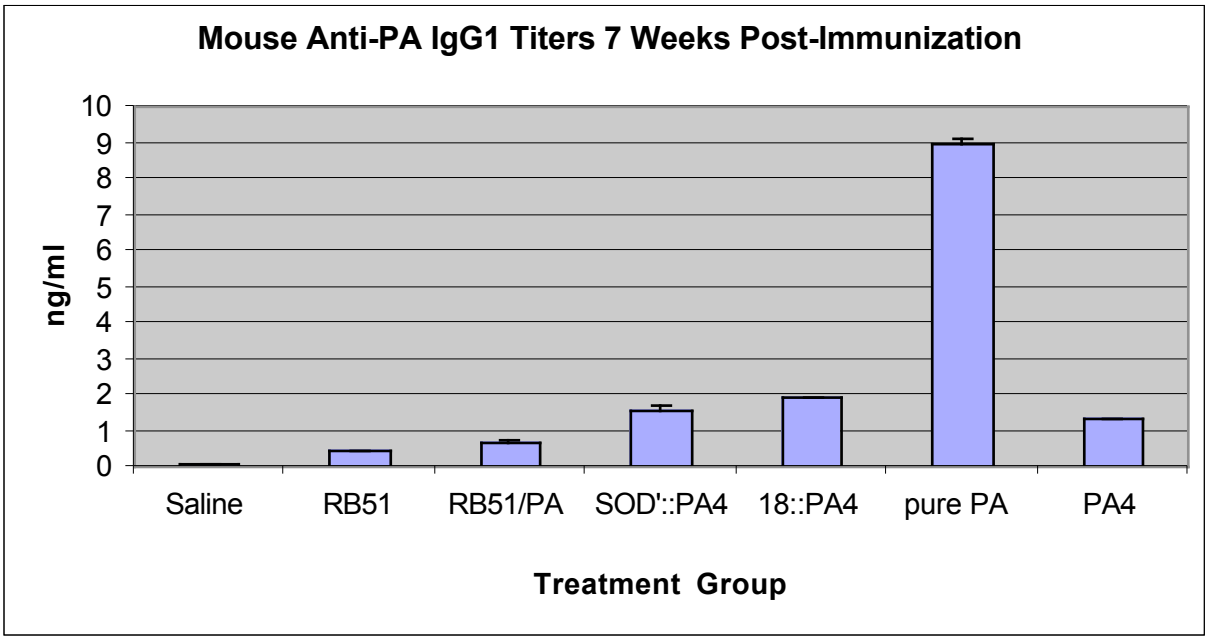

Figure 4.13: IgG1 specific PA antibodies induced by immunization with *Brucella* vaccine candidates with a pure PA boost at week 7.

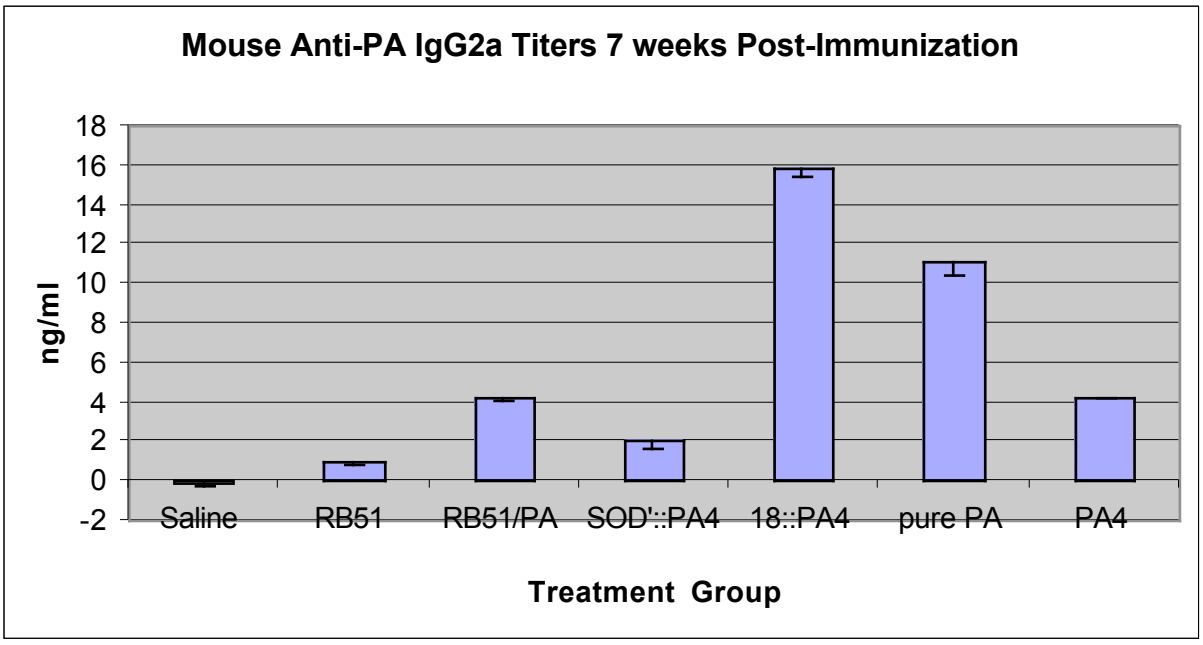

Figure 4.14: IgG2a specific PA antibodies induced by immunization with *Brucella* vaccine candidates with a pure PA boost at week 7.

Challenge with *B. anthracis* Sterne spores.

A/J mice were challenged with $2.4x10^4$ spores ($20xLD_{50}$) of *B. anthracis* Sterne strain. The endpoint of this study was death or survival over a period of 14 days. As expected, no saline or strain RB51 immunized mice survived challenge. However, 4 groups exposed to some form of PA (vaccine strain or purified) plus a PA boost contained survivors. Full protection against challenge was seen in the group receiving PA plus a boost. A/J mice immunized with RB51/PA4 + PA were 60% protected, the group designated as RB51/18::PA4 + PA was 60% protected and the group designated as RB51/SOD'::PA4 + PA was 20% protected (Table 4.5).

Bacillus anthracis Sterne Spore Challenge ($20xLD_{50}$):RB51/PA Immunized Mice

Treatment Group: 1° and 2°	# of Mice Surviving (% Protection)	Mean Days To Death
Saline	0/5 (0%)	5
RB51	0/5 (0%)	5
RB51 + PA	0/5 (0%)	6
RB51/PA4 + PA	3/5 (60%)*	4
RB51/SOD`::PA4 + PA	1/5 (20%)*	6
RB51/18::PA4 + PA	3/5 (60%)*	6.5
PA + PA	5/5 (100%)*	N/A

Table 4.5: Challenge with $2.5x10^4$ *B. anthracis* Sterne spores. Survival over 14 days was observed. The group immunized with PA was fully protected against challenge. In addition, 3 groups displayed partial protection that could be considered superior to immunization with RB51/PA alone. * denotes survival.

Table 4.6 contains a summary of results from outlining the *B. anthracis* Sterne spore challenge in both immunization studies.

	Study 1		**Study 2 (PA Boost)**	
Treatment Group	# Surviving (% Protection)	Mean Days to Death	# Surviving (% Protection)	Mean Days to Death
Saline	0/6 (0%)	4	0/5 (0%)	5
RB51	0/6 (0%)	4	0/5 (0%)	5
RB51/PA	0/6 (0%)	4		
RB51/SOD'::nPA4	0/6 (0%)	3		
RB51/SOD'::PA4/SOD	0/6 (0%)	3		
RB51/SOD'::PA4	0/6 (0%)	5	1/5 (20%)*	6
RB51/PA4	1/6 (17%)*	5	3/5 (60%)*	4
RB51/18::PA4	3/6 (50%)*	6	3/5 (60%)*	6.5
PA	4/6 (67%)*	7	5/5 (100%)*	N/A

Table 4.6 Comparison of spore challenge results from 2 different immunization protocols. * indicates groups with survivors.

4.5 Discussion

Various *B. abortus* strain RB51 vaccine candidates carrying *B. anthracis* PA4 sequence have been generated and shown to express the protective domain in different amounts (Chapter 3). These recombinant vaccines express PA4 fused to 2 different *Brucella* signal sequences in order to localize the protein in different locations for presentation to the immune cells of a host. In this chapter, the ability of these candidate vaccines to induce antibodies consistent with either a antibody-mediated or cellular response was assessed.

A/J mice immunized with these vaccines expressing only the domain 4 of PA induce antibodies that are able to recognize full size PA (Figure 4.2). Antibody levels generated are much higher in the mice vaccinated with strain RB51 candidates expressing only domain 4 than those vaccinated with strain RB51/PA expressing the entire *pag* gene (Figure 4.3).

One explanation for the higher titers could be the result of changing the codon usage of PA4 from its native sequence to the preferred codons used by *Brucella*. In previous work, low PA production and degradation of PA was observed when the whole, native gene was expressed in strain RB51 (Chapter 2). The choice of the shorter domain 4 and switching to the *Brucella* codon usage seems to have overcome this problem (Chapter 3). Presumably the impact of these changes overcame the likely depletion of aminoacyl tRNA pools and subsequent shorter or unstable PA observed on the blots.

The purpose of this study was to assess the efficacies of these new RB51/PA4 vaccines to protect mice from live challenge with either *B. abortus* 2308 or *B. anthracis* Sterne spore injected IP. In the first immunization study, mice were given a single IP immunization with one of the new RB51/PA4 vaccines and bled to assess antibody-mediated and CMI immune responses before challenge.

Western blot showed that mice immunized with these vaccine constructs produced antibodies able to recognize both full size PA and PA4. ELISA results indicate PA antibody levels induced by the RB51/PA4 vaccines are not as high as those in mice immunized with PA alone. However, these PA antibody levels are higher than those induced by strain RB51 expressing the entire *pag* gene. In addition, subisotype ELISA results indicate IgG2a levels are much higher than those of IgG1 suggesting a Th1

response rather than a Th2 response. This would seem to indicate that strain RB51 is responsible for this observation since the mice immunized with PA protein alone appear to have a Th2 biased response as seen from the ELISA results; IgG1 levels are much higher in these mice. Cytokine ELISA's revealed increased INF-γ and low IL-4 levels and correlated to a strong Th1 response as well. In addition, LPA results indicate a CMI response to both strain RB51 and PA as antigens. All groups of mice immunized with RB51 strain had splenocytes that proliferated under exposure to heat killed strain RB51 as expected, since RB51 is a potent CMI inducer. Four groups of mice immunized with some form of PA (either a strain RB51/PA or PA) yielded splenocytes that proliferated after exposure to PA. While comparatively little is known about CMI responses to PA and anthrax, two other studies have also noted a PA specific lymphocyte proliferation (57,71). Ivins et al., (57), and McBride et al., (71), both published studies that included observations of PA specific lymphocyte proliferation even in animals unable to survive spore challenge. While not commented upon by these researchers, it may be that lymphocyte proliferation specific to PA may not be able to induce protection against challenge and thus not important in the immune response. Although, a T cell response to PA may not be an important component of immunity to anthrax challenge, such a response to some other *B. anthracis* protein may be.

Challenge experiments using *B. abortus* 2308 reveal no change in protection produced by the strain RB51 vaccine constructs when compared to strain RB51. However, the protection afforded by the vaccine constructs against a *Brucella* challenge is indeed significant relative to the groups immunized with sterile saline or PA protein.

Thus the expression of PA in strain RB51 does not alter the ability of the strain to protect against brucellosis.

Mice challenged with 20xLD_{50} *B. anthracis* Sterne spores were partially protected in 3 immunization groups: PA, strain RB51/PA4 and strain RB51/18::/PA4. While this was not the expected, i.e., full protection as seen with adjuvanted PA, these mice exhibited better protection than the mice receiving strain RB51/PA seen in the previous study (Chapter 2). Of the groups receiving partial protection against spore challenge, 2 groups had forms of PA or PA4 that would have readily been seen by the mouse immune system, i.e. pure PA or strain RB51/18::PA4 (PA4 on the outer cell membrane of the *Brucella*). This type of exposure to PA (either higher levels and/or less degraded) could be required to induce protective immune responses.

The protection observed in this study either equaled or improved on the previous results (Chapter 2). Strain RB51 expressing the whole PA protein was only able to confer minimal protection (1/6 mice) over *B. anthracis* Sterne spore challenge. While the results of this study are greatly encouraging, further work is needed to improve protection. This could be done by boosting with PA protein or by improving the level of PA expression in the vaccine constructs.

Previously we tested various RB51/PA4 vaccine constructs for their ability to induce protective immune responses against challenge with either *B. abortus* 2308 or *B. anthracis* Sterne spores. These vaccine candidates elicited primarily a Th1 immune response that was fully protective against *Brucella* challenge and in 2 groups (RB51/18::PA4 and RB51/PA4) partially protective against spore challenge. In addition

to a CMI response, antibodies were able to recognize full size PA protein plus the domain 4 of PA.

The challenge results of the previous study did not clearly reveal which RB51/PA4 vaccine strain was most efficacious in inducing protection. In fact, although strain RB51/18::PA4 induced a lower level of PA antibody, it was the most protective vaccine construct. This phenomenon may be explained by the presumed localization of the PA4 in the outer membrane due to the 18kDa signal sequence as evidenced by finding PA in the TCA precipitated insoluble material in the culture medium (Figure 4.12). This location should allow the PA4 to be more readily presented to the host's immune system, but simultaneously may be biased to trigger a CMI response because of strain RB51. This possibility is supported by the subisotype ELISA results which suggest a stronger CMI response to PA4 than an antibody-mediated response. However, after a PA boost, the IgG1 titers rise due to the purified protein's ability to be biased to induce antibody-mediated responses.

While a CMI response has been indicated to be an important component of protection against anthrax, antibodies to PA are essential (15,16,37,99). In order to improve immunity against anthrax, A/J mice were immunized with the RB51/PA4 vaccine constructs and then boosted with PA protein before challenge with Sterne spores. This boost with PA protein slightly elevated anti-PA IgG levels as seen by ELISA (Figure 4.13). However, one notable observation is that the titers of IgG1 and IgG2a have changed. IgG1 levels increased while IgG2a levels in some groups were lowered. This may indicate a better balance between the responses of the two arms of the immune system.

In mice receiving the RB51/PA4 vaccine and then boosted with PA, challenge with 20xLD_{50} of *B. anthracis* Sterne spores revealed some encouraging results. Of the groups immunized and challenged, 4 groups had survivors. When comparing the two challenge studies: the 3 groups containing survivors from the previous study still contained survivors in the second study but the efficacy was improved by boosting with PA protein. Groups RB51/18::PA4 and RB51/PA4 each had mice with a group survival rate of 60%. The group receiving pure PA protein was 100% protected. In addition, RB51/SOD'::PA4 had a survival rate of 20% (Table 4.4).

These results indicate that a strong exposure to PA (i.e., by boosting) is indeed important in inducing protection against anthrax challenge. Boosting with PA resulted in higher survival rates after challenge. Future strain RB51/PA vaccine candidates intended to protect against both brucellosis and anthrax should produce enough PA to induce levels of protection seen by PA injection alone. One way to accomplish this could be to fuse the *pag* gene to a signal sequence which readily locates the protein in the *Brucella* cell for exposure to host immune cells and linking it to a strong *Brucella* promoter (i.e. greater than GroE) for high level expression levels. In addition, converting the entire *pag* gene to the average *Brucella* codon usage and overexpressing it should increase protection since previous studies have shown that while domain 4 antibodies are protective, antibodies to the entire PA are more efficacious (37).

5. Summary and Discussion

PA is a key component of any anthrax vaccine either already licensed for use or in developmental stages due (99) to the fact that the protein plays a major role in both the toxic effects of anthrax seen in the host and in immunity to the pathogen (60,75,100). The protein is capable of effectively inducing protection when employed as a subunit vaccine or as part of a recombinant vaccine (110). While subunit vaccines, such as the AVA licensed for human use, are generally accepted as being safe, they are not as effective as live attenuated vaccines when it comes down to protection against challenge with virulent anthrax (6). Live vaccines such as the spore vaccine, while inducing a lower antibody titer to PA, are much more protective (110). However, use of the live spore vaccine in humans is not widely accepted for fear of a conversion back to a virulent state (98,99). For this reason, other live recombinant vaccines have been developed and tested for their efficacy against anthrax challenge. Bacterial species such as *E. coli*, *S. typhimurium* and *B. subtilis* and viral strains such as vaccinia virus and baculovirus expressing PA have been used to immunize mice, guinea pigs and rabbits (6).

B. anthracis secretes PA, EF and LF into the extracellular environment so the components of the toxin can exert their effects on host cells (79). Therefore, an infected host's immune system is exposed to PA extracellularly, which may be key to the protein's affect on the immune response. Antigen presentation of PA in live recombinant vaccines as a means to enhance protection needs further refinement (124). The requirement of secretion of the antigen, surface-association or intracellular expression for efficient protection against anthrax challenge remains to be determined (124). One goal

of this dissertation was to investigate the role of PA presentation in the recombinant *Brucella*.

In this dissertation *B. abortus* strain RB51 has been engineered to express complete PA (Chapter 2), however, the expression level and stability of PA was less than desirable. Very little full size PA was observed on western blots of extracts prepared from the strains. One reason for the low expression could have been that production of native PA may have been impaired due to depletion of those rare aminoacyl tRNA pools utilized by the *B. anthracis pag* gene leading to truncated protein. The G/C content of DNA differ greatly in *Brucella* and *B. anthracis* (57 and 36% respectively) (84,91). Therefore, the domain 4 of PA was converted from its native codon usage to that of *Brucella* as a means to overcome low expression levels observed in the previous study. In addition, this synthetic PA4 was fused to the *Brucella* signal sequences of either SOD or 18kDa outer membrane protein. These fusions were intended to localize the PA4 to the periplasmic space, outer cell membrane or cytosol (due to lack of signal sequence), respectively. Localization of PA4 to various sites in the *Brucella* cell was done in an attempt to enhance antigen presentation and thus improve the protective immune response in mice.

B. abortus strain RB51 transformed with pBBGroE/PA4 plasmid expresses PA4 protein of the expected molecular weight (~19kDa). The PA4 produced by this RB51 strain was recognized by antibodies to native PA in western blot (Figure 3.9). In addition, western blots of culture media of these RB51/PA4 strains show that PA4 was found outside of the *Brucella* cells grown to mid log phase. No other proteins were seen in a coomassie blue stain of the gel. These results indicate that PA4 synthesized by strain

RB51/18::PA4 was outside the outer cell membrane and is likely falling off of the cell surface during growth. However, the most notable feature of this western blot was finding PA4 in the media of both strains RB51/SOD'::PA4 and RB51/SOD'::PA4/SOD. Although the PA4 in these strains is located in the periplasmic space, the amounts of PA4 in the culture media for each of these strains differed. This observation could be explained in the following way: PA4 and SOD are approximately the same size and could be competing to be "secreted" out of the cell, whereas when PA4 is expressed alone, there is no competition, thus more PA is "secreted". Unpublished studies at Virginia Tech also observed proteins that have leaked out of the cell into the culture media. SOD, when over-expressed in strain RB51 is known to leak out into the culture media (unpublished observation, H. Misra, Virginia Tech). In addition, other as yet unidentified proteins have also been observed in the culture media of *Brucella* as detected by immunoblotting with hyperimmune anti-*Brucella* serum (unpublished observation, A. Bandara, Virginia Tech). Observations such as these are significant since *Brucella* species are not thought to secrete any proteins despite the presence of secretory pathways in the bacterial genome (1,11).

A/J mice were immunized individually with these RB51/PA4 vaccine candidates as a single dose and followed by a PA boost. Their immune responses were monitored using western blot, immunoglobulin subisotype ELISA (IgG, IgG1, IgG2a, and IgM), cytokine ELISA and LPA in study 1. Western blots containing PA and PA4 as target antigens revealed that sera from mice in each immunization group receiving a RB51/PA strain contained specific antibodies able to recognize both forms of the PA (Figure 4.2). PA specific IgG and IgM antibodies were observed in the serum from all mice groups

immunized with RB51/PA (Figures 4.3 and 4.6). Therefore, the level of PA4 expressed in each of the RB51/PA strains was sufficient to induce specific antibody responses in the mice. Subisotype ELISA revealed a Th1 bias based on observing the induction of much higher relative levels of IgG2a than IgG1 (Figures 4.4 and 4.5). However, after a PA boost subisotype ELISA of IgG shows a balancing out of the Th1/Th2 response with the IgG1 and IgG2a levels moving toward each other (Figures 4.14 and 4.15).

Cytokine ELISA results of Il-4 and INF-γ levels support the observations made in the subisotype ELISA. IL-4 levels for all antigens (ConA, PA, RB51 and media) are very low indicating a minimal Th2 response (Figure 4.8). In contrast, corresponding INF-γ levels were much higher suggesting the induction of a Th1 response (Figure 4.9). ConA and strain RB51 induced a high INF-γ level in splenocytes from all strain RB51 immunized mouse. These results were expected since strain RB51 is a potent inducer of Th1 type immune responses (95).

The LPA results supported the cytokine profiles observed for ConA and strain RB51 (Figure 4.10) as the splenocytes from each group were stimulated by ConA. Splenocytes from each group immunized with strain RB51 were stimulated by heat killed strain RB51. One surprising result was the inability of SOD to stimulate splenocytes from strain RB51 immunized mice. However, this result has been observed before by researchers at Virginia Tech (unpublished data) and may be due to use of commercially prepared *E. coli* SOD instead of *Brucella* SOD. As expected, heat killed *B. anthracis* Sterne culture did not stimulate proliferation in any group. However, PA induced proliferation in splenocytes from PA and some RB51/PA immunized mice. This result is somewhat surprising and encouraging since PA is thought to be an inducer of only a Th2

response. It is noteworthy that two published studies assessing the immunological markers of anthrax vaccination using various adjuvants showed PA specific lymphocyte proliferation; unfortunately, this phenomenon was never further studied (57,71). It would appear that anthrax researchers have become fixated on the antibody-mediated immune response to PA and have not taken the time to identify the full complement of immune reactions that occur as a result of live spore vaccination or infection by spores. A protection study involving a toxin-deficient strain of *B. anthracis* showed that three mutant strains deficient in PA production were able to afford partial protection (20-80%) of mice against a spore challenge (85). While a weak antibody response to toxin components in this strain was observed, it seems that protection was more likely afforded from some cellular or antibody-mediated response to the *B. anthracis* antigens unrelated to the toxin (85). Therefore, it seems highly likely that some other as yet identified components of *B. anthracis*, in addition to PA, aids in conferring protection against spore challenge.

PA used as a subunit vaccine is known to be an inducer of a Th2 response; the antibody profile induced by this protein is well documented. IgM antibodies are quickly replaced by the IgG subisotypes, IgG1 and IgG2b, which are seen in antibody-mediated immune responses (13,40,71). Very little IgG2a is observed in response to PA (40). In addition, cytokine profiles typify a Th2 response with high levels of IL-4 and IL-10 and very low levels of INF-□ and IL-2 (13,77). However, immunization of an animal does not involve just the use of PA. PA alone cannot completely protect against anthrax challenge, generally some adjuvant is needed (40,57,71). Most often these adjuvants such as Allhyrdrogel (used in the human AVA vaccine) are employed to induce a

stronger Th2 immune response to PA (57). However, since it has been theorized that some CMI induction is needed to fully protect against spore challenge, adjuvants that induce a Th1 response have also been used with PA. Monophosphoryl lipid A (MPL), QS-21 saponin and Ribi Tri-Mix (Ribi) are all inducers of CMI (57,71). These adjuvants are often used in combination with PA to observe the effects of CMI induction in protection against anthrax spore challenge. These adjuvants, which induce a non-specific CMI response in the mice, often afford greater protection against challenge than do PA vaccines that only induce a antibody-mediated response (54). This observation, in part, is the reason for expressing PA as a recombinant protein in bacteria that induce a CMI response as presented in this dissertation. It is clear that since very little is known about the CMI response of the host to anthrax and its antigens, more research needs to be performed.

One experiment that may clear up the question of the role of CMI in protection against spore challenge may be the use of adoptive transfer in order to attempt to protect mice against spore challenge. Isolation of T cells from the spleens of mice immunized against anthrax could be injected into naïve mice that would then be challenged with spores. Studies have shown that passive transfer of immune components, such as antibodies or B and T cell mixtures, is able to protect mice against spore challenge (14,86). Breedham *et al*., used either passive or adoptive transfer (a mixture of T and B cells) to attempt to protect mice against spore challenge and showed that no significant protection was observed with a mixed immune cell population without the presence of serum antibodies. However, the transfer of cytotoxic T cells (i.e. without B cells) and assessment of protection seems to have been overlooked. Knowledge of the role of PA

antibodies in protection against anthrax is widespread, however, we still need to identify the extent of the role of CMI in protection against anthrax.

In addition, a systematic approach to the identification of potential CMI inducing antigens in *B. anthracis* should be performed. Shot-gun approaches such as testing each antigen for immune responses is a possible alternative, but there are potentially easier approaches. One approach could be to search the sequenced genome of *B. anthracis* for the presence of homologs in other pathogens that are known to induce CMI responses in the host.

Neutralizing antibody assay results showed some protection of J774A.1 mouse macrophages (challenged by LeTx) by sera from strain RB51/PA and PA immunized mice (Figure 4.7). Since neutralizing antibodies most closely correlate to protection of a challenged animal, this result is also encouraging. Challenge by *B. abortus* 2308 revealed no differences in each RB51/PA construct's ability to protect against a virulent *Brucella* challenge compared to the parent RB51 strain (Figure 4.11). Challenge with $20xLD_{50}$ of Sterne spores revealed partial protection of mice in both vaccination protocol studies (Table 4.5). Study 1 had 3 groups that contained survivors: PA (60%), RB51/18::PA4 (50%) and RB51/PA4 (17%) (Table 4.2). Study 2 had 4 groups that contained survivors: PA (100%), RB51/18::PA4 (60%), RB51/PA4 (60%) and RB51/SOD'::PA4 (20%) (Table 4.4). This partial protection by these new RB51/PA4 strains is encouraging but unable to provide over 50% protection in a single immunization, which is one of the ultimate goals of this vaccine research.

The immune responses and protection results observed in this dissertation suggest that CMI is induced by the strain RB51/PA vaccines as predicted. This response is

directed mainly toward *Brucella*, but there are some indications that PA specific CMI may be there as well. This conclusion is supported by the IgG isotype profile, the cytokine ELISA profile and the LPA results indicating a Th1 response. This Th1 response may be triggered by strain RB51 but does seem to aid in protection against spore challenge in A/J mice. More work is definitely required in order to identify the specific anthrax CMI inducers that confer protection against anthrax challenge. Antibodies to PA4 are induced by these vaccine strains that can recognize full size PA in order to block action of the protein as is needed in protection against anthrax; however, this vaccine is not fully protective in a single dose. This may due to amount of PA seen by the host's immune system rather than presentation of the protein to the host. One way to overcome this low amount of PA synthesis may be to identify a stronger *Brucella* promoter and fuse the synthetic PA4 gene to it. This promoter should possibly be either a promoter that produces high amounts of protein constitutively or a promoter that is highly induced when the *Brucella* is inside the macrophage. The development of a strain RB51 that expresses high amounts of PA to immunize an animal against both anthrax and brucellosis in a single dose could be a very important tool for use in developing countries where both diseases still cause economic damage.

Previously published studies of live recombinant vaccines expressing PA have yielded varying results of efficacy against spore challenge. *E. coli* was the first recombinant tested, however, the level of PA expression was considered to be very low and degradation of the protein was observed (96,117). Another version of this recombinant has been shown to express PA well without much degradation and vaccination studies are underway (44). PA expression in *S. typhimurium* is more stable

and vaccine trials comparing this recombinant against adjuvanted PA shows its efficacy to be about the same (33-37%) when administered orally (28). When PA was expressed in vaccinia virus strains efficacy against spore challenge was shown to be about 50% and 60% in baculovirus (48). One of the best candidates for a live recombinant PA vaccine published upon thus far has been that of *B. subtilis* expressing PA (100). This vaccine candidate produces PA levels that are equal to or exceed those seen in *B. anthracis* (76,100). Vaccination trials using this vaccine candidate have shown it to be as efficacious as the live spore vaccine and better that that of the AVA (51,53).

The strain RB51/PA4 vaccine candidates in this dissertation have yielded some promising results when compared to the efficacies of other live recombinant vaccines. Strains RB51/18::PA4 and RB51/PA4 have efficacies of 50% and 17% respectively and 60% when boosted with PA. These efficacies fall in the middle range of the values seen for other recombinant vaccines. In addition, it is possible that the efficacy of these vaccine candidates could be improved by employing one of the strategies mentioned above. This would make the strain RB51/PA vaccine very attractive for use in veterinary medicine since good protection against two potentially economically disastrous bacterial diseases could be obtained in a single vaccine.

Brucellosis is virtually eradicated in the domestic cattle population of the continental United States, Canada, and Western Europe, although it is still endemic in wildlife. However, the same cannot be said for cattle in developing nations where the disease can be considered endemic. The availability of a successful vaccine against *Brucella*, such as strain RB51, is one tool being adopted by some of these countries to eradicate the disease in cattle. Since the vaccine is relatively easy to produce and

requires only one dose for effective immunity, it is a cost effective way to prevent brucellosis (60).

Nations in which brucellosis is endemic often have the same level of anthrax prevalence (60). While the currently available vaccines against *B. anthracis* are effective, they do have some undesirable side effects including virulence in humans and some animal species (53,99).

Since these two diseases tend to be widespread in developing countries, it is often unaffordable for livestock owners to vaccinate against both anthrax and brucellosis (60,118). A desirable alternative would be to develop a dual vaccine that would deliver effective immunity against both diseases using only one or two immunizations. This vaccine would contribute greatly to the eradication of two diseases at one time and allow for economic growth through the ability to trade livestock with nations having disease free livestock. As strain RB51 is such a successful cattle vaccine, it would be a useful platform to serve as a delivery system for other protective antigens (14). This strain has been used successfully in laboratory studies to deliver both homologous and heterologous proteins for immunization of mice (80). Strain RB51 has many of the desirable traits of a live vaccine in that it is generally recognized as safe, possesses intrinsic adjuvanticity and does not induce O-side chain antibodies that confound serodiagnosis (124). Once a method for increasing production and stabilization of PA in strain RB51 is determined, there is every reason to think that the RB51 vaccine strain can be further refined to more efficiently prevent brucellosis and anthrax simultaneously. This vaccine could have the potential to be safe, cheap stable and easy to administer in a single dose as is desired by an ideal anthrax vaccine (124).

LITERATURE CITED

1. Abdallah, A.I, N.J. Commander, M.J Woodward, S. Spencer, C.A. Hart. 2003. Type III secretion homologs are present in *Brucella melitensis*, *B. ovis* , and *B. suis* biovars 1, 2, and 3. Curr. Microbiol. 46: 241-245.
2. Ahmed, S.A., R. Gogal, J. Walsh. 1994. A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes: an alternative to [^{3}H]thymidine incorporation assay. J. Immunol. Meth. 170: 211-224.
3. Ahuja, N., P. Kumar, R. Bhatnagar. 2001. Rapid purification of recombinant anthrax- protective antigen under nondenaturing conditions. Biochem. & Biophys. Res. Commun. 286: 6-11.
4. Ausubel, F., R. Brent, E. Kingston, D. Moore, J. Seidman, J. Smith, K. Struhl. 1993. Current protocols in Molceular Biology. *In* K. Struhl (ed.). Greene Publishing Associates and Wiley-Interscience, New York.
5. Baillie, L., A. Moir, R. Manchee. 1998. The expression of the protective antigen of *Bacillus anthracis* in *Bacillus subtilis*. J. Appl. Microbiol. 84: 741-746.
6. Barnard, J., A. Friedlander. 1999. Vaccination against anthrax with attenuated recombinant strains of *Bacillus anthracis* that produce protective antigen. Infect. Immun. 67: 562-567.
7. Bartkus, J., S. Leppla. 1989. Transcriptional regulation of the protective antigen gene of *Bacillus anthracis*. Infect. Immun. 57: 2295-2300.
8. Bartlett, J., T. Inglesby, L. Borio. 2002. Management of anthrax. Clin. Infect. Dis. 35: 851-857.

9. Benson, E., P. Huynh, A. Finklestein, R. Collier. 1998. Identification of residues lining the anthrax protective antigen channel. Biochem. 57: 5941-5948.
10. Biddle, W. 1995. p. 20-22. In A field guide to germs.
11. Boschirolli, M.L., S. Ouahrani-Bettache, V. Foulongne, S. Michaux-Charachon, G. Bourg, A. Allardet-Servent, C. Cazevielle, J.P. Lavigne, J.P. Liautard, M. Ramuz, D. O'Callaghan. 2002. Type IV secretion and *Brucella* virulence. Vet. Microbiol. 90: 341-348.
12. Bourgogone, A., M. Drysdale, S.G. Hilsenbeck, S.N. Peterson, T.M. Koehler. 2003. Global effects of virulence gene regluators in a *Bacillus anthracis* strain with both virulence plasmids. Infect. Immun. 71: 2736-2743.
13. Boyaka, P., A. Tafaro, R. Fischer, S. Leppla, K. Fujihashi, J. McGhee. 2003. Effective mucosal immunity to anthrax: neutralizing antibodies and Th cell responses following nasal immunization with protective antigen. J. Immunol. 170: 5636-5643.
14. Breedham, R., P. Turnbull, E. Williamson. 2001. Passive transfer of protection against *Bacillus anthracis* infection in a murine model. Vaccine. 19: 4409-4416.
15. Brossier, F., J. Sirard, C. Guidi-Rontani, E. Duflot, M. Mock. 1999. Functional analysis of the carboxyl- terminal domain of *Bacillus anthracis* protective antigen. Infect. Immun. 67: 964-967.
16. Brossier, F., M. Weber-Levy, M. Mock, J. Sirard. 2000. Role of toxin functional domains in anthrax pathogenesis. Infect. Immun. 68: 1781-1786.
17. Brossier, F., M. Mock. 2001. Toxins of *Bacillus anthracis*. Toxicon. 39: 1747-1755.

18. Celli, J., C. Chasteelier, D.M. Franchini, J. Pizarro-Cerda, E. Moreno, J.P. Gorvel. 2003. *Brucella* evades macrophage killing via VirB-dependent sustained interactions with the endoplasmic reticulum. J. Exp. Med. 198: 545-556.

19. Cheville, N., M. Stevens, A. Jensen, F. Tatum, S. Halling. 1993. Immune responses and protection against infection and abortion in cattle experimentally vaccinated with mutant strains of *Brucella abortus*. Am. J. Vet. Res. 54: 1591-1597.

20. Cherwonogrodzky, J. 1993. *Brucella* antigens- old dogmas, new concepts. Rev. Lat-Am. Microbiol. 35: 339-344.

21. Cimons, M. 1998. DoD move forward with anthrax vaccination program. Am. Soc. Microbiol. News 64: 128-129.

22. Cloeckaert, A., V. Weynants, J. Godfroid, J. Verger, M. Grayon, M. Zygmunt. 1998. O-polysaccharide epitopic heterogenicity at the surface of *Brucella* spp. studied by enzyme- linked immunosorbent assay and flow cytometry. Clin. Diag. Lab. Immun. 5: 862-870.

23. Cloeckaert, A., M. Grayon, O. Grepinet, K. Boumedine. 2003. Classification of *Brucella* strains isolated from marine mammals by infrequent restriction site- PCR and development of specific PCR identification tests. Microbes & Infect. 5: 593-602.

24. Colorado Serum Company. Anthrax Live Spore Vaccine Information Leaflet.

25. Compton, J (ed.). 1987. p. 354-361. In Military Chemical and Biological Agents: chemical and toxicologic properties.

26. Corbel, M.J. 1997. Brucellosis: an overview. Emerg. Infect. Dis. 3: 213-321.

27. Cosivi, O., M. Corbel. 1998. WHO consultation on the development of new/improved brucellosis vaccines. Biologicals. 26: 361-363.

28. Coulson, N., M. Fulop, R. Titball. 1994. *Bacillus anthracis* protective antigen, expressed in *Salmonella typhimurium* SL3261, affords protection against anthrax spore challenge. Vaccine. 12: 1395-1401.

29. Dai, Z., T. Koehler. 1997. Regulation of anthrax toxin activator gene (atxA) expression in *Bacillus anthracis*: temperature, not CO_2/bicarbonate, effects AtxA synthesis. Infect. Immun. 65: 2576-2582.

30. Davis, L., M. Kuehl, J. Battey. 1994. Basic methods in molecular biology. Appleton & Lange, Norwalk, CT.

31. Dixon, T., M. Meselson, J. Guillemin, P. Hanna. 1999. Anthrax. N Engl. J. Med. 341: 815-826.

32. Doyle, K. 1996. Protocols and applications guide. Promega, Madison, WI.

33. Dragon, D., R. Rennie. 1995. The ecology of anthrax spores: tough but not invincible. Can. Vet. J. 36: 295-301.

34. Duesbery, N., C. Webb, S. Leppla, V. Gordon, K. Klimpel, T. Copland, N. Ahn, M. Oskarsson, K. Fukasawa, K. Paul, G. Vande Wonde. 1998. Proteolytic inactivation of MAP-kinase-kinase by anthrax lethal factor. Science. 280: 734-737.

35. Dutz, W., E. Kohout-Dutz. 1981. Anthrax. Int. J. Derm. 20: 203-206.

36. Ezzell, J., T. Abshire. 1988. Immunological analysis of cell-mediated antigens of *B. anthracis* . Infect. Immun. 56: 349-356.

37. Flick-Smith, H., N. Walker, P. Gibson, H. Bullifent, S. Hayward, J. Miller, R. Titball, E. Williamson. 2002. A recombinant carboxyl- terminal domain of the protective

antigen of *Bacillus anthracis* protects mice against anthrax infection. Infect. Immun. 70: 1653-1656.

38. Fraser, C. (ed.), A. Mays (ed.). 1986. p. 639-645. *In* The Merck Veterinary Manual.
39. Friedlander, A. 2001. Anthrax. Textbook of Military Medicine.
40. Gaur, R., P. Gupta, A. Banerjea, Y. Singh. 2002. Effect of nasal immunization with protective antigen of *Bacillus anthracis* on protective immune response against anthrax toxin. Vaccine. 20: 2836-2839.
41. Greenfield, R., M. Bronze. 2003. Prevention and treatment of bacterial diseases caused by bacterial bioterrorism threat agents. Drug Discov. Today. 8: 881-888.
42. Gogal, R., S.A. Ahmed, C. Larson. 1997. Analysis of avian lymphocyte proliferation by a new, simple, nonradioactive assay (Lympho-pro). Avian Dis. 41: 714-725.
43. Guidi-Rontani, C., E. Duflot, M. Mock. 1997. Anthrax lethal toxin-induced mitogenic response of human T-cells. FEMS Microbiol. Lett. 157: 285-289.
44. Gupta, P., S.M. Waheed, R. Bhatanagar. 1999. Expression and purification of the recombinant protective antigen of *Bacillus anthracis*. Prot. Express. Purif. 16: 369-376.
45. Gupta, S., T. Ghosh. 2001. Gene expressivity is the main factor in dictating the codon usage variation among the genes in *Pseudomonas aeruginosa*. Gene. 273: 63-70.
46. Halperin, G., H. Marcus. 2001. Application of recovery tests in validation of immunoassays for assessing the immunogenicity of *B. anthracis* PA vaccine. PDA J. Pharm. Sci. & Technol. 55:150-161.

47. Hilleman, M. 2002. Overview: cause and prevention in biowarfare and bioterrorism. Vaccine. 20: 3055-3067.

48. Iacono-Connors, L., S. Welkos, B. Ivins, J. Dalrymple. 1991. Protection against anthrax with recombinant virus-expressed protective antigen in experimental animals. Infect. Immun. 59: 1961-1965.

49. Ibrahim, K., G. Brown, D. Wright, J. Rotschafer. 1999. *Bacillus anthracis*: medical issues of biologic warfare. Pharmacotherapy. 19: 690-701.

50. Inglesby, V., D. Henderson, J. Bartlett, M. Ascher, E. Eitzen, A. Friedlander, J. Hauer, J. McDade, M. Osterholm, T. O'Toole, G. Parker, T. Perl, P. Russell, K. Tonat. 1999. Anthrax as a biological weapon: medical and public health management. Working Group on Civilian Biodefense. J. Am. Med. Assoc. 281: 1735-1745.

51. Ivins, B., S. Welkos. 1986. Cloning and expression of the *Bacillus anthracis* protective antigen gene in *Bacillus subtilis*. Infect. Immun. 54: 537-542.

52. Ivins, B., S. Welkos. 1988. Recent advances in the development of an improved human anthrax vaccine. Eur. J. Epidem. 4: 12-19.

53. Ivins, B. 1988. The search for a new-generation human anthrax vaccine. Clin. Immun. Newsletter 9: 30-32.

54. Ivins, B., S. Welkos, S. Little, M. Crummie, G. Nelson. 1992. Immunization against anthrax with *Bacillus anthracis* protective antigen combined with adjuvants. Infect. Immun. 60: 662-668.

55. Ivins, B., P. Fellows, G. Nelson. 1994. Efficacy of a standard human anthrax vaccine against *B. anthracis* spore challenge in guinea pigs. Vaccine 12: 872-874.

56. Ivins, B., P. Fellows, L. Pitt, J. Estep, J. Farchaus, A. Friedlander, P. Gibbs. 1995. Experimental anthrax vaccines: efficacy of adjuvants combined with protective antigen against an aerosol *Bacillus anthracis* spore challenge in guinea pigs. Vaccine. 13: 1779-1784.

57. Ivins, B., M. Pitt, P. Fellows, J. Farchaus, G. Benner, D. Waag, S. Little, G. Anderson, P. Gibbs, A. Friedlander. 1998. Comparative efficacy of experimental anthrax vaccine candidates against inhalation anthrax in Rhesus macaques. Vaccine. 16: 1141-1148.

58. Kanaya, S., Y. Yamada, Y. Kudo, T. Ikemura. 1999. Studies of codon usage and tRNA genes of 18 unicellular organisms and quantification of *Bacillus subtilis* tRNAs: gene expression level and species- specific diversity of codon usage based on multivariate analysis. Gene. 238: 143-155.

59. Karlin, S., M. Barnett, A. Campbell, R. Fisher, J. Mrazek. 2003. Predicting gene expression levels from codon biases in □-proteobacterial genomes. Proc. Natl. Acad. Sci. 100: 7313-7318.

60. Khanna, H., Y. Singh. 2001. War against anthrax. Mol. Med. 7: 795-796.

61. Kim, C., Y. Oh, T. Lee. 1997. Codon opitimization for high- level expression of human erythropoietin (EPO) in mammalian cells. Gene. 199: 293-301.

62. Koehler, T., Z. Dai, M. Kaufman-Yarbray. 1994. Regulation of the *Bacillus anthracis* protective antigen gene: CO_2 and a trans-acting element activate transcription from one of two promoters. J. Bacteriol. 176: 586-595.

63. Koehler, T.M. 2002. *Bacillus anthracis* genetics and virulence gene regulation. Curr. Top. Microbiol. Immunol. 271: 143-164.

64. Krishnanchettiar, S., J. Sen, M. Caffrey. 2002. Expression of purification of the *Bacillus anthracis* protective antigen domain 4. Prot. Express. & Purif. 27: 325-330.

65. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227: 680-685.

66. Little, S., J. Lowe. 1991. Location of receptor- binding region of protective antigen from *Bacillus anthracis*. Biochem. & Biophys. Res. Comm. 180: 531-537.

67. Lord, V., G. Schurig, J. Cherwonogrodsky, M. Marcano, G. Melendez. 1998. Field study of vaccination of cattle with *Brucella abortus* RB51 and 19 under high and low disease prevalence. Am. J. Vet. Med. 59: 1016-1020.

68. Makoff, A., M. Oxer, M. Romanos, N. Faitweather, S. Ballantine. 1989. Expression of tetanus toxin fragment C in *E. coli*: high level expression by removing rare codons. Nucleic Acids Res. 17: 10191-10202.

69. Maratea, J., D. Ewalt, S. Frasca Jr., J. Dunn, S. DeGuise, L. Szkudlarek, D. St. Aubin, R. French. 2003. Evidence of *Brucella* sp. Infection in marine mammals stranded along the coast of southern New England. J. Zoo. Wildl. Med. 34: 256-261.

70. Marino, O., I. Gallego, E. Rueda, L. Sedano, G. Schurig. 1997. Evaluation of a potential vaccine: *Brucella abortus* RB-51 susceptibility and protection in guinea pigs. Net. Brucell. Res. II 197-211.

71. McBride, B., A. Mogg, J. Telfer, M. Lever, J. Miller, P. Turnbull, L. Baillie. 1998. Protective efficacy of a recombinant protective antigen against *Bacillus anthracis* challenge and assessment of immunological markers. Vaccine. 16: 810-817.

72. McQuiston, J., G. Schurig, N. Sriranganathan, S. Boyle. 1995. Transformation of *Brucella* species with suicide and broad host range plasmids. Electroporation Protocols for Microorganisms 47: 143-148.

73. Michaux-Charachon, S., G. Bourg, E. Jumas-Bilak, P. Guigue-Talet, A. Allardet-Servent, D. O'Callaghan, M. Ramuz. 1997. Genome structure and phylogney in the genus *Brucella*. J. Bacteriol. 179: 3244-3249.

74. Michaux-Charachon, S., E. Jumas-Bilak, A. Alldaret-Servent, G. Bourg, M. Boschiroli, M. Ramuz, D. O'Callaghan. 2002. The *Brucella* genome at the beginning of the post-genomic era. Vet. Microbiol. 90: 581-585.

75. Michigan Department of Public Health. 1987. Anthrax vaccine adsorbed description leaflet.

76. Miller, J., B. McBride, R. Manchee, P. Moore, L. Baillie. 1998. Production and purification of recombinant protective antigen and protective efficacy against *Bacillus anthracis*. Let. Appl. Microbiol.26: 56-60.

77. Misson, J., N. Walker, H. Flick-Smith, D. Williamson, J. Robinson. 2003. Differential processing of CD4 T-cell epitopes from the protective antigen of *Bacillus anthracis*. J. Biol. Chem. 278: 52425-52431.

78. Mizrahi, A (ed). 1990. Bacterial vaccines. p. 105-122. *In* Advances in Biotechnological Processes.

79. Mock, M., A. Fouet. 2001. Anthrax. Annual Review of Microbiology. 55: 746-671.

80. Morton, B., B. So. 2000. Codon usage in plastid genes is correlated with context, position within the gene, and amino acid content. J. Mol. Evol. 50: 184-193.

81. Nicoletti, P., J. Ring, B. Boysen, J. Buczek. 1986. Illness in a veterinary student following accidental inoculation of *Brucella abortus* strain 19. J. Am. Col. Health. 34: 236-237.

82. Noskov, A., T. Kravchenko, V. Noskova. 1996. Detection of functionally active domains in the molecule of the protective antigen of anthrax exotoxin. Mol. Gen. Microbiol. & Virol. 4: 30-39.

83. Novak, J., M. Stein, S. Little, S. Leppla, A. Friedlander. 1992. Functional characterization of protease-treated *Bacillus anthracis* protective antigen. J. Biol. Chem. 267: 17186-17193.

84. Paulsen, I., R. Seshadri, K. Nelson, J. Eisen, J. Heidelberg, T. Read, R. Dodson, L. Umayam, L. Binkac, M. Beanan, S. Daugherty, R. Deboy, A.S. Durkin, J. Kolonay, R. Madupu, W. Nelson, B. Ayodeji, M. Kraul, J. Shetty, J. Malek, S. Van Aken, S. Reidmuller, H. Tettelin, S. Gill, O. White, S. Salzberg, D. Hoover, L. Lindler, S. Halling, S. Boyle, C. Fraser. 2002. The *Brucella suis* genome reveals fundamental similarities between animal and plant pathogens and symbionts. Proc. Natl. Acad. Sci. 99: 13148-13153.

85. Pezard, C., M. Weber, J. Sirard, P. Berche, M. Mock. 1995. Protective immunity induced by *B. anthracis* toxin-deficient strains. Infect. Immun. 63: 1369-1372.

86. Pitt, M., S. Little, B. Ivins, P. Fellows, J. Barth, J. Hewetson, P. Gibbs, M. Dertzbaugh, A. Friedlander. 2001. *In vitro* correlate of immunity in a rabbit model of inhalational anthrax. Vaccine. 19: 4768-4773.

87. Poester, F., E. Ramos, M. Gomes, C. Chiminazzo, G. Schurig. 1997. The serological response of adult cattle after vaccination with *Brucella abortus* strain 19 and RB51. Net. Brucell. Res. II 191-196.

88. Prescott, L.(ed.), J. Harley (ed.), D. Klein (ed.). 1996. p. 567-569. *In* Microbiology.

89. Prince, A. 2003. The host response to anthrax lethal toxin: unexpected observations. J Clin. Invest. 112: 656-658.

90. Price, L., M. Hugh-Jones, P. Jackson, P. Keim. 1999. Genetic diversity of the protective antigen gene of *B. anthracis.* J. Bacteriol. 181: 2358-2362.

91. Read, T., S. Peterson, N. Tourasse, L. Baillie, I. Paulsen, K. Nelson, H. Tettelin, D. Fouts, J. Eisen, S. Gill, E. Holtzapple, O. Okstad, E. Helgason, J. Rilstone, M. Wu. J. Kolonay, M. Beanan, R. Dodson, L. Brinkac, M. Gwinn, R. DeBoy, R. Madpu, S. Daugherty, A. Durkin, D. Haft, W. Nelson, J. Peterson, M. Pop, H. Khouri, D. Radune, J. Benton, Y. Mahamoud, L. Jiang, I. Hance, J. Weidman, K. Berry, R. Plaut, A. Wolf, K. Watkins, W. Nierman, A. Hazen, R. Cline, C. Redmond, J. Thwaite, O. White, S. Salzberg, B. Thomason, A. Friedlander, T. Koehler, P. Hanna, A. Kolste, C. Frasier. 2003. The genome sequence of *Bacillus anthracis* Ames and comparison to closely related bacteria. Nature. 423: 81-91.

92. Rouot, B., M.T. Alvarez-Martinez, C. Marius, P. Menanteau, L. Guilloteau, R.A. Boigegrain, R. Zumbihl, D. O'Callaghan, N. Domke, C. Baron. 2003. Production of the type IV secretion system differs among *Brucella* species as revealed with VirB5- and VirB8-specific antisera. Infect. Immun. 71: 1075-1082.

93. Sakai, H., C. Imamura, Y. Osada, T. Washio, M. Tomita. 2001. Correlation between Shine- Dalgarno sequence conservation and codon usage of bacterial genes. J. Mol. Evol. 52: 164-170.

94. Schurig, G., R. Roop, T. Bagchi, S. Boyle, D. Burhman, N. Sriranganathan. 1991. Biological properties of RB51; a stable rough strain of *Brucella abortus*. Vet. Microbiol. 28: 171-188.

95. Schurig, G., S. Boyle, N. Sriranganathan. 1995. *Brucella abortus* vaccine strain RB51: a brief review. Arch. Vet. Med. 19-22.

96. Sharma, M., P. Swain, A. Chopra, V. Chaudhory, Y. Singh. 1996. Expression and purification of anthrax toxin protective antigen from *Escherichia coli.* Prot. Express. Purif. 7: 33-38.

97. Shlyakhov, E. 1970. Anthrax: biological and immunological principles of diagnosis and prevention. J. Hyg., Epidem., Microbiol. & Immun. 14: 464-468.

98. Shlyakhov, E., E. Rubinstein. 1994. Human live anthrax vaccine in the former USSR. Vaccine 12: 872-874.

99. Shlyakhov, E., E. Rubinstein, I. Novikov. 1997. Anthrax post-vaccinal cell-mediated immunity in humans: kinetics pattern. Vaccine. 15: 631-636.

100. Singh, Y., V. Chaudhary, S. Leppla. 1989. A deleted variant of *B. anthracis* protective antigen is non-toxic and blocks anthrax toxin action *in vivo*. J. Biol. Chem. 264: 19103-19107.

101. Singh, Y., K. Klimpel, C. Quinn, V. Chaudhary, S. Leppla. 1991. The carboxyl-terminal end of protective antigen is required for receptor binding and anthrax toxin activity. J. Biol. Chem. 266: 14593-15497.

102. Singh, Y., B. Ivins, S. Leppla. 1998. Study of immunization against anthrax with the purified recombinant protective antigen of *B. anthracis.* Infect. Immun. 66: 3447-3448.

103. Smith, L., T. Ficht. 1990. Pathogenesis of *Brucella*. Crit. Rev. Microbiol. 17: 209-230.

104. Sterne, M. 1937. Variation in *Bacillus anthracis*. Onderstepoort J. Vet. Sci. & Anim. Ind. 8: 271-349.

105. Sterne, M. 1939. The use of anthrax vaccine prepared from avirulent (unencapsulated) variants of *B. anthracis.* Onderstepoort J. Vet. Sci. & Anim. Ind. 13: 307-312.

106. Sterne, M. 1939. The immunization of laboratory animals against anthrax. Onderstepoort J. Vet. Sci. & Anim. Ind. 13: 313-317.

107. Stevens, M., S. Olsen, G. Pugh, D. Brees. 1995. Comparison of immune responses and resistance to brucellosis in mice vaccinated the *Brucella abortus* 19 or RB51. Infect. Immun. 63: 264-270.

108. Turnbull, P., M. Broster, J. Carman, R. Manchee, J. Melling. 1986. Development of antibodies to protective antigen and lethal factor components of anthrax toxin in humans and guinea pigs and their relevance to protective immunity. Infect. Immun. 52: 356-363.

109. Turnbull, P., S. Leppla, M. Broster, C. Quinn, J. Melling. 1988. Antibodies to anthrax in humans and guinea pigs and their relevance to protective immunity. Med. Microbiol. & Immun. 177: 293-303.

110. Turnbull, P. 1991. Anthrax vaccines: past, present and future. Vaccine. 9: 533-539.

111. Uchida, I., J. Hornung, C. Thorne, K. Klimpel, S. Leppla. 1993. Cloning and characterization of a gene whose product is a trans-activator of anthrax toxin synthesis. J. Bacteriol. 175: 5329-5338.

112. USDA Brucellosis Eradication Program. 1998. APHIS 91-45-011.

113. Varughese, M., A. Teixeira, S. Liu, S. Leppla. 1999. Identification of a receptor-binding region within domain 4 of the protective antigen component of anthrax toxin. Infect. Immun. 67: 1860-1865.

114. Vemulapalli, R., Y. He, S. Cravero, N. Sriranganathan, S. Boyle, G. Schurig. 2000. Overexpression of protective antigen as a novel approach to enhance vaccine efficacy of *Brucella abortus* Strain RB51. Infect. Immun. 68: 3290-3296.

115. Vemulapalli, R., Y. He, S. Boyle, N. Sriranganathan, G. Schurig. 2000. *Brucella abortus* Strain RB51 as a vector for heterologous protein expression and induction of specific Th1 type immune response. Infect. Immun. 68: 3290-3296.

116. Vemulapalli, R., Y. He, N. Sriranganathan, S. Boyle, G. Schurig. 2002. *Brucella abortus* RB51: enhancing vaccine efficacy and developing multivalent vaccines. Vet. Microbiol. 90: 521-532.

117. Vodkin, M., S. Leppla. 1983. Cloning of the protective antigen gene of *Bacillus anthracis*. Cell. 34: 693-697.

118. Wang, X., R. Wattiez, M. Mock, P. Falmagne , J. Ruysschaert, V. Cabiaux. 1997. Structure and interaction of PA63 and EF (edema toxin) of *B. anthracis* with lipid membrane. Biochem. 36: 14906-14913.

119. Welkos, S., T. Keener, P. Gibbs. 1986. Differences in susceptibility of inbred mice to *Bacillus anthracis*. Infect. Immun. 51: 795-800.

120. Welkos, S., J. Lowe, F. Eden-McCutchan, M. Vodkin, S. Leppla, J. Schmidt. 1988. Sequence and analysis of the DNA encoding protective antigen of *Bacillus anthracis*. Gene. 69: 287-300.

121. Welkos, S., A. Friedlander. 1988. Pathogenesis and genetic control of resistance to the Sterne strain of *Bacillus anthracis*. Microb. Pathogen. 4: 53-69.

122. Wesche, J., J. Elliott, P. Falnes, S. Olsnes, R. Collier. 1998. Characterization of membrane translocation by anthrax protective antigen. Biochem. 57: 15737-15746.

123. Winter, A., G. Schurig, S. Boyle, N. Sriranganathan, J. Bevins, F. Enright, P. Elzer, J. Kopec. 1996. Protection of BALB/c mice against homologous and heterologous species of *Brucella* by rough strain vaccine derived from *Brucella melitensis* and *Brucella suis* biovar 4. Am. J. Vet. Res. 57: 677-683.

124. Zegers, N., E. Kluter, H. van der Stap, E. van Dura, P. van Dalen, M. Shaw, L. Baillie. 1999. Expression of the protective antigen of *Bacillus anthracis* by *Lactobacillus casei*: towards the development of an oral vaccine against anthrax. J Appl. Microbiol. 87: 309-314.