Development and Efficacy Assessment of Equine Source Hyper-Immune Plasma against *Bacillus anthracis*

by

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Abstract

The objective of the studies described here was to develop an equine source immune plasma against *Bacillus anthracis* and test its efficacy in two *in vitro* applications; as well as determine its capacity for passive protection in an infection model in mice. Initially, a safe and reliable immunization protocol for producing equine source hyper-immune plasma against *B. anthracis* was developed. Six Percheron horses were hyper-immunized with either the *B. anthracis* Sterne strain vaccine, recombinant protective antigen (rPA) homogenized with Freund's incomplete adjuvant, or a combination of both vaccines. Multiple routes of immunization, dose (antigen mass) and immunizing antigens were explored for safety. A modified automated plasmapheresis process was then employed for the collection of plasma at a maximum target dose of up to 22 ml of plasma/kg of donor bodyweight to establish the proof-of-concept that large volumes of plasma could be safely collected from horses for large scale production of immune plasma. All three immunization protocols were found to be safe and repeatable in horses and three pheresis events were performed with the total collection of 168.36 L of plasma and a mean collection volume of 18.71 L (± 0.302 L) for each event.

Once the hyper-immune plasma had been successfully harvested, two *in vitro* potency tests, an indirect enzyme-linked immunosorbent assay (ELISA) were developed to determine the titer of anti-PA antibodies and a toxin neutralization assay to determine the titer of protective antibodies against lethal toxin. In addition, the performance characteristics of the assay were validated in concordance with standards set forth by the International Conference on Harmonics (ICH) and the Center for Biologics Evaluation and Research (CBER) for analytical procedures.

A working range for this assay was established (73-1581 EU/ml) on the bases of the following parameters: linearity (25 and 1,662 EU/ml, $R^2 = 0.999$, $p < 0.001$), accuracy (94.8 - 105.4 %, recovery within the range of 25 and 1,662 EU/ml), precision (≤ 17.6%CV, repeatability; ≤ 15.7 and ≤ 13.1 % CV, intermediate precision per day and per analyst, respectively), limit of detection (2.25 EU/ml) and limit of quantitation (25 EU/ml). The assay was also demonstrated to be specific for the evaluation of anti-PA antibodies. Based on these performance characteristics it was determined that this assay is adequate for use in *B. anthracis* immunogenicity testing in horses.

Antibody titers against PA were found to be as high as 1:512,000 in some horses. The geometric mean titers for horses hyper-immunized with spore vaccine alone, for horses hyper-immunized with the spore vaccine then hyper-immunized with rPA, for horses hyper-immunized with the spore vaccine then hyper-immunized with twice the volume of spore vaccine, and for horses hyper-immunized with rPA alone was 1:43,031 (SEM±3995, CI 95% 1:35,675 – 1:51,906), 1:213,027 (SEM±32,252, CI 95% 1:152,849 – 1:296,897), 1:83,912 (SEM±16,818, CI 95% 1:54,046 – 1:129,971) and 1:34,912 (SEM±13,961, CI 95% 1:15,035 – 1:81,066), respectively.

Neutralizing antibodies were assessed using mouse macrophage J774A.1 cells in an LF-induced cytotoxicity toxin neutralization assay. Several horses developed neutralizing titers as high 1:1,024. The geometric mean neutralizing titers for horses immunized with the Sterne strain spore vaccine alone, for horses immunized with the spore vaccine then immunized with rPA, for horses immunized with the spore vaccine then immunized with twice the volume of spore vaccine, and for horses immunized with rPA alone was 1: 130 (SEM±2.26, CI 95% 1:126 –

1:135), 1: 964 (SEM±56, CI 95% 1:849 – 1:1,094), 1: 683 (SEM ± 171, CI 95% 1:408 – 1:1,143) and 1:478 (SEM ± 5.5, CI 95% 1:9.5 – 1:68), respectively. Overall, this demonstrates that plasma derived from horses immunized against *B. anthracis* Sterne strain and rPA provides strong *in vitro* correlates of protection.

As a final assessment of the efficacy, hyper-immune plasma and affinity purified immunoglobulins harvested from horses hyper-immunized with *B. anthracis* Sterne strain vaccine was evaluated in the treatment of A/J strain mice intranasal (IN) and subcutaneous (SC) challenge with *B. anthracis* Sterne strain. The treatment of native (un-concentrated) hyper-immune plasma at time 0 hour and 24 hour post-infection following both an IN and SC challenge had no effect on rate of survival to the end of the study period, but did significantly increase mean time to death ($p < 0.0001$) compared to mice treated with *naïve* equine plasma. Mice treated with concentrated immunoglobulins at both time points following IN and SC challenge demonstrated significantly different survival rates ($p < 0.001$) compared to mice treated with *naïve* plasma. *B. anthracis* colony forming units/gram of lung, liver and spleen tissue were also assessed and were not significantly different in mice treated with hyper-immune plasma but were reduced by 4 fold and completely cleared in some cases after treatment with concentrated immunoglobulins ($p < 0.0001$).

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PA	Protective Antigen
LF	Lethal Factor
EF	Edema Factor
LD_{50}	Lethal Dose (50%)
mAb	Monoclonal Antibody
AVA	Anthrax Vaccine Adsorbed
rPA	Recombinant Protective Antigen
EU	Experimental Unit
%CV	Coefficient of Variation
OD	Optic Density
ELISA	Enzyme-Linked Immunosorbent AssaySEM Standard Error of the Mean
PCR	Polymerase Chain Reaction

Chapter 1:

Literature Review

The History of Anthrax

Anthrax as a disease has been known since antiquity. Perhaps the first recorded description of anthrax was provided by Moses detailing the fifth and sixth plagues (the death of livestock and the plague of boils) brought forth on Egypt. Moses declares in ninth chapter of the Book of Exodus,

> "The hand of the Lord will fall with a very severe pestilence (some translations use *murrain*) on your livestock in the fields, on horses, asses, camels, herds and on the flocks." (Exodus 9:3, New American Standard Bible)

In order to deliver this plague Moses was commanded to lift a handful of dust toward the sky;

> "It will become a fine dust over all the land of Egypt and will become boils breaking out with sores on man and beast" (Exodus 9:9, New American Standard Bible)

Other historians and poets have described plagues that may also be interpreted as symbolic of anthrax in humans and animals. The Roman poet Virgil presented a description of a disease similar to anthrax in his third *Georgic*[1];

> "A terrible plague once sprang up there and ranged on through the warmer part of the autumn, not only destroying one flock of sheep after another, but killing animals of all kinds….The steaming ox falls before the heavy plow. Blood mixed with froth issues from his mouth as he groans his last. …..The pelts of diseased animals were useless, and neither water nor fire could cleanse the taint from their flesh. The sheep men could not shear the fleece which was riddled with disease and corruption, nor did they dare even to

touch the rotting strands. If anyone wore garments made from tainted wool, his limbs were soon attacked by inflamed papules and a foul exudate."

The Greek physician Hippocrates was the first to name the disease by coining the term "*anthracites"* (later corrupted to anthrax) a Greek word which translates "coal-like" to describe the black eschars that develop with cutaneous anthrax.[2]

Anthrax was an important cause of death for humans and animals throughout the world until the end of the 19th century. Major epizootics were recorded through the Middle Ages in early veterinary texts such as the "Hippiatrika"[3] and "The medicine of quadrupeds"[4]. These records relate massive losses of domestic livestock in Europe. In France during the 18th century an anthrax outbreak is thought to have decimated half of the sheep population. Similar accounts can be found in Hindu and Chinese texts of the time.[5]

The modern history of anthrax began in 1790 when Chabert provided the first clear clinical description of the disease in animals.[5] In 1863, Pierre Rayer and Casmir-Joseph Davaine two French scientists in the nascent study of microbiology and parasitology consistently observed "filiform bodies about twice the length of blood corpuscles" in the blood of infected sheep, yet not in healthy sheep.[5] This led them to conclude that these "bodies" were the etiologic agent of anthrax and therefore they became the first to associate any disease with a specific microorganism.

Robert Koch in 1876 would further verify these observations through culturing these bacilli from the blood of infected animals on simple blood based agar.[6] He then observed that when injected into naïve animals the disease could be reproduced and the bacilli could be re-isolated. Thus *B. anthracis* served as the model for Koch's famous postulates. Koch's postulates

are a series of steps that could ostensibly assign an etiologic relationship between an organism and a clinical disease. In short, the organism must first be isolated in pure culture from an animal or individual displaying symptoms characteristic of a particular disease, then upon infecting a new and naïve individual with the isolated pathogen the exact disease process is recreated. Finally, to fulfil the postulates the same infecting organism must be re-isolate from the new patient in pure culture. Though no known to be an imperfect model, at the time these guidelines shaped the formation of the germ theory.

In 1881, Louis Pasteur was the first individual to use a live attenuated vaccine when he used his heat attenuated strain of *B. anthracis* to vaccinate a small group of sheep in a village outside of Paris.[2] When these sheep were later challenged with virulent *B. anthracis* all immunized sheep survived.

The incidence of anthrax in humans decreased significantly during the 20th century dropping from an estimated occurrence of 20,000-100,000 cases annually in the first half of the century to less than 2000 in the second half.[5] The majority of these later cases were identified in mill and textile workers. As result of this industrial correlation anthrax became known as "woolsorter's disease" in England. Inhalational anthrax during this time period, as is the case today, had a much lower incidence than the cutaneous form. In a review of inhalational anthrax published in 1980 Phillip Brachman of the Centers for Disease Control and Prevention reported that only 18 cases of inhalational anthrax were reported in the United States from 1900 to the year of publication. This led him to conclude that anthrax in humans was "now primarily a disease of historical interest".[7]

Anthrax as a Bioweapon

The first confirmed allegations of the use of *B. anthracis* as a biological weapon were made against Germany in World War I. German agents were accused of infecting livestock in Budapest in 1916 and France in 1917. Carus describes an account of German agents infiltrating the horse pens in Manhattan's Van Courtland Park as early as 1915 in an attempt to inject the animals with bottles of liquefied *B. anthracis* spores. Following World War I in recognition of the dangerous nature of these weapons the Geneva Protocol of 1925 specifically prohibited "the use of bacteriological methods of warfare".[8,2,9]

Interest in anthrax as a bioweapon resurfaced during the Sino-Japanese War in 1937 when a radical nationalist Japanese military officer and medical researcher named Dr. Shiro Ishii established a complex outside the Manchurian city of Harbin.[2] Dr. Ishii's scientists primarily focused on developing ways to grow and disseminate *B. anthracis* spores. Their attempts included producing a prototype porcelain bomb and candy laced with *B. anthracis* spores. Prisoners of war and Chinese women and children were used as subjects in these experiments.[8] In the summer of 1942 the British began a series of anthrax-bomb tests on the island of Gruinard, an isolated island off the coast of Scotland.[8] Bombs carrying spores were exploded in attempts to infect and kill sheep tethered downwind from the explosions. Because of the success of these tests and others Churchill ordered the production of 500,000 anthrax bombs. There is no record however, of any direct biological attacks ever carried out against Great Britain's enemies.

The United States also began developing a biological weapons program in 1942 at the site that would come later to be known as Fort Detrick in Fredrick, Maryland. The main effort at Fort Detrick focused on the large-scale production of *B. anthracis* and the development of

delivery systems including spray tanks, warheads and cluster munitions.[9] At its peak in the late 1950's the United States biological weapons program extended to three sites; Fort Detrick, Maryland, Fort Terry, Plum Island, and Pine Bluff, Arkansas. Richard Nixon in his "Statement on Chemical and Biological Defense Policies and Programs" in 1971 announced the end of the Unites States offensive biological weapons program and a refocus of the effort into biological weapons defense. This step led to the Biological Weapons Convention and an international treaty in 1972 outlawing the use of biological weapons.

The Soviet Union escalated the development of their biological weapons program during the Cold War and despite signing the Biological Weapons Convention Treaty continued to covertly expanded several agents.[10] Extensive weapons research was carried out by several government ministries under the oversight of the larger umbrella Biological Substance Preparation Program or *Biopreparat*. In addition to *B. anthracis, Biopreparat* research also focused on VEE, *Brucella suis*, small pox (*Variola major), Yersenia pestis,* and Marburg virus. [10] *B. anthracis* was the vanguard of the Biopreparat program. Research and development of *B. anthracis* emphasized the optimization of lethality and the development of antibiotic resistance. Enormous stockpiles of *B. anthracis* spores were assembled. Facilities were constructed exclusively for the purpose of rapidly increasing production following attack or onset of NATO aggression.[10, 11]Evidence of continued covert weapons manufacturing was revealed on April 2, 1979 when an accidental release of *B. anthracis* spores from a biological weapons manufacturing facility into the town of Svderdlosk (now Ekaterinaberg, Russia) and the surrounding area resulted in the largest recorded outbreak of human inhalational anthrax.[12] Aerosolized *B. anthracis* spores were released into the atmosphere from the plant when a clogged air filter was

removed and not replaced between shifts of janitorial staff. Winds carried the spores through six small villages resulting in the exposure of over 16,000 people, 96 confirmed cases of anthrax and the death of 66.[12]

In the early 1990's following the fall of the USSR several high ranking *Biopreparat* officials defected to the United States. One of the defectors was Ken Albiek a physician, microbiologist and First Deputy Director of *Biopreparat*.[11] Alibek provided evidence that large stocks of weaponized agents existed up to the fall of the government and that during the ensuing disorder some stockpiles were released into unknown hands, perhaps representing a means by which terrorist organizations could have obtained the weapons.

In the wake of the Gulf War (1990-1991) rumors of a biological and chemical weapons program developed by Sadam Hussein while president of Iraq surfaced when United Nation weapons inspectors charged with the post-war disarmament of Iraq uncovered confounded evidence of the production of several weapons. These agents included *B. anthracis,* botulinum toxin, aflatoxin, ricin, and *Clostridium perfringens* toxin. Reportedly the Iraqi program assembled several thousand liters of botulinum toxin and weaponized *B. anthracis* spores outfitted for mass dissemination.[13] Continued rumors of basic research in developing weapons of mass destruction by Iraq and Saddam Hussien served in part for the justification for US invasion in 2003.[13]Upon invasion no stockpiles of biological weapons were uncovered and all indications suggest the Iraqi program had been discontinued and evidence of the weapons destroyed years before.

The most recent anthrax bioterrorist attack occurred in October and November of 2001 when letters containing spores were mailed to the offices of Senators Tom Daschale and Patrick

Leahy as well as several news media offices. Known as Amerithrax, the intentional release of *B. anthracis* in this event resulted in the potential exposure of over 10,000 people, 21 infections and 5 fatalities.[14] In 2005, Bruce Edward Ivins, an established biodefense researcher at United States Army Medical Research Institute for Infectious Disease became the major suspect in the federal investigation. The motive behind this offense was never realized as Dr. Ivins committed suicide prior to being fully charged, despite little direct evidence of his involvement. The most condemning evidence uncovered in the course of the investigation was the demonstration through molecular fingerprinting that unequivocally tied the strain used in the attack to strains found in Dr. Ivins laboratory. The impact of this attack resulted in an estimated cost of over $100 million dollars and the inspiration of over 5,000 hoaxes.[14]

B. anthracis spores lend themselves well to aerosolization. Weaponized or milled spores are 2-6 microns in diameter which is an ideal size for efficient suspension in air and inhalation into the lower respiratory tract. A Center for Disease Control and Prevention (CDC) report estimated that following the release of weaponized *B. anthracis* spores under optimal metrological conditions along a 2 kilometer line upwind of a suburban population of 100,000 inhabitants would result in approximately 50,000 cases of inhalational anthrax and 32,875 deaths[15] The cost of such an attack scenario is estimated to be $26.2 billion dollars.[15] The case attack and case fatality rates in this scenario represent far more infections and deaths than predicted for any other Category A agent release. Under these conditions the estimated range where exposure to spores results in 50% case fatality rates is approximately 160 kilometers downwind of the release site.[15] The CDC report may be conservative in its estimates by using an

infectious dose$_{50}$ (ID_{50}) of 20,000 spores where other sources have referenced an ID_{50} dose for humans under these conditions of 8,000-10,000 spores.[16]

Worldwide Incidence of Human Anthrax

Despite these high profile outbreaks and release scenarios the global incidence of anthrax in humans and animals has declined in the last half century. This is thought largely to be the result of vaccination and improved animal husbandry and methods of animal processing. International programs fostered by the World Health Orgnization (WHO) and the World Orgnization for Animal Health (OiE) have been successful in the vaccination of livestock, which is perceived to be the most critical step in reducing anthrax exposure to humans. However, as the incidence of the disease has waned, so has awareness. Recent resurgences in anthrax epizootics in developing nation appear to be fueled by the lack of experience of younger generations of farmers and animal health care providers and failure to recognize and report the disease quickly.

Anthrax is endemic in several tropical areas of the world including sub-Saharan Africa, the Middle East, Southern Europe and Central and South America. Small pockets of endemic locals exist in North America due to favorable soil conditions and repeated livestock infections

perpetually re-contaminating the environment.

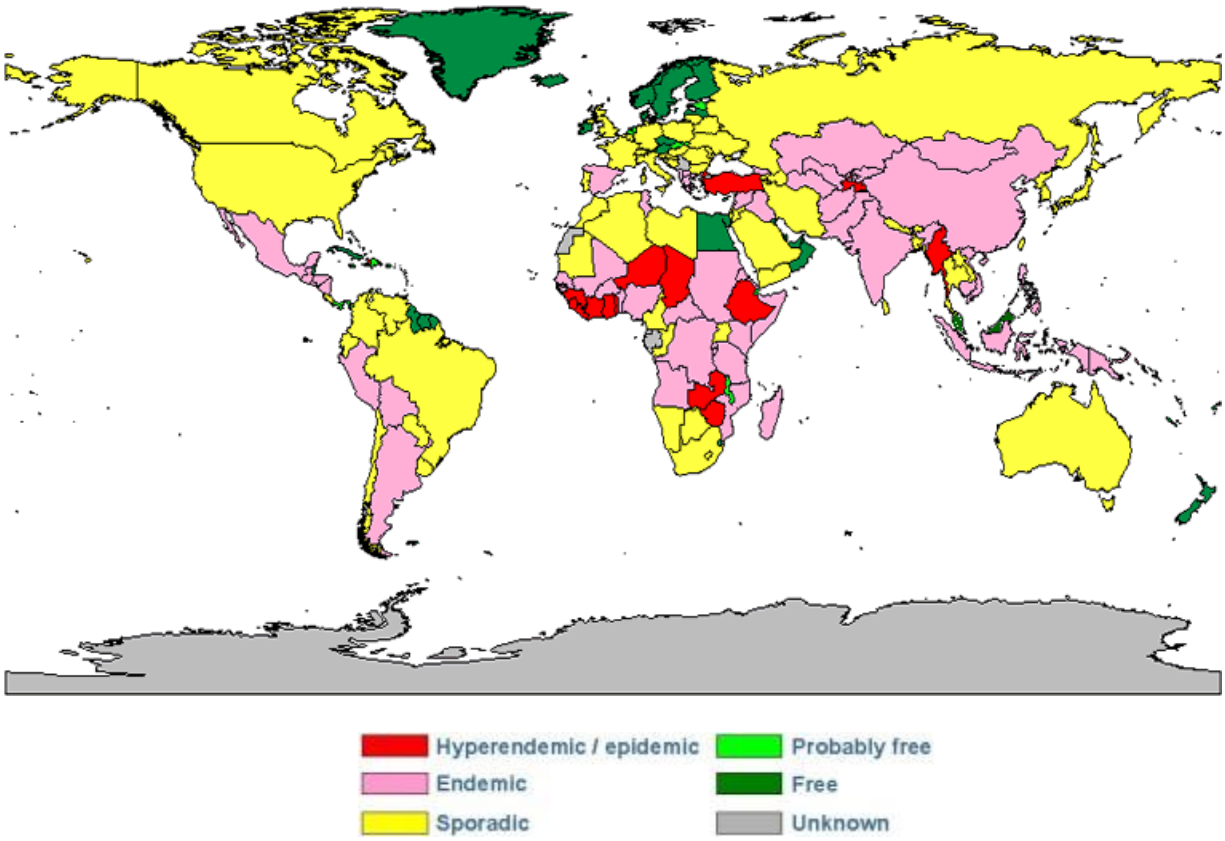

Figure 1.1 Global Prevalence of Anthrax World Health Organization - 2012.

Developing nations have a higher incidence of human anthrax presumably associated with a more agrarian lifestyle and greater contact with infected livestock. The Ivory Coast area of West Africa has been classified as hyperendemic/epidemic by the WHO due to the highest reported incidence of anthrax in animals and humans.[17] Nearly continuous outbreaks of anthrax occur in the wildlife population of that region. Like many epidemic pathogens *B. anthracis* takes advantage of civil unrest. The largest recorded natural outbreak of human anthrax occurred in Zimbabwe, during the time of its civil war. At its peak in 1979 over 10,000 human cases were

recorded. The epidemic was fueled by an unprecedented concurrent epidemic of anthrax in livestock. The full extent of which has not been documented. A smaller outbreak in Thialand highlights the association of natural cases with infected livestock. In that outbreak 76 people from a single village presented with gastrointestinal anthrax following consumption of improperly cooked contaminated meat.[18] In the developed world preventative measures have limited outbreaks to very isolated occurrences often only associated with specific locations where *B. anthracis* spores contaminate the environment. Rarely do these endemic infections affect more than a few individual animals or humans.

Biology of *Bacillus anthracis*

Bacillus anthracis Taxonomy and Phenotype

B. anthracis is a member of the Group 1 bacilli. The type specie for this group is *Bacillus cereus.* Collectively these organisms are non-fastidious, facultative anaerobic, soil organisms with very similar growth characteristics and common natural transformation and conjugation systems. The other members of this group include *B. cereus, B. thuringiensis, B. mycoides, B. pseudomycoides,* and *B. weihenstephanensis*.[19, 20, 21, 22, 23, 24]The defining characteristic of all *Bacillus* species is the ability to produce a resistant endospore in the presence of oxygen. Only three species of *Bacillus* have been reported to be pathogenic: *B. anthracis;* primarily a pathogen of ruminants but can infect any warm blooded animal, *Bacillus cereus;* the most closely related to *B. anthracis* based on sequence homology and the cause of major outbreaks of food borne illness in humans, and *Bacillus thuringiensis;* an important pathogen of insects that also produces pathogenesis through the production of toxins. *B. anthracis* possesses two virulence plasmids, pXO1 and pXO2, that are the defining characteristic that allows differentiation from *B. cereus.*

[25, 26, 27]In addition there are approximately 150 genes that have been shown to differ between the two organisms.

The Group 1 Bacilli have among the narrowest genetic diversity of all Eubacteria.[28] In fact, the three pathogenic members of the genus have nearly exact homology based on DNA-DNA hybridization sequence analysis and electrophoresis profiling.[29] Helgason *et al.* concluded that based MLT studies the Group 1 Bacilli should in fact belong to one species and the current species be reorganized into subspecies based on the current phenotypes. [23] The existing nomenclature has been derived from observations of laboratory culture phenotype and clinical presentation. Traditionally, the presence of the virulence plasmids was sufficient to define specificity for *B. anthracis* but, there are some reports of rare *B. cereus* strains harboring plasmids similar in size and genetic content to pXO1 and pXO2 that manifest anthrax-like illnesses.[30] Enough heterology exists between the RNA polymerase B subunit of each of the species to provide partial laboratory differentiation.[31] More advanced differentiation techniques such as the susceptibility to gamma phages are hoped to provide a field test that is rapid and highly specific for differentiation.

B. anthracis is believed to have split from a common *B. cereus* ancestor relatively recently based on an absence of lateral gene transfer, few single nucleotide polymorphisms (SNPs) and limited genetic diversity within the few clades. *B. anthracis* species are further subdivided into three major clades or lineages (A, B, and C). The A-clade has the greatest worldwide distribution and accounts for >90% of anthrax infections globally.[32] While there is topographical complexity in this group, because of the very limited signature SNPs, it is necessary to conduct whole genome sequences to resolve clade classification. Once these SNPs

226 are verified however, they provide highly dependable signatures for phylogenetic
227 reconstructions.[33,34]

228 The B clade has many fewer members and a limited geographic distribution compared to
229 the A group. There are two distinct subgroups of the B clade; subclade B1 and B2. Subclade B1
230 overlaps in geographic distribution with some members of the A clade within areas of Southern
231 Africa.[32,35,36] The B2 subclade has been isolated most frequently from Southern Europe and once
232 in the Western US. [32,35,36] In the regions where these organisms are found, the B clade is
233 important in the epidemiology of anthrax but do not have the global significance of the A
234 lineage. The fundamentally derived C lineage is rare and does not fall into easily defined spatial
235 order. Only two isolates within this clade have been reported, each independently and from
236 different regions within the US.[32] Some have suggested these represent an entirely distinct New
237 World clade, but widespread distribution of the A clade across the North American continent
238 indicates its presence here for some time and that the two C group isolates simply represent
239 poorly classified outliers.[38,39]

240 The *B. anthracis* genome consists of three structures; the main bacterial chromosome and
241 two virulence plasmids. The nucleotide composition of each of these, like other Gram positive
242 organisms, has a low guanine and cytosine content (approximately 35%). Compared to the
243 limited plasmids found in Gram positive organisms, the plasmids of *B. anthracis* are large and
244 complex with many coding open reading frames. The most important of these are the three
245 virulence genes. The most widely used reference strain in genetic analysis and fully virulent
246 challenges is the Ames strain, a member of the group A clade.[37] Due to the intense investigation
247 into the anthrax letters attack in 2001 in which this strain was used, a highly accurate library of

all the variants of this strain was developed. The genetic reference stock used in these investigations is relatively close to the original 1981 bovine isolate and should still be considered a clinical field isolate representative of wild-type strains with a limited number of laboratory passages separating it from a natural event. The Ames strain genome is 5,503,926 nucleotides in length with an estimated 5775 putative coding genes.[37,38] There are an additional 33 ribosomal genes arranged and 95 tRNA genes found within the chromosome. Together, these genes represent over 95% of the coding capacity of the entire genome. The two plasmids (pXO1 = 181,677 bp; pXO2 = 94,830 bp) house the remaining 5% of genes.

The defining phenotype of *B. anthracis* is the ability to make and excrete the poly-D-glutamic acid capsule and the anthrax toxin proteins. The bacterium produces these virulence factors immediately upon germination and throughout exponential growth on a number of media types. [40,41] Highest toxin and capsule production occurs at 37^0 C and requires dissolved bicarbonate in the medium.[39] These virulence factors are produced early upon germination and reach highest levels of synthesis at the transition from exponential growth to the stationary phase.[40,41] The necessity of bicarbonate for virulence factor production is well described.[40,42-44] Dissolved bicarbonate within the growth media induces a 60-fold increase in the toxin gene and *cap* operon expression.[45] Temperature is also critical for toxin gene expression. When *B. anthracis* is grown at 37 ^{0}C, it exhibits greater than 5-fold higher concentrations of toxin gene transcription than when grown at 28 ^{0}C.[44] Other classic laboratory phenotypes include; the lack of β-hemolysis on ovine blood agar, lack of phospholipase C activity, lack of motility, and sensitivity to penicillin and gamma bacteriophage.[46] There are however exceptions to these characteristics, including motile strains that appear to produce flagella. [47,48] Penicillin-resistant

isolates have been isolated from natural infections and have been produced in laboratory settings.[47,49-51] Resistance to penicillin is imparted by two structural genes for β-lactamase proteins.[38,52] These *bla* genes though present in prototypical strains are transcriptionally silent, but are constitutively expressed in penicillin-resistant isolates. [53,54]

Cell and Colony Morphology

B. anthracis cells viewed under the microscope have a very distinctive morphology. Vegetative bacilli are blunt-ended and approximately 1 by(check some references say 3-5) 5-8 um. The capsule is easily visualized with direct light microscopy using India ink exclusion.[40] *B. anthracis* typically grows as linked planktonic cells in liquid media, but can form more complex structures during static incubation.[55,56] During log phase growth, *B. anthracis* forms long serpentine chains. In infected tissues, however only single cells or short chains of 2-3 cells are observed.[57] When grown on solid media under conditions that support capsule formation, *B. anthracis* colonies appear mucoid due to the moisture-rich capsule, which can be more than 3 μm in thickness.[40] Rough or non-capsular colonies are said to look like "ground glass" and appear off-white to cream in color verticulated edges. When the vegetative cell is stressed, the bacterium is induced to sporulate. There are many established sporulation protocols. All are based on nutrient restriction and some form of temperature stress to induce sporulation.[58-62] Sporulating cells can be visualized with phase microscopy. The forespores form centrally or sub-terminally within the mother cell.[63] Other than a 1 – 1.5 μm refractile oval body, there is no other change in the mother cell during sporulation.

B. anthracis is a facultative anaerobe that is easily grown on a number of laboratory media and use a variety of energy sources. [55,64] Most metabolism studies have focused on the

relationship between nutrient utilization and toxin production. Therefore, the exact nutritional requirements of *B. anthracis* have only been defined by proxy and appear vary by strain. Methionine and thiamine are known to be necessary for fit growth. When *B. anthracis* is cultured in glucose-salts, medium supplementation with multiple amino acids is necessary. Under these conditions nitrogen appears to be a limiting factor forcing the bacterium to de-aminate the amino acids.[65] The optimal temperature for growth is 37^0 C or normal body temperature. Prolonged growth at 42^0 C clears the bacterium of one or both plasmids and renders the organism avirulent. Growth cannot be sustained at temperatures greater the 43^0 C. Cell doubling times on rich media ranges from 30-60 minutes at 37^0 C. [66-68]

The *Bacillus anthracis* spore

Bacillus anthracis exists in two distinct forms; the vegetative form and the spore form. Spores are considered to be the form most frequently encountered in the environment. When exposed to the low oxygen surroundings of the host the vegetative form predominates. The initiating step in the anthrax infection cycle is the entry of the spore into a susceptible host. Upon entry spores germinate and rapidly divide, reaching high numbers in effectively any body tissues. Death of the host and contact of the organisms to air induces spore formation in a proportion of the bacilli completing the infection cycle, whereupon the organism returns to the soil ready to be taken up by the next host. All *Bacillus* species depend on the spore for resistance to harsh environmental conditions. Because *B. anthracis* lives a dichotomous existence between host and environment it has been suggested that survival of the species unconditionally requires spore formation until the next host arrives.[69]

Spore Anatomy

The spore is a series of thin permeable layers enclosing the tougher impermeable core. The core protects the chromosome which exists in a dehydrated tightly folded state surrounded by proteins called small acid-soluble proteins (SASPs). [70,71] This complex of DNA and SASPs, along with the high calcium dipicolinic acid concentrations protects the chromosome for desiccations, heat and radiation. Very little metabolic activity occurs within the core. The spore maintains contact with the outside environment through passive diffusion of ions and small molecules into the core. Juxtaposed to the core is the spore membrane and surrounding it is a peptidoglycan cortex. Both function in restricting diffusion of molecules and in maintaining an appropriately desiccated level of moisture within the core.[72]

Surrounding the cortex is the lamellar protein coat.[73,74] Transmission electron microscopy reveals an appearance of ridges and valleys in the contour rather than a smooth sphere.[74-78] The majority of the spores environmental resistance arises from the protein coat which is accomplished by preventing penetration of large degradative molecules, small reactive molecules (such as gluteraldehyde) and predation by other microbes.[79-83] Despite this apparent robustness, the coat is flexible. As the core swells with water accumulation prior to germination, the ridges of the coat unfold to accommodate the increase in diameter.[84]

The outer most layer of the spore is known as the exosporium, which is present in several other *Bacillus* species.[85-87] The exosporium is composed of two layers; the basal layer and the nap layer. The basal layer is composed of a complex protienaceous shell surrounding the coat. The nap layer forms a series of hair-like extension from the basal layer. A small interspace separates the basal layer from the coat. The contents of the interspace include many enzymes whose independent function has not been fully defined. Some of these enzymes function in

maintaining the spore state, while others once activated by the germination signals function in degrading the cortex and coat.

In recent years the proteins of the exosporium have received considerable attention in hopes of identifying vaccine antigen candidates and ligands for spore detection.[88,89] BclA is a collogen-like glycoprotein that extends out from the nap layer. Attached to its C terminal domain is a separate polysaccarhide residue (known as antharose).[90-94] Together these compounds are the major immunodominant epitopes.[92,95] Thompson *et al.* demonstrated BclA is necessary for interaction with macrophage surface integrin CD11b/CD18.[96] Others have shown that neither BclA nor the exosporium are required for virulence when a large challenge dose is used.[91,93,97-99] In nature, where cutaneous and gastrointestinal infections are more common and the infectious dose is hypothesized to be lower than in laboratory animal infections, the necessity of BclA and the exosporium may be more important. Another collagen-like glycoprotein found in the exosporium is BclB. While not as well characterized as BclA, this protein is known to function as a scaffolding or structural protein during exosporium assembly as *bclB* mutant spores possess a fragile exopsorium.[100]

There are many undefined exosporium proteins in *B. anthracis.* The substantial variation in composition and structure of the exosporium between *Bacillus* species has led investigators to speculate that these distinctive structures contribute to the maintenance of niche populations within complex environments.[79,101] It is logical to speculate that the *B. anthracis* exosporium plays as significant a role in spore resistance and survival within the host, as it does in the environment. Currently, it is unclear which of the proteins making up the exosporium contributes to such a role or if there exist any specific adaption to host defenses.

Sporulation

In the natural setting the process of sporulation is initiated through exposure to highly aerobic environment and starvation of carbon, nitrogen or phosphorus.[99,102-106] Within infected hosts, the low oxygen environment coupled with warm temperatures and easily accessible nutrients permits the nearly exclusive presence of the vegetative form. In contrast, outside the host under hostile conditions the organism can only exist in the spore form. Spore formation (or sporulation) is an intricate synchronized process requiring up to 8 hours under optimal conditions to complete. [107]

The specific triggers for sporulation are have not been fully defined. However, it is known that the initiation of spore formation involves a signal transduction network that integrates information regarding nutrient concentration, cell cycle stage and presence or absence of secreted factors.[104,108] The mechanistic details of this process have largely been extrapolated from studies of *Bacillus subtilis* and are directly applicable to *B. anthracis*.

Seven sporulation stages are described based on their appearance under electron microscopy and analysis of genetic elements involved in the process. During the pre-divisional stage or stage 0 cells are morphologically indistinguishable from actively growing vegetative cells. Near the end of this stage, the nucleoid forms a bulky mass in the center of the cell in preparation for division. In stage I, the nucleoid extends from pole to pole to form the axial filament. At this point, two copies of the chromosome are present to ensure each compartment will inherit a full copy of the genome. Stage II begins with a polar septum dividing the sporangium into two compartments: the spore precursor or forespore and the mother cell. The genome is pulled into the forespore by translocation. A phagocytosis-like envelopment of the

forespore by the mother cell membrane begins stage III. When the engulfment is complete, the outside edges of the mother cell membrane join around the forespore which then pinches away from its polar attachment. At this point, the forespore is released and free within the mother cell cytoplasm. The exterior membrane that originated from the mother cell becomes the outer forespore membrane, while the inner membrane is termed the inner forespore membrane. Stages IV and V contain the synthesis of a modified peptidioglycan layer that will span the forespore membranes and will later become the spore cortex. During stage VI, the complex structure of the spore coat is assembled from more than fifty different proteins assembled by the mother cell. Finally, in stage VII the spore is released following degradation of the mother cell.

The Ecology of *B. anthracis*

There is little well documented evidence for how long *B. anthracis* spores can persist. Jacotot and Viratt demonstrated that anthrax spores grown by Pasteur in 1888 were viable in 1954.[109] Wilson and Russell recover spores that had persisted in dry soil for over 60 years. [110] The longest survival claim to date occurred when spores were recovered from bones carbon-dated at 200 ± 50 years found in an archeological site Kruger National Park, South Africa.[111]

The environmental conditions that appear to favor persistence of spore include dryness, alkaline soil pH and calcium content of the soil. Factors that discourage germination, such as lack of available nutrients and germination determinants appear to be equally important. Even though *B. anthracis* spores are capable of extreme persistence, investigators report that upon germination of such isolates the organism frequently has been cured of one of the virulence plasmids, usually pXO2.[112] Turnbull *et al.* suggested that in the desiccated state the plasmids may not be as well protected as the cell genome.[112] This arrangement may in fact provide an

evolutionary advantage for an obligate pathogen like *B. anthracis* in that the virulence genes are readily accessible upon germination and the vegetative bacilli can rapidly induce pathogenesis.

The influence of climate, rainfall, temperature, soil, vegetation, population density, and host condition on the epidemiology and incidence of anthrax has been thoroughly examined, but few meaningful patterns can be drawn about each condition. Nevertheless, important overarching themes can be identified. It is a consistent observation that anthrax occurs in the hottest times of the year and is especially prevalent in arid climates. Drought conditions first create changes in potential host behavior and susceptibility. For example, herbivores may come in contact with more spores by grazing closer to the ground in periods of sparse forage or by increasing time spent near water where spores also tend to concentrate. An indirect effect of drought is through increased host susceptibility associated with poorer general health and resistance to infection.[113,114]

An association between spore persistence and alkaline soil pH was first made by Higgins in 1916.[115] Van Ness and Stein observed patterns of livestock outbreaks in the US and concluded that endemicity was correlated with soils of pH greater than 6.0 and ambient temperatures above 15.5 ^{0} C.[116] Overlaying a map of the spatial distribution of the soil types found in North America with soils possessing these characteristics confirms that the majority of naturally occurring anthrax infections in the United States occur in the mollisol and aridisol soils.[117] These soil types, particularly chernozan and kastanozem, which have a high pH and are rich in organic matter are found in other anthrax endemic area such as the steppes of Russia and Central Asia.[118-120] In South America the highest incidence of naturally occurring outbreaks are found in intensively grazed areas of Argentina associated with the related phaezoam soils. In Kruger

National Park, South Africa areas with a pH greater than 7.0 and calcium concretions exceeding 15 millequivalents have been associated with anthrax death rates in wildlife seven times higher than in areas with lower values. [121]

Topography greatly influences *B. anthracis* spore survival in areas that are largely lacking favorable soil conditions. An example is in areas of South Texas, shallow depressions or pot holes collect greater amounts of water than the surrounding acidic, sandy-loam and gradually accumulate humus and minerals eventually resulting in a suitable locus for *B. anthracis*.[122] Such places participate in the ecology of *B. anthracis* infection in other ways as well. Because of the greater soil moisture and richness these areas harbor more forage and water longer between rains, thus attracting herbivores and providing exposure to infectious doses. A similar phenomenon has been reported in the bar pits along the Mississippi river delta from which soil was excavated to construct the levees lining the river.[123] The excavation of the soil provided shallow depressions that create similar favorable microcosms for *B. anthracis*. These areas have come to be known as "incubator areas" of anthrax.

Water appears to be a necessary element in the movement of spores. *B. anthracis* spores have a high surface hydrophobicity that results in a high level of buoyancy and permits flotation of the spores during times of heavy rain.[124] Historical examples exist in the United Kingdom where watercourses were implicated in the dissemination of *B. anthracis* spores from tanneries where contaminated effluent was released.[125] Drought ending rains have been implicated in the rise in anthrax infections for similar reasons. This is particularly evident in the annual incidence of anthrax in the wildlife population following monsoon rains in Kruger National Park, South Africa. [126]

Germination

Germination is the return of dormant spores to vegetative metabolically active cells. This process is triggered by multiple germinants including, amino acids, ribonucleosides, and/or peptidoglycan fragments. These compounds are detected by receptors located on the surface of the spore. High concentrations of germinants initiates a mutable cascade of events beginning with the influx of water and rehydration of the core, followed by the disassembling of the cortex and coat. Rehydration is succeeded closely by resumption of metabolic activity and outgrowth of the vegetative cell. There are five independent pathways that *B. anthracis* uses to accomplish germination each associated with different amino acids at varying concentrations. These five pathways are the alanine pathway, the alanine-proline pathway, the aromatic pathway, and two amino acid and inosine dependent pathways (AAID). The AAID pathways depend on the nucleoside inosine in combination with L-alanine, L-proline, L-serine, L-valine, or L-methionine (AAID-1) or inosine combined with any of the aromatic amino acids (AAID-2).[127,128]

L-alanine is among the most potent of germinants for *B. anthracis.* Tight control of this process is critical for the bacterium to ensure germination occurs only in nutrient rich and favorable environments. One key regulatory element is an enzyme called alanine racemace which is found attached to the exosporium. This enzyme converts the active L-alanine to the inactive D-alanine and at low amino acid concentrations prevents premature germination. When environmental L-alanine reaches high concentrations, the racemace enzyme is overwhelmed allowing active L-alanine to penetrate the core and initiate germinations.[129]

The genetic regulation of germination in *B. anthracis* is mediated through the *gerA* family of genes. This regulon contains 6 genes, 5 chromosomal genes and 1, *gerX*, located within

a pathogenecity island on pXO1.[130] Each of the gene products are responsible for sensing one or more of the specific amino acid germinant signals and each appear to be necessary. Disruption of any one gene abolishes the ability to germinate. Null mutation of *gerX* decreases germination efficiency as well as reduces virulence compared to the parent strain.[126]

Pathogenesis

The pathogenesis of *Bacillus anthracis* is entirely responsible for genes found on each of the virulence plasmids and located within four pathogenicity islands. The most pathogenic virulence factors are a poly-D-glutamic acid capsule and two binary toxins called lethal toxin or edema toxin. The toxins are composed of three independent subunits a common transport protein known as protective antigen (PA) and two effector molecules known as lethal factor (LF) and edema factor (EF). Absence of either of these plasmids attenuates the virulence of the bacterium. Curing the bacterium of pXO2 which houses the capsule genes increases the LD_{50} by 6 fold compared to fully virulent strains in mice.[131] One of the most prevalent toxigenic acapsular ($pXO1^+$, $pXO2^-$) strains is known as the Sterne strain and has been used extensively as a live attenuated vaccine in livestock and humans.

Lethal and Edema Toxins

The symptoms associated with anthrax infection are principally the result of toxin production. The genes that encode for each of the toxins are housed on the pXO1 plasmid. The larger of the two plasmids at 181 kb, this plasmid encodes approximately 143 open reading frames many of which the functions are not yet known. All three toxin genes, *pagA*, *lef,* and *cya,* are arranged in a single operon within a 44 kb pathogenicity island flanked by two insertion elements. Though the plasmid shares a similar G/C content with the bacterial chromosome, this

island does not share the same conformity and appears to be the result of a more recent horizontal gene transfer.[132, 133] The plasmids have also been found in the rare isolates of *B. cereus* that demonstrate a similar virulence and clinical manifestation as *B. anthracis*, but it is not clear if these elements have been recently acquired by these strains or if these few isolates are remnants of the parent organism prior to the diversion of *B.anthracis* and *B.cereus*.[31]

The tri-partite toxin is classified as an A-B type toxin. The protective antigen protein is encoded by the gene *pagA*, whose transcription is enhanced by *atxA* and suppressed by *pagR*. Protective antigen serves as the B subunit for both toxins and is responsible for cell surface receptor binding and translocation of the pathogenic A subunits, LF and EF. Protective antigen is a cell-free secreted protein that has a molecular mass of 83kDa and is arranged into four domains of mostly anti-parallel β-pleated sheets.[134]

The host receptors for PA are tumor endothelial marker 8 (ANTXR1) and capillary morphogenesis protein 2 (ANTXR2).[135] These are surface associated proteins with unknown host targets but, each contains a von Wildebrand factor A domain suggesting participation in coagulation.[136] X-ray crystalography of bound PA confirms that domain 4 is responsible for mediating the interaction with these receptors.[134] Once PA binds to these receptors, a furin or furin-like protease cleaves an exposed site at residues 164-167 within the 83 kDa precursor protein.[137] This releases a 20 kDa fragment from the N-terminal end, while the 63 kDa C-terminal end remains adhered to the receptor. Complex interactions between phospholipid rafts in the cell membrane and the interaction of multiple receptors allow bound PA to polymerize into a heptameric pre-pore structure.[138] Once a heptamere is formed EF and LF can competitively bind an exposed binding site on PA through a conserved domain on the amino

terminal of each of the proteins. The congregation of multiple receptor molecules induces a clathrin-dependent endocytosis of each of the heptamere-toxin complexes.[139] When the endocytic vesicle fuses with a cytoplasmic lysosome, a decrease in the pH occurs that leads to a change in the conformation of the heptamer. This allows the pre-pore to insert into the membrane of endolysosome and act as a β-barrel pore through which EF and LF are released into the cell cytoplasm. Initially, it was thought that seven effector molecules could be translocated with each heptamer. Recent studies have demonstrated that due to steric interference only a hypothetical maximum of three subunits can bind the heptamer. When crystallography of the structure was attempted only a single molecule of EF or LF was observed bound to each heptamer.[140]

Edema Factor

Edema factor is a secreted as inactive adenylate cyclase during active bacterial growth.[141,142,143] Similar to the adenylate cyclase in structure and activity to the adenylate cyclase of *Bordetella pertussis*, activation of the enzymatic activity of EF is dependent upon interaction with calcium ions and the host molecule calmodulin.[142] After entry in to the cell, the native EF is bound by calmodulin which induces a 30^0 rotation of a helical domain and allows the protein to then bind a single calcium ion and a single molecule of 3'dATP. This exposes and activates the catalytic site of the enzyme.[144] Once activated, EF converts the bound 3'dATP into cyclic adenosine monophosphate (cAMP). Cyclic adenosine monophosphate is an ubiquitous secondary cell messenger and participates in the regulation of many cellular processes. The enzyme attacks the host cell's ATP stores rapidly consuming the cells energy currency. Within minutes EF converts up to half of the cell's ATP to a concentration of cAMP 10^3 times the normal concentration.[142] The elevated cAMP levels trigger the loss of water and ions from the cell,

causing the accumulation of fluids into surrounding tissues and the characteristic edema found in anthrax infection. Though the extent of cytotoxicity attributed to EF is debated, the enzyme also contributes to virulence in other ways. Purified edema toxin is a potent inhibitor of neutrophil chemotaxis and directly reduces phagocytic ability and oxidative burst.[145, 146] In addition, EF can modulate important pro-inflammatory cytokines and interfere with the innate immune response.[147, 148] Edema toxin was shown to be lethal in BALB/c mice at very low doses.[149] Post mortem analysis revealed accumulation of fluid in several tissues including the gastrointestinal tract, adrenal glands, secondary lymphoid organs, myocardium, and kidneys. The final outcome of intoxication was multi-organ failure.[152] The increased concentration of cAMP in cardiac pacemaker cells have also been implicated with bradycardia and cardiac failure.[150] Lovchik *et al.* generated an isogenic mutant of the Ames strain that expressed only edema toxin but was equally as lethal as the wild-type and lethal toxin expressing strains in a rabbit challenge model.[151]

Lethal Factor

Lethal factor is a zinc-metalloprotease capable of cleaving and inactivating many cellular targets. The most important target for LF is cleavage and inactivation of mitogen activated protein kinase kinase (MAPKKs).[152] The *lef* gene is housed on the pXO1 plasmid and similar to EF its expression is regulated through the *atxA* regulon. The majority of the cytotoxicity observed in anthrax infections has been attributed to lethal toxin. Murine macrophages exposed to lethal toxin undergo apoptosis and show rapid cell lysis.[153] Mortality in mice and rats can be induced within as little as 60 minutes after administration of lethal toxin.[153-155] *B. anthracis* deficient in the *lef* gene have been shown to have a greater the 10^3 increase in the LD_{50} relative

to wild-type strains, whereas disruption of the *cya* gene resulted in only a 10-fold increase in the the LD_{50} in mice. [155, 163]

Macrophages are the primary target of lethal toxin which results in specific and drastic effects. Although other cell types are affected by lethal toxin, only macrophages are lysed by the toxin. Lethal toxin administration to transgenic mice lacking macrophages suffered much less pathology than outbred mice highlighting the central role macrophages play in *B. anthracis* pathogenecity.[153] Specifically, LF targets several of the macrophages' MAPKKs including; MEK1, MEK2, MEK3, MEK4, MEK6, and MEK7.[156,157] Reduction or alteration in these molecules occurs in some continuous macrophage cell lines. These alterations confer *in vitro* resistance to lethal toxin, but have not been correlated with survivability and reduced morbidity *in vivo.*[164, 158] The discordant results suggest that macrophages must first be sensitized or activated before suffering the full cytoxicity caused by LF. Exposure of resistant macrophage cell lines to TNF-α and calyculin A appear to induce sensitivity and result in increased cytotoxicity following exposure to lethal toxin.[166, 159]

Lethal toxin functions to suppress the host innate and adaptive immune response and disrupt cell signaling pathways. Interference with the MAPKK signaling pathways disrupts cytokine production, macrophage activation, neutrophil response, and barrier integrity of lung epithelial cells.[160, 161] Many immune cell types are affected by lethal toxin under *in vitro* conditions. Lethal toxin exposure to dendritic cells reduced TNF-α and IL-10 production.[148] CD4+ lymphocytes exhibited reduced IL-2 secretion and auto-activation following lethal toxin exposure.[162] The apoptotic effects of LF have been attributed to activation of p38 MAP kinase.[163]

Systemically, lethal toxin leads to hypovolemic shock, hypoxia and multi-organ dysfunction and failure. The damage to vascular endothelium leads to systemic vasculitis and leakage from capillary beds. This is believed to be the source of the characteristic pleural and peritoneal effusion compound by severe necrosis of the spleen and liver. [149, 164] Lethal toxin damages endothelial barriers through modification of central actin fibers which alters the shape and integrity of the cells.[165] When combined in a rat constant rat infusion model both toxins produced synergistic virulence leading to circulatory collapse and death. [166]

Capsule and S-Layer

The capsule of *B. anthracis* is composed of repeating polymers of D-glutamic acid. It represents the outermost layer of vegetative cells. The simplicity of repeating D-glutamic acid residues makes the capsule poorly immunogenic.[167] Additionally, the length and structure of the extended glycoproteins serves to cover other antigens on the cell surface and makes the bacterium difficult to phagocytize.[167] These attributes permit successful immune evasion and persistence. The capsule operon consists of five genes (*capA, capB. capC, capD* and *capE*) found on the pXO2 plasmid.[25,40-42,168] CapD is a glutamyl transpeptidase responsible for attaching the capsule to the bacterial membrane. The remaining genes encode for the capsule synthesis proteins.[41] Capsule synthesis is regulated by bicarbonate concentration and *atxA,* as well as two other independent regulatory genes, *acpA* and *acpB.*[40,41,44,45,168-171]

The capsule is necessary for virulence in inhalational challenges of mice by equipping the pathogen with anti-phagocytic attributes.[41] Jang *et al.*. demonstrated that administration of capsule fragments enhanced the cytotoxicity of lethal toxin in J774A.1 mouse macrophages.[172] Loss of the pXO2 plasmid can be induced naturally or experimentally and significantly reduces

the virulence of the strain.[138,173] Recently, the capsule synthesis operon was shown to be essential for trafficking of *B. anthracis* to regional lymph nodes during infection.[174] Strains of *B. anthracis* lacking pXO2, most notably *B. anthracis* Sterne strain, have been used extensively in vaccine production. [175]The capsule itself has been investigated as a vaccine candidate with limited success.[176-178]

The S-layer is a protein layer located between the outer capsule and the peptidoglycan cell wall. This thin layer is made up of two proteins known as extractable antigen 1 (Ea-1) and surface array protein (Sap).[179] Each protein is produced independently and therefore the composition of the S-layer changes depending upon the growth phase of the bacterium. The Sap protein is assembled first upon germination and outgrowth. During the stationary phase Sap proteins are slowly replaced by Ea-1 proteins. As the interface between the capsule and bacterial membrane the S-layer plays a role in maintaining cell shape, inhibition of complement mediated cytolysis and subterfuge of host macrophages.[143] It does not appear to be necessary for virulence as its removal does not affect the LD_{50} in mice following virulent challenge.[180] Because of the interaction with macrophages upon phagocytosis of the bacterium and mediation of resistance to complement defenses, it still may remain important to the course of disease in anthrax infections.[143] The S-layer has been demonstrated to be recognized by the immune system and therefore may have potential as a vaccine candidate.[181, 182]

Virulence Regulation

Virulence expression is regulated by a *trans*-acting global regulator called AtxA (for *A*nthrax *tox*in *a*ctivator) that is encoded on the pXO1 plasmid. The AtxA protein is a global regulator for *B. anthracis* and is involved with regulation of over 70 genes located in the

chromosome and the plasmids.[171,183-185] Null *atxA* mutants display significantly lower transcription of each of the toxin genes and is highly attenuated in murine infection models.[186-188] The precise mechanism by which AtxA exerts its regulation is not clear and there are no reports of direct action with any of its target genes. Direct interaction with DNA appears possible as AtxA possesses several putative DNA binding motifs.[189] Other important structural features of AtxA include two cenrally located phosphotransferase system regulation domains (PRD).[190] The putative function of PRDs is associated with the uptake of specific sugar molecules suggesting that activation of AtxA and virulence expression is linked with carbohydrate availability and resource awareness.

Investigations by Dale *et al.* suggest that steady-state levels of AtxA are necessary for transcription of all three toxin genes.[191] AtxA activates transcription of *pagA* (gene encoding PA) and its repressor *pagR.* PagR in turn provides suppression of *atxA* transcription thereby controlling toxin synthesis through a negative feedback loop.[184] PagR also represses the *sap* gene, while activating the *eag* gene.[192,193] This effect essentially alters the composition of the bacterial surface in favor of Ea-1 surface proteins. AtxA induces capsule synthesis through activation of *acpA* and *acpB.*[40]

Bicarbonate ion concentration also appears to play a critical role in the induction of virulence genes in *B. anthracis*. The optimal bicarbonate ion concentration for toxin expression mirrors the concentration that is found in the bloodstream of mammals which is 48 mM. Though increased levels of bicarbonate ions has been shown to increase expression of *atxA* the regulatory effects of bicarbonate ion on toxin and capsule synthesis appear to be independent of AtxA.[194] In strains cured of pXO1, elevated CO_2 concentration resulted in increased levels of transcription of

the capsule regulatory genes *acpA* and *acpB* to levels observed in wild-type expression.[195] By coupling virulence production to bicarbonate ion concentration, *B. anthracis* limits costly production of its virulence factors until it is necessary in order to survive and thrive in the host.

B. anthracis produces lethal and edema toxin constitutively during vegetative growth, but expression is not consistent at all stages of growth. Toxin production and secretion is highest during exponential growth and in early stationary phase.[196] This is in part due to the actions of two regulatory genes, *abrB* and *spo0A. ArbB* is a transition state regulatory gene that provides constant negative regulation of *atxA* throughout the growth cycle. As the vegetative cells approach late logarithmic growth and begin to sense nutrient depravation the *spo0A* genes are activated.[197] Activation of *spo0A* acts as a repressor of *abrB,* leading to a burst of virulence expression potentially as the host suffers from severe systemic pathology and near the end of the infection phase.[150]

Other Virulence Factors

The plasmids of *B. anthracis* encode for a number of other more recently identified virulence factors. When grown under strict anaerobic conditions, *B. anthracis* can produce the anthrolysins.[198] The anthrolysins are secreted hemolysins that appear to be evolutionary remnants of *Bacillus* virulence factors as homologs of these enzymes have been identified in *Bacillus cereus* and *Bacillus thuringiensis.*[199] They consist of four proteins, Anthrolysin O and three phospholipase C molecules. Expression of the anthrolysins begins early in infection and they are induced soon after phagocytosis.[198] In fact, mutations in the bacterial phospholipase C genes impair the persistence of spores when co-cultured with mouse macrophages.[198] The full catalog

of anthrolysin susceptible cells is not known but an effect on human peripheral blood cells was shown *in vitro*.[200]

Due to its rapid growth during infection and to a partially intracellular phase within macrophages, *B. anthracis* highly depends on two well-defined siderophores for continual uptake of iron.[201,202] Each of these is homologous to siderophores that have been identified in other *Bacillus* sp. Other virulence factors include putative collagen binding adhesins located on the surface of the actively dividing cells.[203] These proteins serve two functions, to aid in adherence to the interstitial matrix and help protect them from attack from the host immune system. *B. anthracis* like other Gram positive organisms possess an elegant mechanism for altering the negative charge on many of its surface macromolecules. The products of the *dltABCD* operon function to covalently attach a positively charged D-alanine to lipoteichoic acid. This alteration in surface charge affords greater defense against lysozyme and β-defensin-1 and other leukocyte antibacterial molecules.[204] Altering the charge on the bacterial cell surface imparts resistance to positively charged antibiotics as well.[204]

Diseases caused by *Bacillus anthracis*

B. anthracis is the causative agent of anthrax. The disease occurs in all vertebrates, but cattle and sheep are most susceptible. Carnivores are relatively resistant to anthrax and humans occupy an intermediate position between herbivores and carnivores. An interesting example of natural resistance occurs in dwarf pigs.[205] In these animals, *B. anthracis* spores do not germinate and are cleared within 24 hours of exposure. The majority of information and data that is known about the pathogenesis and course of disease has been gleaned from laboratory animals and human case reports. Although not every animal model perfectly correlates with the disease as

seen in humans, for the most part it is assumed that the behavior of *B. anthracis* and the pathology of anthrax is the same between humans and animals.

Anthrax in Animals

Naturally occurring anthrax is by and large a disease of herbivores that become exposed to *B. anthracis* spores while grazing low forage over contaminated soils. Other sources of transmission include fodder grown on contaminated soils, bone meal, protein concentrates, and blood, discharge or excreta from other infected animals. Spread of the organism can be accomplished by water, insects, or scavengers. Insects have been demonstrated to transmit *B. anthracis* experimentally, but natural transmission through vectors has not been documented.[206] An exception to this is in tabanid flies where transmission is mechanical only.[207] These infections have resulted in local or cutaneous anthrax.

Ruminants

In cattle only two forms of anthrax occur, the peracute and the acute. Peracute anthrax is characterized by sudden death without premonitory signs. The course of the disease is probably on a few hours. If clinical signs are observed, they are non-specific in nature and include fever, dyspnea, and muscle tremors. Following death, frank blood is discharged from external orifices. The acute from of the disease is characterized by initial clinical signs of fever, severe depression, occasionally a brief period of nervousness, followed by colic, dyspnea, tachycardia, congested or hemorrhagic mucous membranes, anorexia, diarrhea and dysentery.[208] The course of disease normally lasts less than 48 hours. A subacute form has been described in cattle in tropical areas that results following infection of oropharyngeal wounds and involves clinical signs of

submandibular edema. In each case septicemia with massive invasion of all tissues and overwhelming toxemia is the cause of death. Pregnant animals may abort. In these cases, *B. anthracis* can be isolated from fetal tissue. *B. anthracis* does not display any selective tropism for reproductive tissue, more likely extension of the infection to the reproductive tract occurs along with the massive tissue invasion seen in other organ systems.

Horses

Anthrax in horses is always acute and the most common manifestation of disease centers on the intestinal tract. Following germination of *B. anthracis* spores in the intestinal tract, severe enteritis and colitis precede septicemia and result in clinical signs such as colic, diarrhea, fever, and depression. The course is usually less than 96 hours from exposure to death. A localized form has been observed in association with tabanid flies which serve as mechanical vectors. In these cases infection is peripheral and results in painful, subcutaneous edema. Dependent edema can develop in the ventral thorax, abdomen and limbs. These cutaneous lesions are seen most frequently in the neck region.

Pigs

Oropharyengeal involvement with swelling of the head and the head and neck is the most common presentation in swine.[209] In more severe cases, there is fever, depression, and anorexia. Petechial hemorrhages may be present in the skin and mucosa. If these disease extends to caudal portions of the gastrointestinal tract animals may present with dysentery. A pulmonary form of the disease has been observed in neonatal pigs following inhalation of contaminated dust. Lobar pneumonia and exudative pleuritis was characteristic in the single report.

Companion Animals

The morbidity and mortality in dogs and cats is much less than that seen in other domestic species. Various reports indicate that dogs and cats may not develop infection or remain asymptomatic following exposure to *B. anthracis.* Naturally occurring anthrax in free-roaming, captive, or domestics canids or felids is most commonly gastrointestinal in origin, the result of ingestion of meat from infected carcasses. Clinical presentation includes fever, depression, and anorexia as well as local signs of inflammation, edema, and necrosis in the upper gastrointestinal tract. Severe gastroenteritis can spread to local lymphatic organs and lead to fulminant septicemia and death. Experimental inhalational exposure in dogs did not reliably produce clinical disease other than short-term fever and anorexia.[210]

Anthrax in Humans

Cutaneous Anthrax

Cutaneous anthrax accounts for an estimated 95% of all naturally acquired human cases with an estimated annual occurrence of 2000 cases worldwide.[211] There were 224 cases in the US during a 50 year time period from 1944 to 1994.[212] *B. anthracis* is not invasive and cutaneous anthrax only develops when spores passively enter breaks in the skin.[213,214] Areas of skin that are most exposed such as the head, neck, and arms are at greater risk of infection. Once the spores breach the skin, clinical signs commonly develop within 72 hours however in a few cases onset of clinical symptoms was extended beyond 14 days. Prolonged latency of spores does not appear to occur. Germination and toxin production is rapidly initiated once the spore gains access to the subcutaneous tissue.

The first clinical symptom is local edema around the site of infection within 24 hours of germination of the spores. Small, pruritic macules or papules develop shortly following these signs and within 48 hours these lesions enlarge, ulcerate and begin to discharge clear to serosanginuos fluid. Gram stain of the discharge reveals teems of *B. anthracis* vegetative cells. Following ulceration, the lesion desiccates and develops into an eschar. Extensive local and regional edema may persist, but pain and the presence of purulent discharge is absent. By 7-10 days the eschar becomes thick and adherent and eventually loosens and falls away. In many cases, the organisms remain localized to the original lesions and the infection is self-limiting. The development of severe local edema permits systemic extension of the infection via lymphatic circulation. [215] In untreated cases, mortality rate may reach up to 20%.[216]

Gastrointestinal Anthrax

Gastrointestinal (GI) anthrax results from the ingestion of spores which then germinate in the upper and lower intestinal tract. Humans most commonly contract gastrointestinal anthrax after consumption of contaminated undercooked meat. The WHO estimates that 1 case of gastrointestinal anthrax occurs annually for every 30-60 anthrax-infected carcasses consumed.[217] In light of the rapid transit time of the human gastrointestinal tract, some have hypothesized that *B. anthracis* spores would not have sufficient time in which to germinate before elimination from the body. However another hypothesis may be that the apparent infectious dose for GI anthrax is higher than was previously regarded.

There are many attributes of GI anthrax that are poorly understood compared to the inhalational and cutaneous forms of the disease. The incidence of the disease is unknown due to under reporting in the rural areas where it is more prevalent. Large human epidemics have been recorded in Africa and Asia and have been linked to concurrent widespread livestock epidemics and consumption of contaminated meat.[18,211,218] Little information is available about the risk of others source of transmission such as contamination of water or other food sources.

Consumed *B. anthracis* vegetative cells can create more proximal lesions in the oral cavity, causing the oral-pharyengeal form of the disease. Spores more frequently induce disease after germination further distally the intestinal tract. Based on extroplated data from challenge trials in non-human primates the infectious dose is estimated to be at least 10^8 spores. [219] Oral-pharyngeal infections manifest as ulcers in the mouth and proximal esophagus. Later manifestations include lymphadenopathy and edema of the head and neck. Some of these infections will progress to systemic infection and sepsis without intervention.[18,220] Few reports

documenting oral-pharyngeal anthrax exist. One case study involving twenty-four cases occurred in Thailand in 1983.[220] Oral lesions were located on the tonsils in 72% of these cases, 85% of these were unilateral. The evolution of these lesions began with early edematous and congested lesions that developed central necrotic areas containing a whitish plaque or pseudomembrane in 7-14 days. Neck swelling was present in all cases, and submandibular lymph nodes measuring up to 4 cm were noted. Airway obstruction was the only complicating sequelae of infection. The incubation period was approximately 42 hours. The mean interval from the onset of clinical symptoms was 3.5 days and the average hospital stay was 7 days. The estimated mortality rate was 12.5% during this epidemic.

The intestinal form results from *B. anthracis* infection of the mucosa of the small and large intestine.[221,222] In a point source epidemic in Uganda, 155 villagers fell ill after consuming meat from a zebu that was infected with anthrax. Within 15-72 hours, 143 (92%) of the affected people developed anthrax. Of these, 91 % had gastrointestinal complaints, 9% oropharyngeal edema. Nine fatalities resulted from this outbreak, all were children.[223] The mean time to death in these children was 48 hours after onset of clinical symptoms. The primary lesion of intestinal anthrax is ulceration of the intestinal mucosa, principally the ileum and cecum.[224] Initial symptoms include a vague influenza like presentation of nausea, anorexia, vomiting and fever. This progresses within 12 – 24 hours to severe abdominal pain, hematemesis, melena and/or bloody diarrhea.[223] The abdominal pain may be so severe that the patient presents with signs of acute abdomen.[225] The pain may resolve as ascites of large volume develops.

Imaging techniques such abdominal radiographs or computed tomography provides only non-descriptive findings including gaseous distention and evidence of ascites. Exploratory

surgery may reveal mesenteric adenopathy, serosanguinous to hemorrhagic ascites, and mucosal ulceration, edema and necrosis.[226-229] Advanced infections rapidly spread systemically resulting in septicemia and toxic shock. *B. anthracis* has been isolated in the feces of infected individuals, but is too inconsistent to serve as an effective means of diagnosis.[230] A higher diagnostic sensitivity can be obtained from samples of peritoneal fluid and blood for culture.[231]

Inhalational Anthrax

Inhalational anthrax develops following the deposition of *B. anthracis* spores into the alveolar space. It is the rarest form of naturally occurring anthrax, yet is associated with the highest mortality rate. Because of the dynamics of air flow into the respiratory tract spores must be within 1.5 -5 um in order to reach the lower respiratory tract. Spores greater than 5 um are trapped in the upper respiratory tract and effectively cleared by the mucocilliary apparatus. Smaller spores never leave suspension and easily exhaled out of the respiratory tract. The most common source of natural transmission is via contact with infected hides, pelts or fleece. A recent incident of naturally occurring inhalational anthrax occurred in New York where a man became exposed through working with untreated hides used for making drums .[232] Prior to the 2001 attacks, a total of 18 cases of inhalational anthrax had been reported in the United States since 1901.[16] The United States Department of Defense estimates the LD_{50} dose is between 8,000-10,000 spores.[233]

The incubation period from germination to the onset of symptoms lasts up to 6 days. The first stages manifest with non-specific symptoms such as fever, malaise, shortness of breath, chest pain and nausea for an average of 4 days. Often there is a transient improvement in

symptomology lasting less than 24 hours before the second phase begins. Some cases progress from the first phase directly into fulminant disease.

Massive vegetative proliferation precedes the second phase of inhalational anthrax. An explosion of bacterial replication and toxin production overwhelms local innate defenses in the tracheobronchial and mediastinal lymph nodes and leads to lymphadenopathy, edema, hemorrhage and necrosis. The second phase is reported to begin abruptly in most cases with sudden fever, diaphoresis, and dyspnea. The most dramatic development in this phase is the massive hemorrhagic mediastinitis. Damaged endothelium within mediastinal vessels permits the sudden efflux of hemorrhage and pleural effusion into the mediastinal space. Thoracic imaging can be helpful in early diagnosis if the attending physician is aware of the characteristic mediastinal widening in cases of inhalational anthrax. All 11 patients from 2001 attacks had abnormal thoracic radiographs: 7 individuals displayed widening of the mediastinum widening; 7 had evidence of pulmonary cellular infiltrates; and 8 had pleural effusion. The widening of the mediastinum and lymphadenopathy resulted in remarkable stridor in a few cases.[234,235] Respiration is profoundly affected late in the progression of disease. Cardiovascular collapse leads to shock and death within hours of the onset of fulminant disease, in some cases regardless of prompt appropriate treatment.[216,233,236]

Molecular Events during Progression of Systemic Anthrax

Macrophages play a critical role in systemic anthrax infection by serving as the primary site of spore germination. Germination of spores within phagocytic vesicles can take several hours to complete.[237] In that time frame, the activated macrophages have begun to home toward regional lymph centers.[238] This is the basis for the popular theory of systemic dissemination of *B.*

anthracis using macrophages as "Trojan Horses" to deliver the pathogen to centralized lymph tissue.[239] In addition, throughout infection macrophages are the primary target of edema and lethal toxin. By targeting macrophages, *B. anthracis* indirectly manipulates the host immune system and creates an environment that favors its survival. The early events of systemic anthrax infection have been best characterized for inhalational anthrax, particularly in the context of the interaction between *B. anthracis* and the pulmonary macrophage.[240] As result inhalational anthrax provides a model for the progression of the disease from which overarching themes have been applied to the other forms of the disease.

Once in the alveoli, spores are phagocytized by regional innate immune cells and transported to lymph nodes.[241] Alveolar macrophages are the innate immune effector cell in the lungs.[242] Dendritic cells play an important role in the early uptake and have been shown to be more efficient in phagocytosis of spores but because of fewer cell numbers may not contribute greatly to dissemination.[207] Fibroblasts and epithelial cells have been shown *in vitro* to participate in the phagocytosis of *B. anthracis* spores but their contribution to the dissemination and the pathophysiology of the disease is not known.[243,244] Despite the permissibility of these other cell types, it appears alveolar macrophages are the principal location for spore germination.[237,245-247] Spores germinate in the phago-lysosome while in transport from the lungs to mediastinal lymph nodes.[239] Both the lung dendritic cell and the pulmonary macrophage demonstrate sporicidal activity *in vitro*, which likely accounts for a 10-fold reduction in viable colony forming units within the first 2.5 hours following challenge of the cells. The roughly 10% of spores that do persist then rapidly multiply and overwhelm the macrophage eventually

872 inducing cell death. Based on more recent *in vivo* imaging studies, there appears to be limited
873 germination of cells within the parenchyma of the lung.[248]

874 Both the vegetative cell and spore of *B. anthracis* are capable of stimulating a strong
875 innate inflammatory response. Many motifs in both phases are recognized by host pattern
876 recognition receptors. The components of the cell wall including lipotechoic acid and techoic
877 acid stimulate TLR2 receptors.[249] Anthrolysin O stimulates TLR4 receptors.[249] Upon
878 phagocytosis of spores activated pulmonary macrophages and dendritic cells release several pro-
879 inflammatory cytokines.[247,257] In A/J strain mice as little as 100 spores by intranasal challenge
880 elicited a potent IL-1b response.[240] IL-1b and TNF-a recruit and activate phagocytes and promote
881 clearance of un-germinated spores from the alveoli.[250] Mutation of the NF-kappa B gene, master
882 regulator for cytokine expression significantly increased the susceptibility of mouse macrophage
883 cells.[251,252] In spite of this strong response during systemic infection, fully virulent *B. anthracis*
884 quickly begins to counter host defenses as soon as germination id complete and the production of
885 virulence factors begins.

886 Some vegetative cells will escape the lysosome and be into circulation or into the
887 mediastinal lymph nodes.[239,253] Once the vegetative cells begin actively dividing *B, anthracis*
888 begins to express the capsule and toxin proteins. Messenger RNA transcripts for PA, LF and EF
889 appear within 1 hour of infection.[254] This equates with approximately the time period when spore-
890 laden macrophages arrive in regional lymph nodes in the guinea pigs.[253,255] A single putative 'escape'
891 gene located on the pXO1 plasmid appears to be necessary for lysis and release of the organism from
892 the macrophage.[239]

Four hours after challenge vegetative cells and spores were found in the peri-bronchiolar and mediastinal lymph nodes in guinea pigs.[255] In non-human primates this process requires a longer period, up to 18 hours after infection before bacterial cells were isolated from the mediastinal lymph nodes.[255] Following lysis and release into the nodes the bacteria begin to rapidly divide and produce toxin. Though surrounded by activated immune cells the toxins limit any further immune activation.

Immunosuppression can be identified early in the infectious process in some species soon after initial phagocytosis. Lethal toxin abolishes the expression of pro-inflammatory cytokines in cultured macrophages and dendritic cells.[156,256-258] Lethal toxin can also activate cellular apoptosis through disruption of the p38 MAPK system.[163] Edema toxin works in conjunction with lethal toxin to suppress early cytokine responses of other professional phagocytic cells, principally neutrophils. The combination of toxins also inhibits neutrophil activation, chemotaxis, interferon production and the production of NO.[259] Administration of exogenous interferon abrogates the activation of apoptosis in macrophages and improves sporicidal activity.[259] Lethal toxin also targets phospholipase A2 in alveolar macrophages which is known to assist in destruction of spores.[260] Finally, lethal toxin interrupts differentiation of peripheral monocytes into activate tissue macrophages thereby reducing the number of effector cells and promoting bacterial persistence.[261]

In addition to suppression of the innate immune response, the toxins demonstrate suppressive effects on the adaptive immune response as well. Lethal toxin was demonstrated to reduce the expression and localization of co-receptors on antigen presenting cells leading to reduced lymphocyte activation and the development of tolerance.[258] Lethal and edema toxin together reduce T cell activation and proliferation by reducing IL-2 secretion.[166,262] Lethal toxin inhibits the humoral immune response by interfering with B cell activation and MKK expression and reducing IgM production.[258,263] Increased levels of cAMP via exposure to edema toxin forces maturation of T cells

toward a Th2 response, theoretically a less effective immune response against bacterial pathogens. [166,255,264]

This cycle of lysis and release, bacterial division, greater recruitment of immune cells into the lymph node and immune cell death leads to swelling of the node and escape of the pathogen into the bloodstream. *B. anthracis* also passively travels through the lymph to reach adjacent nodes. In the blood, the bacterium replicates unckecked resulting in severe bacteremia and toxemia. Not all immune cells are susceptible to toxin-induced cytopathology, but the capsule also defends the bacterium from phagocytosis and complement mediated lysis. [41,265]

The last stage of the disease is induced by the consequences of systemic toxemia and usually begins12-24 hours after vascular dissemination. Exposure of the toxins to cells of the cardiovascular system results in hemorrhage, clotting disorders, edema and ultimately multi-organ dysfunction and failure. Lethal toxin attacks endothelial cells and induces capillary permeability by rearranging central actin fibers and endothelial structure.[150,153,255] Due to this manipulation, endothelial cells suffer apoptosis or lysis. The severe damage wrought on the endothelium leads to disseminated intravascular coagulation and the characteristic bleeding classically associated with anthrax infections.[266-268] Edema toxin alone is lethal to zebra fish and some in bred strains of mice. [149,150,269] Disruption of cellular homeostasis, swelling and necrosis of cardiac pacemaker cells induced death in mice after intravenous infusion of edema toxin.[151,280] As previously stated, anthrolysin O is considered a hemolysin and actively destroys red cells.[270] Both toxins have been linked to platelet activation and disruption of the clotting cascade. The overall toxin effect on platelet function leads to more severe bleeding and vascular leakage.[269,271] This massive vascular leakage leads to pooling of fluid in the pleural and peritoneal cavity. As the pleural effusion accumulates, affected hosts experience shortness of breath, impaired ventilation and ultimately asphyxia. At the climax of

systemic infection resources and nutrients become limited forcing the vegetative cells in stationary phase growth and initiating the cascade of signals for sporulation. After death of the host autolysis exposes the tissues to higher oxygen concentration and stimulates the bacilli to complete sporulation. This concludes the infection cycle and leaves spores in the environment ready for the next host.

Treatment

Antimicrobial Susceptibility of *B. anthracis*

Most field isolates of *B. anthracis* display susceptibility to pencillin. Because of its inexpensiveness and ready availability, the WHO continues to recommend penicillin based treatment regimens for naturally acquired cases in developing countries. Novel strains isolated from naturally acquired cases consistently remain sensitive to β-Lactam antibiotics. In Western nations current recommended therapy consists of a fluorinated quinolone or doxycycline alone or in combination with an additional antimicrobial that the isolate may be susceptible to.[272] Recent findings have established that *B. anthracis* also displays *in vitro* sensitivity to numerous other antimicrobial classes including glycopeptides such as vancomycin, macrolides, rifampin, fluorinated quinolones and most 3rd and 4th generation cephalosporins. Resistance or intermediate susceptibility to sulfonimdes and earlier generation cephalosporins has also been consistently documented.[273]

Puromycin, an antimicrobial drug from which clindamycin was later derived has been proposed to have particular benefit in the treatment of anthrax on the merit of its ability to limit protein synthesis and specifically abrogate the synthesis of the toxins.[274] Clindamycin may also possess a similar ability to limit toxin synthesis based on clinical findings in toxic shock syndrome produced by streptococcal infections.[275] No efficacy data has confirmed this effect in

anthrax. Brook *et al.* demonstrated that clindamycin in combination with ciprofloxacin was successful in improving the survival of mice challenged with gamma-irradiated spores of *B. anthracis.*[276]

Stepanov *et al.* reported induced resistance in a strain of *B. anthracis* to penicillin, tetracycline, rifampicin, chlormaphenicol, erythromycin and lincomycin through the introduction of a recombinant pTEC plasmid.[277] This finding compelled the Center for Civilian Biodefense Strategies to publish recommendations that ciprofloxacin or other fluorinated quinolones should be the antimicrobial drug of choice in adults and children with inhalational anthrax.[278] They advise that clinicians should assume penicillin- and tetracycline-class resistance in any isolate of *B. anthracis* associated intentional release until sensitivity testing demonstrates otherwise. Shortly following the publication of these recommendations, a study demonstrated the development of *in vitro* resistance of *B. anthracis* Sterne strain to oflaxacin through the relatively simple method of sub-culturing the bacterium through multiple passages in the presence of the antibiotic.[279]

Lincoln *et al.* in early animal studies found that chloramphenicol was not effective in the treatment of systemic anthrax in mice or monkeys.[280] Moreover, due to chloramphenicol's bacteriostatic nature, others have reported that some strains of *B. anthracis* were found to be resistant to the drug.[281] Chloramphenicol has the potential for serious side-effects on bone marrow. Given its safety profile and the lack of therapeutic evidence from animal studies, most health officials have recommended seeking other alternatives.[282]

After the 2001 anthrax attack on the United States, the Center for Disease Control and Prevention released guidelines advocating the use of combination therapy (preferably 2 or 3 anti-

microbial agents) selected on the basis of susceptibility testing. Treatment outcomes from a retrospective case study of the 2001 attack indicate that the intravenous administration of 2 or more antibiotics active against *B. anthracis* resulted in a greater chance of survival.[14] In that study, only 10 cases were analyzed. To date no animal challenge studies have evaluated the effect of multiple therapies on treatment outcomes. Therefore, it remains unclear if combinational therapy truly provides a survival advantage but, the gravity of these infections may overrule empirical evidence.

Recommended Antimicrobial Therapy

For uncomplicated cases of cutaneous anthrax the recommended course of therapy is parenteral penicillin.[206] Alternative therapy includes oral penicillin or amoxicillin. Under these treatment regimens the bacterium is cleared from cutaneous lesions within the first 24 hours. The secondary signs of edema, erythema, and swelling subside within 24 – 48 hours. Prompt antimicrobial therapy does limit the size of the lesion, but does not affect the evolutionary stages it must go through.[283]

In individuals exhibiting signs of systemic involvement intravenous antibiotic therapy is recommended. Given the rapid of onset of disease in these cases prompt antibiotic administration is essential. Delaying therapy for patients with anthrax infection has been shown to decrease the chances for survival.[284,285] Diagnostic testing that is sensitive, specific and rapid is limited. Therefore all persons considered at high risk for anthrax exposure and displaying symptoms of fever or evidence of systemic disease should prophylactically receive antibiotic treatment for potential anthrax while awaiting definitive diagnosis. Recommended combinations for inhalational or gastrointestinal anthrax include doxycycline, ciprofloxacin, penicillin,

clindamycin or clarithromycin. In the treatment of gastrointestinal anthrax in non-human primates, the combination of penicillin and streptomycin has shown synergistic properties.[280] Ciprofloxacin and levofloxacin have yielded clinical success in numerous animal studies. [286,287]

A sequela to systemic anthrax is the development of meningoencephalitis. This complication is considered rare but is associated with a nearly universal mortality rate. Retrospective case analysis indicates an intravenous dose of 20 – 24 million units/day was correlated with survival. The most effective adjunctive antimicrobial agent identified in these studies appears to be intravenously administered rifampicin at 600-1200 mg/day.[282,288] These drugs are synergistic in activity against *B. anthracis* and possess favorable penetration into the central nervous system. Vancomycin has also been recommended in the treatment of meningoencephalitis, but due to its extreme cost is infrequently administered.[282] Though doxycycline has been shown to have high penetration in other tissues, drug concentrations in the cerebral spinal fluid are below the minimum inhibitory concentrations for *B. anthracis.*[288] Lessons from the few reported instances of survival in cases of anthrax meningoencephalitis suggest the dose of intravenous administered penicillin G should approach 20 – 24 million units /day.

Treatment failure rate reported in 4 independent case series was approximately 96% or 45 of 47 cases.[289-291] Delayed diagnosis due to suspicion of more prevalent diseases with neurologic presentations was considered a significant factor in the outcome of these cases by one author.[291] In addition to early diagnosis and antimicrobial therapy, supportive care is very critical in these cases. Critical supportive therapy that is recommended includes ventilator support, ongoing

correction of fluid and electrolyte imbalances, and treatments to resolve cerebral edema and inflammation.

The *B. anthracis* Ames strain isolate used in the 2001 attacks displayed the typical sensitivity profile of naturally occurring strains. However, the isolate did possess two resistance factors; an inducible β-lactamase and a constituitive cephalosporinase. It's unclear what benefit these enzymes provided the bacterium because the isolate demonstrated a high susceptibility to penicillin *in vitro.*[292] None the less, due to the potential for resistance in overwhelming infectious burdens the CDC advises avoiding penicillin as the sole treatment of inhalational anthrax and reconfirms ciprofloxacin and/or doxycycline as mainline therapies.[293]

In controlled causality settings following intentional release (situations where a small number of patients require therapy), the CDC recommends initial intravenous ciprofloxacin followed by a transition to oral ciprofloxacin or doxycycline as the patient stabilizes. In the event of a bioterrorist attack where the number of individuals requiring treatment is high creating an environment where intravenous antibiotic therapy may no longer be logistically feasible the CDC advises the use of oral ciprofloxacin or doxycycline to all exposed individuals. The CDC also advises prolonged courses of antibiotics (at least 60 days post-exposure) based on the findings of two studies that demonstrated antibiotic therapy in experimentally infected animals inhibited the development of an immune response.[293-295] The mechanism of inhibition was not clear in those studies but it suggests that individuals harboring un-germinated spores beyond the duration of treatment may remain at risk of infection.

Pediatric cases of anthrax and the treatment of pregnant women require careful consideration of adverse and teratogenic effects of some antimicrobial agents. In young animals,

ciprofloxacin has been associated with arthropathy and the development of cartilage defects in large weight bearing joints.[296] In spite of this potential concern, the United States Working Group on Civilian Biodefense believes the risk of catastrophic infection outweigh the risk of joint disease and recommends the use of ciprofloxacin in acute anthrax children. Doxycycline is also not generally recommended for children less than 8 years of age due to the adverse effects of enamel staining and inhibition of bone growth.[297] For less severe cases of anthrax in children penicillin is the treatment of choice at the recommended pediatric dosage.

The literature bears no clear recommendation concerning the duration of antimicrobial treatment in all forms of anthrax. Ellingson *et al.* demonstrated that *B. anthracis* cutaneous lesions were bacteriologically cleared within 24 – 48 hours of treatment.[298] During an outbreak in Zimbabwe, treatment antibiotic therapy beyond 4 days provided no benefit in outcomes.[299] In a large case report from Ethiopia, a single intramuscular dose of penicillin resulted in the early discharge of 99 out of 100 patients. Of these 99, 5 patients experienced progression of the lesion and required subsequent follow up and continuation of care.[300] Many physicians have adopted an empirical regimen of a minimum 7 – 14 days of care.

There are no FDA-approved compounds or treatment regimens for the post-exposure prophylaxis of anthrax. Therefore, recommendations for post-exposure prophylaxis are based entirely on the strategies derived for the treatment of anthrax in mass casualty settings. This includes antibiotic therapy for a minimum of 60 days following spore exposure.[293] These guidelines were put into practice following the 2001 attack when over 10,000 US postal workers were potentially exposed. These individuals were provided 60 days of ciprofloxacin or doxycycline for anthrax prophylaxis. Approximately 2% of these individuals sought medical care

for possible adverse effects associated to the medication, but none presented with clinical symptoms typical of anthrax.[301] A retrospective survey including over 6,000 of the individuals provided prophylaxis revealed that less than 45% followed the prophylaxis protocol to its completion. Commonly cited reasons for not finishing the therapy included gastrointestinal upset or other adverse reactions, forgetting to take the medication daily, and belief that there was ever any real risk of contracting the disease.[302,303]

Supportive Care

In animal models, physiologic sequelae of severe anthrax infection have included aberrations in calcium, glucose, acid-base, and potassium regulation as well as respiratory and cardiovascular collapse.[150,304] This suggests that beyond rapid antibiotic administration, mitigation of the effects of systemic toxemia might improve survival if homeostasis can be successfully resurrected. Supportive measures would ostensibly include balanced intravenous fluids, correction of acid-base disturbances, maintenance of appropriate blood glucose concentrations, early ventilatory support and administration of vasopressors. In a rat lethal toxin constant rate infusion model, volume support through crystalloid infusion was shown to decrease the mortality associated with the disease.[305] In a similar study model, vasopressors had no effect on mortality.[306] Of the 22 cases following the 2001 attacks the first 11 presented with severe pleural effusion.[272,307] Seven of these individuals required drainage of the thoracic cavity during the course of therapy and 3 required the placement of chest tubes.[272] Glucocorticoid therapy was found to possess potentially detrimental effects by upsetting the balance of endocrine function and possibly sensitizing susceptible mice to lethal toxin.[308] Turnbull suggested that corticosteroids should be of little value in light of the anti-inflammatory effects of edema

factor.[217] The literature in this area is inconclusive as to the real benefits of corticosteroid with some retrospective case studies revealing a treatment effect while others have found none.[299] In well controlled experimental studies with non-human primates hydrocortisone, did have a statistically significant effect on treatment outcomes.[280] As alluded to previously there does appear to be a beneficial effect of dexamethasone in the treatment of anthrax meningoencephalitis potentially by reducing cerebral edema due to its increased volume of distribution and levels obtained in the brain, but it is difficult to fully substantiate that recommendation due to a lack of clinical data.[289]

Alternative Therapies for Anthrax

Appropriate antimicrobial therapy does not always ensure successful treatment of severe anthrax due to the continued deleterious effects of the toxins despite elimination of the bacteremia. This was first identified in rabbits experimentally infected with the Vollorum strain of *B. anthracis*.[309] In that study, antibiotic therapy initiated late in the course of disease, while effective in eliminating the bacterium from tissue, failed to halt the progression of disease. A retrospective systematic review of cases of human inhalational anthrax evaluated the effect of timing of antimicrobial therapy and the ensuing clinical response.[310] When antimicrobial therapy was administered to individuals during the prodromal stage, there appeared to be a prolongation of that stage from a mean 4.1 days to 5.8 days. Individuals who were treated with antimicrobials or anti-anthrax antiserum during the prodromal phase were less likely to die than those individuals receiving treatment during the fulminant phase or no therapy at all.[310,311] These studies underscore the importance of early antibiotic therapy and illustrate a need for adjunctive therapeutics targeting lethal and edema toxin.

A large number of drugs approved for use in humans display efficacy against one or both of the anthrax toxins under experimental conditions. To date most have been studied in *in vitro* pathogenesis models not therapeutic models. Two calcium channel blockers, verapamil and nifedipine, block the effects of both toxins by limiting the intracellular availability of calcium.[312] Dantrolene, a post synaptic muscle relaxant, and quinacrine, an antimalarial agent, also reduce calcium availability for interaction to lethal toxin through blockades of calcium release for cytosolic stores and phospholipase A_2 activity, respectively.[313,314] Neomycin and protamine inhibited the activity of lethal toxin through competitive inhibition of common binding sites on mitogen activated kinase kinase.[315,316] Adefovir, an antiviral agent used in the treatment of hepatitis B was shown to inhibit the activity of edema toxin through its binding of the enzymes catalytic site.[317] The statins simvistatin and fluvastatin reduced lethal toxin activity by preventing Rho GTPase activity from trafficking lethal toxin throughout the cell.[318]

The available *in vivo* activity of efficacious compounds is very limited. Amiodarone and niclosamide, an anti-arryhtmic and anthelmintic respectively, enhanced survival of rats and mice intoxicated with lethal toxin by raising the endosomal pH and preventing dissociation of the PA heptameric pore from its receptor thereby preventing cellular entry of the toxins.[319,320] N-acetyl-cystine and the antineoplastic agent cisplatin both improved survivability of Balb/C mice following a challenge through distinct, downstream events in the lethal toxin pathogenesis.[153,321,322] N-acetyl-cystine acts as an antioxidant and abrogates the cytolysis produced by reactive oxygen intermediates in way reflective of it use in acetaminophen toxicity. Cisplatin displays a dual role in reducing the pathogenesis of lethal toxin by first preventing PA from forming a heptemere thereby impacting LF and EF translocation as well as

inhibiting LF cleavage of mitogen activated kinase . Select NSAID agents (indomethacin and celecoxib) and mast cell stabilizers (cromolyn) reduced edema toxin morbidity but did not affect mortality outcome in rabbits challenged with edema toxin.[334] The proposed mechanism for each of these compounds was through a reduction in vascular leakage associated with attenuation of edema toxins effects on inflammatory mediators and reduction of mast cell degranulation and histamine release.

Chloroquine, a quinidine derivative molecule approved for us against malaria in humans has demonstrated effect in reversing the pathogenesis of both toxins. Chloroquine administered to mice at clinically relevant doses before and after challenge with lethal toxin a positive effect on survival.[323] On cellular level chloroquine reversed cytotoxicity in T cells after exposure to lethal toxin. Co-administration of chloroquine and amiodarone or furin protease inhibitors produced an additive beneficial effect on T cells.[319,324,325] However, when administered to macrophages at the same time as virulent spores the macrophages displayed reduced sporicidal effect.[326]

The spectrum of unapproved or experimental compounds with activity against the anthrax toxins is extensive. Modification of tetracycline produced the capability to competitively inhibit LF cleavage of MAPKK at concentrations similar to those achieved when the same compound was administered to participants in a phase I and Phase II human cancer trial.[327] Many of these agents display greatest inhibition of LF after the protein has entered the cell. Certain polyphenols that can be extracted from green tea stochastically inhibited the proteolytic activity of LF in mouse macrophages and protected rats from death after lethal toxin challenge.[328]

Some agents have increased toxicity. Aspirin and other anti-clotting compounds increased the mortality of mice after lethal toxin administration and therefore should be avoided the treatment of systemic anthrax.[271] The discovery of new small molecular inhibitors of LF is complicated. In order to effectively inhibit the metalloproteinase function of LF, these compound need to penetrate the cell membrane with high affinity and may inadvertently affect the host enzymes. Many candidate compounds showing promise at the foundational level fail to translate to clinical efficacy by losing potency under normal physiology conditions. Recently the crystal structure of some of basic compounds was resolved which hopefully will inspire the design of improved candidates.[329]

Targeting of the cell surface associated furin protease has been a promising target for novel therapeutics because of its important role in the proteoltyic cleavage of PA following initial interaction with its host cell receptors. Furin is a serine protease and is susceptible to inhibition by inter-alpha inhibitor proteins.[330] Inter-alpha inhibitor proteins are found at high concentrations in human plasma.[331] There general function is to provide regulation over of a broad spectrum of proteases including complement components, granzymes and coagulation and fibronlytic enzymes that are typically present in the pro-inflammatory state. The relationship between inter-alpha inhibitor proteins and mortalitiy in bacterial sepsis has been the subject of several investigations and has the potential to emerge as a therapeutic agent.[332-334] In a study where A/J strain mice were intra-nasally challenged with *B. anthracis* Sterne strain, inter-alpha inhibitor proteins improved survival compared to controls and appeared to act synergistically with anti-microbial therapy.[335] Other protease inhibitors have shown anti-pathogenic potential

through targeting of furin.[336] Alpha antitrypsin blocked furin and inhibited the pathogenic effect of *Pseudomonas auerginosa* A exotoxin in cell culture.[337]

Mutant variants of PA monomers constructed to have dominant negative activity function as decoys and prevent heptameric prepore formation with native PA.[338,339] Deletion of the 2 β_2-2 β_3 loop and point mutations in any of three residues, K397, D425, and F427, produce protective antigen monomers that co-assemble with wild-type protective antigen monomers and block the intracellular delivery of the toxins. These mutant proteins were demonstrated to be very efficient in that only one mutant variant within a heptamere of wild-type proteins was necessary to disrupt translocation of the toxins. Though the dominant negative variants were protective in a rat lethal toxin challenge, there clinical relevance has been called into question due to the timing of administration and the likelihood of a positive effect in the typical diagnostic window of systemic anthrax.[339]

Another novel antimicrobial approach entails the development of bacteriolytic enzymes or lysins derived from double stranded DNA bacteriophages.[340,341] Lysins are enzymes produced by lysogenic phages upon exit of the bacterial cells at the end of the viral reproductive cycle. These enzymes have narrow host ranges and a high specificity for the host's cell wall carbohydrate composition. Investigators used a *B. anthracis* specific γ strain as source of recombinant γ lysin.[342] The γ lysin demonstrated a rapid lytic effect when applied to bacterial lawns of *B. anthracis.* Administration of the γ lysin to Balb/C mice that had been intra-peritoneally challenged with RSVF1, a strain of *Bacillus cereus* that has very high genetic homology to most strains of *B. anthracis*, reduced the level of morbidity and improved survival rates compared with control animals.[343] Lysin therapy is especially attractive because of the low

level of intrinsic resistance and potential for use in genetically engineered, antibiotic resistant *B. anthracis* strains.

Disrupting communication between *B. anthracis* bacilli through inhibition of quorum-sensing is another potential approach. Quorum-sensing systems are bacterial cell surface associated protein complexes that respond to several environmental stimuli, but principally intraspecies cell density, by initiating second messanger cascade that eventually modulates gene expression.[344] Quorom-sensing systems are very important in the regulation of pathogenesis for many bacteria. *B. anthracis* uses a quorum sensing system known as AI-2 that is common to other species.[345] Synthetic halogenated furanones have been identified that are competitive antagonists of this system.[345,346] An *in vitro* study involving the exposure of *B. anthracis* Sterne strain to a naturally occurring AI-2 inhibitor, *(5Z)*-4-bromo-5-(bromomethylene)-3-butyl-2*(5H)*-furanone (fur-1) derived from *Delisea pulchra* a red marine algae, demonstrated a reduction in growth and expression of the *pagA*, *cya*, and *lef* virulence genes.[347]

Knowledge of the amino acid sequence of PA has provided insight into methods of disrupting its function. Fang *et al.* produced a recombinant soluble form of tumor endothelial marker 8 or ANTXR2 that acts as a receptor decoy inhibitor. If applied in animal therapeutic studies these decoys possess the potential to be used as a molecular scrubber to adsorb free form PA monomers.[348] The receptor decoy provided protection from the effects of lethal toxin in RAW264.7 cells, yet failed to improve survival in rats infused with lethal toxin when fused to the F_c portion of IgG1 to increase its circulation half-life. In a similar study, phage display was used to identify small peptide molecules capable of weakly binding the hepatmeric cell-binding subunit of protective antigen.[349] These small peptides, when covalently linked together, as a

polyvalent inhibitor prevented toxin assembly *in vitro*. In lethal toxin studies in rats the polyvalent inhibitor delayed the onset of clinical symptoms and reduced lethality.[349]

B. anthracis initiates pathogenesis production immediately following germination. Therefore the germination process is an attractive target for agents with the potential to abolish this transformation. Inosine, a nucleoside found in high concentrations in tRNA, is a potent germinant for *B. anthracis.* As such the chemotherapeutic agent 6-thioguanosin, an inosine analog, was shown to be effective in blocking spore germination and cytotoxicity in murine macrophages.[350] Other compounds with identified activity against the *B. anthracis* spore include components of non-human primate innate immune defensins. In particular compounds known as theta-defensins or retrocyclins were investigated and demonstrated that in addition to rendering germinating Sterne strain spores non-viable they also blocked the enzymatic activity of lethal toxin and protected murine cells from cytotoxicity.[351] Recently, Crawford *et al.* demonstrated the sporicidal and antimicrobial activity of murine CXC chemokines CXCL9, CXCL10, and CXCL11.[352] A mechanism for this observation was elucidated for CXCL10 through its binding and disruption of FtsX, a putative ATP-binding cassette transporter responsible for translocation of a wide variety of macromolecules.[353]

One of the most devastating end effects of lethal toxin is the activation of the caspase system and induction of apoptosis. Auranofin, an organo-gold compound, blocks the activation of caspase-1 and significantly prolonged mean time to death in rats following intoxication.[354] Though auronofin is the only caspase inhibitor used against anthrax other inflammasome inhibitors have been identified and may have a role in limiting the cellular damage and should be investigated.[355]

Passive Immunotherapy

In 1901, Emil von Behring received the first Nobel Prize for developing polyclonal antibodies (pAbs) and using them as a treatment against diphtheria and tetanus.[356] Around this time an Italian protégé of von Behring, Achille Sclavo, began work on developing a donkey based antiserum against anthrax to stem the effect the disease was creating in Italy at the time. The first report of successful treatment using Sclavo's anti-serum appeared in the *British Medical Journal* in 1905 describing the treatment and recovery of a man with cutaneous anthrax.[357] Following World War I, antibody mediated therapy for the treatment of diseases agents fell by the wayside and were supplanted by antibiotics.[206] However, in the age of bacterial resistance renewed effort is being placed in passive immunotherapy. The risk of antibiotic resistance in *B.anthracis* is acute because if the pathogen is maliciously disseminated it will almost certainly first be selected or engineered for resistance to the common classes of antimicrobial drugs. Revisiting the development of epitope specific antibodies may be a logical countermeasure to this risk. In addition, combined with existing therapies immunotherapeutics act synergistically through distinct mechanisms. Passive antibodies also possess merit as prophylaxis even though the expected half-life in humans is approximately three weeks.[358,359] Specific antibodies that are administered intravenously provide immediate protection that could be easily given to first responders and individuals with the highest risk of exposure. Compared to vaccines which may require a primary and additional booster immunizations while a prepared hyper-immune plasma may only require a single dose. In addition, the antibody titers achieved through passive immunization can be administered in quantities that exceed those that can be achieved with vaccination. This may be of particular benefit in the context of bioterrorism

because of the potentially high infective dose delivered by bioweapons compared with natural exposure.

There are several drawbacks to passive immunotherapy. The production of *in vitro* monoclonal and recombinant antibody technology is expensive. It is currently not known how many doses an individual may need to be administered following a heavy exposure. Immunoglobulins produced in animal species can result in allergic and anaphylactic reactions. Subsequent doses of non-homologous immunoglobulins increase the risk of reaction and likely reduce the effectiveness and circulation as the patient develops a primary immune response against the foreign proteins. Despite these short falls, government agencies and programs such as Bioshield and the Biomedical Advanced Research and Development Authority have pursued refinement of passive immunotherapy and antibody mediated treatments.[360]

The important role for antibodies in protection against anthrax has been confirmed through overwhelming evidence demonstrating neutralizing antibodies as key in conferring immunity to anthrax.[361] From these studies, it is also apparent that an antibody response particularly against protective antigen is sufficient to provide protection.[311,361,362] The only vaccine licensed by the FDA for use against anthrax in humans is known as anthrax vaccine adsorbed (AVA) or Biothrax®. This vaccine has been systematically evaluated and shown to principally induce a humoral mediated response against PA and to a lesser extent the other *B. anthracis* effector molecules.[363] The vaccine is a whole cell lysate containing a large protein fraction of PA and smaller fractions of LF and EF. These components are then adjuvanted with aluminum hydroxide gel to produce the vaccine. Significant limitations in the vaccine's effectiveness include variation in protein content by lot, a slightly unrefined production process

yielding a sometimes highly reactive response in certain individuals and a long course of immunization. The current manufacturer's recommendation for vaccine protection includes 6 immunizations over an 18 month time course. These limitations have instigated a renewed effort in identifying more advanced vaccine candidates with the goal of providing a more robust and rapid response. One of the most promising candidates have been two purified recombinant PA proteins produced from an *Escherichia coli* and an attenuated *B. anthracis* background. [364-366]

Though the AVA vaccine is not licensed for use in post-exposure the scientific literature supports a series of post-exposure immunizations based on several animal studies.[367-370] One principal study evaluated the efficacy of post-exposure vaccination in non-human primates.[371] Among 60 rhesus macaques exposed to a lethal aerosol spore challenge, only animals that received a combination of antibiotic therapy and 2 doses of AVA survived the initial challenge and a subsequent re-challenge 60 days later. In a companion study, a short-course of prophylactic antibiotic treatment (oral ciprofloxacin for 14 days) was compared alone and in conjunction with 3 doses of AVA in rhesus macaques.[372] Four of nine animals receiving ciprofloxacin alone survived inhalational challenge compared to 10 of 10 receiving ciprofloxacin and AVA vaccination.

In the aftermath of the 2001 attacks AVA was not used immediately in individuals exposed to *B. anthracis*. One reason was a lack of available supplies.[373] Later near the end of the 60-day course of prophylactic antibiotic regimen individuals with the highest potential exposure were offered the post-exposure AVA series. The US Working Group on Civilian Biodefense advocates the combination of prophylactic antibiotic therapy and AVA vaccination as the most protective strategy currently available.[373]

It is unlikely that an individual suffering from inhalational anthrax, with the disease's rapid incubation and progression, can be afforded protection through vaccination after exposure. In the situation of a known exposure, prophylactic antibiotics and/or passive immunotherapy are the most effective means of preventing the disease. The epidemiologic data from the limited reports where a large number of people were exposed to aerosolized spores suggests that spores may persist for some time in the lungs. Therefore, the current recommendations for prophylaxis include a 60 day course of antibiotics.[12,374] This approach appeared to be successful in the wake of the 2001 attacks, where close to 10,000 people may have been exposed and were provided a 60 day course of ciprofloxacin or doxycycline. However, a retrospective survey of over 6,000 of these individuals revealed that fewer than 45% followed the prophylaxis protocol to its completion. Commonly cited reasons for not finishing the therapy included adverse reactions, forgetting to take the medication daily, and belief that there was little actual risk of contracting the disease.

Universal administration of post-exposure antibiotics is also difficult when the use of the most commonly recommended antibiotic are contraindicated, such as in pregnant women and children. Perhaps of more concern is the development of multi-drug resistant strains of weaponized *B. anthracis.* Naturally occurring isolates have been identified in a limited number of cases that display resistance to the β lactams.[375,376] In addition, some investigators have demonstrated that simple laboratory techniques such as serial passaging in the presence of doxycycline or ciprofloxacin can generate resistant strains. Others have produced a resistant strain through transfection of resistance genes encoded on plasmids.[279,377] Finally, there is a

critical point in the progression of systemic anthrax when the toxin load is sufficient to continue to cause pathology in spite of appropriate antimicrobial therapy that controls the vegetative cells.

In light of these facts, the development of efficacious alternative modalities in the prophylaxis of inhalational anthrax is imperative. Passive immunotherapy using hyperimmune polyclonal sera or monoclonal antibodies (mAbs) against several *B. anthracis* antigens has been the major focus of the most recent efforts. Specific immunoglobulin therapy is attractive because the immediate and prolonged protection against the toxin components. Many investigations demonstrate the beneficial effect of anti-PA and anti-LF antibodies in several animal models.

Little *et al.* indicated that anti-PA antibodies alone were sufficient to provide protection against an intramuscular challenge of *B. anthracis* Ames spores in guinea pigs.[311] The antiserum was harvested from guinea pigs immunized with rPA and passive transfer significantly protected (approximately 67%) recipients who were challenged at the time of treatment. However, antisera produced against the Sterne strain spore vaccine (demonstrated to possess equal quantities of anti-protective antigen specific antibodies, but greater anti-lethal factor and anti-edema factor antibodies) was not protective in mice (approximately 8% survival), although it did increase the mean survival time to death compared to controls.[378] Based on these early findings, a greater emphasis was placed on the protective capacity of anti-protective antigen antibodies compared to that of the other toxin components. Koblier *et al.* investigated the passive protection of rabbit anit-PA, anti-LF, and anti-Sterne antisera in guinea pigs challenged intranasally with fully virulent Vollurm spores.[379] In that study large volumes of anti-PA antisera administered 24 hours postinfection yielded the greatest protection compared to anti-Sterne and anti-LF antisera (88.8%, 44.4% and 25%, respectively, anti-PA and anti-Sterne were significantly different than

control). When anti-PA antiserum was combined with anti-LF antisera, the percent survival improved to 37.5%. Interestingly, the level of acquired immunity as measured by geometric mean titers against PA and survival following re-challenge at 35 days post-infection was greater in the anti-Sterne antisera treated animals than in the anti-PA treated animals.[379] The same study demonstrated no protective capacity in anti-PA or anti-LF monoclonal antibodies. In contrast to these findings, Staats *et al.* showed that an anti-LF monoclonal antibody was effective in preventing morbidity associated with lethal toxin injection in mice.[380] They also demonstrated that though an anti-PA monoclonal antibody showed higher *in vitro* toxin neutralization, it did not protect mice from lethal toxin challenge. However, addition of the anti-LF monoclonal antibodies in equal volume improved its protective capacity. The importance of antibody mediated immunity over cell-mediated immunity was further demonstrated in a study conducted by Beedham *et al.*. where naïve mice receiving passive transfer of anti-PA antibodies survived longer following challenge with a non-encapsulated toxigenic strain of *B. anthracis* than did mice receiving immunized lymphocytes.[381] These preliminary studies provided the frame work for more detailed experimental designs that move beyond the assertion of the theoretical benefit of passive immunity against *B. anthracis* into investigations of the clinical application of passive immunotherapy.

Monoclonal antibodies possess many advantages compared to polyclonal antibodies including, well characterized specificity, consistent efficacy and higher standards of purity and safety. An engineered affinity-enhanced anti-PA monoclonal antibody was recently produced that provided protection to rabbits before and after inhalation challenge with Ames strain.[382,383] In another study conjugation of high affinity anti-PA specific monoclonal F_{ab} fragments with

polyethylene glycol conferred significant protection against spore inhalation challenge and prolonged circulation half-life.[362]

Murine monoclonal antibodies have long been a potential target for the development of passive immunotherapy. Early studies provided evidence of the benefit of mAbs in neutralizing lethal toxin in cell culture assays and rat toxin challenge models.[384,385] Follow up studies further showed that mAbs specific for PA or LF were equally effective in protection of mice against a virulent spore challenge.[386] However, not all mAbs are helpful as evidenced by an unexpected enhancement of cytotoxicity rather than neutralization through an Fc receptor mediated mechanism.[387] These findings suggest that a shotgun approach where mAbs are generated without regard to specific epitopes and binding affinity may be detrimental and underscores the necessity of identifying and fully characterizing the clinical consequences of mAb therapy.

Animal-derived mAbs have received less attention compared to the chimeric or fully humanized immunoglobulins due to the potential for reaction to foreign protein in patients, especially after repeated administration. There have been endless advances in antibody engineering field that yield human mAbs with longer plasma half-life, enhanced affinity, or conjugation of effector molecules such as drugs, radioisotopes or toxins. The development of phage display to quickly comb through high affinity recombinant candidates has tremendously accelerated the identification of effective mAbs.[388] Wild *et al.* use phage display to isolate two F_{ab} fragments specific for PA that were then proven protective against lethal toxin challenge in rats. Maynard *et al.* constructed single light-chain variable subunits that interfered with the binding of PA to the anthrax toxin receptor then used PCR primers with poor fidelity to prepare

affinity-enchanced clones.[389] The pervading objective of these engineered mAbs has been to demonstrate more effective protection than traditional parent mAbs.

One group of investigators used a more holistic approach to the isolation of fully human mAbs by first collecting lymphocytes specific for *B. anthracis* antigens from the blood of AVA-immunized volunteers and then transplanting these cells into combined immunodeficient (SCID) mice for the purpose of later isolating plasma cell clones specific for PA.[390] Once the plasma cells were isolated they were immortalized as hybridoma cells through fusion with myeloma cells. Another approach produced stable expression of full-length human immunoglobulins by cloning and transfecting the genes into the genes Chinese hamster ovary cells. This method produced an anti-PA antibody with exceptional affinity (K_D = 82 pM) and protected rats from lethal toxin challenge even when administered as early as 17 hr prior to challenge. When the dose of anitbodies used was increased tenfold, 80% of rats survived beyond 7 days. Wang *et al.*. further characterized this mAb and revealed when bound to PA it inhibited heptamer formation and mitigated translocation of the toxins.[391] Later the same mAb was found to fully protect mice following intranasal challenge with Ames strain spores, but only when combined with a low dose of ciprofloxacin.[392] Re-challenge of the surviving mice with same inoculum dose of Ames spores resulted in death of all animals indicating that the immune system had not been stimulated to produce a protective response.[392] Guinea pigs infected following an intranasal Ames challenge demonstrated similar results in that only animals treated with a combination of AVP-21D9 and ciprofloxacin survived.[392] However, AVP-21D9 treatment alone fully protected aerosol challenged rabbits even when administered up to 12 h post-infection.[392] Interestingly the presence of the antibody did not impair a primary immune response to *B. anthracis* as 23 of 24

animals remained resistant to infection when challenged again by aerosol 5 weeks after the primary challenge.[383]

Transgenic mice (HuMab) engineered to express human immunoglobulins have been used to produce specific anti-PA antibodies and screened for *in vitro* activity.[393,394] One monoclonal, mAB-1303, demonstrated high potency and protected rabbits when administered at 1 h and 3 days following inhalation challenge.[393] This mAb also protected nonhuman primates from aerosol spore challenge when administered intramuscularly at 1 h post-infection.[393] When the mechanism was investigated it was discovered that blocking the Fc portion abolished the neutralization capacity as did blocking the Fab portion indicating that both subunits are necessary. This same effect was observed in immunoglobulins isolated from mice, rabbits, and humans.[393] This suggest that administration of the Fab monoclonal fragments may not be as effective as full-length immunoglobulins.

The next iteration for these monoclonal therapies is translation from the basic sciences to commercial application. The Department of Health and Human Services under the umbrella of Homeland Security has been instrumental in funding this endeavor. Four biotechnology companies have begun to develop independent fully human monoclonal into licensed products. Each of these products have successfully navigated Phase I clinical trials and have been awarded Fast-Track and Orphan Drug status by the FDA. One of these monoclonals, raxibacumab, (Abthrax™, GlaxoSmithKline) has received FDA approval for the treatment of inhalational anthrax and the prevention of inhalational anthrax. The FDA approved this drug based on animal studies alone due to the inability to fully determine the effect in humans with anthrax.

Immediately following approval raxibacumab began manufacture to provide 20,000 doses to the National Strategic Stockpile in 2009.

One limitation to anti-PA monoclonal therapies are their monospecific nature and inefficacy against the other toxins produced by *B. anthracis*. This leaves an opportunity to engineer a strain that resists their action. The specific binding epitope for each of the monoclonal products currently under commercial development has not been reported. Simply mutating the binding epitope may reduce the effectiveness of the drug while the other functions of PA such as receptor binding, heptamer formation, and translocation of the EF and LF remain unaffected. One solution to this potential problem is the combination of mAbs with independent epitopes for PA and/or mAbs targeted toward other *B. anthracis* components. Several groups have isolated anti-LF and anti-capsule mAbs that have been demonstrated efficacious in neutralizing lethal toxin *in vivo* and *in vitro*.[395-399]

An alternative approach is the application of polyclonal plasma. Cangene Corporation and Emergent Biosolutions manufacture polyclonal immunoglobulins ('Anthrax Immune Globulin', AIG) harvested from the plasma of human volunteers immunized with AVA. The advantage of polyclonal antibody therapy lies in its breadth of antigenic targets developed during a natural immune response. These multiple specific antibodies have the additional advantage of improving the efficiency of immune effector mechanisms such as opsinization and phagocytosis, complement mediated bacterial lysis, and antibody-dependent cell lysis of intracellularly infected macrophages. There are limitations to human donated plasma however, including limited availability, variation in antibody titers between individuals, the risk of iatrogenic transmission

of blood-borne pathogens, and the high cost of production. Despite these hurdles Cangene has been requested to produce 10,000 doses of AIG for the US National Strategic Stockpile.

Select studies using small animal models have demonstrated a greater protective capacity from polyclonal sera than mAbs.[178] Passive transfer of polyclonal plasma collected from guinea pig immunized against PA or LF protected a larger number of animals following spore compared to monovalent monoclonal passive therapy (67% and 33%, respectively).[177] Monoclonal antibodies have the decided advantage of tailored antigenic specificity compared to polyclonal antibodies. But few studies have evaluated the potential benefits of simultaneously neutralizing multiple epitopes. Monospecific therapy may be rendered ineffective of the epitope can be modified in a way that eliminates the binding affinity. The most significant limitation of polyclonal sera is the inefficiency of production. The ideal passive immunotherapy for anthrax must be robust and highly specific against the most relevant epitopes, non-toxin to humans, long lasting and adaptable to large scale commercial production.

Chapter 2:

The production of hyper-immune plasma from horses serially immunized with a *Bacillus anthracis* Sterne strain spore vaccine and recombinant protective antigen

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Abstract

The objective of this study was to develop a safe and reliable immunization protocol for producing equine source hyper-immune plasma against *Bacillus anthracis*. Six Percheron horses were immunized with either the *B. anthracis* Sterne strain vaccine or recombinant protective antigen (rPA) homogenized with Freund's incomplete adjuvant. Multiple routes of immunization, dose (antigen mass) and immunizing antigens were explored for safety. A modified automated plasmapheresis process was then employed for the collection of plasma at a maximum target dose of up to 22 ml of plasma/kg of donor bodyweight to establish the proof-of-concept that large volumes of plasma could be safely collected from horses for large scale production of immune plasma.[400] All three immunization protocols were found to be safe and

repeatable in horses and three pheresis events were performed with the total collection of 168.36 L of plasma and a mean collection volume of 18.71 L (± 0.302 L).

Introduction

Bacillus anthracis is a gram-positive aerobic rod and the causative agent of anthrax. Anthrax virulence is defined by the production of two toxins known as lethal toxin and edema toxin. Each toxin is composed of two components as effector molecules, lethal factor and edema factor respectively, and a common component known as protective antigen (PA). The toxins gain entry into the cell by means polymerization of PA after binding to cell surface associated receptors and establishing a pore through the cell membrane.[401,402] Vaccination studies have revealed that high antibody titer against PA is directly correlated with survival following lethal spore challenge.[403,404] Moreover, the passive administration of polyclonal antisera against PA protected laboratory animals from inhalational anthrax.[405,406] Those studies established a minimum titer of 1:200 was necessary for protection in guinea pigs. Hyper immune serum or plasma is equally effective in post-exposure treatment experiments.[407] In addition to PA antibodies targeting other epitopes of *B. anthracis* demonstrate protection. When monoclonal antibodies targeting spore antigens were administered to mice following a spore challenge, spores were prevented from germinating.[408] This suggests that polyclonal immunotherapeutics that have a broad range of targets may have greater efficacy through inhibition of *B.anthracis* throughout the infection cycle.

B. anthracis Sterne strain is an avirulent strain cured of the pXO2 plasmid where the genes that encode for the poly-D-glutamic acid capsule found in virulent strains are located.[409]

This strain still possesses the pXO1 plasmid and is hence capable of producing each of the three toxin components. The strain has been used effectively as a live avirulent vaccine worldwide in humans and animals.[17] Currently it is licensed for the prevention of anthrax in livestock species including horses in the United States.[410]

The goal of this study was to establish an effective immunization protocol for the development of equine hyper-immune plasma against anthrax that could be evaluated for protective efficacy in later studies. Here we explored using the Sterne strain spore vaccine administered to horses monthly both subcutaneously (SC) and intramuscularly (IM) for over a year. We also establish the safety of using alternative antigens and an increased dose of spore vaccine in an attempt to maximize specific antibody production. Finally, the successful collection of hyper-immune plasma through automated plasmapheresis and demonstrate proof-of-concept for the large scale inexpensive production of equine anti-*B. anthracis* immune plasma is described in detail.

Materials and Methods

Animals

The work reported herein was performed under the approval of the Institutional Animal Care and Use Committee of Auburn University. Six Percheron draft mares were obtained and determined to be healthy based upon the findings of physical examination, complete blood cell count, and serum biochemistry analysis. Agar gel immunodiffusion tests for each horse were negative for the presences of antibodies against equine infectious anemia. In addition, each horse

was negative for equine herpesvirus-1 via PCR assay. During the study the horses were maintained on mixed grass-Bermuda pastures with *ad libitum* access to water. Each horse was provided approximately 3 kg of 12 % crude protein pelleted feed daily. During the study period, each horse received moxidectin as a routine anthelmintic and was immunized annually against tetanus, rabies, equine herpesvirus-1, equine influenza and *Streptococcus equi* spp *equi*. The horses were immunized twice annually against Eastern and Western equine encephalilits and West Nile virus.100

Antigens

The Sterne strain 34F2 spore vaccine used was the commercially available Anthrax Spore Vaccine (Colorado Serum Company, Denver, CO). Each vial was maintained at 4^0 C protected from light until ready to administer. Recombinant protective antigen (rPA) was obtained from List Biological Laboratories (Campbell, CA). Each vial was reconstituted with 1.0 ml of sterile distilled water to create a concentration of 1 µg/µl. Following reconstitution, the antigen was aliquoted in 20 µl volumes and stored at -80^0 C until ready to use. Each dose of rPA vaccine was prepared the day of immunization as follows; 100 µg of rPA was thawed at 4^0 C overnight and combined with 500 µl Freund's incomplete antigen then homogenized and drawn into a syringe.

Spore Vaccine Immunization Protocol

The following description is provided in outline format in Table 2.1. Each horse was immunized with 1.0 ml of *B. anthracis* Sterne strain spore vaccine administered SC in the neck. A booster dose was administered subcutaneously on Day 21. Following these initial doses two

horses were randomly selected with the aid of computer software and continued to be administered 1.0 ml spore vaccine subcutaneously in the neck monthly from Day 56 to Day 155, while the remaining two horses received 1.0 ml spore vaccine intramuscularly in the neck at the same time periods. Whole blood samples were collected in sodium citrate from each horse on Days 0, 21, 56 and monthly thereafter, and plasma stored at -80 ^{0}C until ready for analysis.

On Day 185, 1 horse from each subset was randomly selected to continue immunizations monthly as described. These immunizations occurred monthly from Day 185 until Day 366. For the remaining two horses immunization was withheld in an attempt to determine the persistence of antibody titers.

2X Spore Vaccine Immunization Protocol

Following the initial immunization protocol, two horses that had previously been immunized against *B. anthracis* according to the schedule above were randomly selected to receive an increased dosage of spore vaccine in an attempt to maximize antibody titer response. Each horse was immunized with 2.0 ml of spore vaccine administered subcutaneously in the neck. A second immunization was administered 14 days after the initial dose and then monthly thereafter for a total of six immunizations, Table 2.2.

Recombinant Protective Antigen Immunization Protocol in Primed Horses

Following the initial immunization regime, the remaining two horses that had previously been immunized against *B. anthracis* according to the schedule in Table 1 were randomly selected to receive immunization with 100 µg of *B. anthracis* recombinant protective antigen

(rPA). First each horse received a single booster immunization of 1.0 ml *B. anthracis* spore vaccine. Thirty days following the booster immunization, each horse was administered 100 µg of rPA homogenized with Freund's incomplete adjuvant was administered intradermally (ID) in the skin along either side of the neck. Intradermal administration of 100 µg rPA was repeated monthly for six total immunizations, Table 2.3.

Recombinant Protective Antigen Immunization Protocol in Non-Primed Horses

To determine the ability of rPA alone to elicit specific antibody titers against *B. anthracis*, two naïve Percheron horses were obtained and determined to be healthy and free of infectious diseases as outlined above. A primary immunization of 100 µg of rPA homogenized with Freund's incomplete adjuvant was administered ID in the neck of each horse. Booster immunizations were administered approximately 30 days apart for a total of six total immunizations, Table 2.4.

Plasmapheresis

Prior to the plasma collection procedure, a physical examination was performed to determine if each horse was suitable for plasma donation. Plasmapheresis was only performed when the horses were deemed healthy based on the finding of physical examination, displayed a rectal temperature < 39.5°C and total plasma protein concentration > 5.5 g/dL, as determined via refractometery. Each horse was weighed in order to determine the maximum target donated plasma volume based on the following equation: 22 ml of plasma/kg of donor body weight. Hair was clipped from a 20 cm x 12 cm area over each jugular vein. The areas were prepared for catheterization by washing with 2% chlorhexidine acetate saturated gauze sponge.

Approximately 1.5 mL of 2% lidocaine hydrochloride was injected intradermally over each catheterization site, and a small skin incision was made with a No. 15 scalpel blade in the anesthetized areas. A 10-gauge, 76-mm catheter was inserted through each skin incision and directed ventrally into each jugular vein of donor horses. The catheters were capped and then secured in place with 2-0 polydioxanone sutures. Plasmapheresis was performed with the horses standing in stocks. The head of each horse was placed in a sling made from padded saddle girths. The catheters of each donor horse were connected to a modified disposable collection set (4R-2252 Plasmacell-C set, Baxter-Fenwal, Lake Zurich, IL.), which was installed on 2 simultaneously operating Auto-C plasmapheresis instruments (Autopheresis-C A-200, Baxter-Fenwal, Lake Zurich, IL) . A tourniquet consisting of an elastic wrap was placed around the neck of donors and was designed to put pressure on only the right jugular vein to prevent venous collapse and ensure adequate blood flow through the catheter during the procedure. Whole blood was continuously extracted from the outflow catheter and infused with sodium citrate solution at a controlled rate, which resulted in an anticoagulant-to-whole blood ratio of 1:16. The anti-coagulated whole blood passed through the modified disposable collection set toward the separation device associated with each instrument. The separation device acted as a rotating membrane filter to separate plasma from cellular components of blood. Plasma exited the bottom port of each separation device, passed through the instrument's refractometer, and entered the 20-L–capacity collection bag. Simultaneously, concentrated blood cells exited the side port of each separation device, then passed through the cell pump and immediately back into the donor via the inflow catheter. Each instrument processed approximately 135 mL of whole blood/min.

Each horse was closely observed throughout the plasmapheresis procedure. Pulse rate, respiratory rate, characteristics of the mucous membranes, and an ECG were recorded at approximately the midpoint and at the end of each plasmapheresis procedure. Donors were administered detomidine (3 to 6 µg/kg, IV) or detomidine and butorphanol (3 to 6 µg of each/kg, IV) respectively during the procedure as needed; sedatives were administered IV through an infusion port located in the modified disposable collection set. At the end of the plasmapheresis procedure, the tourniquet was removed from the neck of each horse. Each horse then received 15 L of fluids IV via gravity flow. Catheters were removed from the jugular veins after fluid administration; hemostasis at the catheterization sites was achieved by use of 4 X 4-inch gauze sponges and slight manual pressure. The donors then were washed, visually inspected, and returned to pasture.

The collection bag was transported to a separate room for further processing. There, the plasma was homogenized and aseptically placed into 1-L high-density polyethylene bottles. Appropriate labels were applied to the bottles; bottles were stored at –80°C in a continuously monitored freezer until ready for use.

Results

Immunization

No adverse events resulting in clinical illness were found following the immunization of horses with each of these antigens. The live attenuated Sterne strain spore vaccine was apparently well tolerated after multiple administrations via both subcutaneous and intramuscular

route. Similarly the administration of 100 µg of rPA appeared to be well tolerated in both previously immunized and naïve horses.

Plasmapheresis

Three successful pheresis events were performed resulting in the collection of a total of 168.36 L of sterile immune plasma, with a mean collection volume of 18.71 L (SEM ±0.302 L), Table 2.5. The described technique was tolerated well by all horses and no adverse clinical events were reported during or following the pheresis procedure.

Discussion

The present study details the methods used in producing hyper immune plasma against *B. anthracis* from horses. The plasma produced in this study was later evaluated for specific anti-PA antibody titers in a validated ELISA, toxin neutralization activity in a mouse macrophage cell culture assay, and passive immunoprotection assays using a homologous lethal challenge model in susceptible mice.

The Sterne strain spore vaccine was chosen, in part, due to its ready availability and prior approval for use in livestock species in the United States, but also because it is a fully toxigenic spore strain potentially leading to a broader response than whole cell lysate vaccines or rPA alone. The manufacturer's adverse clinical event warning describes the potential for light to moderate swelling at the site of injection that is self-limiting.[411] Others report reversion to virulence following use of the vaccine in other species.[412] In the present study, no apparent ill effect was induced by this vaccine at any time point. To the authors' knowledge this is the first

report of horses receiving multiple doses of this vaccine in this frequency. Monthly immunization intervals were chosen for the initiation of immunity in this study, but greater evaluation of titers and duration of immunity are needed to determine if this is the most effective means of establishing and maintaining antibody production.

The technique for automated plasmapheresis in horses as described here has been previously reported and established as a safe and reliable means to collect large volumes of immune equine plasma.[400,413,414] The mean collection volumes recorded in this study were similar to the volumes reported in those works.

Polyclonal antisera have diverse immunoglobulins that target different antigens. Most passive immunization trials evaluating monoclonal preparations against PA reveal a poor correlation of anti-PA affinity and protective capacity indicating that neutralization of multiple epitopes or toxin components are necessary in immunity against anthrax.[311,380,415,416] However, in polyclonal preparations there are immunoglobulins that do not target protective epitopes and the specific neutralizing antibodies are a small fraction of the total immunoglobulin profile. Hence, polyclonal sera tends to have lower specific affinity relative to monoclonal preparations. Other short comings are lot-to-lot variations in the amount of specific antibody and potential immune response against the foreign proteins.[417,418] The advantage of equine based polyclonal antibody production are the rapid induction of immunity in donor horses, the production of large volumes of hyper immune plasma that can be collected as frequently as every two weeks, and the relative lack of cost and infrastructure requirements when compared to the production of equivalent volumes of monoclonal antibodies or human polyclonal preparations.

Table 2.1 Immunization protocol for individual horses. Four horses were immunized with a B. anthracis Sterne strain spore vaccine either subcutaneously or intramuscularly monthly for approximately 1 year. Whole blood samples were collected at predetermined intervals for analysis of specific antibody production.

Horse ID: AX 1	
Day	Immunizing Antigen
1	1.0 ml spore vaccine SC
21	1.0 ml spore vaccine SC
56	1.0 ml spore vaccine SC
91	1.0 ml spore vaccine SC
119	1.0 ml spore vaccine SC
154	1.0 ml spore vaccine SC
184	1.0 ml spore vaccine SC
210	1.0 ml spore vaccine SC
249	1.0 ml spore vaccine SC
275	1.0 ml spore vaccine SC
303	1.0 ml spore vaccine SC
332	1.0 ml spore vaccine SC
366	1.0 ml spore vaccine SC

Horse ID: AX 2	
Day	Immunizing Antigen
1	1.0 ml spore vaccine IM
21	1.0 ml spore vaccine IM
56	1.0 ml spore vaccine IM
91	1.0 ml spore vaccine IM
119	1.0 ml spore vaccine IM
154	1.0 ml spore vaccine IM
184	1.0 ml spore vaccine IM
210	1.0 ml spore vaccine IM
249	1.0 ml spore vaccine IM
275	1.0 ml spore vaccine IM
303	1.0 ml spore vaccine IM
332	1.0 ml spore vaccine IM
366	1.0 ml spore vaccine IM

Horse ID: AX 3	
Day	Immunizing Antigen
1	1.0 ml spore vaccine SC
21	1.0 ml spore vaccine SC
56	1.0 ml spore vaccine SC
91	1.0 ml spore vaccine SC
119	1.0 ml spore vaccine SC
154	1.0 ml spore vaccine SC
184	1.0 ml spore vaccine SC
210	1.0 ml spore vaccine SC
249	1.0 ml spore vaccine SC
275	1.0 ml spore vaccine SC
303	1.0 ml spore vaccine SC
332	1.0 ml spore vaccine SC
366	1.0 ml spore vaccine SC

Horse ID: AX 4	
Day	Immunizing Antigen
1	1.0 ml spore vaccine IM
21	1.0 ml spore vaccine IM
56	1.0 ml spore vaccine IM
91	1.0 ml spore vaccine IM
119	1.0 ml spore vaccine IM
154	1.0 ml spore vaccine IM
184	1.0 ml spore vaccine IM
210	1.0 ml spore vaccine IM
249	1.0 ml spore vaccine IM
275	1.0 ml spore vaccine IM
303	1.0 ml spore vaccine IM
332	1.0 ml spore vaccine IM
366	1.0 ml spore vaccine IM

Table 2.2 Immunization protocol for alternative dosing of Sterne strain spore vaccine in individual horses. Following immunization using the initial protocol two horses were randomly selected to receive an increased dose of Sterne strain spore vaccine in an attempt to maximize specific antibody titers.

Horse ID: AX 2
Previous Immunization Protocol

Day	Immunizing Antigen
1	2.0 ml spore vaccine SC
13	2.0 ml spore vaccine SC
41	2.0 ml spore vaccine SC
70	2.0 ml spore vaccine SC
98	2.0 ml spore vaccine SC
126	2.0 ml spore vaccine SC
160	2.0 ml spore vaccine SC

Horse ID: AX 4
Previous Immunized Protocol

Day	Immunizing Antigen
1	2.0 ml spore vaccine SC
13	2.0 ml spore vaccine SC
41	2.0 ml spore vaccine SC
70	2.0 ml spore vaccine SC
98	2.0 ml spore vaccine SC
126	2.0 ml spore vaccine SC
160	2.0 ml spore vaccine SC

Table 2.3 Immunization protocol for previously immunized horses receiving recombinant protective antigen. Following immunization using the initial protocol two horses were randomly selected to receive 100 µg of recombinant protective antigen (rPA) homogenized with Freund's incomplete adjuvant administered ID in an attempt to maximize specific antibody titers.

Horse ID: AX 1
Previous Immunization Protocol

Day	Immunizing Antigen
1	1.0 ml spore vaccine SC
13	100 ug rPA ID
41	100 ug rPA ID
70	100 ug rPA ID
98	100 ug rPA ID
126	100 ug rPA ID
160	100 ug rPA ID

Horse ID: AX 3
Previous Immunization Protocol

Day	Immunizing Antigen
1	1.0 ml spore vaccine SC
13	100 ug rPA ID
41	100 ug rPA ID
70	100 ug rPA ID
98	100 ug rPA ID
126	100 ug rPA ID
160	100 ug rPA ID

Table 2.4 Immunization protocol for naïve horses receiving adjuvanted recombinant protective antigen. Two previously non-immunized horses were administered 100 µg of rPA homogenized with Freund's incomplete adjuvant ID in an attempt to maximize specific antibody titers.

Horse ID: AX 5	
Day	Immunizing Antigen
1	100 ug rPA ID
13	100 ug rPA ID
41	100 ug rPA ID
70	100 ug rPA ID
98	100 ug rPA ID
126	100 ug rPA ID
160	100 ug rPA ID

Horse ID: AX 6	
Day	Immunizing Antigen
1	100 ug rPA ID
13	100 ug rPA ID
41	100 ug rPA ID
70	100 ug rPA ID
98	100 ug rPA ID
126	100 ug rPA ID
160	100 ug rPA ID

Table 2.5 Mean collection volumes for three plasmapheresis events. Three horses were subjected to plasmapheresis on three separate occasions. No adverse clinical events were reported during or after the pheresis events.

Horse ID	Event 1 (10/19/09)	Event 2 (12/9/09)	Event 3 (4/28/10)		Mean Volume
AX1	18.71 L	19.63 L	20.09 L		18.57 L
AX3	18.02 L	19.05 L	18.92 L		18.74 L
AX4	18.99 L	17.53 L	17.39 L		18.81 L
				Overall Mean	18.71 L

Chapter 3:

Validation of an anti-protective antigen ELISA for the evaluation of hyper-immune plasma derived from horses serially immunized against *Bacillus anthracis* Sterne strain

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Abstract

The potency test for anthrax vaccines has historically involved the challenge of actively or passively immunized laboratory animals with a fully virulent strain of *Bacillus anthracis.* Lethal challenge studies with the archetypal virulent strains such as *B. anthracis* Ames strain present considerable difficulties laboratory management and handling and are too inefficient for the early evaluation alternative therapeutic interventions. An ELISA for the evaluation of antibody response to protective antigen (PA) in horses immunized with the Sterne 34F2 strain spore and recombinant protective antigen (rPA) vaccines was developed. The objective of this work was to study the performance of this assay in terms of the guidelines set forth by the International Conference on Harmonics (ICH) and the Center for Biologics Evaluation and Research (CBER) for analytical procedures. We have demonstrated a working range for this assay (73-1581 EU/ml) on the bases of the following parameters: linearity (25 and 1,662 EU/ml, $R^2 = 0.9988$, $p < 0.001$), accuracy (94.8 - 105.4 %, recovery within the range of 25 and 1,662 EU/ml), precision (≤ 17.6 % CV, repeatability; ≤ 15.7 and ≤ 13.1 % CV, intermediate precision

per day and per analyst, respectively), limit of detection (2.25 EU/ml) and limit of quantitation (25 EU/ml). The assay was also demonstrated to be specific for the evaluation of anti-PA antibodies. Based on the assay performance characteristics it was determined that the assay was adequate for use in *B. anthracis* immunogenicity testing in horses.

Introduction

Bacillus anthracis is the causative agent of anthrax. One important aspect of virulence for *B. anthracis* is the production of three exotoxin proteins that encoded on the pXO1 plasmid. These proteins are known as lethal factor (LF), edema factor (EF) and protective antigen (PA). These three subunits combine in binary fashion to form the active toxins, lethal toxin and edema toxin. The common protein subunit in these toxins is the 83 kDa protein known as protective antigen. After binding to its cell surface receptor PA polymerizes into a heptamer pore and mediates cellular entry of the effector molecules, LF and EF.[134] Targeting PA is key to protection and has been demonstrated in several *B. anthracis* toxin and live virulent challenges.[419-421]

An equine source polyclonal hyper-immune plasma targeted against *B. anthracis* was developed. The hyper-immune plasma was derived from horses hyper-immunized against the acapsular, toxigenic Sterne 34F2 strain of *B. anthracis.* In the development of this plasma, a need arose for the evaluation of the titer response against PA. Historically, potency tests for *B. anthracis* vaccines and passive immunotherapy involved the lethal challenge of passively or actively immunized guinea pigs.[422] These bioassays are expensive and handling fully virulent *B. anthracis* requires specialized containment facilities. Therefore a well-defined immunogenicity assay was needed to overcome this challenge and provide consistent and accurate assessment of

the immune response of horses during the immunization process. Immunogenicity assays are designed to assess antibody response against a set dose of vaccine. Two types of immunogenicity assays have been developed for the assessment of anti-PA antibodies in other species; antigen-specific ELISA and toxin neutralization assays.[423]

In order to assess the antibody titer response in hyper-immunized horses an indirect ELISA was developed and validated. The objective of validation of an analytical procedure consists of defining performance characteristics and determining if the assay consistently meets its intended purpose and pre-determined specifications and quality attributes. The International Conference on Harmonic (ICH) and the Center for Biologics Evaluation and Research (CBER) have set forth guidelines for validating assays which are summarized in the validation of compendial procedures.[423,424] We conducted a series of evaluations designed to examine the assays capacity for specificity, linearity, limit of detection, accuracy, precision and limit of quantitation.

Materials and Methods

Antigens

Recombinant protective antigen (rPA) was obtained from List Biological Laboratories (Campbell, CA). Each vial was reconstituted with 1.0 ml of sterile distilled water to create a concentration of 1 µg/µl and stored at -80^0 C until ready to use. The ovalbumin used as a control in the specificity experiments was obtained from Thermo Scientific (Imject Ovalbumin, Thermo

Scientific, Rockford, IL) Each vial was reconstituted with 1.0 ml of sterile distilled water to create a concentration of 1 µg/µl and stored at -80^0 C until ready to use.

Plasma Samples

Plasma was obtained from horses hyper-immunized with *Bacillus anthracis* Sterne strain 34F2 spore vaccine (Anthrax Spore Vaccine, Colorado Serum Company, Denver Colorado) at monthly intervals for approximately one year. At each sampling 20 ml of whole blood was collected into syringes pre-loaded with sodium citrate as an anticoagulant. Each sample was centrifuged at 5000 x *g* for 30 minutes. The plasma was then aspirated from the red cells and divided into 2 ml aliquots and stored at -80^0 C until ready to use.

Four hyper-immune plasma samples were randomly chosen from the samples collected after 56 days (representing fully primed individuals) and pooled to make a reference plasma sample (REF) that would be used to develop a titration curve and would then serve as the positive standard for each subsequent assay.

Plasma used for negative standards were obtained from two horses presumed to have had no previous exposure to *B. anthracis* or the vaccine (Normal horse plasma, NHP). Samples were collected and processed as described above.

Quantification of anti-PA antibodies

Wells of an Immulon 2 HB, 96-well round bottom microtiter plate (Southern Biological, Birmingham, AL) were coated with 100 µl of a coating solution (rPA diluted at 1 µg/ml in a 0.01

M PBS solution, pH 7.4). Plates were sealed with parafilm and incubated overnight (16 +/- 2 hours) refrigerated at 4^0 C. Following antigen coating the plates were washed three times using 300 µl/well of washing buffer (0.01 M PBS and 0.1% Tween 20) each wash. Unknown, REF, and NHP samples were thawed at 4^0C overnight and kept on ice until ready to use. Unknown samples were pre-diluted 1:500 in dilution buffer and 200 µL were added to wells of the first row. REF samples were also pre-diluted 1:500 in dilution buffer and 200 µL were added to two wells of the first row. NHP samples were diluted 1:50 in dilution buffer and 200 µL were added to the remaining two wells in the first row. 100 µl of dilution buffer (0.01 M PBS, 5% goat serum, and 0.5% Tween 20) was added to all remaining wells. Twofold serial dilutions were made across the plate by mixing and transferring 100 µl of diluted antibodies between adjacent rows. The plate was sealed and incubated at 37^0 C for 1 hour. After the plate was washed as described previously, 100 µl of 1:1000 dilution of goat anti-horse antibody conjugated to HRP (Jackson Immunoresearch Lab, INC, West Grove, PA) in dilution buffer was added to each well and the plate was sealed and incubated again at 37^0C for 1 hour.

Following three additional post-incubation washes, described previously, 100 µl of the substrate ABTS (2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt, Thermo Scientific, Rockford, IL) was added to each well. The plate was sealed and incubated at room temperature protected from light for 20 min. Optical Density (OD) was read at dual wavelength (405 and 550 nm) in a Bio-Tek Elx800 plate reader (Bio-Tek Instruments, Inc. Winooski, VT).

Titration Curve

Eight rPA-coated microtiter plates were prepared as described above. Following overnight incubation, approximately 200 µl of REF diluted 1:500 in dilution buffer was added to the first well of three rows on each plate. Twofold serial dilutions were made across the plate by mixing and transferring 100 µl of diluted antibodies between adjacent rows. In this way each plate contained triplicate REF sample dilutions. The samples were then subjected to the anti-PA ELISA as described. A titration curve was drawn using individual OD values at each dilution point from the 24 assays relating the $\log_{10}$ assigned units/mL vs. $\log_{10}$ OD. Once established, REF was used a positive control in each assay thereafter, Figure 3.1.

Specificity

Two experiments were carried out in order to demonstrate the specificity of this ELISA. In the first, a 750 µl volume of a 1:500 dilution of REF was mixed with an equal volume of rPA at 100 µg/ml and incubated for 1 hour at 37^0 C. After incubation, 100 µl of the mixture were deposited in three wells in each of two rPA-coated microtiter plates. Then, 100 µl of NHP, diluted 1:50 in dilution buffer was added to an additional four wells on each of the plates. Finally, un-treated REF samples, pre-diluted 1:500 in dilution buffer, were added to four wells on each microtiter plate that had been left uncoated.

In the second experiment, two rPA microtiter plates were prepared as described except that ovalbumin (100 µl diluted at 1 µg/ml in a 0.01 M PBS solution), a heterologous protein to *B. anthracis* PA, was used to coat six wells in each plate. After the overnight incubation, one of the

plates was treated with a blocking solution containing 0.5% skim milk and 0.05% Tween 20 in PBS for 1.5 hours at room temperature (Plate I), while the other remained untreated (Plate II). Reference plasma, pre-diluted 1:500 in dilution buffer, were added to two top wells of the plates and serially diluted twofold. In addition, 100 µl of NHP, diluted 1:50 in dilution buffer was added to an additional four wells on each of the plates. Finally, 100 µl of pre-diluted 1:500 REF in dilution buffer was added to each of the six wells coated with ovalbumin on each plate. Both plates were subjected to the anti-PA ELISA as described previously.

Limit of Detection

Two samples of NHP were diluted 1:50 in dilution buffer and subjected to the anti-PA ELISA in triplicate in two microtiter plates, on each of the five days within two weeks. Fresh dilutions and rPA-coated plates were individually prepared and used daily. Limit of detection was estimated by interpolating the mean of 60 OD values, plus three standard deviations, in a titration curve of REF relating $\log_{10}$ assigned units/ml per well vs. $\log_{10}$ OD. The antilog of the interpolated value was subsequently corrected by the dilution of the sample (1:50).

Linearity

Seven unknown plasma samples of varying antibody concentrations, S1 through S7, were used for the assessment of assay linearity. S1 was a randomly selected hyper-immune plasma sample taken for one of the *B. anthracis* horses. Samples S2 through S7 consisted of individually prepared dilutions of S1 in twofold increments, from 1:2 through 1:64. All samples were diluted 1:500 in dilution buffer and subjected to the anti-PA ELISA. All seven samples were randomly

distributed in each of three coated microtiter plates. Log_{10} estimates of antibody concentration per sample (three reads, one per plate) were plotted vs. corresponding log_{10} reciprocal dilution, and the correlation coefficient, the y-intercept, the slope of the regression line and also the *p*-value for the lack of fit were calculated.

Accuracy

Units per ml of each of four freshly made samples (S1, S3, S5 and S6 equivalents to the linearity assay) were estimated by anti-PA ELISA. Each sample was assayed in triplicate in each of three plates. Accuracy is expressed as percent recovery: mean estimated units per ml/assigned units per ml x 100. The assigned concentration of each sample represents the geometric mean estimate of S1 units (n=16), corrected for each sample's dilution.

Precision

Three samples (S1, S3 and S5) were individually prepared and diluted 1:500 in dilution buffer. A triplicate of each sample was serially diluted twofold in each of three microtiter plates and subjected to the anti-PA ELISA on three consecutive days by two analysts. Results are expressed as %CV (SD/mean x 100).

Limit of Quantification

Three samples showing relatively low antibody titers (equivalent to samples S5, S6, and S7) were freshly prepared. Each sample was diluted 1:100 in dilution buffer in triplicate and

serially diluted in each of three microtiter plates. Anti-PA ELISA was performed on three consecutive days. Results are expressed as % CV (SD/mean x 100).

Statistical Analysis

The titration and linearity curves were drawn in Microsoft Excel® 2010 and were analyzed by linear regression analysis. Student's T tests were used compare mean OD values for the specificity experiment. All statistical analyses were conducted using IBM SPSS Statistics version 21.

Results

Specificity

Plasma from *naïve* horses (NHP) demonstrated no significant reactivity with PA in the ELISA comparable to REF plasma when applied to uncoated wells ($p = 0.092$), Table 3.1a. The pre-incubation of free rPA with REF reduced the binding activity to background levels which was not significantly different from the OD values observed for NHP ($p = 0.213$), Table 3.1a. The results of Table 3.1b indicate that a separate blocking step is not required in this system, as the blocking solution tested only resulted in a significant effect for the highest REF dilutions (1:500). In addition, the REF demonstrated no significant difference in binding activity compared to NHP when ovalbumin was used to coat to the wells of the microtiter plate ($p=0.482$), Table 3.1c.

Limit of Detection

Table 3.2a summarizes the OD values obtained from repeated testing in the PA-ELISA of the two NHP samples. The mean OD value for NHP and standard deviation was calculated (0.045 and 0.019, respectively) and interpolated in the titration curve, Table 3.2b and Figure 1. The anti-PA ELISA is capable of detecting antibody levels above 2.25 EU/ml.

Accuracy

Results of the accuracy assessment are presented in Table 3.3. The mean OD value for S1 after 9 assays was 2.494 (Log_{10} = 0.396) which represents an interpolated estimate of 1494 EU/ml (approximately 1500 EU/ml). Based on this observation, the anticipated antibody concentration for S3, S5 and S6 was 375 EU/ml, 93.75 EU/ml, and 46.87 EU/ml, respectively. The percent recovery for S1 ranged from 1456.5 - 1699.5 EU/ml (97.1% - 113.3%). The percent recovery for S3 ranged from 367.87 – 374.62 EU/ml (98.1% - 99.9%). The percent recovery for S5 ranged from 88.2 – 89.62 EU/ml (94.1% - 95.6 %). The percent recovery for S6 ranged from 43.92 – 46.87 (93.7% - 100%). On the basis of the criterion established by the ICH, the assay is accurate between 46 and 1500 EU/ml (94.8 - 105.4 % recovery within this range).

Linearity

In the accuracy experiment the mean estimated EU/ml for S1 was approximately 1500 EU/ml. Table 3.4 displays the predicted EU/ml for each sample (S1 – S7) following twofold dilution. Subjecting each sample in triplicate to the anti-PA ELISA demonstrated the assay to be linear between 25 and 1,662 EU/ml. The relationship between the EU/ml estimated in each

sample and its dilution is significantly linear ($R^2 = 0.9988$, *P* value = 0.000018), Figure 3.2, indicating that the assay accurately predicted the estimated EU/ml at each dilution point, Table 3.4.

Precision

Table 3.5 summarizes assay variability expressed as % CV at two levels of repeatability: intra-plate (triplicate estimates per sample per plate) and intra-day (three assays performed each day) and two levels of intermediate precision: inter-day (three days), and inter-analyst (two analysts on the same day). The assay displayed acceptable repeatability and intermediate precision on the basis of the validity criteria in the 73-1581 EU/ml range establishing this as the reliable working range of anti-PA antibodies for this assay, Table 3.7.

Limit of Quantitation

To define the limit of quantitation, precision was estimated in the range 25-71 EU/ml, Table 3.6. The calculated % CV for this range fell well below that of the acceptable % CV for repeatability establishing 25 EU/ml as the lowest limit anti-PA antibodies can be reliably quantified, Table 3.7.

Discussion

Protective antigen is a critical component of the protective immune response to *B. anthracis* infection. The pursuit of new vaccines and monoclonal and polyclonal immunotherapies requires an efficient and effective assessment of response during the initial

stages of development. We have developed an anti-PA ELISA designed to measure the immune response against PA in horses. This assay was studied using the validation parameters suggested by the ICH and CBER and is consistent with other literature where serologic assays have been used to verify immunologic responses and vaccine analyses.[425] Acceptance criteria for the validation of this assay were based on the performance of similar ELISAs.[426,427] The intended use of the assay is to measure antibodies against PA in the plasma of horses vaccinated with anthrax vaccines. The vaccine used in this study was the *B. anthracis* Sterne strain spore vaccine which produces all three components of the lethal and edema toxins. In addition, other epitopes such as exterior spore antigens and bacterial cell surface antigens are present and have been shown to induce an immune response in other species. Despite the presence of this diverse immune response, it was demonstrated that this ELISA can consistently quantify anti-PA antibodies in the plasma of horses. Saturating a plasma mixture with free PA reduced the level of binding to that equivalent of naïve horse plasma. When ovalbumin was used as a heterologous antigen in the solid phase of the ELISA, plasma from horses immunized against anthrax demonstrated negligible reactivity indicating that the response seen to PA in this assay was not the result of non-specific binding.

The results of the accuracy and precision assessment fell well below the guidelines established by the ICH indicating that this ELISA is both highly repeatable and robust for the range of samples assayed. Some sources recommend a lower standard of 10% CV necessary to define suitable precision. The samples evaluated in the precision assessment represented approximately 100, 80, 50 and 20% of maximal activity. Because both the highest and lowest

concentrations of antibody sampled in the present study were demonstrated to be suitably repeatable the true working range of EU/ml was not fully established. Often extremes in antibody concentration show less precise measurements due to non-uniform error in the assay and further evaluation will be necessary to determine what these levels are for this assay.

The limit of quantitation can be defined as the assay's measure of sensitivity or capacity to measure the smallest amount of target analyte. In the present study the lowest derived antibody concentration was estimated to be 25 EU/ml, which displayed acceptable repeatability. The limit of detection is a means of establishing background levels of reactivity in the plasma of horses expected to be naïve to *B. anthracis.* Based on the results of several repeated assays of two normal horse plasma samples, the lowest detectable anti-PA antibody concentration that was statistically significant from background levels was 2.25 EU/ml. The incorporation of three standard deviations above the mean OD value for NHP yields a 99% confidence level in the difference observed.

Based on these observations, the results of this study validate the anti-PA ELISA described herein ensuring its usefulness in accurately and reliably assessing the antibody response to PA in the plasma of horses.

Table 3.1a Specificity: Free rPA successfully binds with anti-PA antibodies and reduces the interaction of the antibodies with bound rPA. REF plasma was pre-incubated with free rPA for 1 hour and then subjected to the anti-PA ELISA alongside naïve plasma (NHP) and un-treated REF plasma in uncoated wells.

Sample ID	Sample Pre-treatment	Dilution in well	Mean OD values	p value
REF	Pre-incubated with rPA	1:500	0.076	0.21*
NHP	None	1:50	0.064	0.092**
REF	Uncoated Wells	1:500	0.056	

* p value of significance between REF pre-incubated with rPA and NHP

** p value of significance between control wells and NHP

Table 3.1b Specificity: Blocking a microtiter plate with a milk based blocking agent does not significantly alter the OD values obtained. Two microtiter plates were coated with rPA. Following overnight incubation Plate I was treated with a blocking agent while Plate II was left untreated. A sample of REF was serially diluted in duplicate and subjected to the anti-PA ELISA with each plate to determine if a blocking step induced significant changes in OD values.

Sample ID	Sample Pre-treatment	Dilution in well	OD values (mean)		p values*
			Plate I	Plate II	
REF	None	1:500	1.367	1.217	0.021
		1:1000	0.918	1.045	0.166
		1:2000	0.755	0.063	0.345
		1:4000	0.601	0.823	0.366
		1:8000	0.490	0.735	0.343
		1:16000	0.364	0.569	0.295
		1:32000	0.221	0.391	0.381
		1:64000	0.152	0.266	0.380
		1:128000	0.106	0.161	0.413
		1:256000	0.087	0.120	0.374
		1:512000	0.078	0.100	0.444
		1:1024000	0.061	0.186	0.207

*p value of significance between OD values obtained from each plate

Table 3.1c Specificity: REF plasma specifically binds to rPA coated wells but does not bind ovalbumin coated wells.

Sample ID	Plate coating	Dilution in well	OD values (mean) *	p values
REF	Ovalbumin	1:500	0.041	0.482
REF	rPA	1:500	1.292	
NHP	Ovalbumin	1:50	0.038	

*combined means from blocked and unblocked plates.

Table 3.2a Limit of Detection

Two NHP samples were subjected to repeated anti-PA ELISA to establish the lowest limit of detection for this assay.

Day	Sample	OD Values	
		Plate I	Plate II
1	1	0.032	0.025
		0.029	0.028
		0.031	0.026
	2	0.033	0.028
		0.031	0.022
		0.023	0.015
2	1	0.040	0.036
		0.040	0.029
		0.035	0.018
	2	0.079	0.056
		0.063	0.062
		0.055	0.076
3	1	0.085	0.092
		0.090	0.056
		0.086	0.045
	2	0.048	0.062
		0.046	0.063
		0.043	0.061
4	1	0.045	0.072
		0.045	0.07
		0.041	0.069
	2	0.034	0.044
		0.032	0.042
		0.025	0.043
5	1	0.035	0.057
		0.032	0.052
		0.035	0.056
	2	0.041	0.028
		0.028	0.036
		0.021	0.042
Overall Mean		0.045	
St. Dev		0.019	
Mean NHP OD value = 0.045 ± 0.019 LOD = 0.045 + (3 X 0.019) = 0.102			

Table 3.2b Interpolated Limit of Detection

The interpolated limit of detection for the anti-PA ELISA was derived from the addition of the mean OD value for naïve plasma and three standard deviations and the interpolation of this value from the titration curve (**Figure 1**). This measure represents the lowest effective assessment of anti-PA antibody titers (EU/ml) in this assay.

Mean NHP + 3 STD OD Value:	0.102	
Log_{10} (0.102):	-0.9914	
Interpolated EU/ml:	0.045	EU/ml
Corrected for dilution:	2.25	EU/ml

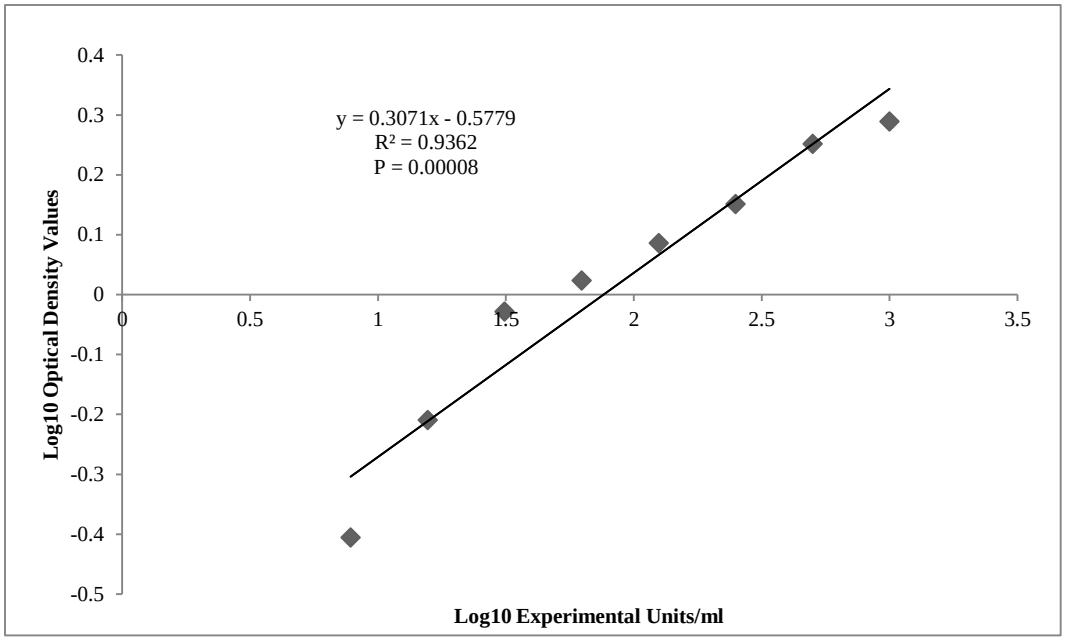

Figure 3.1 Titration Curve

The titration curve was drawn using individual OD values from 24 assays (8 assays in triplicate).

Table 3.3a Accuracy

The mean OD value for S1 after 9 assays was 2.494 (Log_{10} = 0.396). The mean OD value was interpolated to represent an estimated 1494 EU/ml.

Mean S1 OD Value	2.494
Log_{10} OD	0.396
Interpolated Log_{10} (EU/ml)	3.174
Assigned EU/ml for S1	1494 (~1500)

Table 3.3b Accuracy

Units per ml of each of four freshly made samples (S1, S3, S5 and S6 equivalents to the linearity assay) were estimated by anti-PA ELISA. Each sample was assayed in triplicate in each of three plates. Accuracy is expressed as percent (%) recovery: mean estimated units per ml/assigned units per ml x 100.

	Replicates	Target sample (EU/ml)			
		S1 (1500) % Recovery	S3 (375) % Recovery	S5 (93.75) % Recovery	S6 (46.87) % Recovery
Plate 1	1	98.9	98.1	95.0	98.1
	2	106.9	99.1	94.1	98.5
	3	107.4	98.1	95.3	95.2
Plate 2	4	113.3	98.1	94.4	94.1
	5	107.2	99.9	95.6	95.9
	6	102.2	99.5	94.1	98.5
Plate 3	7	113.0	99.1	95.9	97.7
	8	102.5	98.1	95.6	100.0
	9	97.1	99.7	93.8	93.7
Mean		105.4	98.9	94.8	96.8

Table 3.4 Linearity

Comparison of the predicted EU/ml and the assay estimated EU/ml of twofold serially diluted samples.

Sample ID	Predicted EU/ml	Estimated EU/ml
S1	1500	1662
S2	750	742
S3	375	376
S4	188	189
S5	94	91
S6	47	45
S7	23	25

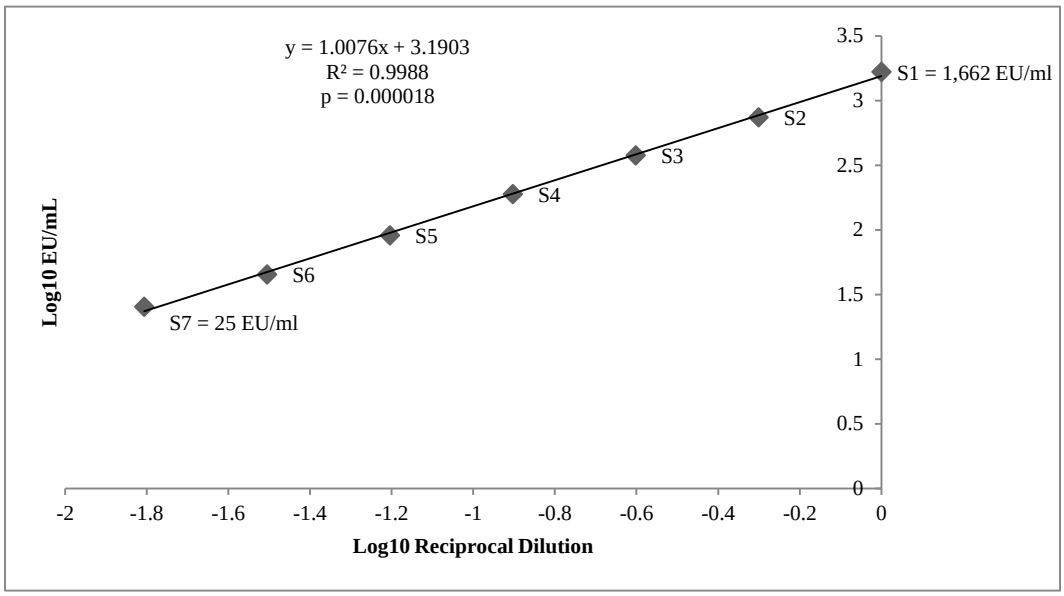

Figure 3.2 Linearity

An unknown plasma sample (S1) was serially diluted twofold (S2 – S7) and subjected to the anti-PA ELISA in triplicate. The observed OD values were used to interpolate the estimated EU/ml for each sample from the titration curve. The $\log_{10}$ EU/ml was then plotted against the $\log_{10}$ of the reciprocal dilution to determine if a linear relationship existed between the estimated EU/ml and the dilution factor.

Table 3.5 Precision

Results of repeated analyses of three samples were compared across multiple plates performed on multiple dates by two analysts to determine the repeatability and robustness of the assay. Results are expressed as %CV (SD/mean x 100).

Sample	Mean (EU/ml)	%CV						Inter-analyst
		Analyst 1			Analyst 2			
		Intra-plate	Intra-day	Inter-day	Intra-plate	Intra-day	Inter-day	
S1	1581	3.3	7.0	9.0	12.6	14.0	15.7	13.1
		4.5			1.3			
		3.9			9.8			
		11.1	9.4		1.3	10.1		
		9.3			7.7			
		4.0			1.7			
		0.9	7.7		6.1	16.4		
		11.1			15.3			
		5.3			16.6			
S3	323	15.9	12.3	10.4	9.2	11.8	10.4	10.5
		7.2			1.3			
		1.9			9.9			
		11.0	9.4		11.2	7.8		
		0.3			6.7			
		5.4			2.8			
		1.7	8.3		6.3	10.5		
		12.0			16.8			
		0.9			7.5			
S5	73	17.6	13.8	10.9	3.7	8.2	10.7	10.8
		9.3			2.5			
		4.9			12.0			
		4.0	9.7		3.0	5.9		
		6.1			3.1			
		10.3			9.3			
		1.2	4.4		1.4	12.8		
		5.8			15.1			
		2.5			7.7			

Table 3.6 Limit of Quantitation

Three samples showing relatively low antibody titers were analyzed across multiple plates performed on multiple days to determine the lowest antibody concentration that demonstrated adequate repeatability. Results are expressed as %CV (SD/mean x 100).

Sample	Mean (EU/ml)	% CV		
		Intra-plate	Intra-day	Inter-day
S5	71	8.1	10.5	10.2
		9.7		
		12.7		
		3.7	10.1	
		10.9		
		10.4		
		4.8	9.1	
		6.2		
		7.5		
S6	44	7.3	5.6	6.6
		3.8		
		4.0		
		5.7	6.5	
		3.8		
		7.4		
		5.2	6.3	
		6.2		
		6.6		
S7	25	1.7	5.5	7.0
		10.4		
		3.0		
		7.0	7.6	
		5.8		
		9.7		
		4.8	7.3	
		5.0		
		6.2		

Table 3.7 Acceptance Criteria and Characteristics of the anti-PA Equine ELISA

Summary of the performance characteristics of the anti-PA ELISA and the acceptance criteria set forth by the ICH.

Assay Characteristics	Acceptance Criteria	Results
Precision-Repeatability		
Intra-plate	% CV < 20	< 17.6
Inter-plate		< 16.4
Intermediate Precision		
Days	% CV < 25	< 15.7
Analyst	% CV < 30	< 13.1
Accuracy	80 – 120 %	94.8 – 105.4 %
Limit of Detection	—	2.25 EU/ml
Limit of Quantification	—	25 EU/ml
Linearity	—	Working Range: 25 and 1,662 EU/ml $R^2 = 0.9988$ *P* value = 0.000018

Chapter 4:

Assessment of protective antigen and toxin neutralizing antibody titers in hyper-immune plasma derived from horses immunized against *Bacillus anthracis* Sterne strain

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Abstract

The *Bacillus anthracis* Sterne strain spore vaccine is currently licensed for use in livestock species in the United States and other counties. Sterne strain is an attenuated toxigenic acapsular ($pXO1^+$, $pXO2^-$) strain of *B. anthracis* that is capable of inducing strong protective responses against protective antigen (PA), lethal factor (LF) and edema factor (EF). Of these antigens numerous natural and experimental infection studies indicate that PA is the immunodominant antigen. In this study, the initial evaluation of the protective capacity of plasma from horses immunized against Sterne strain spore and recombinant PA (rPA) vaccines was determined. Antibody responses to PA were evaluated in an indirect ELISA and were found to be as high as 1:512,000 in some horses. The geometric mean titers for horses immunized with spore vaccine alone, for horses immunized with the spore vaccine then immunized with rPA, for horses immunized with the spore vaccine then immunized with twice the volume of spore vaccine, and for horses immunized with rPA alone was 1:43,031 (SEM±3995, CI 95% 1:35,675 – 1:51,906), 1:213,027 (SEM±32,252, CI 95% 1:152,849 – 1:296,897), 1:83,912 (SEM±16,818, CI 95% 1:54,046 – 1:129,971) and 1:34,912 (SEM±13,961, CI 95% 1:15,035 – 1:81,066),

respectively. Neutralizing antibodies were assessed using mouse macrophage J774A.1 cells in an LF-induced cytotoxicity assay. Several horses developed neutralizing titers as high 1:1,024. The geometric mean neutralizing antibody titers for horses immunized with the Sterne strain spore vaccine alone, for horses immunized with the spore vaccine then immunized with rPA, for horses immunized with the spore vaccine then immunized with twice the volume of spore vaccine, and for horses immunized with rPA alone was 1: 130 (SEM±2.26, CI 95% 1:126 – 1:135), 1: 964 (SEM±56, CI 95% 1:849 – 1:1,094), 1: 683 (SEM ± 171, CI 95% 1:408 – 1:1,143) and 1:478 (SEM ± 5.5, CI 95% 1:9.5 – 1:68), respectively. Overall, this study demonstrates that plasma derived from horses immunized against *B. anthracis* Sterne strain and rPA provides strong *in vitro* correlates of protection and has the potential for further investigation as passive immunotherapy in *in vivo* infection models.

Introduction

Bacillus anthracis is the causative agent of anthrax. *B. anthracis* induces virulence in large part through the production of three toxin polypeptides, lethal factor (LF), edema factor (EF) and protective antigen (PA); all encoded on the pXO1 plasmid. These proteins combine in binary fashion to form two toxins, lethal toxin and edema toxin.[401] The common protein subunit of these toxins is PA, an 83 kDa receptor binding component that mediates cellular entry of the toxin effector molecules.[402] Targeting PA is key to protection and has been demonstrated in several *B. anthracis* toxin and live virulent challenges.[428-432] Targeting PA is key to protection and has been demonstrated in several *B. anthracis* toxin and live virulent challenges.[380,397,433-436]

An equine source polyclonal hyper-immune plasma targeted against *B. anthracis* was developed. Because the *B. anthracis* Sterne strain possesses the pXO1 plasmid all three effector molecules of the toxin are produced during logarithmic growth. Historically, potency tests for *B. anthracis* vaccines and passive immunotherapy involved the lethal challenge of passively or actively immunized guinea pigs with a virulent strain of *B. anthracis.*[437] These bioassays are expensive and handling fully virulent *B. anthracis* requires specialized containment facilities.[438] Therefore well-defined immunogenicity assay were needed to overcome this challenge and provide consistent and accurate assessment of the immune response of horses during the immunization process. Immunogenicity assays are designed to assess antibody response against a set dose of antigen. Two types of immunogenicity assays have been developed for the assessment of anti-PA antibodies in other species; antigen-specific ELISA and toxin neutralization assays.[439,440] In this study plasma from immunized horses was evaluated in each of these assays for the presence of PA specific antibodies and toxin neutralizing activity.

Materials and Methods

Antigens

The *B. anthracis* Sterne strain spore vaccine used was the commercially available Anthrax Spore Vaccine, licensed for use in equine (Colorado Serum Company, Denver, CO). Each vial was maintained at 4^0 C protected from light until ready to administer. The recombinant protective antigen (rPA) and recombinant lethal factor (rLF) was obtained from List Biological Laboratories (Campbell, CA). Each vial was reconstituted with 1.0 ml of sterile distilled water to create a concentration of 1 µg/µl. Following reconstitution, the antigen was aliquoted in 20 µl

volumes and stored at -80^0 C until ready to use. The rPA vaccine was prepared fresh the day of immunization as follows; 100 µg of rPA was thawed at 4^0 C overnight and combined with 500 µl Freund's incomplete adjuvant then homogenized and drawn into a syringe.

Plasma Samples

Plasma was obtained monthly from horses immunized with the *B. anthracis* Sterne strain vaccine and/or rPA homogenized with Freund's incomplete adjuvant. At each sampling up to 20 ml of whole blood was collected into syringes pre-loaded with sodium citrate as an anticoagulant. Each sample was centrifuged at 5000 x *g* for 30 minutes. The plasma was then aspirated from the peripheral blood cells and divided into 2 ml aliquots and stored at -80^0 C until ready to use.

Four hyper-immune plasma samples were randomly chosen from the samples collected after 56 days (representing fully primed individuals based on anti-PA ELISA results) and pooled to make a reference plasma sample (REF PLAS) to develop a titration curve and serve as the positive standard for each subsequent assay.

Plasma used for negative standards were obtained from two horses with no known exposure or history of immunization against *B. anthracis* (Normal horse plasma, NHP). Samples were collected and processed as described for principal samples.

Spore Vaccine Immunization Protocol

The following description is provided in outline format in Table 4.1. Each horse was immunized with 1.0 ml of *B. anthracis* Sterne strain spore vaccine administered subcutaneously

(SC) in the neck. A booster dose was administered SC on Day 21. Following these initial doses two horses were randomly selected with the aid of computer software and continued to be administered 1.0 ml spore vaccine SC in the neck monthly from Day 56 to Day 155, while the remaining two horses received 1.0 ml spore vaccine IM in the neck at the same time periods. Whole blood samples were collected in sodium citrate from each horse on Days 0, 21, 56 and monthly thereafter, and plasma was separated stored at -80 ^{0}C until ready for analysis.

On Day 185, one horse from each subset was randomly selected to continue immunizations monthly as described. These immunizations occurred monthly from Day 185 until Day 366. For the remaining two horses immunization was withheld in an attempt to determine the persistence of antibody titers over time.

2X Spore Vaccine Immunization Protocol

Following the initial immunization protocol, two horses that had previously been immunized against *B. anthracis* according to the schedule above were randomly selected to receive an increased dosage of spore vaccine in an attempt to maximize antibody titer response. Each horse was immunized with 2.0 ml of spore vaccine administered SC in the neck. A second immunization was administered 14 days after the initial dose and then monthly thereafter for a total of six immunizations, Table 4.2.

Recombinant Protective Antigen Immunization Protocol in Primed Horses

Following the initial immunization regime, the remaining two horses that had previously been immunized against *B. anthracis* were randomly selected to receive immunization with 100

µg of *B. anthracis* recombinant protective antigen (rPA). First, each horse received a single booster immunization of 1.0 ml *B. anthracis* spore vaccine. Thirty days following the booster immunization, each horse was administered 100 µg of rPA homogenized with Freund's incomplete adjuvant intradermally (ID) in the skin along either side of the neck. Intradermal administration of 100 µg rPA was repeated monthly for six total immunizations, Table 4.3.

Recombinant Protective Antigen Immunization Protocol in Non-Primed Horses

To determine the ability of rPA alone to elicit specific antibody titers against *B. anthracis*, two naïve Percheron horses were obtained and determined to be healthy and free of infectious diseases as outlined above. A primary immunization of 100 µg of rPA homogenized with Freund's incomplete adjuvant was administered ID in the neck of each horse. Booster immunizations were administered approximately 30 days apart for a total of six total immunizations, Table 4.4.

Quantification of anti-PA antibodies

Wells of an Immulon 2 HB, 96-well round bottom ELISA plate (Southern Biological, Birmingham, AL) were coated with 100 µl of a rPA coating solution (rPA diluted at 1 µg/ml in a 0.01 M PBS solution, pH 7.4). Plates were sealed with parafilm and incubated overnight (16 +/- 2 hours) refrigerated at 4^0 C.

Following antigen coating the plates were washed three times using 300 µl/well of washing buffer (0.01 M PBS and 0.1% Tween 20) each wash. Principal, REF PLAS, and NHP samples were thawed at 4^0C and kept on ice until ready to use. Principal samples were pre-

diluted 1:500 in dilution buffer and 200 µl were added to wells of the first row and performed in triplicate. Reference plasma samples were also pre-diluted 1:500 in dilution buffer and 200 µl were added to two wells of the first row. Normal horse plasma samples were diluted 1:50 in dilution buffer and 200 µl were added to the remaining two wells in the first row. One hundred microliters of dilution buffer (0.01 M PBS, 5% goat serum, and 0.5% Tween 20) was added to all remaining wells. Twofold serial dilutions were made across the plate by mixing and transferring 100 µl of diluted antibodies between adjacent rows. The plate was sealed and incubated at 37^0 C for 1 hour.

After the plate was washed, 100 µl of 1:1000 dilution of goat anti-horse antibody conjugated to HRP (Jackson Immunoresearch Lab, INC, West Grove, PA) in dilution buffer was added to each well and the plate was sealed and incubated again at 37^0C for 1 hour.

Following three additional post-incubation washes as above, 100 µl of the substrate ABTS (2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt, Thermo Scientific, Rockford, IL) was added to each well. The plate was sealed and incubated in the dark for 20 minutes. Optical Density (OD) was determined at dual wavelength (405 and 550 nm) in a Bio-Tek Elx800 plate reader (Bio-Tek Instruments, Inc. Winooski, VT).

Antibody mediated neutralization of LF-induced lysis

Mouse macrophage J774A.1 cells (ATCC TIB-67) were obtained from American Type Culture Collection (Manassas, VA) and grown in high-glucose Dulbecco's modified Eagle medium containing l-glutamine and supplemented with 10% fetal bovine serum and 1%

penicillin-streptomycin (10,000 U/ml penicillin G sodium and 10,000 μg/ml streptomycin sulfate).

For neutralization assays, two-fold dilutions of equine plasma (starting at 1:32) were prepared in supplemented DMEM. Recombinant protein solutions of rPA (1.0 μg/ml) and rLF (1.0 μg/ml) were added to the serum dilutions, and the mixtures were incubated for 10 minutes at 37°C with shaking. J774A.1 cells were diluted to 2×10^6 cells/ml, and 100 μl was added to a sterile 96-well flat-bottomed tissue culture plate and incubated for 2 hours. The media was removed from the cells and replaced with 100 μl of the serum dilutions containing PA plus LF, and the plates were incubated for 4 hours at 37°C in 5% CO_2. alamarBlue (80% solution in Hanks balanced salt solution; Trek Diagnostic Systems Inc., Westlake, OH) was added at 10% of the well volume, and the cells were incubated for 20 h at 37°C in 5% CO_2. Absorbance at 570 nm (to detect oxidized alamarBlue) and 595 nm (to detect reduced alamarBlue) was measured using a Bio-Tek Elx800 plate reader, and the conversion of oxidized alamarBlue to its reduced form was used to determine metabolic activity. Cells lysed by the addition of 10 μl of Triton X-100 were used as negative controls. Assays were performed in triplicate.

Statistical Analysis

Descriptive statistics were compiled in either Microsoft Excel® 2010 or IBM SPSS Statistics version 21. Geometric mean titers were calculated by logarithmic transformation and group means were compared by either repeated measures ANOVA or student's T test by time point. All statistical evaluations were conducted using IBM SPSS Statistics version 21.

Results

Induction of PA antibodies

The *B. anthracis* Sterne strain spore vaccine induced a rapid anti-PA antibody response in each immunized horse, Figure 4.1. The combined geometric mean titer for each time point indicated a plateau of antibody induction was reached following the third administration of the vaccine with no significant increase in antibody production beyond day 50. ($p = 0.171$), Figure 4.2. In order to determine if the plateau of anti-PA antibody titers could be increased, alternate immunization strategies were investigated. Two of the previously immunized horses received a priming dose of 1.0 ml spore vaccine followed by monthly immunization of 100 µg of recombinant PA homogenized with incomplete Freund's adjuvant and administered ID as described previously. Another two previously immunized horses received a priming dose of 1.0 ml spore vaccine followed by monthly immunization of 2.0 ml spore administered SC, as described previosuly. Finally, two horses, naïve to *B. anthracis* and its toxins were immunized with rPA only. Each dose of 100 µg of rPA was adjuvanted with Freund's incomplete adjuvant and administered ID at monthly intervals.

Each of the horses that had been previously immunized against *B. anthracis* displayed relatively high resting anti-PA antibody titers. In addition, following the priming dose a rapid anamnestic response and elevation in titer levels was observed. These horses trended toward higher geometric mean titers when compared to horses immunized with rPA alone. However, there were no significant differences among any the alternative vaccination responses, Figure 4.3.

Antibody-mediated lethal toxin neutralization

For quantification of neutralizing antibodies an assay utilizing J774A.1 mouse macrophages was modified from that previously described by Hanna *et al.*.[441] Lethal toxin induces cell lysis in J774A.1 cells, but this effect has been shown to be ameliorated in the presence of protective antibodies.[439] In this study, protection from lethal toxin induced lysis was determined by monitoring the reduction of alamarBlue as an indicator of cell health and metabolic activity. Healthy cells internalize alamarBlue and convert its oxidized form (which is blue in color) to the reduced form (which is fluorescent pink). The extent to which alamarBlue has been reduced can be measured spectrophotometrically and used to infer the level of cell viability following treatment with lethal toxin.

To assess the ability of the hyper-immune plasma to protect cells from lysis, they were treated with lethal toxin (rLF + rPA) that had been pre-incubated in the presence or absence of the plasma. These preparations of lethal toxin or lethal toxin plus hyper-immune plasma were then over laid on J774A.1 cell monolayers and incubated for 4 hours. Titers were considered protective or neutralizing at the reciprocal dilutions that provided at least 50% protection. In order to ascertain the contribution that neutralization of PA or LF had on the overall protection of the cells the concentrations of LF were varied.

When J774A.1 cells were incubated in the presence of lethal toxin, but not plasma approximately 60% cell lysis occurred when LF was added at 0.1 µg/ml and PA was added at 1.0 µg/ml, Table 4.5. At higher LF concentrations (0.5 µg/ml, 1.0 µg/ml, and 2.0 µg/ml) and PA at 1.0 µg/ml, cell lysis approached 100%. No lysis was observed when either PA or LF was

incubated individually with cells regardless of concentration. When LF and PA were combined at 0.1 µg/ml and 1.0 µg/ml, respectively, and incubated with hyper-immune plasma a higher neutralizing titer was observed than for the plasma samples incubated with higher concentrations of LF (0.5 and 1.0 µg/ml), Figure 4.4. When LF was added at 2.0 µg/ml lower neutralizing antibody titers were achieved and indicating that protection from LF mediated lysis could be overwhelmed.

To determine if pre-incubation of the plasma sample with the lethal toxin was necessary for toxin neutralization, a subset of randomly selected plasma samples were added to media containing 1.0 µg/ml LF and 1.0 µg/ml PA at 0, 1, 2 ,3 and 4 hours after toxin exposure to the cells, Figure 4.5. Protection was conveyed only for 0 hr and some 1 hr time points suggesting that toxin mediated lysis occurs relatively rapidly and rescue from lysis had a defined time limit.

Plasma samples collected from each horse at serial time points were subjected to the toxin neutralization assay under the most sensitive conditions (1.0 µg/ml LF and 1.0 µg/ml PA) to determine the neutralizing antibody titer over time (Figure 4.6). Comparison of horses immunized with the alternate dosages and antigens revealed a pattern similar to that found for anti-PA antibody titers. Horses with previous immunity and then immunized with either 2.0 ml of spore vaccine or 100 µg of rPA displayed a trend toward higher toxin neutralizing titers but did not elcit significantly higher differences compared to horses immunized with rPA alone, Figure 4.7.

Discussion

The use of immunoassays in the field of anthrax vaccine development and characterizations of immunotherapeutics has been well studied in laboratory animal models. However, very little information exists concerning their use in horses. In this study we have modified and adapted an ELISA for the quantitation of anti-PA immunoglobulins and a cell culture toxin neutralization assay for horses vaccinated with the Sterne strain spore vaccine.

The *B. anthracis* Sterne strain vaccine has been shown to induce immune responses against several critical antigens including the effector molecules of the anthrax toxin and spore surface antigens. This diverse immune response has also been shown to be fully protective in both toxin mediated challenge models and fully virulent anthrax infection in several species. In this study, the Sterne strain vaccine produced a strong and rapid anti-PA antibody response in the horses that were immunized with it, yet beyond 50 days there appears to have been no significant elevation in titers regardless of the frequent re-administration of the antigens. *B. anthracis* Sterne strain, while attenuated, is a fully toxigenic strain that produces all 3 toxins molecules during exponential growth phase of the bacterium. The immunizing dose according to the manufacturer recommendations contains $1x10^6$ spores/ml. For antigenic mass to be delivered to the immune system, these spores must first germinate and reach vegetative growth prior to toxin production. Germination of *B. anthracis* can take up to 7 hours to complete. One possible explanation for the plateauing of anti-PA titers is that each horse developed neutralizing antibodies against the spore capsular antigens after the third immunization that limited the number of spores germinating and effectively reduced antigenic mass for the toxin antigens in subsequent doses. In fact, Welkos *et*

al. demonstrated that high anti-PA antibody levels could suppress germination of spores and improve macrophage clearance, thus preventing infections. In order to circumvent a potentially overwhelming response to the spore antigens we immunized two of these horses with 100 µg of rPA and two horses with $2x10^6$ spores (2.0 ml spore vaccine). Both treatments resulted in significantly higher mean anti-PA titers from previous mean titers for each individual suggesting that neutralization of the spore and reduced antigen presentation was likely limiting the continued increase of circulating anti-PA antibodies. The elevation in antibody titers observed in horses receiving $2x10^6$ spores suggests that the increased dose permitted some spores to escape neutralization and produce vegetative bacilli and enough toxins to achieve a successful anamnestic response. It is not clear whether this also resulted in prolonged clearance of the bacterium.

Immunization of horses with recombinant PA alone failed to achieve the same level of anti-PA titers as horses receiving the spore vaccine. One evident factor influencing this result is the lack of previous immunity in horses immunized with rPA compared to that in horses receiving spore vaccine. However, when only the initial immunization time points for horses receiving 1.0 ml of spore vaccine were compared to those time points for horses receiving rPA alone a significantly higher mean anti-PA titer in rPA immunized horses was revealed. This suggests that continued immunization of 100 µg rPA could achieve similar anti-PA titers in time. Because immunization with rPA is an inactivated antigen and antigenic mass and antigen presentation is not reliant upon replication it also possible that horses consistently immunized with rPA alone would not develop the premature neutralization plateau of antigen as seen with horses immunized with the spore vaccine alone.

Assessment of lethal toxin neutralizing titers by the toxin neutralization assay has been shown to correlate with survival post experimental challenge in various animal models of infection. The immunodominant antigen appears to be PA and titers against PA alone are sufficient for protection. If protection was dependent upon neutralization of PA alone, then it is theorized that neutralization titers would be unchanged regardless of the concentration of LF. However, if LF neutralization was important to overall protection then there would be a concentration of LF that could overwhelm the antibody titer in the plasma and result in lysis and protection. We observed that toxin neutralizing antibody titers could be altered by varying the concentration of the LF in the preparation that was overlaid on J774A.1 cells and that at the highest concentration of LF (2.0 µg/ml) titers could be reduced and protection could be overcome. This result suggests a concentration dependent effect of LF on cell lysis and that LF neutralizing antibodies played a role in the overall protection from lysis. However, to fully elucidate this effect depletion of LF antibodies in an individual plasma sample would need to be carried out and the same toxin assays conducted for comparison.

This study is the first to quantify toxin neutralizing immunoglobulins in horses immunized with a live *B. anthracis* Sterne strain vaccine. A similar plateau in toxin neutralizing titers was observed for horses receiving 1.0 ml spore vaccine as seen in anti-PA antibodies. Administration of either rPA or 2.0 ml of spore vaccine in horses previously immunized resulted in significantly higher toxin neutralizing titers ($p = 0.002$ and $p = 0.005$, respectively). Horses immunized with rPA alone displayed significantly lower titers when compared to the initial immunization time points for horses receiving 1.0 ml of spore vaccine. This indicates that the

potentially more diverse immune response to the fully toxigenic spore vaccine, including the LF and EF antigens, conveyed a greater level of toxin neutralization and protection.

In summary, though not exhaustive in its approach, this study indicates that the attenuated *B. anthracis* Sterne strain spore vaccine can successfully and reliably induce anti-PA and lethal toxin neutralizing immunoglobulins in horses. This is an important first step in defining the protective capacity of plasma from horses hyper-immunized against *B. anthracis* and leads to further investigations in animal infection models and ultimately the development of an effective passive immunotherapeutic against anthrax in humans.

Table 4.1 Immunization protocol for individual horses. Four horses were immunized with a *B. anthracis* Sterne strain spore vaccine either subcutaneously or intramuscularly monthly for approximately 1 year. Whole blood samples were collected at predetermined intervals for analysis of specific antibody production.

Horse ID: AX 1

Day	Immunizing Antigen
1	1.0 ml spore vaccine SC
21	1.0 ml spore vaccine SC
56	1.0 ml spore vaccine SC
91	1.0 ml spore vaccine SC
119	1.0 ml spore vaccine SC
154	1.0 ml spore vaccine SC
184	1.0 ml spore vaccine SC
210	1.0 ml spore vaccine SC
249	1.0 ml spore vaccine SC
275	1.0 ml spore vaccine SC
303	1.0 ml spore vaccine SC
332	1.0 ml spore vaccine SC
366	1.0 ml spore vaccine SC

Horse ID: AX 2

Day	Immunizing Antigen
1	1.0 ml spore vaccine IM
21	1.0 ml spore vaccine IM
56	1.0 ml spore vaccine IM
91	1.0 ml spore vaccine IM
119	1.0 ml spore vaccine IM
154	1.0 ml spore vaccine IM
184	1.0 ml spore vaccine IM
210	1.0 ml spore vaccine IM
249	1.0 ml spore vaccine IM
275	1.0 ml spore vaccine IM
303	1.0 ml spore vaccine IM
332	1.0 ml spore vaccine IM
366	1.0 ml spore vaccine IM

Horse ID: AX 3

Day	Immunizing Antigen
1	1.0 ml spore vaccine SC
21	1.0 ml spore vaccine SC
56	1.0 ml spore vaccine SC
91	1.0 ml spore vaccine SC
119	1.0 ml spore vaccine SC
154	1.0 ml spore vaccine SC
184	1.0 ml spore vaccine SC
210	1.0 ml spore vaccine SC
249	1.0 ml spore vaccine SC
275	1.0 ml spore vaccine SC
303	1.0 ml spore vaccine SC
332	1.0 ml spore vaccine SC
366	1.0 ml spore vaccine SC

Horse ID: AX 4

Day	Immunizing Antigen
1	1.0 ml spore vaccine IM
21	1.0 ml spore vaccine IM
56	1.0 ml spore vaccine IM
91	1.0 ml spore vaccine IM
119	1.0 ml spore vaccine IM
154	1.0 ml spore vaccine IM
184	1.0 ml spore vaccine IM
210	1.0 ml spore vaccine IM
249	1.0 ml spore vaccine IM
275	1.0 ml spore vaccine IM
303	1.0 ml spore vaccine IM
332	1.0 ml spore vaccine IM
366	1.0 ml spore vaccine IM

Table 4.2 Immunization protocol for alternative dosing of Sterne strain spore vaccine in individual horses. Following immunization using the initial protocol two horses were randomly selected to receive an increased dose of Sterne strain spore vaccine in an attempt to maximize specific antibody titers.

Horse ID: AX 2
Previous Immunization Protocol

Day	Immunizing Antigen
1	2.0 ml spore vaccine SC
13	2.0 ml spore vaccine SC
41	2.0 ml spore vaccine SC
70	2.0 ml spore vaccine SC
98	2.0 ml spore vaccine SC
126	2.0 ml spore vaccine SC
160	2.0 ml spore vaccine SC

Horse ID: AX 4
Previous Immunized Protocol

Day	Immunizing Antigen
1	2.0 ml spore vaccine SC
13	2.0 ml spore vaccine SC
41	2.0 ml spore vaccine SC
70	2.0 ml spore vaccine SC
98	2.0 ml spore vaccine SC
126	2.0 ml spore vaccine SC
160	2.0 ml spore vaccine SC

Table 4.3 Immunization protocol for previously immunized horses receiving adjuvanted recombinant protective antigen. Following immunization using the initial protocol two horses were randomly selected to receive 100 µg of recombinant proctective antigen (rPA) adjuvanted with Freund's incomplete adjuvant administered intradermally in an attempt to maximize specific antibody titers.

Horse ID: AX 1
Previous Immunization Protocol

Day	Immunizing Antigen
1	1.0 ml spore vaccine SC
13	100 µg rPA ID
41	100 µg rPA ID
70	100 µg rPA ID
98	100 µg rPA ID
126	100 µg rPA ID
160	100 µg rPA ID

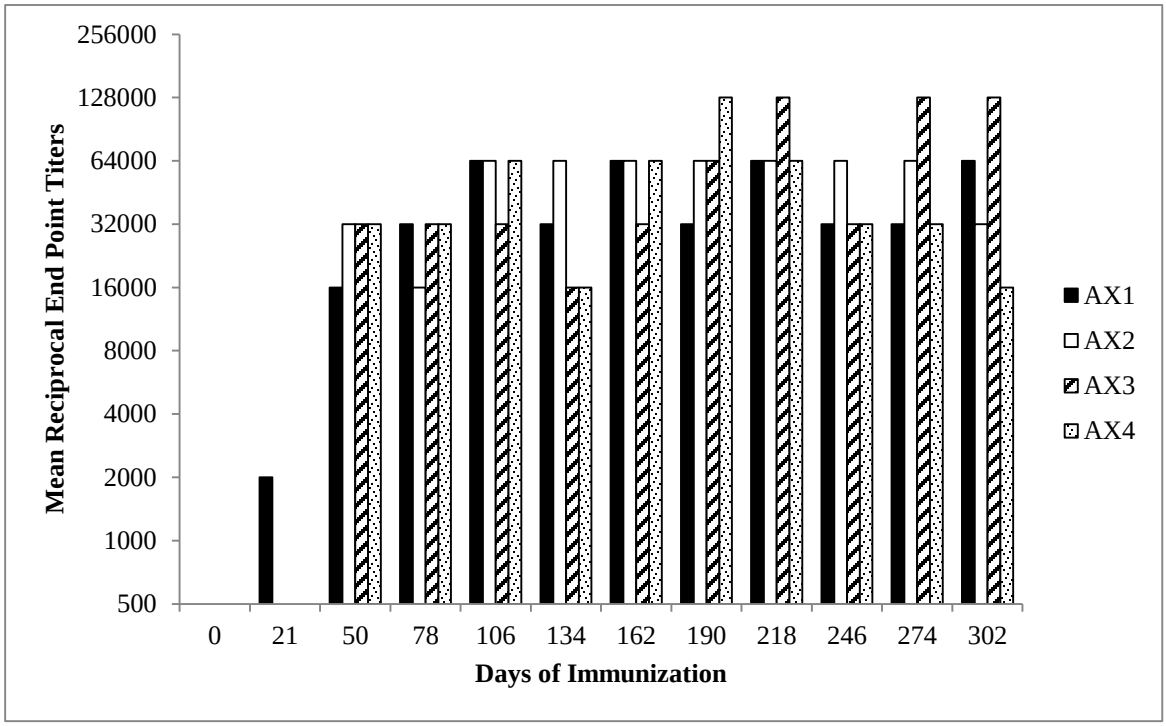
Horse ID: AX 3
Previous Immunization Protocol

Day	Immunizing Antigen
1	1.0 ml spore vaccine SC
13	100 µg rPA ID
41	100 µg rPA ID
70	100 µg rPA ID
98	100 µg rPA ID
126	100 µg rPA ID
160	100 µg rPA ID

Table 4.4 Immunization protocol for naïve horses receiving adjuvanted recombinant protective antigen. Two previously non-immunized horses were administered 100 µg of rPA adjuvanted with Freund's incomplete adjuvant intradermally in an attempt to maximize specific antibody titers.

Horse ID: AX 5	
Day	Immunizing Antigen
1	100 µg rPA ID
13	100 µg rPA ID
41	100 µg rPA ID
70	100 µg rPA ID
98	100 µg rPA ID
126	100 µg rPA ID
160	100 µg rPA ID

Horse ID: AX 6	
Day	Immunizing Antigen
1	100 µg rPA ID
13	100 µg rPA ID
41	100 µg rPA ID
70	100 µg rPA ID
98	100 µg rPA ID
126	100 µg rPA ID
160	100 µg rPA ID



2401

2402 **Figure 4.1 Reciprocal end point titers for horses immunized with 1.0 ml Sterne strain 34F2**

2403 **spore vaccine**

2404 Plasma from each horse was subjected to the anti-PA indirect ELISA for each time point.

2405 Reciprocal titers were achieved from the geometric mean of three replicates of each sample.

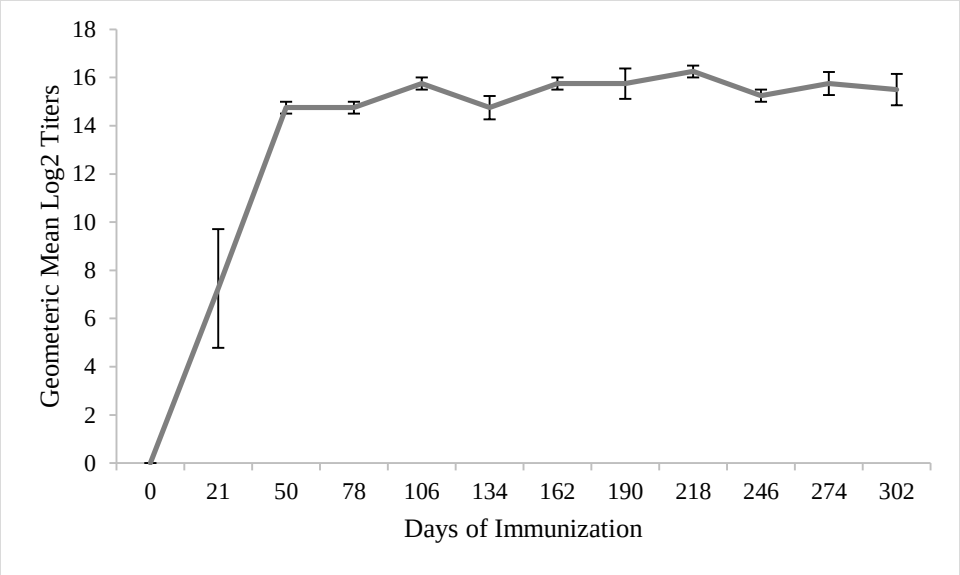


Figure 4.2 Geometric mean titers in four horses immunized with 1.0 ml Sterne strain 34F2 vaccine

Plasma from each horse was subjected to the anti-PA indirect ELISA for each time point. The geometric mean titers are the means for three replicates of all four horses at each time point. A one way repeated measures ANOVA demonstrated no significant differences in mean titers of each horse (p = 0.171).

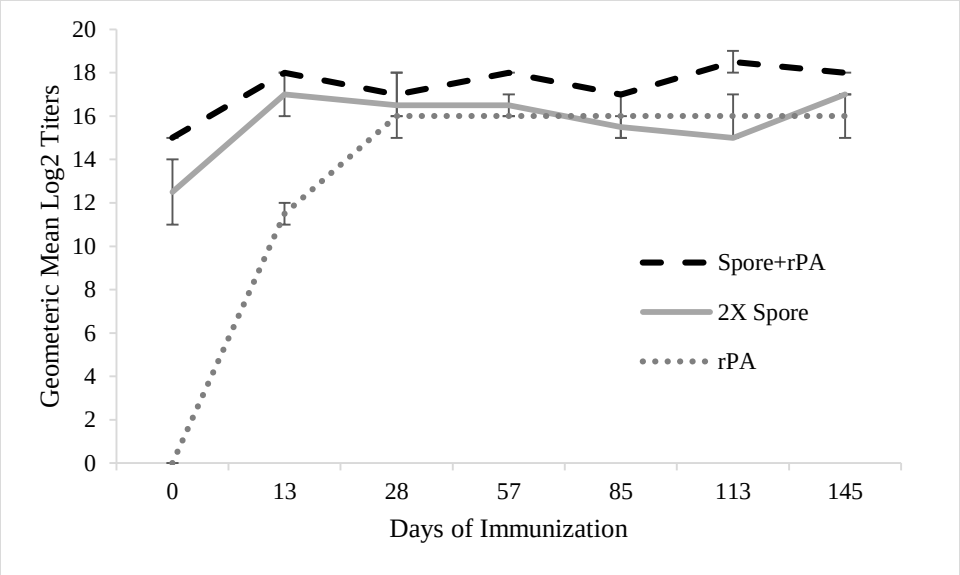


Figure 4.3 Geometric mean titers for horses immunized with spore vaccine and rPA, 2.0 ml spore vaccine, or rPA alone

The geometric means represent three replicates of two horses in each treatment. The solid line represents horses immunized with twice the dose of *B. anthracis.* The small dotted line represents horses immunized with recombinant protective antigen (rPA). The dashed line represents horses immunized first with the labeled dose of spore vaccine then immunized with rPA. A two-way repeated measures ANOVA demonstrated no significant differences in either vaccine response ($p = 0.641$, 0.213 and 1.0) or time ($p = 0.765$).

Table 4.5 Percentage cell lysis induced over varying concentrations of lethal factor (LF)

J774A.1 cells were exposed to *B. anthracis* lethal toxins containing varying concentrations of LF or protective antigen (PA). alamarBlue was added overnight and the amount of reduced alamarBlue was measured as an indicator of metabolic activity. Percent lysis was estimated based on metabolism of alamarBlue and colorimetric analysis compared to a standard curve

PA µg/ml	LF µg/ml	Mean OD Value	% Lysis
1.0	0.1	1.396	60.5
1.0	0.5	1.479	83.5
1.0	1.0	1.487	99.4
1.0	2.0	1.494	100
1.0	0.0	1.249	0
0.0	1.0	1.233	0

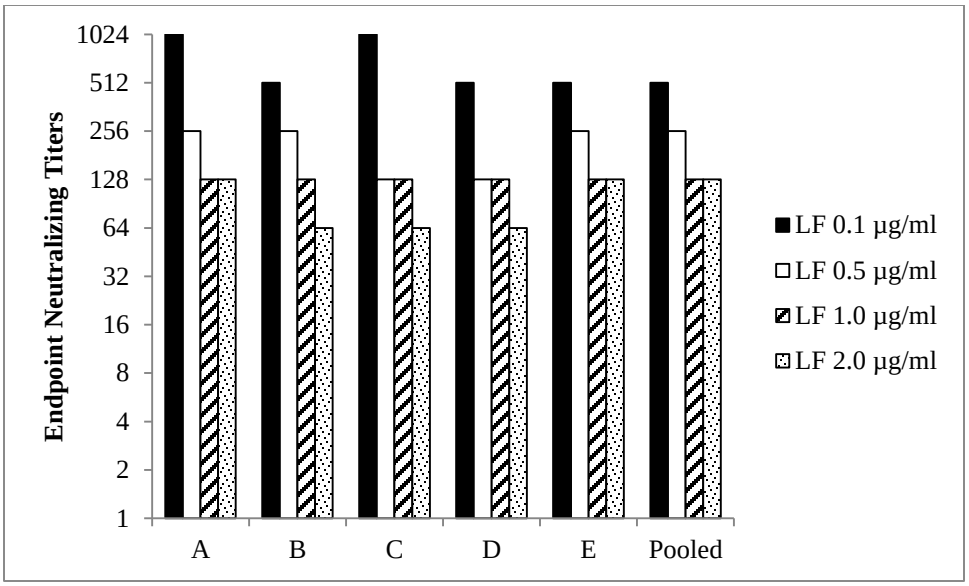


Figure 4.4 Antibody mediated neutralization of LF-induced lysis is influenced by the concentration of LF in the lethal toxin preparation

J774A.1 cells were exposed to *B. anthracis* lethal toxins containing varying concentrations of LF that had been pre-incubated with individual (Samples A-E) and pooled hyper immune plasma samples. alamarBlue was added overnight and the amount of reduced alamarBlue was measured as an indicator of metabolic activity. The neutralization titer, defined as the highest dilution conveying 50% protection from the LF-mediated lysis, is plotted for each plasma sample. The bars indicate different concentrations of LF used to prepare the lethal toxin. Identical results were observed in two independent trials.

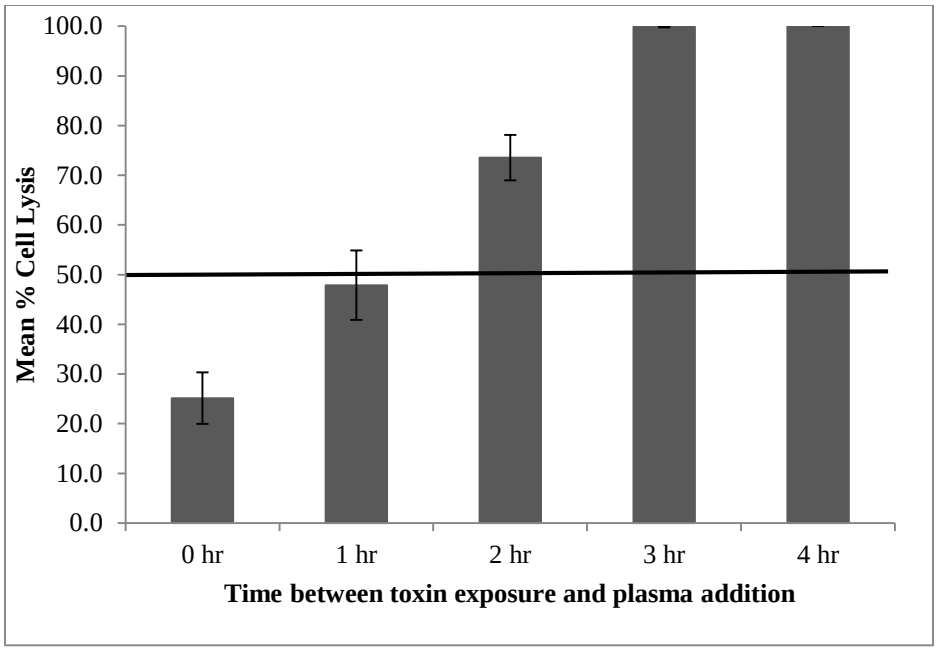


Figure 4.5 Rescue of lethal toxin lysis displays a defined time limit from toxin exposure

J774A.1 cells were exposed to *B. anthracis* lethal toxin (1.0 µg/ml PA and 1.0 µg/ml LF). Cells at the 0 hr time point were exposed to toxin that had been pre-incubated with hyper-immune plasma. At the remaining time points hyper-immune plasma was added to the cells. alamarBlue was added 4 hour after the addition of lethal toxin and left overnight. The amount of reduced alamarBlue was measured as an indicator of metabolic activity. % cell lysis was estimated from the observed OD values compared to a standard curve. The reported % lysis for each time point represents the mean of four independent assays.

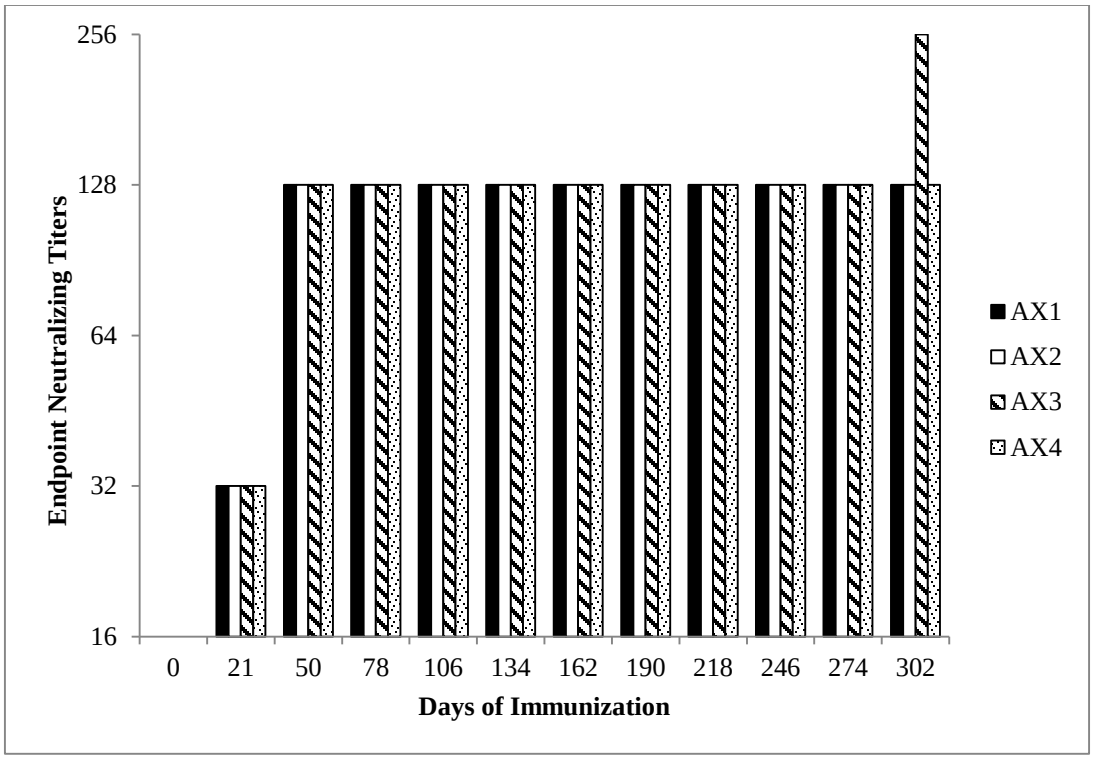


Figure 4.6 Reciprocal endpoint neutralizing titers for horses immunized with 1.0 ml Sterne strain 34F2 vaccine

J774A.1 cells were exposed to *B. anthracis* lethal toxin (1.0 µg/ml PA and 1.0 µg/ml LF) that had been pre-incubated with individual hyper-immune plasma samples. alamarBlue was added overnight and the amount of reduced alamarBlue was measured as an indicator of metabolic activity. The neutralization titer, defined as the highest dilution conveying 50% protection from the LF-mediated lysis, is plotted for each plasma sample at each time point. The bars indicate individual horses. Each titer represents the geometric mean for three replicates.

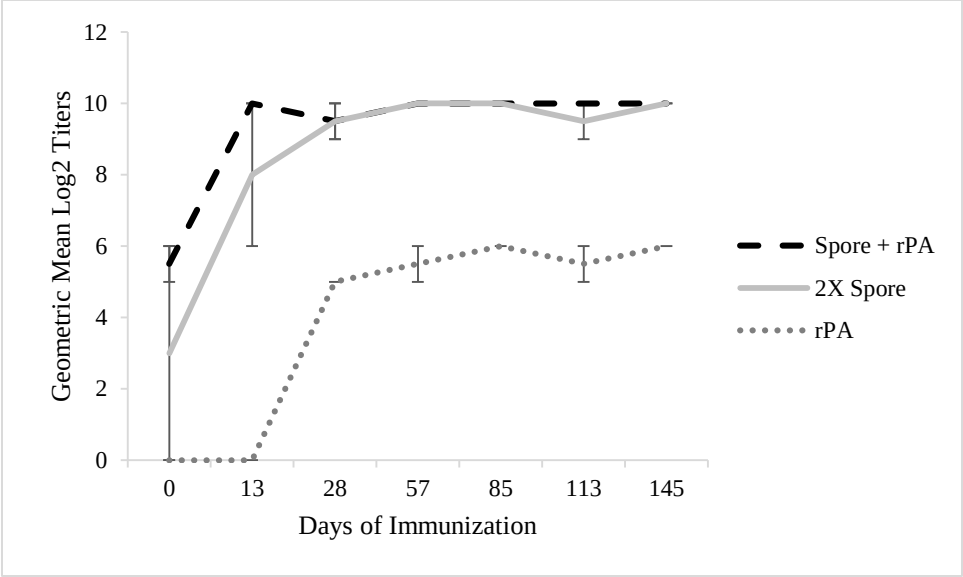


Figure 4.7 Geometric mean neutralizing titers for horses immunized with spore vaccine and rPA, 2.0 ml spore vaccine, or rPA alone

J774A.1 cells were exposed to *B. anthracis* lethal toxin (1.0 µg/ml PA and 1.0 µg/ml LF) and pre-incubated with individual plasma samples from either horses immunized with twice the spore dose (2X Spore, solid line), spore vaccine initially followed by rPA (Spore + rPA, dashed line), and rPA alone (rPA, dotted line). alamarBlue was added overnight and the amount of reduced alamarBlue was measured as an indicator of metabolic activity. The neutralization titer was defined as the highest dilution conveying 50% protection from the LF-mediated lysis. The geometric means represents three replicates of two horses in each treatment. A two-way repeated measures ANOVA demonstrated no significant differences in vaccine type ($p = 0.663$, 0.91, and 0.210) or time ($p = 0.105$, 0.212, and 1.0).

Chapter 5:

Passive protection against anthrax in mice with plasma derived from horses hyper-immunized against *Bacillus anthracis* Sterne strain

Abstract

In this final study, it was demonstrated that hyper-immune plasma and concentrated immunoglobulins harvested from horses immunized with live attenuated Sterne strain veterinary vaccine was successful in passively protecting A/J mice from a homologous challenge. The treatment of mice with hyper-immune plasma at time 0 hour and 24 hour post-infection following both an intranasal and subcutaneous challenge had no effect on survival to the end of the study period, but did significantly increase mean time to death ($p < 0.0001$). Mice treated with affinity purified immunoglobulins at both time points post-infection in both challenge models showed significant increase in survival rate ($p < 0.001$) to the end of the study period. Bacterial loads and colony forming units/gram of lung, liver and spleen tissue were also assessed and were not significantly different in mice treated with hyper-immune plasma but were reduced 4 fold and completely cleared in some cases after treatment with concentrated immunoglobulins ($p < 0.0001$).

Introduction

Bacillus anthracis, a gram-positive, non-hemolytic, non-motile bacterium, is the causative agent of anthrax. *B. anthracis* induces disease through the production of three toxin polypeptides encoded on the pXO1 plasmid and a poly-D-glutamic acid capsule encoded on the

pXO2 plasmid.[442,443] The toxin proteins are constitutively produced during log phase growth of the bacterium and are secreted into the extracellular environment. The three components combine in binary fashion to form two toxins, lethal toxin and edema toxin. Lethal factor (LF) is a zinc protease that inactivates protein kinases, an important mediator of intracellular signaling.[444] The end result of LF exposure is disruption of cytokine expression, impairment of host cell function and induced apoptosis. Edema factor (EF) is an adenylate cyclase that when bound by calmodulin and intracellular calcium generates supraphysiologic concentrations of cAMP.[154] This leads to impairment of other signaling pathways related to water homeostasis in the cell.

Like all members of the *Bacillus* genus, *B. anthracis* produces spores under conditions of resource restriction or vegetative cell stress. Spores are the dormant form of the bacterium and are extremely resistant to desiccation and disinfection. Spores have been documented to remain viable for several decades.[109,110] Exposure to spores is the initiating step in anthrax infection. Once inhaled, ingested or introduced through the skin the spores germinate to produce vegetative organisms. Inhalational anthrax is the deadliest form of the disease in humans due to its vague initial clinical presentation and the rapid replicative capacity of the bacterium. A short 2-5 day incubation period precedes the onset of flu-like symptoms early on. As the organisms overwhelm the innate immune defenses respiratory distress ensues and progresses abruptly to respiratory failure and septic shock. Early recognition of the disease and appropriate antibiotic therapy are critical in successful treatment outcomes. Often despite aggressive antimicrobial therapy the disease progresses due uncontrolled toxemia.

An equine source polyclonal hyper-immune plasma was produced and targeted against *B. anthracis*. The plasma was harvested from horses that were hyper-immunized against the Sterne 34F2 strain of *B. anthracis.* The *B. anthracis* Sterne strain spore vaccine is currently licensed for use in livestock species in the United States and other counties. *B. anthracis* Sterne strain is an attenuated fully toxigenic acapsular ($pXO1^{+}$, $pXO2^{-}$) strain of *B. anthracis* that is capable of inducing strong protective responses against protective antigen (PA), LF, and EF. The principal objective of this study was to determine if equine source hyper-immune plasma specific for *B. anthracis* can protect A/J mice challenged with *B. anthracis* Sterne strain in a well-controlled passive immunization challenge assay.

Materials and Methods

Bacterial Strain

The challenge bacterium used was obtained from the veterinary vaccine *B. anthracis* Sterne strain 34F2 (Anthrax Spore Vaccine, Colorado Serum Company Denver, CO). One milliliter of vaccine was plated onto BHI agar and incubated at 37^{0}C for 24 hours. Colony morphology was typical of an acapsular *B. anthracis* strain and a gram stain demonstrated the cell morphology characteristic of *B. anthracis.* The Miles and Mirsa dilution drop technique was used to verify spore concentration in the vaccine. Briefly 10-fold dilutions were prepared and 20 µl of each preparation were deposited in triplicate onto BHI agar plates and incubated at 37^{0}C for 24 hours. Colonies were only counted for those dilutions where 3-50 full-size discrete colonies could be distinguished. CFU/ml was estimated with the following formula:

CFU/ml = Mean number of colonies for a dilution x 50 x dilution factor

A concentration of 5 x 10^6 spores/ml was verified to be in agreement with published data from the manufacturer.

For preparation of the challenge inocula, single colonies of Sterne strain were taken from BHI agar plates and inoculated in BHI broth for overnight culture at 37^0 C. Each culture was spread onto modified germination (G) medium agar plates [0.2% yeast extract, 0.2% $(NH_4)_2SO_4$, 1.5% Bacto agar, 0.0025% $CaCl_2$ dihydrate, 0.05% K_2HPO_4, 0.02% $MgSO_4$ heptahydrate, 0.005% $MnSO_4$ quatrahydrate, 0.0005% $ZnSO_4$ dihydrate, 0.0005% $CuSO_4$ pentahydrate, 0.00005% $FeSO_4$ heptahydrate]. The plates were incubated at 30°C for 8 to 10 days in the dark. Colonies scraped from the surface of the agar were resuspended in distilled water and were heat treated at 65°C for 1 h to kill any viable vegetative cells. Purification of spores was done with 58% (vol/vol) Renografin (Renocal-76 diluted in dH_2O; Bracco Diagnostics, Princeton, NJ). Spores were layered onto the 58% Renografin and centrifuged at 6,000 × *g* for 30 min in a swinging bucket rotor. The sedimented spores were washed twice with dH_2O. After the final sedimentation, the spores were re-suspended in distilled water to yield a final concentration of 10^9 to 10^{10} spores/ml. The intranasal challenge inoculum dilutions were made to achieve a concentration of 1.2 x 10^5 spores in 30 µl of sterile PBS. Based on preliminary studies, this infectious dose resulted in a consistent delivery of 4 LD_{50} aspirated into the lungs. For the subcutaneous challenge, dilutions were prepared to achieve a concentration of approximately 1 x 10^3 spores in 100 µl of sterile PBS. Stock preparations of both challenge inoculums were verified for spore concentration immediately before and after challenge procedures were performed.

Animals

All procedures described within this study involving the use of animals were approved by Auburn University Institutional Animal Care and Use Committee (protocol #2012-2105). Female A/J strain mice were obtained from Jackson Laboratory (Bar Harbor, ME) and were from 6-12 weeks age during these experiments. Mice were housed in ventilation controlled hepa-filtered biosafety cages with up to 5 animals per cage. All mice were provided *ad libitum* food and water for the duration of the study. Prior to the initiation of the study all mice were randomly allocated to treatment groups (Table

Anti-Bacillus anthracis *Hyper-Immune Plasma*

Hyper-immune plasma was obtained from horses immunized with *B. anthracis* Sterne strain 34F2 spore vaccine (Anthrax Spore Vaccine, Colorado Serum Company, Denver, CO) at monthly intervals for approximately one year. Anti-PA antibody titers and *B. anthracis* toxin neutralization titers were monitored throughout the immunization procedures. Plasmapheresis was performed periodically as described previously. Upon each pheresis event, 20 L of plasma was collected from each horse then aliquoted in 1 liter volumes and sealed aseptically and stored at at -80^0 C until ready for use. Hyper-immune plasma that was used for the treatment of mice in the challenge studies was prepared immediately prior to its administration by pooling four 20 ml aliquots from each of the individual pheresis events that were determined to have the highest anti-PA antibody activity and highest toxin neutralizing activity. Equine plasma that was used for negative controls was obtained from two horses determined to have had no previous exposure to *B. anthracis* or the spore vaccine and no anti-PA antibody activity or toxin neutralizing

capacity as determined by an anti-PA ELISA and lethal toxin neutralization assay. Samples were collected and processed as described for hyper-immune plasma treatments. Individual plasma treatment doses contained 0.8 ml of either pooled hyper-immune plasma or naïve plasma. This volume was chosen as it was considered the maximum volume that could be safely administered intraperitoneal to mice.

Concentrated Immunoglobulin G

The same hyper-immune plasma samples that were used for the treatment of mice in the challenge studies were subjected to polyclonal protein A columns for affinity purification (Pierce Protein A Columns, Pierce-Thermo Scientific, Rockford, IL). Briefly, plasma was diluted 1:1 with Protein A binding buffer and passed through a column of agarose resin beads coupled with Protein A. The solution was allowed to flow through the resin beads by gravity flow and the flow-through was collected from the base of the column. Following a wash with binding buffer to remove unbound protein, the bound immunoglobulins were eluted with elution buffer. The elution fractions were adjusted to physiologic pH via the addition of a neutralization buffer. Elution and protein concentration was monitored through the use of a protein assay (Thermo Scientific BCA protein assay kit, Thermo Scientific, Rockford, IL). Fractions with the highest protein content were pooled and stored at -80^0 C until ready to use. For the preparation of treatment doses, immunoglobulin fractions were thawed and pooled to correspond with the hyper-immune samples used to treat mice in the previous challenges studies.

Lethal Dose$_{50}$ Determination

Seven groups of mice (n = 5) were used for each intranasal (IN) and subcutaneous (SC) LD_{50} challenges. For IN challenges, mice were first anesthetized with an intraperitoneal (IP) administration of xylazine (10 mg/kg) and ketamine (100 µg/kg). Once sedated mice received 30 µl PBS containing log fold dilutions of *B. anthracis* Sterne strain spores from 1 x 10^6 to 10 spores by IN administration. A control group received only 30 µl of sterile PBS administered IN. The droplets were deposited on the external nares and the mice held in dorsal recumbency until all the liquid had been aspirated. Each mouse was then laid in dorsal recumbency until fully recovered from anesthesia. Subcutaneous challenges were carried out with the mice fully awake. Each mouse was administered 100 µl PBS containing log fold dilutions of *B. anthracis* Sterne strain spores from 1 x 10^6 to 10 spores. Inoculations were delivered via 5/8th inch 25 gauge needle injected SC over the shoulders. A control group received only 100 µl of sterile PBS SC. Each of these LD_{50} studies was conducted three times independently. Preparations of all dilutions were verified for spore concentration immediately before and after challenge procedures were performed.

Inoculum Preparation

For the IN challenge dilutions were made to achieve a concentration of approximately 1.5 x 10^4 spores in 30 µl of sterile PBS. For the subcutaneous inoculum dilutions were prepared to achieve a concentration of approximately 2 x 10^3 spores in 100 µl of sterile PBS. Stock

preparations of both challenge inocula were verified for spore concentration immediately before and after challenge procedures. Intranasal challenge preparations ranged in spore concentration from 1.31×10^4 - 1.85×10^4, while spore concentration of the SC inoculum ranged from 1.7×10^3 - 3.0×10^3.

Intranasal and Subcutaneous Challenge of Mice

Intranasal challenges were carried out by first anesthetizing each mouse and depositing 30 µl PBS containing approximately 4 LD_{50} spores deposited on the external nares as described. Sham IN challenged mice were anesthetized and handled in an identical manner, however 30 µl of sterile PBS was deposited on the external nares. For SC infection, mice were injected with 100 µl PBS containing 4 LD_{50} under the skin of the shoulders as described. Sham SC challenged mice were handled in an identical manner but100 µl of sterile PBS was administered SC.

Passive Immunization of Mice

The mice were divided into 7 treatment groups for each route of challenge as described in Table 5.1 and Table 5.2. Briefly each mouse was administered either; 1) plasma collected from horses naïve to *B. anthracis,* 2) hyper-immune plasma from horses immunized against Sterne strain spore vaccine, or 3) concentrated immunoglobulins from horses immunized against Sterne strain spore vaccine. For each treatment 0.8 ml volume was administered into the right rear quadrant of the peritoneal cavity by $5/8^{th}$ inch, 25 gauge needle while the mouse was restrained in dorsal recumbency. Treatments were administered at the time of experimental challenge or 24

hours post-infection. Following each challenge, the mice were monitored twice daily for changes in clinical behavior. An ethogram, Table 5.3, was used to assign a clinical score for four criteria following each observation. Any mouse with a combined clinical score ≤ 3 at an observation would be humanely euthanized. Euthanasia was performed by carbon dioxide inhalation in a euthanasia chamber followed by cervical dislocation according to the "AVMA guidelines for the euthanasia of animals: 2013 edition".

Determination of Tissue CFUs

The lung, liver, and spleen were collected immediately following euthanasia or natural death of each mouse. The tissues were weighed and manually homogenized. Following homogenization, an equal weight by volume of sterile PBS was added to each tissue homogenate and 1ml of the homogenate was serially diluted 1:10 in sterile PBS. Twenty µl of each dilution was plated onto BHI agar plates in triplicates and incubated at 37^0 C for 24 hours. Colony forming units were determined using the Miles and Misra method and calculations made to determine the CFUs/gram of tissue.

Statistical Analysis

Survival rates and LD_{50} was calculated similar to methods described by Welkos.[251] Probit analysis was used to calculate LD_{50} values. Survival rates were compared by Fischer exact tests. Kaplan-Meier survival analysis was used to construct survival curves. Mean time of death was compared using a one way ANOVA with Tukey, LSD, and Bonferroni post-hoc analysis.

Kruskal-Wallis tests were used to compare the colony forming units/gram of tissue. All analyses were conducted using IBM SPSS Statistics version 21.

Results

Estimation of LD_{50} for Sterne strain 34F2 in A/J mice

To determine the number of *B. anthracis* Sterne strain spores needed to induce 100% lethality in A/J mice following both IN and SC challenge models, the LD_{50} dose for each route was established, Tables 5.4 and 5.5, respectively. The combined $\log_{10}$ of the LD_{50} for the IN challenge was 3.572 (lower and upper 95% confidence interval: 3.035 and 4.116) or 3.7×10^3 spores (lower and upper 95% confidence interval: 1084 and 13,072). The combined $\log_{10}$ of the LD_{50} for the SC challenge was 2.738 (lower and upper 95% confidence interval: 2.180 and 3.223) or 5.47×10^2 spores (lower and upper 95% confidence interval: 151 and 1672). Based on these data, it was decided to administer 4 X the LD_{50} dose for subsequent experimental challenges. Therefore, mice exposed to IN challenge received approximately 1.5×10^4 spores and mice exposed to SC challenge received approximately 2×10^3 spores.

Survival of A/J mice following Sterne strain challenge

Table 5.6 and Figure 5.1 show The cumulative survival and Kaplan-Meier survival curve of A/J mice given an IN challenge with 1.5×10^4 spores and administered 0.8 ml of either *naïve* plasma or hyper-immune plasma IP at the same time as challenge or 24 hours post-challenge is demonstrated in Table 5.6 and Figure 5.1. There were no significant differences in the number of mice surviving to the end of the 7 day study period in either treatment groups compared to

controls ($p = 1.000$). However, treatment with hyper-immune plasma at both time points did significantly prolong mean time to death ($p < 0.001$, both treatment groups compared to challenge control), Table 5.7, perhaps indicating an effect of treatment but an insufficient titer of protective antibodies to result in complete survival. Tables 5.8, 5.9 and Figure 5.2 reveal similar results for the SC challenge in that hyper-immune plasma treatments did not result in significantly larger numbers of mice surviving to the end of the study period ($p = 1.000$), but that hyper-immune plasma treatments did lead to significantly longer mean time to death ($p = 0.003$), in this case for the time 0 hour treatment group only.

When mice were administered 0.8 ml of concentrated anti- *B. anthracis* immunoglobulins IP following IN challenge with 1.5×10^4 Sterne strain spores, 86% (13/15) survived to the end of trial when treated at the time of challenge and 80% (12/15) survived to the end of the trial when treated at 24 hours post-challenge, Table 5.6 and Figure 5.3. Treatment with hyper-immune plasma at both time points resulted in significantly more mice surviving to the end of the study period ($p < 0.001$, both treatment groups) and a significantly prolonged survival time ($p < 0.001$, both treatment groups) when compared to mice treated with naïve plasma. Similar results were shown for SC challenged mice when treated with concentrated equine immunoglobulins, Table 5.7 and Figure 5.4. Treatment of mice at the time of challenge resulted in 100% (15/15) survival, while treatment at 24 hours post-challenge resulted in 86% (13/15) survival. These survival rates were significantly higher ($p <0.001$, both treatment groups) than challenge control mice. Both treatments resulted in significantly longer ($p < 0.001$) mean time to death compared to control mice. The lack of morbidity in the treatment control groups for all trials conducted indicates that

the administration of *naïve* plasma, hyper-immune plasma and concentrated immunoglobulins was well tolerated by the mice in this study.

Reduction in tissue CFU

Bacillus anthracis was successfully cultured from the lung, liver and spleen in challenged mice that succumb or were euthanized following IN and SC challenge. The tissues of control mice that received sterile PBS either IN or SC yielded no bacterial growth in any of the conducted trials. Although *B. anthracis* could be successfully isolated from the liver and spleen of mice following IN challenge, only lung tissue homogenates resulted in sufficiently high enough CFUs to accurately estimate bacterial loads. Culture of both lung and liver homogenates yielded sufficient CFUs for estimation in the SC challenge (Table 5.10). Neither challenge route resulted in high enough bacterial loads in the spleen to accurately determine CFU/gram of tissue.

While administration of hyper-immune plasma at the time of IN challenge did not provide sufficient protection to result in survival of infected mice, hyper-immune plasma treatment did result in significantly fewer CFU/gram of lung tissue compared to mice receiving *naïve* plasma, Figure 5.5. On the other hand, administration of native immune plasma at 24 hours post-challenge did not result in significant differences in CFU/gram of lung tissue. When mice were administered concentrated immunoglobulins at the time of challenge and at 24 hours post-challenge bacterial counts were significantly lower than untreated control mice. Interestingly, treatment with concentrated immunoglobulins resulted in only an approximately 4 fold reduction in CFU/gram suggesting that perhaps these mice would have shown morbidity or mortality at a later time point as the concentration of passively acquired immunoglobulins waned.

Following SC challenge, administration of hyper-immune plasma had no effect on CFUs/gram of lung or liver tissue regardless of treatment time, Figures 5.6 and 5.7, Table 5.10. With the exception of the two mice that succumbed to infection, treatment with concentrated immunoglobulins following SC challenge at both time points yielded no bacterial growth from the lung and liver tissue sampled indicating effective clearance of the organism.

Discussion

The use of A/J mice has proven a valuable challenge model for active and passive immunization studies with *B. anthracis*.[251,445] The A/J strain of mice is homozygous for *Naip5*$^{Lgn1\text{-}s}$ which imparts reduced macrophage bactericidal activity and increased susceptibility to bacterial infection.[446] Welkos *et al.* defined this strain's susceptibility to *B. anthracis* Sterne strain by multiple routes of infection.[251] The benefit of this challenge model is chiefly the reduced risk in handling an acapsular strain of the organism compared to fully virulent *B. anthracis* strains. This permits conducting such experiments in BSL 2 laboratory conditions.

The LD_{50} infectious dose for an IN and SC challenge identified in this work were consistent with previously published values.[251] Other investigators using this model have used dramatically higher infectious spore doses (up to 100 LD_{50}), however beyond the inference about the strength of protection provided by the investigated modalities there is little justification for such a heavy challenge.[415] Here the authors chose an infectious dose of 4 LD_{50} (~15,000 spores

IN and ~ 2,000 spores SC) that would be fully lethal. This infectious dose would therefore not result in an overwhelming infection, particularly in the case of the intranasal challenge, and produce spurious results as to the protective benefits of these treatments. Though making comparative correlations between this animal model and natural infections in humans may not be accurate, the estimated median lethal dose for inhalation anthrax in humans is likely within the range of 2,500-55,000 spores and 10 spores or less for cutaneous anthrax.[16]

The lack of survival in both routes of infection (IN and SC) in mice treated with hyper-immune plasma suggests that the titer of transferred antibodies was too low to sufficiently neutralize all the organisms. One hypothesis for the poor protection of these mice is that the passive titers were rapidly consumed by the infection and did not effectively neutralize all of the bacteria. This permitted continued replication and the progression of the disease. This hypothesis is supported by the evidence that treatment with hyper-immune plasma resulted in a significantly longer mean time to death and the overwhelming survival of mice treated with the concentrated immunoglobulins. Presumably the latter resulted from a transfer of a much higher titer of protective antibodies. Another potential mechanism for the poor protection of the hyper-immune plasma is the increased clearance of the foreign immunoglobulins and other plasma proteins in the hyper-immune plasma by the mouse's own immune system. In this study, the titers of specific antibodies present in mouse serum was not determined nor the rate of decay of transferred antibody titers following administration.

Testing both these hypotheses begins with determining the kinetics of passive titers over time in the anti-PA ELISA and toxin neutralization assays. It's difficult to anticipate if the

increased clearance of foreign protein would induce a measurable clinical effect but all treatments were very well tolerated in principal and control groups. The administration of plasma into the peritoneal cavity in mice is a conventionally accepted route of delivering large volumes, but the peritoneum is known in mice and other species to be inherently sensitive to foreign antigen and inflammatory responses. Full necropsies were performed in all mice at the time of death or euthanasia and no pathology consistent with peritonitis was noted in any of these examinations. Perhaps the concentrated immunoglobulin treatment did not induce an antigenic response and increased clearance or at least transferred a titer high enough to effectively neutralize the bacteria in spite of increased clearance.

Ideally, a correlation between the *in vivo* passive protection and TNA and ELISA titers would be made to define the dose of antibodies necessary to provide protection. The failure of the hyper-immune plasma to provide full protection prevented us from developing fully characterized dilutions of plasma to be used for those experiments. Despite not surviving to the end of the study period, treatment with hyper-immune plasma did extend the mean time to death in both experimental challenge routes and time points with the exception of treatment 24 hour after SC challenge. This suggests that multiple treatments of hyper-immune plasma may be necessary to establish protection. Once the half-life of passive antibodies is determined then studies can be conducted to establish a minimum effective dose of concentrated immunoglobulins and/or dosing frequency necessary for successive treatments of plasma. In addition, a combination therapeutic strategy of antimicrobial and passive antibody therapy may provide an additive effect resulting in greater survivability in inhalation *B. anthracis* infection.

It would also be advantageous to define the immunodominant epitopes and the primary factors of protective efficacy in these treatments. Recent studies utilizing combinations of monoclonal antibody therapies or cocktails against LF and/or PA have revealed unexpected mechanisms of protection when compared to single monoclonal therapy.[435] This raises questions about the benefit of monoclonal therapy compared to polyclonal therapy and introduces potential studies designed to elucidate the synergistic effect of multiple protective antibodies.

All mice that died as a result of *B. anthracis* in this study had similar CFUs/gram of tissue of *B. anthracis* regardless of treatment. This may indicate that though the infection may have been restrained by the treatment with hyper-immune plasma and concentrated immunoglobulins the bacterium ultimately gained an advantage and reached a uniform number of bacteria prior to death. Interestingly, treatment with concentrated immunoglobulins only reduced the bacterial load in the lungs of IN challenged mice by 4 fold. Oscherwitz *et al.* demonstrated that immunization of animals with spore antigens induced a protective antibody response that effectively limited germination.[447] The passive treatments used here were derived from horses receiving multiple doses of the *B. anthracis* Sterne strain spore vaccine which may have effectively stimulated similar antibodies. The higher titers provided by the concentrated immunoglobulins may have prevented some of the spores from germinating during the 7 day study period. Alternatively, it has been documented that not all *B. anthracis* spores germinate immediately upon inhalation into the lungs. Cases of inhalational anthrax were reported up to 59 days following the exposure event that occurred in Sverdlosk in the former Soviet Union and up to 21 days following the intentional release of *B. anthracis* in the U. S. mail in 2001.[14,225] If the

observation period for this study had been extended to 14 days as other investigators have done there may have been an observed increase in the morbidity and mortality in the IN challenged mice.

The results of this study demonstrate the protective capacity of hyperimmune plasma and concentrated immunoglobulins against *B. anthracis* harvested from horses vaccinated with the Sterne strain spore vaccine. We have previously shown *in vitro* activity of this plasma in a quantitative anti-PA ELISA and quantitative protective titers in toxin neutralization assays. Successful protection of mice infected with *B. anthracis* described here continues the path in the development of polyclonal hyper-immune plasma to be used as a passive immunotherapeutic agent in humans infected with anthrax. *B. anthracis* remains an important agent of biological warfare and terrorism and much effort and research support has been expended in identifying successful strategies for protecting the public. Vaccination of first responders and military personnel is a key component in the US response to an anthrax attack, but a vaccine that induces rapid and protective immunity suitable for mass immunizations has not been developed. Monoclonal antibodies are a promising new therapeutic avenue for the prevention and treatment of anthrax. Raxibacumab, now ABthrax™ is a PA monoclonal produced by Human Genome Sciences. In mid-December 2012 the FDA approved ABthrax™ for use in patients with inhalational anthrax. Polyclonal antisera harvested from human volunteers vaccinated with the killed anthrax vaccine has also become commercially available from Cangene Corporation and Emergent Biosolutions under the name Anthrax Immune Globulin (AIG). Recently this product received conditional licensure from the FDA for treatment of inhalational anthrax. Passive

immunization using polyclonal or a high-affinity monoclonal antibody may offer adjunctive value to antibiotic therapy. Monoclonal therapy has limitations due to its single epitope affinity, thus making it possible to mutate strains of *B. anthracis* which resist their action. Additionally, manufacturing monoclonal antibodies is a resource dependent endeavor. Polyclonal therapy represents the breadth of the humeral immune response and improves immune clearance of pathogens through more efficient activation effector mechanisms such as opsinization, phagocytosis, and complement mediated bacterial cell lysis. AIG possess several disadvantages however, such as a limited number of individuals who are immunized and willing to donate plasma, inconsistent antibody titers within individuals require post-harvest manipulations to stabilize immunoglobulin concentration and the risk of inadvertent transmission of blood borne infections. Equine source polyclonal plasma resolves these issues by providing a reliable source of large volumes of high affinity plasma that can be produced at an estimated $1/8^{th}$ the cost of human polyclonal plasma. Future directions in developing equine hyper-immune plasma include evaluation of the protection in other animal models and against fully virulent strains, definition of the kinetics of the passively transferred antibodies, and refinement of the appropriate hyper-immune plasma or concentrated immunoglobulin doses alone and in combination with antimicrobial therapy. Additional steps in processing, such as affinity purification and trypsin digestion that are needed to make this product safe for use in humans must be evaluated to determine their effect of protective efficacy. The use of equine antisera for emergent prevention and treatment of infectious diseases has been proven to be an effective and safe strategy. Therefore, immunoprophylaxis with equine hyper-immune plasma might be a viable strategy for anthrax counter measures.

2840

Table 5.1 Allocation of mice into treatment groups for IN challenge.

Mice were randomly allocated into one of 6 treatment groups. Each group except the Tx control group received 1.5 x 10^4 spores suspended in 30 µl of PBS. The 30 µl was deposited on the external nares while the mouse held in dorsal recumbancy until the droplet was inhaled. The Tx control received sterile PBS only. While anesthetized each mouse received an intraperitoneal injection of 0.8 ml the appropriate treatment.

Phase I Hyper-immune Plasma Intranasal Challenge Study

Group Designation	Challenge	Treatment	Volume administered	Number of Mice
Tx Control	Sterile PBS	Hyper-immune plasma	0.8 ml	20
IN Challenge control	1.5 x 10^4 spores	*Naïve* plasma	0.8 ml	20
IN Plasma-0 hours	1.5 x 10^4 spores	Hyper-immune plasma	0.8 ml	20
IN Plasma-24 hours	1.5 x 10^4 spores	Hyper-immune plasma	0.8 ml	20

Phase II Concentrated Immunoglobulins Intranasal Challenge Study

Group Designation	Challenge	Treatment	Volume administered	Number of Mice
Tx Control	Sterile PBS	Hyper-immune plasma	0.8 ml	15
IN Challenge control	1.5 x 10^4 spores	*Naïve* plasma	0.8 ml	15
IN Immunoglobulins-0 hours	1.5 x 10^4 spores	Concentrated IgG	0.8 ml	15
IN-Immunglobulins-24 hours	1.5 x 10^4 spores	Concentrated IgG	0.8 ml	15

Table 6 Allocation of mice into treatment groups for SC challenge

Mice were randomly allocated into one of 6 treatment groups. Each group except the Tx control group received 2.3 x 10^3 spores suspended in 100 µl of PBS. The challenge was injected subcutaneously between the shoulders. The Tx control received sterile PBS only. In addition, each mouse received an intraperitoneal injection of 0.8 ml the appropriate treatment.

Phase I Hyper-immune Plasma Subcutaneous Challenge Study

Group Designation	Challenge	Treatment	Volume administered	Number of Mice
Tx Control	Sterile PBS	Hyper-immune plasma	0.8 ml	20
SC Challenge control	2.3 x 10^3 spores	*Naïve* plasma	0.8 ml	20
SC Plasma-0 hours	2.3 x 10^3 spores	Hyper-immune plasma	0.8 ml	20
SC Plasma-24 hours	2.3 x 10^3 spores	Hyper-immune plasma	0.8 ml	20

Phase II Concentrated Immunoglobulins Intranasal Challenge Study

Group Designation	Challenge	Treatment	Volume administered	Number of Mice
Tx Control	Sterile PBS	Hyper-immune plasma	0.8 ml	15
SC Challenge control	2.3 x 10^3 spores	*Naïve* plasma	0.8 ml	15
SC Immunoglobulins-0 hours	2.3 x 10^3 spores	Concentrated IgG	0.8 ml	15
SC Immunoglobulins-24 hours	2.3 x 10^3 spores	Concentrated IgG	0.8 ml	15

Table 5.3 Clinical Behavior Ethogram

Each mouse was daily observed according to the following parameters. Each subcategory score was tallied for a total clinical score that was used to determine the overall health of the animal and when euthanasia was mandated (total clinical score ≤ 3).

Clinical Observation Ethogram

Parameter	Description	Score
Appearance (Also note if abdominal distention is present)	Normal: bright eyes; shiny, well-groomed hair coat	2
	Abnormal: Unkempt hair coat, dull fur	1
	Abnormal: Hunching, piloerection	0
Natural Behavior	Normal: Active; interactive in Environment	3
	Slight decrease in activity; less Interactive	2
	Abnormal: Pronounced decrease in activity; isolated	1
	Abnormal: Possible self-mutilation; hyperactive or immobile	0
Provoked Behavior	Normal: Quickly moves away	3
	Slow to move away or exaggerated response	2
	Abnormal: Moves away after a short period of time	1
	Abnormal: Does not move or reacts with excessively exaggerated response	0
Body Condition Score	Emaciated	1
	Thin	2
	Normal	3
	Overweight	4
	Obese	5
Total Score		1-13

Table 5.4 Probit analysis for IN LD_{50} determination

Mice were exposed to escalating concentrations of spores suspended in 30 µl sterile PBS. For the challenge each mouse was anesthetized and held in dorsal recumbancy while the 30 µl droplet was placed on the external nares. Once the droplet had been inhaled the mouse was returned to its cage for recovery. The number of dead mice were tallied and probit analysis used to establish the LD_{50}.

Total Dead Mice	Total Mice Exposed	Spore Count
0	15	0
0	15	10
4	15	10^2
8	15	10^3
11	15	10^4
13	15	10^5
15	15	10^6

Probit Analysis

	95% Confidence Limits of Spore Count			95% Confidence Limits of log (Spore Count)		
Probability	Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound	Upper Bound
.500	3731.075	1084.181	13072.463	3.572	3.035	4.116

Table 5.5 Probit analysis for SC LD_{50} determination

Mice were exposed to escalating concentrations of spores suspended in 100 µl sterile PBS. For the challenge each mouse was injected subcutaneously between the shoulders. The number of dead mice were tallied and probit analysis used to establish the LD_{50}.

Total Dead Mice	Total Mice Exposed	Spore Count
0	15	0
1	15	10
6	15	10^2
9	15	10^3
11	15	10^4
14	15	10^5
15	15	10^6

Probit Analysis

	95% Confidence Limits of Spore Count			95% Confidence Limits of log (Spore Count)		
Probability	Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound	Upper Bound
.500	547.467	151.317	1672.243	2.738	2.180	3.223

Table 5.6 Cumulative survival following IN challenge

Mice were IN challenged with 1.5 x 10^4 *B. anthracis* Sterne strain and treated with 0.8 ml of hyper-immune plasma, concentrated immunoglobulins, or *naïve* equine plasma at 0 hours or 24 hours post-infection.

	0 hours	24 hours		p value
Hyper-Immune Plasma	0/20[a]	0/20		p = 1.000[b]
Concentrated Immunoglobulins	14/15	12/15		p < 0.001 [b,c]
Naïve Equine Plasma	–	–	1/35	
Treatment Control	–	–	35/35	

a. Survivors/total

b. One-sided Fischer exact test

c. p <0.001 for both 0 hr and 24 hr treatment groups

Table 5.7 Mean time to death (TTD) following IN challenge

Mice were IN challenged with 1.5 x 10^4 *B. anthracis* Sterne strain and treated with 0.8 ml of hyper-immune plasma, concentrated immunoglobulins, or *naïve* equine plasma at 0 hours or 24 hours post-infection.

	0 hours	24 hours		p value
Hyper-Immune plasma	4.125 days	3.950 days		$p < 0.001^{a,b}$
Concentrated Immunoglobulins	6.467 days	6.833 days		$p < 0.001^{a,b}$
Naïve Equine Plasma	–	–	3.428 days	
Treatment Control	–	–	7 days	

a. Kruskal-Wallis test comparison of means

b. $p < 0.001$ for both 0 hr and 24 hr treatment groups

Table 5.8 Cumulative survival following SC challenge

Mice were SC challenged with 2.3 x 10^3 *B. anthracis* Sterne strain and treated with 0.8 ml of hyper-immune plasma, concentrated immunoglobulins, or *naïve* equine plasma at 0 hours or 24 hours post-infection.

	0 hours	24 hours		p value
Hyper-Immune Plasma	0/20[a]	0/20		p = 1.000[b]
Concentrated Immunoglobulins	15/15	12/15		p < 0.001 [b,c]
Naïve Equine Plasma	–	–	0/35	
Treatment Control	–	–	35/35	

a. Survivors/total

b. One-sided Fischer exact test

c. p <0.001 for both 0 hr and 24 hr treatment groups

Table 5.9 Mean time to death (TTD) following SC challenge

Mice were SC challenged with 2.3 x 10^3 *B. anthracis* Sterne strain and treated with 0.8 ml of hyper-immune plasma, concentrated immunoglobulins, or *naïve* equine plasma at 0 hours or 24 hours post-infection.

	0 hours	24 hours		p value
Hyper-Immune Plasma	2.150 days	1.675 days		p = 0.003a,b p = 0.380a,c
Concentrated Immunoglobulins	7 days	6.833 days		$p < 0.001^{a,d}$
Challenge Control	–	–	1.728 days	
Treatment Control	–	–	7 days	

a. Kruskal-Wallis test comparison of means

b. Significance value for 0 hour native plasma group

c. Significance value for 24 hour native plasma group

d. $p < 0.001$ for both 0 hr and 24 hr treatment group

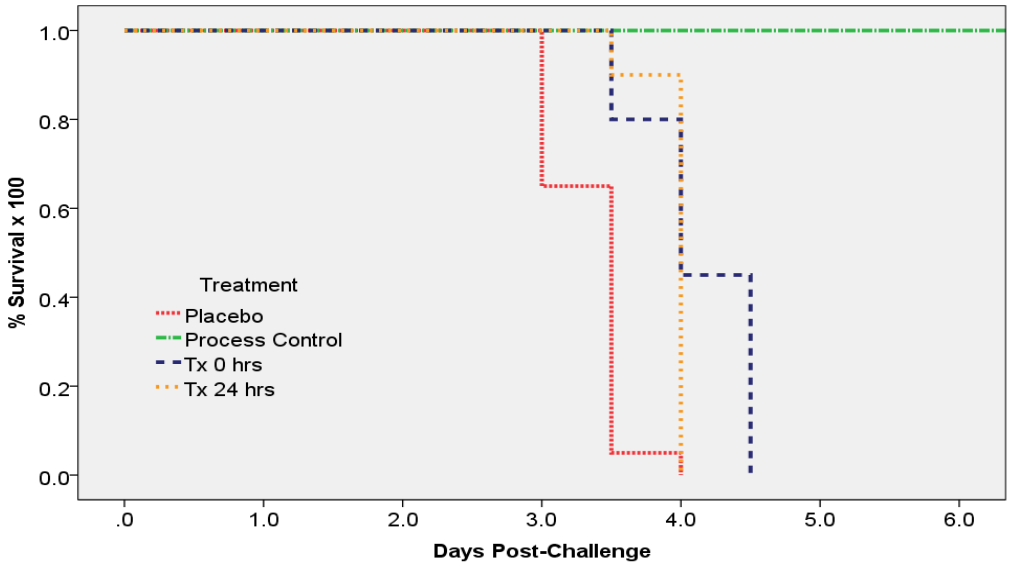
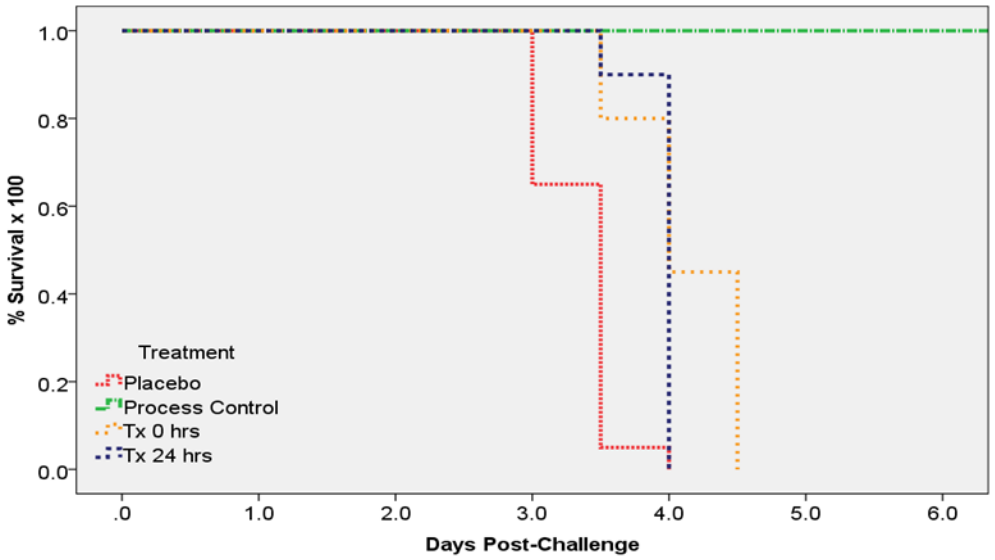


Figure 5.1 Kaplan Meier survival curve following IN challenge and treatment with hyper-immune plasma

Mice were IN challenged with 1.5 x 10^4 *B. anthracis* Sterne strain and treated with 0.8 ml of hyper-immune plasma or *naïve* equine plasma (placebo) at 0 hours or 24 hours post-infection. Process control mice were IN challenged with sterile PBS and treated with 0.8 ml of hyper-immune plasma at 0 hours.



2916

2917 **Figure 5.2 Kaplan Meier survival curve following SC challenge and treatment with hyper-**

2918 **immune plasma**

2919 Mice were SC challenged with 2.3 x 10^3 *B. anthracis* Sterne strain and treated with 0.8 ml of

2920 hyper-immune plasma or *naïve* equine plasma (placebo) at 0 hours or 24 hours post-infection.

2921 Process control mice were SC challenged with sterile PBS and treated with 0.8 ml of hyper-

2922 immune plasma at 0 hours.

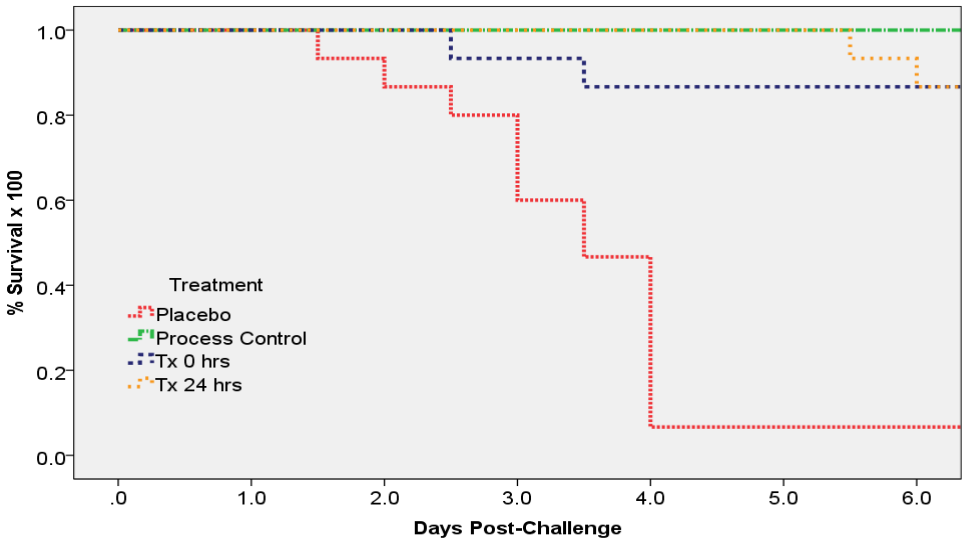


Figure 5.3 Kaplan Meier survival curve following IN challenge and treatment with concentrated immunoglobulins

Mice were IN challenged with 1.5 x 10^4 *B. anthracis* Sterne strain and treated with 0.8 ml of concentrated immunoglobulins or *naïve* equine plasma (placebo) at 0 hours or 24 hours post-infection. Process control mice were IN challenged with sterile PBS and treated with 0.8 ml of hyper-immune plasma at 0 hours.

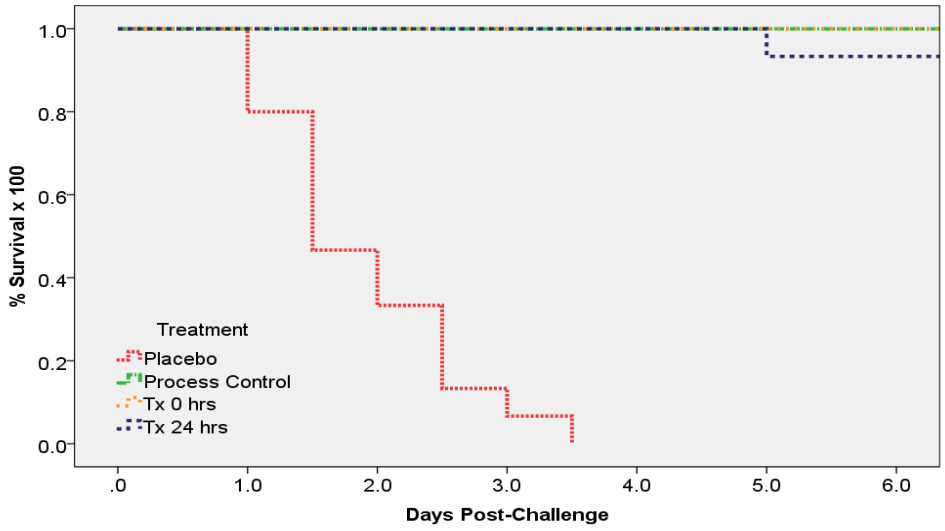


Figure 5.4 Kaplan Meier survival curve following SC challenge and treatment with concentrated immunoglobulins

Mice were SC challenged with 2.3 x 10^3 *B. anthracis* Sterne strain and treated with 0.8 ml of concentrated immunoglobulins or *naïve* equine plasma (placebo) at 0 hours or 24 hours post-infection. Process control mice were SC challenged with sterile PBS and treated with 0.8 ml of hyper-immune plasma at 0 hours.

Table 5.10 Mean colony forming units (CFUs)/ gram of tissue. The lungs, liver and spleen were collected at necropsy from each mouse upon death. Each organ was weighed and homogenized then serially diluted 2 fold into sterile saline. Each dilution was plated onto BHI agar and the quantification of *B. anthracis* vegetative cells or spores was determined by the Miles and Mirsa method. Values represent the mean CFU/gram for each treatment group and challenge, NG = no quantifiable growth, *p* values denote significance compared to challenge control in Kruskal-Wallis comparison of means.

Mean CFU/gram of tissue

	IN Challenge				SC Challenge			
	Lung		Liver		Lung		Liver	
Native Anti-*B. anthracis* Plasma 0 hour Treatment	3.80×10^7	$p = 0.412$	NG		1.25×10^5	$p = 0.844$	7.4×10^4	$p = 0.233$
Native Anti-*B. anthracis* Plasma 24 hour Treatment	4.90×10^7	$p = 0.634$	NG		1.38×10^5	$p = 0.472$	7.75×10^4	$p = 0.380$
Concentrated Immunoglobulins 0 hour Treatment	1.82×10^3	$p < 0.0001$	NG		NG	$p < 0.0001$	NG	$p < 0.0001$
Concentrated Immunoglobulins 24 hour Treatment	9.94×10^4	$p < 0.0001$	NG		NG	$p < 0.0001$	1.22×10^4	$p < 0.0001$
Challenge Control	7.57×10^7		NG		1.15×10^5		3.62×10^5	
Treatment Control	NG		NG		NG		NG	

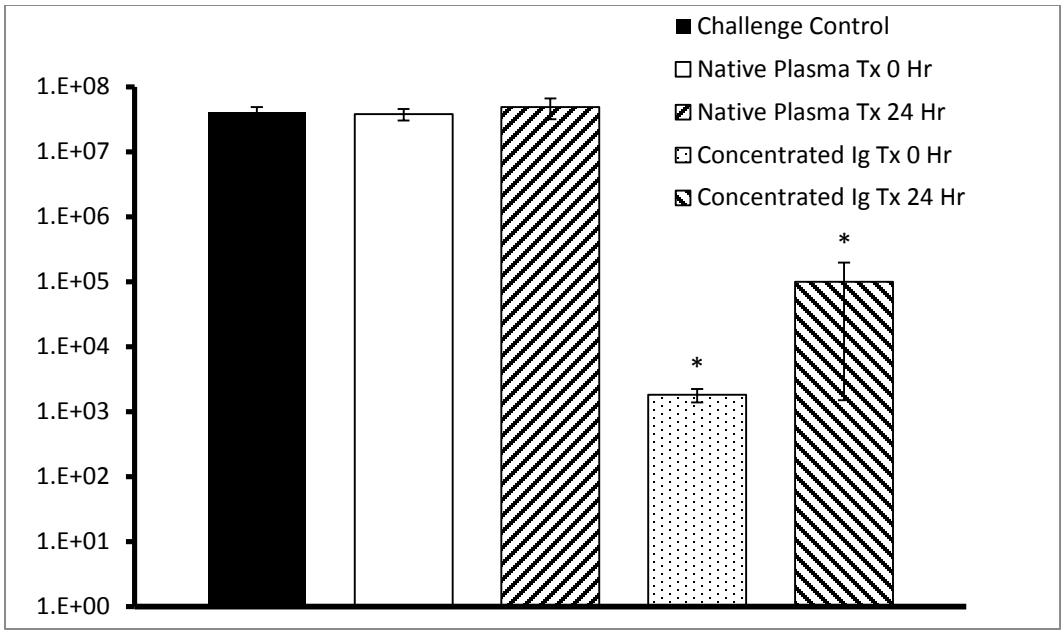


Figure 5.5 CFU/gram of lung tissue from mice following IN challenge

Upon death the lungs of each mouse were harvested. Each organ was weighed and homogenized then serially diluted 2 fold into sterile saline. Each dilution was plated onto BHI agar and the quantification of *B. anthracis* Sterne strain vegetative cells or spores was determined by the Miles and Mirsa method. All groups demonstrated significantly higher bacterial counts than treatment control mice. *$p < 0.001$ compared to challenge control.

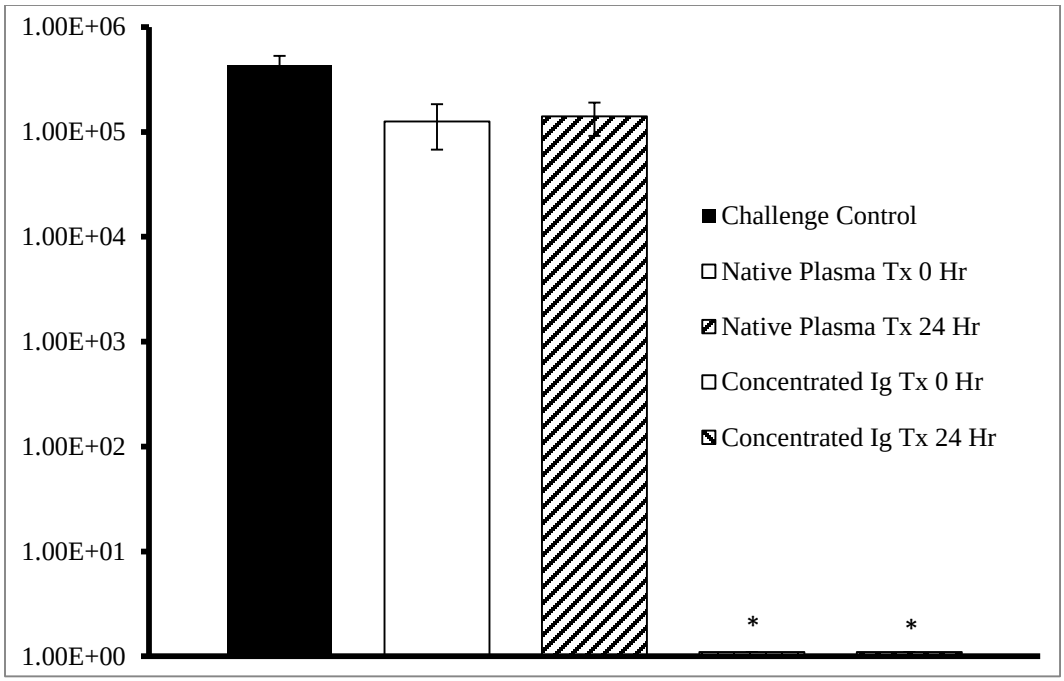


Figure 5.6 CFU/gram of lung tissue from mice following SC challenge

Upon death the lungs of each mouse were harvested. Each organ was weighed and homogenized then serially diluted 2 fold into sterile saline. Each dilution was plated onto BHI agar and the quantification of *B. anthracis* Sterne strain vegetative cells or spores was determined by the Miles and Mirsa method. All mice receiving concentrated IgG were not significantly different from the non-challenged control groups. *$p < 0.001$ compared to challenge control.

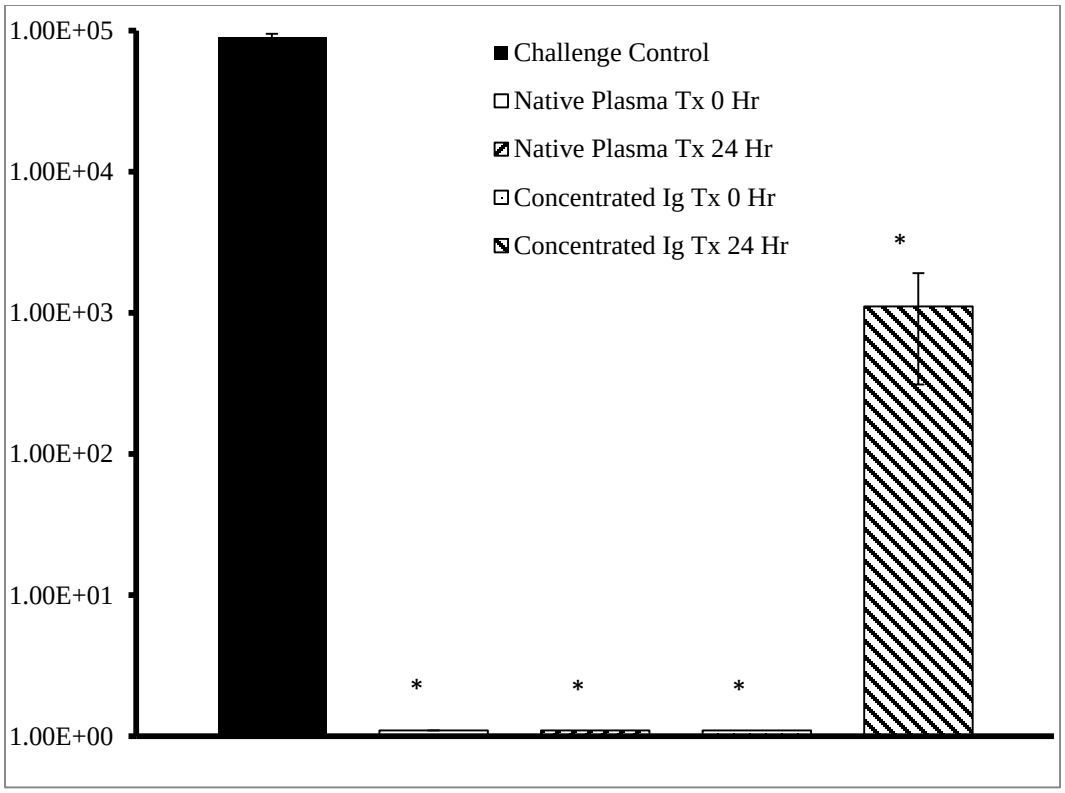


Figure 5.7 CFU/gram of liver tissue from mice following SC challenge

Upon death the liver of each mouse were harvested. Each organ was weighed and homogenized then serially diluted 2 fold into sterile saline. Each dilution was plated onto BHI agar and the quantification of *B. anthracis* Sterne strain vegetative cells or spores was determined by the Miles and Mirsa method. *$p < 0.001$ compared to challenge control.

Chapter 6:

Discussion

Protective antigen is a critical component of the protective immune response to *B. anthracis* infection. The development of new vaccines and immunotherapies require a rapid and accurate assessment of effectiveness during the initial stages of development. The studies outlined here typify the path of development that new modalities can take in the demonstration of effectiveness in preventing or treating anthrax. The use of immunoassays in the field of anthrax vaccine and immunotherapeutics development has been well studied in laboratory animal models, but very little information exists concerning their use in horses. In these studies we have modified and adapted an ELISA for the quantitation of anti-PA immunoglobulins and a cell culture toxin neutralization assay for horses vaccinated with the Sterne strain spore vaccine.

The anti-PA ELISA described here is designed to titer anti-PA IgG in horses. This assay has been established under good manufacturing practices with accompanying documentation and validated according to parameters established by the International Conference on Harmonics (ICH). Acceptance criteria for the validation of this assay were based on the performance of similar ELISAs designed for the measurement of anti-PA immunoglobulins in other species. This ELISA has been used to quantitate anti-PA antibodies in the plasma of horses immunized against the attenuated *B. anthracis* Sterne strain. In addition, the plasma of horses immunized with recombinant PA was also assayed. High anti-PA titers have repeatedly been shown as significant correlates of immunity. ELISAs are fundamentally simple, rapid and require little resource infrastructure for use. In the future, if we or others investigate additional potential

vaccines or immunization routes in horses, this assay has demonstrated repeatability and accuracy and would be the initial screening tool before moving into more expensive evaluations.

One downfall of the anti-PA ELISA is the lack of information provided about antibody affinity and neutralizing capacity of the hyper-immune plasma. Assessment of lethal toxin neutralizing titers by the toxin neutralization assay has been shown to correlate with survival in various animal models of infection. We adapted a practical cell culture assay that was used to assess neutralizing antibodies in the horse. Mouse macrophage J774A.1 cells are exquisitely sensitive to the effects of lethal toxin. Others have used this cell culture system to demonstrate efficacy of anthrax therapeutics before scaling up to live animal use. Briefly, lethal toxin of varying concentrations was pre-incubated with hyper-immune plasma or applied directly to a monolayer of J774A.1 cells. Neutralization of the toxin was then quantitated through the colorimetric change in a cell viability substrate. This study is the first to our knowledge to quantify toxin neutralizing immunoglobulins in horses immunized against anthrax. A key finding elaborated in the course of these investigations was the more diverse antigenic profile of this hyper-immune plasma to the fully toxigenic spore vaccine, including the LF and EF antigens. This likely conveys a greater level of toxin neutralization and protection than other monoclonal preparations.

In the final series of experiments the full protective capacity of the hyper-immune plasma was evaluated in a validated mouse model. The A/J strain of mice is homozygous for $Naip5^{Lgn1\text{-}s}$ which imparts reduced macrophage bactericidal activity and increased susceptibility to bacterial infection. Welkos *et al.* defined this strain's susceptibility to *B. anthracis* Sterne strain by

multiple routes of infection.[251] Interestingly, native or non-concentrated hyper-immune plasma prolonged the life of treated mice compared to non-treated control mice, but did not result in full survival. This suggests that native hyper-immune plasma is appropriate as an adjunct to antimicrobial therapy but not as a solo therapy. Perhaps multiple administrations or continuous rate infusions would provide a longer lasting effect and better treatment outcomes. The prophylactic capacity of this hyper-immune plasma was not addresses in these studies. Prophylactic passive transfer of anthrax specific antibodies has shown promise in other small animal infection models suggesting that the timing and volume of native hyper-immune plasma treatment needs further investigation. Alternatively, concentrated immunoglobulins alternatively was sufficient in protecting treated mice following a single administration at the time of infection or 24 hours post-infection. The concentrated immunoglobulins were produced by passing pooled hyper-immune plasma through a protein A column. Another avenue of continued investigation in this product needs to be a dose titration where the quantity of protective antibodies is established and balanced across treatments. These infection studies were conducted over a 10 day period and no attempt to quantitate the transferred antibodies or determine how long antibodies circulated in treated mice was explored.

Future directions in developing this hyper-immune plasma include many opportunities. Most importantly is the evaluation of protection in other animal models and against fully virulent strains, definition of the kinetics of the passively transferred antibodies, and refinement of the appropriate plasma or concentrated immunoglobulin doses alone and in combination with antimicrobial therapy. Additional steps in processing such as affinity purification and trypsin

digestion that are needed to make this product safe for use in humans need to also be conducted to determine their effect of protective efficacy. In summary, the results obtained in this series of experiments demonstrate the value of *in vitro* assays for the development of equine source immunotherapeutics and indicates that hyper-immune equine plasma is protective in defined anthrax infection models. This work lays the foundation for further development as prevention and treatment of anthrax in humans.

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