THE DEVELOPMENT AND CHARACTERIZATION OF MURINE MONOCLONAL ANTIBODIES RAISED TO RECOMBINANT PROTECTIVE ANTIGEN TOXIN OF *BACILLUS ANTHRACIS*

BY

TONY SALIBA

A Thesis Submitted To
The Faculty of Graduate Studies
In Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

Department of Medical Microbiology
Faculty of Medicine
University of Manitoba
Winnipeg, Manitoba, Canada

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

*****

COPYRIGHT PERMISSION

The Development and Characterization of Murine Monoclonal Antibodies Raised to Recombinant Protective Antigen Toxin of Bacillus Anthracis

by

Tony Saliba

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of

Manitoba in partial fulfillment of the requirement of the degree

of

Master of Science

ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr. Amin Kabani for providing me with the opportunity to work as his student. His guidance and understanding has helped make this project possible. A special thanks to Dr. Jody Berry whose patience, guidance, and support has helped in the development of this thesis project. He has truly been both a mentor and a friend.

I would like to thank the rest of my thesis advisory committee members Dr. Grant McClarty and Dr. John Wilkins for their comments and support.

I would also like to acknowledge all of the work, help, and support offered by the monoclonal antibody unit lab members especially Xin Yong Yuan, Francesca Ranada, and Brigitte Nicolas for their expert technical assistance.

Thanks to Dr. Steven Little from USAMIRIID for contributing hybridomas 2D5 and 3B6 and to Dr. Raymond Tsang for his cooperation.

I would also like to thank my family, whose encouragement and support has given me the motivation needed to complete this project.

I would finally like to thank CRTI for funding this project.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

The interplay between bacterial exo-toxins and host antibody responses represents one of the most stringent examples of pathogen-host co-evolution. The exo-toxin produced by *Bacillus anthracis* is composed of three proteins: lethal factor (LF), edema factor (EF), and protective antigen (PA). Individually, these components are non-toxic, however, the combination of LF or EF with PA forms catalytically active lethal toxin and edema toxin respectively. Therefore, inhibiting PA's functionality may result in the neutralization of both toxins since it is the communal component. Monoclonal antibodies (mAbs) for treatment and detection of anthrax toxin are needed for the clinic and diagnostics respectively. Novel murine mAbs raised to recombinant PA toxin, which neutralize the in vitro cell killing activity of lethal toxin through binding to specific epitopes on PA have been developed and characterized. The immunochemical, biological, and immunogenic properties of these murine mAbs against a recombinant PA immunogen are describe herein. These nine IgG1 / Kappa, mAbs recognize the intact PA83 and trypsin treated PA molecules by western immunoblot, which suggests that all target linear epitopes. Surface plasmon resonance affinity measurements were conducted on the mAbs in order to determine whether their affinities could be correlated with the in vitro neutralization of toxin activity with the J774A.1 macrophage cell-line. The data indicates that the epitope recognized is more important than the affinity. Variable region gene sequencing has determined that the neutralizing mAbs are derived from distinct hybridomas. These antibodies and the epitopes that they recognize will be useful for the development of diagnostic tests, pathogenicity and vaccine studies.

1. INTRODUCTION

1.1 Bacillus Antracis

1.1.1 History

Anthrax is an acute mammalian disease caused by *Bacillus anthracis*. The research conducted on *B. anthracis* has helped shape our understanding of microorganisms and their impact on human life. The first historically, as opposed to scientifically, recorded incident of anthrax is believed to have been approximately 1250 B.C. as the cause of the biblical fifth and sixth Egyptian plagues (Hart and Beeching, 2002; Koehler, 2002). In the 1600's a notable outbreak, named the Black Bane, occurred in Europe that killed 60,000 cattle (Turnbull, 2002). The first scientific reports of anthrax were made in the mid to late 1700's by Maret, Dym, and Fournier who described malignant pustules in humans and by Chabert who described the disease in animals (Wistreich et al., 1973).

Medical research of anthrax truly began in the mid 1800's when Pierre Rayer and Casimir-Joseph Davaine discovered the presence of the bacillus in infected sheep. Davaine believed that these organisms were the cause of anthrax rather than a product of the disease (Sternbach, 2003). It was not until later that century that Robert Koch inoculated healthy animals with cultured *B. anthracis*, causing disease and allowing for the creation of koch's postulates (Smith, 2002). In 1881, Louis Pasteur experimented with a live attenuated vaccine, the first of its kind, and found that the vaccinated animals

survived exposure to virulent anthrax while unvaccinated animals died (Salyers et al., 2002; Sternbach, 2003). Human infection with anthrax was primarily associated with the handling of animal hides and hair.

Through vaccination and improved animal processing and husbandry, the mid to late 20th century experienced a decrease in the frequency of anthrax (Brachman, 2002). During this century, however, perhaps the most frightening development in anthrax research occurred; the increased interest in the use of *B. anthracis* as a biological weapon. It was believed that during World War II, the Germans had been developing the technology for biological weapons. Although this turned out to be false it, nevertheless, sparked the well known anthrax trials on Gruinard Island in 1942. British scientists detonated bombs containing the bacterial spores on the island in an effort to study their effects and methods of decontamination (Manchee, 1983). This period of research spawned a great deal of knowledge about anthrax including the discovery of the 3 part toxin and the production of the human anthrax vaccines (Koehler, 2002).

In 1979, an accidental release of spores occurred at a Soviet Union military facility killing several people (Meselson et al., 1994). In 1993, a terrorist group by the name of Aum Shinrikyo released *B. anthracis* spores from a building in Tokyo. It was later discovered that the strain used lacked the pX02 plasmid, rendering the bacilli harmless (Keim, 2001). The very first intentional release of *B. anthracis* in North America occurred in 2001 when contaminated letters infected several individuals. This most recent bioterrorist attack against the United States has sparked renewed interest in anthrax research resulting in the creation of new methods to control and prevent anthrax.

1.1.2 Bacteriology

Bacillus anthracis is a large, non-motile, gram positive rod. The vegetative bacillus measures between 1-1.5µm wide and 5-8µm long (Koehler, 2002). It is a catalase positive, facultative anaerobe that is non-hemolytic on blood agar. *B. anthracis* grows well on blood agar within 18-24 hours at 35°C (Oncu, 2003). Cultured colonies are generally whitish grey, opaque, and measure between 2-5 mm in diameter (Koehler, 2002; Oncu, 2003). The bacilli tend to group together in long chains, giving them a bamboo stick type of appearance. Although many bacteria have capsules, B. anthracis is unique in that it possesses a poly-D-glutamic acid capsule. The capsule can be seen as a pink structure through the use of an M'Fadyean stain. The bacilli remain relatively sensitive to penicillin and are lysed by gamma phage. They have the ability to form elliptical, subterminal spores to endure harsh conditions. The bacterium can produce three different toxin components that can combine to form two different toxins; lethal toxin and edema toxin.

1.1.3 Ecology and Epidemiology

B. anthracis is a saprophytic microbe. Herbivores are at higher risk due to an increased exposure to the bacilli that are normally found as spores in soil. Grazers may ingest the spores or inhale them when feeding in dry dusty areas. Humans are infected through direct or indirect contact with contaminated animals and animal products.

It has been shown that biting insects, such as stable flies and mosquitoes, are able to mechanically vector *B. anthracis* from one animal to another (Turell and Knudson, 1987). While human to human transmission is extremely rare, some believe that biting insects may play a role in vectoring the bacillus from one individual to another (Davies, 1983).

B. anthracis has a global distribution and is endemic mainly in sub-saharan Africa, central and southern Asia, certain areas in China, and Haiti (Koehler, 2002). Human anthrax infection is very rare in many countries including Canada and the United States. Bioterrorism, however, is not influenced by endemic regions and increases the global risk of contracting the disease. Outside of the bioterrorist risk, people that work with animal products, work in laboratories, and/or live in countries with poor anthrax control are at highest risk for anthrax.

1.1.4 Clinical Manifestations

There are three forms of anthrax; cutaneous anthrax, inhalation anthrax, and gastrointestinal anthrax. Cutaneous and inhalation anthrax are most common and are further discussed in this section. Gastrointestinal anthrax is extremely rare and requires a break in the gastrointestinal lining to allow spore entry into the tissue (Koehler, 2002).

1.1.4.1 Cutaneous Anthrax

Cutaneous anthrax makes up over 95% of all naturally occurring anthrax cases (Koehler, 2002; Hart, 2002; Oncu, 2003). The spores require an opening in the skin in order to establish an infection, as the bacterium is generally regarded as non-invasive. The incubation period is approximately 1 to 12 days followed by the formation of a small papule (Oncu, 2003). A ring of vesicles develops, surrounding the papule. The central papule then breaks down and dries, forming a black eschar. Edema around the lesion is characteristic of cutaneous anthrax. Local lymph nodes can become enlarged (Hart, 2002). It is normally localized but the bacteria can replicate within the lymph nodes and spread systemically leading to sepsis. Without treatment, approximately 20% of those infected succumb to death compared to less than 1% when treated with antibiotics (Oncu, 2003).

1.1.4.2 Inhalation Anthrax

Inhalation anthrax is less common than the cutaneous form but is far more dangerous. The incubation period is between 4 to 6 days and death results in 99% of untreated cases (Koehler, 2002; Schuch et al, 2002). Spores are naturally found in soil, among other areas, that can dry and become aerosolized. Individuals that work with animal hairs and skins are at a higher risk of inhaling spores that can be found within these products. The use of *Bacillus anthracis* as a biological weapon is a current threat and has been responsible for anthrax infection via inhalation. The lethal dose is believed

to be between 8,000 and 50,000 spores (Bossi et al, 2004; Oncu, 2003). Once inhaled, the bacteria settle deep within the lungs and can spread via macrophage movement into the lymphnodes. This disease begins as a flu-like illness and progresses to a respiratory failure and toxemia. Mediastinal widening is quite distinctive of inhalation anthrax and can be seen using chest X-rays (Hart, 2002; Koehler, 2002; Oncu, 2003).

1.1.5 *Bacillus anthracis* as a Biological Weapon

For many decades *B. anthracis* has received a great deal of attention as a possible biological weapon. This is due to several factors that make the organism an ideal choice for both terrorist and warfare use. *B. anthracis* spores can withstand a great deal of environmental abuse and are therefore extremely stable compared to other potential biological warfare agents. The spores have been shown to persist within the environment for decades and pose a threat long after their release. This is due to the possibility of re-aerosolization and is dependent on bacterial concentration and the quality of weaponization. The spores are also more likely to survive dissemination by explosion or high velocity and pressure aerosolization than other possible warfare agents. The relative ease of weapon production is another trait of *B. anthracis* that favors its use as a biological warfare agent. In addition to these factors, anthrax has a very high mortality rate nearing 100% if left untreated. Even with treatment, the mortality rate is quite high because once the symptoms of inhalation anthrax appear the bacteria have already established a life threatening infection. Prior to the 21 century, the reported survival rate of individuals with inhalation anthrax receiving treatment was approximately less than

15% (Brachman, 1980; Meselson et al, 1994). In 2001 there were 11 confirmed bioterrorist related cases of inhalation anthrax and 11 confirmed or suspected bioterrorist related cases of cutaneous anthrax reported in the United States. Improved medical care and awareness resulted in a survival rate of 55% in patients diagnosed with inhalational anthrax (Jernigan et al, 2002).

Although the use of *B. anthracis* as a weapon can be accomplished by trained individuals with relative ease, perfecting the organism for such a task is not that simple. Weaponized *B. anthracis* must have a small spore size in order to increase its spread and the amount of time that it remains suspended in the air. During weaponization, the bacteria tend to develop an electrostatic charge that causes them to clump together, increasing their particle size and therefore reducing their effectiveness. To overcome this, chemical fluidizing agents such as silica or bentonite can be used. These agents negate the electrostatic charge by separating the spores far enough to overcome the Van de Walls forces that link them together, allowing the spores to remain dissociated from one another (Matsumoto, 2003). In 1969, the United States was able to weaponize *B. anthracis* using silica as a fluidizing agent in a process that few other nations could mimic (McAllister, 2001).

Antibiotic resistance remains a relatively low occurrence with naturally found *B. anthracis*. Genetic engineering the bacteria to form antibiotic resistant mutants would further impede the successful control of an anthrax outbreak. The Soviet biological warfare program produced numerous genetically engineered strains of antibiotic resistant *B. anthracis*.

Taken together, all of these factors contribute to *B. anthracis'* voracity as a biological weapon. There is no question that this organism is a real and possible threat to all nations and requires immediate attention and on-going research to help prevent another attack like that seen in 2001.

1.1.6 Virulence Factors

1.1.6.1 Macrophage Interaction

B. anthracis has shown a very tight co-evolution with its host in order to use parts of the host immune system to its advantage. Although the lymph nodes have been described as regions of *Bacillus anthracis'* proliferation and dissemination, they are carried there by macrophages (Koehler, 2002). Simply put, macrophages are Trojan horses, hijacked by the bacteria (Guidi-Rontani, 2002). This is accomplished through the microbe's ability to survive and germinate within macrophages. The lungs are merely a point of entry into the host for the pathogen since germination and proliferation does not occur within the lungs. Once the spores travel deep into the alveoli they are phagocytosed by alveolar macrophages. These macrophages make their way to the regional lymph nodes and mediastinal lymph nodes, where they can travel into the blood stream (Guidi-Rontani, 2002). It has been shown that the spores are able to germinate within vesicles derived from the phagosomal compartment in macrophages (Guidi-Rontani et al, 1999b). It is suggested that the vegetative cells emerge into the macrophages cytoplasm (Guidi-Rontani, 2002). The genes that encode the toxins and the toxin transactivator AtxA are

quickly expressed after germination, allowing the vegetative cells to escape the macrophages (Guidi-Rontani et al, 1999b).

1.1.6.2 Spore Formation

The discovery that *Bacillus anthracis* is able to produce infectious spores marks a milestone in our understanding of anthrax. The spore consists of several layers that have essential functions in maintenance and germination. The inner most layer is the spore core, composed of cytoplasm and nucleoplasm surrounded by membranes (Koehler, 2002). The cortex, found in between the inner and outer membranes, is essential in spore dormancy and heat resistance and resembles the vegetative cell wall (Foster, 1994; Koehler, 2002). The coat that surrounds the outer membrane functions in protecting the spore against degradative enzymes (Moir and Smith, 1990). The exosporium is the outer most layer of the spore. This layer acts as a permeability barrier and contains the spore's surface antigens (Gerhardt, 1967). Spores lacking this layer are just as infectious as those with an exosporium, indicating that it is probably not involved in the protection or infectious nature of the spore (Liu et al, 2004).

Sporulation gives the bacteria the ability to withstand harsh conditions including extreme temperatures, low food availability, and extreme pH variability. It has also made it difficult to eradicate the disease since the spores have a half life of approximately 100 years (Halvorson, 1997). Sporulation allows for the preservation of the bacteria in between hosts and normally occurs in the soil, underneath the dead carcass. It has been shown that this process does not occur in living tissue unless it is exposed to air (Oncu,

2003). The rate of sporulation is dependent on several environmental factors including pH, oxygen availability, and organic matter to name a few (Koehler, 2002).

As previously discussed, germination is vital to *B. anthracis'* pathogenesis and is able to occur within macrophages. It has been shown that several compounds, including L-alanine, adenosine, and DL-tyrosine, have the ability to trigger spore germination (Hills, 1949). Two germination operons have been identified; *gerX* and *gerY* (Guidi-Rontani et al, 1999a). *gerX* is found on the pX01 plasmid in the same region as the anthrax toxin genes and their activators and repressors (Koehler, 2002). *gerY*, on the other hand, is chromosomally based. *gerX* and *gerY* are very good targets for PCR identification because they are likely to be very highly conserved.

The organism's life cycle is strongly based on its ability to form spores. This has a direct impact on the low genetic variability found in this species. It has been suggested that since this bacteria only replicates within its host and stays dormant for the majority of its life in its spore form in between hosts, it does not have as much opportunity to multiply and change compared to other pathogens (Koehler, 2002). This has also lead to a decrease in exposure to mutagens, phages, and other strain varying events.

In contrast, a study conducted by Dr. Hanna on B. anthracis' life cycle showed that the organism can undergo full life cycles in soil without the presence of a host (Hanna, 2004). This suggests that the vegetative form may be able to exchange genetic information with other bacteria and multiply in the environment without the need of a host.

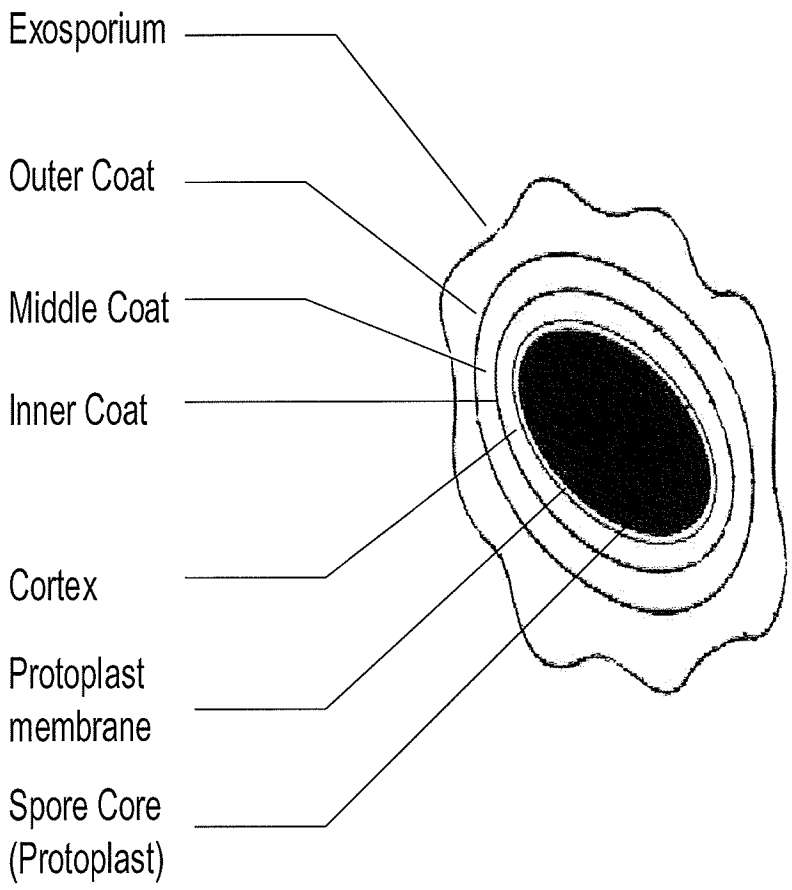

Figure 1 – *Bacillus anthracis* Spore Cross-Section

1.1.6.3 Capsule

Unlike the polysaccharide capsules of other bacteria, the capsule of *B. anthracis* is composed of poly-D-glutamic acid. This capsule is essential in evading the host's immune system. Unlike spores, the vegetative cells must evade phagocytosis to survive and have therefore incorporated the use of this anti-phagocytic capsule as a mechanism. Unfortunately, capsular antigens are non-immunogenic and therefore vaccines that target them are not effective. Antibodies cannot penetrate the capsule and therefore cannot bind to their target proteins. The capsule has also been shown to inhibit the binding of the C3 component of complement (Koehler, 2002).

The *cap* genes encode the capsule and are found on the pX02 plasmid (96kb) (Drysdale, 2005; Salyers and Whitt, 2002). The protein AcpA regulates *cap* gene expression and is encoded by *acpA* and is also found on pX02 (Salyers and Whitt, 2002). AtxA, a protein product of the *atxA* gene, regulates AcpA synthesis. Cross-talk between plamid pX01 and pX02 occurs since *atxA* is found on the pX01 plasmid. It should be mentioned, however, that pX01 is not required for the synthesis of capsule as a pX01 (-), pX02 (+) strain is still fully capable of producing capsule (Alouf and Freer, 1999). CO_2 has been linked with the induction of *acpA* transcription but not *atxA* expression (Vietri et al, 1995).

Virulence is determined by the presence of both capsule and toxins and therefore requires both plasmids. A strain lacking either virulence factor attenuates it (Drysdale, 2005).

1.1.6.4 Exotoxins

Bacillus anthracis is not only unique in having a poly-D-glutamic capsule, but also in possessing the ability to produce three different toxin components that can combine to form two different toxins with one central component. The toxin components include: protective antigen (PA) encoded by the *pag* gene, lethal factor (LF) encoded by the *lef* gene, and edema factor (EF) encoded by the *cya* gene. The *atx* genes (*atxA* and *atxR*) regulate the expression of *pag*, *lef*, and *cya* (Alouf and Freer, 1999; Salyers and Whitt, 2002). Both the regulatory genes and the toxin genes are found on the pX01 plasmid (182 kb) (Drysdale, 2005). The anthrax toxin functions as an A-B type toxin. Both edema factor and lethal factor function as A-components that bind to protective antigen, the B-component.

1.1.6.4.1 AB-Toxins

The term AB-toxin refers to a toxin that contains an A domain that carries out an enzymatic function and a B domain that binds to a receptor on the target cell surface. AB-toxins are further divided into three different structural groups. The first group consists of toxins that carry both the A and B domains on a single polypeptide chain. Diphtheria toxin, botulinum neurotoxin, and *Bordetella pertussis* adenylate cyclase toxin are examples of this type of AB-toxin. The second group is composed of toxins that carry the A and B domains on different subunits that are noncovalently linked with each other. Representatives of this group include the cholera toxin, pertussis toxin, and Shiga toxin.

The third group, known as bipartite or binary toxins, is made up of toxins that have distinct components that carry the A and B domains and are produced as separate peptides that are finally assembled on the surface of their target host cell. Although there are several toxins that fall into this category, including the botulinum C2 toxin, the anthrax toxin is by far the most studied of the group. Most toxins from all three groups undergo proteolytic change in the region between the A and B domains. Once the B domain binds to the cell surface, the toxin is endocytosed and moved to its appropriate destination where the A component is released into the cytoplasm to fulfill its enzymatic function.

	Bacteria	Toxin	Molecular Weight (kDa)	Proteins	Enzymatic Activity
Group I	*Corynebacterium diphtheria*	Diphtheria toxin	62	Single	ADP ribosylation of elongation factor 2
	Clostridium botulinum	Botulinum neurotoxin	150	Single	Zn^{++} dependent protease acts on synaptobrevin at motor neuron ganglioside
	Bordetella pertussis	Adenylate cyclase toxin	45	Single	Local increase in cyclic AMP
Group II	*Vibrio cholera*	Cholera toxin	86	Multiple	ADP ribosylates adenylate cyclase Gs regulatory protein
	Bordetella pertussis	Pertussis toxin	105	Multiple	ADP ribosylates adenylate cyclase Gi regulatory protein
	Shigella dysenteriae	Shiga toxin	68	Multiple	Glycosidase cleavage of ribosomal RNA
Group III	*Clostridium botulinum*	Botulinum C2 toxin: C2I C2II	 49 81	Multiple	ADP rybosylation of actin
	Bacillus anthracis	Edema and Lethal toxins: EF LF PA	 89 90 83	Multiple	 - Adenylate cyclase - Zinc Metalloprotease for MAPKK

Table 1 – Examples of Bacterial Protein Toxins of the AB-Type
Group I toxins carry both the A and B domains on a single polypeptide chain. Group II toxins carry the A and B domains on different noncovalently linked subunits. Group III toxins are produced as separate proteins that are assembled on cell surface. C2I: enzymatic component, C2II: Binds to cell surface, EF: edema factor, LF: lethal factor, PA: protective antigen. (Modified from Alouf and Freer, 1999; Barth et al, 2004)

1.1.6.4.2 Protective antigen (PA)

Protective antigen (PA) has been the focus of many vaccine studies and therapeutics as it plays a central role in *B. anthracis* pathogenesis. PA combines with edema factor (EF) and lethal factor (LF) to form edema toxin and lethal toxin respectively. It functions as a B-component of an A-B toxin, binding to the host cell receptor and internalizing the edema or lethal factors.

PA is an 83kDa protein that is composed of four functional domains (Figure 2). Domain I is an amino terminal domain and is responsible for the activation of the PA molecule, allowing oligomerization of the monomer. PA is activated by a proteolytic cleavage of domain I by a member of the furin class of proteases (Bhatnagar and Batra, 2001). Furin is commonly found on most cells. Domain I can be further subdivided into two sub-domains; sub-domain Ia, which after proteolytic cleavage is released as a 20kDa section, and sub-domain Ib, that remains part of the larger 63kDa portion of PA (Koehler, 2002). The two sub-domains meet between amino acids 164 and 167 at the trypsin-sensitive site, RKKR, where the cleavage takes place (Little and Ivins, 1999; Petosa et al, 1997). This domain also includes the site at which LF and EF bind to PA to form the lethal and edema toxins respectively. It has been shown that LF may indeed interact with two different sites on PA to form lethal toxin (Little et al, 1996).

Domain II extends residues 250 to 487 and is associated with oligomerization and to a greater extent, pore formation (Koehler, 2002; Mogridge et al, 2001). A new study has revealed another function for this domain. Through crystal structure analysis of the binding of PA to CMG2, a host cell receptor, it was determined that a loop extending

from domain II interacts with a pocket on the receptor, increasing the affinity between the two (Santelli et al, 2004). This domain is not essential for the binding of PA to its host cell receptor since a disruption in this domain still allows PA to bind to the target receptor but inhibits the internalization of LF into the cell (Singh et al, 1994).

Domain III extends residues 488 to 594 and is involved in the oligomerization of the PA proteins (Koehler, 2002; Mogridge et al, 2001). A single mutation in amino acid 512 of domain III inhibited the formation of a PA heptamer, indicating its importance in oligomerization (Mogridge et al, 2001).

Domain IV is the carboxyl-terminal end and consists of amino acids 595 to 735 (Koehler, 2002). Unlike domains I, II, and III, domain IV is not intimately associated with the rest of the protein (Bhatnagar and Batra, 2001). This domain is the primary binding site between PA and its host cell receptors TEM8 and CMG2 (Koehler, 2002; Santelli et al, 2004; Varughese et al, 1999). Monoclonal antibodies that target regions within domain IV have been shown to inhibit PA binding to the host cell, reinforcing the stated function of this domain (Little et al, 1988; 1996; 1991). The host cell surface receptors contain a von Willebrand factor A domain that interacts with PA in a fashion that mimics the binding of integrins to their natural ligands (Lacy et al, 2004; Santelli et al, 2004).

A recent study has found that PA associates with the *B. anthracis* spore coat and may be involved in germination (Cote et al, 2005). This adds a significant role for PA in the pathogenesis of the organism. The use of an antibody raised against PA may not only inhibit the function of the toxin but may also inhibit spore germination early on in the infection.

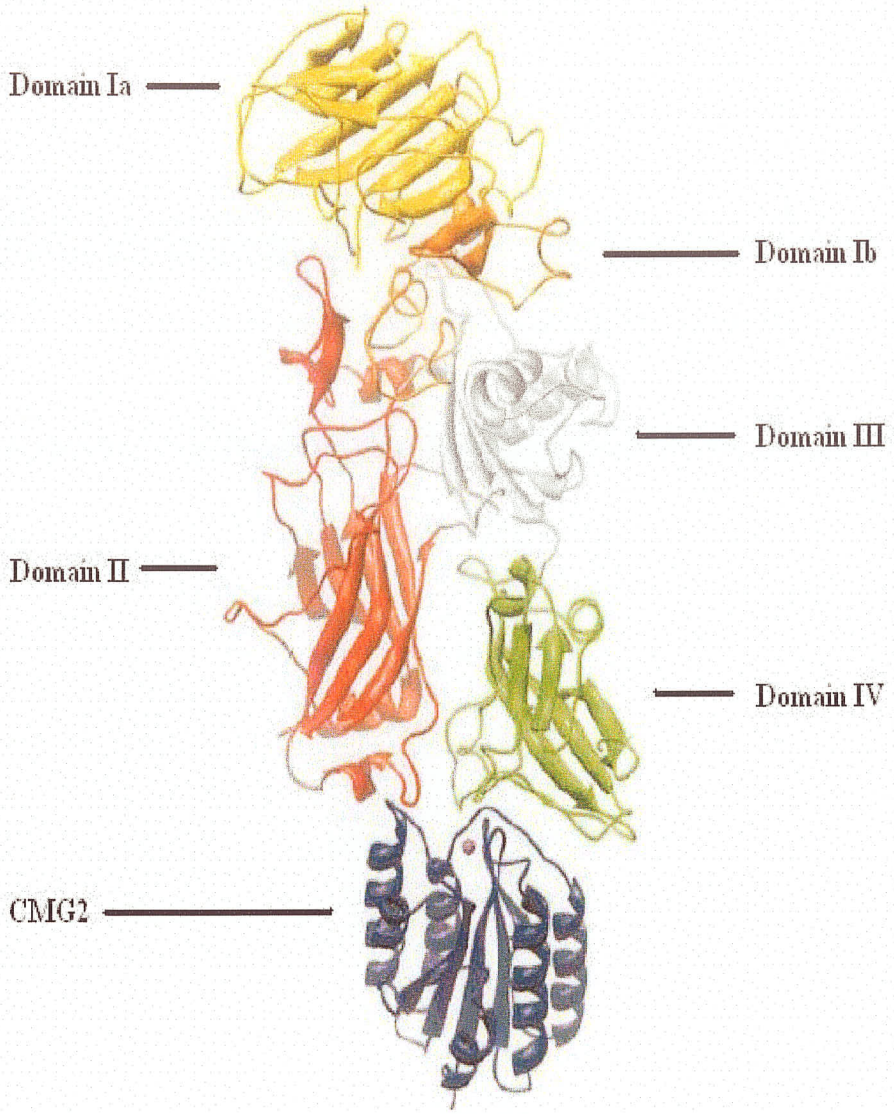

Figure 2 – Structure of Protective Antigen with CMG2 Host Receptor Complex
(Modified from original obtained from Santelli et al, 2004)
Domains II and IV can be seen interacting with the host cell receptor CMG2.

1.1.6.4.3 Lethal Factor (LF)

Lethal factor (LF) is the enzymatic A-component of the lethal toxin. This 90kDa protein binds to the same site on PA^{63} that the EF toxin binds (Collier and Young, 2003). LF and EF are quite homologous in the N-terminal region, which spans 255 amino acids (Alouf and Freer, 1999; Collier and Young, 2003). The N-terminals of both LF and EF are the sites that bind to PA and are known as Domain I. Although polyclonal antibodies have failed to cross-react with LF and EF, several monoclonal antibodies (mAbs) have proven to be successful in doing so (Little et al, 1990).

In order for LF to bind stably to PA and enter the host cell, PA must undergo oligomerization and form a pore in the host cell (Mogridge et al, 2002). Each heptamer is capable of binding up to 3 LF and / or EF competing proteins.

LF functions as a zinc metalloprotease. It cleaves members of the Mitogen-Activated Protein Kinase Kinases (MAPKK), leading to the interference of MAPK signaling and apoptosis (Figure 1) (Collier and Young, 2003; Singh, 2003). Once inside the host cell cytoplasm, LF interferes with three known pathways; the extracellular signal-regulated kinase (ERK), p38, and c-Jun N-terminal kinase pathways (Sanchez and Bradley, 2004). Another study suggests that the interruption of the ERK pathway leads to the apoptosis of endothelial cells causing the vascular damage seen in anthrax (Kirby, 2004). It is possible that LF leads to the destruction of molecules that are essential in the homeostasis of macrophages, leading to their destruction (Tang and Leppla, 1999). Many believe that LF also stimulates the overproduction of cytokines leading to macrophage lysis (Hanna et al, 1993; Singh, 2003). Other studies have contradicted this belief in

reporting that lethal toxin suppresses rather than induces cytokine production (Erwin et al, 2001). This protein can induce the overproduction of cAMP, throwing off the hormonal balance of the cell (Singh, 2003). The true nature of lethal toxin activity requires further study, as there is much to be resolved.

1.1.6.4.4 Edema Factor (EF)

Edema Factor (EF) is the enzymatic A-component of the edema toxin. This 89kDa protein functions by inhibiting phagocytosis and neutrophil function (Singh, 2003). EF is a calcium- and calmodulin-dependent adenylate cyclase (Ascenzi et al, 2002). The production of cAMP within a cell is essential but must be properly regulated. EF, in the presence of calcium and calmodulin, cleaves ATP to form cAMP at an elevated level, leading to impaired maintenance of water homeostasis and edema (Figure 1) (Ascenzi et al, 2002; Leppla, 1982; Singh, 2003). EF activation requires that the three switch loops found on EF undergo a conformational change upon binding to calmodulin, allowing the binding of substrate (Ascenzi et al, 2002). Since high levels of cAMP are not lethal, increased levels of edema toxin do not have lethal consequences (Little and Ivins, 1999).

1.1.6.4.5 Recombinant Technology

Cultured *Bacillus anthracis* produces approximately 20mg, 5mg, and 1mg of PA, LF, and EF respectively per liter of culture supernatant (Leppla, 1988). The use of B.

anthracis to produce the anthrax toxin components requires a great deal of time, funds, and the proper facilities since it is a level three organism and requires an extended period of time to culture. Thanks to recombinant technology organisms like *Escherichia coli* and *Bacillus subtilis* can be used to produce recombinant toxin components. *E. coli* cells, for example, can be made to harbor the anthrax toxin expression plasmids in order to produce the anthrax toxin subunits. Earlier work with recombinant PA toxin production using *B. subtilis* did not generate large quantities of toxin (Baillie et al., 1998). Through improved methods recombinant toxin production now yields large, high purity quantities of anthrax toxin. One group has recently been able to produce approximately 370mg/L of PA protein from *E. coli* (Laird et al, 2004).

1.1.7 Anthrax Toxins: Mechanisms of Action

Bacillus anthracis produces the three toxin subunits, PA, LF, and EF and secretes them into its surrounding environment (Figure 1). PA binds to a type 1 membrane protein receptor, either TEM8 or CMG2 (Figure 1) (Scobie et al, 2003). The CMG2 receptor is present on most tissue cells, whereas the TEM8 receptor is generally found in highest concentration on angiogenic cells in tumours (Bradley and Young, 2003; Santelli et al, 2004). These angiogenic cells provide the tumor with blood for growth and therefore targeting them for destruction would help destroy the tumor. By taking advantage of this and even increasing tumor cell specificity, the use of anthrax toxin as an anti-tumor agent seems quite promising (Duesbery et al, 2001; Liu et al, 2000; 2001).

As previously discussed (Section 1.1.5.4.1), the interaction between PA and TEM8 / CMG2 involves domains II and IV of PA and the extracellular von Willebrand factor A domain of the host cell receptor (Burns et al, 2003). Once bound, PA is cleaved by a member of the furin class of proteases between subdomains Ia and Ib forming a 20kDa segment and a receptor bound 63kDa fragment (Figure 1) (Klimpel et al, 1992). After cleavage, PA63 quickly undergoes heptamerization to form a prepore (Figure 1). It has been shown that both EF and LF require PA oligomerization in order to bind to it (Mogridge, 2002). It has been hypothesized that oligomerization is essential due to the hydrophilic nature of PA (Bhatnagar, 2001). Each PA monomer contains patches of hydrophobic structure that is multiplied through oligomerization, therefore creating a hydrophobic surface large enough to interact with the hydrophobic core of the bilayer. The number of copies of LF and / or EF that can bind to the PA heptamer varies in the literature. Several papers report that the heptamer binds up to three copies of EF and / or LF competitively (Mogridge, 2002). Another study found that each PA subunit in the heptamer can bind one LF molecule, making a total of seven (Singh, 1999). Lipid rafts seem to play an important role in internalization of the heptamerized PA complex into the host cell (Wesche, 2003). This process has been shown to be clatherin-dependant (Abrami et al, 2003). Once the entire complex is endocytosed, it moves to endosomal compartments where the pH is low (Singh, 2003). The initial binding of PA's domain II to the receptor prevents its insertion of the pore forming loop into the plasma membrane (Santelli et al, 2004). The receptor also tethers domains II and IV, limiting their movement. It is not until the pH drops well below 7 that PA undergoes a conformational change allowing the creation of the pore in the endosomal membrane, releasing LF and /

or EF into the cell cytoplasm (Figure 1) (Lacy et al, 2004). EF and LF are then able to fulfill their enzymatic functions, leading to edema and death (Figure 1).

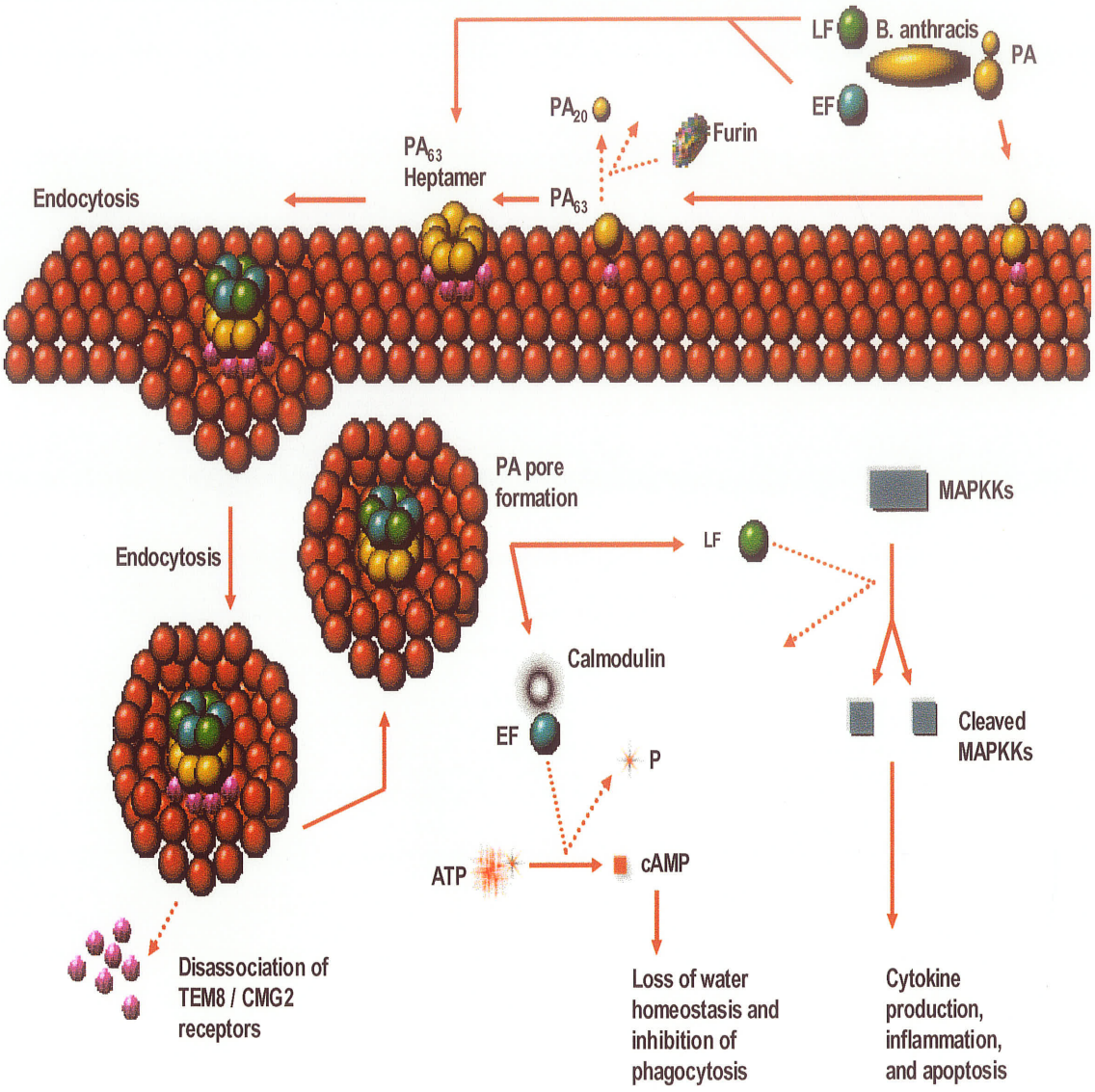

Figure 3 – *Bacillus anthracis* Toxins: Mechanisms of Action

1.2 Anti-Anthrax Strategies

1.2.1 Vaccines

Currently the use of vaccines remains the primary preventative measure against exposure to *Bacillus anthracis*. It is only given to high risk individuals like at risk animal workers and military personnel. The earliest vaccine was given to livestock and created by Pasteur in 1881 (Salyers et al., 2002; Sternbach, 2003). It consisted of a live attenuated *B. anthracis* strain that stemmed from his previous discovery that culturing the bacteria for a prolonged period of time at a high temperature reduced its virulence (Tigertt, 1980).

Live spore vaccines are used on humans in China and parts of the former USSR (Turnbull, 1998). These vaccines are not permitted for use in North America. In 1970, the United States Food and Drug administration approved the human use of Anthrax Vaccine Adsorbed (AVA). The vaccine contains an aluminum hydroxide-adsorbed cell free supernatant from a non-encapsulated strain of B. anthracis. Several studies have shown the vaccine to be quite safe and effective (Sever et al, 2004; Sulsky et al, 2004). On the other hand, there have been several reports of adverse side effects and some supporting studies that strongly suggest the development of safer therapeutics for combating anthrax (Geier and Geier, 2002; Pittman, 2002). In addition to this, AVA requires annual boosters and multiple injections overtime and takes several weeks to establish immunity. The primary effective component of the vaccine is PA (Koehler, 2002).

PA is the central toxin component and has been shown since 1986 to be sufficient on its own to induce a protective immune response to anthrax (Ivins and Welkos, 1986). Since PA is proteolytically cleaved into PA20 and PA63, experimentation has shown that the latter is sufficient for protective immunity (Ivins and Welkos, 1988). Studies are currently en route towards the use of a recombinant PA (rPA) subunit vaccine (Mikszta et al, 2005; Singh et al, 1998). Different aluminum salts have been examined in order to determine their effectiveness as adjuvants (Berthold et al, 2005). It was determined that the amount of adjuvant used had a direct impact on the antibody response that was induced.

An alternative anthrax therapy is the use of polyclonal or monoclonal antibodies. This branch of therapy holds much promise and is currently a very active field of study.

1.2.2 Antibodies

1.2.2.1 Structure and Classes

Antibody structure has been extensively studied (Benjamini and Leskowitz, 1988; Harlow and Lane, 1988; 1999). Antibodies, or immunoglobulins, can be visualized as "Y" shaped structures. The two arms of the "Y" are known as the fragment antigen binding (Fab) fragments. The lower section is known as the fragment crystallization (Fc) portion. The connector between the Fab and the Fc fragments is known as the hinge region. This hinge offers the antibody fragments a little flexibility. Each antibody

contains two light chains approximately 25,000 daltons each and two heavy chains that are approximately 55,000 daltons each. The chains are held together by disulphide bonds that are quite susceptible to proteolysis. There are also intrachain disulfide bonds that form globular loops; two loops in each light chain and four or five loops in each heavy chain.

The first 110 amino acid sequences of the heavy and light chains are extremely variable and are known as the variable regions (Benjamini and Leskowitz, 1988). Both arms of an antibody, however, have identical variable regions. The rest of the heavy and light chains are known as the constant regions. There are two types of light chain constant regions; a κ light chain and a λ light chain. There are five different classes of antibodies based on the type of heavy chain that they possess.

IgG possesses a γ heavy chain. IgG has the longest half life (approximately 23 days) of all classes. It has several functions including opsonization, immobilization, and neutralization of toxins and microbes. It is also the only class of immunoglobulin that can traverse the placenta, transferring immunity to the fetus. It is the ideal antibody candidate for passive immunization due to its mentioned characteristics (Benjamini and Leskowitz, 1988).

Within the variable region there are three amino acid segments, known as complementarity-determining regions (CDRs) that show the most variability. These regions are therefore referred to as the hypervariable regions. The segments between the hypervariable regions are called framework regions. The hypervariable regions are brought together by folds and make up the antigen binding area. This variable region

gives antibodies the specificity to their specific antigen and determines the affinity with which they interact.

1.2.2.2 Therapeutics: Polyclonal and Monoclonal Antibodies

Passive immunization through the use of antibodies targeting toxin subunits is a promising area of research. Polyclonal antibodies offer a mixture of specific antibodies that may have the ability to neutralize anthrax toxin. A study involving polyclonal serum found that it significantly protected guinea pigs from death when challenged with *Bacillus anthracis* spores (Little et al, 1997). One problem with polyclonal serum is the increased risk for side effects due to the presence of unnecessary compounds and non-neutralizing antibodies.

In 1975, a significant paper by Kohler and Milstein was published showing that through a fusion process, antibody-producing cell lines could be established and maintained in vitro (Kohler and Milstein, 1975). These cells produce a specific line of antibodies that are identical in their antigen binding epitopes known as monoclonal antibodies (mAbs).

The production of monoclonal antibodies involves the fusion of antibody-secreting cells from immunized animals with myeloma cells. This fusion immortalizes the antibody-secreting cells, allowing them to produce large amounts of antibody while continually dividing in tissue culture. The fused product is called a hybridoma. The hybridomas are carefully screened to produce individual monoclonal cell lines.

Monoclonal antibodies that target different components of *Bacillus anthracis* have been produced. One group examined a set of monoclonal antibodies that target spore antigens and did not find them to be very robust (Phillips et al, 1988). Several other studies have found that monoclonal antibodies that target functionally relevant epitopes on the anthrax toxin subunits are able to neutralize their toxic activity. In 1988 a set of murine monoclonal antibodies to PA were characterized and two clones were found to inhibit both lethal and edema toxins by preventing PA from binding to its host cell receptor (Little et al, 1988). Since then several mAbs have been identified as anthrax toxin neutralizers including human monoclonal antibodies that have been shown to prevent PA oligomerization (Wang et al, 2004). It is worth mentioning that one study has discovered a population of mAbs that increase, rather than inhibit, the potency of lethal toxin against murine macrophage cell lines (Mohamed et al, 2004). The authors hypothesize that this is due to the binding of the mAb to macrophage bound PA, allowing for the interaction of the mAb with the macrophage's Fcγ receptors. This leads to the stabilization of PA, resulting in an increase in the number of macrophage bound PA.

Due to the central role of PA in the pathogenesis of *B. anthracis*, we have developed and characterized a set murine monoclonal antibodies raised to recombinant protective antigen toxin of *Bacillus anthracis*. Unlike previous mAbs, these novel antibodies were raised against a recombinant form of PA.

2. MATERIALS AND METHODS

2.1 Development of mAbs by Hybridoma Fusion and Isotyping

The hybrydoma fusions, whose products are used in this study, were conducted by Dr. Jody Berry's lab (Saliba et al, 2004). For immunizations, 5-6 week old female BALB/C mice were injected subcutaneously with 5μg of recombinant PA toxin in PBS with an equal part of Complete Freund's Adjuvant (CFA, H37 Ra; Difco) on Day 1. On day 30 the mice received 5 μg of recombinant PA toxin in Incomplete Freund's Adjuvant (IFA). On days 48 and 63, mice received 5 μg PA in IFA. The mice received a final booster injection with 2 μg of purified PA toxin 3 days prior to fusion. Mice were enhanced by anesthesia overdose and exsanguinated by cardiac puncture. The spleens were excised under aseptic conditions. The parental cell-line used for the hybridoma fusion was the P3-X63 Ag8.653 (ATCC). The immunization of mice, removal of spleens, preparation of spleen and myeloma cells, fusion and screening for monoclonal antibodies to PA were performed according to NCFAD standard operating procedures under ISO17025. Hybridomas were cloned out in semi-solid medium essentially as described previously (Berry and Ranada, 2003). Supernatants were screened via ELISA as described below. Isotyping was performed using a commercial dipstick test (Roche) according to the commercial instructions. Hybridomas 2D5 and 3B6 were generous gifts of Dr. Steven Little (USAMIRIID, USA) and are known to be protective in vitro and in vivo (Little et al; 1988). The binding properties of the new mAbs were characterized

essentially as described previously (Berry et al., 2004) with the following changes in brief.

2.2 Concentration of monoclonal Antibodies

The concentration of the anti-PA mAbs was conducted using the Millipore / Amicon Stirred Ultrafiltration Cell according to National Centre for Foreign Animal Disease (NCFAD) Standard Operating Procedure number MC-PR-008-00 found in Appendix A. The mAbs were concentrated using a YM30 filtering membrane. Concentrates that were not immediately used were stored in a -20°C tissue culture freezer.

2.3 Purification of Monoclonal Antibodies

Manual column purification of the anti-PA mAbs were conducted using HiTrap Protein G HP 1ml columns according to National Centre for Foreign Animal Disease (NCFAD) Standard Operating Procedure number MC-PR-008-00 with the following changes in brief. 100µL of protein G binding buffer was added to eight collection tubes. The column was wash with 10ml of binding buffer. 2ml of concentrated mAb sample was applied to the column, followed by a flushing of the column with 10ml of binding buffer. 8ml of elution buffer was applied to the column, adding 1ml fractions per collection tube. The antibody concentration was determined for each fraction using the BioRAD Smartspec 3000 spectrophotometer. Highest concentration fractions were loaded into

Millipore Centriprep Centrifugal Filter Units possessing YM30 membranes along with PBS. They were then centrifuged at 3000 RPM to a desired volume of 1.5ml.

2.4 Determination of Monoclonal Antibodies Concentration

Determination of mAb concentration was carried out using PIERCE BCA Protein Assay Reagent Kit (Product number 23227, Lot number DK578) according to commercial instructions. Albumin (BSA) was used as a standard at 2mg/ml for comparison. The absorbance was measured at 570nm using Dynex Revelation software version 3.04. Linear standard curves were used in the calculation of protein concentration. Average optical densities were used as the samples were loaded in duplicates for increased accuracy.

2.5 Purity Confirmation

mAb purity was confirmed using sodium dodecyl sulfate (SDS) and native poly-acrylamide gel electrophoresis (PAGE) according to National Centre for Foreign Animal Disease (NCFAD) Standard Operating Precedure number MC-PR-009-00. Biorad Kaliedascope prestained protein ladder was used as a molecular weight marker. Beta-Mercaptoethanol sample buffer was used for denaturing SDS gels but not native gels. Samples were loaded in duplicate for increased accuracy.

2.6 Enzyme Linked Immunosorbent Assay (ELISA)

Hybridoma culture supernatants were assayed for binding to recombinant PA toxin in an ELISA assay when the cultured cells were confluent in the culture plates as previously described (Saliba et al, 2004). The Costar 3690 96-well ½ well ELISA plates (Corning, NY) were coated with either bovine serum albumin (BSA) or PA toxin (100ng/well) in PBS overnight at 4°C and then blocked with 0.4% BSA in PBS, for 2 hours at 37°C. The supernatant (30 µl/well) was incubated neat for 1 hour at 37°C. The ELISA plates were washed ten times with distilled water and patted dry on a paper towel. A pan-goat anti-mouse IgG-HRP antibody (Southern Biotechnology Associates, Birmingham, Alabama) was diluted to 1:2000 with 0.2% BSA in PBS, applied to the ELISA plates for 45 minutes at 37°C, and then washed as described above. Positive binding was detected with commercial ABTS used according to the manufacturer's instructions (Roche, Basel, SW). The O.D. was read at 405nm at 15 and 60 minute intervals after addition of the developing reagents. Mouse immune and pre-immune sera were diluted 1:2000 with 2% BSA in PBS for use as positive and negative controls respectively, and for the establishment of the hybridoma screening assay. Quantitative ELISA was done as above using protein G chromatography purified mAbs, starting at an initial concentration of 1 mg/ml.

2.7 Western Immunoblots

Western immunoblots were conducted by Xin Yong Yuan according to National Centre for Foreign Animal Disease (NCFAD) Standard Operating Precedure number MC-PR-009-00 as previously described (Saliba et al, 2004). Whole recombinant PA toxin, at a final total protein concentration of 1 μg per lane, were boiled in SDS-loading buffer for 10 minutes. The samples were loaded in criterion pre-cast gels (BioRad, Mississauga, ON) and electrophoresed at 200V for 30 minutes. The proteins were transferred to Immobilon nylon membranes (Millipore, Billerica, MA) for 2 hours at room temperature at 100 V, or overnight at 27 V at 4°C. Blots were blocked with 3% BSA in TBS, rinsed 3 times with TBS, and reacted with monoclonal antibody overnight at 4°C. The antibody supernatants were reacted neat and the concentrated supernatants were diluted 1:50 in 0.2% BSA in PBS. Blots were washed 3 times with TBS-Tween-20 (0.05%) for five minutes before being incubated with secondary antibody (same as above) at 1:1000 in TBS, 0.2% BSA for 1 hour. The blots were washed as above and developed using DAB insoluble substrate (Pierce, Rockford, IL).

2.8 Variable Region Gene Sequencing

Variable region gene sequencing was carried out by Xin Yong Yuan and used in this study to further characterize the mAbs. The hybridomas were used as RNA sources for V-gene sequencing essentially as described (Gubbins et al., 2004).

2.9 Surface Plasmon Resonance

Biacore2000 was used to conduct all surface plasmon resonance (SPR) experiments according to accepted commercial procedures. The carboxylated dextran matrix on the CM5 sensor chip was coated with recombinant PA toxin using methods essentially as described by (Barbas et al., 1994). During SPR the flow rate over the sensor chip was 5µl/ml. Regeneration of the relative units back to baseline was obtained using 20µl of 1.5pH (Na)glycine. Sensorgrams for each set of mAbs were graphed and KD values were calculated using BIAevaluation 3.0 software. Five runs were done for each clone at five predetermined concentrations. Of the five tests, only the three mid-values of both ka and kd were averaged and used in calculating the affinity. KD (Dissociation constant) = kd/ka. Where ka describes the number of antigen / antibody complexes formed per second in a one molar solution and kd describes the fraction of bound antibody/antigen complexes that detach per second.

2.10 In Vitro Toxin Neutralization Assays

2.10.1 Spot Neutralization Assay

Spot neutralization assays were carried out by Dr. Raymond Tsang's lab and are incorporated into this study as a comparative assay. Lethal toxin neutralization assays were conducted using J774A.1 macrophage cell line essentially as reported by others previously (Little et al., 1990). Raw supernatants for the nine mAbs were combined with

recombinant PA toxin and added to the cells, followed by the addition of MTS tetrazolium (CellTiter 96 AQueous) to determine cell viability according to commercial instructions.

2.10.2 Quantitative Neutralization Assay

Lethal toxin neutralization assays were also conducted, as described above, using purified mAbs rather than supernatant. 96 well plates were incubated overnight at 37°C, 5% CO^2 with approximately 1 x 10^5 viable J774A.1 macrophage cells per well. The concentrations of rPA and rLF that were used in the assay were predetermined through a kill plate experiment to be 1.6 x 10^{-4}mg/ml and 7 x 10^{-3}mg/ml respectively. mAbs, rPA, and rLF were incubated together for 1 hour at 37°C, 5% CO^2. mAbs, lethal toxin and J774A.1 cells were then incubated together for 2.5 hours, followed by the addition of MTS tetrazolium (CellTiter 96 AQueous) to determine cell viability according to commercial instructions. Quantitative neutralizations were done using ½ dilution series starting at 1mg/ml of mAb to determine their neutralization endpoints. Pre-immune and post-immune monkey serum was used as a comparative control. Lanes containing macrophage cells combined with lethal toxin or PBS alone were also used as controls. Experiments were conducted in triplicates.

3. RESULTS

3.1 Monoclonal Antibody Isotypes

All nine novel murine mAb clones have been isotyped as IgG1/Kappa (Table 2).

Anti-rPA mAbs	Isotype
F20G1	Murine IgG1/Kappa
F20G2	Murine IgG1/Kappa
F20G3	Murine IgG1/Kappa
F20G4	Murine IgG1/Kappa
F20G5	Murine IgG1/Kappa
F20G6	Murine IgG1/Kappa
F20G7	Murine IgG1/Kappa
F20G8	Murine IgG1/Kappa
F20G9	Murine IgG1/Kappa

Table 2 – Monoclonal Antibody Isotypes
Isotyping was performed using a commercial dipstick test (Roche) according to the commercial instructions.

3.2 Western Immunoblot

All nine clones were found to recognize both intact PA83 and trypsin treated PA molecules by western immunoblot (Figure 4), suggesting that they all target linear epitopes in the larger fragment.

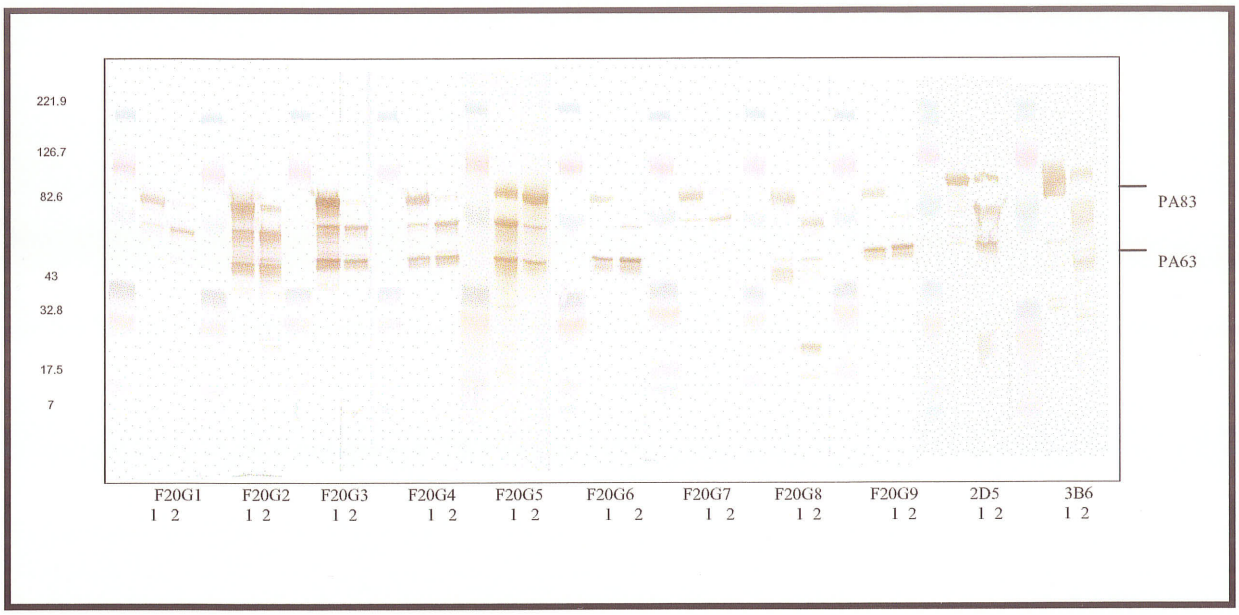

Figure 4 – Western Immunoblot of Monoclonal Antibodies
Western immunoblot was carried out by Xin Yong Yuan and is used in this study to further characterize the mAbs. Western immunoblot of the Protective antigen (PA) toxin or *Bacillus anthracis* probed with mAbs Recombinant PA toxin at a total protein concentration of 1 µg per lane were loaded in criterion pre-cast gels (BIO-RAD) and electrophoresed at 200 V for 30 minutes. The proteins were transferred to Immobilon nylon membranes (Millipore) and then blocked in 3% BSA in TBS. The blots were rinsed three times with TBS, and incubated with monoclonal antibody overnight at 4°C. The antibody supernatants were reacted neat and concentrated supernatants were diluted 1:50 in 0.2% BSA in PBS. Blots were washed three times with TBS/Tween-20 (0.05%) for five minutes before being incubated with secondary antibody (as above) at 1:1000 in TBS/0.2% BSA for 1 hour. The blots were washed again as above and developed using DAB (Pierce) insoluble substrate. The primary mAb used in each blot is listed below each set of lanes. 2D5 and 3B6 were used as comparative controls. Multicoloured bands are from molecular weight markers, whose size (Kda) is listed to the left of the figure. Lanes marked with a 1 contains purified PA toxin, lanes marked with a 2 contain purified PA toxin pre-treated with trypsin.

3.3 V-Gene Sequencing

The V-gene sequencing of F20G1, F20G6, F20G7, and F20G8 reveals that the V-gene usage differs between the clones (Table 3).

Anti-rPA Monoclonal Antibody	V-genes	
	Heavy	Light
F20G1	VHJ558.1 / DHSp2.13 / JH2	Vk19.15 / JK2
F20G2	ND	ND
F20G3	ND	ND
F20G4	ND	ND
F20G5	ND	ND
F20G6	VHF102 / DHSp2.13 / JH2	VkBd-2 / JK5
F20G7	VH36-60 / DHSp2-9.01 / JH4.01	Vk19.15 / JK2
F20G8	VHJ558.1 / DHSp2.13 / JH2	JKBd-2 / JK5
F20G9	ND	ND

Table 3 – V-Gene Sequencing of Monoclonal Antibodies
Variable region gene sequencing was carried out by Xin Yong Yuan and is used in this study to further characterize the mAbs. The hybridomas were used as RNA sources for V-gene sequencing essentially as described (Gubbins et al., 2004). ND signifies unavailable data.

3.4 Affinity Analysis

The use of surface plasmon resonance allowed the comparison of the nine mAbs' affinities to their PA epitopes. The range of their affinities were found to be between 3.20 x 10^{-7} M and 4.10 x 10^{-8} M, keeping in mind that the lower the KD value, the higher the affinity (Table 4, Figure 5). F20G6, F20G7, and F20G8 were found to have the highest affinities at 5.00 x 10^{-8} M, 4.60 x 10^{-8} M, and 4.10 x 10^{-8} M respectively (Table 4, Figure 5).

Anti-rPA Monoclonal Antibody	KD (M) [affinity]
F20G1	1.27 X 10-7
F20G2	9.20 X 10-8
F20G3	1.70 X 10-7
F20G4	8.10 X 10-8
F20G5	1.90 X 10-7
F20G6	5.00 X 10-8
F20G7	4.60 X 10-8
F20G8	4.10 X 10-8
F20G9	3.20 X 10-7

Table 4 – Monoclonal Antibody Dissociation Constants: Quantitative Affinity Measurements
KD values indicate the average affinity between rPA and mAbs interactions. Sensorgrams for each set of mAbs were graphed and KD values were calculated using BIAevaluation 3.0 software. Results are representative of several experiments.

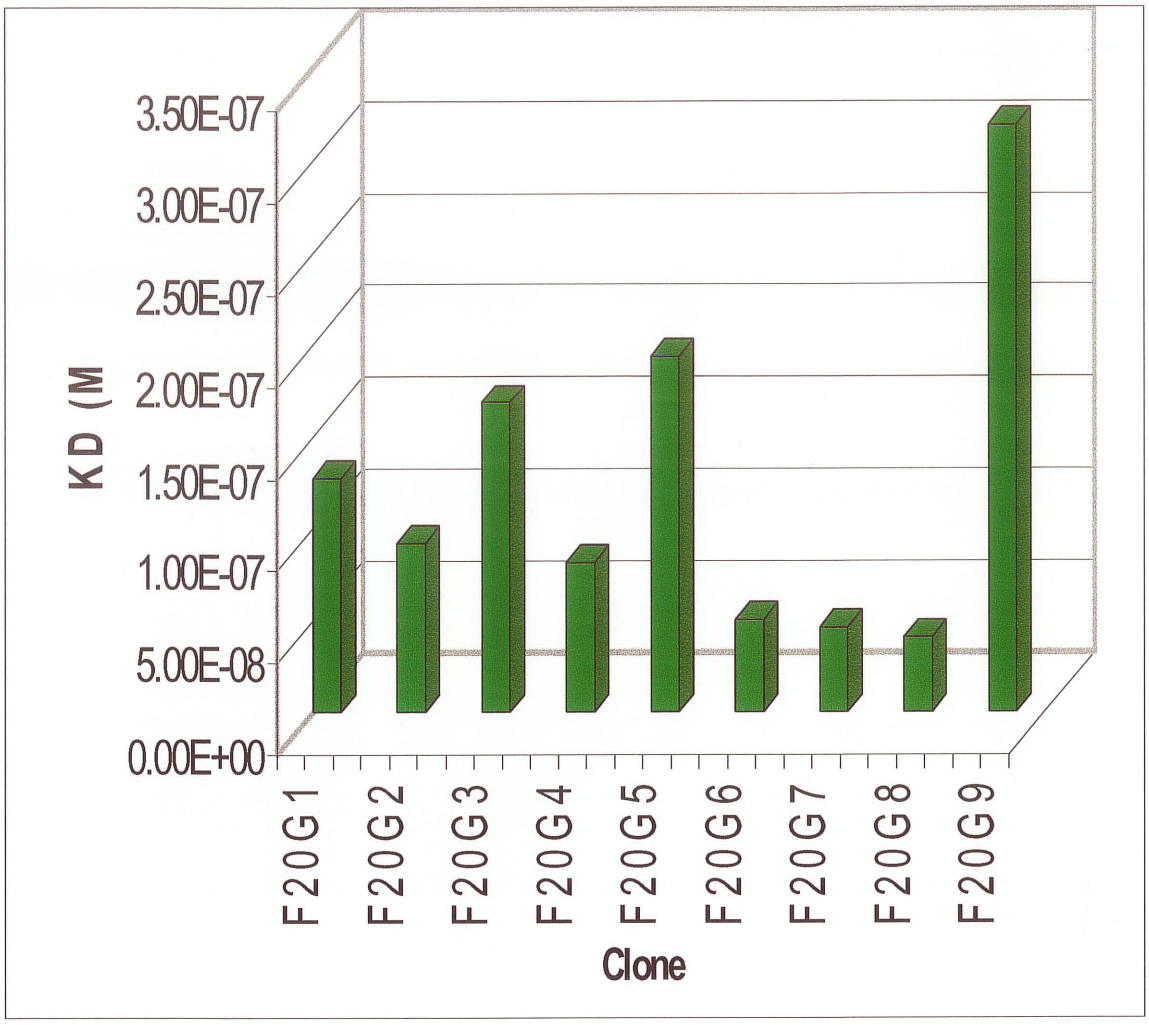

Figure – 5 Comparative Analysis of Monoclonal Antibody Dissociation Constants
KD values indicate the average affinity between rPA and mAbs interactions. Sensorgrams for each set of mAbs were graphed and KD values were calculated using BIAevaluation 3.0 software. Results are representative several experiments.

3.5 ELISA Reactivity

The set of mAbs were tested in ELISA to further assess their affinity and determine their reactivity comparatively (Figure 6 and 7) All nine mAbs were shown to be reactive to rPA (Figures 6 and 7). Initially the results of the nine mAbs were plotted against one and other at all concentrations (Figure 6). However, contrasting all of their optical density values at a mAb concentration of 0.2mg/ml offers a better comparison (Figure 7). There were a few differences between the ELISA and the surface plasmon resonance results but overall the ELISA results confirmed the reactivity of the clones and the results achieved by SPR affinity analysis. F20G6, F20G7, and F20G8 showed the highest affinity in the previous experiment and were among the most reactive in the comparative ELISA assay (Figures 6 and 7).

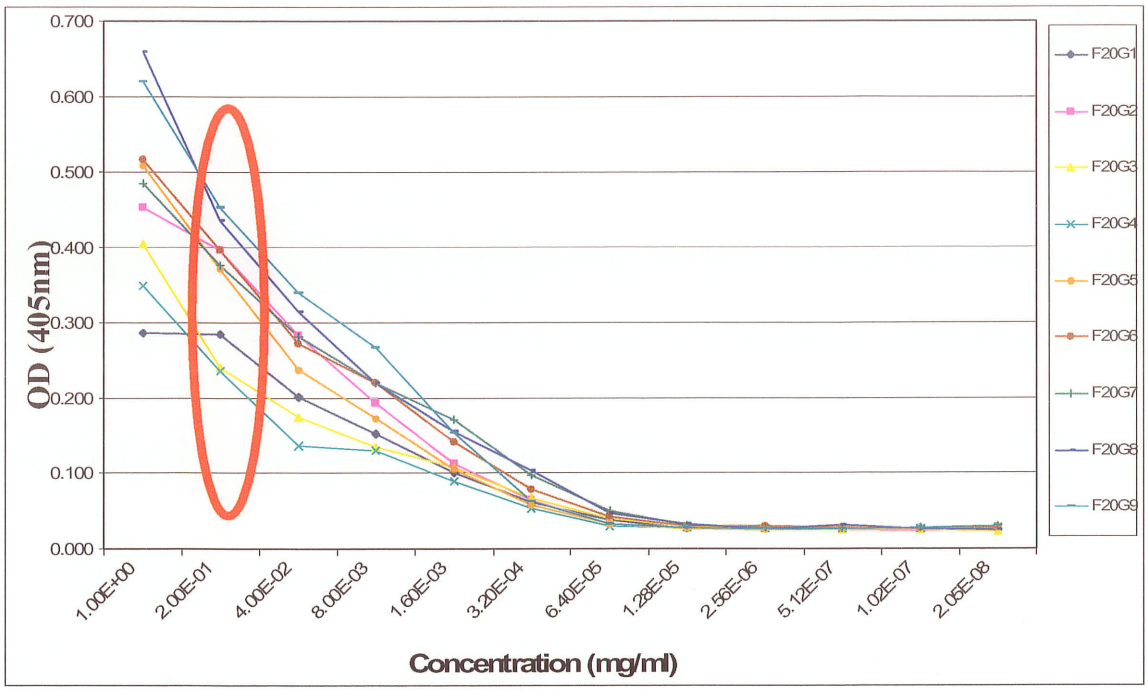

Figure 6 – Reactivity of Monoclonal Antibodies in ELISA
Quantitative ELISA was done as previously described (section 2.6) using protein G chromatography purified mAbs, starting at an initial concentration of 1 mg/ml. The red circle indicates the optical density values at 0.2mg/ml of antibody that were further used for comparison (Figure 4). Experiments were conducted in triplicate.

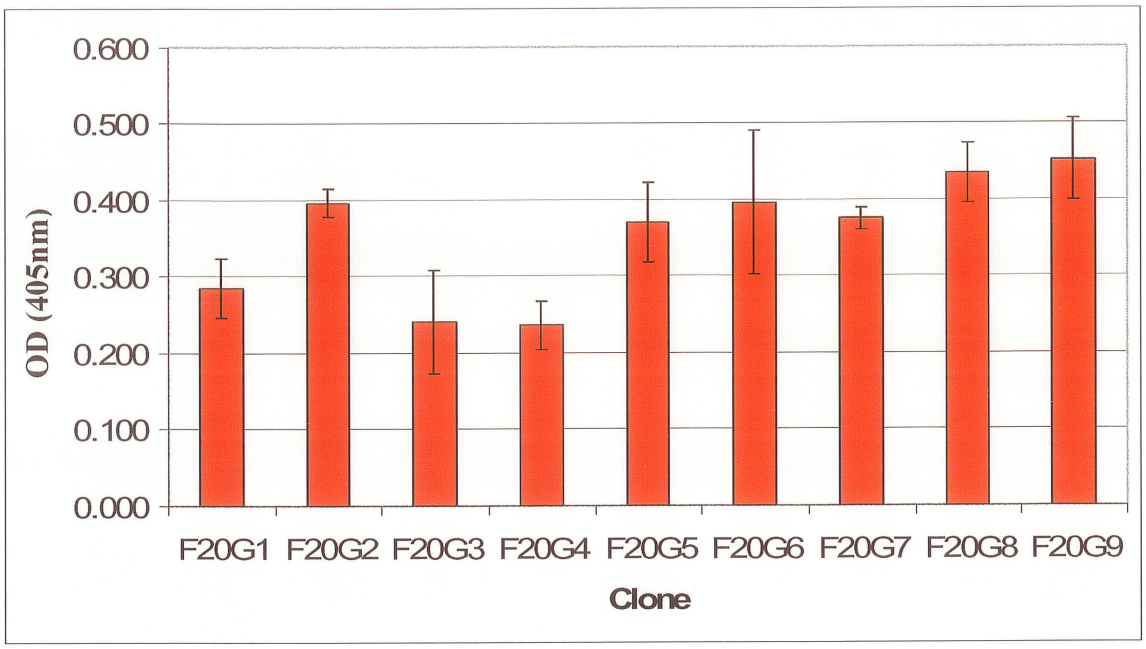

Figure 7 – Reactivity of Monoclonal Antibodies in ELISA at a concentration of 0.2mg/ml

Quantitative ELISA was done as previously described (Section 2.6) using protein G chromatography purified mAbs. The values in this figure are a comparison of the optical density values at 0.2mg/ml of antibody. Experiments were conducted in triplicate.

3.6 In-Vivo Spot Lethal Toxin Neutralization Assay

When tested for their ability to neutralize lethal toxin, three of the nine mAbs were shown to be neutralizing (Table 5). While F20G1, F20G6, and F20G7 were able to neutralize the anthrax toxin, none of the other clones showed any significant neutralizing ability (Table 5). The results of this assay are independent of mAb concentration since raw supernatant was used.

Monoclonal Antibody	Neutralizing
F20G1	Yes
F20G2	No
F20G3	No
F20G4	No
F20G5	No
F20G6	Yes
F20G7	Yes
F20G8	No
F20G9	No

Table 5 – Monoclonal Antibody Ability to Neutralize Anthrax Lethal Toxin in the Spot Neutralization Assay
Spot neutralization assays were carried out by Dr. Raymond Tsang's lab and are incorporated into this study as a comparative assay. Raw supernatants containing the nine mAbs were combined with recombinant PA toxin and added to the cells, followed by the addition of MTS tetrazolium (CellTiter 96 AQueous) to determine cell viability according to commercial instructions. The results of this assay are independent of mAb concentration since raw supernatant was used.

3.7 In-Vivo Quantitative Lethal Toxin Neutralization Assay

In order to quantitatively assess the ability of these mAbs to neutralize lethal toxin, neutralization assays were conducted using purified mAbs. Although all purified mAbs neutralized lethal toxin, it was shown that three of the nine mAbs neutralized the toxin more efficiently than the post-immune polyclonal monkey serum to PA (Figures 8, 9, and 10). These three mAbs, F20G1, F20G7, and F20G8 neutralized lethal toxin at concentrations as low as 1.53×10^{-5} mg/ml, 1.53×10^{-5} mg/ml, and 3.91×10^{-3} mg/ml respectively (Table 6). Note that in the quantitative neutralization assay, unlike the previous spot neutralization assay, F20G8 was found to be neutralizing and F20G6 was not (Table 6, Figures 8, 9, and 10).

Monoclonal Antibody	Neutralization Endpoint (mg/ml) [in vitro, Purified mAbs]
F20G1	1.53×10^{-5}
F20G2	1.56×10^{-2}
F20G3	6.25×10^{-2}
F20G4	6.25×10^{-2}
F20G5	2.50×10^{-1}
F20G6	2.50×10^{-1}
F20G7	1.53×10^{-5}
F20G8	3.91×10^{-3}
F20G9	2.50×10^{-1}

Table 6 – Neutralization Endpoint of the Nine Monoclonal Antibodies
The endpoint values indicate the lowest concentration of mAb required to neutralize the lethal effects of anthrax lethal toxin on J774A.1 macrophage cells in-vivo. The concentrations of rPA and rLF that were used in the assay were predetermined through a kill plate experiment to be 1.6×10^{-4}mg/ml and 7×10^{-3}mg/ml respectively.

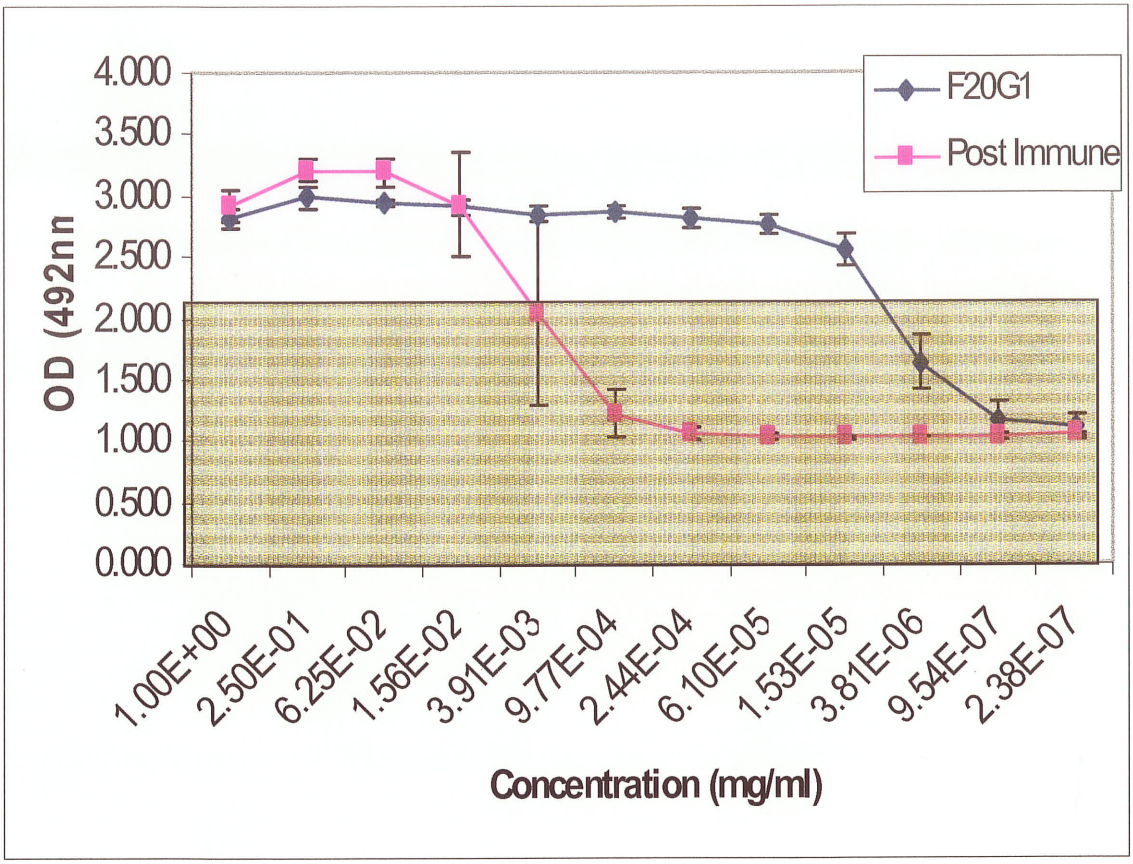

Figure 8 – Quantitative Anthrax Lethal Toxin Neutralization Assay for F20G1
Optical density reading indicates J774A.1 cell survival through colorimetric properties of MTS tetrazolium (CellTiter 96 AQueous). Shaded area denotes J774A.1 cell death (x2 total macrophage cell death baseline value), therefore points within this region are indicative of the mAb's inability to neutralize toxin at that concentration. Post immune refers to serum from a vaccinated monkey containing polyclonal antibodies against PA. Experiment was conducted in triplicate.

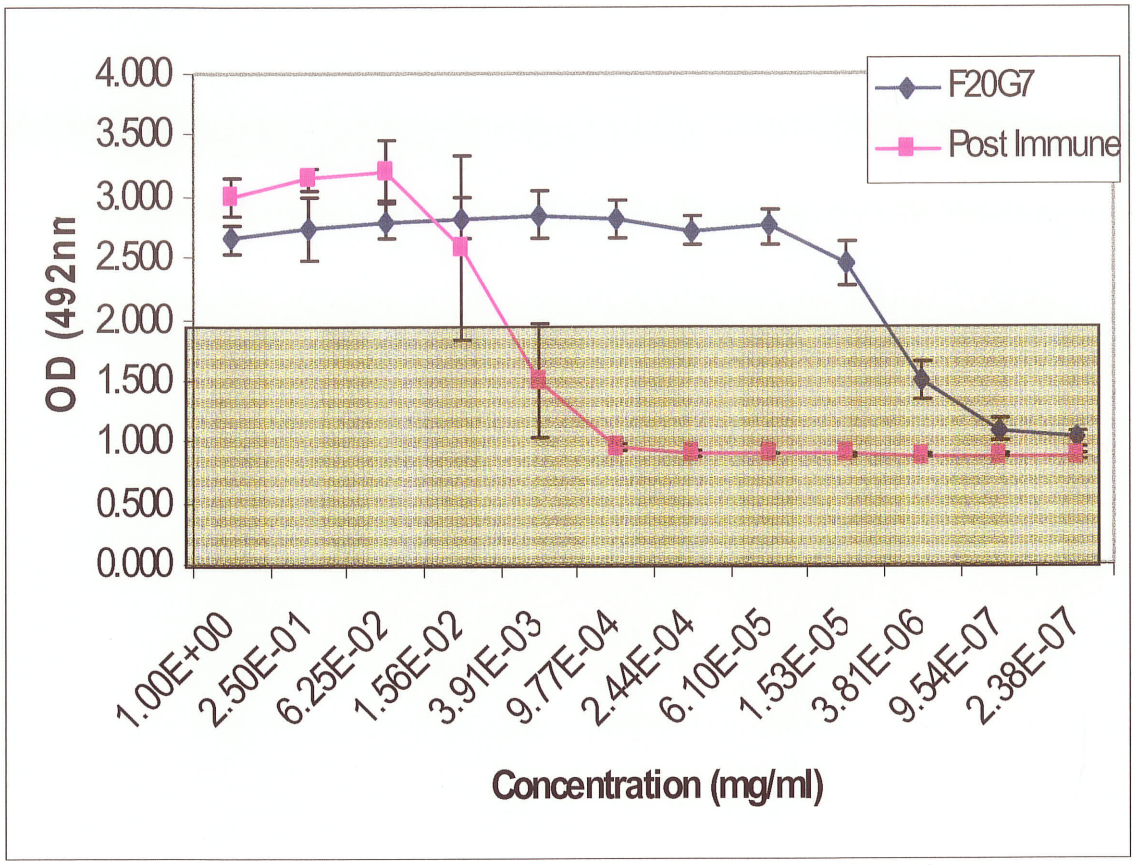

Figure 9 – Quantitative Anthrax Lethal Toxin Neutralization Assay for F20G7
Optical density reading indicates J774A.1 cell survival through colorimetric properties of MTS tetrazolium (CellTiter 96 AQueous). Shaded area denotes J774A.1 cell death (x2 total macrophage cell death baseline value), therefore points within this region are indicative of the mAb's inability to neutralize toxin at that concentration. Post immune refers to serum from a vaccinated monkey containing polyclonal antibodies against PA. Experiment was conducted in triplicate.

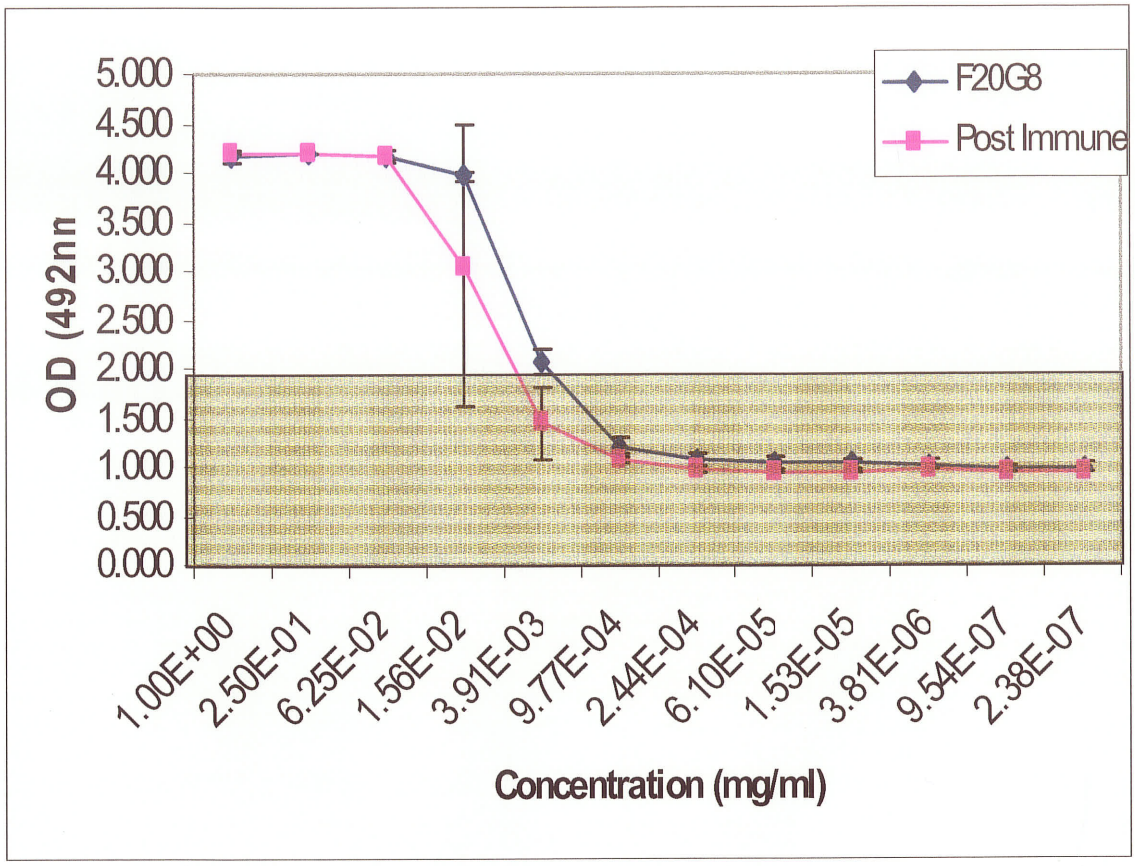

Figure 10 – Quantitative Anthrax Lethal Toxin Neutralization Assay for F20G8
Optical density reading indicates J774A.1 cell survival through colorimetric properties of MTS tetrazolium (CellTiter 96 AQueous). Shaded area denotes J774A.1 cell death (x2 total macrophage cell death baseline value), therefore points within this region are indicative of the mAb's inability to neutralize toxin at that concentration. Post immune refers to serum from a vaccinated monkey containing polyclonal antibodies against PA. Experiment was conducted in triplicate.

4. DISCUSSION

A panel of novel murine mAbs to a recombinant PA toxin were developed and characterized in this study. Today's monoclonal antibody development and characterization requires careful and methodical work as it has from its beginnings in the early 1980's. To our knowledge, this is the first time that recombinant *Bacillus anthracis* toxins have been used to develop murine mAbs by hybridoma technology. Recombinant technology offers several advantages over the production of native toxin as previously described (Section 1.1.6.4.5). The recombinant toxin product is essentially identical to its native counterpart and because of the lack of need for other proteins, for example other bacterial exotoxins, it can be used interchangeably for in-vitro studies.

Due to the many previously mentioned (Section 1.2.2.1) characteristics of IgG, it is the ideal antibody candidate for passive immunization (Benjamini and Leskowitz, 1988). All nine were isotyped as IgG1s with Kappa light chains (Table 2). This was expected since the majority of murine antibodies produced are of the Kappa light chain type.

All nine clones were found to recognize both intact PA83 and trypsin treated PA molecules by western immunoblot (Figure 4). Since trypsin treatment cleaves the proteins on the C-terminal side of the basic amino acid residues lysine and arginine, it removes the protein's tertiary structure. This suggests that all nine mAbs target linear epitopes in the larger fragment rather than epitopes that are conformationally dependant. The fragment pattern is different for many of the mAbs suggesting they target different epitopes on PA.

This was later confirmed for several of these mAbs by synthetic pin peptide (pepscan) epitope mapping (data not shown, private communication – Xin Yuan).

4.1 Surface Plasmon Resonance and Reactivity

ELISA reactivity and surface plasmon resonance interpretation of affinity did not correlate perfectly with each other. This is most likely due to the fact that there is no true method of comparing ELISA reactivity results with the use of surface plasmon resonance affinity measurements. Other studies have used ELISA assays to determine antibody affinity to their target epitopes (Kostolansky, 2000). To obtain a better comparison between the values obtained by both the ELISA and SPR assays, the antigen dilution series for the ELISA assay would have to include concentrations of antibody that would saturate the antigens in order to determine the KD. Since this was not done, it is difficult to compare the ELISA reactivity and surface plasmon resonance affinity results. Overall, however, the results obtained from the ELISA assay show all nine mAbs to be reactive to rPA and generally support the findings of the SPR affinity results (Figures 5 and 7).

SPR affinity measurements use a combination of monovalent and bivalent antibody / antigen interactions. This is due to the random orientation of the antibody as it passes over the rPA coated CM5 chip. As the antibodies flow over the antigen coated chip, they tend to roll and therefore may bind with one of the two Fab fragments or only partially to their epitopes on PA. This phenomenon leads to a decrease in their reported affinities. The use of a system that monitored only bivalent antibody / antigen interactions would allow the determination of the maximum affinity of that complex. The affinity

ratios obtained from the SPR assay offer an accurate method to compare the affinities of each set of mAbs relative to each other.

Several of the murine mAbs efficiently target neutralizing epitopes on PA toxin. Monoclonal antibodies F20G1, F20G6, and F20G7 were found to neutralize recombinant lethal toxin activity in a spot lethal toxin neutralization assay in vitro (Table 5). The significance of these results is limited due to the fact that neutralization could not be linked to mAb concentration. In spot neutralization, a negative or positive toxin neutralization result may be due to the amount of antibody present rather than the antibody's affinity and epitope targeted.

In order to quantitatively assess the ability of these mAbs to neutralize lethal toxin, neutralization assays were conducted using purified mAbs (Table 6, Figures 8, 9, and 10). Although all purified mAbs neutralized lethal toxin, it was shown that F20G1, F20G7, and F20G8 neutralized the toxin more efficiently than the post-immune polyclonal monkey serum to PA (Figures 8, 9, and 10). Since these results are quantified, they offer improved precision and accuracy over the results achieved by the spot neutralization assay. These results reinforce the importance of implicating mAb concentration in the determination of neutralizing capability.

4.2 Proposed Mechanism of Anthrax Toxin Neutralization

This panel of new neutralizing mAbs may reveal important insights on protective domains of PA toxin. Depending on which epitopes these neutralizing monoclonal antibodies are targeting, they can be interrupting several different steps within the anthrax

toxin mechanism of action (Section 1.1.7). By targeting an epitope in domain II, for example, the antibodies may be inhibiting oligomerization of PA, the inner lining of the pore therefore inhibiting pore formation, or even PA antigen binding to TEM8 and CMG2 host cell receptors. Although it has been shown that a disruption in domain II does not inhibit the binding of PA to its host cell receptor because domain IV contains the primary binding epitope, since the domains are so close during binding a large antibody bound to domain II may sterically hinder binding of domain IV to its receptor (Singh et al, 1994). Therefore, the location of the epitopes targeted by the mAbs determines at what point during the toxin's mechanism of action it is interrupted, whether directly or through steric hindrance of a nearby functional domain. Since all neutralizing mAbs interfere with PA's functionality, theoretically they should be equally effective in neutralizing edema toxin as previously discussed (Sections 1.1.7 and 1.2.1).

None of these mAbs map to domain IV which shows that domains other than domain IV contain protective epitopes. This is consistent with results of our collaborators who have found that recombinant domain IV produced in *Caulobacter crescentus* fails to produce toxin neutralizing antisera in animals (L. Hagata, personal communication). A recent study has found that PA is associated with the *B. anthracis* spore and may be involved in the spore's germination (Cote et al, 2005). Anti-PA antibodies were shown to retard spore germination in-vitro. This suggests that the neutralizing mAbs in this study may also possess anti-spore properties, resulting in the inhibition of spore germination and enhancing the phagocytic and sporicidal activities of macrophages. This is proposed to be tested in future studies. Theoretically, if these mAbs are administered during the early stage of infection they may be able to protect against both spore germination and

toxin function. Not only would that decrease the number of systemic *B. anthracis* resulting in a decrease in toxin produced, but it would also prevent the effects of the toxin that is produced.

4.3 Epitope Critical Neutralization

The mAbs target PA toxin with a range of affinities which does not correlate with neutralization activity in vitro (Table 6, Figure 5). These results show that the actual epitope recognized is more important than affinity of the antibody-antigen interaction. How this may translate to an in vivo model of protection is not clear at this point. However, concentration and not avidity (above a minimum threshold), has been shown previously by others to correlate with neutralization in vivo for a cytopathic virus (Bachmann et al., 1997). One group mentioned a correlation between affinity and neutralization using mAbs against PA toxin in their study. However, they selected for certain mAbs to include in their study, but excluded other mAbs with higher affinity that did not neutralize anthrax toxin (Sawada-Hirai et al, 2004). F. Kostolansky et al. have found a strong positive correlation between affinity and viral neutralization for the IIB4 mAb against antigenic site B on influenza A virus haemagglutinin (Kostolansky et al, 2000). Unlike this study however, they only used one mAb and therefore only one epitope was targeted. Kostolansky's study combined with this current study reveals that when comparing antibodies that target different epitopes on the same target molecule the affinity with which the antibodies bind to their target is a determinant of lesser importance in terms of neutralizing ability than the epitopes they bind to. Also, if the

epitopes targeted are identical, then affinity has a greater impact on the antibody's toxin neutralizing ability. Schofield et al. conducted a similar study using several mAbs directed against different epitopes on the haemagglutinin of type A influenza virus and found no correlation between affinity and viral neutralization (Schofield et al, 1997). This supports the findings in this paper that epitope identity is more important in terms of neutralization than affinity.

4.4 B Cell Response

The neutralizing murine antibody response to recombinant PA toxin is encoded by diverse VH + VL domains (Table 3). By analyzing the mAbs' V-gene usage it was determined that, while highly similar, the hybridomas represent distinct clonal lineage. This indicates that multiple B cells respond to PA to produce neutralizing antibodies. Moreover, it is indicative of the fact that the neutralizing mAbs are derived from distinct hybridomas.

4.5 Future Studies

This research has paved the way for future work on these mAbs. Determining the epitope sequences that they bind to on PA and in-vivo animal studies may lead to their use in the human clinical setting.

As comprehensive as this study may be, there remains a great deal of work that can be done to realize the full potential of these mAbs. Since these mAbs can neutralize

lethal toxin at concentrations as low as 0.015μg/ml, they may be relevant clinically as a therapy against *B. anthracis* infection. They could be used in a combination therapy with antibiotics in order to reduce the effects of disease. Since they are murine mAbs, they could only be used once to treat a given patient infected with anthrax. This is because murine mAbs are recognized as foreign entities by the human immune system, resulting in the formation of human-antimouse-antibodies that would prevent their use if re-infection occurs. For this reason, murine mAbs have limited therapeutic utility in the case of re-infection and are rapidly eliminated by the human immune system and may yield adverse side effect. A solution to this would be to create a humanized version of these murine mAbs called chimeric mAbs. This is achieved through the attachment of the murine heavy chain and light chain variable regions to a human constant region. Since the variable regions contain the six complementarity-determining regions (CDR), antigen specificity is maintained. Fully humanizing the neutralizing murine mAbs may prove to be a better option. This is achieved by combining the CDRs from the neutralizing murine mAbs with the four framework regions within each variable region of a human Ab. The use of fully humanized Abs, however, has not officially been proven to be more effective than the use of chimeric Abs (Clark, 2000). Fully humanizing the murine mAbs seems to be a superior approach as newer methods are further reducing their immunogenicity (Tan et al, 2002). In addition, it may be quite beneficial to perform a series of competition experiments using Biacore in order to reveal epitope reactivity by different mAbs.

In summation, the nine mAbs, raised against recombinant protective antigen, that have been developed and extensively characterized and the epitopes which they recognize will be useful for the development of diagnostic tests, pathogenicity,

recombinant antibody, and vaccine studies. While other researchers have developed and characterized mAbs against PA, LF, or EF they were not against recombinant PA. Unlike the panel of mAbs characterized in this study, many of the other mAbs have not been as extensively characterized as these, including neutralization and affinity characterizations (Brossier, 2004; Sastry, 2003). The results of this study indicate that, in terms of lethal toxin neutralization, the epitope identity of the mAb is of primary importance and the mAb's affinity to its antigenic epitope, although significant, is of lesser importance.

5. REFERENCES

Abrami L, Liu S, Cosson P, Leppla SH, van der Goot FG. Anthrax toxin triggers endocytosis of its receptor via a lipid raft-mediated clathrin-dependent process. J Cell Biol. 2003 Feb 3; 160(3):321-8.

Alouf JE, Freer JH. The Comprehensive Sourcebook of Bacterial Protein Toxins. Second ed. San Diago, California: Academic Press, 1999.

Ascenzi P, Visca P, Ippolito G, Spallarossa A, Bolognesi M, Montecucco C. Anthrax toxin: a tripartite lethal combination. FEBS Lett. 2002 Nov 20; 531(3):384-8.

Bachmann MF, Kalinke U, Althage A, Freer G, Burkhart C, Roost H, Aguet M, Hengartner H, Zinkernagel RM. The role of antibody concentration and avidity in antiviral protection. Science. 1997 Jun 27; 276(5321):2024-7.

Baillie L, Moir A, Manchee R. The expression of the protective antigen of Bacillus anthracis in Bacillus subtilis. J Appl Microbiol. 1998 May; 84(5):741-6.

Barth H, Aktories K, Popoff MR, Stiles BG. Binary bacterial toxins: biochemistry, biology, and applications of common Clostridium and Bacillus proteins. Microbiol Mol Biol Rev. 2004 Sep; 68(3):373-402

Benjamin E, Leskowitz S. Immunology: a short course. New York, New York: Alan R Liss inc., 1988.

Berthold I, Pombo ML, Wagner L, Arciniega JL. Immunogenicity in mice of anthrax recombinant protective antigen in the presence of aluminum adjuvants. Vaccine. 2005 Mar 14; 23(16):1993-9.

Berry, J.D., and F. Ranada. [Internet] The production of monoclonal antibody by hybridoma fusion: Immortalization of sensitized B lymphocytes from immune mice; Massachussetts Institute of technology, 2003 – [Cited 2004 Jan 5] Available from: http://iprotocol.mit.edu/protocol/335.htm

Bhatnagar R, Batra S. Anthrax toxin. Crit Rev Microbiol. 2001; 27(3):167-200.

Bossi P, Tegnell A, Baka A, van Loock F, Hendriks J, Werner A, Maidhof H, Gouvras G. Bichat guidelines for the clinical management of anthrax and bioterrorism-related anthrax. Euro Surveill. 2004 Dec 1; 9(12)

Brachman PS. Inhalation anthrax. Ann N Y Acad Sci. 1980; 353:83-93.

Brachman PS. Bioterrorism: an update with a focus on anthrax. Am J Epidemiol 2002; 155:981-7

Bradley KA, Young JA. Anthrax toxin receptor proteins. Biochem Pharmacol. 2003 Feb 1; 65(3):309-14.

Brossier F, Levy M, Landier A, Lafaye P, Mock M. Functional analysis of Bacillus anthracis protective antigen by using neutralizing monoclonal antibodies. Infect Immun. 2004 Nov; 72(11):6313-7.

Burns DL, Barbieri JT, Iglewski BH, Rappuoli R. Bacterial Protein Toxins. Washington DC: ASM Press, 2003.

Clark M. Antibody humanization: a case of the 'Emperor's new clothes'? Immunol Today. 2000 Aug; 21(8):397-402.

Collier RJ, Young JA. Anthrax toxin. Annu Rev Cell Dev Biol. 2003;19:45-70.

Cote CK, Rossi CA, Kang AS, Morrow PR, Lee JS, Welkos SL. The detection of protective antigen (PA) associated with spores of Bacillus anthracis and the effects of anti-PA antibodies on spore germination and macrophage interactions. Microb Pathog. 2005 May-Jun; 38(5-6):209-25.

Davies JC. A major epidemic of anthrax in Zimbabwe. Part II. Cent Afr J Med. 1983 Jan;29(1):8-12.

Drysdale M, Heninger S, Hutt J, Chen Y, Lyons CR, Koehler TM. Capsule synthesis by *Bacillus anthracis* is required for dissemination in murine inhalation anthrax. EMBO J. 2005 Jan 12; 24(1):221-7.

Duesbery NS, Resau J, Webb CP, Koochekpour S, Koo HM, Leppla SH, Vande Woude GF. Suppression of ras-mediated transformation and inhibition of tumor growth and angiogenesis by anthrax lethal factor, a proteolytic inhibitor of multiple MEK pathways. Proc Natl Acad Sci U S A. 2001 Mar 27; 98(7):4089-94.

Erwin JL, DaSilva LM, Bavari S, Little SF, Friedlander AM, Chanh TC. Macrophage-derived cell lines do not express proinflammatory cytokines after exposure to Bacillus anthracis lethal toxin. Infect Immun. 2001 Feb; 69(2):1175-7.

Foster SJ. The role and regulation of cell wall structural dynamics during differentiation of endospore-forming bacteria. Soc Appl Bacteriol Symp Ser. 1994; 23:25S-39S.

Geier DA, Geier MR. Anthrax vaccination and joint related adverse reactions in light of biological warfare scenarios. Clin Exp Rheumatol. 2002 Mar-Apr; 20(2):217-20.

Gerhardt, P. Cytology of *Bacillus anthracis*. Fed. Proc. 1967 26:1504-1517

Gubbins, M.J., Plummer, F.A., Yuan, X.Y., Johnstone, D., Drebot, M.A., Andonova, M., Andonov, A., and Berry, J.D. Molecular characterization of a panel of murine monoclonal antibodies specific for the SARS-coronavirus. Molecular Immunology. 2004 (In the press).

Guidi-Rontani C, Pereira Y, Ruffie S, Sirard JC, Weber-Levy M, Mock M. Identification and characterization of a germination operon on the virulence plasmid pXO1 of *Bacillus anthracis*. Mol Microbiol. 1999a Jul; 33(2):407-14.

Guidi-Rontani C, Weber-Levy M, Labruyere E, Mock M. Germination of *Bacillus anthracis* spores within alveolar macrophages. Mol Microbiol. 1999b Jan; 31(1):9-17.

Guidi-Rontani C. The alveolar macrophage: the Trojan horse of *Bacillus anthracis*. Trends Microbiol. 2002 Sep; 10(9):405-9.

Halvorson HO. Two generations of spore research: from father to son. Microbiologia. 1997 Jun; 13(2):131-48.

Hanna PC, Acosta D, Collier RJ. On the role of macrophages in anthrax. Proc Natl Acad Sci U S A. 1993 Nov 1; 90(21):10198-201.

Hanna, P.C. Evidence towards a complete *B. anthracis* life cycle in soil. American Association for the Advancement of Science annual meeting. 2004 Feb. 14. Seattle.

Harlow E, Lane D. Antibodies: a laboratory manual. Woodbury, New York: Cold Spring Harbor Laboratory Press, 1988.

Harlow E, Lane D. Using antibodies: a laboratory manual. Woodbury, New York: Cold Spring Harbor Laboratory Press, 1999.

Hart CA, Beeching NJ. A Spotlight on Anthrax. Clin Dermatol. 2002 Jul-Aug; 20(4):365-75.

Hills GM. Chemical factors in the germination of spore-bearing aerobes; the effects of amino acids on the germination of *Bacillus anthracis*, with some observations on the relation of optical form to biological activity. Biochem J. 1949; 45(3):363-70.

Ivins BE, Welkos SL. Cloning and expression of the Bacillus anthracis protective antigen gene in Bacillus subtilis. Infect Immun. 1986 Nov; 54(2):537-42.

Ivins BE, Welkos SL. Recent advances in the development of an improved, human anthrax vaccine. Eur J Epidemiol. 1988 Mar; 4(1):12-9.

Jernigan DB, Raghunathan PL, Bell BP, Brechner R, Bresnitz EA, Butler JC, Cetron M, Cohen M, Doyle T, Fischer M, Greene C, Griffith KS, Guarner J, Hadler JL, Hayslett JA, Meyer R, Petersen LR, Phillips M, Pinner R, Popovic T, Quinn CP, Reefhuis J, Reissman D, Rosenstein N, Schuchat A, Shieh WJ, Siegal L, Swerdlow DL, Tenover FC, Traeger M, Ward JW, Weisfuse I, Wiersma S, Yeskey K, Zaki S, Ashford DA, Perkins BA, Ostroff S, Hughes J, Fleming D, Koplan JP, Gerberding JL; National Anthrax Epidemiologic Investigation Team. Investigation of bioterrorism-related anthrax, United States, 2001: epidemiologic findings. Emerg Infect Dis. 2002 Oct; 8(10):1019-28.

Keim P, Smith KL, Keys C, Takahashi H, Kurata T, Kaufmann A. Molecular investigation of the Aum Shinrikyo anthrax release in Kameido, Japan. J Clin Microbiol. 2001 Dec; 39(12):4566-7.

Kirby JE. Anthrax lethal toxin induces human endothelial cell apoptosis. Infect Immun. 2004 Jan; 72(1):430-9.

Klimpel KR, Molloy SS, Thomas G, Leppla SH. Anthrax toxin protective antigen is activated by a cell surface protease with the sequence specificity and catalytic properties of furin. Proc Natl Acad Sci U S A. 1992 Nov 1; 89(21):10277-81.

Kohler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature. 1975 Aug 7; 256(5517):495-7.

Koehler T. Anthrax. New York: Springer-Verlag, 2002.

Kostolansky F, Vareckova E, Betakova T, Mucha V, Russ G, Wharton SA. The strong positive correlation between effective affinity and infectivity neutralization of highly cross-reactive monoclonal antibody IIB4, which recognizes antigenic site B on influenza A virus haemagglutinin. J Gen Virol. 2000 Jul; 81(Pt 7):1727-35.

Lacy DB, Wigelsworth DJ, Melnyk RA, Harrison SC, Collier RJ. Structure of heptameric protective antigen bound to an anthrax toxin receptor: a role for receptor in pH-dependent pore formation. Proc Natl Acad Sci U S A. 2004 Sep 7; 101(36):13147-51.

Laird MW, Zukauskas D, Johnson K, Sampey GC, Olsen H, Garcia A, Karwoski JD, Cooksey BA, Choi GH, Askins J, Tsai A, Pierre J, Gwinn W. Production and purification of Bacillus anthracis protective antigen from Escherichia coli. Protein Expr Purif. 2004 Nov; 38(1):145-52.

Leppla SH. Anthrax toxin edema factor: a bacterial adenylate cyclase that increases cyclic AMP concentrations of eukaryotic cells. Proc Natl Acad Sci U S A. 1982 May; 79(10):3162-6.

Leppla SH. Production and purification of anthrax toxin. Methods Enzymol. 1988; 165:103-16.

Little SF, Leppla SH, Cora E. Production and characterization of monoclonal antibodies to the protective antigen component of *Bacillus anthracis* toxin. Infect Immun. 1988 Jul; 56(7):1807-13.

Little SF, Leppla SH, Friedlander AM. Production and characterization of monoclonal antibodies against the lethal factor component of *Bacillus anthracis* lethal toxin. Infect Immun. 1990 Jun; 58(6):1606-13.

Little SF, Lowe JR. Location of receptor-binding region of protective antigen from *Bacillus anthracis*. Biochem Biophys Res Commun. 1991 Oct 31;180(2):531-7.

Little SF, Novak JM, Lowe JR, Leppla SH, Singh Y, Klimpel KR, Lidgerding BC, Friedlander AM. Characterization of lethal factor binding and cell receptor binding domains of protective antigen of *Bacillus anthracis* using monoclonal antibodies. Microbiology. 1996 Mar; 142 (Pt 3):707-15.

Little SF, Ivins BE, Fellows PF, Friedlander AM. Passive protection by polyclonal antibodies against Bacillus anthracis infection in guinea pigs. Infect Immun. 1997 Dec; 65(12):5171-5.

Little SF, Ivins BE. Molecular pathogenesis of *Bacillus anthracis* infection. Microbes Infect. 1999 Feb; 1(2):131-9.

Liu S, Netzel-Arnett S, Birkedal-Hansen H, Leppla SH. Tumor cell-selective cytotoxicity of matrix metalloproteinase-activated anthrax toxin. Cancer Res. 2000 Nov 1; 60(21):6061-7.

Liu S, Bugge TH, Leppla SH. Targeting of tumor cells by cell surface urokinase plasminogen activator-dependent anthrax toxin. J Biol Chem. 2001 May 25; 276(21):17976-84.

Liu H, Bergman NH, Thomason B, Shallom S, Hazen A, Crossno J, Rasko DA, Ravel J, Read TD, Peterson SN, Yates J 3rd, Hanna PC. Formation and composition of the *Bacillus anthracis* endospore. J Bacteriol. 2004 Jan; 186(1):164-78.

Matsumoto, G. Science [Internet]. Anthrax Powder: State of the Art? Nov. 2003 – [cited 2004 May 28]. Available from: www.sciencemag.org

Manchee RJ, Broster MG, Anderson IS, Henstridge RM, Melling J. Decontamination of *Bacillus anthracis* on Gruinard Island?. Nature. 1983 May 19-25; 303(5914):239-40.

McAllister, N. SFGate.com [Internet]. Murder Will Out: Scientists on the Trail of Killer Bacteria. Nov. 2001 – [cited 2005 June 15]. Available from: http://sfgate.com

Meselson M, Guillemin J, Hugh-Jones M, Langmuir A, Popova I, Shelokov A, Yampolskaya O. The Sverdlovsk anthrax outbreak of 1979. Science. 1994 Nov 18; 266(5188):1202-8.

Mikszta JA, Sullivan VJ, Dean C, Waterston AM, Alarcon JB, Dekker JP 3rd, Brittingham JM, Huang J, Hwang CR, Ferriter M, Jiang G, Mar K, Saikh KU, Stiles BG, Roy CJ, Ulrich RG, Harvey NG. Protective immunization against inhalational anthrax: a comparison of minimally invasive delivery platforms. J Infect Dis. 2005 Jan 15; 191(2):278-88.

Mogridge J, Mourez M, Collier RJ. Involvement of domain 3 in oligomerization by the protective antigen moiety of anthrax toxin. J Bacteriol. 2001 Mar; 183(6):2111-6.

Mogridge J, Cunningham K, Lacy DB, Mourez M, Collier RJ. The lethal and edema factors of anthrax toxin bind only to oligomeric forms of the protective antigen. Proc Natl Acad Sci U S A. 2002 May 14; 99(10):7045-8.

Mohamed N, Li J, Ferreira CS, Little SF, Friedlander AM, Spitalny GL, Casey LS. Enhancement of anthrax lethal toxin cytotoxicity: a subset of monoclonal antibodies against protective antigen increases lethal toxin-mediated killing of murine macrophages. Infect Immun. 2004 Jun; 72(6):3276-83.

Moir A, Smith DA. The genetics of bacterial spore germination. Annu Rev Microbiol. 1990; 44:531-53.

Meselson M, Guillemin J, Hugh-Jones M, Langmuir A, Popova I, Shelokov A, Ampolskaya O. The Sverdlovsk anthrax outbreak of 1979. Science. 1994; 266:1202-1208

Oncu S, Sakarya S. Anthrax- an overview. Med Sci Monit. 2003 Nov; 9(11):RA276-83.

Petosa C, Collier RJ, Klimpel KR, Leppla SH, Liddington RC. Crystal structure of the anthrax toxin protective antigen. Nature. 1997 Feb 27; 385(6619):833-8.

Pittman PR. Aluminum-containing vaccine associated adverse events: role of route of administration and gender. Vaccine. 2002 May 31; 20 Suppl 3:S48-50.

Phillips AP, Campbell AM, Quinn R. Monoclonal antibodies against spore antigens of Bacillus anthracis. FEMS Microbiol Immunol. 1988 Dec; 1(3):169-78.

Saliba, T., Tsang, R., Yuan, X., Schmidt, L., Kabani, A., J. Mogridge and J.D. Berry. Development and characterization of monoclonal antibodies to recombinant PA toxin of Bacillus anthracis. 2004, Immunology 2004, Medimond srl, collective work (in the press).

Salyers AA, Whitt, DD. Bacterial Pathogenesis: A Molecular Approach. Washington: ASM Press, 2002. p. 332-336

Sanchez AM, Bradley KA. Anthrax toxin: can a little be a good thing? Trends Microbiol. 2004 Apr; 12(4):143-5.

Sastry KS, Tuteja U, Batra HV. Generation and characterization of monoclonal antibodies to protective antigen of Bacillus anthracis. Indian J Exp Biol. 2003 Feb; 41(2):123-8.

Santelli E, Bankston LA, Leppla SH, Liddington RC. Crystal structure of a complex between anthrax toxin and its host cell receptor. Nature. 2004 Aug 19; 430(7002):905-8.

Sawada-Hirai R, Jiang I, Wang F, Sun SM, Nedellec R, Ruther P, Alvarez A, Millis D, Morrow PR, Kang AS. Human anti-anthrax protective antigen neutralizing monoclonal antibodies derived from donors vaccinated with anthrax vaccine adsorbed. J Immune Based Ther Vaccines. 2004 May 12; 2(1):5.

Schuch R, Nelson D, Fischetti VA. A bacteriolytic agent that detects and kills Bacillus anthracis. Nature. 2002 Aug 22; 418(6900):884-9.

Sever JL, Brenner AI, Gale AD, Lyle JM, Moulton LH, Ward BJ, West DJ. Safety of anthrax vaccine: an expanded review and evaluation of adverse events reported to the Vaccine Adverse Event Reporting System (VAERS). Pharmacoepidemiol Drug Saf. 2004 Dec; 13(12):825-40.

Schofield DJ, Stephenson JR, Dimmock NJ. High and low efficiency neutralization epitopes on the haemagglutinin of type A influenza virus. J Gen Virol. 1997 Oct; 78 (Pt 10):2441-6.

Scobie HM, Rainey GJ, Bradley KA, Young JA. Human capillary morphogenesis protein 2 functions as an anthrax toxin receptor. Proc Natl Acad Sci U S A. 2003 Apr 29; 100(9):5170-4.

Singh Y, Klimpel KR, Arora N, Sharma M, Leppla SH. The chymotrypsin-sensitive site, FFD315, in anthrax toxin protective antigen is required for translocation of lethal factor. J Biol Chem. 1994 Nov 18; 269(46):29039-46.

Singh Y, Ivins BE, Leppla SH. Study of immunization against anthrax with the purified recombinant protective antigen of Bacillus anthracis. Infect Immun. 1998 Jul; 66(7):3447-8.

Singh Y, Klimpel KR, Goel S, Swain PK, Leppla SH. Oligomerization of anthrax toxin protective antigen and binding of lethal factor during endocytic uptake into mammalian cells. Infect Immun. 1999 Apr; 67(4):1853-9.

Singh S. Biotech Journal [Internet]. San Diego, Mechanism of anthrax toxins. Nov. / Dec. 2003 – [cited 2004 Apr 1]. Available from: http://www.biotechjournal.com.

Smith H. Discovery of the anthrax toxin: the beginning of studies of virulence determinants regulated in vivo. Int J Med Microbiol. 2002 Feb; 291(6-7):411-7. Review.

Sternbach, G. The History of Anthrax. The Journal of Emergency Medicine, 2003; 24 (4): 463-467.

Sulsky SI, Grabenstein JD, Delbos RG. Disability among U.S. Army personnel vaccinated against anthrax. J Occup Environ Med. 2004 Oct; 46(10):1065-75.

Tan P, Mitchell DA, Buss TN, Holmes MA, Anasetti C, Foote J. "Superhumanized" antibodies: reduction of immunogenic potential by complementarity-determining region grafting with human germline sequences: application to an anti-CD28. J Immunol. 2002 Jul 15; 169(2):1119-25.

Tang G, Leppla SH. Proteasome activity is required for anthrax lethal toxin to kill macrophages. Infect Immun. 1999 Jun; 67(6):3055-60.

Tigertt WD. Anthrax. William Smith Greenfield, M.D., F.R.C.P., Professor Superintendent, the Brown Animal Sanatory Institution (1878-81). Concerning the priority due to him for the production of the first vaccine against anthrax. J Hyg (Lond). 1980 Dec; 85(3):415-20.

Turell MJ, Knudson GB. Mechanical transmission of *Bacillus anthracis* by stable flies (Stomoxys calcitrans) and mosquitoes (Aedes aegypti and Aedes taeniorhynchus). Infect Immun. 1987 Aug; 55(8):1859-61.

Turnbull PC. Guidelines for the surveillance and control of anthrax in humans and animals. Third ed. World Health Organization: Department of Communicable Diseases Surveillance and Response, 1998.

Turnbull PC. Introduction: Anthrax history, disease and ecology. Curr Top Microbiol Immunol, 2002; 271: 1-19

Varughese M, Teixeira AV, Liu S, Leppla SH. Identification of a receptor-binding region within domain 4 of the protective antigen component of anthrax toxin. Infect Immun. 1999 Apr; 67(4):1860-5.

Vietri NJ, Marrero R, Hoover TA, Welkos SL. Identification and characterization of a trans-activator involved in the regulation of encapsulation by *Bacillus anthracis*. Gene. 1995 Jan 11;152(1):1-9.

Wang F, Ruther P, Jiang I, Sawada-Hirai R, Sun SM, Nedellec R, Morrow PR, Kang AS. Human monoclonal antibodies that neutralize anthrax toxin by inhibiting heptamer assembly. Hum Antibodies. 2004; 13(4):105-10.

Wesche J. The ELSO Gazette [Internet]. Membrane rafting with anthrax toxin. Issue 14, Apr 2003 – [cited 2005 Jan 9]. Available from: http://www.the-elso-gazette.org.

Wistreich, G. A., and M. D. Lehman. Microbiology and human disease. New York: Glencoe Press, 1973.