Identification of B-cell epitopes on domain 4 of anthrax protective antigen

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Identification of B-cell Epitopes on Domain 4 of Anthrax Protective Antigen

by

Cassandra D. Kelly-Cirino

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Abstract

Protective Antigen (PA) is the receptor binding subunit common to both Lethal (LT) and Edema (ET) toxins, which contribute to the mortality associated with Bacillus anthracis infection. While recombinant PA (rPA) is likely to be an important constituent of second generation anthrax vaccines, evaluating the effectiveness of candidate vaccines is currently difficult, because the specific B cell epitopes involved in toxin neutralization have not been completely defined. The only well characterized antibody, 14B7, has been shown to disrupt the association of PA with the anthrax toxin receptors (ATR) by binding to domain 4 of PA. I hypothesized that other domain 4 epitopes play a critical role in eliciting a protective immune response to anthrax. To test this hypothesis I first identified a novel rPA immunogen capable of eliciting a protective immune response in goats and mice. I next established an LT challenge mouse model to evaluate the ability of antibodies to disrupt the intoxication pathway in vivo. To identify neutralizing epitopes on domain 4 of PA, I screened a collection of murine B cell hybridomas for monoclonal antibodies (MAbs) that reacted with the native PA protein as well as linear peptides within domain 4. Two IgG1 MAbs, 1-F1 and 2-B12, were identified that recognize distinct domain 4 linear epitopes. 1-F1 recognized residues 692-703, part of the ATR recognition region. 1-F1 blocked PA’s ability to associate with ATR in an in vitro solid phase binding assay, and neutralized LT in vitro. 2-B12 recognized residues 716-727, a region not previously known to be a target of neutralizing antibodies. 2-B12 was as effective as 1-F1 in neutralizing LT in vitro, although it only partially inhibited PA binding to ATR. Mice passively administered 1-F1 or 2-B12 were
protected against LT challenge. This data confirm that several epitopes on domain 4 of PA contribute to the protective immune response against anthrax intoxication. The identification of neutralizing MAbs recognizing linear peptides provides us with a powerful tool for identifying the specific epitopes involved in the protective immune response to anthrax and advances our fundamental understanding of the mechanisms by which antibodies neutralize anthrax toxin.
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Dedication

This thesis, in its entirety, is dedicated to the single person who made this journey possible. To my beloved husband Nick Cirino, you started me on this path and it is for you that I have finished it. While you are not here to see the end of this road there is not a single portion of this work, or my life during these years that you did not touch or shape. I can only hope that I have made you proud.
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Chapter 1. Introduction

Anthrax as a continuing public health threat

*Bacillus anthracis* has long been recognized as a public health threat, because of the ease by which spores can enter the respiratory tract, and the high mortality rate that accompanies spore inhalation. These fears were realized in the fall of 2001, when *B. anthracis* spores were disseminated through the United States postal system, resulting in five deaths, twenty-two cases of infection, and the possible exposure of more than 30,000 people (1,19,54).

More recently, two cases of inhalation anthrax infection, and a case of cutaneous infection, have occurred in persons using untreated animal hides to manufacture African-style drums (3,4,8). In 2006 a resident of New York City was confirmed as having contracted inhalation anthrax (3), in 2007 a report of cutaneous anthrax infection was confirmed in a resident of Connecticut (4), and in 2008 a fatal case of inhalation anthrax infection was reported in the United Kingdom (8). While the cutaneous anthrax cases resolved without complication following antibiotic administration (4), the inhalation cases highlight the complexity of diagnosing and treating anthrax infections post-exposure. Both inhalation anthrax infection patients presented to the hospital with 2-3 day histories of fever, rigor, shortness of breath, and general malaise (3,8). Antibiotic therapy was initiated immediately in both cases; however, both patients continued to deteriorate rapidly. Emergency use approvals were obtained for the administration of antitoxin immunoglobulin therapy which allowed for the complete resolution of disease in the New York City patient (122). The patient in the United Kingdom succumbed to the disease within 11 days of hospitalization (8).
Successful post-exposure treatment of inhalation anthrax infection with antibiotics alone, or in combination with a passive immunotherapy, is difficult to achieve due to the rapid onset of generalized symptoms that may go undiagnosed for several days. While routine vaccination of civilians against anthrax is not necessary, certain segments of the population, notably emergency first responders and research laboratory personnel, remain at risk of exposure and are in need of an effective pre-exposure vaccine.

**Current challenges in anthrax vaccine design**

In the United States, the only licensed anthrax vaccine, Anthrax Vaccine Adsorbed (AVA) or Biothrax, has endured years of controversy over its safety (2) and is not considered an ideal vaccine due to its lengthy vaccination schedule (6 injections over 18 months, with yearly boosters). AVA, which consists of formaldehyde-fixed culture filtrate from an attenuated strain of *B. anthracis* adsorbed to aluminum hydroxide, is also inherently difficult to manufacture and standardize. Recent changes in the administration route of AVA has revitalized the potential use of this vaccine for the pre-exposure prophylaxis of high risk populations (73). Protective Antigen (PA) is the common receptor-binding subunit to both Lethal (LT) and Edema (ET) toxins, which are important virulence factors during anthrax infection. While PA has been determined to be the main effective component of AVA (39,53,64) this vaccine also includes additional biologically active toxin components, including Lethal Factor (LF) and Edema Factor (EF). It has been postulated that the overall efficacy of this vaccine may be greater than a recombinant PA subunit vaccine due to its ability to cross-protect against multiple toxin proteins. The presence of functional toxin proteins may also be responsible for the painful side-effects associated with this vaccine. A subset of recipients have complained...
of severe pain, redness and swelling at the site of injection, which may be due to localized formation of functional anthrax toxin causing edema and necrosis (107,108).

In order to decrease the safety and manufacturing difficulties associated with AVA, researchers have sought to develop next-generation vaccine candidates based solely on recombinant PA (rPA). Studies have demonstrated that rPA immunization can successfully protect animals against virulent spore challenge and elicits strong anti-PA titers (6,36,51). The most promising second-generation rPA vaccine, manufactured by VaxGen, was considered a leading candidate to supply the Strategic National Stockpile with 75 million doses. The Department of Homeland Security cancelled its vaccine acquisition contract with VaxGen after issues surrounding vaccine stability were unable to be resolved. For these reasons there is a continued effort to develop a second generation anthrax vaccine based on a defined antigenic composition.

Anthrax Pathogenesis

*B. anthracis* is a gram positive, spore-forming rod which is easily cultivated on standard nutrient media in aerobic or anaerobic environments. Genetically, this bacterium is extremely similar to *B. cereus*, a common environmental bacillus, and *B. thuringiensis*, which is pathogenic to the larvae of *Lepidoptera* (58,112). Animals and humans are susceptible to anthrax infection, although most cases occur in herbivores after ingestion of spores during grazing.

In humans, the anthracis bacilli can cause three types of infections: cutaneous, gastrointestinal and inhalation. The cutaneous form of anthrax develops when the bacterium is introduced through abrasions in the skin. The spores germinate and multiply at the site of inoculation resulting in localized toxin production. A characteristic black
eschar forms as the products of the toxin cause edema and necrosis (61). Cutaneous infection is usually self-limiting and is easily treatable with antibiotics, which reduces the case fatality rate to 20% if diagnosed and treated appropriately.

Gastrointestinal anthrax results after ingestion of contaminated meat and can have an extremely high mortality rate if left untreated. Spores enter through a breach in the intestinal mucosa where they are transported to the mesenteric lymph nodes. After germination within the lymph nodes, the bacteria multiply rapidly causing severe abdominal pain and swelling (61). Complications from this infection include hemorrhagic adenitis and septicemia. If left untreated this form of the disease has case fatality rates approaching 80%, but can be resolved with appropriate antibiotic therapy administered within the first 36 hours of infection.

Inhalation anthrax results when spores less than 5 μm in size are inhaled into the lungs. Preliminary symptoms are “flu-like” and include high fever and chest pain. Early diagnosis of inhalation anthrax is therefore a challenge, and antibiotic therapy is rarely administered within the critical 36 hour time window. Clinically, patients present with a characteristic widened mediastinum visible on chest x-rays. This diagnostic tool has not been very reliable and many cases of inhalation anthrax may go undiagnosed in the early stages (38). After inhalation, spores are phagocytosed by alveolar macrophages where they then germinate and begin to multiply rapidly. Vegetative bacilli traffic to the mediastinal lymph nodes and cause hemorrhagic mediastinitis (61,90). The disease progresses rapidly over 2-3 days to produce a systemic infection resulting in organ failure and shock. Treatment with common antibiotics, such as penicillin and ciprofloxacin, are
generally effective for anthrax infections, but successful treatment requires introduction of the antibiotic before the bacteria have had a chance to multiply to high levels.

The genome of *B. anthracis* is comprised of a 5.2 mega-base chromosome and two plasmids, pXO1 and pXO2, that encode the main virulence determinants. The two major virulence determinants, capsule and toxins, play a critical role in the ability of anthrax bacteria to evade the host immune response. *B. anthracis* lacking either virulence plasmid are avirulent in humans and animals, underscoring the importance the plasmid-encoded proteins play in anthrax pathogenesis. Historically, avirulent strains have been used as vaccines for laboratory workers, first responders and military in both the USA and the USSR. The avirulent Sterne strain is currently used as a livestock vaccine. Concerns over the safety of a live, attenuated, whole-bacterial vaccine have limited their continued use in humans, further highlighting the need to develop a second-generation subunit vaccine.

**Anthrax virulence determinant: Capsule**

The poly-D-glutamyl capsule is encoded by the 60 MDa pX02 plasmid (42). All virulent strains of *B. anthracis* produce this capsule which plays a key role during the invasive stages of the disease. The capsule functions to prevent bacterial killing after phagocytosis by counteracting the bactericidal properties of serum and phagocytes (58,112). The capsule itself is nontoxic and is not overly antigenic although a recent report indicated that immunization with killed-spores provides higher levels of protection than immunization with PA alone (40). Capsule is required for wide-spread dissemination of bacilli in inhalation infections in mice, while noncapsulated bacilli are readily killed by the immune system (33).
Anthrax virulence determinant: Lethal and Edema Toxins

The second virulence determinants, the bipartite toxins, consist of three proteins which are encoded on the pX01 plasmid. The anthrax toxin proteins, protective antigen (PA), lethal factor (LF), and edema factor (EF), are individually secreted and considered components of the classical AB family of bacterial toxins. LF and EF are the catalytically active A subunits which are introduced into the cell after binding to the B subunit. PA, the B subunit, the receptor-binding member, has the ability to bind two distinct A subunits with equal affinity. Lethal toxin (LT) is formed when PA binds LF, and edema toxin (ET) is formed when PA binds EF.

PA is secreted as an 83kDa protein (PA83) that binds two known anthrax toxin receptors (ATR), tumor endothelium marker-8 (TEM-8) and capillary morphogenesis protein 2 (CMG-2) (29,60,102). Following receptor engagement, the amino terminal 20 kDa of PA (PA20) is proteolytically removed by a furin-like protease, exposing a region of PA required to form holotoxin. The membrane-bound 63 kDa PA (PA63) spontaneously oligomerizes into heptamers, and then associates with LF, a zinc-dependent protease (29,34), or EF, a calmodulin-, Ca\(^{+2}\)-dependent adenylate cyclase (31,32), to form LT and ET, respectively. The bound heptamer is then internalized via receptor-mediated endocytosis. The low pH of the endosome causes a conformational change in PA63 so that it is inserted into the endosomal membrane forming a transmembrane pore whereby LF and EF can enter the cytosol to exert their toxic effects (29,59).

The anthrax toxin receptors, TEM-8 and CMG-2, are ubiquitously expressed on the surface of a variety of cells and contain extracellular von Willebrand Factor Type A (VWA) domains. Metal ion-dependent adhesions sites (MIDAS) are characteristic of
these domains and have been shown to play an important role in PA binding to ATR (18,100). PA:TEM-8 interactions are dependent on the binding of metal ions; however, CMG-2 can bind to PA with reduced affinity in the absence of metal ions (105).

LF, a zinc-dependent protease, induces cytokine production in lymphocytes and macrophages (as reviewed in (29)). Once LF is translocated through the pore into the cell, it binds specifically to the N-terminus of mitogen-activated protein kinase kinases (MKK) where it cleaves within a proline-rich region (29,34). This cleavage renders MKKs enzymatically inactive and prevents downstream activation of other MKKs. Loss of MKK signaling has drastic effects on both the innate and adaptive immune response. The specific role that the truncated MKKs play in the pathogenesis of *B. anthracis* has not been fully determined. Currently, the mechanism of macrophage killing by LT is a widely debated topic as discussed by Turk *et al.* (117). It has been reported by several groups that LT caused macrophage lysis and a subsequent burst of cytokine release (16,94). This onslaught of cytokine release was considered to be the cause of shock and subsequent death in animal models. Other groups have observed the opposite effect when cytokine responses were monitored in animal and cell culture models. For example, Moayeri *et al.* (77,78) have conducted extensive characterization of the pathology and cytokine response to LT infection. They report that LT causes tissue hypoxia, liver necrosis and pleural edema independent of TNF-α or cytokine production in two strains of mice (77,95). This conflict in elucidating the cellular role of anthrax intoxication will certainly bring about increased research which will further our understanding of *B. anthracis* pathogenesis with respect to the roles of the bipartite toxins.
EF, a calmodulin-, Ca\(^{2+}\)-dependent adenylate cyclase, has been crystallized with and without bound calmodulin (31,32). Calmodulin binding to EF is required for catalysis and can be inhibited when cellular levels of Ca\(^{2+}\) are too high. Once EF enters the cell an influx of Ca\(^{2+}\) causes intracellular levels of cAMP to rise to 1000 fold above normal levels. Increased cAMP levels have been shown to dampen the host immune response by inhibiting lymphocyte proliferation, phagocytosis, oxidative burst and proinflammatory cytokine release (14). The cellular effects of EF tend to be non-lethal and short-lived due to its short half-life within the cell (~ 2 hr) (62), and has been implicated in tissue damage and lethality in some mouse models (37). Additionally, ET can cause a depletion of ATP reserves in macrophages and neutrophils which may act to inhibit the engulfment process and could result in regional suppression of phagocyte activity (58).

**Anthrax Protective Antigen**

Current second generation anthrax vaccines are focused on PA, which has been established as the primary component of AVA involved in immunity to anthrax infection (64). PA\(_{83}\) is a long, flat molecule (100x50x30 Å) comprised mostly of beta-sheets. PA can be divided into four functional domains (93).

Domain 1 (residues 1-248) contains the furin recognition site (\(^{164}\text{RKKR}^{167}\)), which, upon cleavage, results in the formation of PA\(_{20}\) and PA\(_{63}\). PA\(_{20}\) remains non-covalently attached to PA\(_{63}\) which is bound to the ATR on the cell surface. The remainder of the domain is termed domain 1’ and contains the region of PA that is responsible for binding LF and EF (81). A pair of calcium atoms is tightly bound to
domain 1’ and function in maintaining an appropriate confirmation for self-association and ligand binding (93).

Domain 2 (residues 249-487) functions primarily in the formation of a transmembrane pore. Following heptamerization of PA$_{63}$, a drastic change in pH allows for a conformational rearrangement of the flexible loop located between strands 2$\beta_2$ and 2$\beta_3$ which then forms the transmembrane portion of the pores’ $\beta$-barrel (15,93). Recent evidence suggests that domain 2 may also play a role in PA binding to ATR where a $\beta$-hairpin inserts into a conserved pocket on the ATRs (59,102). Mutations within this $\beta$-hairpin significantly reduced toxicity indicating that this domain plays an important role in determining PA:ATR interactions (82). The crystal structure of PA bound to CMG-2 (PDB 1T6B) was recently identified and shows engagement of domain 2 (residues 259-487) with the receptor (102).

Domain 3 (residues 488-595) is the smallest of the PA domains and forms a ferrodoxin-like fold that resembles domain A of the toxic-shock-syndrome toxin (93). The role of domain 3 has generally been assumed to be structural and no other function had been ascribed to this domain. Recent research identified that mutations within a specific loop of this domain (residues 510-518) resulted in a loss of PA$_{63}$ heptamerization and a subsequent inability to bind LF (79). These are the first results that indicate the involvement of domain 3 in PA self-association and also shed light on the nature of LF/EF binding to PA$_{63}$.

Domain 4 (residues 596-735) constitutes the region of PA involved in receptor recognition and attachment (102,110). Structurally, domain 4 involves a $\beta$-sandwich with an immunoglobulin-like fold. When PA binds to ATR, a hydrophobic core within
the β-sandwich interacts with a hydrophobic ridge adjacent to the MIDAS motif on the receptor (102). The crystal structure of PA bound to CMG-2 (PDB 1T6B) implicates residues 654-662, 681-688 and 714-716 of domain 4 as being in direct contact with the receptor (102,111). Specifically, residue D683 in domain 4 is critical for mediating interaction between TEM-8 and PA (18). Residue D683 projects from the surface of PA and interacts directly with the conserved MIDAS motif on TEM-8 via coordination with divalent cations. This binding interaction is identical to the binding interactions observed for α-integrins and their natural ligands. Deletion of the carboxy-terminal of PA (beginning at residue 608) completely abrogates PA binding to ATR (21,118). Deletion of the large loop within domain 4 (residues 703-722) reduced in vitro toxicity of LT 10-fold, while retaining PA’s ability to bind ATR and LF (21). In contrast, similar studies conducted by Varughese et al. (118), demonstrated that the large loop did not alter PA activity and did not appear to play a role in receptor binding. However, residues near or within the small loop (residues 679-693) were found to be critical for LT toxicity and PA binding to ATR (118). The similarity in these groups findings clearly demonstrate that domain 4 of PA plays a critical role in receptor engagement and is required for toxicity. The opposing findings of these results indentify an important gap in our limited understanding of the critical residues involved in PA:ATR interactions; specifically the individual residues required for binding. Elucidating these intricate details will be important for designing next generation vaccines that specifically target defined regions of PA to ensure that an appropriate and protective immune response is elicited following vaccination.
PA as a target for neutralizing antibodies

It is well established that animals immunized with recombinant PA, or passively administered anti-PA antiserum are protected against LT/ET exposure, or spore challenge (reviewed in (41) and (19)). Antibodies against domain 4 are postulated to be the most effective at neutralizing LT and ET (6), as they are proposed to interfere directly with PA binding to ATR. However, the antibody response to PA is complicated, because, in general, total anti-PA titers, when measured by enzyme-linked immunosorbent assays (ELISA), do not correlate well with protection (97,98). This is likely due to the fact that a large number of anti-PA antibodies are directed against non-neutralizing (or possibly toxin enhancing) epitopes (80,113). Pioneering work by Little and colleagues (69) suggests that there are at least 23 antigen determinants on PA. At present only a handful of these epitopes have been described, and shown to be targets of monoclonal antibodies capable of effective neutralization in vivo or in vitro (6,20,26,43,125,126).

The only neutralizing epitope that has been characterized in detail is recognized by monoclonal antibody (MAb) 14B7 (69,71,100,111). MAb 14B7 was first identified as a MAb capable of blocking PA binding to cells (69). The conformation-dependent epitope was localized to residues 671-721 (71), which includes both the small and large loops within domain 4. Using in silico docking models, Sivasubramanian et al., determined that residues 648-660 and 712-720 were critical for 14B7 binding to PA (111). These findings, combined with the knowledge that the PA:CMG-2 interface spans residues 654-716 (102), supports a steric hindrance mechanism of action for 14B7. Scanning alanine mutation studies revealed that residues 682 and 686 were critical for 14B7 binding to PA and also reduced toxicity 10-100 fold (100). Interestingly, residues
684, 685, 687 and 688 were required for PA recognition by 14B7 but not for PA toxicity. The docking model and the scanning alanine mutation data have helped to refine our understanding of PA:ATR interactions. Differences in these reports remind us that additional detailed information is needed to narrowly define the residues that will be critical for inclusion in a second generation vaccine candidate.

There are certainly additional neutralizing epitopes besides the one recognized by 14B7 on domain 4 (6,20,128). In particular, Abboud and Casadevall suggested that there is a linear epitope (residues 678-697) overlapping the 14B7 binding site that is the target of neutralizing antibodies (6); however, isolation of the MAb recognizing this epitope has yet to be completed. Another domain 4 binding antibody, MAb 7.5, was shown to inhibit PA:ATR interactions and protect mice against Sterne spore challenge (20). The binding of MAb 7.5 has been broadly attributed to residues 608-735 (equivalent to the entire domain 4). Mutational analysis was not able to narrow the residues involved in binding, but indicates that binding does not appear to overlap with the large loop of domain 4 (20). Additional research is needed in order to fully characterize MAb 7.5 and establish that it is distinct from MAb 14B7. Zhou et al. recently identified three conformation-dependent human MAbs that recognize domain 4 and neutralize LT in vitro (128). The authors acknowledge that fine epitope mapping and mutational analysis will need to be completed in the future to fully characterize these antibodies and determine their exact interaction with PA.

The fact that key neutralizing epitopes on PA remain widely unidentified poses a significant barrier to evaluating the efficacy of recombinant PA vaccines in animals and humans. The identification of antibodies that are both linear epitope-dependent and
neutralizing will be extremely useful in defining key residues within PA that contribute to a neutralizing immune response. Additionally, neutralizing linear epitopes can be used as highly specific correlates of immunity and can be incorporated into functional screening assays of potential vaccine candidates.

**Hypothesis**

I hypothesize that other epitopes on domain 4 of PA, in addition to that recognized by MAb 14B7, play a critical role in eliciting a protective immune response to anthrax. Towards this end, I screened a collection of B cell hybridomas produced from PA83 immunized mice for MAbs capable of binding to a domain 4-specific peptide array. Identification of neutralizing MAbs that recognize linear peptides provides us with a powerful tool for narrowly identifying the epitopes involved in the protective immune response to anthrax. In order to best answer the question I specifically focused characterizations of domain 4 MAbs to those that demonstrated conformational and linear peptide reactivity.

I identified two domain 4 reactive MAbs, 1-F1 and 2-B12. 1-F1 recognized a peptide spanning residues 692-703, which falls between the small and large loops of domain 4 and overlaps with 14B7s binding site. 2-B12 recognized a peptide spanning residues 716-727, which corresponds to the large loop on domain 4, a region which has not, until now, been clearly implicated in playing a role in PA:ATR binding (20,118). To date, there have been no antibodies described that bind within this region, making this the first report of a neutralizing MAb that recognizes an epitope distinct from 14B7. Both MAbs neutralized LT* in vitro* and partially protected mice against LT challenge. The discovery of these MAbs is significant in that they delineate specific linear epitopes
within domain 4 that are the target of neutralizing antibodies, furthering our understanding of the role that domain 4 plays in eliciting a protective immune response to anthrax intoxication.

**Applicability to other select agent toxins**

Additional proteins involved in the anthrax intoxication pathway may be important in order to provide a synergistic, and therefore comprehensive, immune response following vaccination. The approach I have described here will be useful for investigating neutralizing epitopes within LF (84) and EF. The protection afforded by the current AVA vaccine may in part be due to the expression of functional LF and EF. Second generation vaccines that focus on using rPA only will exclude antibodies targeting LF and EF and may be less effective at eliciting a neutralizing response. Selectively including multiple epitopes from various toxin proteins in a next-generation vaccine would provide a platform for eliciting a comprehensive response while negating the safety concerns associated with a virulent, live spore-based vaccine. The ability to narrowly define key epitopes that contribute to a neutralizing immune response is critical not just in anthrax pathogenesis but also in other select agent pathogens that are considered high priority for the development of pre- and post-exposure therapeutics.

A major thrust of NIAID research initiatives (i.e.: the Large-Scale Antibody and T Cell Epitope Discovery Program,) focuses on elucidating key epitopes involved in the immune response to a wide variety of pathogens, underscoring the importance that epitope identification plays on a national public health level (106). Results from these funding initiatives are made freely available to the scientific community via the Immune Epitope Database and Analysis Resource (IEDB, www.immuneepitope.org). In fact,
since its inception in 2004, the IEDB has been used to form detailed immune reaction roadmaps for several pathogens including influenza (22), anthrax (125), botulism (125), and tuberculosis (17,35).

Botulism is a neurological disease caused by the production of botulism neurotoxins (BoNT) by *Clostridium botulinum*. The fact that there are seven immunological serotypes of BoNT (A-G) complicates the design of vaccines and therapeutics. Treatment for botulism currently relies on the passive administration of horse antibodies in cocktail mixture specific for several toxin types. Atassi *et al.* have pioneered work into identifying the major epitopes involved in toxin binding to the receptors in order to better understand the challenges experienced in vaccine design as well as what causes the development of resistance to anti-BoNT antibodies (10,11). The identification of several peptides that are recognized by the polyclonal sera from horses, humans and mice following vaccination with toxoid have provided much insight into the role that the various regions of BoNT play in pathogenesis and eliciting an immune response (9).

Research into other pathogens will benefit from the experimental models established here by providing a framework for fine epitope mapping and characterization of a protective immune response.
Chapter 2. General Materials and Methods

Cell lines

J774A.1 murine macrophage-like cell line was obtained from the American Type Culture Collection (TIB-67, Manassas, VA). Cells were cultured in DMEM 1x High Glucose with GlutaMAX (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum, and penicillin/streptomycin at 37°C with 5% CO₂.

Recombinant anthrax toxin proteins

Histidine-tagged, rPA₈₃ and rPA₃₂ were obtained from the Northeast Biodefense Center (NBC) Protein Expression Core. rPA₃₂ is a truncated version of PA₈₃ containing only domains 3 and 4, and was previously shown to be properly folded and capable of binding to anthrax toxin receptor (28). Recombinant lethal factor (rLF) was obtained from BEI Resources (Manassas, VA, Cat# NR-4367). Functional lethal toxin (LT) was produced by mixing rLF and rPA₈₃ at a 1:1 (w/w) ratio in sterile PBS. Toxin stocks were qualified by dose response titrations and used at 2 TCEC₅₀ for in vitro Toxin Neutralization Assays (TNA). Recombinant proteins were stored at -20°C as single use aliquots at a concentration of 1 mg/ml in PBS, except for rLF which was at a concentration of 500 mg/ml in PBS with 1% BSA. PA₈₃ was biotinylated using EZ-Link Sulfo-NHS-LC Biotin (Pierce Biotechnology Inc., Rockford, IL) following the standard manufacturer’s protocol.
Enzyme Linked Immunosorbent Assays (ELISA)

Nunc Maxisorb 96-well microtiter plates were coated with antigen (PA$_{83}$ or PA$_{32}$ or linear peptides) at 0.1 $\mu$g/well overnight at 4°C. Prior to the addition of the analyte (polyclonal serum, 5 $\mu$g/ml; hybridoma supernatant, 0.1 ml/well) plates were blocked with Blocker Casein (Pierce Biotechnology Inc.) and washed 3x with PBS containing 0.05% Tween-20. The plates were probed with HRP-labeled secondary antibodies and developed with SureBlue TMB 1-Component Microwell Peroxidase Substrate (KPL Inc., Gaithersburgh, MD). Microtiter plates were analyzed using a SpectraMax 250 microtiter plate reader (Molecular Devices, Union City, CA).

Anti-serum Titer Determination:

Microtiter plates coated with rPA$_{83}$ (10 nM) in 10 mM carbonate/bicarbonate buffer (pH 8.5) with a final coating volume of 50 $\mu$l. Plates were coated for 1 hr then washed in water and blocked with 5% non-fat milk powder. Antibody titers were measured by reacting (2 hr) serially diluted anti-PA$_{83}$ IgG with the rPA$_{83}$-coated microtiter wells. Secondary antibody was HRP-rabbit anti-goat IgG (Invitrogen Life Technologies). Anti-PA$_{83}$ IgG titers were measured and expressed as the reciprocal of the antibody dilution which produced an absorbance value equal to 50% maximum absorbance.

Linear Peptide ELISAs:

Synthetic peptides, 12-aa long with 4-aa overlaps, spanning PA$_{83}$ (GenBank accession ID: AAA22637) (New England Peptide, Gardner, MA) were used to screen polyclonal sera, B cell hybridomas and monoclonal antibodies. The sequences of the individual peptides and their designated peptide numbers are listed in Tables 1-4. Peptides 1-4 span
the signal sequence region of PA and were included in the studies as negative controls. The sequences of the individual peptides are listed in Table 3. Lyophilized peptides were solubilized in dimethyl sulfoxide (DMSO) at 10 mg/ml, then diluted to 1:5 (v/v) into PBS and frozen at -20°C as single-use aliquots.

Ig Subclass Determination:

The subclass of each MAb was determined by ELISA. NUNC Maxisorb plates were coated with PA₈₃ and then reacted with each MAb for 1 hr. The plates were then washed and incubated with HRP-conjugated goat anti-mouse Ig specific (IgG1, IgG2a, IgG2b, IgG3) antibodies (Southern Biotechnology Associates, Birmingham, AL).

**In vitro Cytotoxicity and Toxin Neutralization Assay (TNA)**

J774A.1 cells were harvested by gentle scraping (no trypsin) and were seeded in 96-well plates at a density of 6x10⁴ cells/well in 100 μl of complete medium. Cells were incubated for 18-24 hr or until >90% confluency had been achieved. Medium was removed, and cells were washed once in sterile PBS before addition of toxin or antibodies. For dose response titrations of LT stocks, 100 μl of LT was added to the cells in 1000 ng, 100 ng, 10 ng and 0.1 ng amounts. The TCEC₅₀ was determined and all subsequent TNA experiments utilized 2x TCEC₅₀ for that particular lot of LT. For TNA experiments, antibodies were incubated with 2 TCEC₅₀ LT at 37°C with shaking for 1 hr. Mixtures were then added to the confluent, washed cell monolayer and incubated for 4 hr at 37°C + 5% CO₂. Cell viability was established using Sigma’s Cell Growth Determination Kit (Sigma-Aldrich, St. Louis, MO). Briefly, 10 μl of MTT dye was added to cells and incubated for 15 hr at 37°C and 5% CO₂. Medium was removed by
tapping and cells were washed gently with 100 μl PBS. Solubilization solution (100 μl) was then added to the wells and the optical density was measured at 570 nm on a SpectroMax 250 microtiter spectrophotometer (Molecular Devices). Control treatments included cells only (PBS, cell viability control), and LT only (cell death control). Percent relative cell viability, for the dose response titrations, was calculated as the ratio between LeTx-treated cells (LeTx) and untreated control cells (100 μl PBS). Percent protection, for the TNA experiments, was calculated as follows: (1-((PBS – Ab)/(PBS – LT))) x 100.
Chapter 3. rPA₈₃-MIS Elicits a Neutralizing Immune Response

Acknowledgments

The novel adjuvant, MIS, used in these studies is proprietary to Virionyx Corporation (Auckland, NZ). Dr. Frank Gelder covalently attached the MIS adjuvant to rPA₈₃. All goats were housed at the Virionyx GMP-certified farm. Immunizations and plasmapheresis of the goats was conducted by staff scientists at Virionyx.

Portions of this work have been published by C. D. Kelly et al. (55).

Introduction

In order to investigate critical epitopes on domain 4 of PA, it was first necessary to identify a relevant antigen capable of eliciting an appropriate immune response following vaccination. The immunogenicity of rPA₈₃ has been well established (53,69), as has the expression and purification of rPA from E. coli (7,24). A novel adjuvant, Microparticle Immune Stimulator (MIS), is a muramyl dipeptide derivative and has previously been shown to be an effective adjuvant when coupled to HIV proteins for the generation of a passive immunotherapeutic for failing-therapy AIDS patients (30). I hypothesized that coupling the MIS adjuvant to rPA₈₃ would elicit an appropriate immune response in animals and provide us with a relevant antigen for investigating individual epitopes involved in immunity to anthrax toxins.

I also investigated the ability of a truncated form of PA₈₃ to elicit a neutralizing immune response. The truncated PA protein, PA₃₂, is a 32-kDa carboxy-terminal fragment of PA₈₃ where only domains 3 and 4 are expressed. Previous work by Cirino et al., has established that PA₃₂ is properly folded, binds to ATR and is internalized.
appropriately (28). I hypothesized that immunization with PA32-MIS would elicit an immune response comparable to that elicited with the full-length rPA83 immunogen. The rPA32-MIS immunogen provided us with a powerful tool to determine the extent that the C-terminal domains of PA play in eliciting neutralizing antibodies.

I expected that a subset of antibodies elicited in response to PA83-MIS immunization would react with linear B cell epitopes. Using an array of synthetic peptides (12-aa in length, 4-aa overlaps) spanning PA83 I investigated the reactivity of the goat anti-PA83 IgG with the linear epitopes. Peptide arrays are increasingly being applied to the assessment of immunity to infectious diseases (83,84), and have important applications for biodefense vaccines. Several groups have used microarrays on derivatized glass slides to quantitate the antibody response to several viruses including cytomegalovirus, herpes simplex viruses and retroviruses (12,75,83). Other groups have used a pin-peptide approach to map reactivity of serum antibodies to peptides specific for domain 2 of anthrax PA (43). Most recently, a solid-phase ELISA method was adopted to screen synthetic peptides for serum reactivity to LF (84). Although many B-cell epitopes are conformation-dependent (49), epitope-based vaccines have a wide range of applications from infectious diseases (48,116), to cancer therapy (27) and are gaining increased interest due to their stability, flexibility and versatility (as reviewed in (96)).

Here I establish that various forms of rPA coupled to the MIS adjuvant successfully elicit a neutralizing immune response in goats. The resulting immune serum is reactive with a series of linear epitopes providing preliminary evidence that linear peptide-reactive antibodies may contribute to a neutralizing polyclonal immune response to anthrax toxins. Further characterization of the specific linear epitopes that contribute
to the neutralizing response will enable us to generate a fine-epitope map of critical residues on PA.

**Materials and Methods**

**Generation of rPA-MIS Adjuvants.** I supplied Virionyx Corporation (Auckland, NZ) with rPA₈₃ and rPA₃₂ for covalent attachment to MIS. MIS was oxidized with sodium meta periodate (0.5M) for 1 hr followed by centrifugation and a water wash to remove any excess. rPA₈₃ or rPA₃₂ (1 mg) in sodium carbonate buffer (0.1M, pH 9.5) was added to 10 mg of activated MIS and incubated overnight at room temperature. The resulting Schiff’s base was reduced by the addition of ascorbic acid to achieve a pH of 7.0.

**Generation of Polyclonal Anti-PA Serum in Goats.** Goats were immunized with 100 μg rPA₈₃- or rPA₃₂-MIS emulsified in Freund’s complete adjuvant. Goats were subsequently boosted three additional times with immunogen in Freund’s incomplete adjuvant over a 13-week period. Hyper-immune plasma was collected from each animal two weeks following the last immunization. Plasma was pooled and IgG was purified using a standard octanoic acid precipitation technique (91). Purified anti-PA₈₃ and rPA₃₂ IgG was supplied at a concentration of 15 mg/ml.

**Evaluation of goat IgG in vitro.** *In vitro* neutralization was evaluated using the standard TNA described in the General Materials and Methods section.
Results

The ability of rPA83-MIS to induce a specific immune response was evaluated in goats (Virionyx Corp.). Animals were immunized four times (days 0, 14, 28, 56) over a period of 56 days and subsequently plasmapheresed on day 94. Total IgG was purified from plasma, and rPA83 specificity was confirmed by Western blot and ELISA (data not shown). Specific rPA83 titers in serum of immunized goats on days 0, 27, 40, 54, 67, and 94 were determined. Antibody titers, as measured by ELISA, reached >10,000 (calculated as the reciprocal of the dilution producing 50% maximum absorbance) within 2 weeks (27 days post-immunization), and reached a maximum of ~16,000 after the fourth immunization (Fig. 1).

The protective efficacy of the anti-PA83 IgG was evaluated using an in vitro toxin neutralization assay (TNA). Cells were treated with 0.5 ng/μl of LT and dilutions of anti-PA83 IgG. Controls included untreated cells (PBS only), cells treated with IgG alone (7.5 μg αPA83 Ig with no LT), or cells treated with 0.5 ng/μl LT alone (LT). LT treated cells demonstrated a statistically significant decrease in cell viability (p<0.001) as compared to the untreated PBS control cells, while anti-PA83 IgG (7.5 - 250 μg) had no effect on cell viability (data not shown). Cells treated with varying amounts of anti-PA83 IgG were protected from LT cytotoxicity in a dose-dependent manner (Fig. 2A). Anti-PA83 IgG (500 nM) fully protected cells against LT mediated cell death, while 63 nM offered minimal protection (35%) over the LT treated control cells. The EC50 for the anti-PA83 IgG was 2.5x10−7 M.

To explore the contribution of PA domains 3 and 4 to the overall neutralizing polyclonal serum I also evaluated goat anti-PA32 IgG. Animals were immunized with
rPA$_{32}$-MIS, and plasmapheresed following the same regimen as described for rPA$_{83}$-MIS. All goats demonstrated significant titers of anti-PA$_{32}$ IgG by day 94, similar to that observed for anti-PA$_{83}$ IgG (data not shown). Next, I evaluated the ability for the anti-PA$_{32}$ polyclonal serum to neutralize LT in vitro. Anti-PA$_{32}$ IgG demonstrated a strong ability to neutralize LT; however, a 10-fold reduction in protection as compared to anti-PA$_{83}$ IgG was observed (Fig. 2B). The EC$_{50}$ for the anti-PA$_{32}$ IgG was 2.5x10$^{-6}$ M. These data confirm that rPA-MIS is a successful immunogen capable of eliciting a neutralizing immune response in goats.
Figure 1. Goat anti-PA\textsubscript{83} serum IgG titers

Serially diluted goat anti-PA\textsubscript{83} IgG reacted with 10 nM rPA\textsubscript{83} in a microplate ELISA. Titer calculated as the reciprocal of the dilution producing 50\% maximum absorbance. Day 0 is 1\textsuperscript{st} immunization with rPA\textsubscript{83}-MIS, asterisks indicate timings of 2\textsuperscript{nd} (day 14), 3\textsuperscript{rd} (day 28) and 4\textsuperscript{th} (day 56) booster immunizations. Purified anti-PA\textsubscript{83} IgG was obtained from plasmapheresed goats on day 94 (time point designated by a square).
Figure 2. Goat anti-PA Ig neutralizes LT in vitro

J774A.1 cells were treated with 50ng (~2.9nM) LT and varying concentrations of goat anti-sera. Cell viability determined by an MTT-based assay. A. Anti-PA$_{83}$ Ig. Data shown are the average ± SEM of five assays each with four replicates. EC$_{50}$ is 2.57x10$^{-7}$M. B. Anti-PA$_{32}$ Ig. Data shown are the average ± SEM of two assays with four replicates. EC$_{50}$ is 2.5x10$^{-6}$M.
Having established that the goat anti-\( \text{PA}_{83} \) and anti-\( \text{PA}_{32} \) IgG was able to protect cells against LT challenge, I predicted that a subset of these antibodies would react with sequential linear B cell epitopes. To test this theory the neutralizing goat sera was reacted with linear peptides spanning \( \text{PA}_{83} \). The amino acid sequences of the peptides are listed in Tables 1-4. As shown in Figure 3, there is a subset of antibodies within the anti-\( \text{PA}_{83} \) polyclonal pool that recognized linear peptides. The average number of reactive peptides was approximately equal when comparing domains 1 and 2 to domains 3 and 4. Polyclonal serum reacted with approximately 30% of the domain 1 and 2 peptides with greater than 50% increase over the background. Similarly, anti-\( \text{PA}_{83} \) IgG reacted with 33% of the peptides in domains 3 and 4. Linear peptide reactivity of the anti-\( \text{PA}_{32} \) IgG was also tested, with nearly identical overlap between reactive peptides observed with anti-\( \text{PA}_{83} \) IgG (data not shown). These data confirm that the polyclonal serum contains antibodies that react with linear B cell epitopes, and although not yet demonstrated, it is expected that a subset of these epitopes will be recognized by neutralizing antibodies.
Table 1. PA$_{83}$ Domain 1 Linear Peptide Array Sequence Information

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Table 2. PA$_{83}$ Domain 2 Linear Peptide Array Sequence Information

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**Table 4.** PA<sub>83</sub> Domain 4 Linear Peptide Array Sequence Information

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</table>
Figure 3. Goat anti-PA\textsubscript{83} Ig reacts with PA\textsubscript{83} epitopes

Goat anti-PA\textsubscript{83} polyclonal (5 \(\mu\)g/ml) serum was reacted with 12-aa long peptides (4-aa overlap) spanning the length of PA\textsubscript{83}. A. Domain 1 peptides. B. Domain 2 peptides. C. Domain 3 peptides. D. Domain 4 peptides.
Discussion

I have demonstrated that rPA\textsubscript{83}-MIS elicits a robust immune response in goats after a series of 4 immunizations. Moreover, the resulting antibody pool was capable of neutralizing LT \textit{in vitro}, confirming my hypothesis that rPA coupled to the MIS adjuvant would provide us with a reliable antigen for investigating antibodies involved in the disruption of the anthrax intoxication pathway.

I have also established that a truncated form of PA, PA\textsubscript{32}, is capable of eliciting a neutralizing response that is comparable to that elicited by the PA\textsubscript{83} immunogen. These data confirm the contribution that domains 3 and 4 of PA play in the immune response to PA immunization, and supports further investigation into identifying domain 4 neutralizing antibodies.

The decrease in neutralization afforded by PA\textsubscript{32} as compared to PA\textsubscript{83} is not surprising since all antibodies directed against domains 1 and 2 are lost in this construct. Other groups have shown that neutralizing antibodies within the N-terminus of PA do exist (43,99) and would therefore contribute to the neutralizing capacity of the polyclonal serum. Based on the linear peptide data, it appears that an equal number of antibodies recognize linear peptides between the N- and C-terminal domains. Assuming that a subset of these linear-peptide recognizing antibodies are also neutralizing, it would be expected that a portion of the neutralizing response would be directed against domains 1 and 2, in support of the decrease in neutralization observed with anti-PA\textsubscript{32} IgG.

Recently, Abboud and Casadevall (6) identified four new linear B cell epitopes within domains 1 and 2 of PA that are elicited following vaccination with full-length PA\textsubscript{83}. The first two epitopes identified span residues 121-150 and 143-158 which correspond to
linear peptides # 19-24 in my peptide array. As shown in Figures 3A and 3B, peptides within this range show moderate reactivity with the anti-PA$_{83}$ serum. The third peptide spans residues 339-359, corresponding to peptides # 46-49. These peptides are non-reactive with the anti-PA$_{83}$ Ig which could be attributable to species differences. This concept is supported by the wide variation in responses observed by Abboud and Casadevall when determining the role that genetic background plays in the host immune response to PA. It is important to note that the authors only identified one species of mice that produced an antibody reactive with this peptide series. The fourth peptide identified spans residues 421-440 which corresponds to peptides # 56-59. These peptides were highly reactive with the polyclonal serum, indicating that this region of domain 2 may be important in neutralizing LT in vivo.

The fact that the PA antiserum afforded a substantial level of neutralization in vitro is in direct contrast to the data presented by Abboud and Casadevall (6). When a construct consisting of only domain 4 was used to vaccinate mice, very little response was observed. Based on the findings reported by Cirino et al. (28) and Leppla et al. (63), expression of domain 4 alone does not appear to be structurally stable and may be unable to bind to ATR. This would account for a domain 4-only construct being unable to elicit an appropriate immune response. Using PA$_{32}$ on the other hand, resolves these issues and clearly elicits a neutralizing immune response in goats.

**Conclusions**

I have confirmed that rPA$_{83}$-MIS generates a neutralizing polyclonal serum that contains a subset of antibodies that are capable of recognizing linear peptides. Specific reactivity within the 4 domains of PA is consistent with data presented by other groups
indicating the involvement of linear epitopes in the generation of neutralizing antibodies (43,99). To date, only limited characterization of the individual antibodies recognizing these linear epitopes has been done. Further characterization of these antigenic regions will advance our fundamental understanding of the mechanisms by which antibodies neutralize anthrax toxin and will have future application in the evaluation of candidate rPA vaccines.
Chapter 4. Establishment of a Lethal Toxin Animal Challenge Model

Acknowledgements

Virulent anthrax spore challenges were conducted by Dr. Johnny Peterson and Laurie Sower at the University of Texas Medical Branch in Galveston, TX. Funding for these studies was provided by the National Institutes of Allergy and Infectious Diseases contract with the University of Texas Medical Branch, Contract # N01-AI-30065 to Dr. Nick M. Cirino, Wadsworth Center.

Financial support for the LT challenge studies was received from the SUNY Albany Foundation through a Ford Foundation IFW Women in Science Fellowship.

All animal work was done with approval from the Wadsworth Center Institutional Animal Care and Use Committee.

Portions of this work have been published by C. D. Kelly et al. (55).

Introduction

The gold standard for evaluating the protective efficacy of polyclonal or monoclonal antibodies involved in anthrax immunity continues to be in vivo challenge studies. Several challenge models have been developed although the majority rely on the use of virulent anthrax spores for challenge (50,51,57,66,92). Use of virulent spores requires Animal Biosafety Level 3 (ABSL-3) containment facilities, highly trained animal care staff and select agent approvals. In order to circumvent the restrictions involved with live spore challenge, I developed an LT challenge model in mice that could be conducted at the Wadsworth Center under BSL-2 conditions. I hypothesized that direct injection of mice with LT would be a valid model for evaluating the ability for
polyclonal and monoclonal antibodies to protect against anthrax intoxication. Such a model would allow us to focus on antibodies that specifically target LT (i.e.: anti-PA antibodies) by negating the use of vegetative bacteria or spores. This is an advantageous model for elucidating the contribution of neutralizing antibodies specific for the intoxication pathway.

Nonetheless, there is some controversy regarding the appropriate animal model for establishing immunity to anthrax. Non-human primates are expected to give the most valuable indication of how an antibody will perform in humans following exposure; however, the costs of these experiments are prohibitive during the early research stages (51,101). Rabbits and guinea pigs are also frequently used although only limited facilities are equipped to house the number of animals needed for a statistically significant experiment (66,68). An alternate challenge model involves the use of mice exposed to B. anthracis spores via droplets introduced into the nares (92). Differences in response to challenge and vaccination have also been reported for rats (87) and mice (6).

One established challenge model, LT administration via the tail vein in rats, has several drawbacks (52,65,103,105). The most significant issue with this model is the rapidity with which the rats succumb to the toxin. Rats die within 2 hours post-challenge, leaving a very narrow time frame for the administration of antibody and for the continued evaluation of their ability to protect. I felt that this narrow time window for evaluating efficacy was too stringent and sought to establish a challenge model where mice would only succumb to the toxin within 24-48 hours post-challenge. More recently several researchers have also turned to using an LT challenge model in mice, although toxin dose and median-time-to-death were not consistent (84,113).
I therefore needed to establish a reliable, consistent and, relatively easy to perform LT mouse challenge model to investigate the role of antibodies in mediating immunity to the anthrax toxins.

**Materials and Methods**

**In Vivo LT Challenge Model.** Female Balb/c mice (Taconic Farms, NY; average weight 17.5 g) were injected with 100 \( \mu \text{g} \) LT (1:1 ratio PA\(_{83}\):LF) in 200 \( \mu \text{l} \) PBS via intraperitoneal (i.p.) injection. Five minutes following toxin injection mice were injected contra-laterally with varying concentrations of polyclonal or monoclonal antibodies in 200 \( \mu \text{l} \) PBS. Control mice received LT followed by PBS injections. Following challenge, mice were evaluated every 2 hr during the first 24 hr then every 8 hr for signs of distress and illness, including muscle weakness, difficulty breathing, and reduced responsiveness to stimuli. Mice were euthanized when they reached a score of 9 on a behavioral scale with 9 representing moribund (adapted from (115)). Death was used as an end-point in this study and all mice were monitored for a total of 15 days. All animals used in this study were housed under conventional, specific pathogen-free conditions and were treated in strict compliance with guidelines established by the Institutional Animal Care and Use Committee at the Wadsworth Center (IACUC).

**Virulent B. anthracis spore intranasal challenge.** The University Texas Medical Branch (Galvaston, TX) performed the spore intranasal challenge studies as follows. Female Swiss Webster mice (average weight 25.2 g) were infected with approximately \( 5 \times 10^4 \) B. anthracis Ames spores (5 LD\(_{50}\)) by 20 \( \mu \text{l} \) installations in each nares. Groups of 10 mice received PBS at 1 hour post-infection or anti-PA83 IgG at 24 hr post-infection.
(32 mg/kg) by i.p. injection. Mice were monitored twice daily for 14d for signs of illness and death.

**rPA₈₃-MIS mouse immunizations.** Female BALB/c mice (8 weeks of age, n = 4) (Taconic Laboratories, Germantown, NY) were immunized by i.p. injection with rPA₈₃-MIS (5 μg or 50 μg), or PBS (toxicity control) in 500 μl total volume on days 0, 10 and 20. Blood was collected from immunized mice via the tail vein on days 17 and 28. On day 35 mice were challenged via i.p. injection with 2LD₁₀₀ LT (200 μg in 200 μl of PBS). Mice were monitored twice daily for 15d for signs of illness and death. Anti-PA₈₃ serum IgG concentrations were established by serial dilution ELISAs with rPA₈₃-coated microtiter wells (10 nM). Anti-PA₈₃ IgG titers are expressed as the reciprocal of the antibody dilution which produced an absorbance value equal to 50% maximum absorbance.
Results

I predicted that challenging mice with LT would provide a reliable model for evaluating the efficacy of anti-PA antibodies in protecting mice against anthrax intoxication. Prior to evaluating LT in the mouse challenge model, all lots of LT were standardized *in vitro* in order to establish a consistent level of cell death. I expected that consistent *in vitro* neutralization would ensure that the specific LT blend would result in similar toxicity between animal challenges. A major hurdle in establishing this animal model was obtaining sufficient quantities of LT that were consistently toxic *in vitro*. I observed that various lots of LT prepared from batches of LF that contained additional bands on SDS-PAGE were more toxic than lots containing more highly purified preparations of LF. These observations are supported by a recent report describing the importance of N-terminal amino acids in establishing LF toxicity (120). It was observed that more homogenous preparations of LF (NR-724) that migrated as a single band on SDS-PAGE were less toxic than preparations that showed additional minor bands (LF NR-142). This report confirmed my observations that non-homogenous preparations of LF are needed to ensure LT toxicity. I was able to obtain sufficient quantities of LF NR-142 (BEI Resources) to conduct all mouse challenge experiments which provided us with consistently toxic LT.

To test the relevance of an LT challenge mouse model I performed dose titrations of LT administered by i.p. challenge. Mice were left untreated (i.e.: no antibodies administrated) in order to determine the appropriate dose to cause death within 24-48 hours. Dose titrations were done for each new batch of LT in order to maintain consistency between experiments (data not shown). The various lots of LT evaluated
were all prepared by mixing PA and LF at a 1:1 ratio (v/v) and their TCEC$_{50}$ was established by repeated testing \textit{in vitro}. A high degree of variability was seen between lots of LT using the \textit{in vitro} TNA. Only LT lots that were able to consistently establish cell death \textit{in vitro} were chosen for evaluation \textit{in vivo}; this was especially challenging as only limited amounts of each LT lot were available for evaluation \textit{in vitro} and \textit{in vivo}. Two-fold dilutions, ranging from 50 $\mu$g to 200 $\mu$g of LT, were administered to 5 mice and mice were observed for 72 hours. The lethal dose for each specific lot of LT was determined to be twice the concentration of LT that resulted in 100% mouse death. The 2LD$_{100}$ concentration was chosen to ensure that the amount of LT administered would be a sufficient challenge to the antibodies under investigation without being so concentrated that protection would not be observable. While a high degree of variability in LT \textit{in vitro} toxicity was observed, very little variability was seen \textit{in vivo} once \textit{in vitro}-established lots were tested. Generally, the 2LD$_{100}$ of the various lots of LT ranged from 150-200 $\mu$g per mouse. This method established a consistent administration route and dosing concentration that resulted in mouse death within 24-48 hours post-challenge. The 24-48 hr extended time window allowed me to evaluate the ability of antibodies to disrupt the anthrax intoxication pathway and protect mice against LT challenge.

Adequate scoring of mice that are passively administered anti-PA antibodies is required by IACUC in order to establish when euthanasia is required to minimize the pain and suffering of the animals. I developed a multi-parameter scoring model (Table 5) by expanding on previously described \textit{in vivo} anthrax intoxication indicators (115). This scoring model used three sets of parameters (appearance, natural behavior, and provoked behavior) to establish the amount of pain and distress animals are experiencing in
response to LT challenge. Each parameter is scored on a 0-3 scale with a total score for each animal reaching a maximum of 9. Animals that score between 0-2 are considered to be normal in appearance and behavior and do not require any intervention. A moderate score of 3-8 indicates that mice are experiencing some changes in behavior and may be experiencing some pain and discomfort associated with the LT challenge. Once animals scored within this moderate range, more frequent observation (every 2-3 hrs) was required in order to prevent extended suffering of the mice. Animals that reached the highest score of 9 were euthanized immediately. Throughout my evaluation of mice challenged with LT this scoring model proved to be reliable and eliminated confusion regarding the outcome of the studies. Control mice that received LT only (no antibody treatments) maintained low scores for the first 12 hours post-challenge. Once they reached a score of 7 they progressed rapidly to a moribund state, usually within 2-4 hrs. Mice which were passively administered anti-PA antibodies demonstrated delayed onset of pain or distress and the majority recovered even if scores of 5-7 were reached. The majority of mice that reached a score of 7 showed the same rapid decline observed with untreated control mice and were generally euthanized within 4-8 hours. However, a minimal number of animals were able to recover from moderate distress scores and, as such, the scoring model was not revised to decrease the total score required for euthanasia intervention. Once mice receiving antibody therapy recovered from LT challenge and returned to normal scores (0-2) they remained healthy for greater than 15 days. Subsequent studies were ended 11 days post-LT challenge. This scoring model established a reliable observation method for determining when mice should be euthanized following LT challenge.
Table 5. Animal Scoring Parameters for LT Challenge Model

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Attribute</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Appearance</strong></td>
<td>Normal: Coat smooth, Eyes/Nose Clear</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Reduced Grooming</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Dull/Rough Coat, Ocular/Nasal Discharge</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Absence of grooming, piloerection, hunched up</td>
<td>3</td>
</tr>
<tr>
<td><strong>Natural Behavior</strong></td>
<td>Normal</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Minor changes, less peer interaction</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Little peer interaction, less mobile and alert, isolated</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>No peer interaction, vocalization, self-mutilation, restless or still</td>
<td>3</td>
</tr>
<tr>
<td><strong>Provoked Behavior</strong></td>
<td>Normal</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Subdued but normal when stimulated</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Subdued even when stimulated</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Unresponsive when stimulated, weak, precomatose</td>
<td>3</td>
</tr>
<tr>
<td><strong>Actions Required Based on Total Score</strong></td>
<td><strong>Normal:</strong> Monitor every 8-12 hours</td>
<td>0-2</td>
</tr>
<tr>
<td></td>
<td><strong>Moderate Distress:</strong> Monitor Frequently (2-3hrs)</td>
<td>3-8</td>
</tr>
<tr>
<td></td>
<td><strong>Moribund:</strong> Euthanize Immediately</td>
<td>9</td>
</tr>
</tbody>
</table>
In the previous chapter I established that the goat anti-PA$_{83}$ and anti-PA$_{32}$ Ig was able to neutralize LT \textit{in vitro}. Expecting that these polyclonal sera would also provide protection \textit{in vivo} I next tested the ability of the goat anti-PA$_{83}$ and anti-PA$_{32}$ Ig to passively protect mice against LT challenge (Fig. 4). Mice were first injected with 2LD$_{100}$ (200 μg LT) of recombinant LT on the left side of the abdomen. After 5 min, mice were injected with approximately 8 mg/kg anti-PA$_{83}$ IgG or anti-PA$_{32}$ IgG on the right side of the abdomen. Control mice received 200 μl of PBS instead of IgG and succumbed to LT by day 2. 80% of mice treated with anti-PA$_{83}$ IgG survived until day 11 post-LT challenge. Anti-PA$_{32}$ IgG treated mice showed a similar survival rate with 60% of mice surviving until day 11. This data confirmed my expectation that \textit{in vitro} neutralizing anti-PA serum would also protect mice against LT challenge. The consistency between the protection efficacies observed for both anti-PA$_{83}$ and anti-PA$_{32}$ Ig supports my hypothesis that domain 3 and 4 antibodies are critical for protection against anthrax toxins.
Figure 4. Anti-PA$_{83}$ and anti-PA$_{32}$ Ig protect mice against LT challenge

Percent survival of female Balb/c mice treated with 100 μg LT by i.p. injection followed 5 minutes later with 8 mg/kg anti-PA$_{83}$ (○) or anti-PA$_{32}$ (□) IgG in 200 μl (5 per group). Control mice (PBS, 3 in group, ■) received 100 μg LT followed by 200 μl Saline. All mice were observed twice daily for signs of illness or distress and all surviving mice were euthanized at day 11 post-challenge.
In addition to establishing that the LT challenge model was appropriate for passive therapeutic studies I also wanted to test the effectiveness of this model following immunization with rPA$_{83}$-MIS. Mice were immunized with 5 μg or 50 μg rPA$_{83}$-MIS on days 0, 10 and 20 via i.p. injection. Serum was collected via tail vein bleeding on days 17 and 28 for anti-PA$_{83}$ Ig serum calculations (Table 6). Mice showed anti-PA$_{83}$ serum titers ranging from 1:200 to 1:25,600 by the second immunization. Serum titers significantly increased following the third immunization (average 1:60,000) indicating that mice may have mounted a sufficient immune response to protect them from LT challenge. On day 35 (15 days following the last boost) mice were challenged with 200 μg LT via i.p. injection. Control mice succumbed to LT challenge within 24 hours as expected. Mice immunized with either amount of rPA$_{83}$-MIS were completely protected against LT challenge for 15 days following challenge (Table 6). One mouse immunized with 5 mg rPA83-MIS had minimal titers even after three immunizations (1:3200) yet survived LT challenge. This observation indicated that this minimal amount of anti-PA Ig required to confer protection may be quite low. These data suggested that the LT challenge model was dually useful for evaluating passive antibody therapy as well as immunization efficacies.
Table 6. Anti-PA$_{83}$ serum titers & mouse survival following LT challenge

<table>
<thead>
<tr>
<th>Group</th>
<th>2$^{nd}$ Immunization Titers$^a$</th>
<th>3$^{rd}$ Immunization Titers$^a$</th>
<th>Days Survived</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 μg rPA$_{83}$-MIS</td>
<td>1:200</td>
<td>1:3200</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>1:1600</td>
<td>1:50,000</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>1:3200</td>
<td>1:13,000</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>ND$^b$</td>
<td>1:26,000</td>
<td>15</td>
</tr>
<tr>
<td>50 μg rPA$_{83}$-MIS</td>
<td>1:25,600</td>
<td>1:100,000</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>1:25,600</td>
<td>1:100,000</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>1:12,800</td>
<td>1:200,000</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>ND$^b$</td>
<td>1:25,000</td>
<td>15</td>
</tr>
<tr>
<td>Unimmunized</td>
<td>Not detected</td>
<td>Not detected</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Not detected</td>
<td>Not detected</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Not detected</td>
<td>Not detected</td>
<td>1</td>
</tr>
</tbody>
</table>

$^a$As determined by ELISA

$^b$Not done
To further validate the LT challenge model, I next tested the anti-PA$_{83}$ Ig in an inhalation spore challenge model. Spore challenge of animals is currently the gold-standard for establishing the efficacy of passive antibody administration or immunization in protecting against anthrax infections. These studies are challenging and costly to perform due to the ABSL-3 laboratory requirement. I used a modified inhalation model where female Swiss Webster mice were challenged with virulent B. anthracis spores via an intranasal infection route (University of Texas Medical Branch, Galvaston, TX). Mice received 5 LD$_{50}$ B. anthracis Ames spores in 20 μl instillations into each nares. Control mice received PBS at 1h post-challenge. Twenty-four hours post-challenge, test groups received 32 mg/kg goat anti-PA$_{83}$ Ig by i.p. injection. At 4d post-infection, only 20% of control mice survived, while 70% of mice treated with anti-PA$_{83}$ Ig were still alive (Fig. 4). By day 6, another 10% of the mice in each group had succumbed to disease and no further mortality was observed through the remaining 14d study. The inhalation spore challenge model results were consistent with the levels of protection seen in the i.p. LT mouse challenge model, confirming that this experimental protocol can be confidently used as a preliminary in vivo challenge model.
Figure 5. Anti-PA$_{83}$ Ig protects mice against intranasal anthrax spore challenge

Percent survival of female Swiss Webster mice, 10 per group, infected with 5 LD$_{50}$ B. anthracis Ames spores by intranasal inoculation. Control mice were treated with PBS (■) 1h post spore challenge via i.p. injection. Mice treated with 32 mg/kg anti-PA$_{83}$ Ig (○) 24 hr post spore challenge via i.p. injection. P $\leq$ 0.05 by the logrank test on day 14.
Discussion

In this study, I developed a reliable LT challenge model that serves as a primary means to evaluate the efficacy of anti-PA$_{83}$ antibodies in protecting against anthrax intoxication. This experimental design is more relevant for my research than traditional spore challenge models, since I was specifically interested in antibodies that bind to epitopes involved in toxin pathogenesis. The LT challenge model consistently provided a 24-48 hr median-time-to-death for untreated mice and required only moderate amounts of anti-PA Ig in order to consistently see protection.

Other groups have also explored the use of LT as a relevant animal challenge model in anthrax pathogenesis studies. These studies are variable in the amounts of toxin required for mouse death and the median-time-to-death for each experimental protocol. A recent study by Staats et al. demonstrated that one MAb (9A11) protects 100% of mice challenged with LT via i.p. injection (113). In this study, mice treated with an irrelevant antibody control succumb to LT within 2 days; however, this level of toxicity required 2-fold more LT than my assay. The difference in amount of LT required may be due to the pre-administration of antibody 24 h prior to injection of LT in the Staats study. A minimum of 750 $\mu$g of MAb 9A11 was needed to achieve complete protection. In my studies complete protection of mice was not achieved, although I administered significantly less total antibody per mouse (8 mg/kg; 140 $\mu$g IgG total). The lowest amount of MAb 9A11 administered in the Staats study was 375 $\mu$g which provided 80% protection (113), which is the same amount of protection observed with 140 $\mu$g of anti-PA$_{83}$ IgG. It is difficult to compare these studies since a monoclonal antibody was used in the Staats study. The majority of MAbs evaluated in protection against anthrax
challenge do not demonstrate significant protection post-exposure and appear to require a blend of several MAbs in order to reduce the mortality associated with anthrax infections (20,97,99). Another recent study evaluating the protective efficacy of anti-LF antibodies also used an LT challenge model. By their administration route not all control mice succumb to the toxin while I have found that administration of LT by i.p. has consistently resulted in 100% fatality within 48 hours.

The rPA$_{83}$- and rPA$_{32}$-MIS immunogens elicit a robust immune response in goats and the resulting polyclonal serum was shown to neutralize LT \textit{in vitro}. Here I demonstrated that the same goat polyclonal serum was capable of passively protecting mice against i.p. LT challenge. 8 mg/kg of IgG conferred 60-80\% protection against 2LD$_{100}$ LT challenge. In addition, three immunizations with as little as 5 $\mu$g of rPA$_{83}$-MIS fully protected mice against LT challenge. The protection data obtained using the LT challenge model was confirmed using the gold standard spore challenge model. The similar protection levels observed between the two \textit{in vivo} passive transfer challenge assays confirmed the applicability of the LT challenge model for establishing the protective efficacy of antibodies directed against the anthrax toxins.
Conclusions

The data presented confirms the utility of the LT mouse challenge model and supports its use for the evaluation of other neutralizing antibodies. In addition, these data demonstrate that the rPA_{83}-MIS induced antibody response is protective \textit{in vivo}, further confirming the utility of this immunogen in investigating the antibody response to anthrax toxins. The ability for anti-PA_{32} IgG to protect mice with similar efficacy as anti-PA_{83} IgG in the LT challenge model supports my hypothesis that the C-terminus of PA is critical for eliciting a protective immune response to anthrax intoxication.
Chapter 5: Identification of MAbs specific for Domains 3 and 4 of PA

Acknowledgements

Ascites were produced by Dr. Stuart Balaban in the Wadsworth Center Immunology Core. IgG purification of the ascites was performed by the NBC Protein Core Facility.

Introduction

I predicted that several epitopes on domain 4 of PA contribute to the immune response required for protection against anthrax infection. One antibody, 14B7, has been well-characterized as a conformation-dependent MAb that disrupts the association of PA with ATR by binding in domain 4 (69,111). It is probable that additional epitopes on domain 4 exist that are recognized by antibodies that will disrupt PA:ATR interactions and be neutralizing in vitro and in vivo. In order to test this hypothesis I produced B cell hybridomas from mice immunized with rPA83-MIS. Previous results indicated that animals immunized with rPA83-MIS produced a protective immune response and that a subset of these antibodies were capable of recognizing short linear peptides. Using this linear peptide array, I screened the mouse-derived hybridomas using a multi-tiered approach, expecting that several monoclonal antibodies will react with domain 4 linear peptides and provide protection against LT in vitro and in vivo.

Hybridomas were screened by testing their reactivity with linear peptides spanning PA83. The identification of MAbs that react with linear peptides allows for fine epitope mapping of the C-terminus of PA by pinpointing specific residues that are involved in PA associating with the cellular receptors. Several groups have used linear peptide screening to broadly elucidate the regions of a protein that are involved in
immunity to a particular toxin. Abboud et al., identified four new linear B cell epitopes within domains 1-3 of PA (6) by screening various mouse polyclonal sera for reactivity against a peptide array. Similar investigations have been completed for botulism toxins by screening the serum from immunized horses for reactivity against peptides spanning the heavy chain of BoNT A (11). While polyclonal serum can provide a large-picture view of the involvement of antibodies in neutralizing toxins, they can not provide an individual map of the involved epitopes. In order to obtain a refined epitope map of PA, the identification of MAbs that recognize linear epitopes is required.

Recently, two groups investigating anthrax immunity identified MAbs that were both neutralizing and reactive with linear peptides. Gubbins et al. identified three MAbs that reacted with the core motif ASFFD, a region within domain 2 of PA (43). This region falls within the 2β2-2β3 loop which has remained unresolved by crystallization presumably due to its flexibility (102). Chemotrypsin cleavage experiments have supported the theory that this region of the loop is solvent-exposed (85,109) although it had not been implicated as an antigenic determinant until this report. These findings will facilitate research into identifying the exact mechanism by which MAbs can disrupt the early steps in the anthrax toxin pathway. A second group evaluated sequential B cell epitopes for reactivity with previously characterized anti-LF antibodies (84). They found that three anti-LF antibodies, 10G3 (70), 9A11 (113) and LF8 (127), were neutralizing in vitro and bound linear epitopes that overlapped with the recognition pattern observed for polyclonal anti-LF antiserum (84). The results of this fine epitope mapping identified key residues involved in substrate recognition, zinc metalloproteinase, and PA-binding domains of LF. This information is important for refining our understanding of LF:PA
interactions, and as additional disruption targets for the development of therapeutics or vaccines against anthrax. These data support the rationale for screening B cell hybridomas for linear peptide reactivity in order to identify MAbs that are domain 4 specific and functionally relevant in the immune response to anthrax infection. Functional analysis of MAbs recognizing linear epitopes on domain 4 of PA will further our understanding of PA:ATR interactions, and will identify key residues that could be incorporated into future peptide-based vaccines.

Materials and Methods

MAb Hybridoma Production. Female BALB/c mice (Taconic Laboratories, Hudson, NY) approximately 8 weeks of age were immunized with PA$_{83}$-MIS ($50 \mu$g) administered by i.p. injection on days 0, 14 and 24, and boosted with a single injection of PA$_{83}$ (100 $\mu$g) without adjuvant 3d prior to B-cell fusion. Mice were sacrificed 3d after the final boost, and total splenocytes were harvested for monoclonal antibody production using the ClonaCell-HY Hybridoma Cloning Kit (StemCell Technologies, Vancouver, British Columbia, Canada).

Ascites Production. Selected hybridomas were produced in ascites by the Wadsworth Center Immunology Core Laboratory and MAbs were purified by standard Protein A purification by the NBC Protein Expression Core (91).

Hybridoma Screening Approach. A multi-tiered screening approach was developed to determine the reactivity of the hybridomas (Figure 8). Initial screening was performed to identify MAbs that react with the native PA$_{83}$ protein. Hybridomas were also screened for their ability to bind to PA$_{32}$, a truncated form of PA$_{83}$ containing only domains 3 and 4, that has been shown to be properly folded and capable of binding to anthrax toxin.
receptor (28). Antibodies that reacted with PA$_{83}$ but not PA$_{32}$ were considered to be specific for domains 1 and 2. Antibodies that reacted with PA$_{83}$ and PA$_{32}$ were considered to be specific for domains 3 and 4. Screening was done by standard ELISA as described in previous chapters.

**LT Toxin Neutralization Assay.** Evaluation of the hybridomas in the LT TNA completed the hybridoma screen. LT TNA was performed as described in previous chapters using 1:2 dilutions of the hybridomas diluted in sterile PBS.

**Linear Peptide Reactivity.** MAbs that recognized the conformational PA$_{83}$ and PA$_{32}$ proteins were reacted with domain 3 and 4 peptides in the PA$_{83}$ linear peptide array as described in earlier chapters.

**Results**

In order to test the hypothesis that several domain 4 epitopes are involved in the protective immune response following anthrax infection, I produced B cell hybridomas from mice immunized with rPA$_{83}$-MIS. Using a tiered screening approach I determined the specificities of the 384 mouse derived hybridomas (Fig. 6). The first tier of the screening approach aimed to establish the reactivity of the hybridomas with PA$_{83}$ and PA$_{32}$. The second tier tested the ability for the PA$_{83}$/PA$_{32}$ reactive antibodies to neutralize LT *in vitro*, while the third tier specifically investigated the reactivity of PA$_{32}$ reactive antibodies with linear peptides spanning domains 3 and 4 of PA. This tiered screening approach was designed to specifically address my hypothesis by identifying hybridoma clones that are specific for domain 4 of PA and are neutralizing *in vitro*.

Hybridomas were first evaluated for their ability to recognize the native proteins, PA$_{83}$ and PA$_{32}$. 37 hybridomas were identified that recognized PA$_{83}$ and were progressed
on for further screening. These 37 hybridomas were next screened for reactivity with PA$_{32}$, a truncated form of PA$_{83}$ that includes only domains 3 and 4 in an appropriately folded protein (28). Hybridomas that recognized PA$_{32}$ were considered to be domain 3 or 4 specific, while hybridomas that did not recognize PA$_{32}$, but did recognize PA$_{83}$, were considered to be specific for domains 1 or 2 or PA. PA$_{32}$ screening identified 6 of the 37 clones to be reactive with the C-terminal residues of PA$_{83}$. The remaining 31 clones reacted with PA$_{83}$ only indicating that they are specific for the N-terminal residues of PA$_{83}$ (Fig. 6).

I was specifically interested in indentifying MAbs that are capable of \textit{in vitro} and \textit{in vivo} protection against anthrax intoxication. I next tested the hybridomas for their ability to neutralize LT in the cell-based toxin neutralization assay (Table 7). Of the 31 domain 1-2 specific clones, 8 were able to completely neutralize LT, 20 were non-neutralizing and 3 appear to actually enhance the toxicity of LT. 6 clones reacted with PA$_{32}$ indicating they were specific for domain 3 or 4 on PA. Of these clones, four (1-F1, 2-B12, 2-C3 and 3-H11) were highly effective at neutralizing LT \textit{in vitro}. The remaining 2 clones (2-A7 and 2-H6) were non-neutralizing (Table 7).
Hybridomas were produced from the spleens of mice vaccinated with rPA83. Each hybridoma was screened for reactivity against PA83, and PA32 prior to being evaluated for their ability to neutralize LT in vitro and reactivity against linear peptides.
Table 7. Hybridoma Domain Reactivity and LT Neutralization

<table>
<thead>
<tr>
<th>Hybridoma&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Domains&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Neutralization&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Hybridoma&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Domains&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Neutralization&lt;sup&gt;c&lt;/sup&gt;</th>
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<tr>
<td>1-A2</td>
<td>1&amp;2</td>
<td>–</td>
<td>2-H2</td>
<td>1&amp;2</td>
<td>+/-</td>
</tr>
<tr>
<td>1-A6</td>
<td>1&amp;2</td>
<td>–</td>
<td>2-H6</td>
<td>3&amp;4</td>
<td>–</td>
</tr>
<tr>
<td>1-A11</td>
<td>1&amp;2</td>
<td>+++</td>
<td>2-H8</td>
<td>1&amp;2</td>
<td>+/-</td>
</tr>
<tr>
<td>1-B5</td>
<td>1&amp;2</td>
<td>+++</td>
<td>3-B2</td>
<td>1&amp;2</td>
<td>–</td>
</tr>
<tr>
<td>1-C4</td>
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<td>+++</td>
<td>3-D1</td>
<td>1&amp;2</td>
<td>–</td>
</tr>
<tr>
<td>1-F1</td>
<td>3&amp;4</td>
<td>+++</td>
<td>3-D4</td>
<td>1&amp;2</td>
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<td>1&amp;2</td>
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<td>3-H11</td>
<td>3&amp;4</td>
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</tr>
<tr>
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<td>4-A9</td>
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<td>1&amp;2</td>
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<tr>
<td>2-B11</td>
<td>1&amp;2</td>
<td>+++</td>
<td>4-C12</td>
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</tr>
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<td>3&amp;4</td>
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<td>4-D1</td>
<td>1&amp;2</td>
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</tr>
<tr>
<td>2-C3</td>
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<td>+++</td>
<td>4-F12</td>
<td>1&amp;2</td>
<td>–</td>
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<td>+/-</td>
<td>4-H9</td>
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<tr>
<td>2-G1</td>
<td>1&amp;2</td>
<td>+/-</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

<sup><small>a</small></sup>Represents plate and well number from hybridoma screens

<sup><small>b</small></sup>Based on reactivity with PA<sub>32</sub> in ELISA; 1&2, non-reactive; 3&4, reactive

<sup><small>c</small></sup>Neutralization of LT <i>in vitro</i> using standard TNA

+++ , highly neutralizing (>60%)
+/- , minimally neutralizing (<25%)
– , non-neutralizing (0 to 50% reduction in cell viability)
– – – , LT-enhancing (>100% reduction in cell viability)
Having identified several clones that were PA$_{32}$ reactive, and capable of neutralizing LT \textit{in vitro}, I next tested their ability to recognize linear peptides on domain 3 and 4 of PA. Linear peptide screening of the 6 domain 3-4 reactive hybridomas identified 3 clones, 1-F1, 2-B12 and 3-H11, which were specifically reactive with domain 4 residues. An additional 2 clones, 2-A7 and 2-H6, were specifically reactive with residues in domain 3 of PA. The final PA32-reactive clone, 2-C3, did not react with the peptide array indicating it was a conformation-dependent antibody. We retested the selected hybridomas for neutralization \textit{in vitro} and confirmed that the domain 4 clones (1-F1, 2-B12 and 3-H11) and the conformation-dependent clone (2-C3) were highly neutralizing, while the domain 3 clones (2-A7 and 2-H6) were unable to protect against LT \textit{in vitro} (data not shown).

In order to effectively evaluate the ability of the hybridoma clones to disrupt PA:ATR interactions and protect mice against LT challenge concentrated amounts of purified antibody was needed. To best address the hypothesis that additional epitopes on domain 4 of PA contribute to the protective immune response, ascites were produced only for clones that recognized linear peptides (i.e.: the conformation-dependent antibody 2-C3 was not evaluated further). Clones 1-F1 and 2-B12 were produced in ascites because they demonstrated high neutralization capacities and recognized linear epitopes on domain 4 of PA. The remaining domain 4 reactive clone, 3-H11, was determined to be a sister clone of 2-B12 and was not chosen for further investigation.

I also predicted that antibodies directed against domain 3 epitopes would not contribute to the neutralizing immune response, in line with the lack of observable \textit{in vitro} LT neutralization for hybridoma clones 2-A7 and 2-H6. To test this prediction I
chose to test one domain 3 reactive clone, 2-A7, in parallel with the domain 4 reactive clones. The selected hybridomas were produced as ascites by the Wadsworth Center Immunology Core Laboratory.

MAbs 2-A7, 1-F1 and 2-B12 were retested for reactivity with linear peptides spanning the C-terminal residues of PA83. Specific reactivity, observed during the hybridoma screen, was reconfirmed with the MAbs and is shown in Figure 7.

MAb 2-A7 recognized a peptide spanning residues 532-543 (peptide 71) within domain 3 of PA83 with greater than 400% increase over background. A second peptide, 72, also exhibited a moderate amount of reactivity (<100%). Since peptides 71 and 72 overlap by residues LQYQ, this data suggested that 2-A7 minimally recognizes amino acids 540-543 on domain 3 of PA.

1-F1 recognized a peptide spanning residues 692-703 (peptide 91) with greater than 500% increase over background. This peptide is within a region of domain 4 known to be an important target of neutralizing antibodies (6,69,111). Several other peptides also showed moderate reactivity with 1-F1, including peptides 79 (<50% increase, residues 596-607), 82 and 83 (~50-80% increase, residues 620-639, common residues 628-631), and 95 (~100% increase, residues 724-735). Since the reactivity of these additional peptides did not exceed 100% increase over background, and there was no similarity in peptides sequences with peptide 91, I concluded that residues 692-703 represented the main reactive epitope for MAb 1-F1.

2-B12 recognized a single peptide, spanning residues 716-727 (peptide 94), with greater than 1000% increase over background. These residues correspond to a region of domain 4 not previously implicated as being a target of neutralizing antibodies (118).
The specificity with which this MAb reacted with a single peptide was surprising considering MAbs 2-A7 and 1-F1 had overlap with neighboring peptides, and low-reactivity with various non-related peptides was seen with 1-F1. Peptide 95, which overlapped with peptide 94 by residues ILIF, and was slightly reactive with 1-F1, yet did not demonstrate any reactivity with 2-B12. The fact that 2-B12 reacted very strongly with residues 716-727 led me to conclude that this MAb exhibits a high degree of specificity for this epitope.

I identified three antibodies that recognize novel, linear epitopes on the C-terminus of PA. The capacity for two of these MAbs (1-F1 and 2-B12) to neutralize LT in vitro is in direct support of my hypothesis that a series of PA domain 4 epitopes may be involved in a protective immune response to the anthrax toxins.
Figure 7. MAbs 2-A7, 1-F1, and 2-B12 react with N-terminal PA epitopes

MAbs 2-A7 (panel A), 1-F1 (panel B), and 2-B12 (panel C) were applied to 96 well microtiter plates coated with overlapping 12-mer peptide library spanning domains 3 (panel A) and 4 (panels B and C) of PA83. The solid black bars represent the percent increase over background. Amino acid sequences are listed in Tables 1-4.
Discussion

The identification of two MAbs (1-F1 and 2-B12) specific for linear epitopes on domain 4 of PA, that were also highly neutralizing in vitro, confirmed my hypothesis that other epitopes, in addition to 14B7, exist on the C-terminal portion of PA that contribute to the overall neutralizing immune response to anthrax infection.

Preliminary characterization of MAbs 1-F1 and 2-B12 showed that they bind to different regions of PA. The residues recognized by 1-F1 (692-703) fall within the established binding region of MAb 14B7 and have been implicated in the disruption of PA’s association with ATR (6,111). I expected that 1-F1 was able to neutralize LT in vitro due to its ability to prevent PA binding to the cell surface receptors. Unlike 1-F1, MAb 2-B12 recognized a series of residues (716-727) that are not involved in neutralizing antibody binding (118). The epitope recognized by 2-B12 overlaps slightly with 14B7’s binding region, indicating that this antibody may also disrupt PA:ATR interactions allowing for the high degree of LT neutralization observed in vitro in my studies.

This is the first report of neutralizing antibodies that recognize linear epitopes on domain 4 of PA. The next chapter will provide an in-depth, functional characterization of these MAbs in order to elucidate their role in disrupting toxin pathogenesis.

I also identified one antibody, 2-C3, which recognized PA32 but did not react with any of the domain 3 or 4 linear peptides, indicating that this antibody recognized a conformation-dependent epitope on the N-terminus of PA. This antibody was highly neutralizing in vitro indicating that it may be highly similar, if not identical, to 14B7.
In line with the prediction that domain 3 of PA would not contribute to the neutralizing response, I demonstrated that MAbs 2-A7 and 2-H6 reacted with domain 3 linear epitopes but did not protect cells against LT. Most reports indicate that domain 3 does not play a significant role in eliciting neutralizing antibodies against PA supporting my findings (79,124).

In the tiered screening of the mouse derived hybridomas, 31 clones that bound to PA\textsubscript{83} but not PA\textsubscript{32} were identified, indicating that these clones specifically recognized the N-terminus of PA, domains 1 and 2. These antibodies demonstrated a varying ability to neutralize LT \textit{in vitro}. The majority of these clones are unable to neutralize LT (~65%); although a subset of clones (~26%) showed a strong capacity to protect cells against intoxication. I hypothesized that the C-terminus of PA is involved in anthrax neutralization; however, I did not ignore the contribution that the N-terminus residues make in a comprehensive immune response. In fact, my previous anti-PA\textsubscript{83} and anti-PA\textsubscript{32} goat Ig \textit{in vivo} protection data established that immunizing with the full-length protein elicited a more neutralizing immune response than immunization with the truncated PA protein. The findings that a quarter of the domain 1 or 2 reactive antibodies were capable of LT neutralization supports my previous conclusion that the decrease in protection observed with anti-PA\textsubscript{32} Ig is due to the exclusion of important N-terminus PA epitopes.

I also found that 3 antibodies recognizing the N-terminus of PA enhanced LT toxicity \textit{in vitro}, a finding that is supported by a report by Mohamed \textit{et al.} (80). The identification of the antibodies that enhance toxicity is important as these epitopes should be excluded from future vaccine designs in order to avoid the production of detrimental
antibodies. In the Mohamed study, they describe the discovery of 29 antibodies that enhance LT activity in vitro (80). They demonstrate that LT-enhancement was Fc dependent and that the antibodies caused an increase in PA recruitment to the cell surface. They also propose that differences in host genetics, adjuvant type, and administration regimen may play a role in the development of LT-enhancing antibodies (80). It is interesting to note that I identified significantly fewer LT-enhancing antibodies (3 vs. 29) than Mohamed et al.; this difference could be attributed to the adjuvant differences between our studies. Both studies utilized female Balb/c mice vaccinated with PA; however, I used the novel MIS adjuvant while Mohamed et al. use CPG as an adjuvant.

While it is not clear what role LT-enhancing antibodies have in a physiological response to anthrax infection, there is substantial evidence that antibody-mediated enhancement (ADE) plays an important role in the immune response to viruses (114). ADE has been documented for dengue virus (45) and yellow fever virus (13), although this mechanism has not be implicated in the immune response to protein toxins. Another report describes an antibody specific to the ricin toxin A chain that blocked enzymatic activity yet enhanced in vivo cytotoxicity (72), similar to my results and those reported in the Mohamed study (80). Regardless of the mechanism by which these antibodies enhance toxicity, the production of LT-enhancing antibodies following PA$_{83}$ vaccination could have a detrimental effect on the overall protective capacity of the immune response. The presence of LT-enhancing antibodies in the polyclonal anti-PA$_{83}$ response highlights the importance of further characterizing specific epitopes in order to better
design next-generation PA$_{83}$ vaccines that would avoid the production of detrimental antibodies.

**Conclusions**

I identified 2 MAbs, 1-F1 and 2-B12, which are specific for linear epitopes on domain 4 of PA that are capable of neutralizing LT \textit{in vitro}. This is the first report clearly identifying antibodies that recognize epitopes distinct from the one recognized by 14B7. This data confirmed my hypothesis that several domain 4 epitopes play a critical role in eliciting a protective immune response following anthrax infection.

Next-generation anthrax vaccines will most certainly rely on rPA (23,36,40,40,55); nevertheless, much remains to be done in terms of identifying the specific regions of PA that elicit neutralizing antibodies in order to fully understand how these antibodies interfere with (or enhance) toxin attachment, oligomerization, and translocation (43,99,126). For example, in the anti-PA$_{83}$ hybridoma screens, 31 clones that reacted with domains 1 or 2 were identified. Preliminary characterization has indicated that roughly 74% of these clones either failed to neutralize LT or actually enhanced LT-induced cell death. In my opinion, a complete and systematic identification of neutralizing, non-neutralizing, and LT-enhancing epitopes is necessary if protective immunity to anthrax toxins is to be fully understood, and if the efficacy of candidate human rPA vaccines is to be properly evaluated. My identification of neutralizing antibodies that bind to linear epitopes within domain 4 of PA was exciting as very little work has been done to characterize the specific antigenic determinants within this region.
Chapter 6. Functional characterization of MAbs 1-F1, 2-B12, and 2-A7

Acknowledgements

SPR analysis was done by Jane Jolly in the Wadsworth Center Immunology Core. MAb 14B7 was kindly provided by Dr. Stephen Leppla, National Institutes of Health. Soluble CMG-2 was kindly provided by Dr. Robert Liddington (Burnham Medical College).

Introduction

The initial intent of my studies was to investigate the contribution of domain 4 epitopes in the anti-PA immune response. A second goal was to perform in-depth characterization of these epitopes in order to further our understanding of the key residues involved in protection against anthrax intoxication. I confirmed that several epitopes existed on domain 4 of PA and that they are recognized by neutralizing antibodies 1-F1 and 2-B12. The fact that these antibodies also recognized linear epitopes provided me with the tools needed to specifically address my secondary goal. I hypothesized that MAbs 1-F1 and 2-B12 are neutralizing because they disrupt initial steps in the anthrax toxin pathway, specifically, PA binding to the cell receptors. I tested this hypothesis by designing a solid-phase binding assay that measures the amount of PA associating with the cellular receptor, CMG-2.

I also chose to test the capacity for MAb 2-A7 to disrupt PA:CMG-2 association. I did not expect that 2-A7 will be able to disrupt this critical association based partly on my observation that this MAb does not neutralize LT in vitro and partly on the fact that domain 4 is the key region of PA involved in PA:ATR binding (102). Domain 4 (residues 596-735) constitutes the region of PA involved in receptor recognition and
attachment (102,110). Structurally, domain 4 involves a β-sandwich with an immunoglobulin-like fold. When PA binds to ATR, a hydrophobic core within the β-sandwich interacts with a hydrophobic ridge adjacent to the MIDAS motif on the receptor (102). The crystal structure of PA bound to CMG-2 (PDB 1T6B) implicates residues 654-662, 681-688 and 714-716 of domain 4 as being in direct contact with the receptor (102,111). Deletion of the large loop within domain 4 (residues 703-722) reduced in vitro toxicity of LT 10-fold, while retaining PA’s ability to bind ATR and LF (21). In contrast, similar studies conducted by Varughese et al. (118) demonstrate that the large loop did not alter PA activity and did not appear to play a role in receptor binding. However, residues near or within the small loop (residues 679-693) were found to be critical for LT toxicity and PA binding to ATR (118).

The well-characterized, domain 4 MAb, 14B7 recognizes a conformation-dependent epitope spanning residues 671-721 (71), which includes both the large and small loops within domain 4. The conformational-dependence of 14B7 was confirmed by its inability to recognize any linear peptides within the array (data not shown). 14B7 has been shown to neutralize LT and ET in vitro, and protect rats against i.v. challenge with LT (69). Using in silico docking models, Sivasubramanian et al., showed that residues 648-660 and 712-720 were critical for 14B7 binding to PA (111). These findings, combined with the knowledge that the PA:CMG-2 interface spans residues 654-716 (102), supports a steric hindrance mechanism of action for 14B7. Scanning alanine mutation studies revealed that residues 682 and 686 were critical for 14B7 binding to PA and also reduced toxicity 10-100 fold (100).
Since MAb 1-F1 was demonstrated to bind to a linear peptide sequence (692-703) that fell within the established binding region for 14B7, I expected that these two antibodies would behave similarly in PA:ATR functional interaction studies. 1-F1’s binding region falls within the PA:CMG-2 interface (102) and in between the large and small loops of domain 4. Binding of 1-F1 to its putative epitope may constrain the large and small loops, preventing them from being able to contact ATR appropriately. Previous reports have established that the large and small loops are critical for PA:ATR interactions and for LT toxicity (21,118). I expected that 1-F1 would prevent PA from binding to CMG-2, most likely due to steric hindrance.

2-B12 recognized residues 716-727 which corresponded to a region of domain 4 not previously implicated as being a target of neutralizing antibodies (118). This region has been shown to be critical for PA:ATR binding (111), and is within the large loop of domain 4. A recent report tentatively demonstrated that mouse anti-domain 4 polyclonal serum reacted with the linear epitope 711-715; however, this serum was not neutralizing in vitro (6). It is difficult to interpret this data as other groups have demonstrated that expression of domain 4 alone generates a protein that is not stable and presumably not properly folded as it can not bind to the cellular receptors (28,63). I expected that 2-B12 would disrupt PA:ATR interactions similar to 1-F1 and 14B7, based on its ability to bind residues within the large loop of PA.

2-A7 was also chosen for further study because it recognized a linear epitope (residues 532-543) within domain 3 of PA. Recent research identified that mutations within residues 510-518 resulted in a loss of PA$_{63}$ heptamerization and a subsequent inability to bind LF (79). Since 2-A7 did not recognize residues within this region, and
there have been no reports that this domain plays a role in eliciting a neutralizing immune response, I did not expect that 2-A7 will play a significant role in altering toxicity in vitro or in vivo. In fact, both domain 3 specific antibodies identified in the hybridoma screen were unable to protect cells against LT intoxication. I expected that 2-A7 would be unable to disrupt PA binding to ATR, compete with 14B7 for binding to PA or protect mice against LT challenge.

This is the first report of MAbs recognizing linear peptides within domain 4 of PA. By conducting functional experiments I hoped to provide a refined epitope map of PA which would narrowly highlight key residues involved in PA:ATR interactions. Characterization of these epitopes will enhance our ability to design next-generation anthrax vaccines that could be either peptide-based vaccines, or subunit PA vaccines.

**Materials and Methods**

**MAb Ig Subclass Determination.** The subclass of each MAb was determined by ELISA. NUNC Maxisorb plates were coated with PA$_{83}$ and then reacted with MAbs for 1 hr. The plates were then washed and incubated with HRP-conjugated goat anti-mouse Ig specific (IgG$_1$, IgG$_{2a}$, IgG$_{2b}$, IgG$_3$) antibodies (Southern Biotechnology Associates, Birmingham, AL).

**MAb Affinity Determination by Surface Plasmon Resonance.** The affinities of individual MAbs for PA$_{83}$ were determined by surface plasmon resonance (SPR) on a Bia3000 instrument (Biacore International AB, Uppsala, Sweden). PA$_{83}$ was immobilized on a CM5 chip using NHS/EDC amine-coupling. Chips were equilibrated for 3 min in running buffer (0.01M HEPES, 0.15M NaCl, 3mM EDTA and 0.005% surfactant P20) prior to injection of MAbs at a constant flow rate of 30 µl/min.
Dissociation was monitored for 10 min. Association and dissociation rate constants were calculated as previously described (86).

**14B7 Competition Assay.** Nunc Maxisorb 96 well microtiter plates were coated with PA$_{83}$ (0.1 μg/well) overnight at 4°C, washed, blocked with casein (1 hr), and then incubated for 2 hours at 4°C with anti-PA MAbs. The plates were washed to remove unbound antibody, and then probed for 1 hr with biotinylated 14B7, followed by avidin-HRP (0.5 μg/ml; Sigma-Aldrich, St. Louis, MO). The plates were developed using SureBlue TMB 1-Component Microwell Peroxidase Substrate, as described above. 14B7 was biotinylated using EZ-Link Sulfo-NHS-LC Biotin (Thermo Fisher Scientific, Rockford, IL).

**CMG-2 solid phase binding assay.** Biotinylated PA$_{83}$ (1 μg) was mixed with MAb supernatants for 1 hr at room temperature, and then applied to wells of a 96 well Nunc Maxisorb plates that had been coated overnight with soluble recombinant CMG-2 receptor (1 μg/well in PBS containing 2mM MgCl$_2$). The plates were incubated for 1 hr at room temperature, washed to remove unbound biotinylated PA$_{83}$ and incubated avidin-HRP, as described previously for ELISAs.

**Results**

Initial characterization of linear peptide reactive antibodies indicated that several were specific for domain 4 and that they were capable of neutralizing LT in vitro. I predicted that antibodies 1-F1 and 2-B12, but not 2-A7, would disrupt the association of PA with the cellular receptors. To test this prediction I investigated the MAbs ability to disrupt PA associating with soluble CMG-2 in a solid-phase binding assay. I also sought to fully characterize the three antibodies by determining their binding affinities for PA,
their ability to compete with 14B7 for PA binding and their capacity to protect mice against LT challenge.

A number of key characteristics of the three MAbs chosen for further study are outlined Table 8. MAb 2-A7 was determined to be an IgG2a that recognized a peptide spanning residues 532-543 (peptide 71) within domain 3 of PA83, an area not expected to contribute to the neutralization of the toxins (79). Biacore analysis revealed that 2-A7 recognized PA83 with a dissociation constant of approximately $1.6 \times 10^{-9}$ M. 1-F1 was determined to be an IgG1 and recognized a peptide spanning residues 692-703 (peptide 91), a region of domain 4 known to be an important target of neutralizing antibodies (6,69,111). 1-F1 bound PA83 with an affinity similar to that of 2-A7. 2-B12, also an IgG1, recognized a peptide spanning residues 716-727 (peptide 94), which corresponded to a region of domain 4 not previously implicated as being a target of neutralizing antibodies (118). Biacore analysis revealed that 2-B12 recognized PA83 with a dissociation constant of approximately $1.2 \times 10^{-10}$ M.

To determine whether the putative linear epitopes recognized by MAbs 2-A7, 1-F1 and 2-B12 were in fact solvent exposed, I modeled the epitope locations on the three dimensional structure of PA83 bound to the cell surface receptor CMG-2 using PYMOL (Fig. 8). This modeling revealed that all three putative epitopes localized to the surface PA83, and were not buried within the tertiary structure of the protein. Of particular interest is the fact that the epitopes recognized by 1-F1 (red, Fig. 8) and 2-B12 (blue, Fig. 8) appeared to be situated within close proximity to the interface between PA83 (yellow, Fig. 8) and CMG-2 (purple, Fig. 8).
<table>
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<tr>
<th>MAb</th>
<th>Epitope</th>
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<th>Domain</th>
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<th>$K_D$ (M)$^b$</th>
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$^a$Sequence of reactive linear peptide
$^b$As determined by SPR analysis
$^c$Previously described by Maynard, J.A. et al. (74)

NA; not available
Figure 8. Modeling the epitopes recognized by 2-A7, 1-F1, & 2-B12 on PA$_{83}$

The location of the linear epitopes recognized by MAbs 1F1, 2A7, and 2B12 were modeled on PA$_{83}$ using PyMOL (http://www.pymol.org). The structure of PA$_{83}$ docked to CMG-2 was derived from PDB accession 1T6B. The image in the right panel is rotated 180° with respect to the image in the left panel. The solid horizontal line on the bottom of the figure represents a cell surface. The anthrax toxin receptor, CMG-2, is depicted in purple. The four domains of PA are colored as follows: domains 1 and 2, gray; domain 3, orange; and domain 4, yellow. The epitopes recognized by the individual MAbs are colored as follows: 2-A7, green; 1-F1, red; 2-B12, blue.
The putative epitope recognized by 1-F1 (residues 692-703) is located between the small and large loops of domain 4 and within the region recognized by 14B7 (residues 671-721). If this model is correct, 1-F1 should competitively inhibit 14B7 from binding to PA. The putative epitope recognized by 2-B12 (residues 716-727) localized within the large loop (704-723), and overlapped 14B7's epitope by 6 residues. Rosovitz et al., demonstrated that only a select few residues were essential for 14B7 binding to PA and these residues are not within 2-B12's recognition region (100). The limitation to this study is that mutational analysis was only performed on the small loop residues and therefore there may be additional residues involved in 14B7 binding to PA that may be within the latter residues of 14B7's epitope (e.g.: overlapping with 2-B12). Based on this information I tentatively predicted that there would be minimal competition between these antibodies.

I tested these predictions using a solid phase competitive inhibition assay in which microtiter plates were coated with rPA, followed by incubation with unlabeled 14B7, 2-A7, 1-F1, or 2-B12 and then probed with biotin-labeled 14B7 (Fig. 9). Unlabeled 14B7 competing with labeled 14B7 served as the competition control and shows 100% competition. The negative control, unlabeled 2-A7, disrupts approximately 50% of 14B7 binding. The binding of biotin-labeled 14B7 was reduced by ~90% in the presence of unlabeled 1-F1, demonstrating that 14B7 and 1-F1 recognize overlapping epitopes. On the other hand, 2-B12 was largely ineffective (~ 40%) at blocking biotin-labeled 14B7 from binding to rPA, indicating that the two MAbs did not recognize critical, overlapping residues. In fact, 2-B12 was no more effective at blocking 14B7 from binding PA than was 2-A7, which recognized an epitope situated within domain 3.
These data confirmed my predictions that 1-F1 and 14B7 overlapped in their recognition epitopes, while 2-B12 and 2-A7 did not appear to recognize similar regions of PA with respect to 14B7.

The capacity of 1-F1, 2-B12 and 2-A7 to neutralize LT in vitro was tested using the previously described TNA. In this assay, the TCEC\textsubscript{50} for 1-F1 was \(~0.002 \mu g/\mu l\) (Fig.10). At amounts greater than or equal to 0.7\( \mu g\), 1-F1 completely protected J774 cells from the effects of LT. This is virtually identical to the protection profile observed for 14B7 (data not shown). The TCEC\textsubscript{50} for 2-B12 was determined to be \(~0.004 \mu g/\mu l\); however, maximal protection conferred by 2-B12 never exceeded 90%. As expected, 2-A7 was completely ineffective at neutralizing LT.
Figure 9. 1-F1, but not 2-B12 or 2-A7, inhibits 14B7 binding to PA$_83$

Microtiter plates were coated with rPA$_83$ and then probed with biotin-labeled 14B7 (0.5 μg/ml) in the presence of unlabeled 14B7, 1-F1, 2-A7 or 2-B12. The results represent the average (+ SEM) of two assays. The horizontal bar at 80% represents an arbitrary cut-off below which MAbs were not considered to be effective competitors of 14B7 (asterisk, P = 0.0016 using a two-tailed t-test).
Figure 10. 1-F1 and 2-B12, but not 2A7, neutralize LT *in vitro*

LT (60 ng; ~2 TCEC₅₀) was mixed with serial two fold dilutions of the 1-F1 (□), 2-B12 (○), 2-A7 (●) at the indicated concentrations and then applied to J774.1 cells in 96-well microtiter plates.
Antibodies directed against domain 4 are proposed to neutralize LT and ET by preventing PA$_{83}$ from associating with anthrax toxin receptors, such as CMG-2, on the surfaces of host cells (111,118). 14B7, for example, inhibits PA$_{83}$ from binding to host cells by more than 95% (69). I used an *in vitro* binding assay to test the effect of 1-F1 and 2-B12 on the association of soluble rPA$_{83}$ with plate bound CMG-2. I expected that 1-F1 would be highly effective at disrupting the association between PA and CMG-2, and predicted that this disruption would be similar in capacity to 14B7.

Biotin-labeled rPA$_{83}$ was incubated with individual MAbs (14B7, 1-F1, 2-B12, or 2-A7) and then applied to microtiter plates coated with CMG-2. 14B7 was used as a positive control for these studies and all data were normalized to the values obtained with this MAb. 1-F1 was as effective as 14B7 in preventing the interaction between rPA$_{83}$ and CMG-2 (Fig. 11). 2-B12, on the other hand, was only 73% as effective as 14B7 at disrupting rPA-receptor engagement. The decreased disruption of PA binding to CMG-2 conferred by 2-B12 was not due to limiting amounts of MAb, as evidenced by the fact that a 20-fold dilution of 2-B12 was sufficient to neutralize LT *in vitro* (Fig. 11). As expected, 2-A7, which is directed against domain 3, demonstrated little capacity to disrupt PA binding to CMG-2.

I wished to test the ability of the MAbs to protect mice against LT challenge. Based on the *in vitro* neutralization data and the varying abilities for PA:ATR disruption I hypothesized that 1-F1 and 2-B12 would protect mice against LT challenge, while 2-A7 would not provide any protection. To accomplish this, BALB/c mice were administered LT (100 μg) by i.p. injection, and five minutes later treated with individual MAbs (550 μg/animal) by the same route. Using this model, I have previously shown that control
animals injected with LT plus saline die within 24 hr, whereas animals treated with goat anti-PA_{83} polyclonal Ig survive 11 days post-challenge. Consistent with my previous findings, all five LT-challenged, saline-treated control animals died within 24 hr (Fig. 12). Mice treated with 2-A7 died at a rate indistinguishable from control animals, confirming that MAbs against domain 3 do not afford any protection against LT \textit{in vivo}. In contrast, both 1-F1 and 2-B12 conferred partial protection against LT. 1-F1 conferred 60% protection at 20 hr, and 40% at 80 hr. 2-B12 conferred 80% protection at 20 hr, and 60% at 80 hr. While I was unable to establish the level of protection for 14B7 in this model due to limited reagent availability, I expected that individual administration of 14B7 would result in levels of protection similar to those reported here (20,71). These results confirmed that 1-F1 and 2-B12 were similarly effective at protecting mice against lethal LT challenge.
Biotinylated PA\textsubscript{83} was mixed with indicated MAbs (100 μg each) for 1 hr before being applied to wells of a 96-well microtiter plate coated with recombinant CMG-2. The plates were then probed with avidin-HRP. The degree to which each MAb interfered with PA binding to CMG-2 (black bars) was normalized to that obtained with 14B7. For comparative purposes, the MAbs (5 μg) were assayed for LT neutralizing activity (grey bars). Each data point represents the average (+SEM) of 4-8 replicates.
Figure 12. 1-F1 and 2-B12 protect mice against LT challenge

BALB/c mice (n = 3-5/group) were administered LT (100 μg) by i.p. injection, and five min later treated with individual MAbs (550 μg/animal). Animals were treated with PBS (■), 2-A7 (●), 1-F1 (□), or 2-B12 (○). Asterisk indicates statistical significance (P<0.025) using the Log Rank Test and a Bonferroni-corrected threshold when compared to the PBS control group.
Discussion

I tested MAbs 1-F1, 2-B12 and 2-A7 in a series of functional assays in order to fully characterize the epitopes on domain 4 that contribute to the neutralizing immune response. I developed a solid-phase binding assay that tests the ability of the antibodies to disrupt the association between PA and CMG-2. I also established the binding affinities for the MAbs, and their capacity for protecting mice against LT challenge. I hypothesized that both domain 4 reactive antibodies, 1-F1 and 2-B12 would behave similarly in the functional assays by disrupting PA:CMG-2 association, and conferring significant protection against LT in the animal challenge model. In contrast, I did not expect 2-A7 to affect PA binding to CMG-2 or to offer any protection against LT intoxication of mice.

Antibodies 1-F1 and 14B7 were capable of disrupting the binding of PA<sub>83</sub> to the soluble cell surface receptor CMG-2 with greater than 60% efficacy. Binding of 1-F1 to PA is possibly disrupting receptor binding via steric inhibition of a solvent-exposed loop within domain 4, residues 679-693, termed the small loop, which has been shown to play a critical role in PA:receptor interactions (118). This prediction is supported by my results demonstrating that 1-F1 recognizes residues that fall within the established binding region of 14B7 and that these antibodies compete for a similar epitope in the competitive binding assay. MAbs 1-F1 and 14B7 were functionally very similar although they demonstrated significant differences in epitope recognition. I have demonstrated that MAb 1-F1 recognized a linear epitope within domain 4 of PA which contributes to the neutralizing polyclonal response and plays a key role in providing protection against anthrax intoxication. 14B7 has been shown to be a conformation-
dependent antibody that recognizes a wide-span of residues on domain 4 of PA (69,111). As expected, 1-F1 was able to partially protect mice against LT challenge.

This is the first clear description of a neutralizing antibody that binds to a domain 4 region completely distinct from 14B7. MAb 2-B12 bound to a previously undescribed critical epitope on domain 4 of PA, spanning residues 716-727. I showed that MAb 2-B12 was able to neutralize LT in vitro and protected 60% of mice challenged with LT. Epitope recognition clearly has a substantial effect on an antibody’s ability to neutralize LT. In the predictive binding model, 2-B12 can be seen binding a region distinct from 1-F1, yet still within the PA:CMG-2 interface. A previous report has indicated that the 2-B12 recognition region (residues 716-727) overlap with the large loop of domain 4, spanning residues 704-722, which did not appear to interact directly with anthrax cellular receptor following alanine substitution of multiple residues within this region (118). In the CMG-2 competitive binding assay I demonstrated that 2-B12 was capable of disrupting 48% of PA from binding to CMG-2. It is possible that the increased affinity 2-B12 exhibits for PA (as compared to 1-F1) compensates for its limited ability to disrupt PA binding to the cellular receptor. The likelihood that 2-B12 may be neutralizing LT via a mechanism other than steric hindrance can not be completely discounted based on my results. However, other work has demonstrated that domain 4 is not in direct contact with the rest of the PA protein and is most likely only involved in direct binding to the toxin receptor (110).

Both 1-F1 and 2-B12 were only able to partially protect mice against LT challenge. When I established this challenge model I was able to obtain complete protection using 8mg/kg of anti-PA_{83} polyclonal goat serum. While I hoped to see
complete protection with the individual administration of the MAbs, it was not surprising that they were only capable of partial protection. The majority of MAbs described in the published literature are not capable of significant protection post-exposure and appear to require a blend of several MAbs in order to reduce the mortality associated with anthrax infections (20,97,99). I would expect that co-administration of 1-F1 and 2-B12 would increase the survival rates of mice following LT challenge; however, I was unable to perform these experiments due to limited amounts of antibody and qualified toxin.

The parallel characterization of an antibody recognizing a domain 3 linear epitope reinforces the critical role that epitopes play in determining neutralization of anthrax toxins. While antigen affinity has been implicated as a key characteristic for neutralization (74), MAb 2-A7 demonstrates that high affinity can not compensate for a non-neutralizing epitope. MAb 2-A7 recognized residues 532-543 which fall within a region (residues 488-595) established as being excluded from the oligomerization interface and not essential to heptamer formation (79). The inability of this antibody to disrupt PA:CMG-2 interaction was expected as domain 3 has no predicted interactions with either toxin receptors and is mainly implicated in self-oligomerization (29). The observed lack of neutralization both 

\textit{in vitro} and \textit{in vivo} for MAb 2-A7 was also expected based on the lack of observed effect when buried residues between 530 and 590 were mutated (79). The MAb 2-A7 data confirms the lack of involvement of domain 3 in PA’s interaction with the toxin receptor and indicates that antibodies in this region may not contribute to the neutralizing antibody response.
Conclusions

The isolation and characterization of two antibodies, 1-F1 and 2-B12, that were protective and able to recognize linear peptides within domain 4 of PA confirmed my hypothesis that other epitopes, in addition to that recognized by 14B7, exist on domain 4 and contribute to the neutralizing immune response to the anthrax toxins. The fact that all fully characterized domain 4 antibodies, 14B7, 1-F1, and 2-B12, bind within the PA:ATR interface suggests that this region is the main antigenic determinant for this domain. Antibodies that disrupt the early stages of the toxin pathway (i.e.: PA binding to ATR) are predicted to be highly effective therapeutics. The ability of 1-F1 and 2-B12 to provide partial protection to mice challenged with LT, even when administered alone, confirmed that these antibodies play a critical role in disrupting the pathogenesis of anthrax intoxication.
Chapter 7. Reactivity of Human Neutralizing Polyclonal Serum with Linear Epitopes on PA$_{83}$

Acknowledgments

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Introduction

A major challenge in evaluating next-generation anthrax vaccine candidates has been defining robust and reliable correlates of immunity. I expected that peptides which reacted with the polyclonal and monoclonal antibodies tested in previous chapters would also react with human anti-AVA serum. If this prediction is correct, these epitopes could be used as correlates of immunity to predict the efficacy of next-generation vaccines.

Current anthrax correlates of immunity to predict the effectiveness of a vaccine candidate rely on establishing total anti-PA serum Ig titers, in vitro TNA evaluation and in vivo animal challenge models (46,88). When evaluating a next-generation vaccine candidate total anti-PA titers and in vitro TNA evaluations are usually undertaken early on during the research phase in order to limit cost and ensure that animal challenges are only performed on established candidates. Unfortunately, these preliminary correlates of immunity are unreliable and do not offer much predictive value in determining the efficacy of a potential vaccine candidate. For example, there is not a definitive correlation between anti-PA serum Ig titers and protection against lethal anthrax challenge (98). In vitro TNA results are more reliable than total anti-PA titers (123)
although neutralization results appear to vary depending on the method used to evaluate neutralization (113). Finally, the applicability of these correlates of immunity appear to vary depending on the animal model chosen for study (67).

I wished to test whether human anti-AVA serum reacts with linear epitopes on domain 4 of PA. Specifically, I was interested to see if the same peptides that reacted with MAbs 1-F1 and 2-B12 would also react with the human polyclonal serum. In order to establish the relevance of these epitopes in a human response I evaluated the ability of anti-AVA Ig to react with the PA83 linear peptide array.

The lack of correlation between anti-PA serum Ig titers, and protection against lethal challenge in most animal models, necessitates the development of a more reliable and predictive correlate of immunity. I proposed that screening polyclonal serum using well-characterized linear peptides could be an effective mechanism to predict the efficacy of next generation vaccine candidates. My characterization of the epitopes recognized by MAbs 1-F1 and 2-B12 established the utility of these peptides for use in such a screening assay. Using this type of screening tool, potential vaccine candidates could be screened for the presence of key neutralizing antibodies to confirm that an appropriate immune response is being stimulated following vaccination. Ideally, multiple linear peptides spanning the entire PA83 protein would be utilized in order to provide a comprehensive reactivity profile for the vaccine candidate. Gubbins et. al identified a domain 2 linear peptide which was associated with a neutralizing response in AVA vaccinated volunteers (43). In a more recent report the same group also established a competitive ELISA assay to screen serum for the presence of antibodies recognizing the domain 2 linear peptide.
(44). I expect that the linear peptides involved in 1-F1 and 2-B12 binding could also be used in a similar assay to screen potential vaccine candidates in the future.

**Materials & Methods**

**Linear Peptide ELISA.** Human anti-AVA serum diluted 1:100 in sterile PBS was reacted with the linear peptides spanning PA$_{83}$, as previously described. Bound antibodies were detected using HRP-labeled goat-anti-human IgG (Invitrogen Life Technologies) at a 1:2000 dilution.

**In vitro TNA.** Human anti-AVA Ig was evaluated in the established *in vitro* toxin neutralization assay as previously described. Varying dilutions of anti-AVA were prepared in sterile PBS and added to LT as previously described in the General Materials and Methods section.
Results

The persistent lack of reliable correlates of immunity to predict the efficacy of potential vaccine candidates has hampered research into next-generation vaccine candidates. I predicted that the peptides recognized by 1-F1 and 2-B12 may also react with human anti-AVA serum, supporting their use as predictive correlates of immunity for assessing protection against anthrax toxicity. To test this hypothesis I tested the serum from an AVA-vaccinated volunteer for epitope recognition using the PA83 peptide array. In order to assess the robustness of the proposed correlates of immunity I compared the reactivity profiles for goat and mouse anti-PA83 Ig with the human anti-AVA Ig.

I first tested the human anti-AVA serum for its ability to neutralize LT in vitro. As shown in Figure 13 the anti-AVA Ig is highly neutralizing in the cell based toxicity assay. A dilution of 1:500 provides 100% protection against LT while 1:1000 only provides 50% protection. Protection dropped significantly (to 10%) when a dilution of 1:5000 is used and reached ~0% protection with a dilution of 1:10,000 (data not shown). This data established that a 1:500 dilution of the human anti-AVA serum contains a significant amount of neutralizing antibody. Based on these findings I decided to test the reactivity of the human polyclonal serum for reactivity with the linear peptide array.
Figure 13. Human anti-AVA Ig neutralizes LT *in vitro*

J774A.1 cells were treated with 50 ng LT and dilutions (1:100, 1:500, 1:1000, and 1:5000) of human anti-AVA Ig. Cell viability determined by an MTT-based assay. Data shown are the average ± SEM of four assays.
Various dilutions of human anti-AVA serum were reacted with the linear peptide array previously used to screen goat anti-PA$_{83}$ IgG and murine hybridomas. Reactivity with the peptide array was strongest with a 1:100 dilution of the serum (Fig. 14). Mouse anti-PA$_{83}$ Ig was also reacted with the linear peptide array for comparative purposes (Fig. 15). The human polyclonal serum recognizes various peptides throughout the 4 domains of PA, as observed for both goat (Fig. 3) and mouse serum (Fig. 15). It is important to note that the goat and mouse profiles were generated using the same rPA$_{83}$-MIS immunogen while the human profile is the result of AVA vaccination.

All three sera showed varying reactivity with the PA$_{83}$ linear peptides. Interestingly, the goat serum reacted with a greater number of peptides within each of the 4 domains as compared to the human or mouse serum. Species differences may account for variability in reactivity although regions of similarity are evident.

Domain 1 (Figs. 3A, 14A and 15A) showed the highest variability between the species with no direct overlaps in peptide reactivity, although regions of interest can be seen, including residues 4-55 (peptides 4-10), 68-135 (peptides 13-20), and 212-255 (peptides 31-33).

Residues 308-319 (peptide 43) within domain 2 (Figs. 3B, 14B and 15B) showed substantial reactivity with both the goat and mouse sera; however, only minimal reactivity was seen with the human anti-AVA serum. All three sera were moderately to highly reactive with residues 404-431 (peptides 55-57) indicating that this region may be highly antigenic across species.

Domain 3 (Figs. 3C, 14C and 15C) exhibited two regions that showed variable reactivity. Residues 492-511 (peptide 66-67), and 588-599 (peptide 78) were moderately
reactive with at least two of the three sera tested. It is interesting to note that residues 532-543 (peptide 71), which reacted with MAb 2-A7, is not noticeably reactive in any of the polyclonal serum tested.

Domain 4 epitopes (Figs. 3D, 14D, and 15D) showed the highest degree of overlap between species. Specifically, residues 596-607 (peptides 79-80) were reactive with all three sets of sera tested. Most importantly, residues 692-711 (peptides 91-92) also demonstrated significant reactivity with all three sera. This is the same epitope that I previously identified as being recognized by MAb 1-F1.
Figure 14. Human anti-AVA Ig reacts with PA$_{83}$ epitopes

Human anti-AVA polyclonal serum, diluted 1:100 in sterile PBS, was reacted with 12-aa long peptides (4-aa overlap) spanning the length of PA$_{83}$. A. Domain 1 peptides. B. Domain 2 peptides. C. Domain 3 peptides. D. Domain 4 peptides.
Figure 15. Mouse anti-PA$_{83}$ Ig reacts with PA$_{83}$ epitopes

Mouse anti-PA$_{83}$ polyclonal serum was reacted with 12-aa long peptides (4-aa overlap) spanning the length of PA$_{83}$. Data represents the average reactivity for 2 mice.

Discussion

A consistent lack of reliable correlates of immunity in anthrax immunology led us to test the utility of linear epitopes for their reactivity across various species. In order for peptides to be used as a robust correlate of immunity they must be of high predictive value; e.g. consistently reactive with various species immunized with the same immunogen. I tested the polyclonal sera from three different species; human, goat, and mouse, for reactivity with the PA$_{83}$ peptide array. Although these anti-sera were not all generated with the same immunogen, I anticipated that regions of similarity would be evident and that these regions may overlap with previously identified epitopes involved in protective immunity (i.e.: epitopes recognized by MAbs 1-F1 and 2-B12).

Domain 1 (residues 1-258) showed the highest variability between the species, with no direct overlaps in peptide reactivity. However, regions of similarity are apparent such as residues 4-55, 68-135, and 212-255. Domain 1 contains the furin recognition site (RKKR$^{164}$), as well as the region of PA that is recognized by LF and EF (81). Abboud and Casadevall (6) recently described the reactivity of mouse polyclonal serum to a linear peptide spanning amino acids 121-150. I have confirmed these results by demonstrating that three separate sera reacted with residues 108-135. In the Abboud study (6) immunization with either full-length PA$_{83}$ or PA1 (domain 1 only) elicited highly reactive sera with these peptides. These sera were also shown to be neutralizing in vitro indicating that this epitope is probably involved in a protective immune response against anthrax infection. In-depth characterization of this epitope would be interesting since domain 1 has been implicated in the generation of antibodies that actually enhance LT toxicity in vitro (80).
Residues 300-319 in domain 2 showed similar reactivity with the human, goat, and mouse sera. Domain 2 (residues 249-487) is implicated in heptamerization, pore formation, and translocation of EF/LF across endosomal membranes (15,76,79,93). The reactive epitope spans amino acids $^{311}$ASFFD$^{315}$ which have been previously shown to react with serum from AVA vaccinated individuals (43). This region is predicted to be a surface-exposed portion of the $2\beta_2$-$2\beta_3$ loop (85,109) that is involved with toxin translocation. The $2\beta_2$-$2\beta_3$ loop is within very close proximity to the $\beta_3$-$\beta_4$ loop that has been shown to interact with the cell-surface toxin receptor, CMG-2 (102). Antibodies recognizing $^{311}$ASFFD$^{315}$ have been reported as neutralizing in vitro (43) and this peptide has been used to develop a competitive screening assay for evaluating the specific reactivity of human anti-AVA immune serum (44). These results show that this epitope is reactive across various species, regardless of immunogen used in the vaccination. This strongly supports the use of this epitope as a correlate of immunity for predicting the efficacy of next-generation vaccines candidates in eliciting an appropriate immune response.

When human, goat, and mouse sera were tested against domain 3 epitopes, two regions of similarity were apparent. These epitopes spanned residues 492-511 and 588-599. Domain 3 has no predicted interactions with the cell-surface toxin receptors and is mainly implicated in self-oligomerization (29). Scanning alanine mutation analysis has recently demonstrated that domain 3 residues 488-595 are not part of the oligomerization interface, and not essential to heptamer formation (79). Based on these reports, it is not expected that the epitopes identified in this screen would contribute to a neutralizing immune response. However, they are likely contributing to the non-neutralizing antibody
pool following PA vaccination. This is supported by the data I have presented with respect to the non-neutralizing MAb 2-A7, which recognized residues 532-543.

Screening of human, goat, and mouse antiserum revealed that two domain 4 epitopes were being recognized, residues 596-615 and 692-703. Domain 4 (residues 596-735) constitutes the region of the PA involved in receptor recognition and attachment (110). Antibodies against domain 4 are postulated to be the most effective in neutralizing LT and ET (6), since they are proposed to interfere with PA binding to ATR. The only neutralizing epitope that has been well-defined is the one recognized by the conformation-dependent antibody 14B7 (71,100,111). A recent report suggested that a linear epitope (residues 678-697), which overlaps the 14B7 binding site, may be the target of neutralizing antibodies (6). The epitope spanning residues 596-615 identified in my research has not been previously implicated as a major neutralizing epitope.

Additional investigations into this region will need to be conducted in order to determine the role that this epitope is playing in the elicited immune response to anthrax vaccination. Peptide screening also identified an epitope, defined by residues 692-703 and directly adjacent to 14B7’s binding site, was consistently reactive with the three species tested. I have established that this is the recognition epitope for MAb 1-F1 as described in Chapters 4 and 5. This epitope also overlaps with the predicted linear epitope described by Abboud and Casadevall (6). These data confirmed that the 1-F1 epitope is sufficiently antigenic to elicit a consistent immune response across various species and with varying immunogens. These data, coupled with the fact that I have shown that this epitope is recognized by a neutralizing antibody, supports the use of this epitope as a reliable correlate of immunity for anthrax infection.
While regions of similarity were evident, variation in epitope reactivity was not unexpected when comparing human, goat, and mouse profiles. Species variability has been implicated as a major challenge for predicting the efficacy of anthrax vaccines (6,25). In addition, the human serum was generated using the traditional AVA vaccine, while the goat and mouse sera were generated from rPA$_{83}$-MIS immunizations. Interestingly, overlap in epitope reactivity between the species/immunogens was observed, indicating that certain regions of PA may be highly antigenic to all species. Two of these epitopes are recognized by antibodies that have been established as neutralizing in vitro and/or protective in vivo. The domain 2 epitope recognized by MAb F20G (43) and the domain 4 epitope recognized by MAb 1-F1 were reactive with the various species tested against the peptide array indicating that they may be reliable predictors of immunity.

Conclusions

A major challenge in evaluating next-generation anthrax vaccine candidates has been defining robust and reliable correlates of immunity. The use of linear peptides as a robust correlate of immunity for predicting the efficacy of an anthrax vaccine candidate has recently been proposed (44). I hypothesized that epitopes that consistently reacted with polyclonal serum from various species would be useful as correlates of immunity to predict the efficacy of next-generation vaccines.

I have verified that two epitopes could be reliable predictors of immunity and therefore useful as a correlate of immunity screening assay. These results confirm that a domain 2 epitope, previously proposed by Gubbins et al. (43,44), reliably elicits a strong immune response following vaccination with AVA or rPA$_{83}$. The domain 4 epitope that I
have described as being recognized by MAb 1-F1 was also consistently recognized by the human, goat, and mouse antiserum. Since I have established that this epitope is recognized by a protective MAb, I suggest that this epitope also be included as reliable correlate of immunity for predicting immunity against anthrax infections. Incorporating multiple peptides into a rapid correlate of immunity screening assay will give researchers an additional tool to evaluate potential vaccine candidates prior to moving into animal experiments.
Chapter 8. Discussion

Current second generation anthrax vaccines are focused on PA, which has been established as the primary determinant involved in immunity to anthrax infection (64). However, evaluating the effectiveness of candidate vaccines has been difficult because the specific PA epitopes involved in anthrax toxin neutralization have not been well defined. Until now, the only well characterized protective antibody (14B7) has been a conformation-dependent, domain 4 specific antibody. I hypothesized that additional epitopes on domain 4 of PA would contribute to eliciting a protective immune response against anthrax. I have confirmed this hypothesis by identifying 2 neutralizing monoclonal antibodies that recognize epitopes on domain 4 that are distinct from 14B7’s recognition site. In order to achieve this goal I tested the ability of rPA$_{83}$-MIS to elicit a neutralizing immune response in goats and mice, established an LT challenge mouse model to evaluate the ability of antibodies to disrupt the intoxication pathway in vivo and screened a collection of murine B cell hybridomas for neutralizing MAbs that reacted with the linear epitopes on the C-terminus of PA. I identified two IgG$_1$ MAbs, 1-F1 and 2-B12, and performed in-depth characterization of their interactions with their specific epitopes in order to elucidate their role in PA:ATR association and disruption of the anthrax intoxication pathway. Although 1-F1 and 2-B12 recognized distinct epitopes on PA, and demonstrated varying capacity for disrupting PA’s association with ATR, they were both able to protect mice against LT challenge. This is the first comprehensive description of epitopes, distinct from 14B7, that are involved in the neutralizing immune response following PA immunization. My findings provide anthrax researchers with
several novel tools that will simplify future investigations of second-generation vaccine candidates and redefine the current concept of anthrax correlates of immunity.

**Elucidating Critical Regions of PA Involved in Immunity to Anthrax**

While investigating the relevance of using an rPA\textsubscript{83} immunogen, linked to a novel adjuvant MIS, I discovered that a truncated form of PA was also effective at protecting against LT challenge *in vitro* and *in vivo*. The truncated PA protein, PA\textsubscript{32}, is a 32-kDa carboxy-terminal fragment of PA\textsubscript{83} where only domains 3 and 4 are expressed. Previous work by Cirino *et al.*, has established that PA\textsubscript{32} is properly folded, binds to ATR and is internalized appropriately (28). Goats immunized with PA\textsubscript{32} developed a robust immune response that passively protected cells and mice against LT challenge. This data established that the C-terminal region of PA was critical for developing immunity against the anthrax toxins. Domain 4 constitutes the region of PA involved in receptor recognition and attachment (102,110). Disruption of the PA:ATR association has been shown to be highly effective at preventing downstream steps required for anthrax intoxication (69,100,118). My discovery of 2 domain 4-specific MAbs that were highly neutralizing, and protective against LT challenge, supports the established dogma that this region of PA is critical for eliciting immunity to anthrax.

In contrast, domain 3 has not been implicated in immunity against anthrax infections and has been assumed to play a strictly structural role. While recent research identified specific residues that were critical for PA\textsubscript{63} heptamerization and LF binding (79), there is no evidence that antibodies directed against this domain are functionally capable of disrupting the anthrax intoxication pathway. I identified two MAbs that bound within domain 3; both of which were ineffective at neutralizing LT *in vitro*. I describe
one antibody, 2-A7, in detail and demonstrate that it is incapable of protecting mice against LT challenge and in disrupting PA’s association with ATR. These data add to the current body of evidence suggesting that PA domain 3 does not play a critical role in immunity against the anthrax toxins.

The ability of the PA32 moiety to elicit a neutralizing response to LT was decreased in comparison to that of PA83. I speculate that this decrease is due to the lack of domain 1 and 2 involvement in the elicited immune response. This hypothesis is supported by my findings that several hybridoma clones specific for the N-terminus of PA were capable of neutralizing LT in vitro. Other groups have also reported that epitopes on domain 1 and 2 are recognized by protective antibodies, indicating that these regions do contribute to the overall neutralizing immune response following vaccination (43,99). Domain 1 contains the furin recognition site (\textsuperscript{164}RKKR\textsuperscript{167}), which, upon cleavage, results in the formation of PA\textsubscript{20} and PA\textsubscript{63}, a step that is critical in the pathogenesis of anthrax. Once cleaved, PA\textsubscript{63} spontaneously heptamerizes and is able to bind the enzymatic proteins LF and EF. It is reasonable to predict that antibodies capable of disrupting either the furin cleavage or the binding of LF/EF would be effective at halting the intoxication pathway and would therefore be protective. Domain 2 is involved in the formation of the transmembrane pore and in PA:ATR associations. Mutations within domain 2 can significantly reduce toxicity indicating that this domain plays an important role in anthrax pathogenesis (82). It is clear that immunization with full-length PA must elicit antibodies directed against these critical regions of the N-terminus of PA that are lacking following immunization with PA\textsubscript{32}. The fact that anti-PA\textsubscript{32} serum only showed a minor loss in neutralization confirms my prediction that
antibodies against the C-terminal region of PA are critical and sufficient for protection against anthrax challenge.

Expansion of the Anthrax Protective Antigen Epitope Map

More than two decades ago, Little and colleagues (69) proposed that there are at least 23 distinct antigenic regions on PA. Identifying the exact epitopes on PA that are recognized by neutralizing MAbs has proven challenging because the most well characterized MAbs have been directed against conformation-dependent epitopes. By simultaneously screening murine anti-PA\textsubscript{83} hybridomas for their ability to neutralize LT \textit{in vitro}, and for reactivity against linear epitopes, I was able to vigorously characterize 2 C-terminal PA epitopes that are involved in the neutralizing response against anthrax toxicity. This level of characterization has not been achievable using conformation-dependent antibodies as their epitopes can only be broadly identified.

The current PA domain 4 epitope map has been elucidated based on the characteristics of one key antibody, 14B7 (69,111). MAb 14B7 was first identified as a conformation-dependent antibody whose binding epitope was localized to residues 671-721, which includes both the small and large loops of domain 4 (69,71). Using \textit{in silico} docking models, residues 648-660 and 712-720 were identified as critical for 14B7 binding to PA (111). Presumably the mode of action for 14B7 is via steric hindrance since this epitope is localized at the PA:ATR interface which spans residues 654-716 (102). My identification and characterization of two novel domain 4 epitopes, residues 692-703 and 716-727, has provided the first definitive expansion of this regions’ epitope map since the identification of 14B7. An additional linear epitope (residues 678-697) was recently identified that appears to be the target of neutralizing antibodies in a
polyclonal pool (6). Isolation of the MAb recognizing this epitope, and subsequent epitope characterization, has yet to be completed.

The epitope spanning residues 692-703 (recognized by MAb 1-F1) is clearly involved in establishing the association of PA with ATR based on the in vitro solid phase binding assay results. This epitope overlaps with 14B7’s binding region suggesting that 1-F1’s ability to protect mice against LT challenge is likely due to steric hindrance. In contrast, the epitope recognized by 2-B12, residues 716-727, does not appear to interact directly with ATR based on the PA:ATR binding assay results. This observation is supported by two recent studies which demonstrated that mutation of multiple residues within this epitope were not able to disrupt PA:ATR association (118), and that deletion of the large loop resulted in reduced in vitro toxicity of LT without any effect on PA’s ability to bind ATR or LF (21). Since binding of 2-B12 to this epitope resulted in effective neutralization of LT both in vitro and in vivo, I conclude that this epitope is critical for maintaining toxicity; however, its mode of action may involve processes other than steric hindrance of PA associating with ATR.

My data clearly advances our understanding of critical domain 4 epitopes and their roles in maintaining anthrax toxin toxicity. Additional investigation into the role that residues 716-727 play in the toxin pathogenesis pathway will be critical for further expanding the current epitope map of PA, and for enabling the targeted design of next-generation anthrax vaccine candidates.

**LT Challenge as a Relevant Anthrax Animal Model**

The gold standard for evaluating the protective efficacy of polyclonal or monoclonal antibodies involved in anthrax immunity continues to be in vivo challenge
studies using virulent anthrax spores. These studies are costly and require ABSL-3 containment facilities, and select agent approvals. In order to circumvent the restrictions involved with live spore challenge, I developed an LT challenge model in mice that can be easily conducted under BSL-2 conditions. This experimental protocol is highly relevant for research focused on elucidating the involvement of antibodies in the anthrax intoxication pathway. The LT challenge model consistently provided a 24-48 hr median-time-to-death for untreated mice and required only moderate amounts of anti-PA Ig in order to consistently see protection.

I tested the LT challenge model by passively administering anti-PA_{83} and anti-PA_{32} goat Ig to mice. 8 mg/kg of IgG conferred 60-80% protection against 2LD_{100} LT challenge. In addition, three immunizations with as little as 5 μg of rPA_{83}-MIS fully protected mice against LT challenge. The LT challenge model was tested head-to-head against the gold standard spore challenge model. The similar protection levels observed between the two in vivo passive transfer challenge studies confirm the applicability of the LT challenge model for establishing the protective efficacy of antibodies directed against the anthrax toxins.

This novel challenge model provides anthrax researchers with a reliable investigation tool for early studies focused on elucidating the protective qualities of antibodies directed against PA, and presumably LF. This model can be used as preliminary evidence for the efficacy of such antibodies, prior to advancing to costly live anthrax spore challenge models in more relevant species including rabbits, guinea pigs and non-human primates (25,51,56,68). A limitation to my experimental challenge model is the reliance on recombinant proteins for eliciting lethal intoxication. The
quality of the proteins used in such studies must be standardized in order to ensure consistency across experiments and to provide a sufficient level of challenge so that high confidence results can be gathered with respect to the neutralization capacity of the antibodies under investigation.

Standardization is Critical for Anthrax Immunity Research

Variability among reports using recombinant anthrax proteins for *in vitro* and *in vivo* challenge assays is common in the anthrax field (84,113). A significant challenge in the research presented here was establishing consistently toxic lots of LT for use in the toxin neutralization assays and the mouse LT challenge model (described in Chapter 3). My investigations into the variability of LT preparations pointed towards differences in the degree of purity of the LF protein. I observed that lots of LT prepared from batches of heterogeneous LF (i.e.: additional bands on SDS-PAGE) were more toxic than lots containing more highly purified preparations of LF. These observations are supported by a recent report describing the importance of N-terminal amino acids in establishing LF toxicity (120). In this report more homogenous preparations of LF (NR-724) that migrated as a single band on SDS-PAGE were less toxic than preparations that showed additional minor bands (LF NR-142). These findings were essential to establishing qualified lots of LT to be used in several different assays such that my results could be confidently compared. A recent interlaboratory comparison of the LT toxin neutralization assay demonstrated that this assay produced high-confidence results when standardized toxin reagents were utilized (88). These studies were completed by large government facilities and/or contract laboratories that have access to different reagent batches of LF and PA than academic institutions. Most academic researchers use small
batches of LF and PA, purified based on reports in the literature (24,89), to conduct their TNA and in vivo experiments. The challenges I faced in this research underscores the difficulty in comparing results across various laboratories without the use of standardized anthrax toxin reagents.

The lack of standardization in the anthrax research field was recently addressed by an American Type Culture Collection Standards Development Organization (ATCC SDO) workgroup. This workgroup brought together recognized anthrax researchers to develop an official standard for the production of the anthrax toxin proteins (PA, LF and EF), and the methods used to qualify the toxicity of LT and ET. The consensus standard, entitled “ASN-0001, Standardization of In Vitro Assays to Determine Anthrax Toxin Activities,” has been submitted by ATCC SDO for publication by the American National Standards Institute (ANSI). This is the first consensus standard developed for use by researchers in the field of microbial pathogenesis. In a guest commentary recently submitted to Infection and Immunity, the value of developing consensus standards as it relates to B. anthracis research is presented and the development process used by the ATCC SDO Anthrax Toxins workgroup is highlighted to provide guidance for other fields that may wish to adopt a similar strategy for establishing continuity of research (Hughes, MA et al., in press).

The ASN-0001 consensus standard for anthrax toxins provides a comprehensive body of work that will allow researchers to use established expression, purification and qualification methods for the generation of reagents critical for anthrax pathogenesis research. The formal standardization of these processes will help to clarify results
reported from various research groups and enable comparison of results from different experimental platforms.

**Future Anti-anthrax Therapies**

Post-exposure treatment options for inhalation anthrax infections are limited in their availability and efficacy. Antibiotic therapy, currently the main treatment option, must occur quickly after exposure in order to offer any survival benefit to the patient. The reason such a narrow time window for treatment exists is due to the rapidity with which the anthrax bacilli replicate and the amount of toxin that is expressed. Antibiotics can reverse septicemia although the patient may still succumb to the infection due to the high levels of ET and LT already causing irreversible effects on the organs. I have shown that passive administration of anti-PA Ig or specific MAbs can protect mice against LT and spore challenge when administered up to 24-hours post-exposure. I expect that a cocktail mixture of highly neutralizing monoclonal antibodies could be effectively used as a post-exposure passive immunotherapy for patients presenting with clinical symptoms of anthrax infection. The inclusion of MAbs 14B7, 1-F1 and 2-B12 would ensure neutralization of the most critical epitopes located on domain 4 of PA.

A polyclonal passive immunotherapeutic was recently used to treat two patients who contracted inhalation anthrax infections following manipulation of untreated animal hides (3,8). Following emergency approvals to administer anti-PA antibodies, one patient began to recover and eventually regained near-normal pulmonary function (122), while the other patient succumbed to the infection (8). The anti-PA passive immunotherapy used to treat the patients was developed from the serum of military volunteers who received the AVA vaccine. A significant limitation of this therapeutic is
the limited amount of product that is available for stockpiling. The preparation of a humanized monoclonal antibody cocktail would provide health officials with a substantial supply of therapeutic that could be easily administered should another intentional release of anthrax spores occur.

Although this is the first report of a symptomatic patient receiving anti-PA immunotherapy, other reports have also suggested that administration of antibody therapy may provide significant clinical benefits, especially when administered prior to the onset of symptoms (47,104). While administration of human antibodies is the goal for any post-exposure therapeutic, obtaining sufficient quantities at a low enough cost to warrant stockpiling is a formidable challenge. The goat anti-PA$_{83}$ Ig tested here was shown to be highly neutralizing against virulent anthrax spores in mice. PEGHRG214, a passive immunotherapeutic for failing therapy AIDS patients, was generated in goats using the approach described here for anti-PA$_{83}$ Ig (30,119). This anti-HIV therapeutic has progressed to phase III clinical trials after being determined to be safe for human administration. I suggest that the rapidity with which the goat anti-PA$_{83}$ Ig can be generated, and the establishment of its neutralizing capacity \textit{in vivo}, warrants its investigation as a potential post-exposure therapeutic for use in humans. In order to augment the safety of the goat therapeutic I have investigated the consequence of removing the Fc portion of the antibodies. Fc removal should decrease any hypersensitivity reactions to the goat-specific portion of the antibodies; however, some groups have proposed that immunity to anthrax is Fc mediated (121). These experiments demonstrated that there was no loss in \textit{in vivo} protection when mice were treated with anti-PA F(ab’)$_2$ versus whole Ig (data not shown).
My research has identified two additional MAbs that could be humanized for inclusion in a cocktail mixture of antibodies for the post-exposure treatment of inhalation anthrax infections. The goat anti-PA$_{83}$ Ig could also be easily manufactured for rapid, high volume production should stockpiling of a polyclonal passive immunotherapy be warranted for ready availability following another intentional release of anthrax spores.

**Future Anthrax Vaccine Candidates**

While the current emphasis of second-generation anthrax vaccine candidates is focused on using rPA immunogens, other avenues are also worth exploring. I have investigated the use of a novel adjuvant Microparticle Immune Stimulator (MIS) which, unlike traditional adjuvants such as alum, is covalently linked directly to the immunogen. MIS has been previously used as an effective adjuvant when coupled to HIV proteins for the generation of a passive immunotherapeutic for failing-therapy AIDS patients (30). I have demonstrated that MIS coupled to rPA$_{83}$ or rPA$_{32}$ elicits a highly neutralizing immune response in goats and mice, and that mice vaccinated with rPA$_{83}$-MIS survive LT challenge. I have also demonstrated that this adjuvant is completely non-toxic to mice when administered in quantities as high as 500 $\mu$g MIS/mouse (data not shown). Further investigations are needed in order to compare the efficacy of rPA$_{83}$-MIS against rPA$_{83}$ with alum to establish the utility of this adjuvant for use in future vaccine candidates.

Several groups have attempted to use truncated forms of PA, expressing only domain 4, as vaccine candidates but have consistently failed to elicit a neutralizing immune response (6,63). Cirino *et al.* previously established that a construct expressing domains 3 and 4 of PA, termed PA$_{32}$, was properly folded, able to bind ATR and was
The protective efficacy observed with AVA may differ from that observed with rPA vaccines due to the inclusion of LF and EF in the original vaccine preparation. A synergistic response to multiple components of the anthrax toxins may be required in order to provide a truly comprehensive immune response and therefore the most efficacious neutralization (36). However, a balance must be achieved with the detrimental effects that would arise following vaccination with multiple full-length anthrax toxin proteins. Vaccination with PA and LF or EF would result in the formation of fully-functional LT and ET with obvious health implications for the vaccinee. In order to circumvent this issue I have begun to evaluate the efficacy of a chimeric protein which links the C-terminus of PA (PA32) with the N-terminus of LF or EF. The N-terminus of LF and EF are expected to comprise several key antigenic determinants as this is the region that is directly involved with binding to PA63 (29,124). In a recent study, 3 anti-LF antibodies were reported as being neutralizing in vitro indicating that this protein is capable of contributing to the protective immune response to anthrax intoxication (84). Interestingly, all 3 antibodies were reactive with linear epitopes on LF (84). Initial studies in my laboratory have indicated that antibodies present in goat anti-LF serum recognize linear peptides similar to that observed for anti-PA Ig.
In order to augment the immunogenicity of these proteins I have coupled the chimeras to MIS, the adjuvant used in the rPA$_{83}$ immunization studies. While additional work is needed to establish that key neutralizing epitopes are located on the N-terminal of LF and EF, I expect that immunization with a chimera expressing portions of the enzymatic anthrax proteins and rPA$_{32}$ will elicit an immune response that will be significantly more neutralizing than rPA$_{32}$ alone. The inclusion of well characterized domain 4 epitopes, coupled with an increase in vaccine safety (due to the use of a non-toxic adjuvant and truncated anthrax toxin proteins) enhances the utility of these chimeras as next generation vaccine candidates.

**Redefining Anthrax Correlates of Immunity**

Reliable anthrax correlates of immunity have remained poorly defined and of limited value in predicting the efficacy of a potential vaccine candidate. Current correlates of immunity rely on establishing total anti-PA serum Ig titers, *in vitro* TNA evaluation and *in vivo* animal challenge models (46,88). The lack of correlation between anti-PA serum Ig titers, and protection against lethal challenge in most animal models (98) necessitates the development of a more reliable and predictive correlate of immunity. I investigated the utility of PA linear epitopes as potential correlates of immunity by testing their reactivity across various species immunized with rPA$_{83}$-MIS or AVA. In order for peptides to be used as a robust correlate of immunity they must be of high predictive value; e.g. consistently reactive with various species immunized with the same immunogen.

I identified two epitopes that were consistently recognized by antibodies present in the human, goat and mouse antiserum. One epitope is localized to domain 2 of PA
spanning residues 300-319, including the $^{311}$ASFFD$^{315}$ site which have been previously shown to react with serum from AVA vaccinated individuals (43). This region is predicted to be a surface-exposed portion of the $2\beta_2$-$2\beta_3$ loop (85,109) that is involved with toxin translocation and PA:ATR interactions (102). Antibodies which bind to this epitope have been reported as neutralizing in vitro (43). The second epitope identified in my screening spans residues 692-703, the exact epitope described here as being recognized by the neutralizing MAb 1-F1. Blocking this epitope by binding MAb 1-F1 caused a significant loss of PA association with ATR confirming the significance of this epitope in eliciting a neutralizing immune response. This epitope also overlaps with the predicted linear epitope described by Abboud and Casadevall (6). These data confirm that both epitopes are sufficiently antigenic to elicit a consistent immune response across various species and with varying immunogens. The fact that I have confirmed one epitope as being recognized by a highly neutralizing MAb supports the use of this epitope as a reliable correlate of immunity for anthrax infection.

In fact, the domain 2 linear epitope has been used to develop a competitive screening assay for evaluating the specific reactivity of human anti-AVA immune serum (44). Incorporating multiple peptides into a rapid correlate of immunity screening assay will give researchers an additional tool to evaluate potential vaccine candidates prior to moving into costly animal efficacy experiments.

**Applicability to Other High Priority Toxins**

The HHS Select Agents and Toxins list is comprised of 31 agents, 11 of which are toxins (5). These toxins are considered high priority due to their extreme toxicity, ease of production from natural sources (e.g.: plants, bacteria and fungi) and the lack of available
treatments or therapeutics. The select agent toxins include the plant-based toxins ricin and abrin, the bacterial-associated botulinum neurotoxins, epsilon toxin, shigatoxins, and staphylococcal enterotoxins. Several of the listed toxins are also A/B type toxins like the anthrax toxins investigated here. Although the mechanisms of action are highly variable amongst these toxins, the tools I have presented should be broadly applicable to lay out a roadmap for the systematic investigation of the critical epitopes involved in immunity to other toxins.

The recent identification of 70 linear epitopes in *C. botulinum* neurotoxins (BoNT) supports this suggestion (125). Generation of a vaccine against *Clostridium botulinum* is inherently difficult to manufacture due to the multitude of distinct toxins types (A-G) that can affect humans. Similar to the anthrax toxins, *C. botulinum* neurotoxins (BoNT) belong to the classic A/B family; however, these toxins are expressed a single peptide which are then cleaved into the separate A and B subunits (14). It is an exciting possibility that 70 linear epitopes may exist on BoNT since this could provide a new avenue for the development of vaccines and novel therapeutics. Investigations into the key antigenic determinants for the various BoNT toxins will enable researchers to specifically target next generation vaccine candidates, as proposed here for the anthrax toxins.

**Conclusions**

I have identified and vigorously characterized two novel epitopes on domain 4 of anthrax PA. These epitopes are recognized by MAbs 1-F1 and 2-B12, which have been shown to be protective against lethal mouse challenge with LT. This is the first comprehensive description of novel domain 4 epitopes that are involved in eliciting a
neutralizing immune response following PA immunization. Characterization of domain 4 epitopes is critical to understanding the key residues on PA that are involved in protective immunity to anthrax toxin, and have important implications in evaluating the efficacy of second generation anthrax vaccines. My findings provide anthrax researchers with several novel tools that will simplify future investigations of second-generation vaccine candidates and redefine the current concept of anthrax correlates of immunity.
Reference List


