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Defensive strategies of *Bacillus anthracis* that promote a fatal disease

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Abstract

Bacillus anthracis is a Gram-positive bacterium that causes anthrax. Bacterial spores that enter the host germinate into metabolically active bacilli that disseminate throughout the body and replicate to high numbers. Two virulence factors are essential for this unrestrained growth. The first is a weakly immunogenic poly γ -D-glutamic acid capsule that surrounds the bacilli and confers resistance to phagocytosis. The second virulence factor, anthrax toxin, disrupts multiple host functions to diminish the immune response.

INTRODUCTION

The *B. anthracis* spore is the metabolically inactive form of the bacterium, which can reside in the environment for years before it enters a mammalian host and initiates an anthrax infection. The type of anthrax that ensues - cutaneous, gastrointestinal, or inhalational - is determined by how the spores enter the body [1]. Cutaneous anthrax, the most common form of disease in humans, occurs after spores breach the skin layer through pre-existing lesions. Gastrointestinal and inhalational anthrax are caused by the ingestion or inhalation of spores, respectively, and are more likely to become systemic than cutaneous anthrax is. Systemic disease is characterized by large numbers of extracellular bacilli in the blood and is almost always fatal if left untreated. The success of *B. anthracis* as a pathogen can be attributed to two major virulence factors that protect the bacilli from the host immune response, a polyglutamic acid capsule and a protein toxin.

ANTHRAX TOXIN

The three proteins that comprise anthrax toxin are secreted individually by the bacilli and associate extracellularly into complexes that intoxicate mammalian cells (Fig. 1) [2]. Protective antigen (PA) is the toxin component that binds cells directly and forms a platform onto which the enzymatic proteins edema factor (EF) and lethal factor (LF) assemble. Blocking steps in the assembly process has become a major strategy for developing novel therapeutics (Table 1).

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Toxin Assembly

PA binds cells through the cellular receptors ANT XR1 and ANT XR2 [3]. These two receptors are structurally similar, containing a cytosolic domain, a single transmembrane domain, and an extracellular I domain that binds PA. I domains, characterized by a single β -sheet surrounded on either side by α -helices, are present in cell adhesion molecules and often serve as protein-protein interaction modules. Interactions are usually mediated through a divalent cation bound to the I domain by residues that make up a metal-ion dependent adhesion site (MIDAS) motif. Structural and biochemical studies indicate that the MIDAS metal is coordinated directly by an acidic residue in PA [4], which suggests that PA competes with natural extracellular matrix ligands for binding ANT XR1/2.

Receptor-bound PA is proteolytically cleaved at a single site by furin or a furinlike protease, which allows the amino-terminal PA₂₀ fragment to dissociate from the carboxy-terminal PA₆₃ fragment and for PA₆₃ to heptamerize on the cell surface. The sole purpose of PA₂₀ is to prevent the premature oligomerization of PA₆₃, as this would likely inhibit its release from the bacterial cell. Processing of PA and oligomerization of PA₆₃ also occurs prior to the binding of PA to cells [5]. The extent to which heptamerization of PA₆₃ occurs in the blood as compared to on the cell surface may be influenced by the concentrations of toxin and host serum proteases. Whether PA₆₃ oligomerizes in the blood or on cells, however, probably does not affect the subsequent steps of toxin assembly and internalization.

Monomers of PA₆₃ self-assemble into a ring-shaped heptamer, the [PA₆₃]₇ prepore, that binds EF and LF with nanomolar affinity [6]. The PA-binding domains of EF and LF are structurally similar and recognize sites on the upper surface of the heptamer that span two PA₆₃ monomers; the requirement of two monomers to form a single binding site limits the number of molecules that can interact simultaneously with the heptamer to three. Thus, a single heptamer can bind one to three molecules of EF alone, LF alone, or combinations of EF and LF.

Toxin Internalization

Monomeric PA remains on the cell surface, whereas [PA₆₃]₇ is rapidly internalized, indicating that toxin oligomerization triggers endocytosis of the receptors. Both ANT XR1 and ANT XR2 are palmitoylated on cysteine residues in their cytoplasmic domains, which appears to retain the receptors on the cell surface [7]. Oligomerization of PA₆₃ clusters the receptors to which they are bound, and by a mechanism that remains unclear, the receptors become depalmitoylated and enter lipid rafts. The raft-associated receptors are ubiquitinated by Cbl and then internalized by clathrin-dependent endocytosis [7]. A co-receptor, LRP6, may be involved in toxin uptake [8], although a recent study suggests that it is not required [9].

The toxin is trafficked first to vesicular regions of early endosomes and then to intraluminal vesicles of multivesicular bodies [10]. It is within acidic compartments only that [PA₆₃]₇ inserts into the membrane because protonation of [PA₆₃]₇ (and possibly the receptors) breaks receptor-[PA₆₃]₇ interactions that hold [PA₆₃]₇ in an uninserted form. This clamping of [PA₆₃]₇ by ANT XR1 is disrupted at pH ~6, which would be encountered in an early endosome, allowing [PA₆₃]₇ to penetrate the membrane and the enzymatic moieties to cross it. A lower pH (pH ~5) is required to induce ANT XR2-associated heptamers to insert into the membrane because ANT XR2 binds PA₆₃ more tightly than ANT XR1 does [11]. Intraluminal vesicles then fuse with the limiting membrane to release the translocated enzymatic moieties into the cytosol. Some studies suggest that following translocation LF disperses throughout the cytosol, whereas EF remains associated with the endosomal membrane [12].

Translocation

The membrane-inserted form of [PA₆₃]₇, termed the pore, is the conduit through which EF and LF translocate the membrane. Conversion of the prepore to the pore requires segments from the outer surface of the ring of the [PA₆₃]₇ prepore to rearrange into a 14-stranded β -barrel that extends below the ring and traverses the endosomal membrane. The ~100 Å long β -barrel aligns with the lumen of the ring to form a water-filled channel through which the enzymatic moieties pass. The channel narrows to an inner diameter of ~15 Å within the β -barrel, which would allow only α -helices or unfolded polypeptide chains to be transported through.

EF and LF sit atop the pore and extend their flexible, positively charged amino-terminal segments of ~27 residues into the negatively-charged opening of the lumen [13]. The acidity of the endosome causes the enzymes to adopt a molten globule state, a compact folding intermediate with a dynamic tertiary structure. The proton gradient across the endosomal membrane is thought to drive the amino to carboxy terminal threading of the enzymatic moieties through the channel of the pore [14]. The pore is not, however, a passive channel through which the enzymatic moieties are electrophoresed. Seven phenylalanine residues that extend into the channel, collectively referred to as the phe-clamp, are critical for translocation [15]. The phe-clamp is thought to function as a chaperone, binding sequential hydrophobic sections of the translocating polypeptide to keep the polypeptide in an unfolded, translocation-competent state. The phe-clamp may also form a seal that prevents the proton gradient from dissipating before the translocation process has completed. As the translocating polypeptide enters the higher pH compartment, anionic sidechains deprotonate, which serves to prevent back-translocation of negatively-charged segments of the polypeptide through the cation-specific pore. This charge-state Brownian ratchet mechanism would be reinforced by the refolding of translocated segments into structures too large to fit back into the channel [14]. This model is sufficient to explain the translocation process without the requirement of mammalian accessory factors, although it is possible that cytosolic factors facilitate the process *in vivo*, as has been observed for other toxins [16].

Edema Toxin

EF is a calcium and calmodulin dependent adenylate cyclase. The protein can be divided into two functional domains, an amino-terminal domain that binds [PA₆₃]₇ and a carboxy-terminal catalytic domain. Calmodulin, a mammalian calcium-sensor, binds the catalytic domain and causes structural rearrangements in EF that allows its interaction with the ATP substrate. Catalysis of ATP to cAMP occurs through a two-metal-ion mechanism that is similar to the mechanism used by mammalian adenylate cyclases even though there is little structural similarity between EF and the mammalian enzymes [17]. The catalytic rate of EF, however, is two to three orders of magnitude greater than that of mammalian adenylate cyclases.

Edema, an excessive accumulation of fluid in tissues, is elicited by edema toxin through an increase in vascular permeability. This increase in vascular permeability does not appear to occur through a direct effect of edema toxin on endothelial cell function, however, but through the stimulation of multiple inflammatory mediators [18]. Inhibition of neurokinins, prostanoids and histamine reduce the vascular leakage caused by edema toxin, although the cell types that release these mediators have not been identified. It is possible that these mediators are also involved in contributing to other pathologies associated with edema toxin. Fluid loss, for instance, may contribute to cardiovascular dysfunction and mortality.

Injection of purified edema toxin into mice causes death preceded by hypotension and reduced heart function [19]. The cause of death is not fully understood, but likely involves direct killing of cells by edema toxin and indirect effects, such as the induction of proinflammatory molecules. Intoxicated animals exhibit general organ damage, and in particular, animals

consistently display necrotic lesions in the cortex of the adrenal glands [19]. Disruption of adrenal gland function could have diverse detrimental effects on the host.

In addition to these gross pathological effects, and perhaps of more importance to the pathogen, are the effects of edema toxin on the immune response [20]. Edema toxin has been shown to inhibit chemotaxis, kill macrophages, reduce phagocytosis by neutrophils and inhibit NADPH oxidase activity - these effects would protect bacilli from destruction by phagocytic cells. Furthermore, edema toxin reduces expression of cytokines by a variety of cell types, which would impair both the innate and adaptive immune responses to infection [20].

Lethal Toxin

LF is a zinc-dependent protease; the amino-terminal domain binds [PA₆₃]₇ and the carboxy-terminal domain contains an HEXXH motif involved in zinc coordination and catalysis. LF cleaves amino-terminal segments from mitogen activated protein kinase kinases (MAPKKs) 1-4, 6 and 7. There is not a strict cleavage motif that LF recognizes, although there is a preference for a hydrophobic residue immediately carboxy-terminal to the scissile bond and basic residues at several positions amino-terminal to the cleavage site [21]. It is currently not known whether LF cleaves other proteins, or whether the cleavage of the MAPKKs accounts for all of the pathological effects caused by lethal toxin.

One of the effects of lethal toxin that contributes to immune evasion is its killing of macrophages and dendritic cells [22]. Intoxicated macrophages and dendritic cells can undergo either apoptosis or cytolysis depending on the source of the cells. The strain-specific difference in how murine macrophages are killed by lethal toxin has been attributed to different alleles of *nalp1b* - lethal toxin causes macrophages that express functional Nalp1b to lyse within a few hours of exposure and those that express mutant Nalp1b to undergo apoptosis [23]. Nalp1b is a member of the NOD like receptor (NLR) family, which comprises cytosolic proteins that detect microbial products or possibly other signals that indicate that the integrity of the cell has been compromised. Lethal toxin activates Nalp1b, probably indirectly, which leads to the assembly of an inflammasome platform that activates pro-caspase-1. Caspase-1 processes the inflammatory cytokines IL-1 β and IL-18; its activity, though possibly not the processing of these cytokines, is required for cytolysis [23]. A recent study by Kim and colleagues indicates that the BH3-only proteins BNIP3 and BNIP3L are also involved in this process [24]. These proteins contain a single transmembrane domain that localizes them to mitochondria; activation causes the mitochondria-associated proteins to insert into the membrane, which can lead to cell death or autophagy, perhaps depending on the activation signal and cell type. The mechanism by which the Nalp1b inflammasome activates BNIP3 and BNIP3L is not known.

In addition to killing some types of cells, lethal toxin facilitates pathogenesis by a number of different mechanisms [25]. As might be expected from a toxin that shuts down signaling through three MAPK pathways (ERK, p38 and JNK), lethal toxin affects a variety of cell types in several ways that could contribute to disease progression and host death. The non-cytotoxic effects of lethal toxin can be divided into three broad categories. First, lethal toxin diminishes the expression of secreted proteins that coordinate the immune response (cytokines and chemokines) or have antimicrobial activity (antibodies, type II-A phospholipase A₂). The inhibition of expression of these genes can occur through decreased transcription or through decreased stability of transcripts containing AU-rich regulatory elements. Second, lethal toxin has the potential to alter immune cell populations by inhibiting proliferation (monocytes, T cells, B cells), surface receptor expression (T cells and dendritic cells) and differentiation (monocyte to macrophage). Third, lethal toxin reduces the bactericidal ability of neutrophils by impairing chemotaxis and superoxide production.

The means by which lethal toxin dampens the immune response appears unrelated to how lethal toxin kills the host. Animals that die from the toxin exhibit hypotension and multi-organ failure, which is not caused by macrophage-killing or cytokine-mediated shock [26]. Hypotension may be a result of increased vascular permeability - lethal toxin has been reported to induce endothelial barrier dysfunction and endothelial cell death. A direct affect of lethal toxin on cardiac function may also contribute to hypotension and host death [27].

CAPSULE

The second major virulence factor of *B. anthracis* is the capsule that surrounds the bacillus [28]. The capsule consists of unbranched chains of poly γ -D-glutamic acid (PDGA) that are covalently attached to the peptidoglycan layer. The polymer chains have molecular weights in excess of 400 kDa and are largely unstructured, although they may form helices under certain conditions [28,29]. PDGA differs from cellular proteins in that it is composed solely of the D enantiomer of glutamic acid, instead of L amino acids, and that the linkage between residues occurs through the γ (side-chain) carbon rather than through the α carbon. Thus, a non-ribosomal synthetic pathway is required to make PDGA.

The capsule biosynthetic operon, *capBCADE*, encodes the proteins required for the synthesis, transport and surface-attachment of PDGA. Although there has been little research into the biochemistry of *B. anthracis* capsule production, some insight has been obtained from genetic studies and from comparisons with related systems. CapB, CapC, CapA and CapE form a membrane-bound complex that is necessary and sufficient to synthesize PDGA. Candela and Fouet have proposed that CapB and CapC are the components of the complex that polymerize PDGA [28]. CapB is a member of the tetrahydrofolate synthase family; it contains a Walker A (ATP-binding) motif and is predicted to use ATP to ligate glutamic acid onto PDGA. Although the exact function of CapC is unknown, it functionally interacts with CapB to facilitate ATPase activity. CapA and CapE genetically interact and are proposed to be the components of the CapBCAE complex that transport the cytoplasmically-synthesized PDGA out of the cell [28].

PDGA that is transported from the cell is covalently attached to peptidoglycan by CapD [30]. CapD is a surface-associated γ -glutamyltranspeptidase, and similar to other γ -glutamyltranspeptidases, CapD is cleaved into two subunits that remain bound to one another after the cleavage event has occurred. Cleavage is required to activate some γ -glutamyltranspeptidases, but this requirement for activity has not yet been determined for CapD. Members of the γ -glutamyltranspeptidase family transfer γ -glutamyl groups to amines (e.g. amino acids or peptides) or water. It has been proposed that CapD attaches PDGA to meso-diaminopimelate in *B. anthracis* peptidoglycan [28]. CapD is also able to use water as an acceptor, which accounts for its ability to depolymerize PDGA. This depolymerization activity of CapD could be exploited as an approach to treating anthrax (Table 1).

The capsule contributes to pathogenesis by helping the bacterium evade the immune response. The structure of the capsule is poorly recognized by the immune system and also serves to shield surface components that are more readily recognized. As a result of these properties, the capsule inhibits phagocytosis, blocks antimicrobial peptides, and reduces immunogenicity. This allows capsulated bacilli to disseminate through the host, while noncapsulated bacilli are destroyed [31].

SUMMARY AND CONCLUSIONS

The severity of anthrax infections can be attributed to two virulence factors expressed by *B. anthracis* that protect the bacteria from being killed, thereby allowing them to grow to overwhelming numbers. As each of these factors can potentially contribute to pathogenesis at

more than one stage of disease and counteract multiple immune defences, it is not a trivial problem to determine how and when the toxin and capsule contribute most to disease progression. Anthrax toxin in particular has been shown to elicit a number of effects that might contribute to one or more stages of disease; other observed effects might not contribute at all. Intoxication of macrophages facilitates escape of intracellular bacilli early in the infection and later could protect the bacteria from phagocytosis. Other toxin effects that have been observed, however, may not aid bacterial survival at any stage of disease or cause disease-associated pathologies; these inconsequential outcomes presumably occur as a side effect of the toxin producing an “intended result” that confers an advantage to the bacteria. For example, the downregulation of some genes not involved in the immune response might occur as a result of the “intended” inhibition of cytokine expression. It has also been noted that certain effects of the toxin may not be important in all infections - disruption of dendritic cell function may influence the outcome of cutaneous anthrax, but might not affect the progression of inhalational anthrax [20]. Thus, unraveling the complexities of anthrax toxin will require continued careful research with particular attention to how the experimental techniques and models used can inform us of natural infections.

The capsule has a more passive, perhaps simpler, but no less important role in pathogenesis than does the toxin. Comprised of monotonous anionic polymers, the capsule acts as a stealth shield that protects the bacilli from being phagocytosed. The capsule might also be an important means of blocking antibodies, antimicrobial peptides and complement. While there is a good understanding of capsule function, our knowledge of the biochemistry of capsule synthesis remains limited. This is unfortunate because the enzymatic machinery responsible for the capsule could be an attractive therapeutic target.

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Figure 1.

B. anthracis is surrounded by an antiphagocytic poly γ -D-glutamic acid capsule. The bacilli secrete the three components of anthrax toxin: edema factor (EF), lethal factor (LF), and protective antigen (PA). PA binds anthrax toxin receptor (ANTXR) 1 or ANTXR2. Furin cleaves PA molecules and the PA₆₃ fragments oligomerize into a [PA₆₃]₇ prepore, which enters a lipid raft and binds EF and LF. The toxin complex is internalized by clathrin-mediated endocytosis. It is trafficked first to an early endosome and then to a multivesicular body. The acidic environment converts the [PA₆₃]₇ prepore into a membrane-inserted pore and causes EF/LF to translocate across the membrane. The intraluminal vesicle that contains EF/LF fuses with the limiting membrane of the multivesicular body and EF/LF are released into the cytosol. EF is an adenylate cyclase and LF is a protease that cleaves mitogen activated protein kinase kinases (MAPKKs).

Table 1Strategies that target *B. anthracis* or anthrax toxin

	Strategic Approach to Target	Expected outcome of intervention at target	Who is working on the target	References
protective antigen	block binding to cells; inhibit translocation	decrease pathologies associated with lethal and edema toxins	Collier Georgiou Kane Manchester Mogridge Young	[32-35]
lethal factor	inhibit enzymatic activity	decrease pathologies associated with lethal toxin	Merck Research Laboratories Montecucco	[36,37]
edema factor	inhibit enzymatic activity	decrease pathologies associated with edema toxin	Tang	[38]
anthrax toxin receptors	block toxin-binding	decrease pathologies associated with lethal and edema toxins	Kane Mogridge	[39]
furin	inhibit enzymatic activity	decrease pathologies associated with lethal and edema toxins	Leppla Lindberg Strongin	[40,41]
capsule	depolymerize capsule	increase susceptibility of bacteria to phagocytosis	Friedlander	[42]
bacterial cell wall	cleave peptidoglycan	lyse bacteria	Fischetti	[43]