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Anthrax toxin: pathologic effects on the cardiovascular system

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1. ABSTRACT

Anthrax is a disease caused by infection with spores from the bacteria *Bacillus anthracis*. After entering the body, the spores germinate into bacteria and secrete a toxin that causes local edema and, in systemic infections, cardiovascular collapse and death. The toxin is a tripartite polypeptide, consisting of protective antigen (PA), lethal factor (LF) and edema factor (EF), which have key roles in the bacterial pathogenesis and disease progression. PA facilitates transfer of LF and EF to the cytosol. Lethal toxin is a zinc metalloproteinase, which has the capacity to inactivate mitogen-activated protein (MAP) kinase kinase (MEK) and stimulates the release of sepsis-related cytokines tumor necrosis factor-alpha and interleukin-1beta. Edema factor is a calmodulin (CaM)-dependent adenylate cyclase, which increases levels of cyclic AMP, causing impaired neutrophil function and disruption of water balance that ultimately results in massive tissue edema. Together, the toxins effectively inhibit host innate and adaptive immune responses, allowing the bacteria to grow unrestrained and overwhelming any resistance. Clinically, inhalational anthrax presents in a biphasic pattern with initial nonspecific "flu-like" symptoms nausea and vomiting 1 to 4 days after exposure, followed by severe illness with dyspnea, high fever and circulatory shock. The latter symptoms represent a terminal stage and treatment is often ineffective when started at that time. Key indicators of early anthrax cardiovascular-related pathogenesis include mediastinal widening in association with pleural effusion and edema. In this review, we describe the current understanding of anthrax toxins on cellular function in the context of cardiovascular function and discuss potential therapeutic strategies.

2. INTRODUCTION

Fatalities associated with inhalational *Bacillus anthracis* during the 2001 bioterrorism attacks spurred a significant amount of research to unravel the complexity of anthrax toxicity. Although effects of anthrax on the hematopoeitic system are well-documented, the specific effects of anthrax toxins on the heart and vasculature are poorly understood. Recent reports investigating the effects of anthrax toxin on the hearts of male Sprague-Dawley rats have reported decreased heart rate with increased left ventricular end-diastolic volume, reduced ejection fraction and decreased contractility. These effects are specifically mediated by anthrax toxin through intracellular signaling mechanisms within two hr post-toxin injection. Below, we discuss the biochemistry and clinical effects of anthrax toxicity and potential effects on the cardiovascular system.

3. STRUCTURE AND BIOCHEMISTRY OF ANTHRAX TOXINS

The anthrax toxin is a tripartite protein, composed of 83-kDa PA (PA_{83}), 90-kDa LF (1) and 89-kDa EF. These toxins conform to the AB model of bacterial exotoxins, where PA is the binding subunit and both LF and EF are alternative catalytic subunits. Separately, LF and EF have no known biological activity, although PA may stimulate cell migration. Lethal toxin (LT) is assembled from PA and LF and is believed to be primarily responsible for the acute effects observed in anthrax, while edema toxin (ET) is assembled from PA and EF and is believed to be responsible for the edematous lesions at the site of infection. These toxins are taken into the cell after PA interacts with its cellular receptor, which leads to receptor-mediated endocytosis followed by toxin translocation into the cytosol. LF has been shown to act as the key virulence factor for *B. anthracis,* since mouse death is seen in the absence of EF, but not in the absence of LF. Another virulence factor and potential target of therapeutic intervention involves the classification of *B. anthracis* as a microorganism that utilizes quorum-sensing signaling pathways to produce auto-inducers of the luxS-signaling system. The autoinducers sense population density and determine gene expression, such as the production of toxins (2).

3.1. Lethal factor

As its name implies, anthrax lethal factor (LF), is thought to be the major virulence factor responsible for impaired immunity, septic shock and death. LF is a zinc-dependent metalloproteinase and has four domains (Figure 1) (1). The N-terminal domain, domain I or LFn, corresponds to residues 1–263. Domain I binds PA_{63}, the membrane-translocating component of anthrax toxin (1). LFn is composed of a bundle of 12 α-helices and 6 β-strands making two sheets on one face of the molecule (3). Domain II (residues 263–550, excluding residues 300–386) is the only part of LF having structural similarities with other proteins. It resembles the fold of the ADP ribosyltransferase of the vegetative insecticidal toxin of *Bacillus cereus* (3). Domain III (residues 300–386) is an α-helical bundle made up of four imperfect sequence repeats from a feature of domain II. It is inserted in domain II and has functional significance in substrate recognition (4). The C-terminal domain, domain IV (residues 551–777), has the zinc protease site. The active site is similar to that of the thermolysin protease family with the HEXXH motif located in an α-helix neighboring a four-stranded β-sheet. The substrate binding site is a long cleft formed by domain II and parts of domains III and IV (5, 6). Domain IV also resembles domain I (1). The structure thus reveals a protein that has evolved through a process of gene duplication, mutation and fusion.

3.2. Edema factor

Edema factor (EF) is an important virulence factor of *B. anthracis.* EF is an active Ca^{2+} and CaM-dependent adenylyl cyclase consisting of an N-terminal PA-binding domain, a central catalytic core and a C-terminal helical domain (Figure 1) (7, 8). Once EF enters the cytosol and binds with CaM, it induces a prolonged increase in intracellular cAMP. This strategy has also been successfully evolved by several other bacteria (e.g. *Bordetella pertussis, Pseudomonas aerunginosa, Yersinia pestis*) to disrupt a wide array of physiological processes. EF has been shown to have ~1000 fold greater activity than eukaryotic forms of the enzyme (9). The N-terminal 300 amino acids of EF is homologous in sequence and structure to the corresponding domain from LF (10) and is

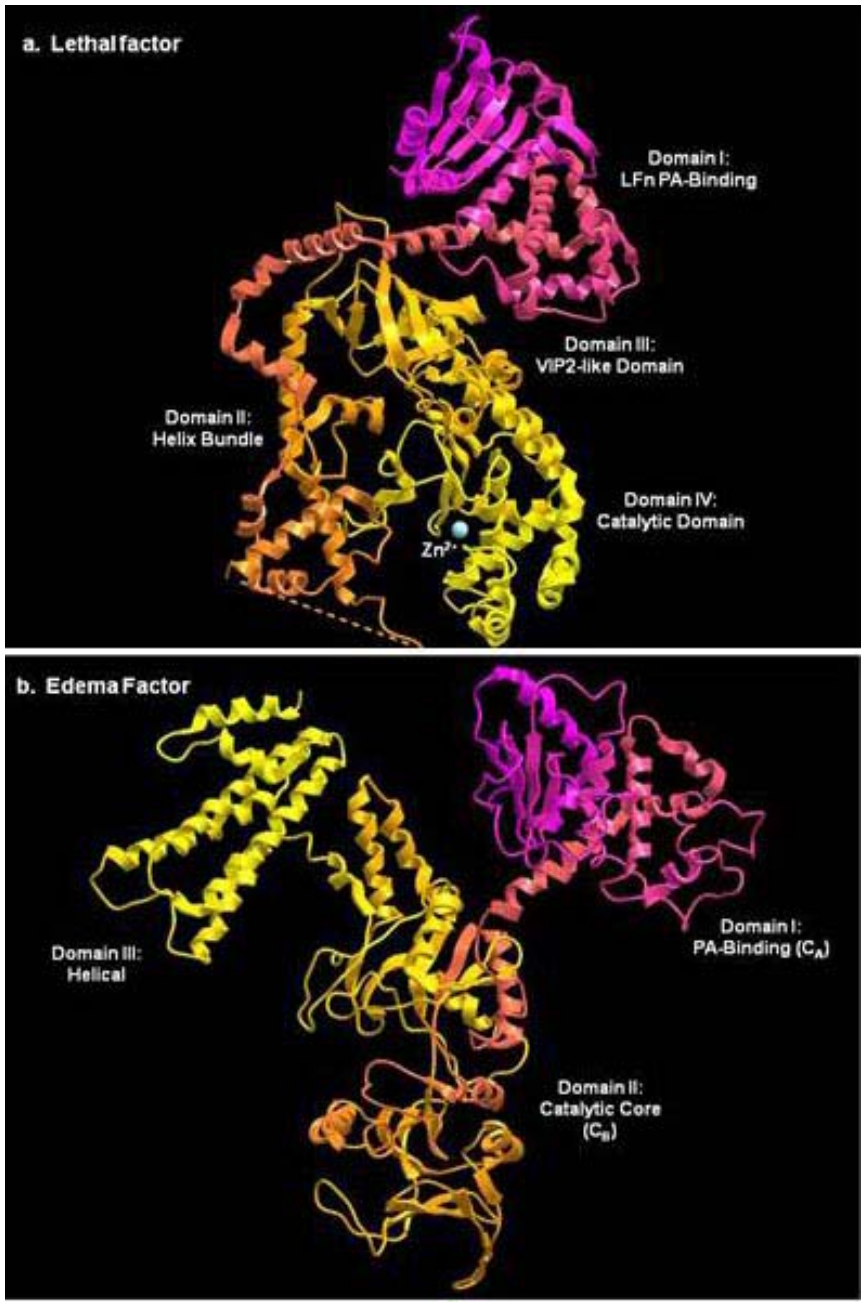

Figure 1. Structure of Anthrax Toxins. (a) LF is a zinc-dependent metalloproteinase with four domains. The N-terminal domain, domain I or LFn, corresponds to residues 1–263 and binds PA_{63}. LFn is composed of a bundle of 12 α-helices and 6 β-strands forming two sheets on one face of the molecule. Domain II (residues 263–550, excluding residues 300–386) is the only part of LF having structural similarities with other proteins. Domain III (residues 300–386) is an α-helical bundle that consists of four imperfect sequence repeats from a feature of domain II. It is inserted in domain II, and it has functional significance in substrate recognition. The C-terminal domain, domain IV (amino acids 551–777), includes the zinc protease site. (b) EF is an adenylyl cyclase, which requires activation by calmodulin. The protein is 800 amino acids (minus 33 a.a. signal sequence) in size. A truncated form of the enzyme lacking the amino-terminal 290 amino acids is sufficient to reproduce the calmodulin-activated adenylyl cyclase activity and has been used to elucidate the structure and activation mechanism of the toxin. EF is comprised of three globular domains. The amino acid chain of domain I (termed C_A) is the PA-binding domain, and is homologous to the PA-binding LFn domain of LF from amino acids 10 through 280 of LF and 50 through 271 of EF. Domains I and II, termed C_A and C_B, respectively, form the catalytic core of the enzyme. The carboxy-terminal portion of C_A is connected by a linker to domain III. A critical aspect of EF activation is opening of the interface between domains I and III by calmodulin binding. The molecular structures for LF and EF were adapted from PDB ID 1J7N (1) and 1XFY (7), respectively.

considered to be the domain that binds to PA_{63}. EF has a large proportion of highly charged amino acid residues at the N-terminus. Recently it has been reported that alanine substitution of residues Tyr^{137}, Tyr^{138}, Ile^{140} and Lys^{142} resulted in defective binding of PA_{63} to EF, and is thus incapable of inducing cAMP toxicity in CHO cells (11). The catalytic domain of this enzyme begins immediately following the binding domain, and lies entirely within the 262-767 sequence. The catalytic activity of EF requires association of the eukaryotic calcium binding protein, calmodulin (CaM). However, the Ca^{2+}/CaM complex is a much more potent activator of EF than CaM alone. The catalytic domain of EF shares homology with other bacterial adenylyl cyclase toxins, including *Bordetella pertussis* CyaA and *Pseudomonas aerunginosa* ExoY. In an inactive state, the unique helical domain of EF is arranged against the catalytic core. CaM inserts between the helical and catalytic domains in an extended conformation, separating them and inducing a 30° rotation of the two domains relative to one another. The extensive interactions between CaM and EF induce conformational changes in the linker region between the two domains and within two regions of the catalytic domain. These interactions correctly position many of the residues involved in substrate binding, thus activating the enzyme.

4. ANTHRAX TOXIN RECEPTORS

4.1. Basic structure of TEM8 and CMG2 receptors

B. anthracis uses two different α-integrin-like cellular receptors (12) to mediate anthrax toxin entry to the cells: anthrax receptor 1 (ATR1), also called tumor endothelium marker 8 (TEM8) (13) and ATR2, also known as capillary morphogenetic protein 2 (CMG2) (14). Like many α-integrins, the extracellular portions of TEM8 and CMG2 have a single Von Willebrand factor A (VWA) domain, which contains the typical metal ion-dependent adhesion site (MIDAS) motif. This site mediates specific binding to collagen IV and laminin (15, 16), suggesting that TEM8 and CMG2 function as adhesive molecules mediating cell and extracellular matrix adhesion. However, recent identification of CMG2 mutations as the causes of two related human conditions, juvenile hyaline fibromatosis (17) and infantile systemic hyalinosis (18), indicates that TEM8 and CMG2 have different functional roles.

4.2. Expression and function of TEM8 (ATR1)

The TEM8 gene is expressed as three alternatively spliced transcripts that share a common extracellular domain. These have been characterized as long (TEM8, 564 amino acids), medium (ATR1, 368 amino acids) and short (333 amino acids) isoforms (13). The long and medium isoforms are transmembrane proteins which contain I-domains and function as PA receptors. The extracellular domain of the short isoform is identical to long and medium isoforms, but lacks a transmembrane domain. Thus, the short putative secreted form does not appear to function as a PA receptor. TEM8 is selectively and highly expressed in the epithelial cells lining organs that represent the major uptake routes of *B. anthracis* spores. Increased sensitivity of certain organs, such as lung, skin and intestine to anthrax infection correlates with high expression levels of TEM8 receptor (19), suggesting that the initial entry sites of the bacterium also represent sites for increased anthrax toxin uptake. High levels of anthrax toxin receptors on lung epithelium may be responsible for edema formation and the accumulation of pleural fluids. The long isoform has been shown to be predominantly expressed in the lung, spleen and kidney and to a lesser extent in the heart and ovary (19). The medium isoform is absent from the lung, but highly expressed in the small intestine and to a lesser extent in the skin and liver. The short isoform is highly expressed in the heart, brain and kidney, but its significance remains to be determined.

4.3. Expression and function of CMG2 (ATR2)

The CMG2 gene is located on human chromosome 4q21, and recent analysis of CMG2 cDNAs and the genomic sequence shows the presence of a 1.46 kb open-reading frame, which codes for a 488 amino-acid protein (18). TEM8 is the only known paralog of CMG2, which shows 56% overall amino acid identity (14). Sequence comparison of CMG2 and TEM8 indicates that the highest level of conservation (80%) occurs at residues 338–421, which are in the intracellular region of the protein. Semi-quantitative analysis using reverse-transcriptase polymerase chain reaction shows expression of the CMG2 transcript in all tissues, except brain. Although the precise function of CMG2 receptor remains to be determined, this protein appears likely to have an important role in endothelial cell function and maintaining the integrity of the basement membrane matrix. This is evidenced by marked upregulation of CMG2 in endothelial cell morphogenesis (15) and the deposition of hyaline membrane material between the endothelial cells and pericytes in juvenile hyaline fibromatosis and infantile systemic hyalinosis (17, 18), presumably due to leakage of plasma components through the basement membrane into the perivascular space. Subsequent studies will be required to better understand the role of CMG2 in endothelial cell function and how this may be altered by PA alone and in combination with anthrax toxins LF and EF.

4.4. Ligand binding and anthrax receptor function

The soluble extracellular domain of TEM8 binds to collagen type 1 and gelatin (20) and can bind cleaved C5 domain of α_3 (VI) collagen chains (16). *In vitro*, TEM8 mediates cell spreading on immobilized PA and collagen I (21). The cell spreading on PA occured in the presence of α_1 integrin blocking antibodies, suggesting that TEM8 function is independent of α_1 integrins. CMG2 is markedly induced during capillary-tube formation in collagen gels and has a more ubiquitous expression pattern than TEM8 (15). The CMG2 extracellular domain shows binding activity to collagen type IV and laminin (15). Although TEM8 and CMG2 can interact with specific molecules of the extracellular matrix, the physiological consequences of this adhesion process remain to be determined. TEM8 was identified by its increased expression in tumor-associated endothelial cells (22) and, therefore, proposed as a candidate molecule to target anti-tumor therapies (23).

Signaling pathways activated by PA binding to TEM8 and subsequent cellular migration are poorly understood. The cytoplasmic tail of TEM8 is much larger than that of other cell-surface tumor endothelial markers and contains several potential phosphorylation sites, suggesting that TEM8 may be involved in signaling pathways that regulate tumor-specific angiogenesis. Although PA triggers a pro-inflammatory response via toll-like receptors 2/6 heterodimers, these responses are not evoked when complexed with LF (24). This suggests that the heptamerization of PA and subsequent association with LF changes the conformation of PA and conceals epitopes that trigger an innate immune response.

4.5. LPR6 as a co-receptor for TEM8 and CMG2

Low-density lipoprotein-receptor-related protein (LRP6) has recently been shown to mediate internalization and lethality of anthrax toxin (25). LDL receptor-related proteins (LRPs) are macromolecules that interact with and deliver ligands into cells via endocytosis (26). LRPs serve as multifunctional receptors, which bind to lipoprotein lipase, apoE-rich remnant lipoproteins, α2-macroglobulin/protease complexes, plasminogen activator/inhibitor complexes, active protease tissue-type plasminogen activator, and the surface binding domain of exotoxin A from *Pseudomonas aeruginosa* (27). The LRP6 has been previously known to be a co-receptor for the Wnt signaling pathway. Wei and colleagues (25) demonstrated that downregulation of LRP6 or co-expression of a truncated LRP6 dominant-negative peptide inhibits cellular uptake of complexes containing PA and protects targeted cells from death, as did antibodies against epitopes in the LRP6 extracellular domain. However, the precise role of LRP6 in mediating binding and uptake of anthrax toxins is poorly understood and in some cases does not appear to be required for intoxication (28). Immunoprecipiation experiments reveal that LRP6 interacts with TEM8 and CMG2, but not PA (25). LRP6 initiates Wnt signaling when it enters cells together with other members of a Wnt-based complex (29). However, it remains to be determined whether LRP6 may function similarly in the heart and vasculature, as well as other organs, to promote internalization of PA and its receptors.

5. INTERNALIZATION OF ANTHRAX TOXINS

5.1. Protective antigen and internalization of lethal factor and edema factor

Internalization of anthrax toxin requires the presence of TEM8 or CMG2 receptors on the cell surface. A recent review describes toxin receptor binding, internalization, pore formation, and translocations of LF and EF in detail (30). The sequential order of PA heptamerization and ligand binding, as well as the exact mechanism by which anthrax toxin gains entry into the host cells, remain elusive. The current model of anthrax toxin self-assembly and entry into host cells includes initial binding of the full length 83-kDa PA (PA_{83}) to cell surface receptors followed by its cleavage by cellular furin-family proteases (Figure 2). A 20 kDa amino (N)-terminal fragment of PA (PA_{20}) dissociates into the extracellular medium and subsequently plays no further known role in the toxin action. The remaining carboxyl (C)-terminal, 63-kDa fragment (PA_{63}) then assembles into a homo-heptameric ring structure accompanied by the clustering of the receptors into lipid raft domains of the plasma membrane. Proteolytic release of the N-terminal fragment of PA exposes a high affinity binding site for the LF and EF enzymatic moieties on the surface of PA_{63}. Once assembled, the toxin complex is internalized by receptor-mediated endocytosis and is trafficked into the endosome via clathrin-coated pits. A stable complex between the PA heptamer and EF and/or LF contributes to the overall toxicity of the complex. The tight association between PA and EF or LF results in an enhanced effective concentration of the toxin on the cell surface and improved efficiency of internalization, allowing the binding of up to four molecules of EF or LF per PA heptamer. The acidic environment of the endosome induces the complexes to undergo a conformational transition from a prepore to a pore, leading to formation of a 14-stranded membrane-spanning β-barrel through which LF and EF are released into the cytosol (31). The pore formation occurs in early or late endosomes, depending on whether the toxin is bound to TEM8 or CMG2 (32, 33). EF and LF avoid transport to late endocytotic compartments containing hydrolases by inserting into membranes of intraluminal vesicles, from where they are released (34). For final release into the cytoplasm, anthrax toxin relies on back-fusion of the intraluminal vesicles with the limiting membrane (34). Once translocated into the cytosol, EF and LF catalyze reactions leading to toxic responses (30).

5.2. Molecular aspects of anthrax toxin internalization

Refinements of the major internalization steps have been investigated at the atomic, molecular, and amino acid residue levels using a combination of crystallographic and molecular modeling techniques. One study began with a 3.6 Å crystallographic model and utilized non-crystallographic replacement symmetry to build a 4.3 Å molecular model of the prepore complex, including key amino acid residues of PA with the CMG2 VWA domain and the locations of seven pore-forming loops that form the transmembrane β-barrel pore (35). Further studies utilized crystallographic analysis to identify amino acid residues of the CMG2 VWA domain at 1.5-1.8 Å resolution and of the metal ion-adhesion site (MIDAS) motif (DXSXS…T…D), where X is any amino acid (35). A 2.5 Å crystal structure of PA_{63} complexed with the CMG2 receptor was used to refine by molecular modeling the regions of interaction of PA with CMG2 to the atomic resolution. In addition, molecular dynamics simulation showed that low pH protonation of His^{121} and Glu^{122} of CMG2 breaks a salt bridge between PA and CMG2 to release PA to form a pore (36).

Structure-based mutagenesis studies have identified Gly^{153} and Leu^{154} in the β4-α4 loop of CMG2 I-domain as being responsible for the low pH requirement for pore formation of PA_{63}. From these studies, a space-filling atomic model of PA domain 2 residues in contact with a space-filling mesh overlay of CMG2 residues Asp^{152}, Gly^{153} and Leu^{154}, and additional projection of residues that may be involved in upstream contact was developed (37).

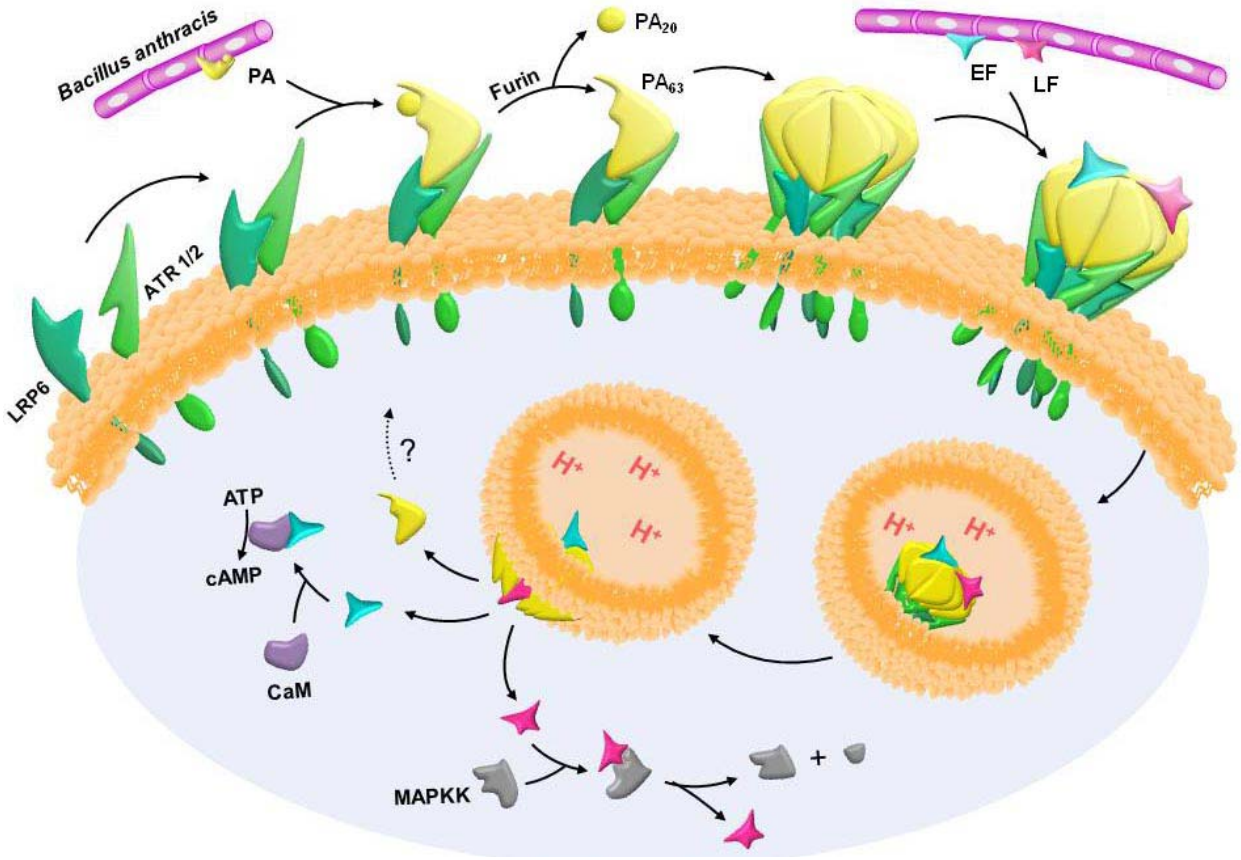

Figure 2. Internalization of Anthrax Toxins. PA is produced by *B. anthracis* as a protein of 764 amino acids (85.8 kDa). Cleavage of a 29-residue signal sequence releases the mature PA_{83}. In this state, PA is inactive, but has binding affinity for the TEM8/ATR receptor. PA_{63} heptamerization causes the receptors to cluster in detergent-resistant membrane microdomains (lipid rafts). Following lipid-raft association, anthrax toxin-receptor complexes are internalized through clathrin coated pits and trafficked to early endosomes. Binding to LF or EF is only possible after PA_{83} has been cleaved to PA_{63} by furin. LF is a zinc-dependent metalloproteinase of 809 amino acids (minus 33 a.a. signal sequence). In the cytosol, the proteinase cleaves dual specificity MEKs. Cleavage of MEKs occurs in the N-terminal proline-rich region preceding the kinase domain, which disrupts the protein-protein interactions necessary for the assembly of signaling complexes. EF is 800-amino acid adenylyl cyclase that binds calmodulin to induce cAMP second messenger activity.

Mutagenesis combined with chemical modifications identified a heptad of Phe^{427} residues on PA_{63} β loops that form the pore (Figure 3). ESR and single channel conductances showed that upon lowering the pH, these phenyl rings converged from over 15 Å to less than 10 Å apart. This arrangement allows the formation of a ~100 Å long by ~15 Å wide pore which contains a hydrophobic aromatic iris of phenyl rings all aligned at the same cross-section of the pore, to create a so-called "φ clamp", that would permit translocation of an α helical structure through the pore under the proper conditions. With its N-terminus leading, LF moves through the pore by nonspecific hydrophobic effects involving π-π and cation-π interactions allowing forward, but not backward, translocation by a Brownian ratchet motion (38). Therefore, following the incorporation of PA_{63}/LF/EF complexes via clathrin-coated pits into endosomes, LF and EF may escape into the surrounding cytosol by utilizing the mechanisms described above (30) (Figure 2). Once in the cytoplasm, EF binds to the N-terminus of calmodulin (CaM), locking it into a closed conformation, and triggers the insertion of the C terminus of CaM into EF with tight binding (Kd = 20 nM). In this configuration, His^{351} of EF can interact sufficiently close to the 3'OH of ATP (4-8 Å) to facilitate hydroxide-mediated deprotonation of this group, a step in the pathway to cAMP formation (7). As described in more detail below, cytoplasmic LF catalyzes the cleavage of MEKs to inactivate their kinase activity.

6. GENERAL EFFECTS OF ANTHRAX TOXICITY

6.1. Clinical presentation of anthrax toxicity

Known since ancient times, human anthrax disease begins with exposure to the spores of *Bacillus anthracis*. Spores vegetate, bacillus multiplies and its antiphagocytic capsule facilities local spread (Figure 4). The three major clinical anthrax syndromes are cutaneous, inhalational and gastrointestinal (39), which reflect the initial route of infection. Although each syndrome has unique clinical characteristics, each is associated with

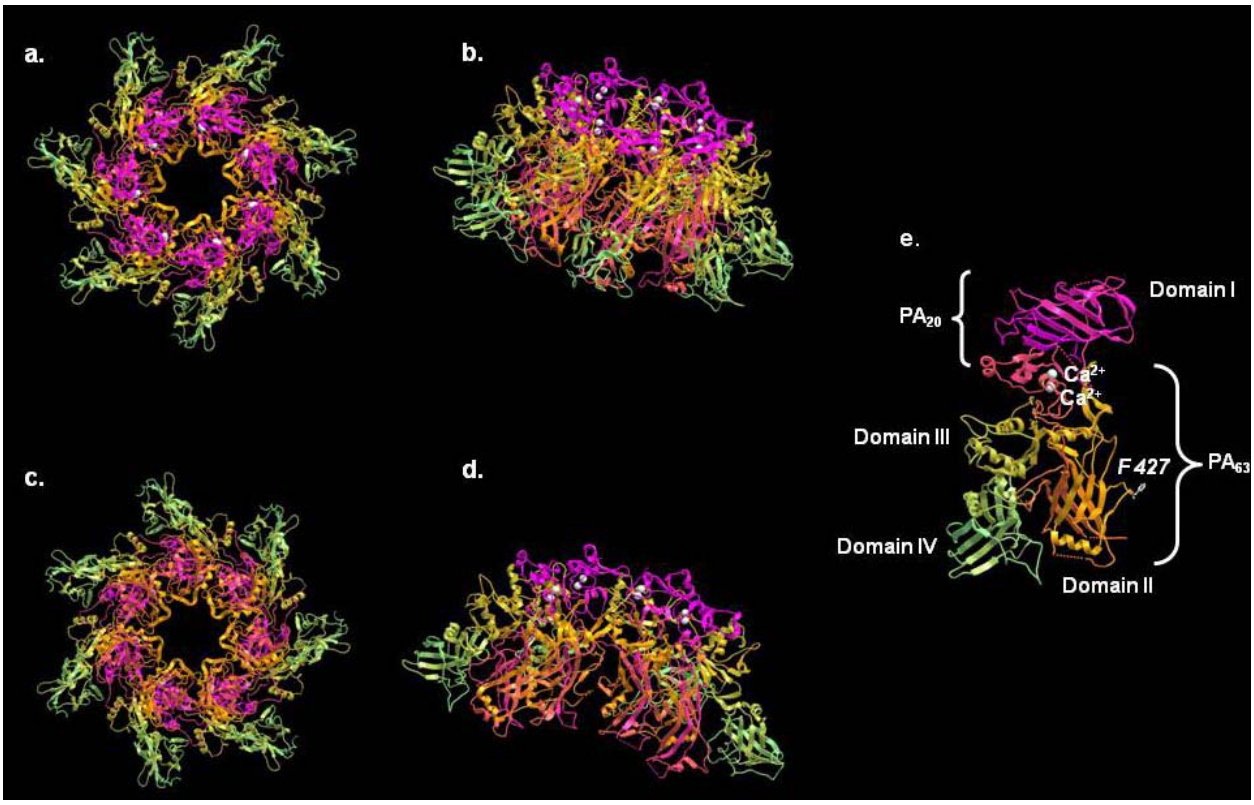

Figure 3. Structure of the Protective Antigen Prepore. The Protective Antigen (PA_{63}) prepore is composed of a heptamer of PA molecules, forming a central tunnel by which bound LF and EF are pulled by π- π interactions. (a) The top view of the pore (N-terminus) is where LF and EF bind at the surface of the PA heptamer. (b) The bottom view (C-terminus) binds to TEM8/ATR. (c) The side view of the PA_{63} pore shows the consistent heptameric structure and (d) the inner surface of the pore. PBD ID: 1TZO (35) (e) A single PA_{83} molecule with the phenylalanine residue shown in white that interacts with six other residues along the inner surface to form the "φ clamp". PA_{20} includes part of Domain I, the furin cleavage site, while PA_{63} is comprised of domains II, III and IV. Domain III may regulate oligomerization during pre-pore formation. Domains II and IV mediate binding to the cellular receptors TEM8/ATR. PBD ID: 1ACC (141).

severe cardiovascular symptoms in later stages.

6.1.1. Cutaneous anthrax

Cutaneous anthrax develops after spores of *Bacillus anthracis* are introduced subcutaneously, usually from contact with infected animals or animal products. Incubation period is typically one week, after which the first manifestation is a small, painless and often pruritic papule. The papule enlarges and develops a central vesica or bulla. Erosions then lead to a painless necrotic ulcer with a black, depressed eschar. Toxin release stimulates extensive edema with regional lymphadenopathy and lymphangitis. The cutaneous lesion can be associated with systemic symptoms of fever, malaise and headache. Untreated, the mortality may be as high as 20%. With antibiotic therapy, the case fatality rate is less than 1%.

6.1.2. Inhalational anthrax

Anthrax spores can be inhaled when working with animal products such as animal hides or hair. Anthrax spores less than 5 μm in diameter are deposited in the alveolar ducts. There they germinate and release toxins, causing hemorrhagic necrosis, into the thoracic lymph nodes draining the lungs and result in hemorrhagic mediastinitis. The incubation period during the 2001 bioterrorism event average 4.5 days. Early symptoms of inhalation anthrax are nonspecific, presenting a complex differential diagnosis that includes community acquired pneumonias, influenza, adenovirus, parainfluenza virus, pneumonic tularemia, histoplasmosis, legionnaire's disease, coccidiomycosis and malignancy. Descriptions of inhalation anthrax typically include patients presenting with cough, dyspnea, or chest pain and found to have abnormal lung examination results with pleural effusions or enlarged mediastinum. Consistent with the ability of lethal factor to cleave MEKs, clinical and experimental evidence indicates that an excessive inflammatory response does not contribute to shock with LT. Thus, the immunosuppressive effects of LT could promote infection, whereas direct effects on endothelial dysfunction may promote shock. Two fatal cases of inhalation anthrax were reported that had nonspecific prodromal illnesses (40). One patient developed predominantly gastrointestinal symptoms, including nausea, vomiting and abdominal pain. The other patient had a "flu-like" illness associated with myalgias and malaise. Both patients ultimately developed dyspnea,

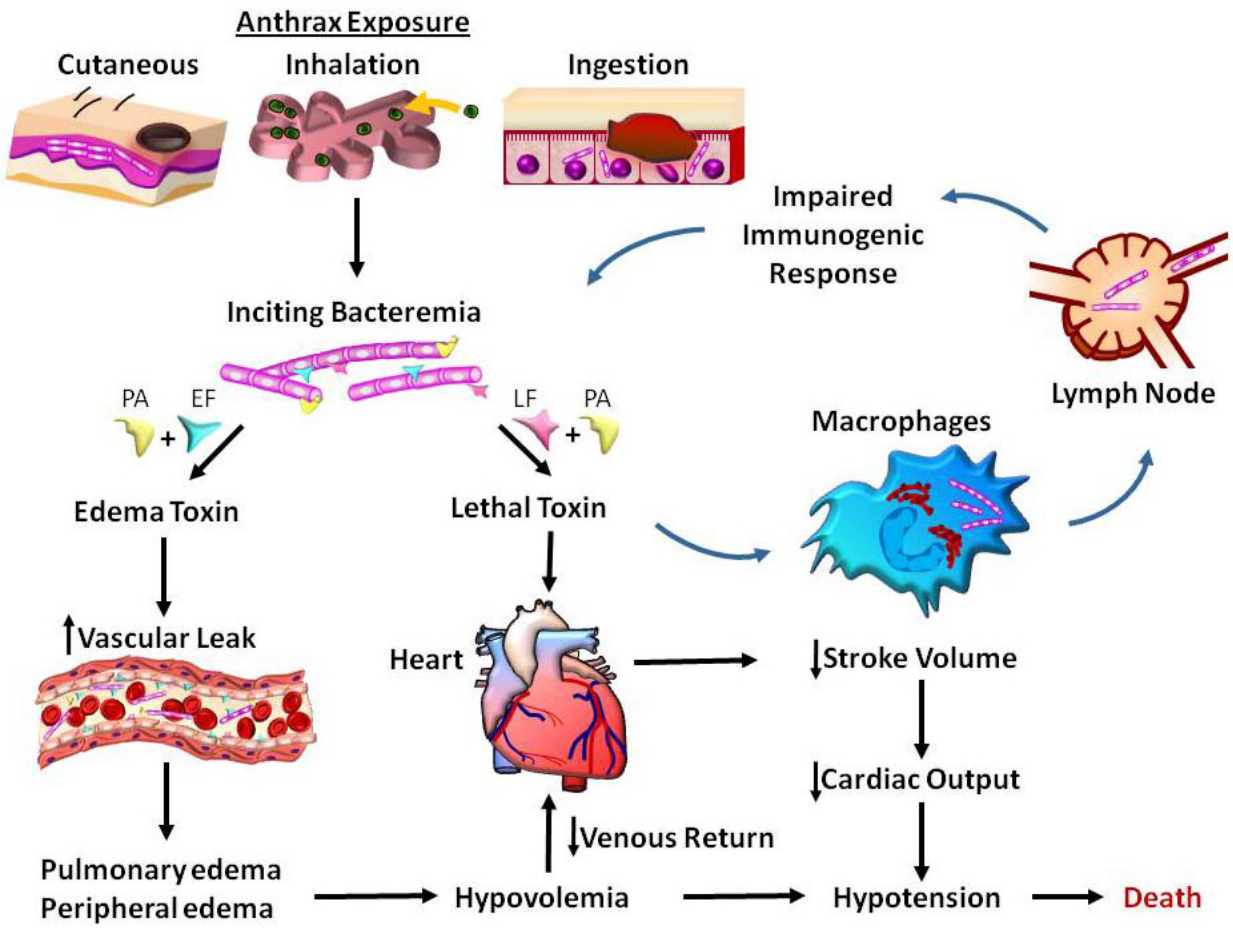

Figure 4. Cardiovascular Effects of Anthrax Toxin. Bacteremia is induced by inhalation, ingestion or cutaneous exposure to *B. anthracis* spores. Once inside the body, the spores germinate and produce bacterial virulence factors ET and LT. ET induces increased vascular leak, leading to pulmonary and peripheral edema and resulting in systemic hypovolemia. LT has a devastating effect on immunity, impairing the immunogenic response by destroying macrophages, which release the bacteria into lymph, and induce further spread of *B. anthracis* and septicemia. Most importantly, the cellular effects of LT on the heart result in decreased stroke volume and cardiac output, which are further diminished by the effects of hypovolumia, resulting in hypotension and death.

retrosternal chest pressure and respiratory failure requiring mechanical ventilation. The durations of illness were 5 to 7 days from onset of symptoms to death. Mina *et al.* (41) observed a 61-year-old woman who was a New York City hospital employee that developed fatal inhalational anthrax, but with an unknown source of anthrax exposure. The patient presented with shortness of breath, malaise and cough that had developed 3 days prior to admission. Within a few hours of presentation, she developed respiratory failure and septic shock and required mechanical ventilation and vasopressor therapy.

6.1.3. Gastrointestinal anthrax

Consuming undercooked meat from animals infested with anthrax is the etiology of oral pharyngeal and intestinal anthrax. After an average incubation of 6 days, the pathogenesis begins with infection in the gastrointestinal tract epithelium, which spreads to the mesenteric lymph nodes. The early symptoms include abdominal pain, anorexia, nausea, vomiting and fever. The symptoms later progress to include hematemesis, bloody diarrhea, abdominal distention and hemorrhagic ascites. Terminal manifestations are toxemia, cyanosis and shock. The oro-pharyngeal form is similar to cutaneous manifestations but located on the oro-pharyngeal epithelium. Edematous lesions of the oropharynx progress to necrotic ulcers covered with a pseudomembrane. Localized and cervical adenopathy can be accompanied by pharyngitis and fever.

6.2. Cellular effects of lethal toxin

6.2.1. Effects of MAP kinase kinase (mek) cleavage

Anthrax lethal toxin is a protease that targets the MAP kinase cascade at the level of MEK (42, 43). This was first discovered in 1998 by George Vande Woude's laboratory (43), in which it was demonstrated that LF was able to cleave the N-terminus of MEK1 and MEK2, resulting in inactivation of MEK1 and inhibition of the ERK1,2 signal

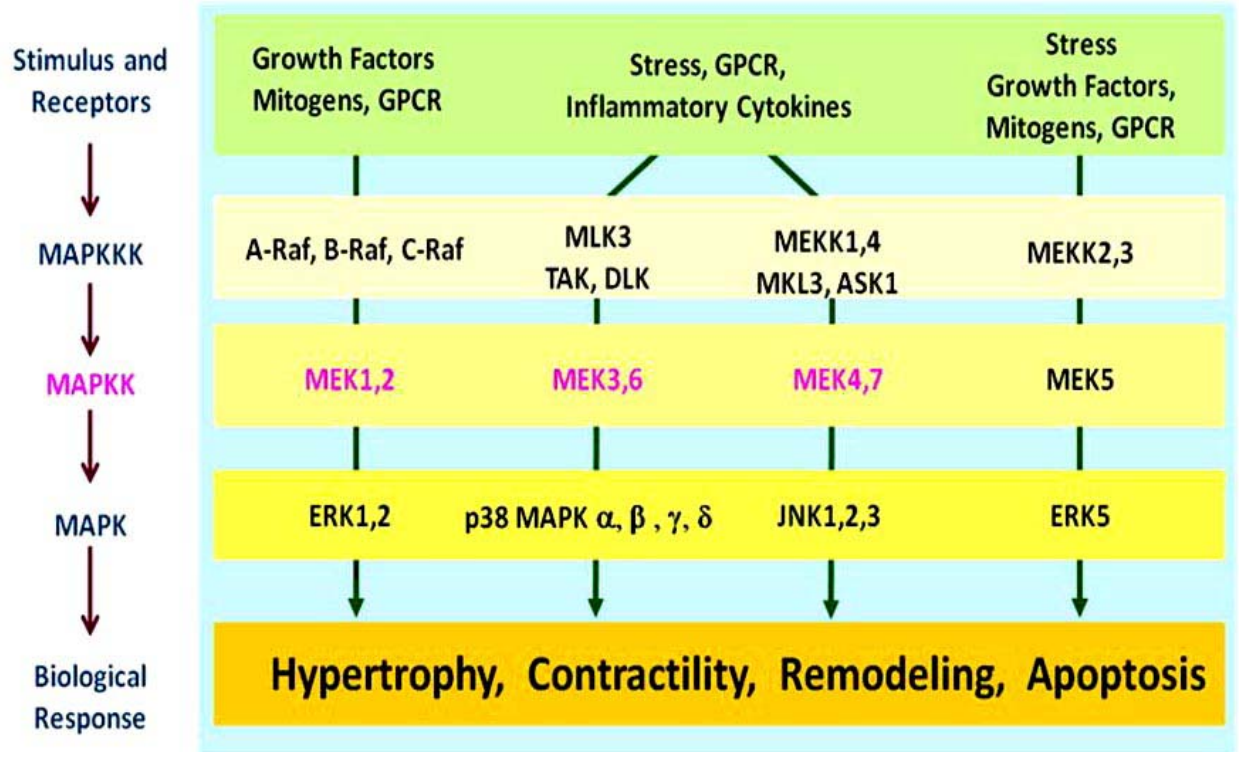

Figure 5. Mitogen-Activated Protein Kinase Signaling Cascades. MAP kinases belong to a highly conserved family of Ser-Thr protein kinases in the human kinome, and have a role in various physiological functions. The four best characterized MAPK cascades are Erk 1/2, p38, JNK and Erk 5. MAP kinase signaling is regulated through a three-tiered cascade (MAPK cascade), composed of MAP kinase, MAP kinase kinase (MKK or MAP2K), and MAP kinase kinase kinase (MKKK or MAP3K). Anthrax lethal toxin targets MAP kinase cascade at MKK level. MKKs targeted by lethal factor are shown in pink.

transduction pathway. It has since been shown that LF cleaves all MEK members, except MEK5, and occurs in multiple cell types, including macrophages (44), platelets (45) and CD4+ T-cells (46).

MAP kinases belong to a highly conserved family of Ser-Thr protein kinases and play a critical role in intracellular signal transduction and regulation. MAP kinases require dual phosphorylation of their Thr (Glu/Pro/Gly) Tyr motif in the kinase domain to become catalytically active. Classical MAP kinases, including extracellular signal regulated kinases (ERK1,2), p38 MAP kinase (α, β, γ, δ), c-Jun N-terminal kinases (JNK1, 2 and 3) and big MAP kinase (BMK or ERK5), which have been implicated in a wide range of cellular processes including cell growth, proliferation and apoptosis (47). At the molecular level, each of the four classic MAP kinase subfamilies has a clearly delineated activation cascade mediated by specific upstream MAP kinase kinase kinases (MEKK) and MAP kinase kinases (MEK) (Figure 5). Thus, MEKs 1 and 2 regulate the activity of ERK 1 and 2, MEKs 3, 4 and 6 have been implicated in regulation of p38 MAP kinase, MEKs 4 and 7 regulate the activity of JNK, while MEK 5 regulates the activity of ERK 5. All MEKs are structurally characterized by a conserved kinase domain and a divergent N-terminal proline-rich extension that contains localization signals and mediates protein-protein interactions that are important in the assembly of specific signaling complexes and nuclear translocation (48-50).

Cleavage of MEK results in removal of a short N-terminal sequence, leaving the bulk of the protein, including the protein kinase domain, intact (42, 43) (Figure 6). Binding and docking studies have established that LF-cleavage products of MEKs are impaired in their ability to bind to their MAP kinase substrate, suggesting a common mechanism for the LF-induced inhibition of signaling (51). Thus, LF cleavage disrupts the ability of MEKs to phosphorylate and activate MAP kinases, and disrupts modulation of cellular responses to various stimuli, including pathogens. (43, 51-56).

6.2.2. Effects on neutrophil function

MAP kinases protect against pathogens by inducing inflammatory changes in macrophages, leading to production of proinflammatory cytokines, activation of the oxidative burst pathway and the release of oxygen intermediates. By deletion-mutation analysis and site-directed mutagenesis (57) an LFIR (LF interacting region) has been identified in the carboxyl-terminal kinase domain of MEK1 adjacent to the proline-rich region, which is essential for LF-mediated proteolysis of MEK. LFIR are conserved among all the MEKs. Point mutations in this region abolish LF-mediated MEK proteolysis, but do not alter the kinase activity. Similar mutations in MEK6 also

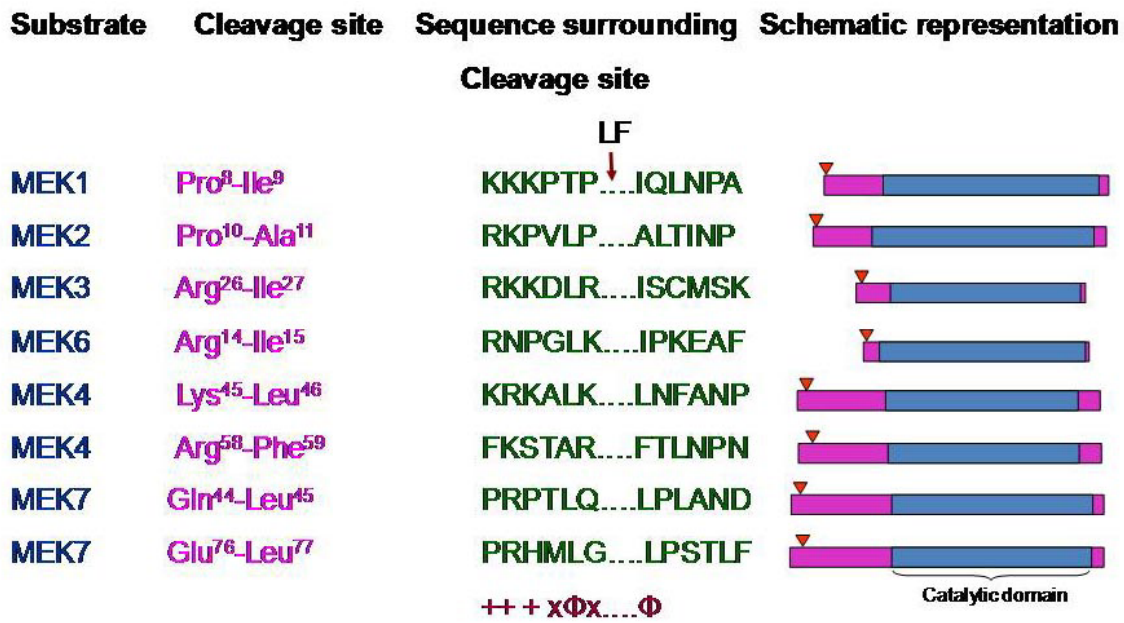

Figure 6. Cleavage of MEKs by Anthrax Lethal Factor. Known LF cleavage sites in mammalian MEKs. All cleavage sites are N-terminal to the catalytic domain (represented schematically on the right) so that cleavage removes a small N-terminal stretch but leaves protein kinase domain intact. Substrate amino acid residues (single letter codes) surrounding LF cleavage sites are shown and the cleavage sites are indicated by dots (….). The consensus residues are shown at the bottom and marked as either basic (+) or hydrophobic (Φ).

prevent proteolysis, indicating that this region is functionally conserved among MEKs. In addition, amino-terminal proteolysis of MEK1 by LF was found to reduce not only the affinity of MEK1 for its substrate mitogen-activated protein kinase but also its intrinsic kinase activity (57). Therefore, the amino-terminal end of MEK is important not only for substrate interaction, but also for catalytic activity. LT has recently been shown to have profound effects on heat-shock protein-27 (Hsp27) phosphorylation and actin assembly (58), which emphasizes the importance of the p38 MAP kinase signal-transduction pathway and Hsp27 for actin-based motility. Targeting of this important pathway by LF may be how *B. anthracis* paralyzes neutrophils. Impairment of this critical component of the innate immune system could explain how this pathogen is able to rapidly multiply in the host. Blockade of the p38 MAP kinase pathway affects other actin-mediated functions, including platelet spreading and hemostasis, as well as vascular endothelial junction integrity. In cardiac and vascular tissue, the downstream effects of MEK inhibition remain to be determined.

6.3. Cellular effects of edema toxin

Lethal toxin was found early during anthrax research to recapitulate the systemic pathophysiological characteristics of the infection; therefore ET has received less attention than LT (59). Recent work, however, has elucidated the involvement of ET and suggests that this toxin, either alone or acting in concert with LT, as would occur during systemic infection, may play a more significant role than was previously considered in the pathogenesis of anthrax. Unlike the rapid cytotoxicity observed with LT, ET induces dramatic increases in levels of cAMP in mammalian cells, resulting in tissue edema (9) and the dysregulation of cytokine networks that might suppress host immune responses (60). Downstream effects of increased cAMP in host cells have been characterized among immune cells, such as neutrophils (polymorphonuclear leukocytes (PMNs)), T lymphocytes, bone marrow-derived dendritic cells and monocytes. PMNs intoxicated with ET demonstrate increased chemotaxis but reduced phagocytosis and chemiluminescence (61-63). ET induces interleukin (IL)-6 production in human monocytes and significantly decreases lipopolysaccharide-induced monocyte tumor necrosis factor alpha (TNF-α) production (60). *Et al*so impairs antigen receptor activation of T cells and production of cytokines (IL-6, IL-10, IL-2) in bone marrow-derived dendritic cells (63). In addition, ET has also been shown to inhibit human PMN NADPH oxidase activity, thus reducing production of reactive oxygen species (ROS) (62). Despite these investigations, the mechanisms underlying the clinical findings of ET-induced edema remain unknown. Tessier and colleagues (64) have speculated that release of preformed or rapidly generated mediators of vascular leakage, such as histamine or prostaglandins, may play a role for ET-induced edema.

7. CARDIOVASCULAR EFFECTS OF ANTHRAX TOXICITY

7.1. Hemodynamic effects of inhalational anthrax

In the anthrax bioterrorism episode in 2001, patients with inhalational anthrax also developed refractory hypotension (41, 65). In most patients, due to the acuteness and severity of the events, full hemodynamic and molecular studies were not carried out. Concerning the report of two

fatal cases that progressed to respiratory failure and refractory hypotension (40), echocardiography to differentiate hypovolemic, myocardial or tamponade etiologies was not done. For the Mina *et al.* case (41), the patient's respiratory and hemodynamic status rapidly deteriorated after admission. Initial echocardiography plus right heart hemodynamics indicated hypovolemic shock. Final echocardiography indicated cardiac tamponade contributed to patient's refractory hypotension. The 2001 bioterrorism event suggested that the diagnosis could be made earlier by the combination of chest computed tomography, showing mediastinal lymphadenopathy in the context of postal exposure. Confirmed diagnosis of clinical anthrax requires isolation of *B. anthracis* from the patient. Immunohistochemical staining of tissues, anti-PA-IgG enzyme-linked immunoassay and Laboratory Response Network for Bioterrorism (LRN) polymerase chain reaction offer retrospective support of the diagnosis of clinical anthrax. Ten patients of the 2001 bioterrorism event had inhalation anthrax and six survived. Patient isolates were sensitive to ciprofloxacin, doxycycline and several other antimicrobial agents (66).

7.2. Cardiovascular effects observed in animal studies

7.2.1. Whole anthrax toxin studies

Beall and Dalldorf (67) found a preparation of anthrax toxin where 0.4 ml intravenously was 100% lethal for the Fischer 344 rat. One ml of whole anthrax toxin intravenously killed Fischer 344 rats in 80 to 90 min and 2 ml killed rats in 60 to 70 min. Intraperitoneal (IP) injection of 2 ml of this preparation of whole anthrax toxin killed rats in three to six hr. The time to death in the Fischer 344 rat was found to vary in a uniform manner with the amount of anthrax toxin injected (68). The response to toxin was not as uniform with Sprague-Dawley and Osborne-Mandel strains as observed in the Fischer 344 rat. The rats injected with anthrax toxin did not appear sick until 60 min after injection, after which animals became cyanotic and died with a frothy liquid emerging from their nostrils (68). Pulmonary edema and hydrothorax were found at necropsy. Pulmonary edema deaths were observed with Sprague-Dawley and Osborne-Mandel strains, but not NIH black rats. Further investigation of alveolar capillaries, using light microscopy, demonstrated the presence of only a few erythrocytes, which were often bridged by delicate, pale-staining membranes (67). Electron microscopy revealed these membranes to be reflections of the cytoplasmic processes of the capillary endothelial cells. This marked alteration of pulmonary capillary endothelium was followed by widespread capillary thrombosis.

7.2.2. Lethal toxin studies

In the early stages, LF helps the bacteria evade the host immune system, promoting its survival and release from macrophages where the spores germinate. After the infection becomes systemic, LF targets endothelial cells resulting in vascular barrier dysfunction (69). LT in rats, mice, and rabbits is lethal (70-72) and in rats, LT causes circulatory shock, pleural effusions, and hemorrhages (71-73), similar to the effects of late stage inhalational anthrax observed in humans. The pharmacodynamics of PA, LF and EF, following intravenous (IV) injection, has been recently determined in the Sprague Dawley rat (71). Higher doses resulted in increased serum concentrations of PA, with peak levels in the ug/mL range and half-lives in the order of 10-20 min. Comparing IV bolus injection of LT to infusions over a range of zero to 5,000 μg/kg LF and 10,000 μg/kg PA, in contrast to lipopolysaccharide, shows that excessive inflammatory cytokine and nitric oxide release does not appear to contribute to the circulatory shock and lethality occurring with PA/LF in a rat model (73). No significant differences in the heart for congestion, hemorrhage, necrosis or inflammation were found by light microscopy. These results imply a possible cardiogenic pathophysiology for LT-associated shock. Cui *et al.* (74) demonstrated in a rat model that mortality, heart rate and blood pressure improved by administration of protective antigen-directed monoclonal antibody up to 6 hr (and possibly 12 hr) after starting an infusion of LT.

A study of the cytokine effects LT in BALB/cJ and C57BL/6J mice revealed that of 50 cytokines analyzed, BALB/cJ mice showed rapid but transitory increases in specific factors including KC, MCP-1/JE, IL-6, MIP-2, G-CSF, GM-CSF, eotaxin, FasL, and IL-1β (70). No changes in TNF-α occurred. The C57BL/6J mice did not mount a similar cytokine response. These factors were not induced *in vitro* by LT treatment of toxin-sensitive macrophages. The evidence presented shows that LT kills mice through a TNF-α–independent, FasL-independent, noninflammatory mechanism that involves hypoxic tissue injury but does not require macrophage sensitivity to toxin. In addition, chemical protease inhibitors (phosphoramidon and 1,10-phenanthroline), as well as immune sera against M4 and M9 proteases of *B. anthracis*, were used to treat mice challenged with *B. anthracis (*Sterne) spores (75). These substances demonstrate a substantial protective efficacy in combination with ciprofloxacin therapy initiated as late as 48 h post spore challenge, compared to the antibiotic alone.

The effects of LT on general hemodynamic responses and left ventricular (LV) systolic and diastolic functional performance were longitudinally assessed in conscious, chronically instrumented dogs (76). The experimental animal received an IV bolus of LT (PA: 0.265 mg/kg and LF: 0.265 mg/kg mixed in 10 ml of saline), and the control animal received 10 ml bolus of saline. Data and blood samples were collected at 1, 6, 24, 48, 72 and 96 hr. Compared with controls, there were serially progressive decreases in LV contractility (assessed by the slopes of LV pressure (P)-volume (V) relations) and slowed LV relaxation during the transition from LV dysfunction to failure with LT treatment. The cardiac depression was apparent about 6 h after initiating LT infusion, but progressed through the subsequent 72-96 hr. After LT infusion for 72 hr, the heart rate (86 vs. 125 bpm), the end-diastolic P (21.1 vs. 9.0 mmHg) and the time constant of LV relaxation (39.7 vs. 29.9 msec) were increased, and the early diastolic portion of the LV P-V loop was shifted upward with marked reductions in left ventricular ejection fraction (LVEF), stroke volume (9.0 vs. l6.8 ml), stroke work and the LV end-systolic P (94 vs. 114 mmHg). In addition, the cardiac mechanical efficiency, as measured by SW/PVA, was also markedly decreased (0.58 vs. 0.45).

Compared with an age-matched normal control dog, there was a progressive LV spherical dilatation after LT treatment for 72 to 96 hr. Compared with normal myocytes, in the LT-treated cardiomyocytes, the length of myocyte was also increased (196 $\pm$ 8 vs. 124 $\pm$ 4 μm), and the length-width ratio was greater than the normal cells. At 96 hr after LT infusion, SV and LVEF were further decreased, accompanied by the development of lactic acidosis/metabolic acidosis, vascular leak and cardiac failure. At this stage, IV infusion of dextran and dopamine were ineffective, while dobutamine (6.0 μg/kg/min) was associated with an adverse reaction. In contrast, a new myofilament Ca^{2+} sensitizer, levosimendan, showed evidence of transient clinical improvement prior to the animal's death. The authors concluded that the early and progressive decrease of LV contractility after LT treatment may be an important contribution to the development of heart failure, circulatory shock and death.

The mechanism of death from intravenous anthrax toxin has been described for primates (77), including respiratory failure of central nervous system origin. These effects were amplified by administration of anthrax toxin into the cerebrospinal fluid of monkeys, in which death was due to terminal anoxia mediated by the central nervous system (78). The cardiac changes were late and secondary to systemic hypoxia. Thus, the mechanism of death from anthrax toxin may be different in primates compared to rats.

7.2.3. Edema toxin studies

Little information is available on the effects of ET on the cardiovascular system. Recently, we and others have demonstrated that ET infusion results in early and pronounced decrements in mean arterial blood pressure and tachycardia, which leads to hypotension (72, 79). Recent studies have suggested that the hypotensive effect induced by ET is not due to excessive release of inflammatory cytokines and nitric oxide (79). In contrast to LT, hemoglobin levels were not increased with ET, which suggests that it did not produce substantial extravasation of fluid. It is very likely, however, that hypotension with ET is partly related to the adenyl cyclase activity of EF (9, 80). Another speculation for ET-induced hypotension is that excessive production of cAMP in vascular smooth-muscle cells and cardiac tissue would cause subsequent activation of cAMP-dependent protein kinase A (PKA) (Figure 7). Activation of PKA leads to the phosphorylation of several regulatory proteins involved in cardiac excitation-contraction (EC) coupling and energy metabolism, including L-type Ca^{2+} channels, the sarcoplasmic reticulum (SR) membrane protein phospholamban (PLB), myofilament proteins and glycogen phosphorylase kinase. Cardiac PLB is a 52-amino acid phosphoprotein located in SR membranes (81). Two adjacent residues in the cytoplasmic domain, Ser^{16} and Thr^{17}, have been identified as the phosphorylation sites for PKA and CaMKII, respectively (82). It has been proposed that a physical protein–protein interaction between the cytoplasmic domains of PLB and SR Ca^{2+}–ATPase is critically involved in its inhibitory effect on the Ca^{2+} pump (83). The physical interaction may promote the aggregation of SR Ca^{2+}-ATPase molecules into catalytically unfavorable conformations, thereby suppressing pump activity. PLB phosphorylation may disrupt the interaction of PLB with the pump, leading to a reversal of its inhibitory effect (84). Excessive phosphorylation of these cytosolic proteins may then result in sequestration of free cytosolic calcium by the sarcoplasmic reticulum and dephosphorylation of the myosin light chain, followed by vascular dilation and hypotension (13, 80).

7.3. Cardiovascular effects of MAP kinase kinase (MEK) cleavage

7.3.1. Effects on vascular function

Although it is likely that the disruption of MAP kinase signaling cascade by LF is the central mechanism in anthrax pathogenesis, the downstream molecular mechanism and the sequence of events leading to death are still unclear and controversial. MAP kinase members are ubiquitously expressed and this particular pathway is operational in most cell types and organ systems. These facts suggest that LF mediates multisystem dysfunction by disruption of MAP kinase pathways, which serve essential functions such as proliferation, survival and inflammation in all cell types. Pathophysiological manifestations in mice injected with LT included loss of plasma proteins, decreased platelet count, slower clotting times, fibrin deposits in tissue sections, and gross and histopathological evidence of hemorrhage. Clinical observations of infected human subjects have shown signs of vascular damage such as tissue hemorrhages and gastrointestinal bleeding (85). Autopsy findings also show destruction of both large and small blood vessels (85). These findings suggest that blood vessel leakage and hemorrhage lead to disseminating intravascular coagulation and/or circulatory shock as an underlying pathophysiological mechanism (86).

7.3.2. Effects on cardiac function

Although circulatory shock plays a major role in the lethality of anthrax lethal toxin and MAP kinases are known to be a central pathway for regulation of cardiovascular pathophysiology, only a few studies have examined the direct role of anthrax toxin on the cardiac system. Recently Watson *et al.* (71) have shown that LF leads to a significant reduction in ejection fraction (systolic dysfunction), decreased velocity of propagation (diastolic dysfunction), decreased velocity of circumferential fiber shortening (decreased contractility) and increased LV systolic area (pathophysiology). Watson *et al.* (71) further report a doubling of the velocity of propagation and 20% increases in LV diastolic and systolic areas in LF-treated rats. Together these results suggest that LF reduced left ventricular systolic function (71).

MAP kinases are highly expressed in the heart, and their specific function in the heart has been summarized in several excellent recent reviews (47, 87-90). All cells of the cardiovascular system require MAP kinase pathways for proper function. ERK1,2 becomes activated in cardiac myocytes in response to virtually every type of stress stimulation examined to date (91). Cardiomyocyte death resulting from various stresses is a major cause of functional deterioration, local inflammation and irreversible

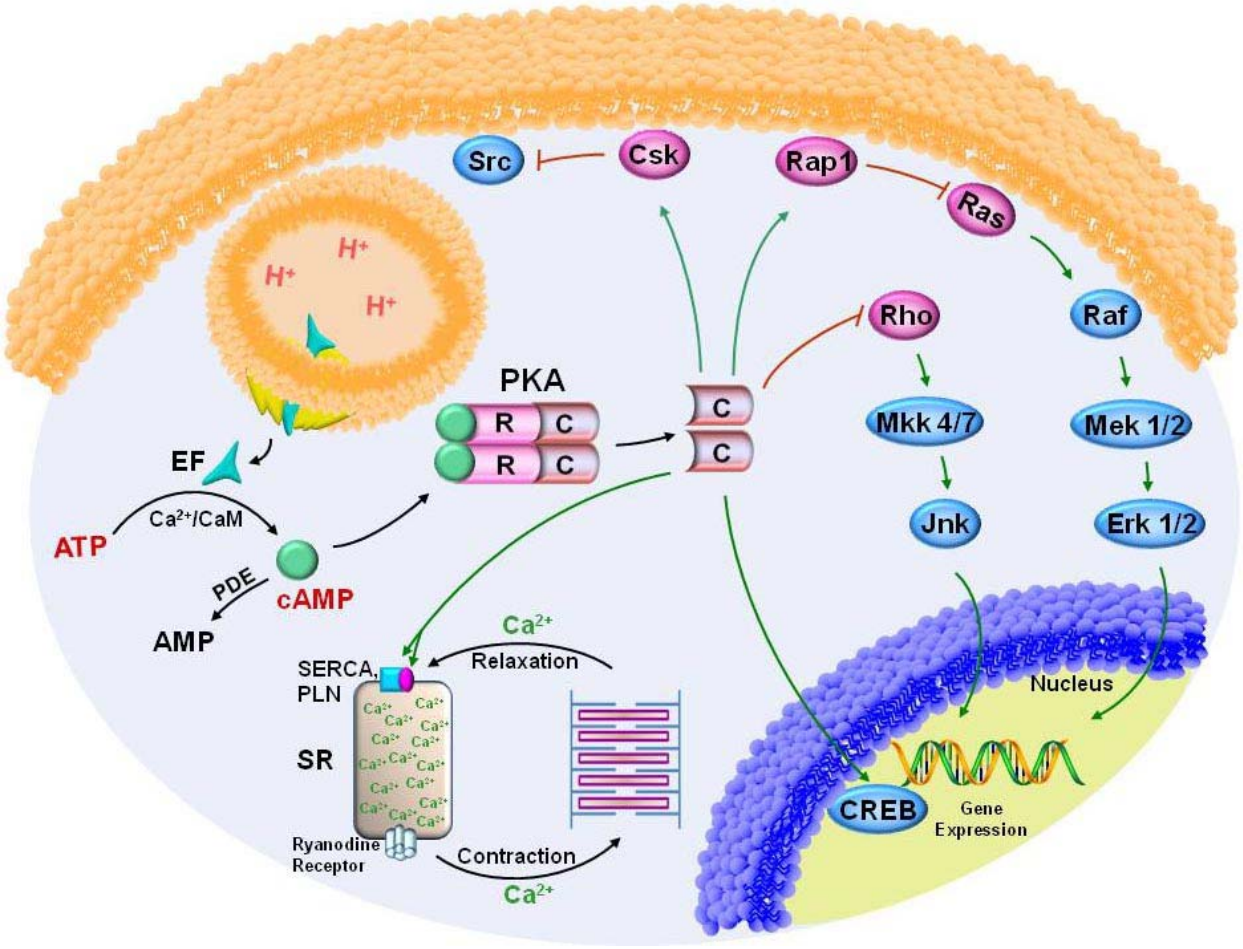

Figure 7. Putative Roles of Anthrax Edema Toxin in Signaling and Contraction**.** The acidic environment of the endosomal lumen (shown as H^+) causes a conformational change of the PA prepore, resulting in the insertion of PA heptamers into the membrane and the formation of a channel that assists the membrane translocation of EF into the cytosol. EF catalyzes the conversion of ATP to cAMP, and calcium and calmodulin act as positive catalysts for this reaction. EF inhibits MAPK-dependent gene expression through multiple pathways involving PKA, whose catalytic subunit (C) dissociates from the regulatory subunit (R) following cAMP binding. PKA phosphorylates a negative regulatory residue of Raf and thus inhibits the Raf–MEK–Erk pathway. Direct activation of Rap1 (a small GTPase) by PKA results in the inhibition of Ras signaling. This is achieved by phosphorylation and activation of EPAC, a Rap1 GTP exchanger. In addition, PKA inhibits the MKK4–JNK and MKK7–JNK pathway by inhibiting Rho GTPase. Finally, PKA inhibits signaling through receptors coupled to Src family protein tyrosine kinases by activating the specific inhibitory kinase Csk. PKA-dependent phosphorylation of phospholamban (PLB) results in activation of SERCA receptor in the endoplasmic reticular membrane of cardiac membrane followed by sequestration of Ca^{2+} and may lead to muscle relaxation, although this has not been experimentally demonstrated. PKA can also modulate gene expression by translocating into the nucleus and phosphorylating cAMP-responsive transcription factors, such as cAMP response-element binding protein (CREB).

fibrotic remodeling. Genetic inhibition of cardiac ERK1,2 promotes stress-induced apoptosis and heart failure (92). *In vivo*, MEK transgenic hearts are protected from ischemia/reperfusion injury and myocyte apoptosis (93). In contrast, inhibition of Raf-1 by the expression of a dominant-negative mutant or cardiac-specific knockout promoted myocyte apoptosis and decompensated heart failure (94, 95). Similarly, genetic inactivation of ERK2 promotes cellular injury and death in response to ischemia/reperfusion (93). These findings suggest that the Ras-Raf-MEK-ERK pathway has an important cardioprotective role. Anthrax LF cleaves MEK1,2 and severely impairs ERK pathway signaling, which is an important cardioprotective mediator in response to pathologic stimuli (92).

In cardiomyocytes, p38 MAP kinase and JNK, two stress-activated protein kinases (SAPK) of the MAP kinase family, are rapidly activated by stress both *in vitro* and *in vivo* (96, 97). A number of recent studies in genetically modified animal models have shown a cardioprotective role of these two cascades (96). Pressure overload–induced cardiac hypertrophy appeared to be enhanced further by dominant-negative mutants of p38

MAP kinase, revealing an inhibitory function of the kinase on cardiac hypertrophy (98). *In vivo* JNK activation in transgenic animal models developed lethal restrictive cardiomyopathy and a significant induction of fetal gene expression (99). JNK1 has shown to be important in maintaining cardiac contractility and prevention of heart failure under sustained mechanical overload (100). JNK can also phosphorylate and inhibit calcineurin activity, which in turn attenuates hypertrophy signaling (101). Thus, present findings suggest that all three MAP kinase pathways known to be affected by anthrax lethal toxin play a cardioprotective role, which suggests that anthrax toxin-induced lethality may be due to cardiac failure. The direct effects of anthrax toxins on cardiac cell death and apoptosis and the exact contribution of the MAP kinase pathways in this event are unclear and require further study.

8. THERAPEUTIC APPROACHES

The terrorist attacks of 2001 involving anthrax underscore the imperative that safe and effective medical countermeasures should be readily available. Although current FDA-approved treatments for anthrax infection involve antibiotics that kill the bacteria, these agents do not neutralize or prevent the biological effects of the toxins already released into the body. Protection of the cardiovascular system and tissues requires that either the toxin be neutralized after release by the bacteria, prevented from gaining entry into cells and/or inhibited at the cellular level. Therapeutics which prevent MEK cleavage by LF would be expected to be cardioprotective, whereas agents which target EF would be more beneficial for protecting the vascular system.

Vaccines, antibodies and inhibitors have been three major approaches used to prevent or minimize cellular damage resulting from anthrax toxicity. Presently, only vaccines have been utilized as a therapeutic approach with varying disadvantages, and a clinical trial is in progress with a recombinant anthrax vaccine developed by Vaxgen, Inc. Although effective, vaccines require generation of an immune response against the toxin and therefore do not provide immediate cardiovascular protection. As discussed below, several alternative approaches are being developed which target various mechanisms responsible for mediating the cellular effects of anthrax toxins. Results from *in vitro* and animal studies are promising, however, the efficacy of these agents in mediating cardiovascular protection under emergency conditions of anthrax exposure remains to be determined.

8.1. Vaccines

One approach to prevent the adverse effects during an infection is to block cellular entry of the toxin. Two virulence factors are produced by *B. anthracis*, the poly-d-glutamic acid capsule (102) and the tripartite toxin (103). PA is the major component of the only approved human anthrax vaccine. Studies have shown that a strong antibody response to PA is protective against anthrax (104, 105). This protection could be mediated through several different mechanisms including inhibition of the activity of both PA/LF and ET or through anti-spore activity, facilitating phagocytosis and spore killing, and inhibition of spore germination (106-108). Two vaccines are currently licensed for human use. In the United States (US), the Anthrax Vaccine Adsorbed (AVA, BioThrax™, Bioport Corporation, Lansing, Michigan) is in use consisting mainly of PA from cultures of the unencapsulated, toxin-producing *B. anthracis* V770-NP1-R strain adsorbed onto aluminum hydroxide (AI, 600 μg/dose) and administered by the sub-cutaneous route. In the United Kingdom (UK), the Anthrax Vaccine Precipitated (AVP), which is an alum-precipitated filtrate of a *B. anthracis* Sterne strain culture, is administered by intra-muscular injection. Vaccination appears to be the most effective form of mass protection against a biological attack. However, the two vaccines have a number of disadvantages including minor reactions at the injection site with the US vaccine (109, 110) and transient reactogenicity associated with the UK vaccine (111-113). Also, both vaccines require frequent boosting over an 18 month period for maintaining immunity (114, 115). Because PA in AVA and AVP are derived from supernatants of modified *B. anthracis* cell cultures, concentrations of PA in AVA vary among lots. These drawbacks of current vaccines justify the enormous amount of effort currently being put into developing more effective vaccines and other treatment modalities, such as antibodies and inhibitors. For these reasons, there has been considerable interest in developing safer and more effective vaccines that could be used to immunize civilian and military personnel before or after exposure to these agents.

Vaxgen, Inc. (Brisbane, CA) have reported the results of a phase 1 clinical trial (116) designed to assess the safety and immunogenicity of their recombinant anthrax vaccine candidate, rPA102. The purified rPA102 protein is derived from the culture supernatant of a modified *B. anthracis* strain that contains a recombinant plasmid encoding PA, adsorbed to Al adjuvant with a final concentration of only 82.5 μg per dose. In the double-blinded trial, 100 adult healthy volunteers were randomized to receive 5, 25, 50, or 75 μg of rPA102 at 0, 4, and 6 wks or AVA at 0 and 4 wks. In pre-clinical studies, rPA102 protected rabbits and non-human primates from an inhalational challenge with *B. anthracis* (117). This clinical trial shows that the rPA102 vaccine candidate was well-tolerated. Immunogenicity at higher rPA102 doses was not statistically inferior to that of AVA, despite a much larger amount of Al adjuvant in the AVA. Overall, these results of the rPA vaccine are encouraging. However, additional trials are necessary to identify optimal formulations and immunization regimens for pre- and post-exposure prophylaxis. Although the vaccine candidate rPA102 is not a significant step forward with regard to protecting citizens rapidly in the event of an attack, since it requires multiple doses and needs to be administered intramuscularly, it is reassuring to have more than one producer of anthrax vaccine in the United States. In November 2004, the Department of Health and Human Services awarded a contract to VaxGen for 75 million doses of rPA102 for the Strategic National Stockpile at a price of $877.5 million (118). Future licensure of rPA102 and other candidate vaccines will depend on their safety

and immunogenicity profiles in humans, and their ability to confer protection in animal models of inhalational anthrax.

8.2. Antibodies

Although active vaccination against PA is a straightforward protection strategy, its therapeutic feasibility is limited as post-exposure vaccination is generally not applicable because of the rapid onset of the disease. In victims of bioterrorism-associated anthrax exposure, anti-PA IgG is first detectable in serum samples collected 11–22 days after the onset of symptoms (119). Another approach is to develop antibodies against anthrax toxins, which would confer instant protection and be equally effective against antibiotic-resistant strains, as well as effective in later stages of the disease (120, 121). Passive administration of polyclonal antibodies raised against recombinant PA has been shown to be protective in mice (122) and Guinea pigs (123). Animals that received immune serum providing a titer >1:200 were fully protected. Polyclonal equine antiserum raised against the *B. anthracis* Sterne strain have been proven effective in treating inhalational anthrax in nonhuman primates when administered intramuscularly once (day 1) after aerosol exposure or twice (days 1 and 6) after exposure; 40 to 45% survival (compared with 90% lethality in controls) was achieved, and mean time to death was delayed from 5 to more than 20 days (124).

However, horse-derived proteins in the sera trigger local immune reactions ("serum fever") with symptoms that include malaise, fever, skin rashes and joint pain. As an alternative to horse serum, human recipients of anthrax vaccine have been recognized as a potential source of hyperimmune plasma and fractionated immunoglobulin for therapy and prophylaxis. In 2002, a collaborative program involving the U.S. Centers for Disease Control and Prevention, the Department of Defense and the National Institutes of Health (NIH) was established to procure anthrax immune plasma for assessing efficacy in animal models and to make available a therapeutic agent for contingency use in humans under an investigational protocol (125). In a recent study by Pittman (126), individuals vaccinated with AVA vaccine were shown to produce normal titers of PA immunoglobulin. Although these results are promising, the efficacy and potency of anthrax immune plasma and/or immune globulin in treating systemic anthrax disease in humans remains to be demonstrated in controlled studies.

8.3. Types of anthrax toxin inhibitors

Animal studies have demonstrated that inhibiting toxin activity prevents morbidity. The development of anti-toxins to treat anthrax has been recently reviewed (127). Several years ago, Rainey and Young (127) outlined major target areas for antitoxin strategies, many of which are continuing to be pursued. Approaches under investigation include receptor decoys, dominant-negative inhibitors of translocation, substrate analogues, and small molecule inhibitors.

8.3.1. Furin inhibitors

Inhibition of furin, the metalloproteinase responsible for cleaving PA to allow pore formation, is a potential therapeutic target. Furin is a calcium-dependent serine protease encoded in the *fur* gene that appears to be ubiquitously expressed in all cell types and can recycle from the cell membrane to the endosome (128). The substrate specificity for furin is an R-X-X-R motif (128). Because PA requires cleavage by furin before the remaining product, PA_{63}, can begin pore formation, either non-competitive or competitive inhibitors of furin could provide mechanisms for preventing anthrax intoxication. Three synthetic substrates, In-2-LF ($AcGYA_b$ $(R)_8VLR$-NHOH), D6R ($(DR)_6$-NH_2) and D9R ($(DR)_9$-NH_2), have been shown to be effective inhibitors of furin and LF (129), with In-2-LF originally discovered as an inhibitor of LF (130) (Figure 8a). Combining D6R and In-2-LF in the same treatment yields the most effective inhibition of both furin and LF (129).

8.3.2. Lethal factor inhibitors

Because of its vital role in anthrax pathogenesis, much work has focused on finding potent inhibitors of LF (Figure 8b). For instance, a library of peptide hydroxamates based on metalloprotease peptide substrates was screened, and one was found to inhibit LF with a K_i value of 0.6 ± 0.22 mM (130). A library of known zinc-metalloprotease inhibitors was screened against LF, and a subsequent structure–activity study produced a small molecule hydroxamate termed LFI, with IC_{50} values of 54 nM and 210 nM for the *in vitro* and cell-based assays, respectively (131). Polyphenolic compounds in green tea were tested for inhibition of LF, and a catechin gallate (EGCG) was found to be a potent inhibitor of LF with IC_{50} values of ~100 nM and <1 μM for *in vitro* and cell-based assays, respectively (132). These studies led to the identification of several non-competitive LF inhibitors with galloyl moieties generated via a Pictet–Spengler reaction followed by *in situ* screening (K_i = 2–124 μM) (133). Several potent LF inhibitors are functional *in vitro*. However, there are significantly fewer inhibitors with cell-based activity, an important point since LF functions in the cytosol (56). Therefore, it is essential that agents not only inhibit LF cleavage of substrate, but are also active in a lethal toxin challenge of macrophages. Identifying compounds that inhibit LF, yet are able to enter and remain active in cells, constitutes a major challenge in developing effective anti-LF therapeutics. Screening of chemical libraries using a combination of *in vitro* and cell based assays have identified several factors that can rescue cells from LT-mediated cell death (134). Promising agents include N-oleoyldopamine (OLDA, IC_{50} = 15 μM), which confers inhibition of LF through the catechol moiety binding to the zinc metal cation in the LF active site.

8.3.3. PA association inhibitors

Another chemical approach to preventing anthrax intoxication is the design of polyvalent molecules that could prevent the association of LF to PA_{63}. Gujraty, *et al.*, (135) synthesized poly (N-acrylocycloxysuccinimide) (pNAS) and covalently attached it to multiples of a 13-residue peptide sequence that was known to be an inhibitor of toxin assembly (Figure 8c). A pNAS polymer with approximately 6 peptides attached to it was found to be effective in preventing Fisher rats from intoxication by LF (135).

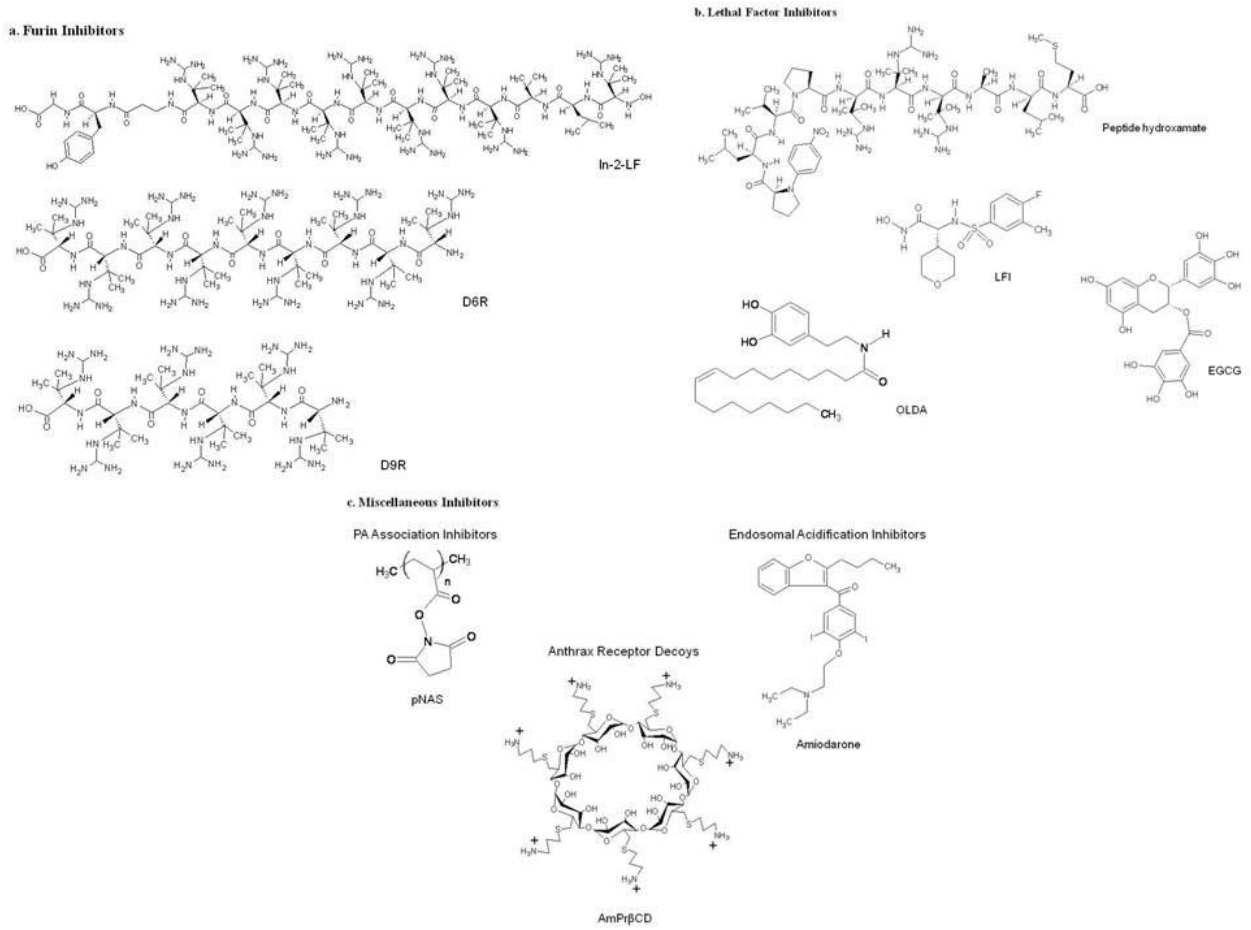

Figure 8. Inhibitors of Anthrax Lethal Factor. (a) Furin Inhibitors: Furin inhibitors include the synthetic substrates In-2-LF (Acetate-Gly-Tyr-beta-Ala-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Val-Leu-Arg-NHOH), D6R ($(D\text{-}Arg)_6\text{-}NH_2$), and D9R ($(D\text{-}Arg)_9\text{-}NH_2$), which may inhibit both LF and furin, thereby preventing cleavage of PA_{20} (129). (b) Lethal Factor Inhibitors: Potent lethal factor inhibitors include LFI, EGCG, OLDA and the peptide hydroxamate metalloprotease, para-nitroaniline-Pro-Leu-Val-Pro-Arg-Arg-Arg-Ala-Leu-Met-acetic acid, which was found to inhibit LF with a Ki of 0.6 ± 0.22 mM (130). The Zn-metalloprotease inhibitor LFI, (2R)-2- ((4-fluoro-3-methylphenyl)sulfonylamino)-N-hydroxy-2- (tetrahydro-2H-pyran-4-yl) acetamide, inhibited LF with IC_{50} values of 54 nM and 210 nM for the *in vitro* and cell-based assays (135). The catechin gallate EGCG, (2R,3R)-2- (3,4,5-trihydroxyphenyl)-3,3-dihydro-1 (2H)-benzopyran-3,5,7-triol-3- (3,4,5-trihydroxybenzoate), inhibited LF with IC_{50} values of ~100 nM and <1 μM for *in vitro* and cell-based assays (136). OLDA (N-oleoyldopamine) is a promising agent that may reverse toxin-mediated cell death. (c) Miscellaneous Inhibitors: In order to prevent the association of LF to PA_{63}, the polymer pNAS (poly (N-acrylocycloxysuccinimide)) was designed to covalently bind LF and thus prevent LT assembly (131). AmPrβCD (per-6- (3-aminopropylthio)-beta-cyclodextrin) acts as an anthrax receptor decoy by completely blocking the PA_{63} pore (133). Amiodarone, (2-butylbenzofuran-3-yl)- (4- (2-diethylaminoethoxy)3,5-diiodo-phenyl)-methanone, is a polycyclic antiarrythmic drug that blocks endosomal acidification, which prevents formation of the pore.

8.3.4. Anthrax receptor decoys

Another major therapeutic target could be the availability of soluble forms of anthrax toxin receptors to serve as receptor decoys. A soluble form of the CMG2 receptor, sCMG2, has been shown to be effective in protecting rats against lethal toxin challenge, making it one of the most effective anthrax antitoxins tested (136). One of the potentially most attractive and effective approaches to preventing anthrax intoxication is the design of small molecular weight, non-metabolic, non-antigenic and non-toxic blockers of the PA_{63} pore. Using rational drug design (137), AmPrβCD was designed, synthesized, tested and found to completely protect Fischer F344 rats from lethal toxin (Figure 8c).

8.3.5. Endosomal acidification inhibitors

The polycyclic Type III antiarrythmic drug amiodarone, a cationic amphiphilic derivative of benzofuran, was found to be effective in protecting Fischer 344 rats from LT intoxication (138) (Figure 8c). Its mechanism of action appears to be to block endosomal acidification. However, due to its many modes of action, side-effects that may be fatal and interactions with other drugs, amiodarone is only for use in life-threatening situations, which would be anthrax intoxication.

Interactions with other drugs could have severe consequences. Thus, amiodarone therapy would require careful prescreening and careful monitoring due to potential drug toxicity.

8.3.6. Catalytically inactive LF and EF

It has been recently shown that when structurally conserved regions of LF and EF are mutated (LFE687A and EFH351A, respectively), the variants are non-toxic, compete with native LF and EF and provide protection under *in vitro* and *in vivo* experiments (139). *In vitro* inhibition required a 10-fold excess of mutant toxin, where *in vivo* studies required a much greater amount of competitor. These results suggest that these agents could be defensive during the early stages of infection. If the LF and EF variants generate an immune response, antibodies to LF and EF would also augment toxin neutralization by preventing the association of PA with LF and EF, thus protecting the host from serious manifestations of anthrax.

8.4. Novel Anti-anthrax approaches

B. anthracis produces two siderophores, bacillibactin and petrobactin, which scavenge iron from the environment. It has recently been demonstrated that petrobactin is essential for anthrax virulence (140), because siderophore bacillibactin is targeted by mammalian siderocalin, which forms part of the innate immune response by targeting siderophores. Since petrobactin is not produced by other Bacillus species, targeting of petrobactin synthesis and uptake may be a feasible anti-anthrax therapeutic strategy.

9. SUMMARY AND FUTURE PERSPECTIVES

Anthrax caused by *B. anthracis* is one of the oldest diseases in cattle and humans. Although the disease has now become obscure, anthrax has recently received widespread publicity as a biological weapon. Since the terrorist attacks of 2001, progress on anthrax research has focused on the possible development of improved human anti-infective strategies targeting *B. anthracis* spore components, as well as strategies based on host–pathogen interactions. Review of clinical symptoms, together with results from animal studies, indicates that the cardiovascular system is a critical target of anthrax toxins. The current recommended post-exposure treatment is a combination of an antibiotic (e.g. ciprofloxacin) and human vaccine AVA and rPA102. Although early treatment with antibiotic is essential, results from animal studies suggest that once levels of toxin have accumulated to lethal levels, antibiotics are no longer effective. Recent insights into the structure and function of these toxins have increased the understanding of both the pathogenesis and treatment of anthrax. Therapeutic approaches which take into account cardiovascular effects of LF and EF would be expected to have the most significant clinical benefit. A number of therapeutic approaches, which target various steps in the cellular uptake and function of LF and EF, have been shown to be effective under *in vitro* and *in vivo* models. Although most therapies have focused on prevention and acute rescue, long-term effects should also be considered since anthrax toxin may trigger the onset of long-term complications. Thus, understanding how the toxin specifically affects the cardiovascular system is an important goal for future research.

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Abbreviations: ADP: Adenosine diphosphate**;** ATR: Anthrax receptor; CaM: Calmodulin; CaMKII: Calcium/calmodulin-dependent kinase II; CMG2: Capillary morphogenetic protein 2; CREB: cAMP response element binding protein; EC: Excitation-contraction; EF: Edema factor; EPAC: Exchange protein directly activated by cAMP; ERK: Extracellular signal-regulated kinase; ESR: Electron spin resonance; ET: Edema Toxin; JNK: c-jun N-terminal kinase; LF: Lethal factor; LFIR: LF-interacting region; LFn: Lethal factor N-terminal domain; LRN: Laboratory Response Network for Bioterrorism; LRP6: Lipoprotein-receptor-related protein; LT: Lethal toxin; LV: Left ventricular; LVEF: Left ventricular ejection fraction; MAPK: Mitogen-activated protein kinase; MEK: Mitogen-activated protein kinase kinase; MIDAS: Metal ion-dependent adhesion site; PA: Protective antigen; PKA: Protein kinase A; PLB: Phospholamban; PMN: Polymorphonuclear leukocytes; RyRs: Ryanodine receptors; SAPK: Stress-activated protein kinases; SERCA: Sarcoplasmic/endoplasmic reticulum calcium ATPase; SR: Sarcoplasmic reticulum; SV: Stroke volume; TEM8: Tumor endothelium marker 8; VWA: Von Willebrand factor A

Key Words: Anthrax, Anthrax receptor 1/2, Calmodulin, Cardiomyocytes, Edema factor, Heart failure, Hypotension, Lethal factor, Mitogen-activated protein kinase, Phospholamban, Protective antigen, Reivew

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