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Molecular determinants for a cardiovascular collapse in anthrax

Jurgen Brojatsch[1], **Arturo Casadevall**[1], and **David L. Goldman**[1,2]

[1]Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, NY

[2]Department of Pediatrics, Children's Hospital at Montefiore/ Albert Einstein College of Medicine, Bronx, NY

Abstract

Bacillus anthracis releases two bipartite proteins, lethal toxin and edema factor, that contribute significantly to the progression of anthrax-associated shock. As blocking the anthrax toxins prevents disease, the toxins are considered the main virulence factors of the bacterium. The anthrax bacterium and the anthrax toxins trigger multiorgan failure associated with enhanced vascular permeability, hemorrhage and cardiac dysfunction in animal challenge models. A recent study using mice that either lacked the anthrax toxin receptor in specific cells and corresponding mice expressing the receptor in specific cell types demonstrated that cardiovascular cells are critical for disease mediated by anthrax lethal toxin. These studies are consistent with involvement of the cardiovascular system, and with an increase of cardiac failure markers observed in human anthrax and in animal models using *B. anthracis* and anthrax toxins. This review discusses the current state of knowledge regarding the pathophysiology of anthrax and tries to provide a mechanistic model and molecular determinants for the circulatory shock in anthrax.

Keywords

Anthrax; shock; MAPK; Nod-like receptor; apoptosis; Review

2. CIRCULATORY COLLAPSE IN ANTHRAX

2.1. Anthrax associated shock

The clinical manifestations and mortality of human anthrax depend on the route of inoculation with pulmonary disease producing the highest mortality, followed by gastrointestinal and skin disease (1). Anthrax-associated shock generally occurs in the context of pulmonary or gastrointestinal disease. Though precise incidence of shock in association with infection is not well defined, it is clearly associated with a high mortality. The anthrax shock is generally marked by high bacterial loads, hemorrhage, edema, inflammation, hypotension and ultimately a hemodynamic collapse (1). Bradycardia, hypotension and EKG abnormalities are characteristic of *B. anthracis*-induced shock in a variety of animal models and are consistent with a cardiovascular collapse in human anthrax (2-4).

Send correspondence to: David L Goldman, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY, Tel: 718-430-2399, Fax: 718-430-8968, david.goldman@einstein.yu.edu.

A distinguishing feature of human anthrax disease is vascular dysfunction (1). Anthrax victims of the Sverdlovsk epidemic in 1979 showed arterial damage (vasculitis), fibrin-rich edema, pleural effusion and hemorrhagic lesions (5). The vascular collapse in human anthrax is often accompanied by hemoconcentration, pleural effusions, and hemorrhage into body fluids (e.g. CSF and pleural fluid) and organs (regional lymph nodes, lung, and gastrointestinal tract) (6-8). Increased vascular permeability has been linked to the hemodynamic collapse and death in anthrax. While tissue specimens from the 2001 anthrax attack victims showed no signs of vascular leakage, the tissue samples exhibited evidence of organ hemorrhage (7, 9). Intriguingly, anthrax associated shock is generally resistant to the standard combination of antimicrobial therapy and supportive measures (e.g. fluid resuscitation and pressor support).

2.2. Toxins are the main virulence factors of *B. anthracis*

Multiple studies indicate that *B. anthracis* releases the two bipartite toxins contribute significantly to anthrax-associated lethal effects. The bipartite anthrax toxins consist of three proteins: the protective antigen (PA), necessary for toxin uptake into host cells, and the toxic moieties, lethal toxin (LF) and edema factor (EF). In combination with PA, the toxic subunits LF and EF form lethal toxin (LT) and edema toxin (ET), respectively. The uptake of the anthrax toxins into target cells has been studied in detail. The binding of the PA subunit to two alternative cell cellular receptors (TEM-8 and CMG2) initiates uptake of the anthrax toxins into target cells (10, 11). Following receptor binding, a cell-associated furin cleaves PA into the PA20 and PA63 subunits. PA63 fragments multimerize into an oligomer on the cell surface, which recruits the LF and EF subunits (12). Following endocytosis, the PA heptamer forms a channel in the acidic environment of endosomes resulting in the release of LF and EF into the cytosol. The released moieties then cause the toxin-induced downstream events and disease.

Several studies illustrate the importance of the anthrax toxins in anthrax-mediated shock. For example, anti-PA antibodies block not only uptake of LT and ET into target cells, but also the circulatory shock in anthrax (13). For this reason, PA is the prevailing antigen used in anthrax vaccine formulations, and has been accordingly termed the protective antigen. Moreover, small-molecule inhibitors of LF (the enzymatic component of LT) prevent the circulatory shock associated with *B. anthracis* infections in rodent models (14-17). Accordingly, anthrax toxin receptor deficiency has been shown to prevent toxin uptake and shock mediated by toxins and *B. anthracis* spores (18). In addition, most markers of vascular dysfunction in human anthrax can be reproduced in animal models using LT alone, or *B. anthracis* challenge. LT-induced vascular defects range from changes in the transendothelial electrical resistance (TEER), a loss of macromolecules and fluids (vascular leakage), to a loss of whole blood caused by hemorrhage via damaged blood vessels. As LT challenge largely replicates the vascular collapse associated with anthrax infection, LT has been used as a model system for anthrax disease. Taken together, these findings suggest that LT is a major – if not main – virulence factor for anthrax-mediated disease.

2.3. Lethal toxin targets MAPK signaling pathways

While it is clear that the cardiovascular system is critical for LT-mediated shock, the mechanism by which LT targets cardiovascular cells and causes the pathological effects is poorly understood. The zinc protease lethal factor (LF) is the toxic subunit of anthrax lethal toxin. Point mutations that eliminate the activity of LF prevent shock mediated by LT and *B. anthracis* spores indicating the importance of LF's proteolytic activity for disease induction (19). LF has been shown to cleave two cellular substrates: mitogen-activated protein kinase kinases (MAPKKs) and the Nod-like receptor Nlrp1b (as described below) (20, 21). By cleaving MAPKK 1 to 7, except for 5, LF blocks multiple MAPK signaling pathways resulting in cell cycle arrest and cell death (22-24). Consistent with these findings, agents that block MAPK pathways could trigger cell death and heart failure (25).

While the role of MAPK signaling pathways in LT-induced shock remains to be shown, it is established that MAPK signaling pathways play a significant role in controlling immune responses (26). By blocking MAPK signaling pathways, LT prevents the induction of TLR-associated inflammatory cytokines and thereby disrupting the adaptive immune response. This LT-induced block of MAPK signaling pathways shuts down the adaptive immune response, which causes the high bacterial loads that are characteristic of anthrax-associated shock (27, 28).

2.4. Cardiovascular cells are critical for lethal toxin-induced shock

LT triggers a circulatory shock, pleural effusions, and hemorrhages in rodent models, similar to late stage inhalational anthrax observed in humans. While LT results in multi-organ failure, the cardiovascular collapse has been widely suggested to be the lethal events in human anthrax and in LT-treated mice.

Animal studies with LT from the 1960s first called to attention the potential role of cardiac dysfunction in anthrax-associated shock (29, 30). Subsequent animal studies, however failed to demonstrate significant cardiac pathology in association with *B. anthracis* toxin challenge (3, 31, 32). Likewise human studies failed to demonstrate significant cardiac pathology in association with the Sverdlovsk epidemic of 1979 (5). More recent studies, however, suggest that LT-induced cardiac dysfunction is central to the pathogenesis of anthrax-associated shock. For example, molecular markers of cardiac failure are hallmarks of LT-mediated shock suggesting that the heart might be directly targeted upon LT exposure (1). LT has also been shown to produce increased left ventricular compliance and resulting ventricular dysfunction in rats (33).

Consistent with this theory, a recent landmark study by Liu *et al.* demonstrated that cells of the cardiovascular system are critical for mortality in mice challenged with LT and by *B. anthracis* (34). Using mice lacking the main anthrax toxin receptor, CMG2, in specific cell types the authors demonstrated that anthrax receptor expression in cardiomyocytes and vascular smooth muscle cells is required for LT-induced lethal effects (34). Corresponding expression of CMG2 in cardiovascular cells in CMG2-deficient mice reconstituted LT toxicity. While mice express beside CMG2 an additional anthrax receptor (TEM8), these studies indicate that CMG2 expression is necessary and sufficient for LT/ET-mediated

toxicity (34). These studies demonstrate the importance of cardiovascular system in LT-treated mice, consistent with an increase of markers for cardiac failure in human anthrax and in LT-treated mice.

2.5. Role of endothelial cells in the anthrax shock

Analysis of the 2001 anthrax victim revealed lesions in multiple organs, including lymph nodes, lungs, kidneys and heart, associated with necrotic lesions in arteries and veins (5). Recent studies have focused on these cytotoxic lesions in the circulatory collapse in anthrax. While it is clear that LT kills many host cells, it is unclear whether cytopathic effects actually contribute to LT-induced disease. LT triggers apoptotic cell death in human endothelial cell lines suggesting that EC killing might play a role in a cardiovascular collapse (35, 36). However, mice lacking the anthrax receptor CMG2 in endothelial cells (ECs) are susceptible to shock mediated by moderate LT concentrations, suggesting that ECs are dispensable for LT-induced mortality (34). Yet at high LT concentrations, CMG2 expression in ECs enhances LT susceptibility significantly (34). Notably, LT triggers a loss of vascular integrity in zebrafish that is independent of endothelial cell death, suggesting that LT might trigger cardiovascular changes without inducing any cytotoxic effects in the endothelium (37). Taken together, cardiovascular cells and the endothelium are targeted in model systems using LT.

2.6. Liver damages drive disease mediated by anthrax edema toxin

The second toxin associated with *B. anthracis*, edema toxin (ET), has also been linked to the vascular collapse in anthrax. Edema toxin is a Ca^{2+}− and calmodulin-dependent adenylate cyclase that increases intracellular cAMP levels (27). ET is highly toxic *in vivo*, and causes extensive necrotic lesions in multiple organs, including lymphoid organs, kidney, and the gastrointestinal mucosa (38). ET appears to act synergistically with LT by causing vascular leakage and reduced ventricular volumes (33, 39, 40). While some studies (but not all) suggest that ET increases vascular permeability, these findings are challenged by the fact that increased intracellular levels of cAMP are generally associated with decreased vascular permeability (41). Consistent with these studies, ET did not induce hemoconcentration in rats (40). Recent studies have focused on the role of ET-induced arterial dilation in anthrax-associated shock. ET challenge in rats, rabbits and dogs results in an acute onset of hypotension (40, 42, 43). In the dog model, this correlates with a decrease systemic vascular resistance and central venous pressure. These findings are consistent with ET-induced arterial dilation (43). In support of this hypothesis, ET was noted to impair the contractile response of aortic rings to phenylephrine of and related PE stimulated cells (38).

More recent evidence suggests that the primary role of ET may be outside of its effects on the cardiovascular system. For example, mice expressing CMG2-deficient hepatocytes showed a significant reduction in ET-induced mortality (34). These studies suggest that the liver is the main target of ET toxicity *in vivo* (34). CMG2 expression in hepatocytes was critical for ET toxicity and edema formation, a hallmark of anthrax disease and ET-induced pathologies. Accordingly, biomarkers for necrotic cell death and liver failure, such as aminotransferase (ALT) and LDH, are significantly increased in ET-treated mice (31). Importantly, the ET-mediated increase in ALT and LDH levels did not occur in mice

expressing CMG2-deficient hepatocytes following ET exposure (34). As liver failure and edema formation have been associated with anthrax disease, these effects might be linked to ET toxicity. and related PE stimulated cells (38).

While these studies demonstrate the cytotoxic potential of ET, it is unclear to what extent ET actually contributes to anthrax disease progression. Analysis of LF and EF levels revealed a significant LF excess over EF (5:1 ratio of LF:EF) in animal models following *B. anthracis* challenge (44). In addition, significant higher ET than LT concentrations are required to induce lethal effects in mice (44). This is consistent with studies demonstrating that small LF inhibitors that specifically target LT are sufficient to block the *B. anthracis*-mediated shock, which leaves ET active in this process (14-17) . While these studies suggest that LT is the main virulence factor in *B. anthracis*, recent findings suggest that ET still contributes to the anthrax disease (40, 44). For example, it has been shown that LT and ET effects are additive and, depending on the readout, even synergistic (40, 44). In addition, both LT and ET injections of mice cause hypotension, a hallmark of inhalation anthrax in the 2001 anthrax attacks (8, 33, 45). Taken together, LT and ET appear to work in conjunction, and it remains to be shown whether and to what extent ET either directly or indirectly contributes to the circulatory shock in anthrax.

2.7. Cell death-independent events mediated by anthrax toxins

While anthrax toxins have been shown to trigger cytotoxic events, multiple studies have linked cell-death-independent events to shock mediated by LT and ET. For example: LT triggers a decrease in transendothelial electrical resistance (TEER) of endothelial barriers *in vitro* (46, 47).

LT triggers intradermal leakage *in vivo*, which could be blocked by the inhibitor of mast-cell degranulation, ketotifen (48). However, LT-mediated intradermal leakage is strain-dependent in mice, and some murine strains are killed by LT without showing any signs of intradermal leakage (48).

LT-mediated targeting of p38 signaling pathways has also been linked to endothelial barrier disruption, platelet aggregation and increased gap formation (49, 50).

Multiple studies have linked ET to a vascular shock. For example, ET has been shown to trigger an artery widening and hypotension (1). The ET-induced rise in cAMP levels has also been reported to suppress platelet aggregation, potentially aiding hemorrhage in anthrax (51).

3. CYTOKINE STORM AND CIRCULATORY SHOCK

3.1. The Nod-like receptor Nlrp1b controls cytokine release and disease progression

Murine strains differ in their susceptibility to anthrax lethal toxin. Genetic crossbreeding experiments between susceptible and resistant murine strains have led to the identification of the LT susceptibility factor, Nlrp1b (52). Nlrp1b is an intracellular surveillance receptor belonging to the family of nucleotide-binding oligomerization domain (NOD)-like receptors. LT susceptibility has been mapped to the N-terminal region of Nlrp1b (52-54). LT triggers

Nlrp1b cleavage in susceptible cells and sequential recruitment of downstream proteins (55-57). This results in activation of caspase-1, which then triggers interleukin (IL)-1β and IL-18 processing, and induces necrotic cell death (46). Caspase-1-mediated cell death, also termed pyroptosis, is a distinguishing feature of LT-susceptible macrophages (58, 59). Pyroptosis is also induced in cells challenged with a range of microbial pathogens, ranging from Salmonella, Shigella, Legionella, Franscisella to Burkholderia (60-65).

Expression of a dominant Nlrp1b isoform renders rodent strains highly susceptible to LT-mediated cell death and disease progression (54). Accordingly, inhibition of caspase-1 activation blocks LT-mediated cell death and rapid circulatory shock in susceptible rats (66). LT triggers a cytokine storm and significantly faster disease progression in rodent strains expressing the dominant form of Nlrp1b (22, 32, 67). The LT-induced cytokine storm appears to exacerbate disease progression in mice expressing the dominant Nlrp1b isoform (2, 32, 67). However, LT does not trigger a cytokine storm in C57BL/6 mice, which are nevertheless susceptible to LT-mediated cardiovascular shock (2, 32, 67). In addition, the LT-mediated block of MAPKK signaling generally tempers a cytokine storm in anthrax (as described above). Taken together, these findings suggest that a cytokine storm is not necessary for anthrax disease in rodents expressing the recessive form of Nlrp1b and in humans, which also express a resistant NLRP1 isoform.

3.2. Role of cytokines in LPS-induced shock

Whereas cytokines appear to be dispensable for anthrax-associated cardiovascular shock in humans and most rodent strains, they play an important role in classic bacterial shock. Intriguingly, LPS – like LT - activates a Nod-like receptor. While LT triggers the NLR Nlrp1b, LPS activates the NLR Nlrp3 (68). However, LT activates only the dominant isoform of Nlrp1b, LPS induced Nlrp3 in all rodent strains tested (and in humans). Recent studies using Nlrp3-deficient murine strains have shown that Nlrp3 expression contributes significantly to an LPS-induced toxic shock (68). Nlrp3 triggers two cellular events: processing of specific cytokines (IL-18 and IL-1β), and necrotic cell death. The cytokines IL-18 and IL-1β appear to be dispensable for the endotoxin shock, because deficiency in those cytokines does not prevent the LPS shock (69, 70). Intriguingly, LPS does not trigger a cytokine storm and endotoxin shock in mice deficient in TLR4 or MyD88 (70, 71). These studies suggest that a cytokines, other than IL-18 and IL-1β, play a significant role in LPS-mediated sepsis. Recent studies also suggest that the second activity associated with Nlrp3 signaling – necrotic cell death – might also be involved in the Nlrp3-mediated shock. Support for a role of necrotic cell death comes from LPS studies focusing on HMGB1, a cytosolic protein that is released from necrotic cells and elevated in septic patients. Intriguingly, anti-HMGB1 antibodies have been shown to block LPS-induced shocks (72-74). Taken together, these studies suggest that a cytokine storm and Nlrp3-mediated necrotic cell death contribute to LPS-induced shock.

4. ROLE OF THE CAPSULE AND CELL WALL IN ANTHRAX-ASSOCIATED SHOCK

4.1. The capsule

While the late stage circulatory shock appears to be driven by the anthrax toxins, the capsule of *B. anthracis* is an additional virulence factor in anthrax pathogenicity. The capsule of *B. anthracis* is encoded by the pXO2 plasmid and composed of a D-glutamic acid polymer, which imparts a highly negative charge to the bacterium. Similar to the capsule of other microbes, the capsule of *B. anthracis* is believed to contribute to pathogenesis by blocking complement-mediated phagocytosis (75, 76). Accordingly, acapsular strains of *B. anthracis* (e.g. the Sterne strain) exhibit markedly reduced virulence compared to capsule-containing strains, and acapsular strains are therefore used as attenuated vaccine vehicles (77). Accordingly, removal of the capsule by using capsule depolymerases significantly reduces the pathology associated with *B. anthracis* Ames strain infections (78). Consistent with these findings, anti-capsular antibodies protect mice from inhalational anthrax presumably by promoting phagocytosis of the bacterium (79). Taken together, these studies demonstrate that the capsule is, in addition to the anthrax toxins, a major virulence factor of *B. anthracis* (78).

4.2. Cell wall components

The cell wall of gram-positive organisms can trigger inflammatory cytokines, an endotoxin-like shock state and lethal effects in rats. Cell wall components of *B. anthracis*, such as lipotechoic acid and peptidoglycans, have been implicated in the pathogenesis of anthrax. For example, peptidoglycans (PGN) triggers a series of inflammatory responses, including a decrease in platelets and fibrinogen, and an increase in prothrombin (80). Injections of *B. anthracis* PGNs alone trigger hypotension, hypoxemia, and increased production of pro-inflammatory cytokines and nitric oxide. The impact of PGN-mediated inflammatory responses may be limited in the case of anthrax, as LT suppresses MAPK-mediated and PGN-induced signaling pathways (80-82). Accordingly, *B. anthracis*–derived PGN triggers injurious inflammation only when used in isolation, but not in the context of LT-expressing *B. anthracis* strains. In fact, PGN might decrease the lethality of *B. anthracis* infection counteracting some of the LT effects (83).

5. THERAPY

As the shock in anthrax differs significantly from endotoxin-mediated shock, standard sepsis-based therapies fail in anthrax. Therapeutical approaches have focused instead on blocking the main virulence factors, the anthrax toxins. Several agents have been developed that target the anthrax toxins. For example, antibodies targeting LF and especially PA (see above) have been proven effective against anthrax. Antibody therapy with LF and PA-specific antibodies consistently reduces mortality in anthrax animal models. While antibiotic therapies of already infected hosts show only minimal efficiencies, late administration of PA-neutralizing antibodies can improve hemodynamic function and survival in anthrax rat model (84). For example, PA antibodies were protective in rabbits even when administered 3 days after inhalational challenge (85, 86). In fact, a phase 1 clinical trial has been

completed showing the efficacy of a human (or humanized) anti-PA antibody raising hopes that antibody-based therapeutics may be an useful option for human therapy (87). Alternatively, monoclonal antibody therapy directed at the capsule (as noted above) may also present a significant option in modifying the course of anthrax-associated shock (79). Recently, an anti-PA human monoclonal antibody has been licensed by the FDA for the treatment and prophylaxis of anthrax (88).

Traditionally therapies supporting cardiovascular function have failed once the shock in anthrax was developing (89, 90). As the shock in anthrax is much rather a circulatory shock than a septic shock, hemodynamic support with fluids fails to provide improvements in anthrax. In contrast, hemodynamic fluid support has been proven useful in the treatment of LPS-induced septic shock. Cardiac protection in anthrax, however, might require beta-adrenergic inhibitors or angiotensin receptor blockers. Taken together, management of anthrax-associated shock and cardiovascular collapse might require a combination of antimicrobial, immunoadjuvant therapy to alter the pathogenesis of infection and supportive therapy.

6. CONCLUSIONS

B. anthracis and LT trigger a circulatory shock that is distinct from the septic shock generally associated with infections by bacterial pathogens. As the anthrax toxin LT is the main virulence factor of *B. anthracis*, the enzymatic activity of the zinc-protease LF appears to be critical for anthrax disease. One of the characteristics of the protease is it's ability to kill a variety of specific target cells. Recent studies suggest that it is rather the LT effect on cardiovascular and endothelial cells, than on macrophages that drive the disease. While a cytokine storm is associated with anthrax disease, recent findings indicate that a cytokine storm is dispensable for the toxin-induced shock. The circulatory shock in anthrax is associated with high mortality, despite anti-microbial therapy. An increased understanding of the pathophysiology of the cardiovascular collapse is essential to the design of new agents that can reduce the high mortality of this disease.

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