

Pathophysiology of anthrax

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1. ABSTRACT

Infection by *Bacillus anthracis* in animals and humans results from accidental or intentional exposure, by oral, cutaneous or pulmonary routes, to spores, which are normally present in the soil. Treatment includes administration of antibiotics, vaccination or treatment with antibody to the toxin. A better understanding of the molecular basis of the processes involved in the pathogenesis of anthrax namely, spore germination in macrophages and biological effects of the secreted toxins on heart and blood vessels will lead to improved management of infected animals and patients. Controlling germination will be feasible by inhibiting macrophage paralysis and cell death. On the other hand, the control of terminal hypotension might be achieved by inhibition of cardiomyocyte mitogen-activated protein kinase and stimulation of vessel cAMP.

2. INTRODUCTION

Because of its antiquity and central role in modern microbiology and immunology, extensive knowledge has been achieved on the history, biology, natural history of the illness and biochemistry. In contrast, little is known about the events in the late stages of anthrax.

2.1. History

Bacillus anthracis chromosomal DNA is closely related to *Bacillus cereus* DNA. Both organisms may have evolved a symbiotic relationship with plants to avoid mammal and insect consumption, respectively (1). The earliest recorded cases of anthrax may be the “boils” on Pharaoh’s cattle in a 1500BC papyrus (2). Virgil’s *Georgics* written in 30BC described cattle anthrax as well as its transmission to people. Outbreaks of anthrax were described in records from 14th century Germany and 17th

century Russia and central Europe. In 1769, Jean Fournier reported the black anthrax lesions originated from human exposure to animal skins and wool. In 1850, Rayer, Davain and Pollander showed filliform bodies in the blood of anthrax victims and suggested these “bodies” transmitted the disease. In 1876, Robert Koch isolated the *Bacillus anthracis* organism. Between 1880 and 1881, Greenfield and Pasteur perfected heat-killed vaccines for the disease. In World War I and II, Germany and Japan used anthrax spores as bioweapons (3). In 1979, airborne spores were accidentally released at Sverdlosk, Russia with 97 victims. In 2001, letters contaminated with spores in the U.S. led to 22 cases and 5 deaths. The World Health Organization reported in 1970 that aerosol release of spores by an aircraft over a 5 million population urban area would cause 100,000 casualties and cost \$26 billion in medical costs.

2.2. Biology

The spore is synthesized inside the mother cell in response to environmental signals and consists of a central protoplast with dipicolonic acid, a cortex with peptidoglycan and cortex-hydrolyzing enzymes, and the exosporium containing at least 21 proteins including an arginase and small amounts of protective antigen (4). Spore germination is triggered in part by inosine through membrane receptors. Vegetative organisms produce a polyglutamic acid capsule and the tripartite toxin comprised of protective antigen, lethal factor and edema factor. Macrophages in the skin, gastrointestinal tract or lungs phagocytose spores. In a contest between the spore and the macrophage, the macrophage employs toll-like receptors, myeloid differentiation factor 88 (MyD88), NFκappaB transcription factor, pro-interleukin-1beta protein, nucleotide-binding oligomerization domain (NOD)-like receptors, IRAC3, beta-interferon, interferon receptor, signal transducers and activator of transcription (STAT)1, caspase 1, and processed interleukin-1beta to react to the organism and produce local nitric oxide (5). The spore exosporium contains small amounts of the tripartite toxins and arginase to block these macrophage steps and prevent macrophage defenses and block nitric oxide damage, respectively. Once the spore has escaped from the macrophage and produced vegetative offspring and toxins, symptoms and signs of anthrax develop.

2.3. Natural history of infection

Patients exhibit non-specific symptoms at presentation (6). Cutaneous anthrax patients will have an ulcerated small erythematous lesion resembling a spider bite. Gastrointestinal anthrax patients have abdominal discomfort, nausea, vomiting, diarrhea. Pulmonary anthrax patients have fever, cough, dyspnea, chest pain, malaise, diaphoresis, headache and may also have nausea and vomiting. Males are more commonly affected than females (3:1). Treatment with antibiotics and antisera and pleurocentesis reduces mortality. The fulminant phase occurs after 3-5 days and is short with irreversible shock. Unfortunately, detailed hemodynamic monitoring of patients during the “honeymoon” period has not been reported.

2.4. Biochemistry

Protective antigen is an 83 kDa protein with four domains (Figure 1a) (7). Domain 1 has a beta-sandwich jelly roll topology with four small helices and two calcium ions. Furin cleavage of RKKR at residues 164-167 removes this domain. Domain 2 has a beta-barrel with excursions and a modified Greek-key topology and forms the transmembrane pore when the 2beta2 and 2beta3 loops are unfolded in acidic conditions. Domain 3 has a ferridoxin-like fold and mediates oligomerization by inserting into a cleft in domain 1' of the adjacent PA63 subunit. Domain 4 is a beta-sandwich with an immunoglobulin-like fold and binds the receptor. Lethal factor is a 90 kDa protein with four domains (Figure 1b) (8). Domain I binds protective antigen. Domains II-IV create a long deep groove which holds the N-terminal tail of mitogen-activated protein kinase (MAPKK, MEK or MKK). Domain IV has the zinc metalloprotease catalytic center. Edema factor is an 89 kDa protein with the protective antigen binding domain (PABD) and a calmodulin-activated adenylyl cyclase domain (Figure 1c) (9). Protective antigen binds receptors present on most cells (10). The receptors are tumor endothelium marker 8 (TEM8) and capillary morphogenesis gene 2 (CMG2). Both receptors contain a von Willebrand factor type A domain with calcium at the binding site-metal ion-dependent adhesion site (MIDAS). Protective antigen domain IV has an aspartate which forms a salt bridge with the receptor calcium ion. Protective antigen domain II has a short alpha-helix which interacts with a hydrophobic cleft on the receptor. After cell binding, protective antigen is cleaved by cell-surface furin and the 63 kDa protective antigen fragments heptamerize and bind 3 molecules of lethal factor or edema factor. The complex moves to lipid rafts and undergoes receptor-mediated endocytosis. In acid endosomes, the protective antigen separates from the receptor with the assistance of protonated His-121 of the receptor. The protective antigen 2beta2-2beta3 loops unfold in acid with protonation of four His in the loops and form a 14-strand beta-barrel pore. Lethal factor and edema factor are unfolded in the low pH endosomes. The N-termini of unfolded lethal factor and edema factor enter the pore first. Translocation is effected by the pH gradient between the endosome and the cytosol. Since the pore is cation-selective, peptides with acidic residues can only enter the low pH side. Phe-427 of protective antigen forms a clamp at the pore entrance to help chaperone the unfolded lethal factor and edema factor to the pore lumen and block ion passage. Once the lethal factor and edema factor travel through the pore to the cytosol, they refold and proteolyze MEKs and generate cAMP, respectively.

3. GERMINATION VERSUS SPORE DESTRUCTION

The interplay of the spore and macrophage determines the fate of the host organism. The spore needs time to germinate. The host needs to rapidly respond by either triggering superoxide-mediated destruction of the spore in the phagosome or exuberant cytokine and chemokine signaling to bring fresh innate and adaptive immune cells to the site. Recent data suggests toxins secreted by both the germinating spore in the

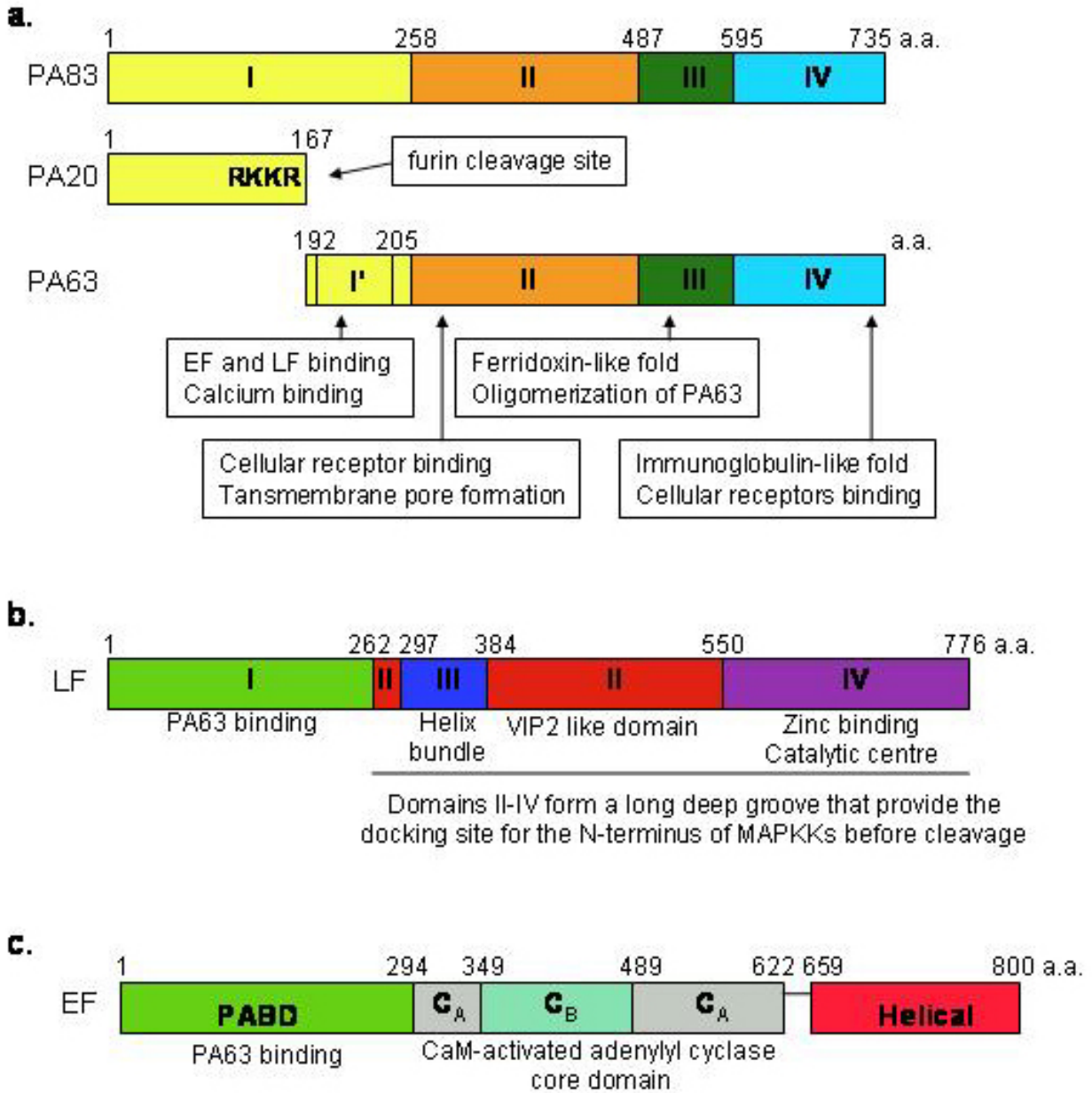


Figure 1. Schematic diagram of domain organization of anthrax toxin molecules. The amino acid (a.a.) boundary is indicated above each domain. (a) protective antigen (PA). (b) lethal factor (LF) (c) edema factor (EF).

phagolysosome and toxins from surrounding vegetative organisms can impact the outcome. Immune paralysis clearly favors bacterial survival. Rapid macrophage lysis may adversely impact the organisms by not allowing sufficient time and proper environment for germination. Most studies have examined the effects of toxins on macrophages and spore-macrophage combinations in tissue culture.

3.1. Macrophage response

The host macrophage engulfs the spores and reacts by recognizing pathogen associated molecular

patterns with its toll-like receptors and NOD-like receptors as described above. The defense by the organism to macrophage signaling is complex. Germinating spores may release toxins intracellularly which modify macrophage functions (11). Vegetative bacilli also release lethal toxin; the toxin enters macrophages and inhibits further spore phagocytosis (12). The lethal toxin also inhibits macrophage release of cytokines (13). Lethal toxin prevents up-regulation of co-stimulatory molecules (14). Lethal factor cleaves MKK3 and MKK6; p38 stress-activated protein kinase fails to be activated; interferon-regulatory factor 3 is not phosphorylated and activated;

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chemokine and nitric oxide synthetase proteins are not expressed (15). Edema toxin triggered increased toxin receptor expression and inhibited some cytokine secretion (16). Both toxins inhibit cell migration (17).

3.2. Macrophage cell death

Anthrax lethal toxin causes rapid macrophage cell death in some rodent species (18). Slower and less potent cytotoxicity is observed with human monocytes and endothelial cells (19). Several experiments show cell death depends upon the lethal factor enzyme activity (20), Nalp1b protein (18), potassium ion fluxes (21), proteasome activity (21), and caspase 1 (21). How these different events are tied together is currently unknown. Conversely, non-human primate cells are less sensitive to lethal toxin (12).

4. HEMODYNAMIC RESPONSE TO SECRETED TOXINS

Systemically administered anthrax toxins reproduce many of the symptoms, signs, laboratory abnormalities, hemodynamic changes and pathology of the late stages of anthrax (22, 23). In addition, infection of animals with mutant *Bacillus anthracis* which lacked toxin genes (pOX1⁻) yielded a thousand-fold reduction in toxicity (24). Animals were protected from death due to anthrax bacilli infections by prophylactic treatments with anti-toxin antibodies (25, 26). Further, immunization with protective antigen protected animals from anthrax (27), and vaccine protection correlated with titers of anti-protective antigen antibodies (28). Thus, secreted toxins may be responsible for the late lethal stage of anthrax, particularly in cases where antibiotics reduced growth of vegetative organisms.

4.1. Lethal toxin-induced shock

Anthrax lethal toxin has been administered to mice and rats. Equal amounts of protective antigen and lethal factor given at 1-4 mg/kg each to Balb/c and C57BL/6J mice produced malaise and death by 60-100 hours (23). Similar doses produced death within 24-72 hours in CAST mice (29). Blood tests showed hypoalbuminemia, hypofibrinogenemia, thrombocytopenia, elevated prothrombin time, and elevated partial thromboplastin time. Circulating inflammatory cytokines were not present. Pathology showed disseminated fibrin deposition, hemorrhage, tissue hypoxic damage, and evidence of vascular leak consistent with shock. Corticosteroids and aldosterone did not prevent the shock or lethality suggesting endocrine dysfunction was not responsible (30). Rats received twice as much protective antigen as lethal factor in a series of studies. Doses of 0.04mg/kg to 10mg/kg protective antigen and half as much lethal factor given intravenously to Sprague-Dawley and Fischer rats produced death in 2-21 hours with refractory hypotension, lactic acidosis, bradycardia, pleural effusions and secondary tissue hypoxic injury (26, 31). Fisher rats treated with a mutant form of protective antigen which only binds the CMG2 anthrax receptor combined with lethal factor developed hypotension, bradycardia and death similar to rats treated with wild-type protective antigen and lethal factor (32). Saline infusions failed to reverse lethal

toxin-induced shock in rats (33). Thus, dehydration or hypovolemia alone does not account for the shock. Animals infected with *Bacillus anthracis* spores produce blood levels of protective antigen and lethal factor prior to death similar to those observed with toxin infusions (34, 35). Interestingly, anti-protective antigen antibodies could be given to rats even after the onset of lethal toxin-mediated shock and both blood pressure and survival improved (26). Thus, the observations with purified toxin should have relevance to clinical anthrax disease—particularly in the late stage. Importantly, patients with late stages of pulmonary anthrax often have refractory shock unresponsive to antibiotics, pressors or volume replacement—a scenario similar to the toxin-treated rodents described above (6, 36).

4.2. Edema toxin-induced shock

Edema toxin was administered to Balb/c mice with equal doses of protective antigen and edema factor ranging from 1-4mg/kg of each protein (37). Death occurred within 60 h and profound hypotension occurred. Pathology showed fluid accumulation in the intestines, adrenal hemorrhage, lymphocytolysis, osteoblast necrosis, cardiomyocyte necrosis, elevated transaminases, lactate dehydrogenase, urea nitrogen, phosphorus and amylase. The results were consistent with irreversible shock and hypoxic tissue injury. Rats treated similarly with 0.2-0.8mg/kg edema toxin developed hypotension and tachycardia (38). The combination of anthrax lethal toxin and edema toxin in rats gave additive effects.

5. HYPOTHESES FOR MOLECULAR PATHOGENESIS OF SPORE AND BLOOD PRESSURE CONTROL

The clinical course of patients exposed to *Bacillus anthracis* spores depends upon several critical events. First, spores are phagocytosed by macrophages in the skin, gastrointestinal tract or lungs and must germinate. Second, vegetative organisms release toxins which incapacitate the host. The first event is a contest between the macrophage and spore. The traditional view is that immune paralysis and cell death are equivalent and favor the pathogen. We hypothesize that rapid macrophage lysis does not provide sufficient time for spore germination and will favor the host. If valid, then hosts with sensitive macrophages will tolerate higher spore numbers and have better survival than hosts with resistant macrophages. Monocytes were isolated from the blood of human, baboon and rats by density gradient centrifugation and plastic adherence and then cultured with human or rat granulocyte-macrophage colony-stimulating factor for one week. Then aliquots of cells were incubated for 48 h with protective antigen (different concentrations) and lethal factor (1nM). ³H-leucine was added for an additional 18 h and then cells harvested and counted. The IC₅₀ is the concentration of protective antigen yielding a 50% reduction in protein synthesis. Rat macrophages had a median IC₅₀ of 60pM; baboon macrophages had a median IC₅₀ of 180pM human macrophages had a median IC₅₀ of 200pM. Published data on spore sensitivity estimates of the human, monkey and rat aerosol spore number leading to death were 10, 50 and

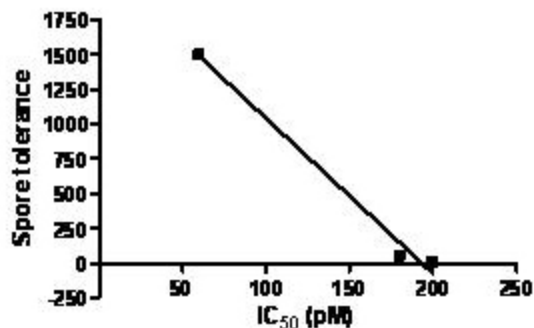


Figure 2. Plot of macrophage IC_{50} (pM) vs. spore tolerance based on experiments above and spore data from Young (39).

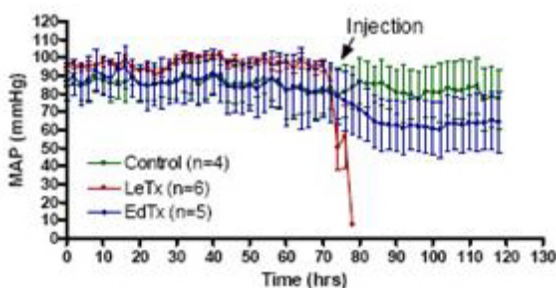


Figure 3. Effect of bolus anthrax toxins on mean arterial pressure (MAP). MAP in rats was recorded continuously via telemetry before and after toxin injection.

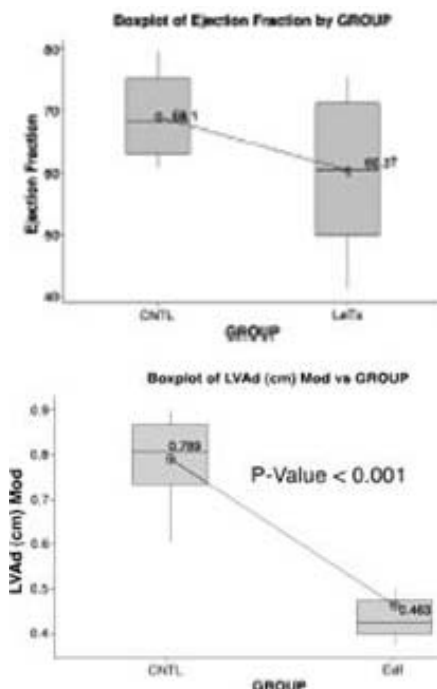


Figure 4. Rat echocardiography studies performed as described (41). A. Ejection fraction of lethal toxin treated rats; B. Left ventricular end-diastolic area of edema toxin treated rats.

1500, respectively (39). A plot of macrophage sensitivity vs. spore tolerance shows an inverse relationship with an $r^2=0.99$ and a p value of 0.07 (Figure 2).

Shock associated with the late stages of many bacterial pathogens is associated with an inflammatory cytokine storm and responsiveness to volume replacement and pressors. In contrast, anthrax shock lacks these properties. We hypothesize that anthrax lethal toxin induces cardiogenic shock, and anthrax edema toxin worsens this state by vascular dysfunction leading to preload reduction. Cohorts of Sprague Dawley rats were treated with 0.1mg/kg protective antigen plus 0.045mg/kg lethal factor or 0.45mg/kg protective antigen plus 0.225mg/kg edema factor and monitored by telemetry (40). These doses yielded 50% mortality within 4 days. Serum protective antigen levels reached 1.5 μ g/mL. Mean arterial pressure, systolic blood pressure and diastolic blood pressure were significantly lowered by lethal toxin and edema toxin (Figure 3). These results confirm the incidence of refractory hypotension with anthrax toxins.

We then administered similar doses of anthrax toxins to rats and performed echocardiography at various times post-toxin infusion (41). Lethal toxin reduced ejection fraction by 30% in 11/14 surviving animals (Figure 4A). Edema toxin did not reduce ejection fraction in any animal. There were associated increased left ventricular end-systolic areas but not increased end-diastolic areas in treated rats. Heart-rate-corrected-velocity of circumferential fiber shortening (v_{cf}) was decreased indicating decreased left ventricular contractility. Edema toxin did not reduce ejection fraction but did decrease left ventricular end-systolic and diastolic areas consistent with decreased preload (Figure 4B). These results are consistent with lethal toxin effects on the heart and edema toxin effects on blood vessels.

We followed up this study with an examination of treated rat chemistries and tissue pathology (Figure 5) (42). Lethal toxin produced transaminasemia, elevated lactate dehydrogenase and elevated hematocrit. Edema toxin produced transaminasemia, elevated lactate dehydrogenase and lymphopenia. The only observed pathology was in the lungs. Lethal toxin produced pulmonary edema and edema toxin produced pulmonary vascular hemorrhage. Protective antigen, edema factor and lethal factor alone had no effect on chemistries or pathology.

These results are all consistent with a model of anthrax lethal toxin-induced cardiogenic shock and anthrax edema toxin-induced vascular damage and preload reduction. However, our results were limited to rodents. To extend these findings, we treated dogs with 0.27mg/kg protective antigen and 0.14mg/kg lethal factor and monitored hemodynamics periodically (43). After 72 h, heart rate was 129bpm vs. 95bpm control. Left ventricular end-diastolic pressure of treated vs. control was 21mmHg vs. 9mmHg, time constant of relaxation was 41ms vs. 30ms, stroke volume was 8.4mL vs. 15.8mL, end-systolic

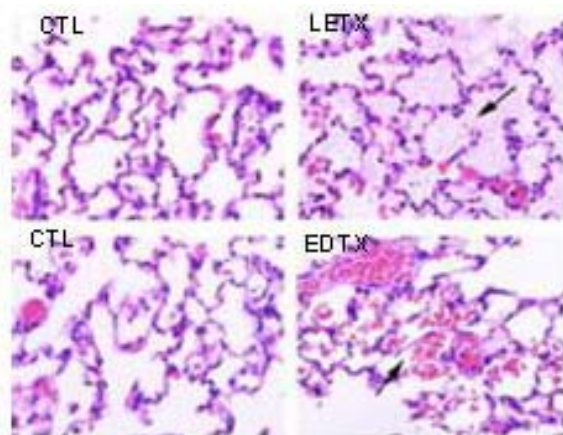


Figure 5. Lung pathology of control, anthrax lethal toxin and anthrax edema toxin treated rats. Arrows point to interstitial protein/fluid with lethal toxin and vascular disruption/hemorrhage with edema toxin.

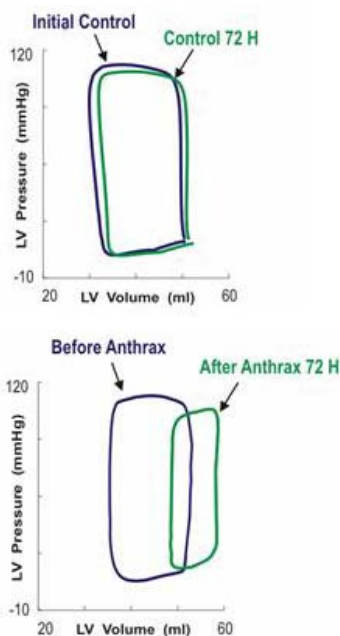


Figure 6. Dog left ventricle pressure-volume loops after anthrax lethal toxin infusion

pressure was 92mmHg vs. 118mmHg, left ventricular contractility was 4.1mmHg/mL vs. 8.1mmHg/mL, and MSW was 42.mmHg vs. 88.9 mmHg. There was a rightward shift in the left ventricular pressure-volume loops (Figure 6). At 96 h, MSW, systolic volume and ejection fraction were further decreased accompanied by severe heart failure. Histology showed left ventricular dilatation. Toxin treated dogs died by day 4. Thus, the cardiovascular effects of anthrax lethal toxin were reproducible in a non-rodent species.

With confirmation of the physiological effects of lethal toxin *in vivo*, we next wanted to examine whether the effects were due to direct action on cardiomyocytes and vascular cells or indirect effects. Myocytes were isolated

as previously described by collagenase digestion of left ventricular biopsies (44). Lethal toxin-treated myocytes had an increase in length to 201 μ m vs. 122 μ m control. Toxin-treated myocytes had a change in cell contraction with DL/dt_{max} of 87 μ m/s vs. 148 μ m/s control. Examples of analog recordings in electrically-stimulated myocytes are shown in Figure 7. Compared to control myocyte, lethal toxin treated myocytes had reductions in SA, dL/dt_{max} , and dR/dt_{max} . All these findings suggest a direct effect of lethal toxin on cardiomyocyte contractile function.

Proof that anthrax toxins directly alter hemodynamics will require prolonged incubations of toxins with isolated normal cardiovascular tissues. These studies will help provide data for a molecular mechanism of toxin

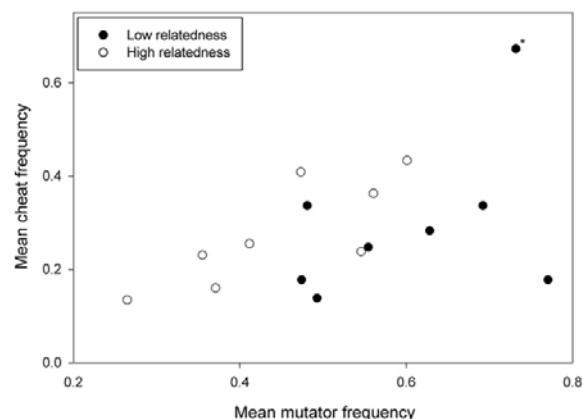


Figure 7. Isolated dog cardiomyocyte *in vitro* contractility parameters. Cells were from anthrax toxin treated or control dogs.

induced shock and also facilitate testing of novel anti-toxin therapeutics which may be employed during late stage anthrax. Evaluation of the proposed molecular pathophysiology of anthrax should generate advances in both microbiology and medicine.

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