Commentary

Exposing a Killer: Pathologists Angle for Anthrax

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The grim reality of bioterrorism struck the United States in 2001 in the wake of the horrific terrorist attacks on the World Trade Center and the Pentagon. Weaponized *Bacillus anthracis* was directed through the postal service to targeted individuals in media and government institutions resulting in 22 confirmed cases of anthrax with five fatalities, the latter being associated with inhalational exposure.¹ Until the attack, general interest in anthrax was relatively subdued, being largely a veterinary concern, but that has clearly changed as reflected in the pre- and post-2001 publication rates on this topic (Figure 1). In the battle against weaponized anthrax, the skills and techniques of pathology have stepped up to the challenge.

Anthrax is among the diseases that have shaped human history. It holds a place in the Judeo-Christian tradition, being purported to be the agent of the sixth of the ten plagues of Egypt, its cutaneous form was described by the Roman poet, Virgil, and through the efforts of Robert Koch and Louis Pasteur it launched broad acceptance of germ theory and vaccination.²⁻⁴ Military strategists quickly recognized the potential of anthrax as a biological weapon with evidence of attempted application at least as early as World War I.⁵ Despite international bans on biological warfare, governments with the means have continued research to accentuate the delivery and pathogenicity of this agent. Despite our long historical relationship with anthrax there are still challenges with respect to its biology and pathophysiology that have yet to be overcome; however, the knowledge that has accrued reveals why this organism can be so effectively applied as a bioweapon.

Bacillus anthracis is a non-motile, gram-positive bacillus that can survive in spore form in soil for decades. On infecting a mammalian host the spores germinate into vegetative forms at 37°C and if unrestrained, will grow at a tremendous rate leaving a rotting carcass seething with sporulating organisms. Thus, the bacteria only require the living host as a temporary "incubation chamber". Fortunately, humans are relatively resistant to disseminated infection under conditions of natural exposure but several forms of infection do occur. Bacteriological and

clinical aspects of anthrax have been extensively reviewed^{2,6,7} and will only be briefly summarized here.

The major clinical forms of anthrax are cutaneous, gastrointestinal, inhalational, and meningeal, the latter being the potential result of secondary lymphatic or hematogenous spread after primary infection at one of the other sites. Cutaneous anthrax is the most commonly encountered clinical manifestation under conditions of natural environmental exposure.⁸ The spores enter a cut or abrasion where they develop into vegetative forms, causing development of a localized papule followed by non-suppurative vesiculation, ulceration, and finally, formation of a black eschar, which is responsible for the organism's name, anthracis, meaning "coal-like". Although cutaneous anthrax can sometimes progress to disseminated disease it is usually self-limited, suggesting that host immunity has adapted to contain the organism when exposed by that route.

Gastrointestinal anthrax is considered to be of lower incidence than the cutaneous form but it has been argued that this is due mainly to poor recognition of the disease.⁹ However, it is generally agreed that this form of the disease is associated with greater morbidity and mortality. Humans acquire the disease through the ingestion of undercooked *B. anthracis*-contaminated meat. Spores can penetrate at any site from the oropharynx to the cecum. Analogous to cutaneous anthrax there is ulceration at the site of primary infection with spread to regional lymph nodes and associated hemorrhage and enlargement. As might be predicted, the clinical presentation differs depending on the site of primary penetration. Oropharyngeal anthrax presents with upper gastrointestinal symptoms such as sore throat, dysphagia, fever, edema, and cervical lymphadenopathy. Gastrointestinal anthrax results in a gastroenteritis picture presenting with anorexia, abdominal pain, nausea, vomiting, fever, and diarrhea, which can progress to bloody diarrhea, hematemesis, and ascites, with eventual septicemia. Mortality rates for gastrointestinal anthrax are not clearly established due to lack of controlled epidemiological studies, and reported values range from 4% to 50%.⁹

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The studies with larger patient numbers suggest that lower values are more accurate, indicating some degree of resistance to gastrointestinal exposure.

Inhalational anthrax is by far the most fatal form of primary infection, approaching nearly 100% mortality without medical intervention. Although spores are of ideal size to reach alveolar spaces on inhalation, the lung is likely not a normal route of primary infection under natural conditions. Analysis of airborne spores around fields with anthrax-infested carcasses indicated that spores were associated with larger particles and airborne spore counts were low, making airborne spread of disease unlikely.¹⁰ However, rare episodes of occupational exposure of the lung is well recognized among individuals handling contaminated animal products in closed environments, a condition previously known as "Wool Sorter's Disease."¹¹ Efficient exposure of the lung requires purposeful weaponization of spores so that they remain dispersed and airborne. The high susceptibility of the lung route of infection is likely related to the lack of host organ-based innate resistance and optimal conditions for application of bacterial virulence factors. Hence, weaponized *B. anthracis* is a highly effective killer on inhalation.

Inhaled *B. anthracis* spores are rapidly phagocytosed by alveolar macrophages and transported to draining lymph nodes of lung and mediastinum. Thus, unlike many other bacteria, *B. anthracis* uses the lung as a portal of entry rather than a site of primary infection. Within hours after phagocytosis, spores begin to germinate into vegetative cells within lymph nodes; however, spore forms may persist for more than a month (providing the rationale for prolonged post-exposure antibiotic therapy). Vegetative cells multiply rapidly at body temperature with concomitant induction of plasmid-encoded virulence factors.¹² Two plasmids, pXO1 and pXO2, carry the genes for these factors. Plasmid pXO1 codes for three exotoxin components, protective antigen (PA), lethal factor (LF), and edema factor (EF), which combine to form two binary forms, lethal toxin (PA+LF) and edema toxin (PA+EF). The PA is a 735 amino acid polypeptide that facilitates transfer to the cytosol, where the toxins act. Lethal toxin, a zinc metalloproteinase, has the capacity to inactivate mitogen-activated protein kinase kinase (MAPKK) and stimulate the release of sepsis-related cytokines tumor necrosis factor- α and interleukin-1 β . Recently, lethal toxin

was demonstrated to impair dendritic cell maturation, potentially disrupting the link between innate and adaptive immune recognition.¹³ Edema toxin, a calmodulin-dependent adenylate cyclase, increases levels of cyclic AMP, causing impaired neutrophil function and disruption of water balance, resulting in massive tissue edema. The pOX2 plasmid encodes three genes involved in synthesis of a polyglutamyl capsule that protects the vegetative bacteria from phagocytosis. 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 shock. The latter symptoms represent a terminal stage and treatment is often ineffective when started at that time. A key radiological finding purported to aid early recognition is mediastinal widening in association with pleural effusion, but the interpreting clinician must also have a high index of suspicion for anthrax exposure.^{1,14} Unfortunately, as evidenced in 2001, that suspicion may be absent in the initial days following a bioterror attack and ultimate diagnosis will depend on the investigations of pathologists and laboratorians.

Inhalational Anthrax: Pathological Findings and Challenges

Until the attack in the fall of 2001, much of our knowledge regarding the pathological manifestations of inhalational anthrax in humans arose from experience with 68 deaths following an accidental release from a bioweapons facility in Sverdlovsk, in the former Soviet Union.^{15,16} The consistent finding in those necropsy studies was hemorrhagic thoracic lymphadenitis and hemorrhagic mediastinitis. Organisms were demonstrable by tissue gram staining. In addition, there was profound edema of the mediastinum and serosanguinous pleural effusions. In many cases, there was hematogenous spread to the leptomeninges and gastrointestinal tract. An interesting observation in cases of inhalational anthrax is the lack of pneumonitis, which is analogous to the nonsuppurative ulcers in cutaneous anthrax and is likely related to the anti-neutrophil effects of anthrax virulence factors.

The 2001 bioterror attack in the United States resulted in eight cases of inhalational anthrax of which five resulted in death. Like the Sverdlovsk incident, a confirmed anthrax etiology was not made until after the first deaths. The presenting signs and symptoms of inhalational anthrax invoke a broad differential diagnosis (Table 1).⁶ Because symptoms of initial exposure are so nonspecific and the disease progresses with such rapidity, diagnosis is difficult and the time of optimal intervention is brief. Thus, the primary challenge for medical personnel is early recognition. Unlike the Soviet incident, state-of-the-art antibiotic therapy was quickly introduced in suspect cases and provided prophylactically to those who had potential exposure; this probably limited the potential

Figure 1. Annual publication rates of journal articles regarding anthrax. Derived from the National Library of Medicine reference database.

Table 1. Conditions Potentially Mimicking the Clinical and Radiologic Findings of Inhalational Anthrax

Viral pneumonia
Acute bacterial mediastinitis
Mycoplasma pneumonia
Legionnaires' disease
Psittacosis
Q fever
Tularemia
Viral pneumonia
Histoplasmosis
Coccidioidomycosis
Silicosis
Sarcoidosis
Ruptured aortic aneurysm
Superior vena cava syndrome

morbidity and mortality. A complicating aspect of empirical antibiotic therapy is that ultimate diagnosis may be prevented or obscured by altering typical pathological findings and/or by sterilizing tissues containing diagnostic organisms. Because of the urgency "to shoot and ask questions later", pathologists and laboratory technologists will simply have to deal with this challenge.

Detection and Identification of Bacillus anthracis

Traditionally, laboratory-based diagnosis of anthrax had depended on biochemical characterization of gram-positive bacilli cultured from infected tissues and fluids. Serological tests have also been available but have only retrospective value because there is minimal antibody response in the acute stages of the disease. More recently, molecular diagnostic methods using polymerase chain reaction (PCR) detection of anthrax-specific genes have been used.¹⁷⁻¹⁹ This approach has several advantages, such as rapid turnaround time, high specificity/sensitivity, application to field samples as well as to clinical specimens and archived fixed tissue. However, the technique requires special extraction procedures, may not detect degraded DNA sequences and provides no morphological information. The latter disadvantages have been largely overcome by the recent application of immunohistochemical staining for anthrax-specific antigens.

In the August 2003 issue of *The American Journal of Pathology*, the Inhalational Anthrax Pathology Working Group¹ present their findings using an immunohistochemical (IHC) approach to detect antigens of *B. anthracis* in tissues from victims of inhalational anthrax infected during the Fall 2001 bioterror attack. Using two reagents consisting of highly specific IgM monoclonal antibodies directed against either *B. anthracis* cell wall or capsule, they demonstrate the presence of intact and degraded organisms in formalin-fixed, paraffin-embedded tissues. Distribution of organisms was fully consistent with patterns of gross and microscopic pathology. The pulmonary route of infection was confirmed by abundant staining in mediastinal lymph nodes, adjacent soft tissues, and pleura. In addition, evidence of hematogenous dis-

semination was evident by positive staining within blood vessels and sinusoids of lung, liver, spleen, and intestine. The effects of antibiotic therapy were also implied as those individuals with longer courses of therapy had fewer intact extracellular organisms and more intracellular granular bacterial fragments. Also, the meningeal spread reported as common among victims of the Sverdlovsk incident was not a prominent feature, likely due to the restraining effect of antibiotics.

Immunohistochemical detection of *B. anthracis* offers the advantage of direct identification of the etiological agent within infected tissues, and if applied to frozen biopsy specimens or smears potentially could provide a rapid diagnostic procedure. Other advantages include no requirement for specialized equipment, the option to work with fixed, non-infectious tissue, and the ability to perform retrospective studies of archived tissue, allowing for more detailed characterization of the disease process and incidence. In addition, as noted by Guarner et al,²⁰ IHC studies can provide important information regarding the route of exposure to help health investigators track potential sources of infection. These workers also point out the importance of using IHC in concert with other methods such as PCR, because IHC has potential pitfalls due to non-specific staining of closely related antigens.

In conclusion, pathologists and laboratory professionals play a critical role in the diagnosis and investigation of bioterror incidents, as evidenced in the anthrax attack of 2001.^{21,22,23} Weaponized anthrax, an unfortunate product of human fear and fanaticism, is a highly effective killer that will need to be combated on all fronts. Immunohistochemical detection of *B. anthracis* provides another important tool for the pathologist in dealing with this challenge.

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