Anthrax

How it kills, and how it can be stopped

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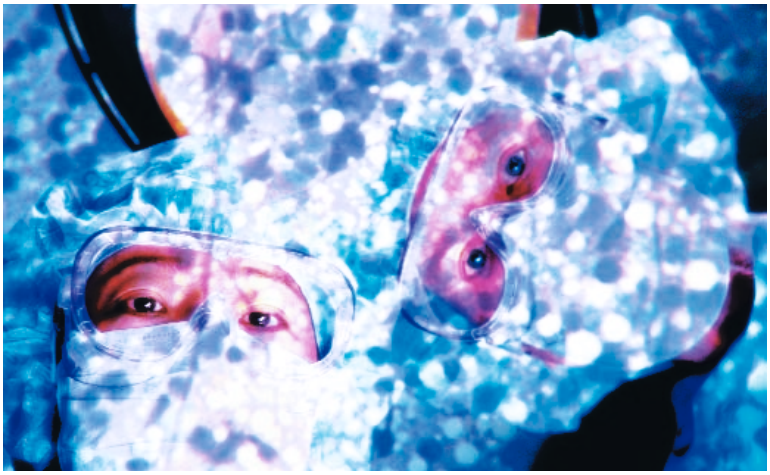

The intentional release of *Bacillus anthracis* spores in the USA during the autumn of 2001 alerted the public to the possibility of future attacks using biological weapons. It also underscored the importance of understanding the pathogenesis of the organism and the need for developing new therapeutics and vaccines. Fortunately, there has been a resurgence in anthrax research. This review focuses on the characteristics of *B. anthracis* that have led to its use as a biological weapon and the approaches that are being taken by medical researchers to minimize the impact of another release.

B. anthracis

In Nature, *B. anthracis* exists in the soil as metabolically inactive spores, which remain viable for decades because their structure confers resistance to extreme environmental conditions. Our understanding of these spores is based on extensive studies on a related bacterium, *B. subtilis*[1]. Spores of *B. subtilis* are surrounded by a multi-layered protein armour that provides structural stability and prevents entry of lytic enzymes into the spore interior. The chromosome within the spore is bound by specialized proteins, SASPs (small, acid-soluble spore proteins), which protect it from damage by noxious chemicals, heat, and UV. In addition, low water-content in the spore interior is associated with resistance to heat, possibly by decreasing thermal denaturation of spore proteins. These properties contribute to making spores "the hardiest known form of life on Earth"[1] and make *B. anthracis* spores, in particular, amenable for use as an agent of biological terrorism.

Although spores can remain quiescent for long periods of time, they can detect conditions that are favourable for growth and then initiate a process called germination, in which the spores develop into metabolically active cells[2]. *B. anthracis* spores that enter the lungs are engulfed by alveolar macrophages (Figure 1), which not only fail to destroy the spores, but also transport them to regional lymph nodes and trigger their germination. The cells escape from the macrophages and then spread from the lymph into the circulatory system. Each rod-shaped cell is surrounded by a capsule that is essential for its survival. The non-immunogenic capsule consists of chains of poly-γ-D-glutamate and allows the bacterium to evade phagocytosis by immune cells. The bacilli proliferate and can reach titres of 10^8 cells/ml of blood. Death of the mammalian host occurs from a combination of the large numbers of bacilli in the blood and from a toxin (discussed below) secreted by the cells. After an infected animal dies, nutrients become limiting, and the cells are exposed to air causing them to undergo sporulation. The spores then return to the soil where they await another host to infect.

Anthrax toxin

The key factor responsible for the deadly nature of *B. anthracis* is anthrax toxin[3]. It is a combination of three proteins released by the bacterium that inflicts damage on the host. One of the three proteins, protective antigen (PA), binds a protein receptor (ANTXR1 or

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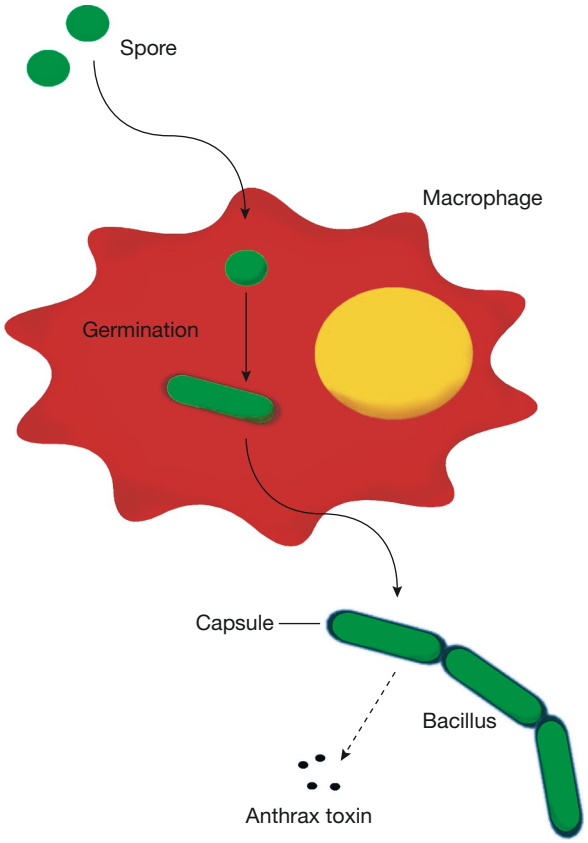

Figure 1. Germination of *B. anthracis* spores in macrophages

ANTXR2) on mammalian cells and is recognized, and then cut, by a mammalian protease at a single position in its peptide backbone (Figure 2). One of the resulting fragments, PA_{20}, is released, while the second fragment, PA_{63}, remains associated with the cell. The PA_{63}–receptor complex can move laterally in the membrane allowing PA_{63} to interact with other molecules of PA_{63} to form a heptameric complex $[PA_{63}]_7$. The dissociation of PA_{20} reveals a surface on PA_{63} that binds the two other components of anthrax toxin, oedema factor (EF) and lethal factor (LF). A maximum of three molecules of EF, LF or a combination of these proteins can bind $[PA_{63}]_7$, and these complexes are internalized by receptor-mediated endocytosis. The acidity of the endosome triggers a conformational change in $[PA_{63}]_7$, which inserts a loop from each monomer into the endosomal membrane to form a 14-stranded β-barrel. Concomitant with β-barrel formation, EF and LF cross into the cell cytosol by a process that remains unclear.

EF is an adenylate cyclase that becomes active when it binds to the mammalian protein calmodulin. The increased intracellular level of cAMP does not kill cells, but it does impair the ability of neutrophils to phagocytose bacteria. LF is a protease that cleaves N-terminal fragments from mitogen-activated protein kinase kinases and perhaps from other proteins that have not been identified. The proteolytic activity of LF results in the death of macrophages, but not other cell types that have been tested. In addition to killing macrophages, the mixture of PA and LF (called lethal toxin) kills animals. Death occurs suddenly and is accompanied by shock-like symptoms, but it is not known precisely how lethal toxin causes death or whether macrophage death plays a role.

Inhalational anthrax

It has been estimated that 'weapons-grade anthrax powder' contains 1 trillion spores/g and that only about 10 000 inhaled spores are sufficient to kill 50% of exposed people[4]. Symptoms may appear within days of exposure, but because spores can remain dormant for extended periods of time in the lungs (weeks or even months), the onset of symptoms may be delayed significantly. Patients who develop the disease first manifest flu-like symptoms, such as fever, fatigue, headache and cough. This phase of the disease can last from 1 to 4 days before a second phase, characterized by fever, breathing difficulties and heavy sweating, begins. Death can occur as soon as several hours or as long as several days after the onset of the second phase.

Therapeutic strategies

Natural isolates of *B. anthracis* are susceptible to penicillin, doxycycline (a tetracycline-class antibiotic) and ciprofloxacin (a fluoroquinolone). A strain has been engineered, however, to resist both penicillin and tetracycline classes of antibiotics, and it has been reported that ciprofloxacin-resistant strains are relatively easy to construct. Alternative therapies are therefore being developed.

One innovative approach is to use phage lysins to attack the bacilli[5]. Lysins are a class of protein produced by bacteriophage after they have entered and replicated in a bacterial cell. Lysins are inserted into the bacterial cell membrane and punch holes in it to release the bacteriophage. Researchers have determined recently that these lysins can kill bacteria if added exogenously to cells and that they can protect animals from infection by lysin-sensitive bacteria. Promisingly, it appears that constructing a lysin-resistant strain of *B. anthracis* could be very difficult.

Another strategy for treating anthrax is to target the toxin instead of the bacterial cell. It has long been known that there is a 'point of no return' in an anthrax infection after which antibiotics lose efficacy; even though the bacteria in the body can be killed after this point, the large amount of toxin remaining in the blood is sufficient to cause death. Blocking toxin action in addition to antibacterial treatments may significantly improve the prognosis of late-stage anthrax and would also be effective at treating antibiotic-

resistant strains of *B. anthracis*.

One approach to blocking toxin action is to develop molecules that prevent its assembly. In one study[6], a peptide was identified from a phage display library that binds to $[PA_{63}]_7$ and prevents the association of LF. The affinity of the isolated peptide to $[PA_{63}]_7$ was too weak for it to inhibit the intoxication of cells effectively, so multiple copies of the peptide were attached to a flexible polymeric backbone. A single molecule presenting many peptides was predicted to increase the apparent affinity of the interaction and, therefore, to be better able to prevent toxin assembly. Indeed, it was demonstrated that attaching the peptide to a polymer increased its inhibitory activity 7500-fold and the resulting molecule could block lethal toxin in rats.

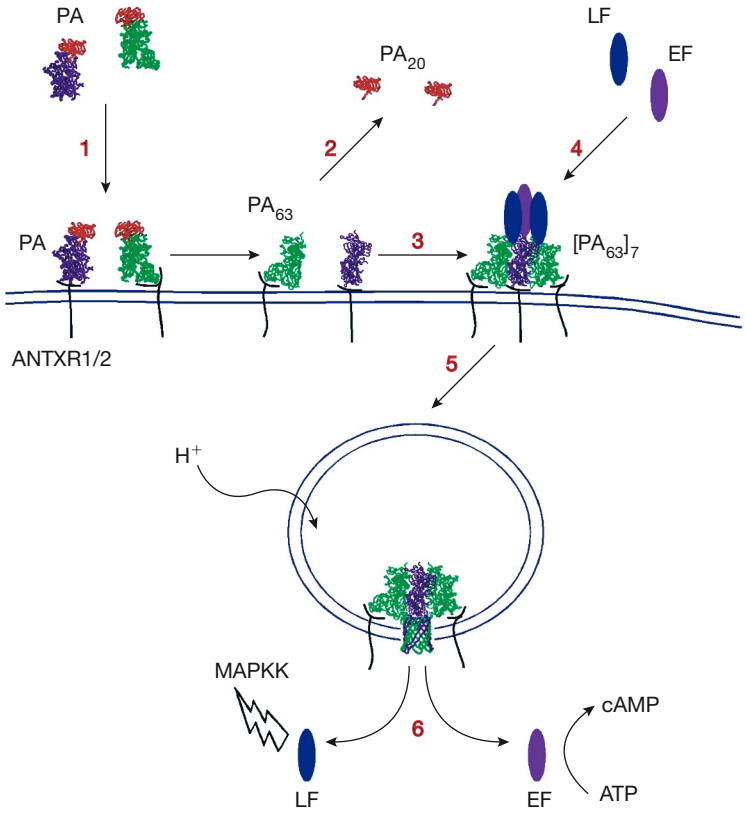

Figure 2. Intoxication model. PA binds cellular receptors ANTXR1 and ANTXR2 (step 1) and is cleaved by a protease. PA_{20} dissociates (step 2) and PA_{63} self-associates to form heptamers (step 3). LF and EF bind $[PA_{63}]_7$ (step 4) and these complexes are internalized (step 5). Acidification of the endosome triggers translocation of EF and LF to the cytosol (step 6). MAPKK, mitogen-activated protein kinase kinase.

Vaccines

The anthrax vaccine that is used at present is an aluminium sulphate precipitate of PA from non-encapsulated *B. anthracis* supernatants. It protects monkeys from inhalational anthrax and is safe for humans, with only a small percentage of people developing minor side effects (such as headache and fever). This vaccine is not ideal, however, because there are inconsistencies associated with its production and the immunization protocol consists of six injections followed by yearly boosters. Substantial efforts are being made to develop new vaccines, with a notable example being a dually active vaccine consisting of PA conjugated to poly-γ-D-glutamate[7,8]. This conjugate elicits antibodies against PA and, in addition, against the normally non-immunogenic poly-γ-D-glutamate. Thus this vaccine elicits antibacterial and antitoxin responses.

Conclusions

In nature, the inability of anthrax bacilli to spread directly from host to host means that *B. anthracis* gains no advantage from keeping the host alive, but benefits from replicating to very high numbers before returning to the soil as spores. Its strength as a pathogen lies in its ability to overwhelm the host, whereas its weakness is perhaps in finding the next host. Not surprisingly then, the effective dissemination of spores is the limiting step in a terrorist attack and weaponization of spores is focused on producing a form that can be aerosolized and delivered deep into the lungs. Countermeasures include increased levels of funding and research that are facilitating discoveries into the basic mechanisms of anthrax pathogenesis and the development of new vaccines and therapeutics.

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Jeremy Mogridge completed a PhD in 1998 at the University of Toronto. He then joined John Collier's laboratory at Harvard Medical School, where he began studying anthrax toxin. In 2001, he joined the Department of Laboratory Medicine and Pathobiology at the University of Toronto as an Assistant Professor. He holds the Canada Research Chair in Bacterial Pathogenesis.

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