REVIEW ARTICLE

ANTHRAX TOXIN CHARACTERIZATION

Jiří Patočka[1], Miroslav Špliňo[2]

Purkyně Military Medical Academy, Hradec Králové: Department of Toxicology[1], Department of Epidemiology[2]

Summary: The anthrax toxin comprises three proteins. When they work together, they can kill humans, especially after spores of the bacteria have been inhaled. One anthrax protein, called protective antigen (PA), chaperones the two other toxins into human or animal cells and shields them from the body's immune system. The second, lethal factor (LF), destroys the white blood cells that hosts send in defence. The third toxin molecule, edema factor (EF), hijacks the signaling system in the body. This disrupts the energy balance of cells and leads to them accumulating fluid and complete destroy of cells.

Key words: *Bacillus anthracis; Anthrax toxin; Structure; Mechanism of action*

Introduction

The anthrax bacillus, (*Bacillus anthracis*) was the first bacterium shown to be the cause of a disease when in 1877, Robert Koch grew the organism in pure culture, demonstrated its ability to form endospores, and produced experimental anthrax by injecting it into animals.

The concept on which medical microbiology are based arose from its work on this bacterium (13). At that time, 125 years ago, anthrax was significant mainly as an economically damaging disease of domesticated animals. Today anthrax is important agent of biological terrorism and threats to civilization. *B. anthracis* causes three forms of disease, cutaneous, pulmonary and gastro-intestinal. The pulmonary form is the most dangerous and may lead to death merely one to two days after onset of severe symptoms. This is due to the rapid growth and release of several potent toxins that engage the immune system and promote tissue destruction.

Bacillus anthracis

Bacillus anthracis is large, non-motile encapsulated gram-positive spore-forming rod, 1–1.2 μm in width and 3–5 μm in length with square or concave ends. Genotypically and phenotypically it is very similar to *Bacillus cereus,* which is found in soil habitats around the world, and to *Bacillus thuringiensis,* the pathogen for larvae of *Lepidoptera.* The three species have the same cellular size and morphology and form oval spores located centrally in a non-swollen sporangium (11).

B. anthracis is capable of producing aerobic endospores that are highly resistant to desiccation and capable of surviving for decades in soil. Soil is the natural reservoir for *B. anthracis*, but its mere presence in soil does not necessarily result in infection of grazing animals. Under favorable circumstances, spores enter a vegetative phase and multiply to levels high enough to infect grazing herbivores. Anthrax is mainly a disease of animals, but in rare cases, it can spread to people and cause life-threatening illness. *B. anthracis* may cause a systemic infection in animals causing their death which results in the return of a new aerobic endospores population. The stability and infectivity of *B. anthracis* along with its ability to produce a rapidly lethal pneumonia has drawn attention to its potential as a weapon capable of mass casualties (5).

Anthrax Toxin

The toxigenic properties of *B. anthracis* were discovered in 1954. Prior to that time, it was assumed that death was due to blockage of the capillaries by large amount of spores. But experimentally it was shown that only about 3×10^6 cells.ml^{-1} are necessary to cause death of the animal. These observations led to the conviction that a specific anthrax exotoxin plays a major role in the pathogenesis of disease. Death from anthrax in humans or animals frequently occurs suddenly and unexpectedly. The level of the lethal toxin in the circulation increases rapidly quite late in the disease, and it closely parallels the concentration of organisms in the blood.

Production of the anthrax toxin is mediated by **plasmid pX01**, of 110 MDa (16). Anthrax toxin is not single compound, but it is complex substance composed of three antigenically distinct components: Factor I, edema factor (EF), factor II, protective antigen (PA) and factor III, lethal factor (LF). Each component of the toxin is a thermolabile protein with a molecular mass of approximately 80 kDa.

The EF and the LF are internalized into the eukaryotic target cells via the protective antigen (2).

Edema factor (EF) is necessary for the edema producing activity of the toxin. EF from *B. anthracis* is a 89 kDa secreted adenylate cyclase exotoxin and is activated by the host-resident protein calmodulin. (1). Calmodulin is a ubiquitous intracellular calcium sensor in eukaryotes and activates edema factor nearly 1000-fold upon binding (6). EF is known to be an **inherent adenylate cyclase**, similar to the *Bordetella pertussis* adenylate cyclase toxin. The **edema toxin** rise by binding of PA to EF (PA+EF) and this complex induces edema (12).

Protective antigen (PA), 83 kDa protein, induces protective antitoxic antibodies in guinea pigs (3). PA binds to specific cell surface receptors and when cleaved to a 63-kDa fragment forms a channel in the cell's membrane, which allows EF and LF to enter the cytoplasm (18). Seven molecules of the PA toxin subunit bind to one another to form a pre-pore on the cell's surface. The other toxin subunits, LF and EF, bind to the pre-pore, and this complex leak into cell by the help of endocytosis. In the acid environment of the endosome, subsequently, the pre-pore inserts into the membrane and allows LF and EF to enter the cytoplasm, where they destroy the cell (Fig. 1). (14). PA and LF act together to form **lethal toxin (LT)** which is primarily responsible for the death of infected animals.

Anthrax LT is a virulence factor responsible for the major pathologies seen during systemic anthrax infections. Injection of sterile LT into test animals mimics the shock and sudden death seen during active bacterial infections. Once large levels of LT are produced within the body, destruction of bacteria by administration of antibiotics is usually unsuccessful. The LT is believed to be secret into the bloodstream where it circulates freely throughout the body and binds and enters host cells. Once in the cytoplasm, the lethal factor acts as a zinc-metallo-protease disrupting normal homoeostatic functions. Low levels of LT induce macrophage production, in vitro, of the shock-inducing cytokines TNF and Il-1beta. Higher levels of LT cause overproduction of reactive oxygen intermediates, bursting of macrophages and release of mediators of shock (10).

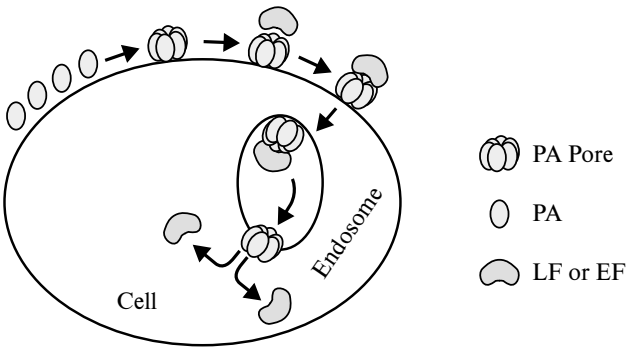

Fig. 1: The illustration shows the assembly and entry of anthrax toxin into the cell. Single molecules of the protective antigen (PA) toxin subunit bind to one another to form a pre-pore (PA Pore) on the cell's surface. The other toxin subunits, lethal factor (LF) and edema factor (EF), bind to the PA Pore, and this complex is endocytosed. In the acidic environment of the endosome, the PA Pore inserts into the membrane and allows LF and EF to enter the cytoplasm, where they evoke destruction of the cell. Illustration by Hana Patočková.

The LF acts as a zinc metallo-protease, which inactivates the mitogen-activated protein kinase (MAPK) pathway used by cells to control cell growth, maturation, and development.

LF appears to proteolytically cleave MAPK-activating enzymes (MAPK kinase 1, MAPKK1 and MAPKK2) leading to inhibition of the MAPK pathway. LF cleaves NH_2-terminal amino acids from MAPKK1 and MAPKK2 preventing activation of the MAPK pathway (7). Although it is clear that LT kills animals and that LF plays a major role in the animal's death, it remains to be proven that MAPK pathway inhibition is the immediate cause of death (8).

Lethal factor (LF) is essential for the **lethal effects** of the anthrax toxin. LF is a protein (relative molecular mass 90 kDa) that is critical in the pathogenesis of anthrax (15). This protein is a highly specific protease that cleaves members of the mitogen-activated protein kinase kinase (MAPKK) family near to their amino termini, leading to the inhibition of one or more signalling pathways. LF comprises four domains: **domain I** binds the membrane-translocating component of anthrax toxin, the protective antigen (PA); domains II, III and IV together create a long deep groove that holds the 16-residue N-terminal tail of MAPKK-2 before cleavage. **Domain II** resembles the ADP-ribosylating toxin from *Bacillus cereus*, but the active site has been mutated and recruited to augment substrate recognition. **Domain III** is inserted into domain II, and seems to have arisen from a repeated duplication of a structural element of domain II. **Domain IV** is distantly related to the zinc metalloprotease family, and contains the catalytic centre; it also resembles domain I. The structure thus reveals a protein that has evolved through a process of gene duplication, mutation and fusion, into an enzyme with high and unusual specificity (15).

Apart from their antigenicity, each of the three factors exhibits no significant biological activity in an animal. However, combinations of two or three of the toxin components yield the following results in experimental animals.

PA+LF combine to produce lethal toxin
EF+PA combine to produce edema toxin
EF+LF is inactive
PA+LF+EF produces edema and necrosis and is lethal

These experiments suggest that the anthrax toxin have the familiar A-B enzymatic-binding structure of bacterial exotoxins with PA acting as the B fragment and either EF or LF acting as the active A fragment (4).

Combination EF+PA has been shown to elevate cyclic AMP to extraordinary levels in susceptible cells. Changes in intracellular cAMP are known to affect changes in membrane permeability and may account for edema. In macrophages and neutrophils an additional effect is the depletion of ATP reserves which are needed for the engulfment process. Hence, one effect of the toxin may be to impair the activity of regional phagocytes during the infectious process (9).

The virulence of *B. anthracis* is attributable to three bacterial components:

1. EF component of exotoxin
2. LF component of exotoxin
3. Capsule component

The EF and LF components have been already characterized. Concerning capsular material, it is known that *B. anthracis* forms a single antigenic type of capsule consisting of a poly-D-glutamate polypeptide. All virulent strains of *B. anthracis* form this capsule. Production of capsular material is associated with the formation of a characteristic mucoid or "smooth" colony type. "Smooth" (S) to "rough" (R) colonial variants occur, which is correlated with ability to produce the capsule. R variants are relatively avirulent. Capsule production depends on a 60 MDa **plasmid pX02** (17).

The capsule of *B. anthracis*, composed of poly-D-glutamic acid, serves as one of the principal virulence factors during anthrax infection. By virtue of its negative charge, the capsule is purported to inhibit host defence through inhibition of phagocytosis of the vegetative cells by macrophages. The toxin-encoding plasmid pX01 and capsule-associated plasmid pX02 are required for full virulence of *B. anthracis* in some animals.

The poly-D-glutamyl capsule is itself nontoxic, but functions to protect the organism against the bactericidal components of serum and phagocytes, and against phagocytic engulfment. The capsule plays its most important role during the establishment of the infection, and a less significant role in the terminal phases of the disease, which are mediated by the anthrax toxin (1).

Both the capsule and the anthrax toxin can play a role in the early stages of infection, through their direct effects on phagocytes. Virulent anthrax bacilli multiply at the site of the lesion. Phagocytes migrate to the area but the encapsulated organisms can resist phagocytic engulfment, or if engulfed, can resist killing and digestion. A short-range effect of the toxin is its further impairment of phagocytic activity and its lethal effect on leukocytes, including phagocytes, at the site. After the organisms and their toxin enter the circulation, the systemic pathology, which may be lethal, will result. *B. anthracis* coordinates the expression of its virulence factors in response to a specific environmental signal. Anthrax toxin proteins and the antiphagocytic capsule are produced in response to growth in increased atmospheric CO_2. This CO_2 signal is thought to be of physiological significance for a pathogen, which invades mammalian host tissues.

Acknowledgments

This study was supported by the grant of Ministry of Defense, project No. 6602129911.

References

1. Bhatnagar R, Batra S. Anthrax toxin. Crit Rev Microbiol 2001;27:167–200.
2. Brossier F, Mock M. Toxins of Bacillus anthracis. Toxicon 2001;39:1747–55.
3. Brossier F, Weber-Levy M, Mock M, Sirard JC. Protective antigen-mediated antibody response against a heterologous protein produced in vivo by Bacillus anthracis. Infect Immun 2000;68:5731–4.
4. Cao H, Agrawal D, Kushner N, Touzjian N, Essex M, Lu Y. Delivery of exogenous protein antigens to major histocompatibility complex class I pathway in cytosol. J Infect Dis 2002;185:244–51.
5. Dragon DC, Rennie RP. The ecology of anthrax spores: tough but not invincible. Can Vet J 1995;36:295–301.
6. Drum CL, Shen Y, Rice PA, Bohm A, Tang WJ. Crystallization and preliminary X-ray study of the edema factor exotoxin adenylyl cyclase domain from Bacillus anthracis in the presence of its activator, calmodulin. Acta Crystallogr D Biol Crystallogr 2001;57:1881–4.
7. Duesbery NS, Webb CP, Leppla SH et al. Proteolytic inactivation of MAP-kinase-kinase by anthrax lethal factor. Science 1998;280:734–7. Comment in: Science 280:1673–4 and Science 1998;280:676.
8. Duesbery NS, Vande Woude GF. Anthrax lethal factor causes proteolytic inactivation of mitogen-activated protein kinase kinase. J Appl Microbiol 1999;87:289–93.
9. Guidi-Rontani C, Weber-Levy M, Mock M, Cabiaux V. Translocation of Bacillus anthracis lethal and oedema factors across endosome membranes. Cell Microbiol 2000;2:259–64.
10. Hanna P. Lethal toxin actions and their consequences. J Appl Microbiol 1999;87:285–7.
11. Helgason E, Okstad OA, Caugant DA et al. Bacillus anthracis, Bacillus cereus, and Bacillus thuringiensis – one species on the basis of genetic evidence. Appl Environ Microbiol 2000;66:2627–30.
12. Kumar P, Ahuja N, Bhatnagar R. Purification of anthrax edema factor from Escherichia coli and identification of residues required for binding to anthrax protective antigen. Infect Immun 2001;69:6532–6.
13. Mock M, Fouet A. Anthrax. Annu Rev Microbiol 2001;55:647–71.
14. Nassi S, Collier RJ, Finkelstein A. PA63 channel of anthrax toxin: an extended beta-barrel. Biochemistry 2002;41:1445–50.
15. Pannifer AD, Wong TY, Schwarzenbacher R et al. Crystal structure of the anthrax lethal factor. Nature 2001;414:229–33.
16. Sirard JC, Guidi-Rontani C, Fouet A, Mock M. Characterization of a plasmid region involved in Bacillus anthracis toxin production and pathogenesis. Int J Med Microbiol 2000;290:313–16.
17. Welkos S, Little S, Friedlander A, Fritz D, Fellows P. The role of antibodies to Bacillus anthracis and anthrax toxin components in inhibiting the early stages of infection by anthrax spores. Microbiology 2001;147:1677–85.
18. Zhao J, Milne JC, Collier RJ. Effect of anthrax toxin's lethal factor on ion channels formed by the protective antigen. J Biol Chem 1995;270:18626–30.

Submitted March 2002.
Accepted April 2002.

Prof. RNDr. Jiří Patočka, DrSc.,
Purkyně Military Medical Academy,
Department of Toxicology,
500 01 Hradec Králové,
Czech Republic.
e-mail: patocka@pmfhk.cz