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Minireview

Anthrax toxin: a tripartite lethal combination[1]

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Abstract Anthrax is a severe bacterial infection that occurs when *Bacillus anthracis* spores gain access into the body and germinate in macrophages, causing septicemia and toxemia. Anthrax toxin is a binary A–B toxin composed of protective antigen (PA), lethal factor (LF), and edema factor (EF). PA mediates the entry of either LF or EF into the cytosol of host cells. LF is a zinc metalloprotease that inactivates mitogen-activated protein kinase kinase inducing cell death, and EF is an adenylyl cyclase impairing host defences. Inhibitors targeting different steps of toxin activity have recently been developed. Anthrax toxin has also been exploited as a therapeutic agent against cancer.

Key words: Anthrax toxin; Edema factor; Lethal factor; Protective antigen; *Bacillus anthracis*

1. Introduction

Horrendus magis est perimit qui corpora, carbo:
urit hic inclusus, vitalia rumpit apertus.[2]

Anthrax is an often fatal, rather uncommon, bacterial infection caused by the rod-shaped Gram-positive bacterium *Bacillus anthracis*. After entering into the body through skin abrasions, inhalation or ingestion, *B. anthracis* spores are phagocytosed by macrophages, which either remain in the primary infection site or migrate to regional lymph nodes. The spores germinate in the macrophages and become vegetative bacteria which lyse the macrophage, multiply in the lymphatic system, and then enter the bloodstream, causing massive septicemia and toxemia which has systemic effects that can lead to the death of the host. *B. anthracis* continues its proliferation until the nutrients are terminated, triggering sporulation which generates an immense number of highly resistant spores. These are dispersed in the environment by natural agents and remain intact for dozens, perhaps hundreds of years, ready to infect animals and humans, restarting the cycle of the pathogen [1–8].

Anthrax is an epizootic disease to which most mammals, especially grazing herbivores, are considered susceptible. On rare occasions, anthrax can be transmitted to humans, usually through contact with infected animals or their products; there are no known cases of human-to-human transmission. Cutaneous anthrax, the most common form of the disease, is easily curable and usually heals spontaneously. However, a small percentage of cutaneous infections becomes systemic and these can be fatal. Cutaneous anthrax is initially characterized by an edematous–necrotic lesion that evolves into a black eschar. The color and the appearance of the black eschar give the disease its name 'anthrax', which is derived from the Greek word for coal. Systemic anthrax resulting from the inhalation of the microorganism (inhalation anthrax) has a mortality rate approaching 100%, with death usually occurring rapidly after the onset of symptoms. Based on primate data, the LD_{50} for humans (i.e. the dose sufficient to kill 50% of the people exposed to it) is 3×10^3 to 5×10^4 inhaled *B. anthracis* spores, varying in relation to the strain virulence and the delivery system. The rate of mortality among persons with infection resulting from ingestion of *B. anthracis* spores (gastrointestinal and oropharyngeal anthrax) is variable, depending on the outbreak. The involvement of meninges (anthrax meningitis) is a rare but almost always fatal complication of systemic anthrax. Whatever the portal of entry, systemic anthrax involves massive bacteremia and toxemia with unnoticed initial symptoms until the onset of hypotension, shock, and sudden death. Manifestations of the advanced disease, including shock and sudden death, are believed to result from the *B. anthracis* toxin action [1–8].

The major virulence factors of *B. anthracis* are the (exo)-toxin that causes cell death and impairs host defences, and the

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[1] This paper is dedicated to the memory of the victims of bioterrorism.

[2] 'Much more aggressive is the deadly anthrax: concealed it burns slowly, once overt it ravages vital organs'. From *Liber Medicinalis* by Quintus Serenus Samonicus (250–350 CE), Chapter XXXVIII, *Carboni tollendo* (Anthrax therapy).

Abbreviations: ATR, anthrax toxin receptor; CaM, calmodulin; EF, edema factor; EF_{58}, C-terminal catalytic portion of EF; ET, edema toxin; LF, lethal factor; LT, lethal toxin; MAPK, mitogen-activated protein kinase; MAPKK, mitogen-activated protein kinase kinase; PA, protective antigen; PA_{20}, proteolytically cleaved N-terminal fragment of PA; PA_{63}, proteolytically activated C-terminal fragment of PA; $[PA_{63}]_7$, heptameric PA_{63}

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capsule that inhibits the phagocytosis of vegetative bacilli. The toxin-coding plasmid pXO1 carries the genes (*cya*, *lef*, and *pag*) making up the secreted toxin, and the capsule-coding plasmid pXO2 carries the genes (*capB*, *capC*, and *capA*) involved in the synthesis of the poly-D-glutamyl capsule. The expression of *B. anthracis* toxin and capsule genes is mediated by the transcriptional activator AtxA, whose activity appears to be affected by host-specific factors such as elevated temperature (≥ 37°C) and carbon dioxide concentration ($\geq 5\%$) and by the presence of serum components. The expression of the capsule gene is also controlled by its own transcriptional regulator, AcpA [1–8].

2. The *B. anthracis* toxin

2.1. A tripartite lethal combination

The *B. anthracis* toxin belongs to the family of bacterial binary A–B toxins characterized by an A moiety, which acts within the cytosol of target cells and by a B moiety that binds target cells and translocates the A moiety into the cytosol. It is composed of a single B unit called protective antigen (PA), and two alternative A subunits: the lethal factor (LF) or the edema factor (EF). The three toxin components combine to form two binary toxins, the lethal toxin (LT, i.e. LF+PA) and the edema toxin (ET, i.e. EF+PA). PA binds directly to the so-called cell-surface anthrax toxin receptor (ATR), a type I membrane protein with an extracellular Willebrand factor A-binding domain, and so mediates the entry of LF and EF into the cytosol of host cells. LF is a zinc-dependent endopeptidase which removes specifically the N-terminal tail of mitogen-activated protein kinase kinases (MAPKKs), leading to macrophage lysis. EF is a calcium- and calmodulin- (CaM-) dependent adenylyl cyclase which elevates the intracellular cAMP concentration, leading to impairment of host defences [1–12].

2.2. The anthrax PA: toxin assembly and translocation

Anthrax PA, so-named for its use in vaccines, is the central component of the tripartite protein toxin secreted by *B. anthracis*. PA mediates the entry of LF and EF into the host cell (Fig. 1), but can also translocate chimeric proteins containing PA-binding determinants, and is being evaluated for use as a general protein delivery system, mainly for therapeutic purposes [1–8,11,13,14].

Monomeric PA displays four structural domains, mostly composed of antiparallel β-sheets. Domain 1 contains two calcium ions and the cleavage site for activating proteases. Domain 2 (i.e. pore formation) contains a large flexible loop implicated in membrane insertion. The small domain 3 (i.e. oligomerization) is involved in heptamer formation. Domain 4 (i.e. receptor binding) binds to ATR (Fig. 2) [5,15,16].

After binding ATR on the cell surface, PA is proteolytically activated by a furin-like cell-surface membrane protease (Fig. 1). The enzymatic cleavage of PA occurs at a surface loop within domain 1 (Fig. 2) with the release of the proteolytically cleaved N-terminal fragment of PA (the PA_{20} fragment) in the extracellular medium. PA_{20} plays no further role in anthrax intoxication. Loss of PA_{20} leads the proteolytically cleaved C-terminal fragment of PA (PA_{63}) to self-associate, forming symmetric, ring-shaped, membrane-inserting heptamers ($[PA_{63}]_7$) (Figs. 1 and 2). PA_{63} monomers pack like pie wedges, with domains 1′ (the remainder of domain 1) and 2 on the inside of the $[PA_{63}]_7$ ring and domains 3 and 4 on the outside. $[PA_{63}]_7$ shows a negatively charged lumen, and exposes a large hydrophobic surface for competitive binding of the anthrax toxin enzymes LF or EF (Figs. 1 and 2) [15].

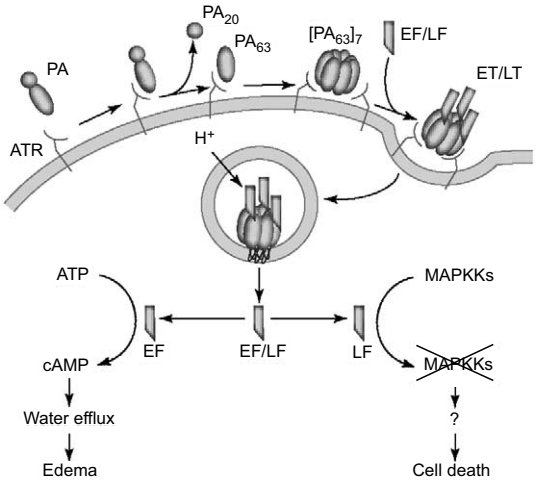

Fig. 1. Model of anthrax toxin action. PA secreted by *B. anthracis* binds to ATR. Then, PA is cleaved by a furin-like cell-surface membrane protease, releasing the PA_{20} subunit in the extracellular milieu. The remaining PA_{63} subunit oligomerizes to form $[PA_{63}]_7$ that binds a maximum of three molecules of LF or EF. The assembled toxins (ET and LT) are endocytosed and trafficked to endosomes. There the low pH triggers a conformational change(s) in $[PA_{63}]_7$ promoting the translocation of LF and EF across the membrane. LF is a highly specific zinc metalloprotease that cleaves members of the MAPKK family within their N-termini. This cleavage initiates a poorly characterized chain of events leading to the death of the host. EF is a calcium- and CaM-dependent adenylyl cyclase that causes an increase in intracellular cAMP concentration resulting in water efflux and edema. Modified from [7] with permission of the authors.

The LF and EF binding site was found to span the interface between two adjacent PA_{63} subunits (Fig. 2), explaining the dependence of LT and ET formation on PA oligomerization. The location of residues comprising the LF and EF binding site (Fig. 2) suggests that a single LF or EF molecule sterically occludes two adjacent sites, consistent with the finding that $[PA_{63}]_7$ binds a maximum of three LF or EF molecules. The resulting $[PA_{63}]_7$–LF and $[PA_{63}]_7$–EF complexes are taken up into cells by ATR-mediated endocytosis and enter the lumen of the acidic endosomal compartment (Fig. 1). Low pH triggers a conformational change(s) of $[PA_{63}]_7$, leading to its insertion into the membrane and the formation of a membrane-spanning cation-selective channel, based on a 14-stranded β-barrel (Fig. 1). This conversion drives the translocation of LF and EF across the endosomal membrane into the cytosol (Fig. 1) [4,5,7,10,15,17–20].

2.3. The anthrax LF: a zinc metalloprotease

Anthrax LF is a highly specific, four-domain zinc metalloprotease that cleaves members of the MAPKK family within their N-termini. This cleavage initiates a poorly characterized chain of events leading to host death (Fig. 1) [1–8,11,21,22]. The correlation between MAPKK cleavage and the reported LF-dependent inhibition of the release of pro-inflammatory mediators nitric oxide, tumor necrosis factor-α, and interleukin-1β or macrophage cytotoxicity is the subject of intensive investigations [1–8,23–26]. A rapid and selective apoptosis of lipopolysaccharide-activated macrophages has

been correlated to the LF-induced cleavage of the N-terminal portion of MAPKKs with activation of the p38 mitogen-activated protein kinase (MAPK) [27]. By inducing apoptosis of activated macrophages, LT could prevent the release of chemokines and cytokines that alert the immune system to the presence of infecting *B. anthracis*, thereby facilitating the systemic spread of the infection [27]. This result is difficult to reconcile with the previous, well documented, LT-induced cytotoxicity and with the fact that the final stages of anthrax have all the characteristics of a septic shock triggered by an extensive release of vasoactive mediators of inflammation [1–8,23–26]. Perhaps LF also cleaves other intracellular substrates or it exhibits additional functions in host cells, which could account for these pathophysiological observations [4].

The three-dimensional structure of LF reveals a protein that has evolved through a process of gene duplication, mutation and fusion, into an enzyme with high and unusual specificity. LF comprises four domains, with the prevalence of α-helices. Domain 1 (i.e. PA binding) binds the $[PA_{63}]_7$ component of LT. Domain 2 (i.e. VIP2-like) resembles the ADP-ribosylating toxin VIP2 from *Bacillus cereus*, but the active site has been mutated and recruited to participate in substrate recognition. Domain 3 (i.e. helix bundle) is inserted into domain 2, and seems to have arisen from a repeated duplication of a structural element of domain 2. Domain 4 (i.e. catalytic center) is distantly related to the zinc metalloprotease family and contains the active site (Fig. 2) [28].

The active site zinc ion is coordinated tetrahedrally to the LF domain 4 by a water molecule and three side chains (i.e. His686, His690, and Glu735), in a structural arrangement typical of the thermolysin family. Glu687 acts as a general base to activate the zinc-bound water molecule during catalysis and Tyr728 (located on the opposite side of Glu687) probably functions as a general acid to protonate the leaving amino group [28].

The LF broad deep groove holding the substrate (~40 Å long) is contiguous with the active site center (Fig. 2). The groove has an overall negative electrostatic potential containing clusters of Glu/Asp as well as Gln/Asn residues. The alignment of the N-terminal region of MAPKKs made it possible to determine a consensus motif for the cleavage site: positively charged residues are located at positions P_7–P_4 and hydrophobic residues at P_2 and P_1'. It is worth noting that the EF:MAPKK-2 substrate adduct (Fig. 2) is the first example of a protease in complex with its uncleaved substrate and might represent a 'pre-cleavage' adduct [28,29].

2.4. The anthrax EF: a calcium- and CaM-activated adenylyl cyclase

Anthrax EF is a calcium- and CaM-dependent adenylyl cyclase that takes ATP, clips off two phosphates and forms cAMP. EF floods the cell with cAMP, upsetting water homeostasis and destroying the delicate balance of intracellular signaling pathways. Moreover, EF impairs neutrophil function(s) and it is believed to be responsible for the edema found in cutaneous anthrax (Fig. 1) [1–8,11].

The C-terminal catalytic portion of EF (i.e. EF_{58}) shares no significant structural homology with mammalian adenylyl cyclases or any other protein. EF_{58} comprises three globular domains (i.e. 1 or C_A, 2 or C_B, and 3 or helical) and three switch loops (i.e. A, B and C) (Fig. 2). Domain 1 binds the $[PA_{63}]_7$ component of ET. Remarkably, EF_{58} and LF share

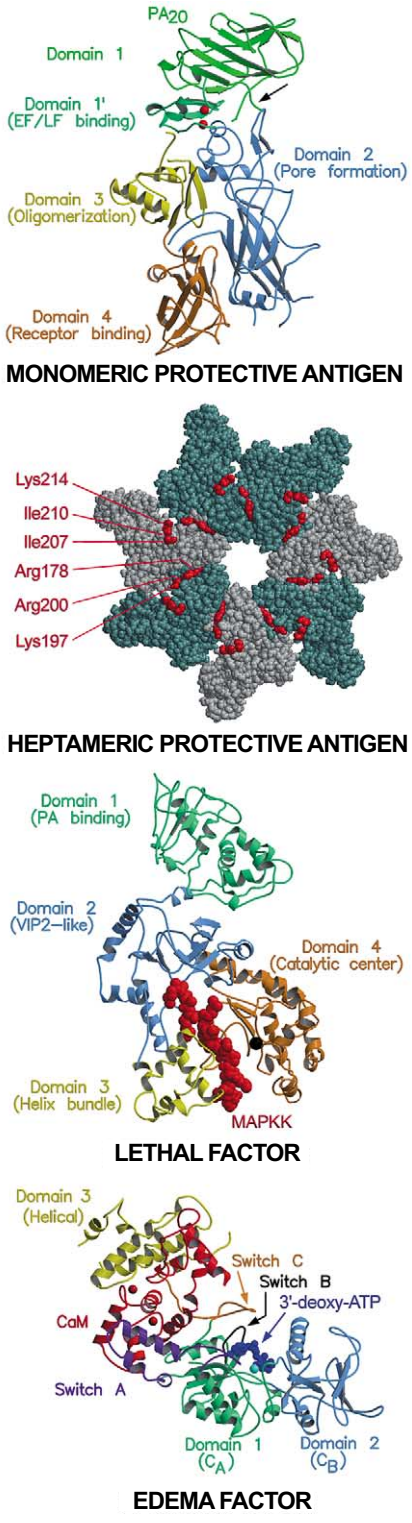

Fig. 2. Crystal structures of the anthrax toxin components PA_{63} [15], $[PA_{63}]_7$ [7], LF [28], and activated EF_{58} [30]. Critical residues of $[PA_{63}]_7$ involved in binding to LF and EF lie at the interface between two adjacent PA_{63} monomers. Residues Arg178, Lys197, Arg200, Ile207, Ile210, and Lys214, when mutated, prevent LF binding. PA_{63} monomers are colored gray and green for easier recognition of individual subunits. The black arrow indicates the proteolytic cleavage site of PA resulting in PA_{63} generation. Red, black, and light blue spheres indicate calcium, zinc and metal ions, respectively. PA_{63}, LF, and activated EF_{58} were drawn with MOLSCRIPT [48]. For further structural details on individual protein regions, see [7,15,28,30]. The crystal structure of $[PA_{63}]_7$ was reproduced from [7] with permission of the authors.

significant homology at the level of domain 1, competing for the same binding site on $[PA_{63}]_7$ (Figs. 1 and 2) [5,7,17–20]. The EF_{58} active site lies at the interface of domains 1 and 2, which together constitute the catalytic core (Fig. 2) [30].

The structure of EF_{58} alone differs significantly from that of calcium- and CaM-activated EF_{58}, but there are essentially no differences between the structures of EF_{58}–CaM with and without the non-cyclizable substrate analog 3′-deoxy-ATP. CaM occupies much of the same volume taken by domain 3 in the structure of EF_{58} alone. To accommodate CaM, the EF_{58} domain 3 undergoes ~15 Å translation and ~30° rotation away from the EF_{58} catalytic core, such that domains 1 and 3 and the switch loop C (composed of residues from the linker that connects domains 1 and 3) form a large clamp that almost completely encircles CaM (Fig. 2). In addition to the domain 3 displacement, the three switch loops A, B and C undergo large conformational changes in response to CaM binding, leading to EF_{58} activation and permitting substrate binding. CaM bound to EF_{58} shows an extended conformation (Fig. 2), very different from the collapsed geometry observed in other structures of CaM bound to effector peptides [30].

The location of 3′-deoxy-ATP and of the single metal ion in the catalytic site of activated EF_{58}–CaM (Fig. 2) is in keeping with a mechanism centered on residue His351 acting as the catalytic base. Such a mechanism differs from that proposed for mammalian adenylyl cyclases which are thought to require two metal ions [30].

3. Fight against anthrax

B. anthracis strains are generally sensitive to a variety of antibiotics, including penicillin, doxicyclin, and fluoroquinolones. However, the paucity of early symptoms and the rapid course of the disease make the antimicrobial therapy ineffective, especially in the case of inhalation anthrax, calling for alternative therapeutic strategies [1–8,31,32].

Vaccination has protected cattle and select humans (e.g. the military personnel) by eliciting a strong humoral response primarily directed to the neutralization of PA activity, but it is not recommended for population-wide prophylaxis [1–8,32–38]. Animal studies also indicate that passive immunization with monoclonal antibodies against PA provides some protection to the lethal challenge with *B. anthracis* [39,40].

At present, pharmacological inhibition of toxin activity seems to be the major (if not the only) therapeutic option for severely ill patients, especially in the unlucky eventuality of a bioterroristic attack involving inhalation anthrax [4,32,41,42]. Notably, a soluble extracellular ATR domain comprising the PA binding site prevents PA binding to ATR and protects cultured cells from toxin action [9]. Furthermore, a peptide that binds $[PA_{63}]_7$ and inhibits the interaction with LF (i.e. impairs LT assembly) protects rats from LF-induced death [43]. Accordingly, the mutation of LF residues involved in the interaction with PA abrogates LT assembly and intoxication [17]. Rats and cultured cells challenged with LT are also protected by mutated PA_{63} that binds cells, co-oligomerizes with wild-type PA_{63} and binds LF, but is unable to form channels and translocate LF into the cytosol. A single mutated PA_{63} monomer per heteroheptamer is sufficient to abolish LF translocation [33,34]. The inhibition of the LF catalytic activity is an additional step of anthrax toxin therapy; peptides impairing LF activity were recently found to abrogate LF cytotoxicity in a macrophage cell line [44]. Finally, protection from LT might be achieved by interfering with the function(s) of Kif1C, a kinesin-like motor protein which mediates mouse macrophage resistance to LF [4,45]. The redundancy of this microtubule-based molecular motor protein in mammals [46] may be of advantage as a therapeutic approach.

4. The therapeutic use of the anthrax toxin

Since MAPKKs regulate MAPKs, the discovery that LF cleaves MAPKKs hinted that LT could be used as a therapeutic against cancer cells in which MAPKs are activated by oncogenic proteins such as Ras. In fact, intratumorally injected LT has been shown to inhibit the growth of Ras-transformed cells implanted in athymic nude mice, with no apparent systemic toxicity. Moreover, LT greatly reduces tumor neovascularization, a critical step in tumor growth and spread [47]. Furthermore, mutated PA molecules were engineered by replacing the furin site with recognition sites for matrix metalloproteases and urokinase plasminogen activator, which are overexpressed in a variety of tumor tissues and cell lines. Consequently, only tumor cells overexpressing these proteases could activate PA mutated at the furin site. The engineered PA directs toxicity towards tumor cells without affecting non-transformed cell lines [13,14]. Future design of such therapeutic agents will take advantage of both the unique properties exhibited by cancer cells and the powerful catalytic activity of LF and EF. These features ensure selective damage of tumors even at very low dosage, thereby improving the therapeutic index of these drugs.

There is some good in every ill.

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