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New aspects of the infection mechanisms of *Bacillus anthracis*

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Abstract

Articles concerning new aspects of *B. anthracis* mechanisms of infection were reviewed. It was found, that the hair follicle plays an important role in the spore germination process. The hair follicle represent an important portal of entry in the course of the cutaneous form of disease infections. After mouse exposition to aerosol of spores prepared from *B. anthracis* strains, an increase in the level of TNF-α cytokines was observed. The TNF-α cytokines were produced after intrusion into the host by the microorganism. This process may play a significant role in the induced migration of infected cells APCs (Antigen Presenting Cells) via chemotactic signals to the lymph nodes. It was explained that IgG, which binds to the spore surface, activates the adaptive immune system response. As a result, the release C3b opsonin from the spore surface, and mediating of C3 protein fragments of *B. anthracis* spores phagocytosis by human macrophages, was observed. The genes coding germination spores protein in mutant strains of *B. anthracis* MIGD was a crucial discovery. According to this, it could be assumed that the activity of *B. anthracis* spores germination process is dependent upon the *sleB*, *cwlJ1* and *cwlJ2* genes, which code the GSLEs lithic enzymes. It was also discovered that the specific antibody for PA20, which binds to the PA20 antigenic determinant, are able to block further PA83 proteolytic fission on the surface of cells. This process neutralized PA functions and weakened the activity of free PA20, which is produced during the PA83 enzyme fission process. Interaction between PA63 monomer and LF may be helpful in the PA63 oligomerization and grouping process, and the creation of LF/PA63 complexes may be a part of an alternative process of assembling the anthrax toxin on the surface of cells. It was found that actin-dependent endocytosis plays an important role in the PA heptamerisation process and leads to blocking the toxin activity. Chaperones, a protein derived from host cells, may be helpful in ATP and cytosolic factors translocation, and in this way increase the translocation of diphteria toxin A domein (DTA) and substrate of fusion protein LF_N-DTA.

Key words

Cutaneous anthrax, anthrax toxins, inhalational anthrax

INTRODUCTION

Bacillus anthracis is a Gram-positive rod-shaped bacteria with a characteristic capsule, which plays an important role in the initial stage of infection, protecting the pathogen from phagocytosis [1]. In the environment outside the host organism, most frequently in soil, *B. anthracis* produces spores. After entering the body through the skin, gastrointestinal tract, or by inhalation, the spores can multiply either at the site of infection, causing local changes, or may penetrate the lymph nodes, thereby initiating an infection. Depending on the route of infection, there are three basic forms of anthrax: cutaneous, pulmonary and oral [2].

Cutaneous anthrax. Cutaneous anthrax occurs as a result of contamination of skin with the bacterial spores, due to its mechanical abrasion or damage caused by insect bites. Although the most common form of anthrax is skin infection, the process of germination of spores on the skin is unknown. In experiments on mice infected with *B. anthracis* spores in the epiderma, it was shown that abrasion of the skin is essential for the development of the infection. On intact skin, although there is a spore germination, the process does not lead to further development of the infection. It is assumed that the germination of spores, which are inoculated on the abraded skin, does not require phagocytosis by macrophages, however, some studies have shown that the spore germination is dependent on phagocytosis [3].

After experimental intradermal injection of monkeys and mice with *B. anthracis* a large number of bacilli was observed in the infected skin, but their presence could result from the digestion of spores by macrophages, intracellular multiplication and their escape with the extracellular fluids of the host [3]. It was found that after applying the filter that separated the spores from the phagocytes, an extracellular germination of spores occurred. The mechanism of macrophages action probably differs between the skin and inhalation form of infection. Spores getting into the body via the pulmonary route encounter the pulmonary alveolar macrophages, while the neutrophils that constitute the majority of cells present in the tissue fluids of damaged skin may accumulate there intensively, making the contact of macrophages with damaged skin difficult, and inhibiting germination of the spores. The presence of substances that stimulate germination in extracellular fluids of damaged skin is also important. A small number of ungerminating spores can penetrate the damaged skin, and initiate the infection thanks to the presence of macrophages.

It was shown that after infection of damaged skin of mice, the extracellular germination occurred on the surface, before the reaction from the phagocytes was initiated. The spores

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stained with malachite green began to germinate 1-3 hours after inoculation. The skin abrasion is necessary for the germination of *B. anthracis* spores, although it is not the only condition determining the development of infection [3]. Extracellular spore germination, as well as the production of large numbers of bacilli on the skin surface, does not determine the further development of infection [3].

In recent years, another mechanism initiating the infection was discovered, which involves the participation of hair follicles. A series of inoculations was performed on mice of the DBA/2 and C57BL/6 strains in the inflammatory foci of hair follicles, and after inoculation, histological examination of the epidermis was performed in the central and outer parts of the hair follicles. Analysis showed an extensive spread of the infection, indicating that the hair follicles may also be a possible route of infection. After the experimental infection of mice with *B. anthracis* via hair follicles, the progression of anthrax disease and death of the animals were observed [4]. The multiplication of *B. anthracis* bacilli in fragments of the epidermis and hair follicles may constitute an important way for the development of the skin infections. In the intradermal infection of immunized C57B /6 mice with *B. anthracis* spores the animals were shown to survive the infection [4].

Pulmonary anthrax. The initial stage of pulmonary (inhalation) anthrax is the accumulation of *B. anthracis* spores in the space of bronchial pneumonia, and then germination of the scattered bacilli inside the host organism. Internalization of "dormant" spores inside the lungs can occur in different types of host cells and is probably a key step in the transition from localized to disseminated infection phase [5]. In this phase, alveolar macrophages are involved in the transport of spores from the lung to regional lymph nodes and dendritic cells. It was shown that internalization of spores took place in the epithelial cells lining the alveoli [5].

B. anthracis spores located in the bronchioles and alveoli are phagocytosed by antigen presenting cells (APCs) and moved to the regional lymph nodes. Lysis of the infected host cells in the mediastinal lymph nodes causes a release of the *B. anthracis* bacilli, which penetrate into the bloodstream, leading to the development of bacteremia, and in consequence, evoking damage of the blood vessels and oedema, haemorrhage and thrombosis, and finally, death of the host [6]. It has been proved that *B. anthracis* spores do not germinate in the lungs, since both spores and bacilli are found in the lymph nodes on the day of infection. The lungs simultaneously play the role of an input port and a lock through which spores are able to enter the body, while the spore germination and rapid growth of bacteria occurs in the pulmonary lymph nodes which, on the other hand, are also the site of the host's immune response after recognition of the agent [6]. After aerosol infection of mice with *B. anthracis* spores, an increase in the level of TNF-α was observed. TNF-α is a known strong anti-inflammatory cytokine produced after the entry of a pathogen into the host organism by the reaction of Toll-like receptors. Perhaps this phenomenon plays an important role in inducing migration of infected APCs to the lymph nodes. It was shown that TNF-α levels in the lymph nodes decreased during a simultaneous increase in the number of bacteria (CFU – colony-forming units) in the liver and spleen. The levels of TNF-α and other inflammatory cytokines (interleukin-1 and interleukin-6) increased at the beginning of *B. anthracis* infections in mice. After aerosol infection of mice of the A/J strain, changes in the levels of TNF-α in the lungs, liver and lymph nodes were observed in early stage of the disease. The level of TNF-α in the liver of the infected mice was twice as high in comparison to the uninfected animals at the beginning of infection, and the value of CFU (bacterial loads) in the livers of dying mice increased 1 and 3 days after infection [6].

Alveolar macrophages play a key role in the pathogenesis of anthrax inhalation. The role of macrophage receptors that mediate phagocytosis of spores of *B. anthracis* is still not fully elucidated [7]. In order to clarify whether immunoglobulins present in the lungs are involved in phagocytosis, the binding of human IgG and C3 to the surface of *B. anthracis* spores was examined at different concentrations of human serum and in the presence of monocytes. It turned out that opsonin C3b bound with *B. anthracis* spores was activated by the binding of IgG to the surface of spores. Additionally, it was demonstrated that the C3 opsonin facilitated the occurrence of phagocytosis and augmented the phagocytosis by human macrophages. It was also shown that native IgG in serum, although associated with spores of *B. anthracis*, were not able to influence the phagocytosis. IgG bound to the surface of the spores activated the specific immune response. As a result of the activation of the IgG binding to the surface of the spores, a release of the C3b opsonin from the spores' surface was observed. In consequence, the associated C3 fragments on the *B. anthracis* spores mediated the phagocytosis by human macrophages [7].

The outer layer of *B. anthracis* spores (exosporium) has a nap formed by BcIA glycoprotein on its surface, which is similar to the structure of hair [8]. Recognition of BcIA by Mac-1 integrin (a receptor playing a role in cellular signaling) leads to the absorption of bacterial spores and growth of this pathogen in distant lymph nodes. It was shown that CD14 associates with rhamnose residues of BcIA, acting as a co-receptor in spore binding by Mac-1. In this process, CD14 induces signal transduction mediated through TLR2 and PI3K, which stimulates the activation of Mac-1, thereby enhancing internalization of spores by macrophages. Rhamnose binding to CD14 induces signals from TLR-2, leading to activation of PI3K and conversion of Mac-1 into an active receptor for *B. anthracis* spores. Mac-1-dependent etching of spores show independence from the CD14 receptor. Binding and absorption of *B. anthracis* spores by phagocytes is a dynamic process involving multiple receptors and signaling pathways [8].

In recent years, genes essential for intracellular germination of *B. anthracis* spores have been identified; 18 loci were identified using the mini – transposon library for *B. anthracis,* and mutants causing the intracellular germination delay were screened using a mutated transposon. Fourteen transposon mutants were identified among genes that had not been previously suspected to be related to germination of *B. anthracis*. The other four mutants, which had insertional transposons in the genes: gerHA, gerHB, gerHC encoding spore germination proteins: GerHA, GerHB and GerHC, respectively, were identified in the mutants of the *B. anthracis* MIGD 101 strain [1]. Strain MIGD 101 (macrophage intracellular germination defective 101) has a transposon insertion in an intergenic region between BAs2808 and BAs2807, which can be helpful in clarifying how intergenic regions affect the ability of *B. anthracis* germination [1].

Expression of the lethal factor (LF) and edema factor (EF) in the early phase of infection is probably necessary to initiate germination, and thus important in weakening the phagocytosis of bacteria, which enables protection from the damaging effects of macrophages and their release [9].

B. anthracis spore cortex, which is the main component affecting its stability and hibernation before germination, is hydrolysed by specific lytic enzymes GSLEs (Germination-Specific Lytic Enzymes), which allow the initiation of this process and the multiplication of vegetative cells. The activity of *B. anthracis* spore germination is dependent on the genes encoding GSLEs (SleB, CwlJ1, Cw1J2): sleB, and cwlJ2 cwlJ1. In addition, it was demonstrated that removal of any of these genes was not decisive in stopping the process of spore germination, suggesting that the proteins encoded by these genes may also have other functions [10]. The process of spore germination is induced by nutrients (food germinants), which are associated with the spore inner membrane receptors, and cause accumulation of dipicolinic acid (DPA) and Ca^{2+} in the spore cortex [11]. Spore cortex is a thin layer consisting of peptidoglycan (PG), responsible for its resistance to environmental factors. When the spore's protective barrier is broken, activation of specific lytic enzymes occurs, leading to rehydration and rapid growth of bacteria.

After inhalation into the lungs, the spores remain dormant for up to several weeks before they are engulfed by macrophages and transported to the pulmonary lymph nodes [10] where they germinate and multiply. An important role in this process is played by GSLEs: SleB, and to a lesser extent CwlJ1 CwlJ2. It was found that the removal of all three enzymes resulted in blocking germination *in vitro* [10].

As mentioned earlier, germination requires degradation of the peptidoglycan of the spore cortex by four specific germination lytic enzymes: CwlJ1, CwlJ2, SleB and SleL. The function of all four GSLEs was examined *in vivo* by combining them and creating deletion mutations in all possible double, triple and quadruple mutations. It is thought that lytic transglycosylase SleB may facilitate the digestion of the cortex peptidoglycan (PG) into fragments of different sizes: tetra and octasaccharides, some of which are released, and the remaining part is accumulated inside the spores. CwlJ1 affects the completion of germination, but the mechanism of its enzymatic activity remains to be explained. CwlJ2 contributes to the final digestion of the spore cortex, playing a complementary role in the process of germination [12].

Anthrax toxins. *B. anthracis* spores after entering the body, germinate in favourable conditions, converting into a vegetative state capable of producing toxins that play a crucial role in the pathogenesis of the disease. The main role of these toxins in the development of anthrax has been demonstrated with the use of highly purified toxins which, when administered to animals, caused symptoms typical for anthrax [13]. Application of antibodies against the anthrax toxins protected the animals against infection [13].

In recent years, much new information concerning the mechanisms and regulation of anthrax toxins action, as well as their expression, has been revealed [13]. On the basis of the latest findings, a new line of research has been developed devoted to the methods of anthrax toxins neutralization and inhibition of their activity [13].

The process of intoxication begins when PA83 (Protective antigen, 83kDa) binds to cell receptors: TEM8 (Tumor Endothelium Marker 8) also called ANTHRAX1 and CMG2 (Capillary Morphogenesis Protein 2) – ANTHRAX2 [14]. Then a 20kDa N-terminal fragment of PA83 (PA20) is released by a membrane endoprotease of the furin family [15]. Until recently it was thought that the PA20 does not play a significant role in the pathogenesis of *B. anthracis* infections. In the *in vitro* studies, using PBMC (Peripheral Blood Mononuclear Cells), several genes regulated by rPA20, which are associated with PBMC apoptosis, were discovered. It is believed that rPA20 may be responsible for functions previously assigned to PA63/LF complex, such as binding to the LF that occurs in the host cells [16, 17]. It is possible that PA20 fragments are not covalently bound to PA63 retaining the ability to bind to LF molecule, and creating proper conditions for the creation of ligand. PA63 heptamer can tolerate one or more fragments of PA20, which could participate in the hook to pull LF from the extracellular environment [17]. Such interaction, coupled with the development LF/PA63 complexes, may be a part of an alternative process of anthrax toxins accumulation on the cell surface [17]. An inhibitory effect of rPA20 on the expression of CD38, an integral membrane receptor type II and an adhesion molecule leading to cytotoxic cell death, was observed [16]. Studies have shown that the PA20 fragment induces apoptosis of peripheral blood leukocytes, in the absence of other PA components. It was also found that rPA20 has the ability to reduce the activity of caspase-3, an enzyme participating in the final stages of programmed cell death, in PBMC. It is worth noting that the measurement of the activity of caspase-3 is used to assess the intensity of apoptosis in cells [16]. The LF-rPA20 complex may also play other, so far unrecognized functions, involved in the pathogenesis of anthrax [16].

PA20 epitope as a immunodominant determinant causing a strong immune response may have a significant impact on the effectiveness of anthrax vaccines based on PA [18]. It is specific in the binding of antibodies to individual domains (paratopes). To date, a few paratopes specific for determinants associated with the PA20 region of the PA monomer have been found [18]. Studies on the PA20 fragment have shown that most paratopes occurring after vaccination are specific for determinants in the amino-terminal PA20 region of PA monomer toxin. 62% of isolated monoclonal antibodies were specific for determinants associated with this fragment. It is possible that antibodies that recognize epitopes in the region of monomer PA-PA20 can be deprived of the ability to effectively neutralize the toxins; therefore, research is being carried out to find monoclonal antibodies for other PA regions involved in mediating cytotoxicity neutralization [18].

Antibodies that bind specifically to PA20 epitopes are capable of blocking the proteolytic cleavage of PA83 on the cell surface which, in consequence, leads to the neutralization of PA functions. It is possible that antibodies specific against PA20, which block the cleavage of furin, may decrease the activity of free PA20 produced during intoxication. Studies on mice have shown that PA is cleaved *in vivo* independently of binding to the cell surface, and its proteolytic fragments of PA20 and PA63 circulate in the blood vessels. The C-terminal 63kDa fragment – PA63, preserved after enzymatic cleavage, undergoes oligomerization into heptamers, known as prepores, that can associate to form complexes with EF

or LF [2, 19]. A recent investigation on the binding of the PA63 monomers with LF or LF-N domain, have shown that oligomerization of PA is not a necessary condition to create complex PA/LF, or PA/LF-N, and PA63 monomers as well as LF mutually interact. Nowadays, it is believed that the complex of LF with monomeric PA63 is transient, and mediates the process of accumulation of toxins on the surface of host cells [17].

The initiation of ligand formation through binding of the PA63 monomer with LF could be helpful in PA63 oligomerization and clustering [17, 18]. It has been shown that the PA20 fragment interacts with the LF-N domain, which may be an alternative process during accumulation of the anthrax toxin on the surface host cell. Although the model of spontaneous creation of PA63 heptamers, which then bind to the LF and/or EF molecules has been accepted, it has been suggested recently that LF could first associate with the monomeric form of PA63, and then bind with a second molecule of PA63 via interactions of this complex [17].

PA oligomerization is initiated by dimeric complexes formed by anthrax toxin receptor on the cell surface. Through interactions of the complexes with the receptor, resulting in the formation of oktameric and heptameric PA oligomers, which are similarly stabilized. In view of the instability of PA heptamer and its extracellular accumulation in plasma, it was possible to explain the regulation of the toxin's concentration gradient around the sites of infection in the pathogenesis of anthrax. This is probably due to the role of PA ANTXR in collecting and determining the degree of stabilization in communicating with two different oligomers in their focus. Octamer toxins circulating in the plasma are stable, while the heptametric toxin is unstable, forming aggregates in the early formed channel. The cell surface is probably the site of the accumulation of two different oligomeric forms of PA7 and PA8, which are stabilized by the toxin receptor [20].

Two forms of oligomeric PA have been discovered, one of which is formed on the cell surface and the other in the plasma. In each of these environments, PA oligomerizes into heptameric and octameric forms, which then bind to the LF and EF. While the PA octameric form dominates in the plasma under physiological conditions (pH 7.4, 37°C), the heptameric PA is the predominant form on the cell surface [20]. Anthrax toxin receptor (ANTXR) binds with PA on the cell surface, and its extracellular domain stabilizes the toxins' complexes by protecting the premature formation of a channel by PA in the process of toxin inactivation. The role of ANTXR in the oligomerization of PA and stability of toxins' complexes containing octameric PA is not completely understood [20].

The mechanism of anthrax toxin endocytosis and the influence of various factors (actin and clathrin adapters unconventional AP-1, AP-2) on PA oligomerization process, which leads to blockage of the toxin's activity, has been investigated. The mechanism of endocytosis is based on ubiquitination of the cellular receptors CMG-2 and TEM8-1, requiring a heterotetrameric adaptor AP-1 and clathrin for its activity. In the next step, the oligomerization of PA is necessary, independently of the CMG-2 receptor (receptors are not linked to actin), but dependent on the TEM8 receptor-1 (receptors are linked to actin). In the light of these data, endocytosis is strongly dependent on actin, which plays an important role in the process of heptamerization of PA [14].

In understanding the pathogenesis of anthrax, it is important to determine how the PA pore mediates the translocation of enzymatic components through the membrane. Katayama et al. (2010) performed a study in which PA-pore membrane complexes were constructed and visualized using an electron microscope. Two populations of PA pores were observed: vesicle-inserted and nanodisc-inserted, allowing the reconstruction of two virtually identical structures of the PA pores, at 22Å resolution. Reconstruction of a domain 4-truncated PA pore inserted into nanodiscs enabled showing that this domain has only a slight influence on the pore structure. Further observations using a method of normal mode flexible fitting of the x-ray crystallographic coordinates of the PA pre-pore, allowed the authors to show that a prominent flange seen within the pore lumen is formed by the convergence of mobile loops carrying Phe427, which is a residue known to catalyze protein translocation [21].

Multimeric pores formed in the membrane by PA of the endosomal anthrax toxins enable the enzymatic moieties of the toxin to translocate to the cytosol of mammalian cells. A specific phenylalanine residue (Phe427) within the lumen of the cap of the PA pore, near the junction of the cap with the stem, plays a key role in the transportation functions of PA [22]. It has been reported that replacing Phe427 in a single subunit of the predominantly heptameric pore with a basic or an acidic amino acid results largely in the formation of non-functional oligomers, causing inhibition of cytotoxicity through inhibition of toxin translocation in the planar phospholipid bilayer system. Ablation of the cytotoxicity was most pronounced when Phe427 was replaced by His and Glu, which caused ablation of both cytotoxicity and translocation. The presented study has shown that basic residues at position 427 prevented the Phe clamp from interacting with a translocation substrate to form a seal against the passage of ions, and accelerated dissociation of the substrate from the pore. On the other hand, acidic residues allowed the seal to form and the substrate to remain firmly bound; however, they blocked its passage via electrostatic interactions with the positively charged N-terminal segment [22].

Electrophysiological studies of anthrax toxin in planar lipid bilayers showed that the PA pores play an active role in the translocation of LF and EF across the endosomal membrane. The PA pore is a mushroom-shaped structure with a globular cap and a 100Å long stem with a transmembrane segment. Enzymatic fragments bind to the "cap" by the homologous N-terminal domain (LF_N and EF_N). An unorganized segment is characterized by a high density of both acid and base residues present at the N-terminal part of LF_N and EF_N. This segment is able to initiate the N-terminal to C-terminal translocation [22]. The blockage of ion conductance through the pore, when the proteins LF_N and EF_N bind, depends largely on this highly charged segment. Translocation of the bound ligands through the PA pores may be obtained by application of a pH gradient, or a cis-positive potential, resulting in the relief of channel blockage [22]. Specific phenylalanine residue (Phe427) within the pores cap, near its junction with the stem, plays a key role in the transportation function of PA.

Phe427 mutation affects the ability of PA to mediate toxicity. Mutations of Phe427 to Ser, Thr, or His inhibit the translocation functions of PA without affecting the conformational transformation of the prepores into pores,

while several other mutations at this site have an influence on the pore formation. Inhibition of the ionic conductivity in planar bilayers by translocatable ligands, such as LF_N, also depends on Phe427 [22]. The complexes formed by PA and LF, or PA and EF, undergo endocytosis, and a low pH of the cell alters the conformation of the complex enabling the formation of a cation-specific channel and translocation of EF or LF into the cytosol [13]. Production of cholesterol-dependent cytolysin and anthrolysin by *B. anthracis* facilitates the escape of bacteria from the cell's phagolysosome [2].

The EF factor, which is a calmodulin-dependent adenylate cyclase, causes elevation of the levels of cAMP in the cell, leading to disturbance of the water homeostasis and disruption of intracellular signaling pathways. Calmodulin is an intracellular calcium binding EF activating cofactor [13, 15, 19].

The LF factor is a zinc endoprotease, which undergoes cleavage to a N-terminal MEK fragment (mitogen-activated protein kinase kinases) showing an ability to inactivate components of the protein kinases cascade [13, 19]. As a result of the translocation of the toxin complexes into the cytoplasm, a deregulation of numerous vital cell processes is observed, leading to abnormal ion transport, loss of electrolytes and water, and blockage of cellular signal transduction pathways necessary for proper functioning of the body's immune response. As a consequence of the biological activity of anthrax toxins, an Anthrax Associated Shock (AAS) is evoked.

The results of some recent investigations have allowed a better understanding of the structure of the LF and EF factors. Two receptors have been identified which seem to be important for molecular binding and accumulation of the anthrax toxin, endocytosis, cavity formation, and enzymatic properties of EF and LF [2]. However, despite significant progress, the mechanisms of translocation and regeneration of LF and EF have still not been fully elucidated [13].

It is extremely important to clarify which targets in the cell are essential for toxin activity in the infection process, and in the mechanisms of the toxin's action that leads to increased concentrations of cAMP and MEK kinases cleavage. Besides edema formation, it is crucial to understand other mechanisms that may be important for the infection, such as the synergistic action of toxin's LF and EF on the organism [13].

The essential processes for proper cell function, such as protein secretion and degradation, require the molecular mechanism of protein folding and movement through the membrane, as well as inside the proteolytic complexes. Proteins' translocation is also important in the pathogenesis of the disease, since bacteria can use the transport channels for translocation of protein toxins into the cell. The above-mentioned proteins PA, LF and EF are involved in the toxin translocation [23].

Anthrax toxin forms a complex of active holotoxins comprising PA oligomers in the form of a ring, which are associated with multiple copies of LF and EF. These complexes undergo endocytosis into the host cells, where PA forms a connection with the protein translocation channels. Under the influence of proton movement, unfolding and translocation of LF and EF takes place through the PA channel. Proteins that are folding are thermodynamically stable under normal conditions of living cells; thus, the molecular sources of energy are necessary for the unfolding and translocation of protein substrates. Initially, the protein substrate is unfolded and needs an energy source for further movement, which allows the expanded polypeptide chain to be translocated through a narrow channel. The unfolded peptide chain reveals the changing configuration of chemical residues, possible translocation configurations of the protein backbone and the diffusive motion, which could be a result of the counterproductive backsliding of the polypeptide [23].

Other models propose multiple non-specific binding sites as critical features of the unfolding machinery. These non-specific binding sites provide a solution to the configurational and chemical variability of the unfolded state by recognizing general features of an unfolded chain, such as uniformly shaped α-helical secondary structure, backbone hydrogen bonds, or hydrophobic groups. In addition, binding the polypeptide in multiple locations helps stabilizing unfolded intermediates, productively resists backsliding (retro-translocation), and allows the available energy to be more efficiently converted into a productive mechanical force.

Several discrete clamping sites have been observed in the PA channel that address these aspects of translocation, especially the α clamp, the Φ clamp, and an anion-charge-repulsion site, which are implicated in ΔpH-driven translocation. The α clamp is not only important in the unfolding of the first helix of LF_N, but it may also play a role in unfolding the later domains of a translocating substrate. Another clamping site seen in the PA channel is the Φ clamp, which can stabilize further unfolding by non-specific binding to the sequences, while limiting the diffusive movement that causes retro-translocation. As a result, under appropriate ΔpH-driving-force conditions, Brownian motion can be captured by a change of protanation state of acidic residues in the translocating chain. It has been shown in the Atx system that the electric potential and the formation of ΔpH leads to the unfolding of LF and EF, where the ΔpH is considered to be a more effective driving force in this process [23].

Under low ΔpH conditions at room temperature, LF requires about 100s to translocate through the heptameric or octameric PA channel. Despite this, auxiliary factors from host cells, called chaperones (proteins stabilizing the structure of a polypeptide during its transportation to various places), may assist in translocation. ATP cytosolic factors constitute a good example of such assistance, as they may increase the translocation of diphteria toxin A domain (DTA) and the LF_N-DTA fusion protein substrate. Nevertheless, the role of chaperones in the translocation pathway remains unclear; the enhacement of enzymatic activity could either indicate that the chaperones assist during unfolding and translocation, or they function post-translocationally to properly fold the translocated substrate. Mechanisms of protein processesing explain the formation of the PA channel and protein translocation [23].

Lethal toxin (LeTx) is anthrax virulence factor determining the course of *B. anthracis* infection. It plays a key role in all stages of infection, from germination until the death of the host [9]. In the early phase of infection, LeTx inhibits the processes of immune response of the cells and cytokine production [15] which contributes to the dynamic growth of bacteria. In further stages of the disease, the toxins induce cytokine-independent SLD (Shock-Like Death) [9]. A better understanding of the mechanisms of circulatory shock-induced lethal toxin formation is important for the selection of appropriate therapy, as well as development of effective

methods to prevent the consequences of its actions. Studies on Fischer and Sprague-Dawley rats showed that inhibition of the inflammatory response and cytokine release, resulting from production of nitric oxide by macrophages under the influence of LeTx, is similar to the shock induced by LPS or other bacterial toxins [24].

Lethal toxin has a disarming effect on many parts of the immune system, *inter alia* cleavage of MEK protein kinases by inhibition of cellular responses to cytokines, inhibition of dendritic cells, as well as B and T cell responses. These modes of LeTx action allow bacteria to survive in the host cells.

Production of edema toxin also prevents phagocytosis and cytokine secretion through induction of cAMP. Lack of immune response of the host leads to bacteremia, during which the bacteria disposes its envelope and cell wall. As a result, macrophages can become sensitized, leading to destruction of bacterial cells in the presence of high concentrations of toxins secreted into the blood in the last stages of the disease. In this way, in the later stages of infection, lethal toxin disarms the immune system through the destruction of macrophages. LeTx also induces other lethal molecular factors directed towards as yet unidentified cell targets, leading finally to vascular leakage, hypoxia, and SLD [9].

SUMMARY

The presented study describes three major steps in the course of anthrax infection; an invasion phase, which occurs in the lung, in which toxins have a short-term effect on lung phagocytes; a phase of bacillus proliferation, in which in the mediastinal lymph nodes the process of bacillus proliferation with toxins local effects occur; and a terminal, diffusion phase, characterized by long-term effects of toxins, leading to host death.

Continuation of research is essential to discover the mechanisms of immunity to *B. anthracis,* and to improve the effective method of diagnosis, treatment, and protection against the disease.

The understanding of the molecular changes induced by anthrax toxin in different cells at each stage of infection will be crucial in discovering the bacterium pathogenesis. Future studies on *B. anthracis* and its virulence factors will be likely to need to rely increasingly on *in vivo* models to achieve a complete understanding of this process.

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