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REVIEW ARTICLE

Anthrax Pathogenesis as Overcoming Lymph Node Macrophage Barrier

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Introduction

Anthrax is especially dangerous infectious disease caused by the gram-positive spore-forming microorganism *Bacillus anthracis*, and with expressed toxic syndrome and high mortality of infected animals or humans [1,2]. *Bacillus anthracis* belongs in the *Bacillus cereus* group according to biotaxonomic characteristics [3,4]. The of these two microorganisms is more than 90%, and from the evolution *B. cereus* is considered as a precursor of the anthrax pathogen [5].

A single point mutation in the gene of the global transcription regulator plcR induced the "silence" of a large gene cluster responsible for the synthesis of more than 20 proteins produced by *B. cereus* and the *Bacillus anthracis* forming [6,7]. Two high-molecular plasmids pX01 and pX02 were necessary for formation as new bacilli species [8-10]. These plasmids determined the new phenotype and the ecological niche of *Bacillus anthracis* - a highly virulent spore-forming bacilli with a powerful pathogenicity complex for animals and human [11,12].

The pX01 plasmid contains the structural genes of the exotoxin *pag, lef, and cya*, and gene of new global trans-activator atxA that regulates the transcription of the toxin and capsule formation genes, as well as the additional operon *gerX* encoding three germination genes A, B, and C [13-16]. These genes are located on a fragment of 44.8 thousand base pairs flanked by inverted IS-elements and called the "pathogenicity island " [17].

pX02 plasmid contains the cap operon that includes the *capB, capC, capA,* and *capD* genes responsible for the synthesis of D-glutamyl polypeptide and capsule assembly [18], as well as

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the dep gene encoding the capsule degradation process [19], and the regulatory genes *acpA*, *acpB*, and *abrB* [20,21] pX02 plasmid contains also the *amiA* gene that controls the peptidoglycan hydrolysis as important and needing stage at spore germination when envelope is destroying [22].

The global transcription regulator atxA is strongly dependent from CO_2 (5-10%), and this played a crucial role in the *in vivo* selection of new bacilli [23-26].

In nature the anthrax pathogen infecting occurs only by spores [1,2,28] and therefore transmission of infection is possible only after the death of the host and the spore formation in it.

Some pathways of spore entry into the host body are described: subcutaneous, oral-intestinal, intranasal and aerogenic.

Peroral-intestinal infection occurs with the help of water by washing out the spores from remains of anthrax died animals and eating the meat of these animals. The lethal dose ranges from several hundred spores and the mortality rate reaches 40% [27,28]. This infection pathway is the most significant in the spread of anthrax from an epidemiological point of view.

As for subcutaneous pathway, the possibility of such infection for animals is minimal for animals [26,28]. People are infected most often subcutaneously when are working with infected animals at enterprises engaged in the processing of animal hair and leather raw materials. Domestic infection is usually associated with butchering the meat of animals that have died from anthrax. The anthrax cutaneous form is expressed in inflammation of closest regional lymph nodes and is often accompanied by the formation of suppurations and ulcers. Human subcutaneous infection is characterized by a low mortality rate, since in this case the disease does not reach the stage of generalized infection [1,26,28]. The mouse model of infection with subcutaneous injection is the most effective for determination of the degree of pathogenicity of various *Bacillus anthracis* strains. Death of animals occurs usually on 2-3 days and lethal doses range from 1-10 spores. This pathway is used in laboratory researches as it makes it possible to control the conditions of the experiment to the greatest extent. Significant destruction of the skin occurs during skin infection in humans and this leads to a powerful inflammatory reaction. As a result, while the phagocytosed by macrophages pathogen is localized in the lymph node, an inflammatory zone is formed due to vasoconstriction of blood vessels, blocking the pathogen's progress into the blood. Therefore, the pathogen that has multiplied in the lymph node still needs to overcome the inflammatory zone. If the pathogen fails to do this inflammatory barrier, then skin cells lysis as ulcers occurs. The infectious disease prognosis is favorable in this case.

Aerogenic infecting is used only in experimental conditions. Lethal doses for aerogenic infecting range from 7000 to 50,000 spores according to various authors [28,29]. This parameter varies depending on the type of infected animals used in experiments, as well as on the volume of aerosol and other numerous conditions. However, the lethal dose in this case does not fall below several thousand spores [1,28,29]. Such high lethal doses are associated with most die of the germinating spores in alveolar macrophages, as was found on similar mouse adenocarcinoma cells J774.A [30,31].

B. anthracis spores, regardless of the method of entry into the host body, are phagocytosed by macrophages and transported to the lymph stream to the regional lymph node [32-36]. Spores germinate only in macrophages [37-42]. The components of the surrounding nutrient medium penetrate through the swollen shell into the spore and the GPR germination

protease begins to hydrolyze SASP proteins that compactly pack DNA [43]. Degradation of protective proteins leads to the release of DNA regions carrying ger operon genes responsible for germination, as well as toxin formation genes [2,40].

The germinating spores are sensitive to aggressive phagosome conditions: rapid acidification and action of proteolytic and oxidative systems [44,45]. The rapid lysis of the phagosome membrane is most effective pathway to survival of germinating spores. The bacteria escape from phagosome to the macrophage cytoplasm during phagocytosis was shown firstly for listeria producing the pore-forming protein Listeriolysin O [46,47]. This protein lysed phagosomal membrane and thereby accelerated the bacterial escape from the phagosome to the cytoplasm.

Bacillus anthracis produces two pore-forming proteins: anthrolysin O [48,49] and protective antigen [50,51]. Anthrolysin synthesis is constitutive while bacterial survival on germination stage connects early expression of toxin formation genes [11]. It has been established also that the activity of the *atxA* (inducing toxin synthesis) that is necessary for the survival of germinating spores inside macrophages [2,15,17]. The *atxA* coordinates the germinating spore's responses to external signals, primarily to elevated CO_2 levels [14,25]. Toxin production is regulated in vitro and *in vivo* through the positive control of the *pag*, *lef*, and *cya* by *atxA* [14,24]. This indicates the direct involvement of the toxin in the survival of germinating spores during phagocytosis. However, for the conversion of PA83 into the pore-forming protein PA63 capable to lysing the cell membrane is necessary also hydrolysis it by proteases [52].

From our point of view, immediately after the start of synthesis and secretion, the molecules of the protective antigen PA83 diffuse through the peptidoglycan layer, in that they undergo hydrolysis by bacterial proteases to PA63. So, it was demonstrated that immune inhibitor A1 (InhA1) is a secreted metalloprotease that is unique to pathogenic members of the Bacillus genus and has been associated with cleavage of host and bacterial proteins during infection [53].

Acidic phagosome conditions in the surface layer of peptidoglycan create to rapid oligomerization of PA63 molecules [50]. These oligomers are bound with the phagosome membrane and form pores in it without interacting with specific receptors [51]. The first oligomer sharply reduces the rate of acidification as protons escape from the phagosome through forming PA63 pores. Next PA63 oligomers bind with the phagosome membrane and destroy it. Thus, the survival of germinating spores during phagocytosis depends on the rate of synthesis of the protective antigen PA83 and its hydrolysis by bacterial proteases to PA63. Furthermore, swelling of germinating spores occurs unevenly and only in the part that is most likely adjacent to the phagosome membrane [41]. The remaining part of the spore shell protects the germinating spore that not yet covered a full-scale bacterial wall (Figure 1).

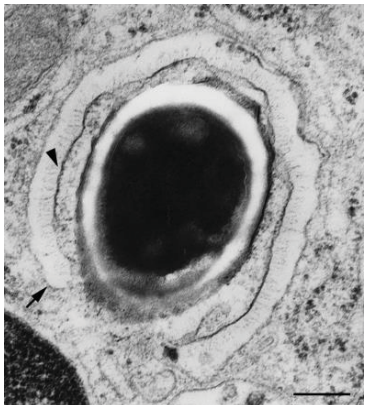

Figure 1 Transmission electron microscopy of B. anthracis 7702 spores in peritoneal macrophages. Peritoneal macrophages were infected for 5 h with 7702 spores. Monolayers were then washed, fixed and processed for electron microscopy. After infection, the exosporium (arrow head) and the phagolysosomal membrane (arrow) were closer. Scale bar.200 nm [41].

PA63-oligomer destroys phagosome membrane that connected with swelling part of germinating spore and it escape from phagosome.

Unusual and extremely interesting data was obtained at the study of spore survival with different genotypes during phagocytosis by macrophage-like J774A cells (Table 1).

Germinated spores of vaccine strain STI-1 ($pXO1^+pXO2^-$) survived better then spores high virulent strain 81/1 ($pXO1^+pXO2^+$): 5,12 KOE against 2,87 KOE according data at 2 hour. The analysis of PA synthesis data put everything in place. STI-1 synthesized PA significantly more than 81/1, accordingly 520 ng/ml and 380 ng/ml for 3 hours. But to 24 hours of cultivation, the difference decreased, accordingly 24.6 mkg/ml and 19.3 mkg/ml.

Thus, the effective survival of germinating spores in macrophages at an early stage of phagocytosis depends from the rate of PA83 synthesis (Figure 2).

The investigation of the pathogen multiplication within macrophages showed the STI-1 vaccine strain vegetative cells multiplied in macrophages more 24 hours of observation. Macrophages not lysed although bacterial cells synthesized LF. Usually, the macrophages are lysed within one hour at lethal toxin (PA+LF) addition [30]. Therefore, the macrophages should have lysed within 1-2 hours after infection by spores of toxin-producing strains.

The macrophage lysis absence directly indicates that monomeric LF molecules, synthesized by vegetative cells, are not effective. Previously us was proposed translocation model according to which only the multi-oligomeric complex 7×PA17+7×LF/EF penetrates into the target-cell [55].

This oligomeric complex induces high and rapid cytotoxicity and not monomeric LF molecules. These results cast doubt on the proposed "unfolding-folding" as mechanism for the penetration of LF individual molecules into target cells [56]. Also, it is also questionable the supposed intracellular target for LF, although it was demonstrated LF hydrolyzes MAPK-kinases in solution and in macrophages [57,58] but not in Bacillus anthracis infected macrophages (Figure 3) [31,53,59,60].

Further, it was demonstrated the virulent strain 81/1 vegetative cells escape macrophages to 4-6 hours observation and the macrophages maintained their integrity at the same time [31,53,60] (Figure 4).

This moment should be discussed in more detail as it is fundamental for bacterial escape from macrophages. This phenomenon is the exocytosis of bacteria using pore-forming

Table 1: Spore survival of different strain during phagocytosis by macrophage-like cell J774.1.[31].

N/N	STI-Rif $pXO1^-$ $pXO2^-$	ΔAmes $pXO1^-pXO2^+$	81/1 $pXO1^+pXO2^+$	71/12 $pXO1^+pXO2^{-/+}$	STI-1 $pXO1^+pXO2^-$
***LD50**	$>10^9$	$>10^7$	1-10	10^2	10^5
PA83/ml, 3 h	< 5 ng	< 5 ng	380 ng	310 ng	520 ng
PA83/ml, 24 h	< 5 ng	< 5 ng	19.3 mkg	15.4 mkg	24.6 mkg
Time, h	**Seeding from macrophages, log CFU**				
0,5 (Control)	5,99	5,98	6,03	5,99	6,01
2	0,68	1,25	2,87	3,65	5,12
4	0,16	0,42	1,6	2,29	5,76
6	0,15	0,24	0,42	1,58	5,43
12	0,11	0,24	0,29	1,26	5,34

*LD_{50} for white inbred mice by subcutaneous injection.

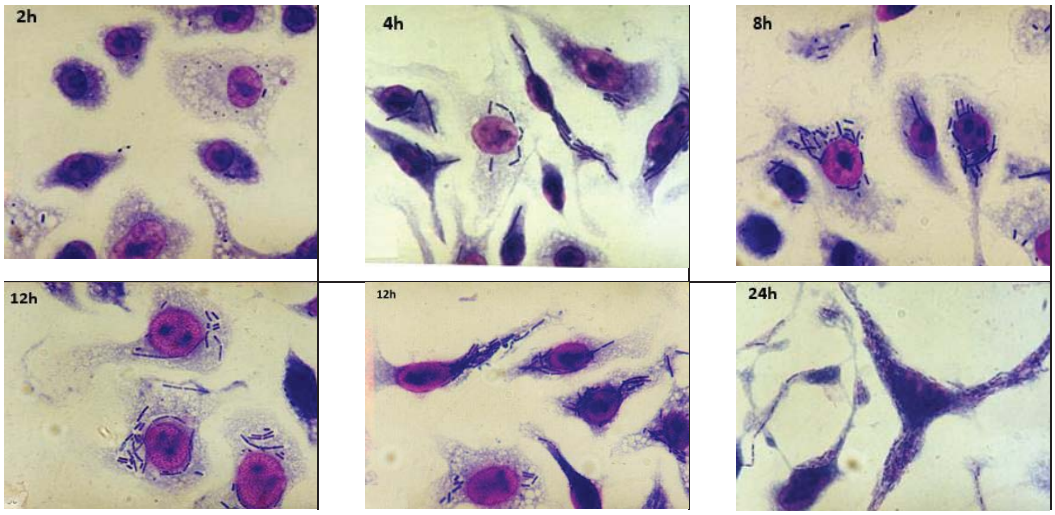

Figure 2 Long-term multiplication of vaccine strain STI-1 vegetative bacterial cells [37,81]. Cells are colored by Romanosky-Gimza. Light microscopy by Carl Zeiss Axioplan 2 imaging system, 15×100. Macrophage-like J774A cells were infected by vaccine strain STI-1 spores during 30 minutes and ×3 washed by CO2 condition medium. Infected macrophage-like J774A cells cultivated during from 2 to 24 hours into CO_2-incubator.

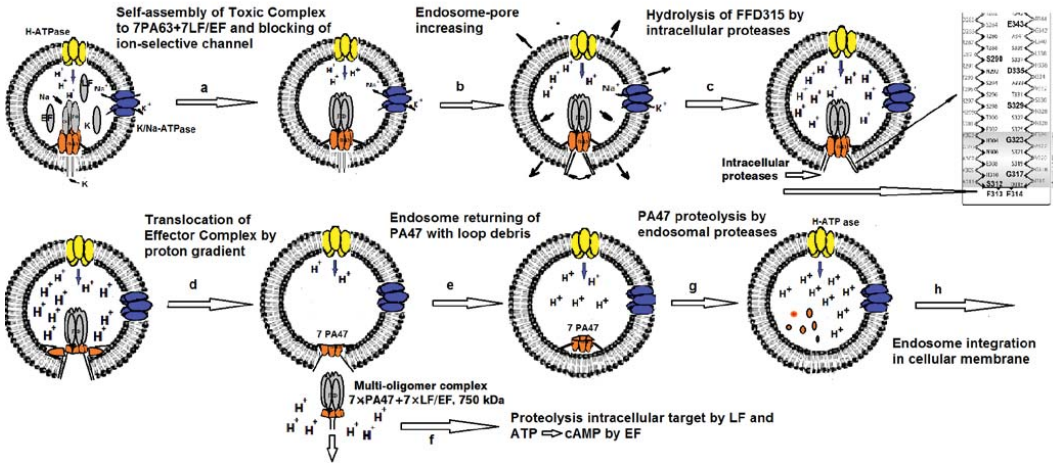

Figure 3 Main stages of anthrax toxin translocation from endosome into cytoplasm of target-cell. The self-assembly of Toxic Complex to 7PA73+7LF/EF and blocking of ion-selective channel (a). Endosome and pore increasing (b). Hydrolysis of FFD315 in PA63 by intracellular proteases (c). Translocation of Effector Complex by proton gradient (d). Endosomal returning PA47 with loop debris (e). Hydrolysis PA47 by endosomal proteases (g). Proteolysis of intracellular target by LF and ATP ⇨ cAMP by EF (f). Endosome integration into cellular membrane (h).

proteins. Numerous pores on the side of the inner membrane induce exocytosis and bacteria escape macrophages. The membrane closes behind the bacterial cells and the eukaryotic cell remains intact.

Bacterial exocytosis is a common and effective mechanism bacterial escape macrophages and other cells using pore-forming proteins (*Bacillus*, *Listeria*, *Staphylococci*, *Streptococci* etc.).

Concerning the *Bacillus anthracis*, only capsule-coated and toxin-synthesizing bacteria escape the macrophages. After synthesis and secretion, the molecules PA83 are hydrolyzed by bacterial proteases to PA63, that are oligomerized to 7×PA63 diffusing in the acidic capsule space (poly-D-glutamic acid) [26,61].

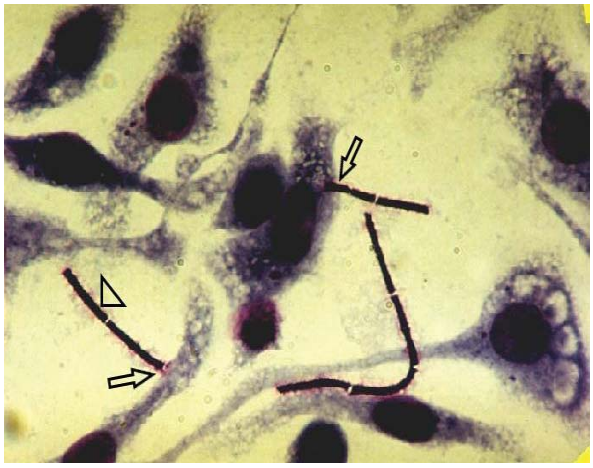

Figure 4 Escape (shown by arrow) of capsule covered bacterial cells from macrophages to 4-6 h by light microscopy Carl Zeiss Axioplan 2 imaging system, 15×100. [31]. The triangle indicates rose color of covered by capsule bacterial cells. Cells are colored by Romanosky-Gimza. Macrophage-like J774A cells were infected by vaccine strain STI-1 spores during 30 minutes and ×3 washed by CO2 condition medium. Infected macrophage-like J774A cells cultivated during from 2 to 24 hours into CO2-incubator.

Therefore, pore-forming proteins 7×PA63 come out of the capsule and interact with the membrane inducing exocytosis. At this stage of infection, the capsule plays not a protective but a functional role as a space in that PA83 is activated.

Concerning the Bacillus anthracis, only capsule-coated and toxin-synthesizing bacteria escape quickly the macrophages by means exocytosis. With our point of view synthesized the PA83 molecules are hydrolyzed by bacterial proteases to PA63 that are oligomerized to 7×PA63 at diffusing in the acidic capsule space (poly-D-glutamic acid) [26,61]. Therefore, 7×PA63 pore-forming proteins diffuse from capsule and interact with the membrane inducing exocytosis. At this stage of infection, the capsule plays not a protective but a functional role as a space in that PA83 is activated.

Thus, vegetative cells of virulent strains escape quickly macrophages, while vaccine strain cells ($pXO1^{+}pXO2^{-}$) remain in macrophages for a long time.

Concerning non-toxigenic capsule strains ($pXO1^{-}pXO2^{+}$), they escape more slowly macrophages with exocytosis induced by pore-forming anthrolysin O. For the oligomer formation is required acidic conditions that appear at diffusing in the acidic capsule space. Certainly, antrolysin accelerates the escape of capsule-coated and toxin-synthesizing cells ($pXO1^{+}pXO2^{+}$).

These processes take place in the lymph nodes during infection. Therefore, vegetative cells of virulent strains, protected by capsule from rephagocytosis [62], escape quickly macrophages, multiply within lymph node and synthesize exotoxin. This exotoxin attacks and lyses macrophages and cells of the epithelial-endothelial barrier (EEB) [63]. As a result, the EEB is destroyed and bacterial cells penetrate into bloodstream. Using methods *in vivo* imaging with bioluminescent, it was found that during 30-35 hours after phagocytosis of spores, bacteria are detected only in one or more lymph nodes [64,65].

But why do bacteria multiply only in the lymph nodes during more 30 hours, and not disseminate to the lymphatic system with the lymph flow?

From our point of view, lymph node blockade is one of the key moments in anthrax pathogenesis (and other infections as plague, tularemia, and caused viruses etc.) and is induced by cytokines in response to the appearance of infected macrophages. During the reproduction of bacterial cells in macrophages, infection markers appear on their surface in the form of MHCI with peptides from secreted pathogen proteins. In the lymph node, these structures interact with T-cell receptors of T-cells. This interaction induces IL-1, TNFa, IL-2 and INFg synthesis by macrophages and T-cells. Synthesized cytokines stimulate the phagocytosis, maturation and proliferation of cytotoxic lymphocytes (CTL) to destroy the

infectious agent. The cytokines induce also vasoconstriction of lymphatic vessels to lymph node blockade. Key moment, the immune system blocks the infected macrophages for pathogen elimination, so that it does not escape beyond the lymph node. IL-1 is a pro-inflammatory cytokine and its action is associated with endothelial cells. IL-2 responsible for cell proliferation.

Probably, TNFa or INFg (TNFa + INFg) can act as vasoconstriction inductors of lymphatic vessels. In addition, the synthesized cytokines penetrate to the bloodstream at toxin epithelial-endothelial barrier destruction and cause a prodromal state (first non-specific symptom) as a predecessor of cytokine storm.

Essential note, anthrax pathogenesis proceeds asymptomatic during 30-35 hours up to pathogen penetration into bloodstream and yet more 6-8 hours to first detected symptoms as temperature, frequent pulse and breathing.

Bioluminescence was used to reveal how bacterial cells multiply in mice immunized with a protective antigen. In immunized mice, a small bacterial growth observed at first, which quickly decayed. [64,65]. This allows us to conclude that infection of the lymph node occurs regardless of the presence of antibodies to PA that blocked the cytotoxic effect on all sensitive cells: macrophages and cells of the epithelial-endothelial barrier [64,65]. As a result, the pathogen is destroyed cooperatively in the lymph node by immune system cells and cannot enter the bloodstream although anti-PA antibodies do not have a direct antibacterial effect. Therefore, all used today vaccines induce PA-antibodies [66-68]. An important moment - phagocytosis and spore germination, and the penetration into the macrophage cytoplasm, do not depend on the presence of anti-PA antibodies.

Penetrated into bloodstream bacterial cells of virulent strains, and covered with a capsule and therefore protected from rephagocytosis, multiply rapidly. The infected host is converted in a kind of fermenter, in that a huge pathogen mass produces the exotoxin. The concentration of exotoxin can reach 20 mg/ml at the stage of bacteremia in 10^9 CFU. This is despite the fact that lethal doses for macrophages are about 1 mkg/ml [30]. The exotoxin attacks macrophages and endothelial cells of micro-vessels that leads to impaired gas exchange in the lungs, disruptive at work of the heart and other vital organs.

Thus, toxemia is ended as a powerful toxic and cardiovascular shock inducing death of infected organism [69,70].

After infected host death and a body temperature decreasing, ideal conditions for spore formation are created. The vegetative cell multiplication is stopped, genes responsible for capsule depolymerization are activated, toxin synthesis is inhibited, cell chains are shortened, etc. [68].

The died from anthrax animals, both in their natural environment and into burial grounds, are sources of long-term preservation of the spore form.

Conclusion

The spores, after entering the host body, are phagocytized by macrophages that migrate to near regional lymph node. Phagocytosed spores germinate in phagosomes and destroy the phagosome membrane by means PA63 oligomers that are formed during proteolysis by bacterial proteases PA83. Germinated spores penetrate to macrophage cytoplasm and transform into vegetative cells covered with a poly-d-glutamine capsule. Synthesized and secreted PA83 molecules diffuse through the acidic capsule space and are undergo proteolysis by bacterial proteases to PA63. The PA63 molecules form oligomers that interact with the macrophage membrane, form pores in it, and induce the exocytosis of vegetative cells. This mechanism of bacterial exit from macrophages

is common to intracellular pathogens with pore-forming activity. The covered with a poly-d-glutamine capsule vegetative cells multiply rapidly in the lumen of the lymph node. The exotoxin synthesized by bacterial cells attacks and lyses macrophages and epithelial-endothelial barrier cells. Multiplied bacterial cell after destruction of the barrier between lymph and blood enter into the bloodstream. The rapidly increasing bacterial mass synthesizes a huge amount of exotoxin, which attacks and lyses macrophages and endothelial cells that form blood vessels. Final pathogenic stage is toxic and cardiovascular shock that culminating in the rapid host death.

Thus, the Bacillus anthracis spore form ensures the survival of this biological species in the harshest environmental conditions, and controlled by "quorum system" synthesis of pathogenic factors such as capsule, exotoxin and proteases make this pathogen the most dangerous for human and animal life.

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