Bacillus anthracis as a biological warfare agent: infection, diagnosis and countermeasures

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ABSTRACT

AIM: Bacillus anthracis is a causative agent of zoonotic anthrax disease. In the last years, significant progress in therapy and diagnosis of anthrax was made. Concurrently, knowledge about anthrax progression, molecular pathology and release of anthrax toxin during the disease has improved. This review covers the recent progress in this field.

METHODS: In this review, specifications of B. anthracis, anthrax disease, medical and biomedical countermeasures and diagnostic tools were surveyed. The actual literature was summarized and relevance of the microorganism as a biological warfare agent and the ways how to reduce its impact including therapeutic protocols were written and discussed.

RESULTS: Currently, the microorganism is considered one of the top biological warfare agents due to lethality, long term stability of spores, easy dissemination and production. The recent research is focused on countermeasures suitable for reduction of consequences by a misuse of the microorganism in form of biological weapon (Tab. 3, Fig. 1, Ref. 101). Text in PDF www.elis.sk.

KEY WORDS: anthrax, bacillus anthracis, biological warfare agent, biological weapon, detection, diagnosis, infection, therapy.

Introduction

Bacillus anthracis is a well-known biological warfare agent and many people without experience in the field of biological weapons probably call back the knowledge about B. anthracis as it was presented in popularized fiction stories and movies. Though B. anthracis is a very dangerous biological agent and the disease anthrax epidemic can be followed by a high mortality, there is a higher number of microorganisms that can represent relevant military threat or at least could be used by a military or a terrorist under some circumstances. Along with the other biological warfare agents, it is named in the international convention called “The Convention on the Prohibition of the Development, Production and Stockpiling of Bacteriological (Biological) and Toxin Weapons and on their Destruction” from 1972.

Thought the international convention contains the list of microorganism species counted in dozens, only few of them are considered seriously dangerous and relevant as a tool in a warfare attack. The category “A” according to the Center for Disease Control and Prevention (Atlanta, GA, United States) is frequently used for the indication of the most dangerous biological warfare agents. B. anthracis is considered as one of the top biological warfare agents from the A category. Beside B. anthracis, microorganisms Francisella tularensis and Yersinia pestis, viruses Variola major and hemorrhagic fevers viruses like Ebola, Lassa, Machupo and Marburg, and Clostridium botulinum with its botulinum toxin are in the same category (1–5). Beside of biological warfare agents, there is a high number of microorganisms that are able to cause serious infections, but they are not considered as biological warfare agents because of their slow limited tactical impact. Toxoplasmosis or AIDS (Acquired Immuno-Deficiency Syndrome) can be exampled as relevant diseases, but their progression is slow and their spreading requires specific conditions (6–9). They are not considered a significant military or terrorist threat due to the above mentioned reasons. Anthrax is another story because of high mortality and fast disease progression. Relevance of B. anthracis for warfare can be also learned from the act that both superpowers from the Cold War era produced and stockpiled large number of weapons based on B. anthracis. The danger coming from B. anthracis used for a bioterrorist purpose can also be learned from the case of so called Anthrax letters in 2001 (10, 11).

In this review, a survey of actual literature on B. anthracis and the disease anthrax is provided and the mechanism of anthrax pathological effect, medical countermeasures and the threat coming from its misuse as biological warfare agent is surveyed. The aim is also to summarize and describe the relationship between the recent findings and progress in research on anthrax disease.
**Bacillus anthracis**: a causative agent of anthrax

*B. anthracis* is a rod shaped gram positive bacterium forming spores growing under both anaerobic and aerobic conditions (12). The cells of *B. anthracis* are rod shaped and quite large with the size variation of the rods from 1×3 up to 1.3×10 μm (13). The microorganism is non-motile in semi-solid media and it is also catalase positive, these two specifications are used for microbiological identification (14–16). Horse blood agar can be used for the growth purpose, but agar based on polymyxin, lysozyme, ethylenediaminetetraacetic acid and thallous acetate (known as PLET agar) is also suitable (17). Hemolysis on 5 % sheep blood agar, gelatin hydrolysis and mucoid colonies on agar in form of string of pearls are another characteristic signs for *B. anthracis* (18). Spreading of *B. anthracis* is possible by its spores that exert enormous resistibility to the ambient environment and can persist in soil, water etc. for a long time (19, 20). The long-term stability and resistibility of the spores contribute to its virulence and they are significant properties making this microorganism a relevant threat, when misused for biological warfare purposes. Important specifications of *B. anthracis* are given in the Table 1.

*B. anthracis* is a causative agent of anthrax disease, which is a zoonosis manifested in various forms. Cutaneous form starts, when spores or cells penetrate skin; gastrointestinal form follows ingestion of cells or spores and pulmonary form is the last and the most lethal type of anthrax following inhalation of spores or cells (21, 22). Each of the anthrax forms can lead to a very serious meningitis and bacteremia (23). The meningitis is typical for at least one third of patients and is manifested by a severe headache, mental problems and other related neurological signs (24). A detailed manifestation of anthrax can be learned from the work of Chen and coworkers, where fever, malaise, nausea, vomiting, tachycardia, tachypnea, edema, mental problems and other related neurological signs (24). Apart from the above mentioned forms of anthrax, serious anthrax disease with a high bacteremia can start under specific conditions as a consequence of contaminated surgery tools and other similar invasive tools kept in an insufficient purity. Heroin associated anthrax epidemic can be mentioned as an example of potential problems caused by contaminated needles (26).

The ability of *B. anthracis* to survive in the host organism and the lethality of the infection is supported by production of anthrax toxin. The production of anthrax toxin is a substantial condition for germination of anthrax spores and progression of the anthrax disease (27). Hosts express anthrax toxin receptor, which is a cellular transmembrane protein naturally involved in angiogenesis, cell migration, skin elasticity and other functions to keep homeostasis (28–30). In reality, two proteins, tumor endothelium marker-8 and capillary morphogenesis protein-2, stand behind the name anthrax toxin receptor (31–33). Anthrax toxin is a protein with three subunits each having specific task in toxicokinetics and toxicodynamics. Protective antigen, edema factor and lethal factor are distinguished (34–36). The individual protein subunits are not toxic, but all the subunits together form the toxicity (37–39). While protective antigen is responsible for stabilization, hiding before the host immune system and transfer, edema factor is an adenylate cyclase producing cAMP to abnormal levels and in the final consequence causing disbalance in the host cell and evading the immune system. Lethal factor is a zinc dependent endoprotease, which interferes with signaling pathways and finally damages the cells. Macrophages are the major target for the both anthrax toxin and the microorganism itself (40–42). Lethal factor of anthrax toxin namely deactivates ERK (a group of Extracellular Signal-Related Kinases), p38 Mitogen Activated Protein Kinases (a group of kinases known under acronym MAPK) and c Jun N-terminal kinase by their proteolysis (43). *B. anthracis* can grow in the other phagocytes as well. Neutrophils are for instance another target of this pathogen (40, 44). Apart from the anthrax toxin, other factors are irreplaceable for the ability to survive within macrophage cell. Production of nitric oxide is another key quality of *B. anthracis* and the produced nitric oxide contributes to killing of macrophages by S-nitrosylation of proteins, necessary for macrophage metabolism (45). Spores of *B. anthracis* must stand harsh conditions and germinate there. Activation of specific genes like yhgC (46) and protection from superoxide in phagosomes (47) are substantial for the bacterium survivability. Acquiring of necessary nutrients from the ambient environment is another specification of *B. anthracis* to germinate the spores (48). Once the bacterium suppresses the host cell, it spreads itself over the organism. While the infected host cells perish, bacterial burden increases and bacteremia is detectable and
systematic spreading of infection follows, when the therapy is not sufficient or immune system not strong enough (49). B. anthracis also acquires nutrients at the expense of host. Proteolysis of human hemoglobin and acquiring of the liberated amino acids is another impact of anthrax on host organism (50). Scheme of B. anthracis life cycle and its interaction with host is depicted in the Figure 1.

**Anthrax therapy**

There are more approaches to reduce anthrax impact and both prophylactics as well as therapeutic means are available. The infection can be resolved under standard conditions by antibiotics. Amoxicillin, doxycycline, ciprofloxacin and penicillin G are recommended as the prime choice for chemotherapy of anthrax (51). The occurrence of B. anthracis strains resistant to the standard antibiotics like ciprofloxacin results in the necessity to investigate and introduce combination of antibiotics (52). Research on new derivatives of the common antibiotics like tetracycline derivative omadacycline (53) and aminomethyl spectinomycins (54) is ongoing. Antibiotics are also applicable for the prophylaxis of anthrax and they can be employed as a cost effective measure preceding anthrax epidemic in emergency situations (55–57). Overview of anthrax therapies are given in the Table 2.

Mortality caused by anthrax would be reduced by blocking the anthrax toxin. Though the idea appears quite promising, the efficacy is not so high to replace standard antibiotics. The effect of antibodies specific to protective antigen of anthrax toxin was investigated by Tournier and coworkers (58). The authors discussed the effects of the antibody-based therapy and problems with evaluation of the therapy efficacy using an animal model. Testing of a new drug called Obiltoxaximab, which is a chimeric monoclonal antibody against protective antigen of anthrax toxin proved some effect and improved the chance to survive the infection (59–62). The drug is under clinical trials. Its effect can be further improved by combination with standard antibiotics. Comparing to the standard antibiotics, the therapy by antibodies against anthrax toxin can be employed for prophylactic purposes because patients can exert good tolerability to such therapy. The applied antibodies can be specific to edema and lethal factor of anthrax toxin and the neutralization of the whole toxin is principle of the therapy (63, 64). Antigen binding synthetic fragment of an antibody with a high affinity to edema factor appears promising (63). Combination of an antibiotics with an antitoxin (antibody specific to anthrax toxin) is recommended as a highly effective way how to protect from anthrax (65).

Active vaccination is not a therapeutic process, when the infection already starts but it is an effective tool for either avoiding or at least ameliorating the disease progression. The parts of anthrax toxin, such as protective antigen, spore specific antigens and inactivated spores can be chosen for vaccination purpose (66, 67). Commercial Anthrax Vaccine Licensed containing culture supernatant of a non-encapsulated strain producing toxin is effective for eliciting antibodies response. Current research is focused on production of vaccines containing recombinant proteins like Near-Iron Transporter having a good efficacy in the immunization of tested animals (68). The vaccination has also its limits. For

| Tab. 2. Basic therapies for anthrax. |
|------------------------------------|
| Type of therapy | Drug | References |
|----------------|------|------------|
| antibiotics | standard drugs: amoxicillin, doxycycline, ciprofloxacin, penicillin G and others | (51) |
| application of antibodies against protective antigen of anthrax toxin | Obiltoxaximab – under clinical trials | (59–61) |
| application of antibodies against edema factor of anthrax toxin | fragment of a synthetic antibody | (63) |
| vaccination | immunization by parts of anthrax toxin, spore specific antigens and inactivated spores, commercially available Anthrax Vaccine Licensed containing culture supernatant of a non-encapsulated strain producing toxin | (66) |
instance, Glinert and coworkers tested a protective antigen based vaccine and found that it was effective against subcutaneous spore challenge but the efficacy was limited for systemic challenges like intravenous application of virulent strains or mutants with deficient anthrax toxin (65). Therapies of infectious diseases can be further improved by nanoparticles serving like drug carriers, supporting material for antigen presentation during vaccination or material releasing antibiotics for a wider time span (69–76). Though there are no relevant studies on anthrax, the applicability of nanoparticles in the therapy can be inferred for the future.

**Diagnosis and detection techniques**

Determination of a biological warfare agent or a causative agent of an infection is an important step during emergency situations. Either a direct recognition of the microorganism and/or toxin or recognition of markers can be used in revealing of an attack by biological warfare agent like anthrax. A successful diagnosis confirmed by identification of the causative microorganism is the best situation and afterwards an optimal therapy can be chosen.

The microorganism itself can be differentiated from the other microorganisms by the aforementioned cultivation tests. Though the cultivation is a standard method and it is necessary for confirmation of results, it has limited use for first response countermeasures because the cultivation protocols take one or two days. Simple devices like various hand-held devices, biosensors etc. are suitable for direct detection of *B. anthracis* spores or grown cells. Hand-held devices working on the principle of lateral flow immunochromatography assay are quite popular due to price, simplicity, portability and no need of an instrumentation because a coloration is evaluated by a naked eye and they also appears to be suitable for detection of anthrax cells, spores, antigens or anthrax toxin (3, 77, 78). They can be also employed for the diagnosis of the disease by specific antibodies assay (79). There is also an advantage that more biological warfare agents can be determined in a single step. The device called Pro Strip by Advt Biotechnologies, LLC (Phoenix, AZ, USA) is for instance able to contemporary detect *Bacillus anthracis* with limit of detection $1.5 \times 10^4–8.3 \times 10^8$ cells or spores per ml, ricin with limit of detection 10 ng/ml, butulinum toxin with limit of detection 33–500 ng/ml, *Yersinia pestis* with limit of detection $10^6$ cells/ml, and *staphylococcal enterotoxin B* with limit of detection 10 ng/ml in an assay lasting 15 minutes. A single channel version of the before mentioned is called Biowarfare Agent Device. Its limit of detection for the single biological warfare agents and time per assay are the same as in the previous case. Another company, Alexeter Technologies (Wheeling, IL, USA), make devices working on lateral flow immunochromatography assay principle as well. Their RAID 5 and RAID 8 devices are able to contemporary measure five respective eight biological warfare agents. The manufacturer exerts the limit of detection $10^8$ spores/ml for *B. anthracis*. As seen from the aforementioned examples, lateral flow immunochromatography assay is a method for the detection of wide number of biological warfare agents. On the other hand, the limits of detection are quite high and they are not able to recognize low number of vital microorganism or spores, which are still dangerous for humans and able to initiate development of disease. In the current time, there is an effort to introduce biosensor devices to be an alternative to the standard bioassays, for instance optical and electrochemical biosensor for the detection of *B. anthracis* are developed (80–83). There is also a development of simple analytical tools allowing identification of *B. anthracis* by measurement of a specific metabolic reaction. It can be depicted on the work by Robinson and Bishop, who prepared the gel releasing fluorescent methylumbellifereone by enzymatic activity of a-glucosidase from *B. anthracis* spores, when the microorganism is presented in tested sample (84). The simple disposable gel is able to disclose as low as $5 \times 10^4$ CFU stuck on reaction test well with square 0.32 cm$^2$.

In the standard laboratory praxis, there is a typical identification of *B. anthracis* based on characterization of their DNA and polymerase chain reaction (PCR) method is the most common. PCR can be of course used for the determination of microorganism isolates in vital form as well as spores, *B. anthracis* cells in swabs used for surface stirring, environment samples like soil, but it can be also employed for determination of viable cells and spores in clinical samples (85–90). Because PCR amplify DNA from a collected sample, one cell or spore can be theoretically determined by this method. Chromatography with a simple detector and combination of chromatography and mass spectrometry are universal analytical methods suitable for the determination of wide number of analytes. They can be used for measurement of specific antigens like glycoproteins on *B. anthracis* (91) and anthrax toxin (92–94).

Both antibodies specific to *B. anthracis* and antigens from *B. anthracis* itself can be recognized by standard immunochemical tests like Enzyme-Linked Immunosorbtent Assay (ELISA). Though the assay is a standard one and it may appear that there is no improvement in this, the contrary is true because new types of antibodies and antigens are prepared resulting in better analytical properties than exert the predecessor assays. Even standard ELISA has quite good analytical properties including a very low false-positivity and false-negativity (95, 96). Varshney and coworkers, for instance, prepared ELISA based on chimera protein contain-

| Principle of assay | Detected part of *B. anthracis* or marker | References |
|-------------------|------------------------------------------|------------|
| Lateral flow immunochromatography assay | whole cell or spore – limit of detection around millions of spores per milliliter | (3,77,78) |
| Polymerase chain reaction (PCR) | whole cell or spore in clinical or environmental samples, theoretically one cell or spore can be detected | (85–90) |
| Chromatography and/or mass spectrometry | specific antigens like glycopeptides, anthrax toxin | (91–93) |
| Enzyme-Linked Immunosorbtent Assay (ELISA) | antibodies specific to *B. anthracis* from a host organism and antigens from *B. anthracis* | (95–99) |
ing parts of protective antigen and lethal factor of anthrax toxin expressed in Escherichia coli (97). This ELISA served for anthrax diagnosis by recognition of specific antibodies against the parts of anthrax toxin. The use of recombinant proteins as a platform for revealing the antibodies against B. anthracis by ELISA was also chosen in the work by Simbote and coworkers (98) and Ghosh and coworkers (99). Methods like various fluorescent immunosor- bent assays (100) or enzyme linked immunospot (101) are functional alternatives to the standard ELISA (Tab. 3).

Conclusions

B. anthracis is a serious threat, when misused for military or terrorist activities. It can cause a lethal impact on human population even though therapies and prophylactic countermeasures are available. In the current approach, a proper diagnosis of the disease, timely recognition of causative agent, prophylaxis of personnel providing help and a suitable therapy to the victims are the crucial steps. Further improvements of the diagnostic and detection platform are necessary. Effective passive and active vaccines are also highly desired. Though antibiotics are available and effective for therapy, further research on the new drugs with minimal side effect could bring an opportunity to protect general population with minimal harm in the case of false positive report of an anthrax attack and a good efficacy of therapy in the case of real emergency situation.

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