1. Introduction

*Bacillus anthracis* is a gram-positive, aerobic, spore forming bacterium that is the etiological factor of anthrax, causing disease in animals and humans. Due to its health effects, this bacterium is included in the 3 categories of biological hazard. Among animals, ruminant infections are the most common. Human infections occur mainly through contact with infected animals or animal products. The main routes of infection are microtrauma, alveoli, and the gastrointestinal tract. There are three main forms of anthrax: cutaneous, pulmonary, and food [21]. *B. anthracis* can be transmitted to humans by direct contact, ingestion, aerosolization or injection of vegetative cells or spores. *B. anthracis* spores were used in a bioterrorist attack in 2001 [8]. The event prompted the issuing of recommendations concerning, among other things, diagnostics, treatment, and prevention in the event of a terrorist attack [17, 35]. Recommendations developed in 200 by a working group of 23 experts representing medical and military centers, scientists, and emergency response offices contain recommendations on the use of antibiotic therapy [13]. These recommendations refer to the use of ciprofloxacin and doxycycline in post-exposure prophylaxis [27]. Full post-exposure prophylaxis, in the case of the likelihood of inhalation of *B. anthracis* spores, lasts 60 days, the minimum duration of antibiotic therapy is 10 days. The authorized services decide about the necessity to extend the therapy to a full 60 days. The 60-day therapy is conditioned by the period of spore germination, after which time a full-blown disease may develop [8, 13]. Even though antibiotics were administered before or immediately after the exposure to spores, in case of exposure to dangerous spore aerosol, inhaled spores may dwell in dormancy in mediastinum lymph nodes or can germinate immediately and release toxin after interruption of antibiotic treatment, causing severe disease or death. It is estimated that about 70% of cases can be prevented if the mass distribution of antimicrobials was commenced during the six days of initial exposure [3].

For this reason, it is important to combine antibiotics with vaccination in post-exposure therapy [3]. The alternative to antibiotic treatment [2] may be pre and post-exposure prevention with the use of vaccines. Post-exposure use of vaccines may shorten the prophylaxis time required. Terrorist attacks have forced scientists to take steps to search for an effective, safe, easy-to-administer vaccine, the use of which...
will provide long-term protection. Currently, the AVA vaccine is available on the US market and the UK AVP [8, 9, 12, 23].

2. Toxins in pathogenesis as a vaccine targets

The full virulence of *B. anthracis* is determined by the presence of the ternary toxin and the coat that protects bacterium against phagocytosis [11].

Anthrax spores enter the body, where they duplicate and transform into vegetative forms capable of producing a toxin consisting of three synergistically acting proteins: protective antigen (PA – 83 kDa); lethal factor (LF – 87 kDa) and edema factor (EF – 89 kDa). LF in combination with PA form lethal toxin and EF in combination with PA form edema toxin [8, 16, 39].

According to the latest research, PA binds to the ATR (anthrax toxin receptor), which is a cellular receptor encoded by the ANTXR1 gene present on the surface of the host cell and is digested by the protease resulting in the removal of 20 kDa fragment. The obtained C-terminal PA fragment (PA63 kDa) acquires the ability to polymerize by forming heptamer. The next step is binding of factors LF and EF. Once (PA63) 7 – LF and (PA63) 7 – EF complexes are formed, they penetrate via ATR-mediated endocytosis into the acidic environment of the endosome. [39]. The acidic pH in the endosome induces the incorporation of the PA heptamer into the endosome membrane and translocation of the EF factor into the cytosol of the attacked cell [24].

3. Current vaccines

Presently, the vaccines against anthrax are produced in the Great Britain and in the USA. The British vaccine AVP (anthrax vaccine precipitated) and the American AVA (anthrax vaccine adsorbed) are licensed.

Both vaccines containing PA (protective antigen), a highly immunogenic protein and small amounts of LF (lethal factor) are culture filtrate with aluminum hydroxide [9, 12, 23].

The AVP vaccine was first licensed in the UK in 1979 and is administered in three doses every three weeks and fourth dose after six months with an additional boost dose every twelve months. The AVA vaccine was registered in 1970 in the USA, at the Michigan Biologic Products Institute, Lansing and it is a cell-free culture adsorbed on aluminium hydroxide. The vaccine was named Biothrax in 2002 [3, 7, 9, 22, 28].

AVA requires a long vaccination schedule which is troublesome to perform and it is marginally reactogenic. It is administered six times in three doses every two weeks, and then three additional doses after six, twelve and eighteen months; plus yearly boosters. Up to 01.03.1999, around 590,000 doses of the anthrax vaccine were administered to American soldiers with no vaccine side effects noted [7, 14, 38].

In research on primates it was shown that administering two doses with two weeks between them, fully protected the animals from infection with air-suspended spores of *B. anthracis* in the 8th and 38th week from the vaccination, and after a hundred weeks the immunity was 88% [7, 14, 40].

In the future, post-exposure antibiotic therapy should be shortened and combined with vaccination which guarantee protection against bacteraemia. A study conducted by Williamson et al. on rabbits has shown the effectiveness of antibiotic therapy combined with the rPA vaccine. In this research rabbits were infected with *B. anthracis*. Rabbıts without rPA inoculation, were treated with antibiotics. Survival rate amounted to 56% of rabbits, in addition 50% of rabbits had bacteraemia detected in their blood. The study shown that maintenance dose of antibiotic levofloxacin combined with reduced rPA vaccine, increased animals survival to 89% and 11% of animals which had bacteraemia [36].

The US Food and Drug Administration accepted AVA (Anthrax Vaccine Adsorbed BioThrax) as anthrax vaccine. In post-exposure prophylaxis (PEP) AVA is recommended to administered three times subcutaneously: zero, fourteen and twenty-eight days, in combination with sixty days of antibiotic therapy [28, 29].

The Advisory Committee on Immunisation Practices (ACIP) recommends that one combines antibiotic therapy followed by vaccine administration in two weeks in post-exposure regimen [3].

The Centre for Disease Control and Prevention provides Biothrax in 5 ml dose vials. as the cell-filtrate of strain *B. anthracis* cultures adsorbed on aluminium hydroxide in 0,85% saline [3].

A study by Sivko et al. was conducted in order to assess the potential for shortening the time of antibiotic treatment. Research indicated the efficacy AVA PEP vaccination against inhalational anthrax in NHP (non-human primates) after anthrax exposure.

Group of forty-eight cynomolgus macaques were randomized to five group. Next, the AVA vaccine was administered in various mixtures on days zero and fourteen. Then Ames spores of *B. anthracis* were used for exposure on day twenty-eight at the dose of 200 LD50 [29]. Subjects who survived were tested for the presence of anti-PA IgG and the levels of Toxin Neutralising Antibody (TNA). Survival increased from 24 to 100% and it was correlated with pre-challenge humoral response. Animals which survived and were vaccinated had increased levels anti-PA IgG and TNA [29]. They did not show any signs of infection. Bacterial cells were not found in the blood, but spores were discovered in
the lung tissues. The body’s immune response consist of antibodies and the cellular immune response constituting a complex process. Antibodies are needed to defend the body against infection, and their level in the blood can be determined. However, their level does not reflect the body’s resistance [15, 32]. Increasing the effectiveness of a new generation of vaccines will depend on the possibility of stimulating the cellular response together with inducing humoral response to the PA antigen [15].

4. Immunogenicity of anthrax vaccine candidates

Ndumnego et al. conducted research to determine the level of humoral response of B. anthracis protective antigen in African goats to collagen protein similar to anthracis collagen, spores of B. anthracis strain inactivated in formaldehyde (FIS), and vegetative antigen prepared from a capsule and toxin deficient strain (CDC 1014). Booster vaccination gave a higher titer of anti-FIS, anti-rPA and also lethal toxin-neutralizing antibody titer than a single dose of vaccination. A single and double dose of SLVS vaccine (Sterne 34F2 Live Spore Vaccine) gave the highest level of anti-FIS IgG to the other analyzed rPA (recombinant protective antigen) and rBcIA (recombinant bacillus collagen – like protein of anthracis) antigens. A second dose of vaccination resulted in an increase in antibody titers of 350 in the case of anti-FIS and 300 antibodies against rPA. SLVS vaccine administration did not affect rBcIA antibodies. A single dose of vaccine increased the neutralizing lethal toxin titer by 80 and second dose by 700. Vaccination with SLVS protected most goats treated with 800 B. anthracis spores. These results suggest that a second dose of vaccination may be given less than three months after the first dose [25].

Animals susceptible to infection can be protected by an approved Sterne vaccine containing live B. anthracis spores. However, this vaccine causes side effects in sensitive species. During the outbreak of the epidemic does not provide full protection and it incompatibility with antibiotics.

New generation vaccines containing recombinant peptides can provide solutions to these problems. Recently, the proposed antigen was rPA, BcIA, FIS. The ability to elicit a cellular response by selected antigens was tested on goats and with using a mouse model in vivo. Goats that had been given all three antigens showed the highest antibody titers. One dose of recombinant peptides observed a weaker effect, while second vaccination effected highest increase antibodies titers. The survival of mice given the rPA, rBcIA antigen mix after exposure to B. anthracis was 73%, while 68% of the exposed mice survived when the combination of the two rPA and rBcIA antigens was administered.

A 5% increase in goat survival was observed when FIS was added to the rPA and rBcIA mixture. These results confirmed earlier studies in which goats were vaccinated with rPA and rBcIA as well as rPA, rBcIA with the addition of FIS. The vaccine consists of three antigens for greater protection against B. anthracis infection, and the survival rate was 80%, while the rPA and rBcIA vaccinated goats had a 50% survival rate [26].

New generation vaccines based on recombinant forms of anthrax toxin are unstable during storage. Research shows that the response to this type of vaccine is directed at non-functional, non-neutralizing sections of anthrax toxin. Neutralization of anthrax toxin occurs by blocking the functional regions and epitopes, antibodies that comprise the PA domain [22]. The AVA and rPA vaccines with Alhydrogel adjuvant used in phase 1 clinical studies in humans and animals gave similar levels of antibodies [22].

As a result of these concerns, a lot of effort has been invested in production and testing of the immunological efficiency of rPA (recombinant PA protein).

The vaccine containing the recombinant PA protein elicited a strong immune response producing a high neutralizing antibody titer providing protection against attenuated B. anthracis strain A16R that was obtained by mutagenesis of wild-type B. anthracis A16 which can synthesize the exotoxin, but without a capsule [18].

The rPA protein can be used to produce anthrax vaccine due to its in vivo and in vitro biological activity. Solubility is another feature of this protein that supports its use in the vaccine. Vaccination with rPA mice two or three times provided 100% protection against 10 or 50 LD50 doses of B. anthracis strain 16R [19, 20].

5. Bivalent vaccines

Both inhalation anthrax and pneumonic plague can be prevented simultaneously using a new form of vaccine presented in another research publication. A new study reports the development of a vaccine which allows targeting three antigens, F1 and V from Yersinia pestis and PA from B. anthracis, using a triple antigen consisting single recombinant vaccine. The functionality and immunogenicity characteristics of all three antigens are combined in the triple antigen. Results present a comprehensive prevention against inhalational form anthrax and pneumonic plague, along with strong antibody response in animals (rats, mice and rabbits), by using two dosages of the immunogen along with an Alhydrogel adjuvant.

Tested animals proved a full protection from a simultaneous challenge with Y. pestis and the lethal toxin of B. anthracis. Finally, due to demonstrated strong immunogenicity proved in the human trials against two of
the bio-terrorist agents, the bivalent anthrax-plague injection becomes a promising nominee for further production [30].

Another example of a nanoparticle vaccine directed against both anthrax and plague is a product based on bacteriophage T4 as a platform. The strong immune response, which is specific for anthrax and plague, was triggered by virus particles. Moreover, nanoparticles tested on animal models such as rabbits, mice and rats, protected them against inhalational anthrax and pulmonary form of plague.

Despite administrating simultaneously a lethal dose of both anthrax toxin and Y. pestis CO92 bacteria, the animals survived as a result of complete protection. Two doses of this vaccine tested in animal models allowed a full protection against both inhalational anthrax and pneumonic plague when tested on animal models of: BALB/c mice, brown Norway rats, and New Zealand white rabbits. The T4 bacteriophage nanoparticles elicit a strong immune response to the pneumonic plague and inhalational anthrax. Use of modern platforms of nanoparticles such as phage T4, allow for the development of multipotent vaccines that could fight high-risk pathogens and help in fighting potential bioterror attacks [31].

The mucosal and systemic compartments were tested for the immunological memory response by Sun-Je Woo et al., who have investigated intranasal immunisation of rPA of B. anthracis. Use of the cholera toxin (CT) paired with rPA in a form of intranasal immunisation, resulted in long-term (6 months) response of PA-specific antibodies in lung, nasal, washes and serum. After booster immunisation a strong PA-specific memory B cells induction was noticed in lung, spleen and cervical lymph nodes (CLNs).

Moreover, use of the mentioned intranasal immunisation of rPA and CT, resulted in formation of effector memory CD4+ T cells in the lungs. As the result Th1 and Th17 – type cytokines in the lungs expanded their expression. However, this do not hold for the spleen or CLNs. From the results it can be assumed that the PA-mediated immunity through nasal route, may become an promising agent to protect from anthrax, as there is direct correlation between protective immunity and the PA-specific antibodies. In conclusion, nasal route appears to be the most promising platform for the vaccine delivery, and produces a long-term immunity against anthrax [37].

6. Capsule conjugated vaccines

Vaccination with the anthrax capsule – a naturally occurring component of the bacterium that causes the disease – completely protected monkeys from lethal anthrax infection. These results indicate that anthrax capsule is a highly effective vaccine component that should be considered for incorporation in future generation anthrax vaccines [33].

Research concerning the new anti-capsule vaccine development becomes an interesting topic, judging by the current results. Significant antibody responses were observed for the outer membrane protein complex (OMPC) of Neisseria meningitidis, and partial for the anthrax challenge, by using the capsule conjugate vaccines where the outer membrane protein complex (OMPC) is connected to the capsule. Moreover, in a cutaneous infection model a full protection was observed for the capsule connected to peptidoglycan [5].

Currently most of the available anthrax vaccines work only against the single PA immunogen of B. anthracis. This led to the development of a vaccine that can work against possible resistant strains, which is composed of anthrax polyglutamic acid capsule being covalently conjugated to the N. meningitides serotype B outer membrane protein complex (OMPC). The results showed that only partial protection of rhesus macaques against inhalational anthrax was achieved using two doses of 2.5 µg of the vaccine.

However, the higher dosage of 50 µg of the capsule conjugated vaccine resulted in a full protection of rhesus macaques against inhalation anthrax. Therefore, the promising results indicate that this conjugated capsule vaccine could become a promising drug in fighting anthrax challenge.

From the current studies, it can be observed that large doses of conjugate capsule-OMPC vaccine, can deliver a full protection in the rhesus macaques, animal model of inhalation anthrax. In fact, the promising results of the non-human primate model can suggest that the conjugated vaccine of immunogenic capsule with PA in prospect anthrax vaccines can possibly boost the effectiveness of PA-based vaccines [5, 6].

7. Domain's and epitopes of PA

Vaccines development, diagnostic assays and the post exposure therapy are the main areas PA antigen is targeted in research. It takes ten to eleven days from the first symptoms of cutaneous anthrax for the immune system reaction to be detectable, and it continues to be recognizable for the following eight to sixteen months.

Intoxication by anthrax toxin is a large process where each part of PA has a defined role. The second domain (PAD2), which is responsible for membrane insertion and heptamerisation of PA, generated the largest immune response. Another significant response was detected against domain 4 (PAD4), that has generally higher presence in the course of heptamerisation. However, the highest immune response was caused
by the domain 1 in a mouse model, in comparison to domains 2 and 4 which were significantly less efficient in triggering the larger antibody titer.

In conclusion, the individual domain 2 and 4 may become interesting targets for vaccine development through generating the chimeric protein with different applicable proteins, due to significant immunoreactivity observed on human cutaneous anthrax [34]. The results of the research of Gubbins et al. showed that the domain 2 of the PA protein as an immunogenic agent can neutralise the lethal \textit{in vitro} toxin using monoclonal antibodies, which are a key factor in the response to \textit{B. anthracis} infections [10]. The goal of the research of Gubbins et al. was to obtain PA specific monoclonal antibodies and the recognition of specific epitopes by these antibodies, where the epitopes protect the PA83 protein from being cleaved into PA63 and PA20. Brossier et al. created two monoclonal antibodies which, when bonded with domains 2 and 4 of PA83, neutralised anthrax toxin [4]. They obtained monoclonal antibodies 7.5 and 48.3 anti-PA, capable of both \textit{in vitro} and \textit{in vivo} neutralisation; the antibody 7.5 bonded with the domain 4 and protected it from being joined to the cell receptor. The antibody 48.3 bonded to the domain 2 and blocked the cleaving of PA83 into PA63 and PA20. The PA83 domains were, therefore, recognised by Mab 7.5 and 48.3 [4].

The main disadvantage of PA based vaccines is a short shelf life which restrains their usage, although they are currently the most efficient drug fighting against \textit{B. anthracis}. Usage of ID II – ID III region of PA and the N-terminal region of LF in the development of an epitope-based chimeric vaccine (ID-LFn), was described in the research prepared by Aggarwal and colleagues. In contrast to well-known PA-based vaccines, use of the ID-LFn injection on mice model resulted in both high immunisation and longer shelf life. Therefore, the ID-LFn as the vaccine becomes a more promising candidate for a drug against \textit{B. anthracis}, in contrast to standard subunit vaccines. Moreover, neutralisation of anthrax lethal toxin and elicitation of cytokine response, along with protective responses can be all mediated by immunodominant protective epitopes, namely ID I, ID II and ID III.

In conclusion, this study described the development of a new generation vaccine for fighting against anthrax, by chimeric protein fusion of immunodominant epitopes of PA and N-terminal domain of LF (LFn) [11].

8. Summary

The threat of \textit{B. anthracis} spores use at a mass scale against humankind triggers the need to elaborate an effective countermeasure strategy. The research focuses on numerous ways of protection including effective vaccination and methods that would insure protection even if we are faced with antibiotic – resistant \textit{B. anthracis} strains. Anthrax is still a serious and often incurable disease, which requires fast and effective treatment methods. Due to a better understanding of the mechanisms of \textit{B. anthracis} infection, new possibilities have emerged to solve important problems on the way to produce safe and effective formulations. Animal studies to date have shown that promising treatments to anthrax that include combination therapies with vaccines and new antibiotics that stimulate immunity against \textit{B. anthracis} spores as well as toxins and vegetative forms.

References


