

## [Anthrax]

Anthrax is far from a new bacterium, in fact, it has been around for quite some time. However, modern American society is newly familiar with this particular strain of bacteria because of the severe bioterrorism threats that it possesses. The general public is targeted more than any group because the ways that civilians can be attacked is more diverse than the ways that the military can be attacked – the release of biochemical weapons in a civilian setting is more potent because of the chaos that it causes (Lane 2007). It is dangerous because it is almost completely undetectable as an odorless powder that may be incorporated into food, water, or any other ingestible source. The article goes on to say that biomedical research and discoveries can potentially hurt the United States.

There are many ways to become inflicted with anthrax, including cutaneous anthrax (through ‘abrasions, cuts, or possible insect bites’ etc), gastrointestinal anthrax (‘from the ingestion of undercooked meat’), inhalation anthrax (‘industrial exposure to spores’), and anthrax meningitis (‘the appearance of blood in cerebrospinal fluid’) (Spencer 183). Thus, it is quite easy to contract this pathogen; however, the key lies in prevention and detection.

*Bacillus Anthracis* is a gram positive, non-motile, spore forming, rod shaped bacterium. Gram positive bacteria are those which have a thick peptidoglycan layer exterior to the plasma membrane and have no outer membrane. There are a lot of characteristics that one can visually see with this species – as Fischetti (2000) states in his article, Gram-positive Pathogens, there are bamboo-like structures that extend from the

pathogen that are very characteristic to it. Another visual characteristic of *B. anthracis* is the location of the endospores, which are centrally located within the cell and elliptical in shape. Finally, when grown under certain conditions, *B. anthracis* colonies have a mucous-like appearance, which is due to the capsule covering each bacterium – this is perhaps the most important characteristic.

The *Bacillus anthracis* capsule is unusual among bacteria and most directly responsible for its virulence. It has been demonstrated that mice inoculated with *B. anthracis* that lack the ability to make a capsule do not cause disease to the extent that would be seen with the capsule. This is because, without the capsule, the bacterium has more of a chance of getting phagocytosed and killed by the immune system's macrophages. In addition to covering the cells, antigen studies performed by Salyers (2002) has shown that when a pathogen has a capsule, in this case specifically *B. anthracis*, the immune response is weak and barely protective.

Virulent strains of *B. anthracis* carry two large plasmids which encode and regulate virulence factors. Plasmid PX01 (182 Kb) encodes the genes for the toxin proteins EF, LF and PA. Plasmid PX02 (93 Kb) encoded genes include those required for synthesis of the poly-D-glutamic acid capsule. Expression of capsule and toxin genes by *B. anthracis* during growth in media is enhanced by the presence of bicarbonate and elevated CO<sub>2</sub> (Fischetti 524). The concentrations of bicarbonate and CO<sub>2</sub> that up-regulate toxin and capsule production are similar to those in the human body. Finally, bacteria grown at 37°C as opposed to 28°C also produce more toxin and capsules, illustrating another regulation mechanism of virulence genes.

As stated previously *B. anthracis* has the ability to form an endospore which is complex and highly resistant to destruction. The outermost layer, the exosporium, is a thin proteinaceous covering. Under the exosporium lies the spore coat, comprising layers of spore-specific proteins. Beneath the spore coat lies the cortex, a layer of peptidoglycan that is less cross-linked than normal cell peptidoglycan. Finally, central to the spore is the core which is comprised of the bacterial cell and all its components.

The mechanism of anthrax pathogenesis is mediated by 3 toxins produced by the bacteria. Tournier et al. (2007) state these toxins as the protective antigen (PA), the lethal factor (LF), and the edema factor (EF). The mechanism of the most deadly type of infection, inhalation anthrax, begins with spores entering the lungs and then being phagocytosed by alveolar macrophages and lung dendritic cells. Subsequently, the engulfed spores become vegetative cells which use the macrophages and dendritic cells to carry themselves to the lymph nodes.

The protective antigen PA is non-pathogenic by itself; rather, its function is to aid in transporting EF and LF into the target cells. Once bound, PA is cleaved by a cellular furin-like protease releasing a 20 Kd fragment that functions to keep the PA units from self-assembling. The functions of the toxins vary between the separate toxins – LF cleaves protein kinases, EF increases concentrations of cAMP which results in problems with cytokines and also helps to increase the rate at which the toxins are internalized (Tournier et al. 2007). In addition, LF causes apoptosis, inhibits macrophages and dendritic cells from secreting inflammatory cytokines, inhibits the recruitment and activation of neutrophils and also inhibits the differentiation of monocytes (Tournier et al. 2007).

In the late stage of the anthrax infection, the anthrax bacilli form long chains in the bloodstream and significant amounts of the three toxins are released into circulation. The effect is endothelial wall (blood vessel) disruption and subsequent hypoxia leading to death. The time from initial infection is so short that the effect of the adaptive immune system is largely unknown. A study had been conducted of the patients who had been exposed to the inhalational form of the bacteria, and none of the patients who had passed away due to infection had any noticeable amount of the antibody in their blood samples (Tournier et al. 2007).

Spores formed by *Bacillus anthracis* when conditions are less than favorable can be eaten by cattle and cause anthrax infection. Infections of cattle and humans are common in Central and South America, Southern and Eastern Europe, Africa, and the Middle East. Cattle are commonly vaccinated in America and Europe so the disease tends to be rare in those countries. Anthrax is mainly transmitted from animals to humans through three separate ways: inhalation, intestinal, and subcutaneous. Anthrax can not be spread person to person, but is spread from animal to person through spores. *Bacillus anthracis* is fairly flimsy and responds well to basic antibiotics like penicillin and tetracycline. However, in the event of a terrorist act, ciproflaxin is administered because of the possibility of resistant strands. With high doses of antibiotics administered within 48 hours, even inhalation anthrax is survivable (Shafazand et al. 1999).

*Bacillus anthracis* is most commonly a bacterium that infects animals, but has spread from animals to humans through exposure to infected animals or animal products. Thus the vaccine first created in the late 19<sup>th</sup> century was for the protection and

prevention of the disease in animals. In the 1940s, the idea that anthrax could be used for bioterrorism started to emerge, which jolted scientists into creating an effective vaccine for human use (Joellenbeck et. al. 2002). The vaccine was distributed only to small populations throughout the 20<sup>th</sup> century, the most of them being people who were regularly exposed to animals that may carry the disease and to military personnel. Because of the premeditated release of *Bacillus anthracis* spores in the United States in 2001, the vaccine was also used to treat a large number of individuals who were exposed. Although this vaccine has been used over the course of the 20<sup>th</sup> century, the efficacy and safety of the vaccine is still under scrutiny. A huge safety concern that has pressured researchers is the vaccine has seemed to cause many acute and chronic health problems, ranging from fever and malaise to other more serious, and even fatal, diseases (Joellenbeck et. al. 2002). The vaccine is also used as an option to treat infection, but it is not a recommended treatment. An anthrax infection can be treated using antibiotics, but most times the disease is not caught early enough to administer them. Once the bacteria starts creating toxins, the antibiotics can no longer do anything.

Our group chose the particular pathogen *Bacillus anthracis* because of its bioterrorism potential in modern society. The current vaccine is semi-effective, though a poor solution due to a various amount of problems such as unknown long-term effects, the manner in which it is administered, and its inefficacy in protecting against the inhalational form of anthrax. Also, the vaccine does not protect against mutagenic forms of the bacterium (Joellenbeck et. al. 2002). Anthrax is a dangerous threat because of the many ways of contracting its many forms. *B. anthracis* is easy to colonize and reproduce, and has a fairly simple chemical makeup, relatively speaking. This means it

can easily find its way into the wrong hands, which could potentially pose a major threat. The subject population of target is military and service personnel. As long as our military remains unharmed, the vaccine can perhaps be mass-produced to be sufficient in case of another bio-terrorist attack. Keeping supply for the armed services is crucial for the protection and future endeavors of our country.

There are approximately four types of vaccines commonly used, including inactivated, live-attenuated, toxoid, and subunit. Each provides unique characteristics to serve its purpose. In addition, there are three other types that are more recent but not yet standard, including conjugate, recombinant vector, and DNA vaccinations. For this particular anthrax bacterium, choosing the DNA vaccination seemed most appropriate. An inactivated vaccine would not be effective because it instigates a short-lived immune response and needs booster shots, which would not be conducive to a mass-produced and mass-used vaccine. A toxoid vaccine would not be appropriate either because it provides protection only against the toxins created by the bacterium and not the bacterium itself. A subunit vaccine would also be ineffective because it provides protection against the capsular protein and not anything else.

In DNA vaccination, a DNA protein from the infectious agent is injected into the subject or patient. This quickly induces the cell-mediated immune response, which is ideal for *Bacillus anthracis*, which contains intracellular components. DNA plasmids, which are what have been injected, have been engineered so they code for the specific antigens tested. According to Donnelly et. al, DNA immunization is extremely helpful specifically in regards to cytotoxic T-cells which recognize and destroy intracellular invasions (Donnelly et. al, 1997). Other cells, such as B-cells for example, recognize the

proteins as foreign from bodily cells, then surmount an attack, creating antibodies which reside in its host to prevent future infections by the same bacteria. Antibodies, as well as memory cells, are important in the creation of an effective vaccine. Another reason why DNA vaccinations are a good choice is due to the fact that DNA vaccines are easy to produce and store, which is necessary when there is to be mass production.

Immunity will be induced to three specific antigens: the protective antigen, the lethal factor, and the edema factor (elements of a tripartite toxin). The edema factor and the protective antigen suppress neutrophil function and impair the host's resistance. (O'Brien, 1985). The lethal factor, as stated earlier, does a variety of things, such as causing apoptosis, inhibits the release of inflammatory cytokines, inhibits the activation of neutrophils, and also inhibits the differentiation of monocytes (Tournier et al. 2007). These three antigens were chosen because they are what solely contribute to the virulence of *Bacillus anthracis* (O'Brien, 1985).

Adjuvants are absolutely necessary in the preparation of a vaccine. They do not induce any specific antigen-like responses like the pathogen does, but they still fuel the immune system. One of the main functions of adjuvants is that they increase the innate immune response because they inflate the behaviors of the antigen presenting cells, such as dendritic cells, lymphocytes, and macrophages. Adjuvants let the antigen have a prolonged delivery to the immune system, which is helpful because it gives the immune system more time to manufacture and regulate the B and T cells that make the vaccine effective. The prolonged delivery also helps with memory T cells and IgG (Gupta et. al 1995). The vaccine is not a real, natural infection of the pathogen in the body but the adjuvant mimics what would happen if it was, which causes an immune response to

occur. The adjuvant that would be most effective in the case of an anthrax vaccine would be a virosome adjuvant. At this moment, with the current vaccine, an aluminum hydroxide adjuvant is being used. Recent studies have shown that aluminum adjuvants raise safety issues (they may cause neuron death). As stated by Gupta et. al, aluminum adjuvants are sometimes used under less than favorable conditions due to the fact that there may be nothing else to use.

Virosome adjuvants are helpful because they stimulate the immune system more than aluminum salt adjuvants do. Aluminum salts induce the immune response by activating a localized inflammatory response, while virosomes do not. Virosomes are also effective because they help the uptake of the antigen in the antigen presenting cells, which is necessary for an adaptive immune response. According to Gluck et. al, virosomes fuse with the immune system cells, such as macrophages and monocytes, and transfer their contents through this fusion directly into the cells that are being activated. This, then, provides great protection even in patients whose immune systems have been previously compromised (Gluck et. al, 2005). Virosomes are also particularly helpful because they activate cell-mediated *and* humoral immunity.

Cell-mediated immunity is derived from T-cells without any use of antibodies involving cells such as cytotoxic T-cells and helper T-cells which activate macrophages. Adjuvants help with this form of immunity because they inflate the behavior of antigen presenting cells. Cytotoxic T-cells are important in intracellular pathogen infections as they are attracted to the altered MHC on the surface of infected cells and then induce apoptosis within these cells. TH1 cells signal macrophages to fuse lysosomes with the vesicles inside with carry the toxins and then destroy them. The tripartite toxin-specific

TH2 cells secrete cytokines that signal B-cells to create antibodies to the specific antigen. Tripartite toxin specific TH-2 cells are very effective for a vaccine as they provide for a production of memory cells.

Humoral immunity refers to the protection that one gets from antibodies. It related to cell-mediated immunity on the subject of TH-2 cells that activate B-cells. It is crucial for memory cell generation. Since inhalational anthrax is the most potent form, IgA is the most common antibody produced after isotype switching. Initially, IgM is secreted and isotype switching of IgG soon follows. These particular antigens are T-dependent, therefore isotype switching and memory cell production occur.

Problems possibly to be encountered include having immuno-suppressed patients having difficulties producing antibodies. Virosomes help this problem by attaching directly to target cells and injecting it's components directly as well. This eliminates a portion of the problem. Also, malnourished individuals may have to undergo protein therapies so they can more readily accept the vaccination.

IgA is generally secreted by mucosal lined tracts of the body. These tracts of course work to defend against pathogen. IgA neutralizes against pathogens and their toxins. In this case, against toxins released from B. anthracis that are so harmful to it's host. Testing for presence of IgA required an ELISA to be performed. ELISA, which stands for Enzyme-Linked ImmunoSorbent Assay, is an immunological test that can be used to determine the quantity of an antibody in a patient. The specific antibody that will be noted in an inhalational anthrax infection would be IgA, therefore the antibody that is being noted in an ELISA of someone who has recently been immunized with an anti-anthrax vaccine would also be IgA (7-10 days after vaccination). First, the microtiter

plate is covered with purified antigen, which in this case would be protective antigen, lethal factor, and edema factor. A serum sample from the patient that is to be tested is then added. Finally, anti-human alpha chain is added so that it can bind to the Fc region of the specific antibody, which would be human IgA. Chromogenic substrate is then added for a color change that can be measured in a spectrophotometer - the more color noted, the more specific antibody is present in the sample. A further test to ensure the presence of anthrax, is to amplify the PA gene by PCR. This test is safe and easy to perform and is capable of identifying environmental factors as well.

Since anthrax is so dangerous if used, and because of widespread fear of use as a bioterrorist weapon, eventually the vaccine should be administered not only to military, but to the general public. Like chicken pox or mumps vaccinations, anthrax should be administered in the first few years of life so the child will never be at risk to its effects. Since our vaccination adjuvant is a virosome, and ideally more effective than the aluminum hydroxide, and would not have to be administered nearly as often. Currently, the anthrax vaccine is given six times, at week 0, 2, and 4, and then at 6, 12, and 18 months. This new vaccine would be administered between ages 0-6, and then again at about 12 to ensure complete resistance to *B. anthracis*.

Flow cytometry can be used to measure T cell memory because it measures the frequency of the cell requested (memory T cell). T cells cannot bind to antigen directly, therefore a complex including the tripartite-toxin-peptide has to be introduced to the serum from the patient for T cell-antigen binding to occur. Memory T cells contain a complex of self-peptide and MHC I that binds to the tripartite-toxin-peptide complex that is introduced. The antigen complex is then fixed with fluorochrome and sent through the

flow cytometer - the reading it provides is the frequency of memory T cells. This test should be performed a month after injection, to prove efficacy.

Possible problems with the vaccine include that the newly created vaccine is to be administered just twice in a human lifetime. Currently, the vaccination is being administered six times in the course of 18 months. Perhaps the vaccine is not strong enough or will not provide enough memory cells to cause complete immunity to the particular strain of bacteria. It is possible that there may need to be booster shots administered as well, to insure health. Tests would enable proof of the vaccine efficacy as previously mentioned. Also, the idea of an anthrax patch is being looked into, as a potential patch is safe and does not involve a needle. Though, this idea becomes far more complicated in logistics of entering the human body, attempting to first trigger mucosal membrane reactions.

For the clinical trial of this new DNA vaccine, approximately 400 healthy individuals will be used. Their physical and mental well-being as well as organ function will be tested prior to their acceptance into the trial. A general physical exam will also be administered prior to acceptance. The individuals will be given the first vaccine injection upon acceptance into the trial - if a serious reaction occurs to the vaccine, medicinal help will be provided at the site.

Approximately 7 to 10 days following the injection, the individuals will be brought in to extract a sample to perform an ELISA test to see if antibodies are being produced. Every 6 months for four years the individuals will come in for another ELISA test and also for a flow cytometry test to study the activation of memory T cells. Hopefully the vaccine is effective enough that even after four years, the antibodies and memory T cells

are still around. This vaccine, though completely different from others, incorporates DNA, making its possibilities endless. The thought of eliminating anthrax and its effects could be life changing, and make the country safer against bioterrorist threats.

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