

## A Step Closer for Anthrax Vaccine: How does It Work?

*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. Visually, “the rod-shaped cells form long bamboo-like structures characteristic of the species” (Fischetti, 520). 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 much disease. “Most bacterial capsules are composed of polysaccharides” (Salyers 335); however, the capsule of *B. anthracis* is a “polypeptide composed of gamma-linked alpha peptide chains of 50 to 100 D-glutamic acid residues” (Fischetti 523). The cell is completely covered by this capsule making it “strongly resistant to phagocytosis” (Fischetti 523).

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 lays 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 cells and all its components.

The mechanism of anthrax pathogenesis is mediated by 3 toxins produced by the bacteria. “These three factors are the protective antigen (PA), the lethal factor (LF) and the edema factor (EF)” (Jean-Nicolas Tournier et al). 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. “LF cleaves most mitogen-activated protein kinase (MAPK) kinases (MKK); whereas EF increases intracellular cAMP concentration leading to various disruptions of cytokine secretion and an increase in ANTXR expression resulting in an

increase in the rate of toxin internalization” (Jean-Nicolas Tournier et al). 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 (Jean-Nicolas Tournier et al).

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. “In the follow-up study of the patients with bioterrorism-related inhalational anthrax, none of the five patients who died had any detectable anti-PA antibody in their initial blood samples” (Jean-Nicolas Tournier et al).

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, ciprofloxacin is administered because of the possibility of resistant strands. With high doses of antibiotics administered within 48 hours, even inhalation anthrax is survivable (Shirin et al., pp. 1369-1376).

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 potential agents and circumstances of bioweapon attacks in civilian settings are more diverse than those directed at the military; attacks against civilians are usually intended to cause widespread panic and terror” (Lane 1271). 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. “The ultimate goal of biomedical research is to advance the public health of society. In this regard, the biomedical research community clearly has a responsibility to participate in the current and future struggle against bioterrorism” (Lane 1273).

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 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. Around the 1940s, work on anthrax vaccines “suitable for human use gained urgency...with fears that anthrax would be used as a biological warfare agent” (Joellenbeck et. all, p.40). 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. all, p. 85). 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 bio-terrorism potential in modern society. The current vaccine is semi-effective, though a poor solution. 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.

### *Description of vaccine*

Studies have shown that immune response to PA and capsule produce the best outcome for a vaccine (J. Chabot et al,2004). To create an immune response to PA, it was cloned in to a plant virus and expressed on the surface of the viron. Furthermore for the capsule a protein carrier, Bovine serum albumin, were covalently linked to enhance B cell activation. The two parts will be injected as a dual part vaccine in the blood stream.

To illicit the necessary anti-PA antibodies we will create a recombinant plant virus expressing a portion of PA on its surface. Cowpea mosaic virus has a 9.8Kb, segmented ss(+)RNA genome (Boshuizen et al., 1997). The viron is icosahedral made up of 60 copies each of a small and large capsid protein (Boshuizen et al., 1997). It has been demonstrated that cowpea mosaic virus is an efficient carrier of foreign proteins which generate a humoral response (Boshuizen et al., 1997). It has also been demonstrated that the entire anthrax PA protein is not necessary to illicit a humoral immune response. In fact, the D4 sequence of PA which is 140 amino acids in length generates the best neutralizing antibodies and Th2 cells when expressed on a virus surface (Calkins et al., 2006). To generate a recombinant cowpea mosaic virus expressing PA D4 it will be necessary to insert the D4 sequence inside the small viral capsid protein cDNA. Once created, the recombinant viron will contain the small capsid-D4 fusion protein. This fusion protein will be expressed 60 times on the viron surface in a very ordered fashion. Finally, we will use aluminum salts to enhance the immune response; this adjuvant has been show to work with the type of vaccine (Bendig et al., 1999)

The goal of this part of the vaccine is to produce both neutralizing antibody producing B cells and antigen specific Th2 cells. Antigen specific B cells should recognize D4 on the surface of cowpea mosaic virus however, to be activated the B cell needs a signal from an antigen specific Th2 cell. The virions will be phagocytosed by dendritic cells which will degrade the virions and load short peptides derived from D4 onto MHC class II which will be expressed on the surface of the dendritic cell. A Th2 cell will recognize this MHC-peptide complex and be activated with help from the dendritic cell. The activated Th2 cell will then encounter an antigen specific B cell which has phagocytosed the viron and is expressing it on MHC class II, the Th2 cell will then activate this B cell creating the desired antibody response as well as memory Th2 and B cells. Specifically however, we wish to illicit primarily IgG antibodies. Finally this vaccine will not create a significant CD8 T cell response because little if any D4 peptide will be expressed on MHC I because the viron is not infectious.

This type of recombinant plant virus expressing a portion of PA was chosen for several reasons. First, PA was the chosen antigen because studies and old vaccines have shown that PA is essential to forming immunity. Anti PA antibodies neutralize PA before it can form pores which the other two toxins, LF and EF need for entry into host cells where they execute their toxic effects. Therefore, blocking PA essentially negates all the toxic effects of anthrax because EF and LF are harmless outside of host cells (Turnbull, 1991). Furthermore, cowpea mosaic virus was chosen as a vector because previous use as a vector for expressing other antigens and because of its relative safety. This virus is not known to be infectious toward mammalian cells (Bendig et al., 1999). The virus's innocuous nature makes it safer than attenuated bacteria or animal viruses which can revert and become highly infectious. Moreover, as with all viruses, antigen-capsid proteins will be expressed in an ordered fashion which is believed to stimulate B cells better than soluble antigen (Calkins et al., 2006). This is probably because the rigid capsid-antigen complexes are in close proximity and cause the B cell receptors to crosslink which amplifies the signal sent to the B cell nucleus. Finally, this part of the vaccine will be cost effective because it can be grown to high titers inside plants and then easily purified (Bendig et al., 1999).

There are a few hurdles that could be encountered in making this vaccine. Firstly, a sequence as large as 140 amino acids has never been inserted into the genome of cowpea mosaic virus. It would prove difficult to successfully insert this sequence and get it to correctly fuse with the small capsid protein.

Immunity to the capsule may enhance the protection afforded by protective antigen vaccines against anthrax if opsonizing antibodies are produced. In this report we show that vaccination with the capsule is protective against lethal infection. A target would be for the capsule to opsonizing it so it can be phagocyted by macrophages. To have an inactive and not lethal capsule representation in this study a conjugate vaccine for the capsule are used.

Bovine serum albumin is one of the most widely studied proteins and is the most abundant protein in plasma. Bovine serum albumin is also used to describe a protein or a group of proteins defined by solubility in water for example the albumin fraction of wheat which makes it easier to circulate in the blood system. BSA is stable, it lacks of effect in many biochemical reactions, and its low cost since it is readily available in large

quantities as it is purified from bovine blood. This is a good criterion for a cheap and safe vaccine. Why this could be a good carrier it is less of a risk factor, because it is the most abundant protein in the circulatory system and contributes 80% to colloid osmotic blood pressure (<http://www.proliantinc.com/biologicals/main/Biologicals.asp>).

A conjugate vaccine is created by covalently attaching a poor antigen to a carrier protein, thereby conferring the immunological attributes of the carrier on the attached antigen. Fragments of the capsule is taken and conjugated with BSA. For this epitope to be recognized by T-cells at least 10 residue of the peptides of the saccarids are conjugated (J. Chabot et al,2004). What also will be connected to the residue is a transmembrane. The transmembrane helps to make a stronger expression of the antigen on the cell membrane when it is expressed to the T cells. The type of transmembrane being used is a type Beta-barrels which is the protein found in only outer membrane of gram negative bacteria as anthrax , cell wall of Gram-positive bacteria, and outer membranes of mitochondria and chloroplasts. Beta-barrels are as stable as BSA and soluble almost as BSA (DeGrado W.F., Gratkowski H. and Lear J.D. 2003. How do helix-helix interactions help determine the folds of membrane proteins?).

The problem could be if the foreign molecule is not a protein, then proteolytic digestion can not occur and the T-dependent pathway described above does not operate. An alternate stimulation can still occur if the antigen has a repeating structure, as in these case polysaccharides, however because of the lack of T cell help polysaccharides are poor vaccines that do not produce an anamnestic response. They are T-independent antigens.

By using these properties of the BSA, the capsule can be conjugated to the BSA as a carrier of the antigen. BSA and the capsule are similar in properties which makes BSA as a good carrier protein of the capsule antigen. The capsule as mentioned above coated by a layer of the capsule polysaccharides. This capsule cloaks antigenic proteins on the bacterial surface that usually provokes immune response and thereby opsonization of the bacteria. What also occurs is by linking these outer coats to BSA the immune system can be led to recognize the polysaccharide as if it were protein antigen. The capsular is like the BSA water soluble, and acidic. BSA is a membrane protein that will transport the capsule in the blood across the cell membrane. This is transportation is possible through that BSA transport the capsule against the concentration gradient in to the cell membrane so called facilitated diffusion. Which is a transport from higher concentration to lower concentration across the membrane. Larger molecules diffuse through carrier proteins, in these case BSA, that change shape as the molecules are carried through, for example glucose and amino acids. After binding to the molecule, the protein changes shape and carries the molecule across the membrane, where it is released. The protein then returns to its original shape, to wait for more molecules to transport (Chemistry & Chemical Reactivity, Sixth Edition, Thomson Brooks/Cole, 2006) .

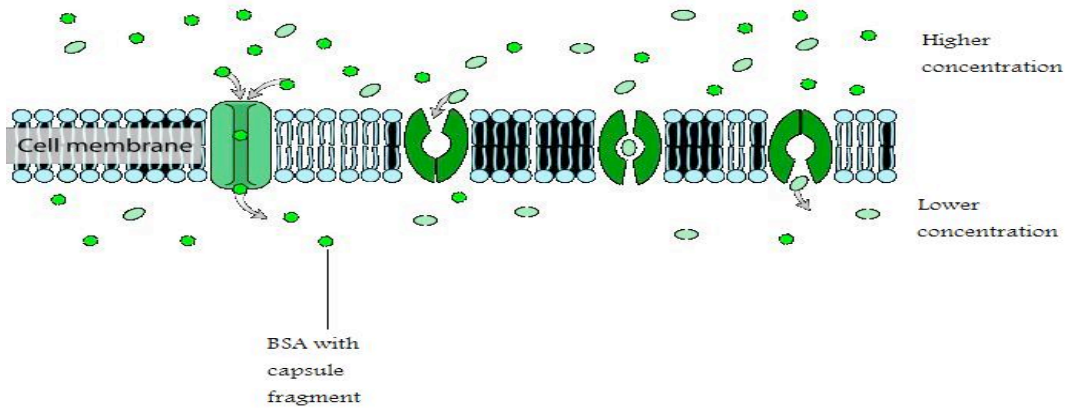


Fig 1: BSA diffusion from a higher concentration to lower concentration.  
[http://en.wikipedia.org/wiki/Image:Simple\\_diffusion\\_in\\_cell\\_membrane.svg](http://en.wikipedia.org/wiki/Image:Simple_diffusion_in_cell_membrane.svg)

The next step after diffusion is Phagocytes of the antigen to represent this for T cells. Ingests a pathogen, the pathogen becomes trapped in a food vacuole, This vacuoles immigrates to the cell surface and are been expressed on the MHC class II.

One way in immunity response is B-cells direct recognizes the antigen on the BSA. Polysaccharide conjugates bind to polysaccharide-specific pre-B cells and processed by so called proteolysis into peptides. When they are inside the cell, the protein part is digested to release T cell epitopes that result in T cell help. The polysaccharide is converted to a T-dependent antigen by the simultaneous presence of the carrier protein. The mechanism is when a B cell ingests a pathogen, it expresses the pathogen's proteins to a class II MHC protein. This is expressed on the cell membrane, which then can be recognized by a T lymphocyte, which is compatible with similar structures on the cell membrane of a B lymphocyte.

To have an optimal working antibody, B cells must be activated by T cell. The first signal comes from antigen cross linking the B cell receptor (BCR) and the second signal comes from co-stimulation provided by a T cell. MHC class II with the antigen presented on present this protein to a special subtype of T cell called a Th2 cell. When a B cell processes and presents the same antigen to the primed Th cell, the T cell secretes cytokines that activate the B cell. These cytokine makes the B cell to proliferate and differentiate into plasma cells. Isotype switching to IgG, and memory cell generation occur in response to T-dependent antigens. This isotype switching is known as Class Switch Recombination (CSR).

BSA is a carrier protein and can penetrate cell membranes and it can also be phagocytosed by macrophages/dendritic cells which also are called antigen presenting cells. When this occurs a second way of immune response is induced. These results in some of the peptides fragment of the capsule on the BSA are expressed on the cell surface on their MHC class II. MHC class II is recognized by the T cells and activation of CD4+ T cells. This occurs through both the T cell receptor and CD28 on the T cell by the MHC peptide and B7 family members on the APC, respectively. Both of this is required for production of an effective immune response. In the absence of CD28 co-stimulation,

T cell receptor signaling alone results in anergy. The first signal must be provided by binding of the T cell receptor to a short peptide presented by the MHC on another cell. This connection makes sure only a T cell with a T cell receptor (TCR), specific to that peptide is activated. In this case it's presented by APCs, B cells, and infected cell. Cells that have penetrated the antigen through diffusion are presenting their peptides to CD4+ cells by MHC class II molecules. The second signal comes from co-stimulation, in which surface receptors on the APC are induced by a relatively small number of stimulation, usually products of pathogens. The only co-stimulatory receptor expressed constitutively by naïve T cells is CD28, so co-stimulation for these cells comes from the CD80 and CD86 proteins on the APC. Without it, the T cell becomes anergic and it becomes more difficult for it to activate in future. Th1 cells produce Interferon-gamma, which activates the bactericidal activities of macrophages, and induces B-cells to make opsonizing (coating) antibodies, and leads to cell-mediated immunity. However Th2 cells produces Interleukin 4, which results in the activation of B-cells to make neutralizing (killing) antibodies, leading to humoral immunity. In this case Th1 cells are more effective against intracellular capsule that has been diffused. Like cytotoxic T-cells, most of the CD4+ helper cells will die upon resolution of infection, with a few remaining as CD4+ memory cells.

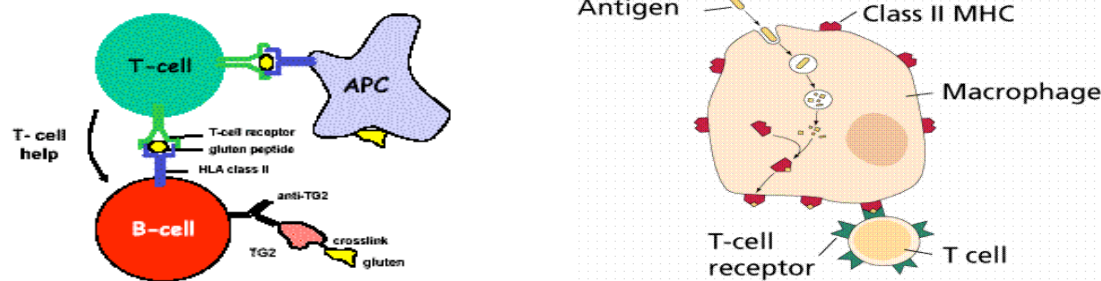


Fig 2: To the left B cell activation by a T cell . To the right antigen presentation by a professional antigen presentation cell to a T cell. (<http://www.cartage.org.lb/en/themes/sciences/lifescience/generalbiology/Physiology/LymphaticSystem/Antibodymediated/tcellrecept.gif>, <http://www.med.uio.no/rh/forskning/tematisk/cevi/ill/project10-1.gif>)

### Assays

Several studies and developments have been done on vaccine based in live live attenuated vaccine for B. anthrax. Most of them have been successful but not fully protective. In this study a single one dose injection of a dual part vaccine will be administered in to the blood stream. This gives optimal an immune response sense we are targeting bacteria once they are in the tissues. To make sure of optimal results antibody levels were measured two weeks after the injection. This is because it takes around two weeks to produce high titers of antibodies. For optimum T cell assay it is better to measure it 8-10 days after injection.

Since PA is primarily released in tissues and serum the most important protective immunoglobulin that will neutralize PA will be IgG. To quantify the amount of anti-PA IgG in the serum of vaccinated individuals, an indirect ELISA will be performed. First, purified PA will be added to a well plate and then washed. Next skim milk will be added to the plates and washed in order to bind all the spots where PA hasn't. Next, serum from

vaccinated individuals will be added and any PA specific IgG will bind PA and any that doesn't will be washed out. Later, rabbit-anti human gamma-globulin covalently linked to an enzyme will be added which will bind the Fc region of anti-PA antibodies bound to PA. After that, a chromogenic substrate will be added that once acted on by the enzyme will change from clear to colorless. Lastly the color can be measured by a spectrophotometer which after calculation will quantify the amount of specific IgG the individual's serum. A negative control must also be done to make sure that it is only PA that is being bound, to accomplish this PA is not included in the well plate; a colorless result confirms that the results were correct. In addition a positive control should be run which to compare the results and make sure the reagents are working properly, this is done by using a serum that is known to have anti-PA antibodies. To measure the number of anti-poly D glutamic acid IgG antibodies the same procedure used above for quantification of anti-PA antibodies will be followed but with anthrax capsule as the antigen instead of PA.

It is important to show that the anti-PA antibodies are capable of neutralizing PA so that it cannot form pores through which LF and EF can gain entry into cells. In order to determine if the antibodies are neutralizing LF+PA will be added to and mixture mouse macrophage cells and serum from immunized individuals in vitro. If the antibodies are not neutralizing, then PA+LF will kill the macrophage cells. However, if anti-PA inhibits PA from forming pores than the cells should survive. It is important to run a positive control with no antibody to make sure that the LF+PA toxin is lethal (Pichichero et al. 2007).

To measure PA specific CD 4 T cells we will utilize a self MHC II-peptide tetramer labeled with a fluoro-chrome and detect it by flow cytometry. The tetramer will be composed of self MHC II and peptides from the D4 domain of PA. Antigen specific Th2 cells from immunized patient serum will recognize the self MHC II-peptide complex and will bind to the tetramer with high enough affinity enabling it to stay attached. Finally, fluoro-chrome tagged MHC II-peptide tetramers bound only to antigen specific Th2 cells will be detected and counted by the flow cytometer's laser, thus quantifying the amount of Th2 in the immunized individual's serum. To detect poly D-glutamic acid specific Th2 cells it will be necessary to repeat the above steps substituting D-glutamic acid peptides for PA peptides. To make sure there is no non-specific binding negative controls should be done using an unrelated peptide. In addition, positive controls using Th2 cell lines known to be peptide specific should be used to ensure the reagents are all working.

One way of examining the macrophage response to the vaccine is having macrophages mixed with a serum of the patient. Putting this sample under a microscope for further observation of phagocytosis by the macrophages of the bacteria by the naked eye.

Criteria for a successful trial would be the presence of opsonizing antibody to the D-glutamic capsule, neutralization of PA by antibody, high antibody titers and high levels of specific Th2 cells. Following the vaccination these will last for three months, to test for any statistically significant adverse reactions.

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