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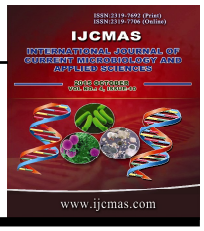

Original Research Article

In-silico Design and Antibody Response to the Peptide Sequences from Protective Antigen and Lethal Factor Toxins of *Bacillus anthracis*

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Keywords

Bacillus anthracis, ELISA, lethal factor, protective antigen, western blotting

A B S T R A C T

Vaccination is a key approach to control and prevent the inhalational form of anthrax. Current FDA approved anthrax vaccine adsorbed (AVA), even though being protective against anthrax, causes adverse immunological side effects and includes a long immunization schedule. Considering the above fact, together with variations in batch to batch production of AVA, there is a need for a stable and safe alternative. Recent studies on anthrax vaccine suggests that neutralizing the toxin molecules during the infection by developing neutralizing antibodies against them is the best way to prevent the disease from establishing. In this regard, an approach for peptide vaccine have been considered by *in-silico* designing of antigenic peptide sequences from the *Bacillus anthracis* toxin molecules viz Protective antigen (PA) and Lethal factor (LF). Twenty six domain specific peptide molecules, fifteen from PA and eleven from LF were designed based on their specificity and surface accessibility. In addition, all the peptides were software-predicted B-cell epitope. The peptides were custom synthesized and immunized in mice individually to evaluate their ability to induce humoral immune response. It was found that, five peptides from PA and two from LF were able to produce specific, high antibody titre against their respective toxin as determined by ELISA and western blotting. These peptide induced antibodies will be further tested for their toxin neutralizing ability and subsequent protection studies against lethal *Bacillus anthracis* challenge. The present study provides a promising platform for the development of an alternative vaccine against anthrax.

Introduction

Bacillus anthracis is the only pathogen known to be highly infectious with potential biothreat in the family Bacillaceae. The organism in its endospore form has the potential to get aerosolized and infect large population resulting in panic and mass causality (Wilkening, 2006). Because of this reason, Center for Disease control and prevention (CDC) has listed *Bacillus anthracis* as category 'A' agent. The major

virulence factor in *Bacillus anthracis* are the exotoxins components comprised of three proteins viz protective antigen (PA), lethal factor (LF) and edema factor (EF). These proteins assemble in two different ways to form lethal toxin (LeTx) and edema toxin (EdTx). Basically anthrax toxin belongs to A-B toxin superfamily where the B moiety binds to cell surface receptor and translocates the A moiety inside the cell (Odumosu *et al.,* 2010). In case of anthrax, PA acts as B moiety and LF/EF acts as A moiety. Once LF/EF is inside the cell they can exert their enzymatic effects leading to anthrax related symptoms (Friedlander *et al*., 1986; Hanna *et al*., 1992; Friedlander *et al*., 1993; Szarowicz *et al*., 2009 and Newman *et al*., 2010).

The disease can be managed with vaccine and antibiotics at pre and post exposure stages respectively (Chitlaru *et al*., 2010). Antibiotics work by killing the circulating bacterium but fail to protect at later stage of infection when toxin molecules are produced in huge amounts (Altboum *et al*., 2002). Anti toxin antibodies and small molecule inhibitors have made their way in recent times to combat anthrax toxin molecule but still under clinical trials (Hu *et al*., 2008 and Malkevich *et al*., 2013). As far as prophylaxis is concerned, vaccination is the only approach which is known to provide protection against the disease. Developed during the early 1970's, Anthrax Vaccine Adsorbed (AVA), the only FDA approved anthrax vaccine for humans is still in use due to its robust protective efficacy in spite of its adverse immunological side effects (Vietri *et al*., 2006 and Weiss *et al*., 2007). Progress in this regard to obtain better vaccine molecule that reduces the immunization schedule and has no immunological side effects has been carried out from past few years. Recombinant subunit vaccine consisting of only PA protein of *Bacillus anthracis* has been under clinical trial and is likely to replace the existing vaccine (Bellanti *et al*., 2011). Since cold chain storage and long lasting memory to the anthrax vaccine molecule is a major area of concern, research is focused towards safe alternatives, like DNA and peptide based vaccine approach. Even though DNA and peptide vaccine overcomes the above said hurdles, most of the times these vaccines fail to elicit sufficient immunological response primarily due to the lack of proper adjuvant formulation and right mode of delivery.

In the recent times considerable progress has been made towards peptide vaccine for anthrax and is showing promising results (Kaur *et al*., 2009 and Ascough *et al*., 2014). Peptide vaccine consisting of B cell epitopes are known to be effective against anthrax as these epitopes enhances humoral antibody response that is sufficient in providing protection against the disease. The availability of protein sequence information and large number of computational methodologies to predict B and T cell epitopes have made it an easy approach for developing therapeutics and vaccine molecules against infectious diseases. *In silico* design of epitope vaccine has been proved to be a promising approach for combating many diseases like malaria, cancer, multiple sclerosis etc (Knutson *et al*., 2001; Lopez *et al*., 2001 and Bourdette *et al*., 2005).

In the present study, we have designed a series of 15mer peptide sequences from *Bacillus anthracis* lethal toxin components PA and LF which are predicted to be antigenic and acts as B cell epitopes as determined by protein sequence information and the web servers. Further, these peptide sequences were immunized to Balb/c mice and the sera was tested by ELISA and

western blotting to determine the immunodominant epitopes that could be used as a potential vaccine molecule against anthrax.

Materials and Methods

***In silico* designing of peptide sequences:** The coding sequences for PA and LF were retrieved from National Centre for Biotechnology Information (NCBI), USA. The accession numbers for PA and LF were P13423 and P15917 respectively. A series of 15 mer peptide sequences were generated that overlapped 14 amino acids and represented the whole protein. A total of 750 and 794 peptide sequences from PA and LF were obtained which were 15 amino acids in length. All the peptide sequences were subjected to protein BLAST to obtain the sequences with 99% specificity to their respective native proteins which reduced the peptide sequences to 111 and 96 for PA and LF respectively. Further the peptides were selected based on their physicochemical properties like surface accessibility, antigenicity and B cell epitope characteristics with the help of web servers Immune Epitope Database (IEDB): www.iedb.org and BCePred: www.imtech.res.in/raghava/bcepred (Saha and Raghava, 2004). The final screening of the peptides was done based on the epitope interactive sites on individual proteins PA and LF (Varughese *et* al., 1999; Cunningham *et* al., 2002; Melnyk *et* al., 2005; Oscherwitz *et* al., 2009 and www.uniprot.org/uniprot/13423). By employing the above approach 15 specific and antigenic peptide epitope sequences were obtained for PA and 11 for LF respectively. Theses peptides were custom synthesized from Biolinkk India Pvt Ltd, India and used for immunization purpose.

Immunization of Balb/c mice: The peptides were dissolved in sterile water to obtain 2mg/ml stock solution. 100 µg of each peptide sequences were immunized to a group of three mice in emulsion with Incomplete Freund's adjuvant three times with an interval of 2 weeks between each schedule. Mice were bled one week after the final immunizations, each group sera was pooled together and stored at -20 ^{o}C until further use.

Anti body response of peptide immunized mice sera:

Indirect-Enzyme linked Immunosorbent Assay: Two 96well plates were coated with 5 µg ml^{-1} of PA and LF respectively in 0.1 M carbonate buffer and kept overnight at 4 ^{o}C. Next day, both the plates were blocked with 5% skim milk at room temperature for 2 hours following which, PA and LF peptide immunized sera at 1:100 dilution was added to PA and LF coated plates respectively. After incubation for one hour at room temperature, plates were washed four times with TBS-T and further incubated with anti mouse IgG-HRP conjugate at 1:10,000 dilution (A4416, Sigma Aldrich Chemicals, Bengaluru, india) at room temperature for one hour. The plates were washed with TBS-T and developed with ortho-phenylenediamine dihydrochloride (OPD) substrate and the reaction was stopped with 2 N H_2SO_4. The absorbance was recorded using UV-Vis microplate reader (BioTek, Winooski, VT) at 495 nm. All the experiments were done in triplicate and the sera from unimmunized mice was taken as negative control.

Western blotting: Western blotting analysis of high antibody titer immunodominant PA and LF peptide sera was performed against their respective native proteins and their domain. Briefly, PA and LF along with their domains were resolved in 10% sodium

dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred on to nitrocellulose membrane using semi dry blotting system (Hoefer TE70X, San Francisco, USA). After blocking with 5% skimmed milk for one hour at room temperature, the membrane was incubated with PA and LF reactive peptides sera. Following washing with PBS-T and PBS, the membranes were further incubated with anti mouse IgG HRP conjugate (A4416, Sigma Aldrich Chemicals, Bengaluru, India) at the dilution of 1:5000 and finally developed with 3,3'-Diaminobenzidine (DAB) substrate (D 4418, Sigma Aldrich Chemicals, Bengaluru, India).

Statistics: Statistical comparison for IgG titer of PA and LF peptide immunized mice sera with control mice was performed. Analysis was done by one way ANOVA using GraphPad Prism demo version 6.0. The *p* value greater than 0.05 was considered statistically significant.

Results and Discussion

Antigenic peptide sequences: *In silico* screening of PA and LF protein sequences for surface accessibility, antigenecity and B cell epitope characteristics was carried out using web servers and Uniprot protein information database along with available literatures. A total of 26 antigenic 15mer peptide epitope sequences, 15 from PA and 11 from LF were designed as listed in Table 1. In specific, peptide sequences PA 1-4 were from PA domain 1 region, PA 5 from PA domain 2 and PA 6-15 from PA domain 4 region. Similarly, peptide sequences LF 1-3 were from LF domain 1while peptide sequences LF 4-11 belongs to LF domain 4 region.

Selection of Immunodominant peptide sequences: All the 26 peptide sequences were immunized to a group of three Balb/c mice and their sera was collected for performing Indirect ELISA. Serum antibodies of five peptide sequences from PA and two from LF were found to be highly reactive to their respective native proteins as evident by high antibody titer in ELISA (Figure 1). The list of immunodominant peptide sequences are given in Table 2. The remaining peptides immunized sera and the sera from control group failed to evoke any immune response to PA and LF.

Western Blotting: Sera from all the seven immunodominant peptide epitope immunized mice, five from PA and two from LF were analyzed by western blotting. Peptide sequence PA 1 sera could react with native PA and PA domain 1 while peptide sequences PA 11 – 14 sera reacted to both full length PA and PA domain 4 (Figure 2). Similarly, peptide sequence LF 1 could react with full length LF and LF domain 1 and LF 4 peptide sera reacted with full length LF (Figure 3).

Recent years has witnessed considerable advance in the field of anthrax research largely due to the aftermath of 2001 US mail attack. With the availability of protein sequence information and computer aided vaccine designing platform, development of epitope based peptide vaccine has become easy in terms of time and cost. Although present vaccine AVA is known to confer protection against anthrax, its long immunization schedule, undefined composition and immunological side effects have made researchers to look for safer alternative. Peptide vaccines are one such approach which is being investigated in this regard.

The objective of this study was to

investigate the immunogenic proteins PA and LF of *Bacillus anthracis* and find out the immunodominant B cell epitopes that could serve as peptide vaccine. For this purpose, we first screened the entire length of both PA and LF proteins to obtain a series of 15mer peptide sequences which were specific, antigenic, surface accessible and B cell epitope using web servers and protein data base. A total of 26 peptide sequences were generated and custom synthesized. On immunization of these peptides to Balb/c mice, only seven peptides were able to elicit significant level of antibody response against their respective native proteins as determined by ELISA.

Table.1 Peptide sequences finalized from PA and LF after in silico screening and analysis

Peptide sequences from PA	Peptide sequences from LF
PA 1 - SAGPTVPDRDNDGIP	LF 1 - PVLVIQSSEDYVENT
PA 2 - GIPDSLEVEGYTVDV	LF 2 - ALNVYYEIGKILSRD
PA 3 - SLEVEGYTVDVKNKR	LF 3 - IKNASDSDGQDLLFT
PA 4 - VDVKNKRTFLSPWIS	LF 4 - RNDSEGFIHEFGHAV
PA 5 - GNAEVHASFFDIGGS	LF 5 - EGFIHEFGHAVDDYA
PA 6 - ESVVKEAHREVINSS	LF 6 - GFIHEFGHAVDDYAG
PA 7 - AHREVINSSTEGLLL	LF 7 - LTSYGRTNEAEFFAE
PA 8 - NSSTEGLLLNIDKDI	LF 8 - NEAEFFAEAFRLMHS
PA 9 - SSTEGLLLNIDKDIR	LF 9 - EAEFFAEAFRLMHST
PA 10 - KEVINDRYDMLNISS	LF 10 - EFFAEAFRLMHSTDH
PA 11 - QDGKTFIDFKKYNDK	LF 11 - NAPKTFQFINDQIKF
PA 12 - GKTFIDFKKYNDKLP	
PA 13 - KTFIDFKKYNDKLPL	
PA 14 - FKKYNDKLPLYISNP	
PA 15 - AVTEKNTIINPSENG	

Table.2 Immunodominant peptide sequences from PA and LF reactive to their native proteins determined by Indirect ELISA

Immune dominant PA peptide sequences	Immune dominant LF peptide sequences
PA 4 - VDVKNKRTFLSPWIS	LF 1 - PVLVIQSSEDYVENT
PA 11 - QDGKTFIDFKKYNDK	LF 4 - RNDSEGFIHEFGHAV
PA 12 - GKTFIDFKKYNDKLP	
PA 13 - KTFIDFKKYNDKLPL	
PA 14 - FKKYNDKLPLYISNP	

Figure.1 Serum IgG titer of PA and LF peptide sequences immunized mice sera as determined by Indirect ELISA. (A) PA protein reactive peptides sera. (B) LF protein reactive peptide sera. Data represented in triplicate of pooled sera and analyzed by One way ANOVA using GraphPad Prism Demo version 6.0. $p > 0.05$ was considered statistically significant

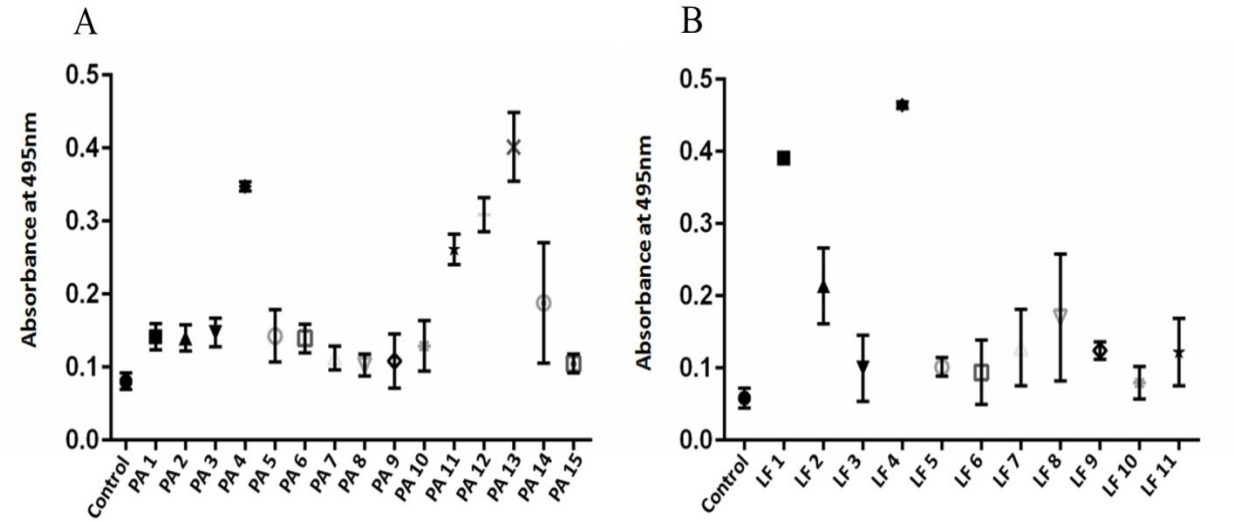


Figure.2 Western blotting of PA peptide sera with native PA and its domains. PA and its domains PA-D1 and PA-D4 were resolved in 10%SDS PAGE and transferred to nitrocellulose membrane. (A) PA 4 peptide sera reacting to PA and PA-D1. (B – E). PA and PA-D4 proteins reaction with peptide sequences PA 11, 12, 13 and 14 respectively. Sera reactive protein bands are indicated by black arrows

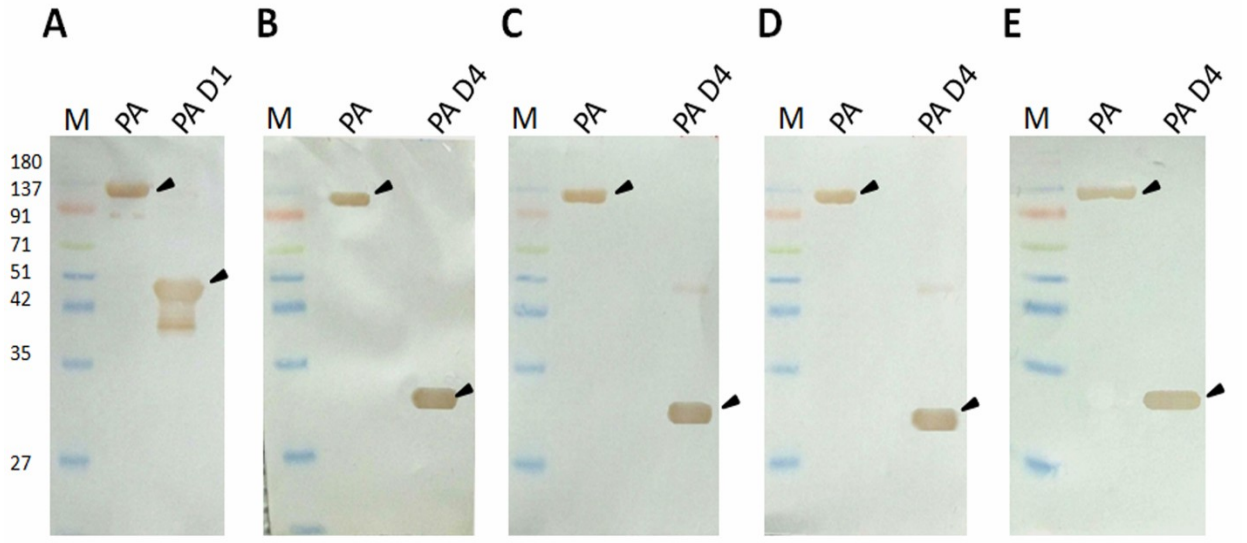
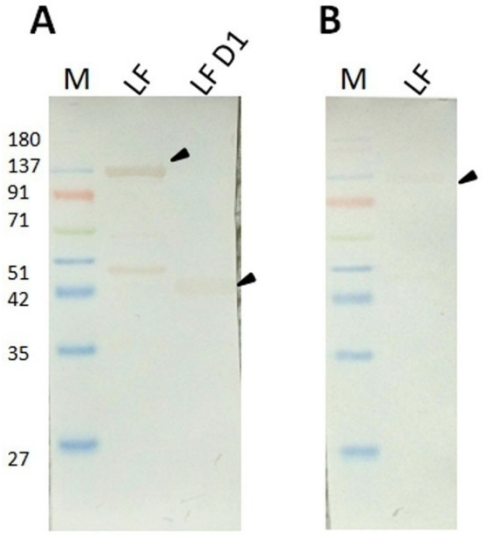


Figure.3 Western blotting of LF peptide sera with native LF and its domains. LF and its domains LF-D1 was resolved in 10% SDS PAGE and transferred into nitrocellulose membrane. (A) LF 1 peptide sera reacting to LF and LF-D1. (B) LF 4 peptide sera reaction with full length LF. Sera reactive protein bands are indicated by black arrows



Five out of seven epitope sequences were from PA while two from LF. The four sequences of PA were from domain 4 spanning the amino acid residues 698 to 721 in native protein. These are the regions of PA which are known to bind the cell surface receptors during intoxication (Varughese *et al*., 1999). The fifth immunodominant epitope sequence of PA was found to be the region from PA domain 1 adjacent to furin cleavage site. The two epitope sequences of LF were found to be from LF domain 1 and 4 which corresponds to PA binding site and catalytic site respectively. Western blotting analysis of these peptide immunized sera confirmed the fact that each sera could specifically bind to its respective domain along with their full length proteins.

The peptide sequences in the present study were finalized based on careful sequence analysis, consistent output data from two well known web servers and *in vitro* analysis of immunized peptide sera. Hence these epitope sequences were found to be immunodominant and can be a good candidate for peptide vaccine that can trigger an effective immune response against anthrax *in vivo*. Further advancement in this regard can be achieved by suitable adjuvant formulation and mode of delivery to get better and sustained immunological response. In addition, the antibodies derived by immunizing the peptide sequences PA 1, 11,12,13,14 and LF 1 and 4 could recognize their respective full length proteins in domain specific manner. This feature can be very well exploited in developing antigen based diagnostic tools. Finally to conclude, the immunodominant peptide sequences identified in this study can be used both as vaccine and diagnostic molecules against anthrax.

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Reference

Altboum, Z., Gozes, Y., Barnea, A., Pass, A., White, M., Kobiler, D. 2002. Postexposure prophylaxis against anthrax: Evaluation of various treatment regimens in intranasally infected guinea pigs. *Infect Immun* . 70: 6231-6241

Ascough, S., Ingram, R. J., Chu, K, K., Reynolds, C. J., Musson, J. A., Doganay, M., Metan, G., Ozkul, Y., Baillie, L., Sriskandan, S., Moore, S. J., Gallagher, T. B., Dyson, H., Williamson, E. D., Robinson, J. H., Maillere, B., Boyton, R. J., Altman, D. M. 2014. Anthrax lethal factor as immune target in humans and transgenic mice and the impact of HLA polymorphism on CD4+ T cell immunity. *PLoS Pathog* 10(5): e1004085. doi:10.1371/journal.ppat.1004085

Bellanti, J. A., Lin, F. Y. C., Chu, C., Shiloach, J., Leppla, S. H., Benavides, G. A., Karpas, A., Moayeri, M., Guo, C., Robbins, J. B., Schneerson, R. 2011. Phase I study of recombinant mutant protective antigen of *Bacillus anthracis*. *Clin Vaccine Immunol*. 19: 140-145

Bourdette, D. N., Edmonds, E., Smith, C., Bowen, J. D., Guttmann, C. R., Nagy, Z. P., Simon, J., Whitham, R., Lovera, J., Yadav, V., Mass, M., Spencer, L., Culberstone, N., Bartholomew, R. M., Theofan, G., Milano, J., Offner, H., Vandenbark, A. A. 2005. A highly immunogenic trivalent T cell receptor peptide vaccine for multiple sclerosis. *Mult Scler*, 11: 552–561.

Chitlaru, T., Altboum, Z., Reuveny, S., Shafferman, A. 2010. Progress and novel strategies in vaccine development and treatment of anthrax. *Immunol Rev*. 239: 221-236

Cunningham, K., Lacy, D. B., Mogridge, J., Collier, R. J. 2002. Mapping the lethal factor and edema factor binding sites on oligomeric anthrax protective antigen. *Proc Natl Acad Sci*. 99: 7049-7053

Friedlander, A. 1986. Macrophages are sensitive to anthrax lethal toxin through an acid- dependent process. *J Biol Chem.* 261: 7123–7126.

Hanna, P.C., Kochi, S., Collier, R.J. 1992. Biochemical and physiological changes induced by anthrax lethal toxin in J774 macrophage-like cells. *Mol Biol Cell.* 3: 1269–1277.

Hu, M., Li, J., Yao, S.Q .2008. In situ Click assembly of small molecule matrix metalloprotease inhibitor containing Zinc chelating groups. *Org Letters* . 10: 5529-5531

Kaur, M., Chug, H., Singh, H., Chandra, S., Mishra, M., Sharma, M., Bhatnagar, R. 2009. Identification and characterization of immunodominant B cell epitope of the C terminus of protective antigen of *Bacillus anthracis*. *Mol Immunol*. 46: 2107-2115

Knutson, K. L., Schiffman, K., Disis, M. L. 2001. Immunization with a HER-2/neu helper peptide vaccine generates HER-2/neu CD8 T-cell immunity in cancer patients. *J Clin Invest.*107: 477–484.

Lopez, J. A.,Eilenman, C., Audran, R., Roggero, M. A., Bonelo, A., Tiercy, J. M., Spertini, F., Corradin, G. 2001. A synthetic malaria vaccine elicits a potent CD8(+) and CD4(+) T lymphocyte immune response in humans. Implications for vaccine strategies. *Eur J Immunol.* 31: 1989-1998

Malkevich. N. V., Basu, S., Rudge, T. L., Clement, K. H., Chakrabarti, A. C., Aimes, R. T., Nabros, G. S., Skiadopoulos, M. H, Ionin, B. 2013.

Effect of anthrax immune globulin on response to biothrax (Anthrax vaccine adsorbed) in Newzeland white rabbits. *Antimicrob Agents Chemother*. 57: 5693-5696

Melnykk, R. A., Hewitt, K. M., Lacy, D. B., Lin, H. C., Gessner, C. R., Li, S., Woods, V. L., Collier, R. J. 2005. Structural determinants for the binding of anthrax lethal factor to oligomeric protective antigen. *J Biol Chem.* 281: 1630-1635

Newman, Z. L., Crown, D., Leppla, S. H., Moayeri, M. 2010. Anthrax lethal toxin activates the inflammasome in sensitive rat macrophages. *Biochem Biophys Res Commun.* 398: 785-789.

Odumosu, O., Nicholas, D., Yano, H., Langridge, W. 2010. AB toxins: A paradigm switch from deadly to desirable. *Toxins*. 2: 1612–1645. doi:10.3390/toxins2071612Friedlande r, A. M., Bhatnagar, R., Leppla, S.H., Johnson, L., Singh, Y. 1993. Characterization of macrophage sensitivity and resistance to anthrax lethal toxin. *Infect Immun*. 61: 245–252.

Oscherwitz, J., Yu, F., Jacobs, J. L., Liu, T. H., Johnson, P. R., Cease, K. B. 2009. Recombinant vaccine displaying the loop-neutralizing determinant from protective antigen completely protects rabbits from experimental inhalation anthrax. *Infect. Immun*. 77: 3380-3388

Saha.S and Raghava G.P.S. 2004. BcePred:Prediction of Continuous B-Cell Epitopes in Antigenic Sequences Using Physico-chemical Properties. In G.Nicosia, V.Cutello, P.J. Bentley and J.Timis (Eds.) *ICARIS* 2004, LNCS 3239, 197-204, Springer.

Szarowicz, S.E., During, R.L., Li, W., Quinn, C.P., Tang, W.J., Southwick, F.S. 2009. *Bacillus anthracis* edema toxin impairs neutrophil actin-based motility. *Infect Immun*. 77: 2455–2464.

Varughese, M., teixeira, A. V., Liu, S., Leppla, S. H. 1999. Identification of a receptor binding region within domain 4 of protective antigen component of anthrax toxin. *Infect Immun.* 67: 1860-1865

Vietri, N. J., Purcell, B. K., Lawler, J. V., Leffel, E. K., Rico, P., Gamble ,C. S., Twenhafel, N. A., Ivins, B. E., Heine, H. S., Sheeler, R., Wright, M. E., Friedlander, A. M. 2006. Short-course postexposure antibiotic prophylaxis combined with vaccination protects against experimental inhalational anthrax. *Proc Natl Acad Sci*. 103: 7813–7816.

Weiss, M. M., Weiss, P. D., Weiss, J. B. 2007. Anthrax vaccine and public health policy. *Am J Public Health*. 97: 1945–1951

Wilkening, D.A. 2006. Sverdlovsk revisited: Modeling human inhalational anthrax. *Proc Natl Acad Sci*. 103: 7589-7594