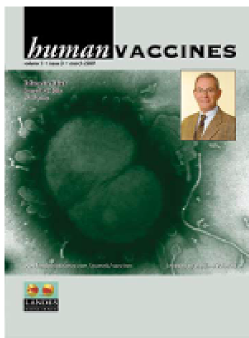

Human Vaccines

ISSN: 1554-8600 (Print) 1554-8619 (Online) Journal homepage: http://www.tandfonline.com/loi/khvi19

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To cite this article: Diane R. Bienek, Lawrence J. Loomis & Raymond E. Biagini (2009) The anthrax vaccine: No new tricks for an old dog, Human Vaccines, 5:3, 184-189, DOI: 10.4161/hv.5.3.7308

To link to this article: http://dx.doi.org/10.4161/hv.5.3.7308

Published online: 01 Mar 2009.

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[Human Vaccines 5:3, 184-189; March 2009]; ©2009 Landes Bioscience

Commentary

The anthrax vaccine

No new tricks for an old dog

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Key words: anthrax, vaccine, immunity, protective antigen, antibody

The original license for production of the anthrax vaccine, Anthrax Vaccine Adsorbed (AVA), was issued in 1970. Since that time, over 8 million AVA immunizations have been administered to 2+ million individuals. In 2002, the National Academy of Sciences, Institute of Medicine, reviewed the safety and efficacy of AVA. They concluded that the vaccine is acceptably safe and effective in protecting humans against anthrax. The vaccine should protect people against all known strains of anthrax bacteria, as well as against any strains that might be created by potential terrorists or others. Although the Institute of Medicine concluded that AVA was reasonably safe, they noted that it is fairly common for people to experience local reactions (e.g., redness and swelling at the injection site) and for a smaller number to experience systemic reactions such as fever and malaise, within hours or days of vaccination. Results of animal studies done previously and subsequent to this report are generally in agreement. For instance, AVA vaccination increases the level of anthrax anti-protective antigen IgG (anti-PA IgG), which is thought to be one possible correlate of protection (although absolute protective concentrations have not been identified in humans). Anthrax lethal factor neutralization has also been identified as possibly being an important additional correlate of immunity. Future vaccine research efforts include developing a recombinant anthrax vaccine and anthrax monoclonal antibodies to block the anthrax toxin(s). It is projected that the next-generation vaccine will elicit a markedly increased anti-anthrax immune response within a shorter time period and consequently, will enable the easier inoculations of individuals working within high-risk areas.

Anthrax Bacteria Characterization

The Gram-positive, rod-shaped bacterium *Bacillus anthracis* is the causative agent of anthrax. There are three manifestations of anthrax infection in humans: (1) cutaneous anthrax resulting from dermal penetration of the bacteria through a cut or abrasion in the skin; (2) gastrointestinal anthrax resulting from ingestion of contaminated meat products; and (3) pulmonary anthrax resulting from the inhalation of anthrax spores, the most lethal form. The prescribed medical treatment for all forms of the disease is high doses of antibiotics, most commonly penicillin and ciprofloxacin hydrochloride.[1] However, bacterial resistance to third-generation cephalosporins has been documented; further thwarting attempts to quickly treat this infection.[2] Moreover, antibiotics are effective against the vegetative form of *B. anthracis*, but are not effective against the spore form of the organism. Although antibiotics are available to treat anthrax infection, their effectiveness is limited, in part due to delays from the time of exposure to the initiation of treatment.

The principal virulence factors of *B. anthracis* are encoded on two plasmids, one involved in the synthesis of the polyglutamyl capsule that inhibits phagocytosis of vegetative forms and the other bearing the genes for the synthesis of the exotoxins it secretes.[3] The exotoxins are binary, composed of a B (binding) protein that is necessary for entry into the host cell and an A (enzymatically active) protein. The B component is known as the protective antigen (PA) and is common to both toxins. The A component of the edema toxin is the edema factor (EF), a calmodulin-dependent adenylate cyclase that is responsible for the prominent edema at sites of infection, the inhibition of neutrophil function, and the hindrance of the production by monocytes of tumor necrosis factor and interleukin-6.[4] The A component of the second toxin, lethal toxin, is lethal factor (LF), a zinc metalloprotease that inactivates members of the mitogen-activated protein kinase family, leading to the inhibition of intracellular signaling. Lethal toxin stimulates the release by macrophages of tumor necrosis factor-α and interleukin-1β, a mechanism that appears to contribute to sudden death.[5] The current Food and Drug Administration (FDA)-cleared anthrax vaccine, Anthrax Vaccine Adsorbed (AVA), contains various amounts of PA, LF and EF depending on the lot preparation.[6] However, it is unclear if LF or EF antibodies contribute to protection in humans.

Anthrax Vaccine Adsorbed (AVA)

In 1970, the license for production of the current anthrax vaccine was issued to the Michigan Department of Public Health by the Division of Biologics of the National Institutes of Health. The production plant, production line and intellectual property were sold to a private company,[7] Emergent BioSolutions, Inc.,

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Submitted: 09/09/08; Accepted: 11/02/08

Previously published online as a *Human Vaccines* E-publication:
http://www.landesbioscience.com/journals/vaccines/article/7308

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(Rockville, MD), formerly BioPort Corp., Lansing, MI. The AVA (Biothrax®) that is presently licensed for human use in the US, is a cell-free filtrate of microaerophilic cultures of an avirulent, nonencapsulated strain of *B. anthracis.*[8] The 83 kDa PA protein is the principal immunogen of the current vaccine.[7] The FDA-cleared vaccination regimen consists of six subcutaneous injections, with the first three doses administered at two-week intervals, and dose four to six given at six-month intervals. Thereafter, boosters are administered annually.[8]

Anthrax Vaccine Immunization Program (AVIP)

The Department of Defense (DoD) Immunization Program for Biological Warfare Defense (DoD Directive 6205.3) specifies that personnel assigned or scheduled for deployment to a high-threat area should be immunized against validated biological warfare threat agents for which suitable vaccines are available in sufficient time to develop immunity before deployment to high-threat areas. To counter the growing threat to military personnel deployed overseas, the Secretary of Defense ordered the AVIP in December 1997. Under this guidance, all military members would receive the anthrax vaccine. In early 2000, most of the AVIP was suspended because of the short supply of available vaccine, but was resumed in 2002. In response to a FDA Citizen Petition filed by military members, US courts have since evaluated the legality of mandatory administration of the anthrax vaccine. In December 2003, the US District Court in Washington issued a preliminary injunction on the mandatory administration of AVA to military personnel. For about eight days in October, the AVIP program was resumed, but then the injunction was re-instated. The basis of the injunction was that the AVA was not proven to work against inhalational anthrax. Early in 2005, the injunction was modified to allow issuance of an Emergency Use Authorization for the administration of AVA. Under the Emergency Use Authorization, individuals who were deemed by DoD to be at heightened risk of exposure due to attack with anthrax could receive the vaccination series; however, the AVIP was revised to give personnel the option to refuse. On October 16, 2006, DoD announced its intentions to resume vaccinations for select personnel, but the vaccinations remained voluntary. By February 2007, the military resumed mandatory vaccinations of certain troops. As part of that implementation, each individual continued the vaccination series where they left off. Given this, administration of the next AVA dose may have been delayed up to 24 months. To date, over 8 million AVA immunizations have been administered to over 2 million troops.

Vaccine Effectiveness

Presently, there is little direct scientific evidence that humans vaccinated with AVA are protected against anthrax. The only human study that addresses vaccine effectiveness was an epidemiological evaluation of vaccinated and unvaccinated employees in four textile mills in the 1950's and the incidence of anthrax among these at-risk workers.[9] The anthrax vaccine in use at the time predated the AVA, which was reformulated for human use around 1960. More documentation exists for vaccine protection against cutaneous anthrax, the most common form of the disease,[10] with very little direct proof of effectiveness against the inhalation form of infection.

Indirect evidence of vaccine effectiveness has been gathered in several studies that measured human immunoglobulin G (IgG) antibody titer in recipients of the US-licensed anthrax vaccine, given at the FDA-licensed intervals. The blood anti-anthrax PA IgG level is considered to be an effective indicator to assess vaccine efficacy.[11] By the indirect hemagglutinin assay, 83% of 190 vaccinees seroconverted (≥1:8 anti-PA titer) 2 weeks after administration of the third dose. Other data indicated that 2 weeks after an annual booster immunization, all 85 vaccinees had seroconverted.[12] A report which compared a direct enzyme-linked immunosorbent assay (ELISA) with a capture ELISA for the ability to determine the anti-PA antibody titers in sera samples from 32 anthrax vaccinees indicated that specific antibody concentrations increased after administration of each AVA dose. Examination of data suggest that the highest antibody titers were achieved on days 364 and 475 (administration of dose five and six, respectively).[13] Pittman et al. (2006) characterized the antibody profile during and after the six-dose primary vaccination series in 86 human volunteers. After the second and third AVA dose, 93% and 100%, respectively of the recipients developed IgG antibodies to *B. anthracis*. Peak anti-PA antibody concentrations were observed after the fourth injection.[14]

Other indirect evidence of vaccine effectiveness has been provided in studies where many of the subjects had not completed the entire AVA series. Lininger et al. (2007) reported that the mean antibody concentration increased in an approximate linear dose-response manner ($r = 0.73$, $p \leq 0.001$) as a result of increasing numbers of vaccinations. Additionally, these authors reported a marked difference between anti-PA concentrations at three and four AVA inoculations and an apparent threshold distinguishing individual responses to the primary one to three vaccinations from those receiving four to six doses.[15] Sera obtained from vaccinated military personnel demonstrated that the anti-PA levels varied considerably. For instance, individuals receiving five doses had an anti-PA antibody index (n-fold over cutoff value) between 1.2 and 3.0.[16] A significant positive correlation ($r = 0.73$, $p < 0.0001$) between the PA-specific antibody response observed in serum and saliva has been reported. When compared to the unvaccinated population, a seven- and ten-fold increase was observed in the mean salivary antibody response of individuals that received three and four to six vaccinations, respectively.[17] Accordingly, whole saliva may have the potential of being an alternative to serum for antibody testing.

Reports pertaining to the duration of immunity after AVA administration appear to be discordant. Anti-PA IgG serum concentrations are present at low levels (5.9 ± 6.43 μg/ml) for at least ~30 months after one to two AVA injections[15] and appear to peak after the fourth AVA injection.[14,15] There is also evidence that there is a reduction in anthrax immunity over time without yearly AVA boosters.[18] This is supported by a report by the National Academy of Sciences, Institute of Medicine indicating either non-existent or low levels of anti-*B. anthracis* IgG antibodies two years after initial vaccinations with AVA.[7] Other investigators have shown that the half-life in humans of anti-PA IgG after AVA vaccination is ~64 days.[19]

Adverse Reactions

In 2002, an Institute of Medicine review reported that AVA was reasonably safe although they indicated it is fairly common for people to experience local reactions (e.g., redness and swelling at the injection site), and for a smaller number to experience systemic reactions such as fever and malaise, within hours or days

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of vaccination. The Institute of Medicine noted a gender difference (female predominance) in the occurrence of injection site reactions, although the etiology of this finding was unknown.[7] In a recent report of AVA vaccinated women, obesity was associated with arm soreness and decreased pre-vaccination serum progesterone levels were associated with increased rate of arm swelling.[20]

Assays to Assess Antibody Response

In response to the anthrax terrorist attacks of 2001, the Centers for Disease Control and Prevention undertook accelerated development of a quantitative ELISA for detection of anti-PA specific IgG in human serum and the development of a competitive inhibition assay to enhance diagnostic specificity.[21] This assay was shown to have a diagnostic sensitivity of 97.8%, and a diagnostic specificity of 97.6%. Pre-adsorption of sera with PA enhanced the diagnostic specificity to 100%.

A potential limitation of ELISA is that it is a monoplex technology. Only one analyte can be measured per assay and measurement of numerous analytes necessitates either simultaneous or sequential assays. When the number of analytes becomes large, resource and manpower limitations can occur. An alternative to the ELISA is an assay that can multiplex analytes (i.e., measure numerous analytes simultaneously). Fluorescent covalent microsphere immunoassay (FCMIA) is a technology that can accomplish this using uniquely dual-stained microspheres for the measurement of up to 100 analytes simultaneously.[22] Biagini et al.[18] described a newly developed FCMIA and subsequently compared it to a specific, sensitive and quantitative ELISA for anti-PA IgG and also presented multiplexed data for measuring anti-PA and anti-LF IgG in serum.[23] The FCMIA minimum detectable concentration (MDC) was 0.006 μg/ml of anti-PA IgG, the reliable detection limit was 0.016 μg/ml anti-PA IgG, and the whole-serum equivalent MDC was 1.5 μg/ml anti-PA IgG. The dynamic range was 0.006 to 6.8 μg/ml. Evaluation of the association of anti-PA IgG concentrations measured by FCMIA and ELISA (20 sera) yielded a highly significant correlation ($r^2 = 0.852$, $p < 0.001$). The MDC for anti-LF IgG was 0.001 μg/ml while the RDL was 0.003 μg/ml anti-LF IgG. Benefits of the anti-PA IgG FCMIA over ELISA include greater analytical sensitivity, greater dynamic range (less serum dilutions are necessary to gain a result), long term stability of reagents, and overall assay time. This technology has also been used to measure serum antibody responses to numerous bioterrorism agents simultaneously.[24]

Quantification of anthrax lethal toxin (LT) neutralization activity (TNA) is pivotal in assessing protective antibody responses to anthrax vaccines and for evaluation of immunotherapies for anthrax.[25] A range of LT-sensitive cell types have been used in these assays, most notably the murine monocyte/macrophage J774A.1 and RAW264 cell lines. The reporter systems have included monitoring release of lactate dehydrogenase and use of colorimetric reporters, most commonly the tetrazolium salt, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide or alamarBlue.[25] Using sera from anthrax vaccinated donors, TNA and ELISA results have been shown to be highly correlated ($r = 0.870$, $p < 0.0001$).[19]

Anti-PA antibody levels are not routinely measured either after the AVA injection series or after booster injections. One possible explanation for this is that anti-PA IgG measurements are laboratory-based, necessitating specific equipment and trained personnel.[21,23] Unfortunately, these tests are impractical in field settings. With advancing technology, the development and commercialization of screening tests is moving toward rapid point-of-care assays. Lateral flow assays are semi-quantitative colorimetric tests that are well-established in the public sector (e.g., to test for pregnancy, drugs of abuse and HIV). To detect anti-PA antibodies in serum and whole blood, Biagini et al. (2006) developed a lateral flow blood-based assay.[26] In that study, samples from 18 vaccinated individuals with relatively high concentrations (52 to 340 μg/ml) of PA-specific IgG were tested. With these samples, the diagnostic sensitivity and specificity of the lateral flow assay were 100%, using ELISA-measured anti-PA as the standard. Additionally, a human anti-anthrax vaccine serum standard was used, which determined the visual detection limit of the lateral flow assay to be 2.8 μg/ml of PA-specific IgG. A subsequent study evaluated this lateral flow assay with samples from a cohort with a broad range of anthrax-specific antibody concentrations [≤ the ELISA minimum detection concentration (MDC; 3 μg/ml PA-specific IgG) and MDC to 708 μg/ml]. The anthrax lateral flow assay had a 92% diagnostic sensitivity and 98% diagnostic specificity at a maximal diagnostic efficient cutoff (based on ELISA anti-PA IgG) of 11 μg/ml. At this concentration, the receiver operating characteristic analysis yielded an area under the curve of 0.988, suggesting the lateral flow assay is an extremely accurate diagnostic test.[27]

With this rapid point-of-care test it would be easy to screen vaccinated individuals to ensure that antibody levels of deferred vaccinees have achieved a level of PA-specific IgG that is comparable to recipients of an uninterrupted AVA regimen. Nonetheless, this test cannot presently determine if a protective level of antibodies has been attained in the vaccinees, as a quantitative correlation needs to be established between the level of antibody response in animals and its corresponding protective value compared to the antibody response that occurs in humans.[7]

Animal Models

To help define the correlate of immune protection in humans, there are extensive data in the literature describing anti-AVA antibody titer and conferred protection in several animal models challenged with various *B. anthracis* strains. Guinea pigs have commonly been used as a model to evaluate vaccine effectiveness. Results of studies conducted with this model show that AVA gives variable protection when the animals are challenged intramuscularly with anthrax spores. In these studies, 0% to 100% of the immunized animals survived challenge with various spore strains.[28-32] Also, AVA did not provide good protection in the guinea pig, with only 26 and 20% of the animals surviving the first and second aerosol spore challenge, respectively.[33] It was proposed that this animal model may respond poorly to the AVA vaccine itself.[28]

In studies examining survival rates of immunized guinea pigs challenged intramuscularly with the Ames spore strain, anti-PA antibody titers were also measured and found to be an accurate predictor of survival in one study,[34] but not an accurate predictor in other studies.[29-32] The latter studies found no significant correlation between anti-PA antibody titer and survival using human vaccines isolated from culture filtrates. However, it should also be noted in studies conducted before 1990, the human vaccine used was not optimized for PA expression. The type of adjuvant used also influenced the protective effect in experimental vaccines and affected the amount of anti-PA produced against the vaccine.[35]

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A study using rabbits supports the conclusion that antibody levels to PA after AVA immunization predicted protection in animals receiving an aerosolized Ames spore challenge.[36] A protective effect of 90% or greater was also realized in AVA-immunized rabbits challenged by aerosol with spores from six *B. anthracis* isolates that are highly virulent.[28]

It is the work conducted with non-human primates that is generally accepted to most closely predict the protection afforded humans against anthrax spore inhalation. The applicability of this model is based on data demonstrating the similarities in pathogenesis, clinical course and tissue pathology.[1] The FDA has articulated this to be one of the preferred animal models necessary to meet what is commonly called the "two animal rule." To better define immune correlates, a Rhesus macaque non-human primate inhalational anthrax model was inoculated with AVA and subsequently challenged with *B. anthracis* aerosol (200 to 400 LD50 equivalents).[37] When compared to the placebo group (16% death), animal groups inoculated with vaccine dilutions of 1:1, 1:5, 1:10, 1:20 and 1:40 exhibited 2%, 0%, 9%, 10% and 7% death, respectively. These vaccine dilutions were highly correlated with survival (Chi-square, $p < 0.0001$). Moreover, vaccine dilution was highly correlated with anti-PA IgG antibody concentration at week 30 ($r = 0.88$). In another study, all Rhesus macaques vaccinated with AVA survived a subsequent aerosol challenge of lethal doses of Ames strain spores, while all control animals perished.[38] One hundred percent and 80% protection against an aerosol spore challenge was observed in AVA-inoculated Rhesus macaques, which were challenged by aerosol with spores of either *B. anthracis* isolates Namibia or Turkey, respectively.[28]

The variability of survival outcomes in animal models may be a direct effect of the differing virulence of the challenge strains and the types of vaccines used. Therefore, the results are inconclusive because vaccine efficacy in one animal model cannot be compared to the protection afforded other animals immunized with the same vaccines or challenged with the same anthrax strains. This also demonstrates the inherent difficulty in extrapolating results of anthrax vaccine protection observed in animals to that in humans. In the absence of any controlled human trials of vaccine effectiveness, the best animal model is the non-human primate. Clearly, additional work needs to be conducted with this model.

Although some time has passed since the Institute of Medicine Committee assessed the safety and efficacy of the anthrax vaccine,[7] it seems that their recommendations have still to be entirely fulfilled. Specifically,

(1) Additional active protection studies should be conducted or supported to develop data that describe the relationship between immunity and both specific and functional quantitative antibody levels, including studies of:

- The relationship between the vaccine dose and the resulting level of antibody in the blood of test animals that protects the animals from challenge;
- The relationship between the level of antibody that protects animals from challenge and the level of antibody present in humans vaccinated by the regimen currently recommended for the licensed product; and
- The vaccine dose that results in a level of antibody in the blood of human volunteers similar to that in the blood of protected animals.

(2) DoD should support efforts to standardize an assay for quantitation of antibody levels that can be used across laboratories carrying out research on anthrax vaccines.

It may not be possible to fully achieve these objectives and unequivocally define the level of antibody that is protective against challenge, inasmuch as a variable immune response is elicited in the human population. As the correlates of protection against *B. anthracis* continue to be defined, it may be useful to develop multiplexed assays, such that a single sample could be simultaneously screened for anti-PA, anti-EF and anti-LF antibodies. There may also be merit in evaluating antibody classes and subclasses, which are associated with the anthrax-specific response.

Evolution of Vaccine Regimen and Vaccine Candidates

With the advancement of technology and continued assessment of AVA recipients, it is likely that the vaccine regimen will change. A recent human clinical trial indicated that the number of priming doses may be reduced by omitting the week-two dose.[39] In addition to reducing the prevalence of adverse events at the injection site, this study also demonstrated that a reduced dose schedule and intramuscular administration elicited a serum response at month 7 that was similar to regimens containing four doses of AVA, administered subcutaneously or intramuscularly. Nonetheless, at an earlier time point (week 8), the anti-PA response appears to be affected by gender, age and ethnicity. With regard to the overall anti-PA response, these data are supported by studies performed at the US Army Medical Research Institute of Infectious Diseases, which showed seroconversion in volunteers administered AVA in alternate dosing regimens[40] and in Gulf War veterans (1991) tested 18 to 24 months following vaccination with one, two and three AVA doses.[41] Moreover, altering the time intervals between the doses can enhance the PA-specific IgG response. In this study, sera of AVA-vaccinated at-risk laboratory workers and individuals receiving the second vaccination at 2, 3 and 4-week intervals after the initial dose showed positive titers in 46, 78 and 100% of the individuals tested, respectively.[42]

The route of administration is another likely change to the present regimen of the currently licensed vaccine. A recent human clinical trial demonstrated merit in changing the regimen from subcutaneous to intramuscular.[39] In the future, it is possible that vaccination will occur via intranasal inoculations, as recent publications demonstrated that mucosal anthrax immunization induces antibody production in both the systemic and secretory-excretory compartments (i.e., saliva, vaginal fluid, respiratory lavages or fecal extracts).[43-49]

In 2002, the National Institute of Allergy and Infectious Diseases emphasized the need for the continued development of anthrax vaccine candidates. Ideally, the new candidates should: (1) confer protection against inhalational anthrax; (2) be administered within three or fewer doses; (3) have shorter administration time; and (4) offer increased safety. For new anthrax vaccine candidates, it is expected that PA will be a component, as anti-PA is a major correlate of protection. On September 26, 2008, it was announced that a three-year Federal government contract was awarded to Emergent BioSolutions, Inc., to develop the next-generation anthrax vaccine candidates. This will include developing a recombinant anthrax vaccine (AV7909) and an anthrax monoclonal antibody to block the anthrax toxin. The AV7909 is reported to consist of AVA and

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an adjuvant called CPG 7909, licensed from Pfizer, Inc., (NY, NY). It is projected that the next-generation vaccine will elicit a markedly increased anti-PA response within a shorter period and consequently help the US military more easily inoculate individuals working within high-risk areas.

Conclusion

Taken together, there are gaps of information that shadow the vaccination-protection scenario of the present anthrax vaccination regimen. Valuable information should emerge from efforts that address the: (1) variable immune response elicited in vaccinees; (2) effect of unavoidable interruptions in the timing of the vaccine doses; (3) monitoring of the anti-PA antibody levels after the primary series and after booster injections; and (4) conflicting data yielded from vaccination-challenge studies and subsequently define the correlate of immune protection (i.e., level of anthrax-specific antibody needed to confer protection in recipients of the currently licensed anthrax vaccine).

Note

Since the submission of this manuscript to *Human Vaccines*, the FDA approved a change in route of administration for the anthrax vaccine adsorbed (AVA) from a subcutaneous injection to intramuscular. The FDA also approved a change in the vaccination series by removing the 2 week dose. This approval occurred on December 11, 2008.

Acknowledgements

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