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Development of Equine Anthrax Antitoxin for Disease Treatment - an *In vivo* A/J Mice Passive Protection Assay for Evaluation of Antitoxin Potency

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Background: Anthrax Immune Globulin (AIG) is a polyclonal anthrax immunoglobulin product developed by Cangene (Winnipeg, Canada) and Emergent BioSolutions (Rockville, MD). It is derived from plasma of individuals who have been vaccinated with BioThrax™. It is a candidate for intravenous post-exposure treatment in patients with symptoms of anthrax disease. This study used the equine antitoxin as an alternative for anthrax medical counter measure. **Methods:** Horses were immunized intramuscularly with AVA (Anthrax vaccine adsorbed; BioThrax™). The sera were examined with anti-PA (Protective Antigen) ELISA and TNA (Anthrax toxin neutralization assay). The passive protection test was performed with ATCC 14186 strain spores and equine sera in A/J mice model. **Results:** The sera from horses immunized with AVA have high titer in anti-PA ELISA, and stable neutralization activity in TNA *in vitro*. In a well-controlled A/J mice animal model, the serum repeatedly and accurately protects A/J mice from exposure to a lethal challenge of the ATCC 14186 strain of *Bacillus anthracis*. **Conclusions:** Anthrax equine antitoxin shows a high titer and high neutralization activity, both *in vitro* and *in vivo*.

Key words: anthrax, equine, antitoxin, A/J mice, passive protection

INTRODUCTION

The deliberate dissemination of *Bacillus anthracis* spores via the US mail system in 2001 confirmed their potential use as a biological weapon for mass human casualties. This dramatically highlighted the need for specific medical countermeasures to protect individuals from a future bioterrorism attack.[1-3] Although vaccination appears to be the most effective and economical form of mass protection, current vaccines have significant drawbacks that justify the immense research effort devoted to developing improved treatment modalities.[4-8] After 12 years, only marginal progress has been made in the development of effective therapeutics.[9-12]

Bacillus anthracis, the causative agent of anthrax, is a Gram-positive, rod-shaped bacterium that forms highly resistant spores under conditions of environmental stress.[13] Spores represent a dormant, non-reproductive form of the bacterium that is resistant to UV light, desiccation, extreme temperatures and other environmental conditions.[14] Spores can persist in nature for many decades, primarily in soil, and are very difficult to eradicate.[15] Owing to the soilborne nature of *B. anthracis*, anthrax affects mainly grazing animals but all mammals are susceptible to the disease. Inhalation anthrax is the deadliest form of the disease in human because it is difficult to diagnose in a timely manner and exposure via the inhalation route has the potential to affect a large number of individuals in the event of a deliberate release.[16] After the initial spore uptake by local tissue macrophages, the organism germinates into its vegetative, replicative form,[17-19] and rapid generation contribute to its capacity to overwhelm innate host defenses.[20] The vegetative bacilli enter the bloodstream and ultimately cause sepsis. The disease has a typical incubation period of 1-6 days and begins with relatively mild, flu-like symptoms such as malaise, fatigue and slightly elevated temperature. This is followed by respiratory distress, which progresses abruptly and rapidly to respiratory failure and shock despite of aggressive antibiotic treatment. The anthrax genome comprises a single, covalently closed chromosome accompanied by two essential virulence plasmids: pXO1 and pXO2. The smaller, 95.3 kilobase pair pXO2 carries the capBCAE gene operon, responsible for the synthesis of the poly-c-D-glutamic acid exocapsule .The 184.5 kbp pXO1 plasmid carries the atxA regulatory gene and three others responsible for toxin synthesis.[21] Two viru-

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lence factors, the capsule, which allows the bacterium to evade phagocytosis, and two AB-type exotoxins, lethal toxin and edema toxin, are associated with anthrax pathogenesis.[22,23] The B moiety, protective antigen (PA), represents the cell-binding component required for the entry of the enzymatic A moieties, lethal factor (LF) and edema factor (EF).[24] LF is a zinc protease that cleaves several mitogen-activated protein kinase kinases leading to blockage of signaling pathways by which immune cells respond to pathogens.[25-28] EF is a calmodulin-dependent adenylate cyclase that generates unphysiologically high levels of cAMP.[29] This leads to impairment of intracellular signaling pathways, interference with phagocytosis by macrophages and disruption of water homeostasis with resulting edema.[30,31]

Anthrax vaccine adsorbed (AVA; BioThrax™) was licensed in the United States in 1970 for prevention of anthrax in humans.[6,32-34] AVA is prepared from sterile culture filtrates of the toxigenic, nonencapsulated *Bacillus anthracis* strain V770-NP1-R grown under microaerophilic conditions in a chemically defined protein-free medium. The final product is formulated to contain 1.2 mg/ml aluminum (as aluminum hydroxide) in 0.85% sodium chloride, with 25 g/ml benzethonium chloride and 100 g/ml formaldehyde added as preservatives.[34] The primary immunogen in AVA is anthrax toxin protective antigen (PA). Anti-PA IgG antibodies are thought to protect against anthrax by neutralizing the toxin, inhibiting spore germination, and enhancing phagocytosis and killing of spores by macrophages.[33,35,36] The recently approved AVA schedule is three 0.5-ml intramuscular (i.m.) injections, at 0, 1, and 6 months, with subsequent boosters at 12 and 18 months and annually thereafter for those at continued risk of infection. A major drawback of the AVA vaccine is its lot-to-lot variation, and the lengthy course of administration.

Given the short incubation time and rapid disease progression of inhalation anthrax, vaccination is unlikely to afford protection after an individual has been exposed to aerosolized spores. In this situation, antibiotics administered soon after exposure and prior to the onset of symptoms are the most effective means of preventing disease. Since spores can remain dormant in the lungs for an extended period of time,[37-39] a 60-day course of oral antibiotics is recommended. This type of prophylactic treatment was effective in the aftermath of the anthrax attacks of 2001, in which close to 10,000 individuals were thought to have been exposed to airborne *B. anthracis* spores and were offered a full course (60 days) of the antibiotics ciprofloxacin or doxycycline. However, a follow-up survey of more than 6000 of these individuals revealed that adherence to the drug regimen was poor. Only 44% of the surveyed individuals followed the prophylaxis protocol correctly whereas others forgot, cited side effects or stopped because they thought they were not at personal risk.[40] The poor compliance is troubling and suggests that additional measures of protection need to be considered in the event of a future mass exposure. Although monoclonal anti-PA antibodies represent a welcome addition to the arsenal of prophylaxis and treatment options for inhalation anthrax,[41-48] their mono-specific nature makes it possible to develop *B. anthracis* strains that resist their action. By mutating the epitope to which the antibody binds, the drug will lose its effectiveness. Meanwhile, Cangene Corporation (Winnipeg, Canada) and Emergent Biosolutions both manufacture polyclonal immunoglobulin or 'Anthrax Immune Globulin' (AIG) from plasma of human volunteers who have been vaccinated with AVA. The advantage of AIG is that its antibody composition reflects the breadth of the natural immune response and therefore offers the possibility of a more efficient induction of effector mechanisms such as complement-induced bacterial cell lysis, antibody-dependent cellular cytotoxicity, phagocytosis, and so on. There are also clear disadvantages, such as the limited availability of donor blood, batch-to-batch variation, risk of infectious disease transmission and high cost of production.[49] The US government has announced plans to purchase 10,000 doses of AIG manufactured by Cangene for the National Strategic Stockpile.[50] AIG has recently been administered under an Emergency Investigational New Drug use protocol to a patient who had naturally acquired inhalation anthrax.[51] The patient presented to a local hospital with symptoms of mild respiratory distress and initially received aggressive antibiotic treatment as well as other critical support. When the patient's condition deteriorated, AIG was added to the treatment protocol according to a recommendation by the CDC. LF in serum plasma and pleural fluid dropped sharply after administration of AIG, suggesting that it had a beneficial effect. The patient eventually recovered.[51]

Taiwan does not have the anthrax vaccine immunization program; hence, there is no stable source of donor blood. Instead, equine antitoxin is used as an alternative. The use of equine antisera for emergent prevention and treatment of infectious diseases has been proven to be an effective and safe strategy.[52-57] Therefore, immunoprophylaxis and treatment with equine hyperimmune globulin might be a viable strategy for anthrax medical counter measure.

MATERIALS AND METHODS

Bacterial strains and culture

B. anthracis (ATCC 14186), which expresses functional lethal toxin (LT) and edema toxin (ET), was grown and maintained as previously described.[58,59] Bacteria were stored in 50% medium and 50% glycerol solution in freezers at -80°C before use. To reactivate bacteria from frozen stocks, 25 ml bacterial stock solution was transferred to a test tube containing 5 ml of freshly prepared culture medium and then incubated at 30°C or 37°C under agitation overnight (16-18 hr). Overnight tryptic soy broth cultures of *B. anthracis* were diluted to about 10^7 CFU/ml in phosphate-buffered saline, and 0.1-ml aliquots were inoculated onto blood agar plates. The agar plates were incubated at 25-37°C until 90-99% phase bright spores were observed by phase-contrast light microscopy. Spores were harvested and washed with cold sterile distilled ionized (DI) water and stored in DI water at 4°C for up to 2 weeks with the water changed at least once a week, or in the freezer at -20°C for up to a month until use.

Mice

A/J strain mice were purchased from The Jackson Laboratory, USA. The mice were housed in the animal facility of the Institute of Preventive Medicine (IPM) at the National Defense Medical Center (NDMC). All mice experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the IPM at the NDMC.

Immunizations and blood sampling

For antitoxin studies, horses were immunized intramuscularly with 5c.c.of AVA emulsified 1:1 in complete Freund's adjuvant (Sigma) at week 0, and then boosted three times with 2.5c.c. of AVA emulsified 1:1 in incomplete Freund's adjuvant (Sigma) at weeks 2, 4 and 7. Blood sampling was carried out on immunized horses once every week since first immunization. Follow-up immunization program was similar to the previous one.

ELISA

PA-specific serum antibody titers were determined by end-point dilution enzyme-linked immunosorbent assay (ELISA) of sera from individual animals. A 96-well immunoplate (Nalge Nunc International, Rochester, NY, USA) was coated by overnight incubation at 4°C with 50 μL of PA solution (10 μg/mL) in PBS. Wells were blocked with 200 μL PIERECE-37515 SuperBlock blocking buffer for 1 h at 37°C. The plate was incubated with 100 μL of serial dilutions of sera for 1 h at 37°C, and bound antibody was detected using AP-conjugated goat anti-horse IgG antibody, followed by incubation with PNPP (p-Nitrophenyl Phosphate) buffer as the chromogenic substrate. The absorbance at 450 nm was measured using an universal microplate reader (Labsystems Multiskan MS).

Anthrax toxin neutralization assay (TNA)

Antibodies to lethal toxin were measured by the ability of sera to neutralize the cytotoxicity of lethal toxin for J774A.1 cells. Antisera were diluted in cell culture medium,added in triplicate to cell culture-treated 96-well microtiter plates (Corning Costar, Acton, MA), and then pre-incubated with PA+LF (1.2 μg/ml and 0.6 μg/ml, respectively) for 1 h at 37 °C. A plate-to-plate transfer from the titration plate to another 96-well plate containing a monolayer of J774A.1 cells, plated the day before the assay at 1×10^5 cells per ml, was then performed. Plates were incubated at 37 °C for 3 h in 5% CO_2. Cell viability was determined by WST-1 (100 μL; Roche, Nutley, NJ, USA) WST-1 was added to each well and incubated at 37 °C for 2 h. Optical density at 450nm (OD450) was measured using a Labsystems Multiskan MS microplate reader. Two wells containing only medium served as medium controls, and three wells containing only lethal toxin served as blanks. The percent neutralization (P) for each diluted sample was calculated as follows.

P = (OD450 of sample mean - OD450 of blank mean) / (OD450 of medium control mean - OD450 of blank mean)×100.

ED50 TNA titer was determined as the reciprocal of the serum dilution that resulted in 50% toxin neutralization.

Passive protection test

At various times pre-challenge, 0.5 ml of anti-AVA horse serum was administered intraperitoneally. Dilutions of this serum were made in 0.9% NaCl. The animals were challenged subcutaneously with 100 LD50 (2×10^5 spores) of ATCC 14186 strain in 0.2 ml of sterile water for injection. The animals were challenged 4 h after treatment with immune serum.

RESULTS

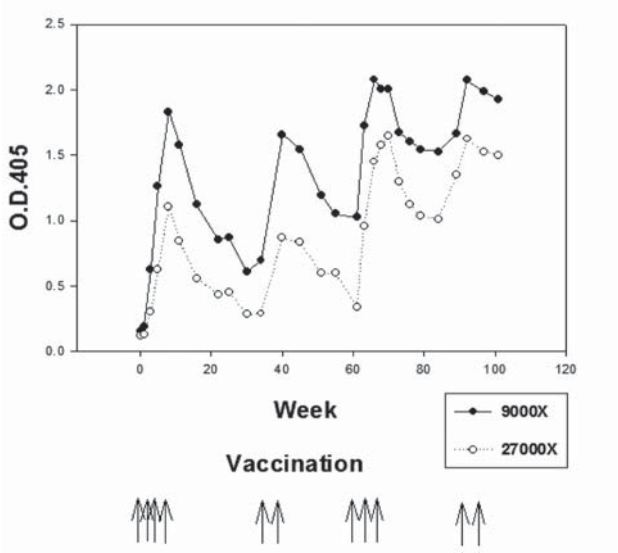

Fig. 1 ELISA (IgG to PA) for anti-anthrax horse serum

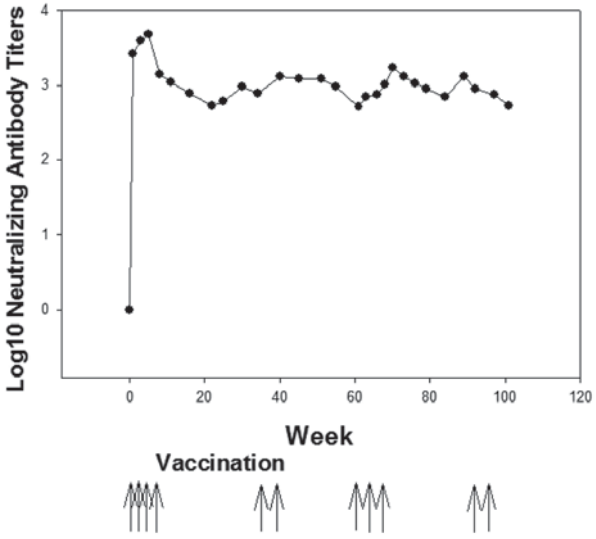

Fig. 2 *In vitro* Toxin Neutralization Assay (TNA) for anti-anthrax horse serum

Horses were immunized with Anthrax Vaccine Adsorbed (AVA) purchased from Emergent BioSolutions and their blood samples were monitored every week. The major component of AVA is PA (protective antigen), so the anti-PA ELISA is taken as the first index of the immune response. As seen in Fig. 1, after each vaccine boost, the anti-PA titer increases substantially followed by a gradual decline over time until the next boost.

Secondly, toxin neutralization assay (TNA) with J774A.1 cells can reflect the ability of the serum to neutralize the lethal toxin (PA+LF) cell cytotoxicity *in vitro*. As seen in Fig. 2, the neutralization titer is the highest after the first boost. The data show an increase in titer after each boost though never as high as the initial rise. Despite of the rise and fall after every boost, the titer remained at a relatively stable level. The antibody neutralization titers in log10 value were between 2.7 and 3.7.

Different concentrations of serum and lethal toxin were used in toxin neutralization assay (TNA) to verify whether there is a dose-dependent relationship. As shown in Fig. 3, the neutralization effect of serum on toxin is indeed dose-dependent.

In most cases, ELISA and TNA can be good indexes for verifying the immune response in anthrax study, but still need a good *in vivo* animal model. Because of the difficulty for using P3 animal lab when challenged with wild type anthrax spore and the inconsistency between some animal models with ELISA and TNA, the US Army Medical Research Institute of Infectious Diseases (USAMRIID) developed an *in vivo* animal model using A/J strain mice and Stern strain anthrax spore in 2008.[60] Hence, this study used this A/J mice animal model to verify the equine serum neutralization ability *in vivo*.

First, the *Bacillus anthracis* ATCC 14186 strain ($pXO1^+$ $pXO2^-$) spores LD50 in A/J mice was tested. Similar to Stern strain, ATCC 14186 strain lacks pXO2 plasmid and the exocapsule. Its LD50 is twice as that of Stern strain, 2×10^3 spores (data not shown). In the passive protection test, A/J mice were pretreated i.p. with serial dilutions of anti-anthrax equine serum, and then challenged with 100 LD50 ATCC 14186 spores. Mortality of mice were monitored daily for 14 days post inoculation. As seen in Fig. 4, there were significant differences in survival in mice treated with up to a 1:64 dilution of the serum, compared with corresponding controls. The 1:16 dilution of the serum protected 87.5% of mice against a 100 LD50 challenge of the *B. anthracis* ATCC 14186 strain. Dilutions of 1:32 and 1:64 each protected 62.5% of the mice. No mice survived when treated with a 1:256 dilution of human anti-AVA horse serum. Compared with the USAMRIID analysis data of anti-AVA plasma from individuals who had received between 4 and 26 injections of AVA BioThrax™,[60] the neutralization activity of anti-AVA equine serum observed in this study is relatively stronger.

DISCUSSION

Results of this study show that the serum from horses immunized with AVA (anthrax vaccine adsorbed, Bio-

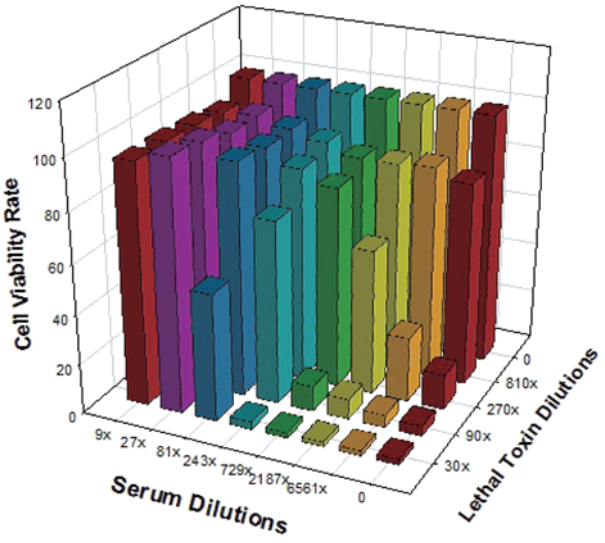

Fig. 3 Toxicity of anthrax lethal toxin to mouse macrophage cell J774.A1 neutralized by a variety of anti-anthrax horse serum dilutions

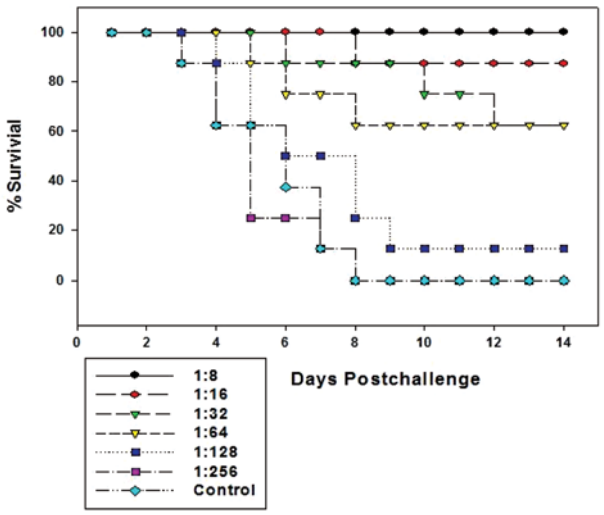

Fig. 4 Survival of A/J mice treated i.p. with 1-ml serial dilutions of anti-anthrax horse serum and challenged s.c. with 100 LD50 spores of *Bacillus anthracis* ATCC 14186

Thrax™) have high titer in anti-PA ELISA, and also stable neutralization activity in TNA *in vitro*. In a well-controlled A/J mice animal model, the serum repeatedly and accurately protects A/J mice from exposure to a lethal challenge from the ATCC 14186 strain of *Bacillus anthracis*. The challenge dose is the same as that used by the USAMRIID,[60] 100 LD50, and the spores are the ATCC 14186 ($pXO1^+$ $pXO2^-$), same as the Stern strain. Although rare as a natural disease in humans, anthrax has gained substantial attention because its spores can be utilized as an agent of biological warfare and terrorism. *B. anthracis* spores have several characteristics that make them the leading threat among bioweapons, and their intentional release in the US mail system in 2001 increased public vulnerability to anthrax bioterrorism. Passive immunization using a polyclonal or a high-affinity monoclonal antibody may offer adjunctive value to antibiotic therapy. Meanwhile, Cangene Corporation (Winnipeg, Canada) and Emergent Biosolutions both manufacture polyclonal immunoglobulin or 'Anthrax Immune Globulin' (AIG) from plasma of human volunteers who have been vaccinated with AVA. Raxibacumab, now called ABthrax™, is a PA monoclonal antibody produced by Human Genome Sciences (HGS). Officials of the US Food and Drug Administration (FDA) in mid-December 2012 approved ABthrax™ for use in patients with inhalational anthrax. This approval is the first under the agency's 'animal rule', established for evaluating the efficacy of products that would be unethical or impossible to test in humans. ABthrax™ is licensed for use as an adjunct to conventional antibiotics such as Cipro (ciprofloxacin). This is also the first time FDA approved a mAb for an antibacterial indication. The US Department of Health and Human Services contracted for 10000 AIG (Cangene) and 20000 ABthrax (HGS) treatment courses, at a total cost of USD 310 million. Therefore, the current study offers a potential alternative treatment for anthrax attack on the army and civilians in Taiwan.

DISCLOSURE

All authors declare that this study has no conflict of interest.

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