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Examination of serological memory in rabbits injected with *Bacillus anthracis* protective antigen adsorbed to Alhydrogel ☆

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ABSTRACT

Serological memory after inoculation of protective antigen (PA) combined with Alhydrogel adjuvant (PA/Alhydrogel) was examined in New Zealand white rabbits, an animal model for anthrax. A threshold dose of 0.1 μg of PA/Alhydrogel was identified which resulted in an ELISA titer 2 weeks after a primary immunization of only 0.168 μg anti-PA IgG per ml and a toxin-neutralizing antibody titer (TNA ED_{50}) of 1.8 ($n = 40$). A significant increase in anti-PA IgG and TNA ED_{50} titers were measured ($p < 0.0001$) 2 weeks after a booster immunization with 0.1 μg of PA/Alhydrogel at 14 days ($n = 10$; 40.9 μg anti-PA IgG per ml; 522 TNA ED_{50}) and 28 days ($n = 10$; 63.8 μg anti-PA IgG per ml; 501 TNA ED_{50}). At this threshold dose of PA/Alhydrogel, protection against an aerosol exposure to *Bacillus anthracis* Ames spores improved as the booster immunization was administered from 4 days (40% survival), to 8 days (50% survival), and to 12 days (80% survival) before challenge. The partial protection of rabbits, even in the absence of protective antibody titers (0.9 μg anti-PA IgG per ml and 26 TNA ED_{50}) when the booster immunization was administered 4 days before challenge, suggested a protective potential for serologic memory.

1. Introduction

Human infection with *Bacillus anthracis*, which generally is caused by contact with infected animals or spore-contaminated animal products, may be manifested as cutaneous, inhalational, or gastrointestinal anthrax, suggesting the route of exposure. The establishment of anthrax is generally based upon the presence of two major virulence factors, a poly-γ-D-glutamic acid capsule and a tripartite exotoxin. The capsule enhances virulence by inhibiting phagocytosis of the bacterium, resulting in a massive bacteremia [1]. The tripartite exotoxin is formed by competitive binding of either lethal factor (LF) or edema factor (EF) with protective antigen (PA) to form lethal toxin (LeTx) or edema toxin (EdTx), respectively. PA, an essential component in the formation of each toxin, binds to cell surface receptors, oligomerizes after undergoing proteolytic activation, then binds and translocates LF and EF into the cytosol [2,3]. LF is a metalloprotease that targets most isoforms of mitogen-activated protein kinase kinase [4,5] and EF is a calmodulin-dependent adenylate cyclase [6]. Early establishment of infection is effected by both LeTx and EdTx by impairing the immune system [3,7]. As the infection progresses, LeTx targets cardiomyocytes and vascular smooth muscle cells while EdTx causes edema within the intestinal lumen and liver [7–9].

People at risk of infection in the United States are immunized with a chemical-based vaccine, anthrax vaccine adsorbed (AVA BioThrax) while anthrax vaccine precipitated (AVP) serves as the clinical vaccine in the United Kingdom. The major protective immunogen in these vaccines is PA [10,11]. Recombinant PA vaccines are currently in clinical trials [12–14]. Antibody responses measured by an anti-PA IgG enzyme linked immunosorbent assay (ELISA) and an in vitro LeTx neutralizing antibody (TNA) assay, in which the functional activity of antisera are evaluated in a macrophage cytolysis assay, have been identified as serological correlates of immunity in non-human primates, rabbits, and guinea pigs [15–20].

The current study was conducted to further develop the rabbit animal model for anthrax by examining serological memory by identifying a threshold dose of PA which does not produce an

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antibody response after the primary immunization but does after a booster injection. The protective capacity of serologic memory at the threshold dose was examined relative to the timing of a booster injection against an aerosol spore challenge. Also, the primary and secondary antibody response profiles in rabbits injected with a protective dose of PA were examined.

2. Materials and methods

2.1. New Zealand white rabbits

An equal number of male and female New Zealand white (NZW) rabbits (3.0–3.5 kg) (Harlan Bioproducts for Science, Indianapolis, IN and Covance Research Products, Denver, PA) were used in the studies.

2.2. Immunization screening

The threshold dose of PA for rabbits was chosen by injecting rabbits intramuscularly (i.m.) on days 0 and 14 with either 10 μg, 1 μg, 0.1 μg, 0.01 μg, or 0 μg of PA (List Biological Laboratories, Campbell, Calif.) diluted in PBS in a volume of 0.5 ml, each adsorbed to 1 mg of aluminum per ml (Alhydrogel, 2% Al_2O_3; HCl Biosector, Frederikssund, Denmark; PA/Alhydrogel) for >2 days at 4 °C before use. Control rabbits were injected with PBS adsorbed to Alhydrogel (PBS/Alhydrogel). Two weeks after the primary injection and booster injection (days 14 and 28), blood was collected and serum was tested for the presence of antibodies to PA ELISA and TNA titers. After determining the threshold dose of PA for rabbits (0.1 μg PA/Alhydrogel, see Section 3), additional booster injections of the threshold dose of PA used in the primary injection were given at either 28 days, 84 days, or 168 days after the primary injection to examine the effect on the memory antibody response. Blood was collected 2 weeks after the booster injection (day 42, day 98, and day 182, respectively) and serum was tested for the presence of antibodies to PA ELISA and TNA titers.

The primary and secondary antibody response profiles were examined by injecting rabbits i.m. with 50 μg of PA/Alhydrogel on days 0 and 21 and collecting blood periodically to test serum for the presence of antibodies to PA and TNA titers in the serum.

2.3. Protection against aerosol exposure

Rabbits were injected i.m. with 0.1 μg of PA/Alhydrogel and boosted with 0.1 μg of PA/Alhydrogel 4 days (day 86), 8 days (day 82), or 12 days (day 78) before aerosol exposure on day 90 following the primary immunization. The initial immunizations were staggered to permit challenge of 10 rabbits from each group on the same day for all the groups. The remaining 4 rabbits from each group were used for serology testing after the challenge date. Challenge controls ($n = 8$), which received PBS/Alhydrogel, were likewise divided; 6 were challenged and 2 were used for serology testing. Aerosol exposure (head only) was performed with a lethal dose of spores from the Ames isolate of *B. anthracis* as previously described [15]. Survival was noted for 21 days after challenge. Moribund animals were humanely euthanized. The aerosol LD_{50} of Ames spores in NZW rabbits is 1.1×10^5 spores [15]. The inhaled dose of spores (average $LD_{50} \pm SD$) for the two hood lines were 139.6 ± 64.23 spores and 82.2 ± 44.24 spores (total average 110.9 ± 61.64 spores).

2.4. Serological analysis of antibodies

A quantitative anti-PA IgG ELISA was performed as described [17]. Briefly, PA-coated plates were sequentially incubated with dilutions of each sample in triplicate, HRP-conjugated goat anti-rabbit IgG(H + L), then ABTS substrate. Samples were diluted in PBS, 0.1% Tween 20, 5% non-fat dry milk (PBSTM) and plates were washed between steps using PBS, 0.1% Tween 20 (PBST). Titers were calculated from the average absorbance value for triplicate wells of each sample with the absorbance values of the affinity purified rabbit anti-PA IgG standard curve by linear regression analysis and reported as micrograms of anti-PA IgG per ml (KC4 software, BioTek Instruments, Winooski, Vt.) as described [17] and recorded as the geometric mean (GM) and standard error of the geometric mean (SEM) from triplicate dilutions [21]. The lowest sample starting dilution ratio was 1:50. Negative samples that were not extrapolated from the standard curve were set at 0.036 μg of IgG per ml.

For the TNA assay, J774A.1 cells were exposed to final concentrations of 100 ng of PA per ml and 50 ng of LF per ml (List Biological Laboratories) in the presence of antibody as described [17]. After incubating for 4 h, MTT was added for 2 h before lysing the cells and solubilizing the reduced formazin. The percent viability, determined using the following formula, % viability = (((test avg − LeTx avg)/(media avg − LeTx avg)) × 100), was plotted against each respective test dilution using a 4-parameter logistic equation algorithm and TNA assay titers were expressed as the reciprocal of the dilution of antiserum that neutralized the cytotoxic activity of LeTx on J774A.1 cells at 50% of control values (ED_{50}) using XLfit software (IDBS, Inc., Emeryville, Calif.) as described [17]. TNA titers were recorded as the GM ED_{50} (SEM) [21]. The starting dilution for the TNA assay was 1:50 and negative samples that could not be quantified from the standard curve were assigned an ED_{50} value of '1'.

2.5. Avidity measurement

Antibody avidity was measured by modifying the ELISA [17]. After incubating twofold sample titrations on the ELISA plates for 2 h, the plates were washed and triplicate sample titrations were incubated for 15 min with either 1.5 M sodium thiocyanate or PBSTM. The plates were washed and the ELISA procedure resumed by adding the secondary antibody. The avidity was evaluated by comparing the respective absorbance values (A_{405nm}) of the 1:800 dilutions of sera samples incubated in either 1.5 M sodium thiocyanate or PBSTM.

2.6. Data analysis

ELISA titers, TNA ED_{50} titers, and avidity absorbance values were log 10 transformed. ELISA and TNA ED_{50} titers were compared using either unpaired *t* test with Welch's correction (to examine samples between groups) or paired *t*-test (to examine samples within a group), as appropriate. Comparisons between avidity absorbance values within each group were performed using repeated measure one-way ANOVA with the Greenhouse-Geisser correction and Bonferroni's multiple comparison test or paired *t* test, as appropriate. Comparisons between absorbance values between groups were performed using one-way ANOVA with Tukey's multiple comparison test. The antibody response over time was analyzed against the preceding primary or booster immunization by one-way ANOVA with repeated measures using Holm-Sidak's multiple comparison test. Survival curves, which plotted the percent survival as a function of time, were compared by log-rank (Mantel-Cox) test and MTTD were compared by unpaired *t* test with Welch correction. Statistical analysis was performed by using GraphPad Prism 6 (GraphPad Software, La Jolla, Calif.) and *p*-values were two-sided.