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Mucosal priming of newborn mice with *S.* Typhi Ty21a expressing anthrax protective antigen (PA) followed by parenteral PA-boost induces B and T cell-mediated immunity that protects against infection bypassing maternal antibodies

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ABSTRACT

The currently licensed anthrax vaccine has several limitations and its efficacy has been proven only in adults. Effective immunization of newborns and infants requires adequate stimulation of their immune system, which is competent but not fully activated. We explored the use of the licensed live attenuated *S.* Typhi vaccine strain Ty21a expressing *Bacillus anthracis* protective antigen [Ty21a(PA)] followed PA-alum as a strategy for immunizing the pediatric population. Newborn mice primed with a single dose of Ty21a(PA) exhibited high frequencies of mucosal IgA-secreting B cells and IFN-γ-secreting T cells during the neonatal period, none of which was detected in newborns immunized with a single dose of PA-alum. Priming with Ty21a(PA) followed by PA-boost resulted in high levels of PA-specific IgG, toxin neutralizing and opsonophagocytic antibodies and increased frequency of bone marrow IgG plasma cells and memory B cells compared with repeated immunization with PA-alum alone. Robust B and T cell responses developed even in the presence of maternal antibodies. The prime-boost protected against systemic and respiratory infection. Mucosal priming with a safe and effective *S.* Typhi-based anthrax vaccine followed by PA-boost could serve as a practical and effective prophylactic approach to prevent anthrax early in life.

1. Introduction

Concern over the illicit use of the bacterium *Bacillus anthracis*, a potential bioterror agent and the causative agent of anthrax, has heightened in recent years. The deliberate dissemination of anthrax spores through the US postal service in 2001 and the resulting harm and disruption it caused illustrated our vulnerability to such an attack and the need to develop effective and safe diagnostic, therapeutic, and prophylactic tools [1].

The anthrax vaccine currently available for use in humans in the U.S. consists of a cell-free culture filtrate containing *B. anthracis* protective antigen (PA) adsorbed to aluminum hydroxide (AVA-BioThrax®). PA is the non-toxic cell-binding component of the organism's tripartite toxin and the pathogen's major virulence factor. A similar cell-free vaccine consisting of alum-precipitated culture filtrate containing PA (AVP) is available in the U.K. [2].

While animals studies support the immunogenicity and protective efficacy of AVA, the extent to which this vaccine prevents disease in humans has been less clear. The immunization schedule is lengthy, consisting of five intramuscular injections over a period of 18 months followed by yearly boosters [3]. Local adverse reactions can occur that intensify with successive injections, and most importantly data demonstrating the ability of AVA to protect human against inhalational anthrax is lacking [reviewed in [4,5]]. In addition, the vaccine is perceived by the public (including high-risk groups) as unsafe and ineffective [6–8], and as a consequence its use has been limited to military personnel who have received it reluctantly [9].

There is indeed a pressing need to develop vaccines and immunization strategies capable of inducing rapid and effective protection, which can be safely given to all members of the population including vulnerable high-risk groups such as infants and young children who are particularly susceptible to bacterial infection. Anthrax has a rapid onset and progression in young children and severe complications have been described [10,11].

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Furthermore, infants and young children cannot be easily treated with antibiotics, let alone the aggressive and prolonged antibiotic therapy needed to effectively treat inhalational anthrax [2,11]. Even if alternative therapeutic antimicrobials become available in the near future, the rapid course of infection suggests that post-exposure therapy alone would be insufficient to prevent mortality [12,13]. Thus, safe and effective prophylactic vaccines capable of protecting the pediatric population against biological warfare are urgently needed.

A successful immunization strategy for infants will have to overcome several major obstacles, including: (1) the low levels of activation or "inexperience" of the neonatal/infant immune system, (2) a bias towards Th2-type responses, and (3) the presence of maternal antibodies that can oftentimes interfere with successful immunization. An ideal vaccine for this age group would be capable of inducing long-lasting protective levels of anthrax toxin neutralizing antibodies and robust mucosal and cell-mediated immunity following minimal dosing via a user-friendly route of immunization.

Our group was the first to demonstrate that attenuated strains of *Salmonella enterica* serovars Typhi and Typhimurium expressing a foreign vaccine antigen could prime robust immune responses in newborn mice following mucosal delivery despite the presence of high levels of maternal antibodies [14]. In subsequent studies we showed that unlike conventional subunit vaccines, live attenuated *Salmonella* has the capacity to enhance the activation and maturation of neonatal DCs in vivo, thus favoring more efficient T cell priming and ensuing adaptive immunity [15]. We also found that neonatal responses can be further enhanced by employing a heterologous prime-boost regimen; newborn mice primed with *S.* Typhi expressing *Yersinia pestis* F1 and boosted (as infants) with F1-alum developed protective immunity against systemic plague infection [15].

In this study, we examined the immune responses and protective efficacy afforded by neonatal mucosal priming using the licensed live attenuated typhoid vaccine strain Ty21a expressing *B. anthracis* PA followed by a parenteral PA-alum boost. Ty21a was chosen in preference to other strains because of its excellent record of safety, tolerability and immunogenicity in humans including school-age children [16,17], toddlers [18–20] and infants [18]. We also examined the ability of the neonatal *S.* Typhi priming-PA-boost immunization strategy to elicit B and T cell responses in the presence of maternal antibodies.

2. Materials and methods

2.1. S. Typhi Ty21a expressing B. anthracis PA

Plasmid pSEC91-83 encoding PA83 from *B. anthracis* [21] was electroporated into *S.* Typhi vaccine strain Ty21a. Transformants were recovered on Luria–Bertani (LB) agar plates containing kanamycin (10 μg/ml). PA expression was assessed by SDS-PAGE and immunoblot as described previously [15] using an anti-PA monoclonal antibody (Abcam, Cambridge, MA) followed by HRP-labeled goat anti-mouse IgG (Roche, Indianapolis, IN) and ECL Plus detection system (Amersham Biosciences, Buckinghamshire, UK). Master and working cell banks were produced from single colonies and stored at −70 °C. The vaccine strain *S.* Typhi Ty21a(pSEC91-83) is henceforth referred to as Ty21a(PA). Ty21a(PA) and Ty21a used for immunization were grown at 37 °C in LB broth supplemented with kanamycin as required. The immunizing dose was verified by plating serial dilutions onto LB agar with and without antibiotic. In vivo vaccine distribution following immunization was examined in a similar manner, by plating serial dilutions of homogenized tissue.

2.2. Immunoflourescence

Ty21a(PA) and Ty21a were grown overnight as described above and incubated with anti-PA monoclonal antibody (Abcam) in PBS, 0.1% BSA and 0.01% NaN_3 for 1 h at room temperature followed by FITC anti-mouse IgG (Invitrogen, Carlsbad, CA) as previously described [15]. Stained bacteria were visualized using a Nikon Eclipse 2000-E UV fluorescent microscope.

2.3. Mice and immunizations

BALB/c (Charles River Laboratories, Wilmington, MA) and A/J mice (The Jackson Laboratories, Bar Harbor, ME) were bred as previously described [14]. Newborns were primed intranasally (i.n.) with one or two doses of Ty21a(PA) or Ty21a (1×10^9 CFU in 5 μl volume) administered on day 7 or on days 7 and 15 after birth. All animals were boosted intramuscularly (i.m.) on day 22 after birth with 2 μg PA (List Biologicals, Campbell, CA) adsorbed to 0.5% aluminum hydroxide (Alhydrogel®, Brenntag Biosector, Frederikssund, Denmark), hereafter referred to as "alum". Control groups received PBS or PA i.m. on day 7 after birth followed by PA-boost on day 22. One-week-old mice were used in these studies as they best reflect the maturity of the immune system in human newborns (1–28 days of age) and their responses to vaccines [22]. Blood was collected at different time points as previously described [15]. To study interference by maternal antibodies, female breeders were immunized twice, 14 days apart, with 2 μg of PA-alum via i.m.; the last dose was administered 2 weeks before mating. Blood was collected before immunization, at the time of breeding and immediately after delivery. Litters from immune mothers were cross-fostered to naive surrogate females immediately after birth as previously described [14]. All animal studies described were approved by the University of Maryland Institutional Animal Care and Use Committee.

2.4. PA-specific IgG, IgG subclasses and IgG avidity

PA-specific serum IgG, IgG1 and IgG2a were measured by ELISA as previously described [23] using HRP-labeled anti-mouse IgG, IgG1, and IgG2a (Roche) conjugates and TMB Microwell Peroxidase substrate (KPL, Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). End-point titers were calculated as the inverse of the serum dilutions that produced an absorbance (A_{450}) value of 0.2 above the blank [15]. IgG avidity was measured by ELISA using 6 M urea as a chaotropic agent as previously described [24].

2.5. Antibody-secreting cells (ASC) and memory B cells (B_{Mem})

PA-specific ASCs were measured in the nasal-associated lymphoid tissue (NALT), lung, and bone marrow (BM) by ELISPOT as previously described [14,24]. Briefly, fresh cells resuspended in complete RPMI [RPMI 1640 supplemented with 10% FCS (Hyclone, Logan, UT), 200 mM glutamine, and gentamicin 50 μg/ml, all from Invitrogen-Gibco, Gand Island, NY] were added in serial two-fold dilutions (5×10^5–6.25×10^4) to Immulon II plates previously coated with PA (5 μg/ml) and incubated overnight at 37 °C, 5% CO_2. HRP-labeled goat anti-mouse IgA (Zymed Laboratories, San Francisco, CA) and IgG (Roche) were used as conjugates, followed by True Blue substrate (KPL) in agarose overlay [15]. BM PA-specific and IgG^+ B_{Mem} lymphocytes were measured by ELISPOT after polyclonal expansion and overnight antigen recall as previously described [15].

2.6. Toxin neutralization and opsonophagocytic assays (OPA)

Anthrax toxin neutralizing antibodies were measured in individual samples using the method developed by Quinn [25,26].