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Comparisons of the humoral and cellular immunity induced by live A16R attenuated spore and AVA-like anthrax vaccine in mice

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ABSTRACT

The live attenuated anthrax vaccine and anthrax vaccine adsorbed (AVA) are two main types of anthrax vaccines currently used in human. However, the immunoprotective mechanisms are not fully understood. In this study, we compared humoral and cellular immunity induced by live A16R spore vaccine and A16R strain derived AVA-like vaccine in mice peripheral blood, spleen and bone marrow. Both A16R spores and AVA-like vaccines induced a sustained IgG antibody response with IgG1/IgG2b subtype dominance. However, A16R spores vaccine induced higher titer of IgG2a compared with AVA-like vaccine, indicating a stronger Th1 response to A16R spores. Using antigen-specific ELISpot assay, we observed a significant response of ASCs (antibody secreting cells) and IL4-CSCs (cytokine secreting cells) in mice. Specially, there was a positive correlation between the frequencies of antigen specific ASCs and IL4-CSCs in bone marrow derived cells, either by A16R spore or AVA-like vaccine vaccination. Moreover, we also found A16R spore vaccine, not AVA-like vaccine, could induce sustained frequency of IFN-γ-CSCs in bone marrow derived cells. Collectively, both the vaccines induced a mixed Th1/Th2 response with Th2 dominance in mice and A16R spore vaccine might provide a more comprehensive protection because of humoral and cellular immunity induced in bone marrow.

1. Introduction

Anthrax is a fatal zoonotic disease caused by the spore-forming bacterium *Bacillus anthracis*. Despite epidemic mainly in wild and domestic herbivores, the *B. anthracis* can also invade the human beings with deadly consequences through several potential routes, such as the consumption of contaminated food or direct contacting with infected animals [1,2]. In addition, the anthrax spores, the dormant form of the bacteria, have widely been considered as one of the most harmful bio-terror agents that can be maliciously used as biological weapon, because of the strong resistance to environmental insults and almost 100% mortality rate in the unprotected populations [3]. The primary virulence factors of *B. anthracis* include lethal toxin (LT), edema toxin (ET), and a poly-γ-D-glutamic acid capsule. The protective antigen (PA) is the common component of the LT and ET by binding the lethal factor (LF) and edema factor (EF) to mediate their entry into host cells [4].

There are two types of anthrax vaccines currently available for human use, the live attenuated anthrax vaccine consisting of avirulent spores from *STI-1* (in Russia) or *A16R* (in China) strain and the aluminum absorbed or precipitated PA-based subunit vaccine (also known as AVA licensed in the USA and AVP in the UK) [5,6]. The attenuated *B. anthracis* A16R strain with the $pXO1^+$ and $pXO2^-$ genotypes (exotoxin-producing and noncapsuled) is derived from the wild-type A16 strain by UV-induced mutagenesis and continuous subculture and was used for the production of the live spore vaccine for humans in China [6,7]. Both vaccines can elicit humoral immune response and provide sufficient protection against the lethal *B. anthracis* challenge. Notwithstanding slight LF and EF

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antibodies production, the circulating anti-PA IgG antibodies are generally considered as the predominate contribution to the protection [5,8,9]. However, the comparative studies of live spore vaccine and PA-based vaccine demonstrated that the anti-PA antibodies were not sufficient to provide full protections against certain virulent strains of *B. anthracis* in mice and *guinea* pigs [9,10]. In addition, PA-based vaccine did not protect mice and *guinea* pigs against 9602P, a capsulated PA-deficient *B. anthracis* strain, of which toxicity was primarily due to the capability of causing septicemia [11]. However, formaldehyde-inactivated spores (FIS), on which surface presented spore antigens, could provide partial protection in mice and complete protection in *guinea* pigs against 9602P lethal challenge [11]. In contrast, the asporogenic KBMA (killed but metabolically active) anthrax vaccine derived from *Sterne* strain exhibited relatively lower protective efficacy comparing with the *Sterne* live spore vaccine against lethal *Ames* spore challenge in *guinea* pigs [9,12]. Furthermore, Liu *et al.* [13] identified 50 immunogenic proteins in A16R spores and vegetative cells, which might contribute to the immunogenicity and protective efficacy of A16R spore vaccine. These studies suggested that the presence of spore antigens in live spore vaccine could induce a greater variety of immune responses than the PA subunit vaccine, and implied the importance of T and B cell function in the protection of *B. anthracis*.

Recently, growing recognition that cellular immune responses involved $CD4^+$ T helper cells also contributed significantly to protective immunity of *B. anthracis* spore vaccines and PA subunit vaccines [14–18]. The Th1-skewing cells with IFN-γ secretion were reported predominantly mediating the FIS induced immunity and protecting mice from infection challenged with a capsulated non-toxinogenic *B. anthracis* [18]. In addition, several studies also demonstrated the AVA could elicit a mixed Th1/Th2 response with Th2 dominance, which was essential for sustained production of the PA-specific functional antibodies in human and nonhuman primate [19,20]. Our previous work had demonstrated that both live A16R spore vaccine and A16R strain derived AVA-like vaccine could induce the antigen-specific Th1/Th2 response as well as antibody-secreting cells (ASCs) activation in the peripheral blood [21]. However, the relationship between Th1/Th2 response and humoral immunity induced by live A16R spore vaccine and AVA-like vaccine in peripheral blood, spleen and bone marrow is largely unknown. Studies of humoral and cellular immune response to anthrax antigens will help to inform new insight into early evaluation of vaccination efficacy and clinical development of novel vaccines.

Here we systematically assess and compare humoral and cellular immunity induced by live A16R spore vaccine and AVA-like vaccine, and provide a detailed evaluation of magnitude and duration of live A16R spore or AVA-like antigen specific humoral and cellular immunity profiles during the early onset period after vaccination in peripheral blood, spleen or bone marrow of mice.

2. Materials and methods

2.1. Mice, vaccine preparation and immunization

Female BALB/c mice at 6–8 weeks old were purchased from the Institute of Jing Feng Medical Laboratory Animal and housed under BSL2 pathogen-free conditions. The animal research herein reported was approved and conducted in facilities with programs accredited by the Animal Subjects Research Review Board of the Beijing Institute of Microbiology and Epidemiology, and was carried out in strict accordance with the recommendations in the Guide for Care and Use of Laboratory Animals of the National Institutes of Health. All surgery procedures were performed under sodium pentobarbital anesthesia to minimize suffering.

The anthrax supernatant antigen isolated from *Bacillus anthracis* A16R strain was absorbed to alhydrogel (National Vaccine and Serum Institute, NVSI) to generate a AVA-like vaccine, in which contains anthrax supernatant antigen 600 μg/mL and alhydrogel 1.2 mg/mL. The attenuated A16R spore anthrax vaccine, obtained from the China Medical Culture Collection, was manufactured as previously described [13] and maintained in our laboratory [21]. The mice were vaccinated with live A16R spore vaccine and AVA-like anthrax vaccine, respectively. The vaccinated mice ($n = 5$ per group) received 0.5 ml of AVA-like vaccine (300 μg) on a 3-dose (0, 2 and 4 weeks) *i.m.* schedule, or 0.5 ml of live A16R spore vaccine (about 5×10^6 CFU of spores) on a 2-dose (0 and 2 weeks) *s.c.* schedule. The mice injected with PBS were used as control.

2.2. Isolation of splenocytes and bone marrow

Splenocytes and bone marrow cells were isolated and purified from immunized and control mice as previously described [18,22]. Briefly, the spleens were harvested and homogenized by mechanical disruption, and the splenocytes were resuspended in complete RPMI 1640 medium as single cell. Bone marrow cells were flushed from the femurs and tibias by the RPMI 1640 medium. Erythrocytes were removed using red blood cell lysing buffer (0.16 M NH_4Cl, 0.17 M Tris-HCl, pH 7.4).

2.3. Preparation of PBMCs

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh blood following instructions of the manufacture. The erythrocytes were removed by lysing buffer (BD, Pharmingen, USA) incubation for 10 min and washed 3 times with 1640 medium containing 1% fetal calf serum and resuspended in 1640 medium with 10% fetal calf serum and antibiotics (penicillin 100 U/ml and streptomycin 100 μg/ml) for immediate use.

2.4. ELISA for antibody analysis

Blood samples were collected on days 7 and 14 after each immunization, respectively. The antibody levels in sera were determined by indirect ELISA using 96-plates coated with predetermined concentrations of AVA-like antigens (2.5 μg/ml) and inactivated spores (2×10^7/mL), and were detected with HRP conjugated anti-mouse IgG, IgG1, IgG2a and IgG2b (BETHYL, USA) as appropriate. Each sample was repeated in triplicate. The absorption value was measured at 450 nm and the positive thresholds were set as 2 times higher than the mean value of negative controls.

2.5. Frequencies of IgG Antibody Secreting Cells (ASCs)

To determine frequencies of antigen specific IgG antibody-secreting cells (ASCs), the enzyme-linked immunospot assay (ELISpot) was performed as described previously [23]. Briefly, nitrocellulose-bottom 96-well multiscreen HA filtration plates (Millipore Corporation, San Francisco, Calif.) were coated at 100 μL/well with PBS containing 7.5 μg/mL AVA-like antigens or 10^8/ml inactivated spores and incubated overnight at 4 °C. Wells coated with 7.5 μg/mL BSA were used as irrelevant antigen negative control. The plates were washed and blocked, and incubated with a total of 5×10^5 cells per well at 37 °C for 4–6 h. The biotinylated mouse IgG (BD, Pharmingen, USA) (at 1: 250 diluted in PBS-T containing 1% FCS) and HRP-conjugated avidin D (at 1:100 diluted in PBS-T containing 1% FCS) were used to detect the captured specific ASCs in the plates, and the spots were enumerated using an automated ELISpot reader (Cellular technology Ltd.,