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Protective effect of *Bacillus anthracis* surface protein EA1 against anthrax in mice

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ABSTRACT

Bacillus anthracis spores germinate to vegetative forms in host cells, and produced fatal toxins. A toxin-targeting prophylaxis blocks the effect of toxin, but may allow to grow vegetative cells which create subsequent toxemia. In this study, we examined protective effect of extractable antigen 1 (EA1), a major S-layer component of *B. anthracis*, against anthrax. Mice were intranasally immunized with recombinant EA1, followed by a lethal challenge of *B. anthracis* spores. Mucosal immunization with EA1 resulted in a significant level of anti-EA1 antibodies in feces, saliva and serum. It also delayed the onset of anthrax and remarkably decreased the mortality rate. In addition, the combination of EA1 and protective antigen (PA) protected all immunized mice from a lethal challenge with *B. anthracis* spores. The numbers of bacteria in tissues of EA1-immunized mice were significantly decreased compared to those in the control and PA alone-immunized mice. Immunity to EA1 might contribute to protection at the early phase of infection, i.e., before massive multiplication and toxin production by vegetative cells. These results suggest that EA1 is a novel candidate for anthrax vaccine and provides a more effective protection when used in combination with PA.

1. Introduction

Bacillus anthracis, a Gram-positive, spore-forming, rod-shaped bacterium, is the causative agent of anthrax, which is primarily a disease of livestock. Its spores are highly resistant to adverse conditions, capable of surviving for years. Spores enter the host through injured skin, or through the gastrointestinal or respiratory tracts [1]. The high mortality rate of inhalational anthrax is associated with not only a potent virulence of the pathogen but also delays in proper diagnosis and treatment. Initial symptoms of inhalational anthrax are non-specific and similar to flu-like illness, and no rapid diagnostic test is available in the early stages of inhalational anthrax.

Inhaled spores are engulfed by macrophages, which are the primary site for spore germination [1]. After germination and multiplication, they disperse into the blood stream with aggressive extracellular multiplication, and secrete a cytotoxic toxin. The major virulence factors of *B. anthracis* are three toxin components (lethal factor, LF; edema factor, EF; protective antigen, PA), and a poly-γ-D-glutamic acid polymer capsule [1,2]. Both LF and EF require PA to exhibit their cytotoxic effects. PA interacts with receptors on host cell surfaces and delivers LF and EF into the cytosol [2]. Two licensed, PA-based cell-free vaccines, anthrax vaccine adsorbed (AVA) and anthrax vaccine precipitated (AVP), have been available in US and UK for human use [3]. Frequent intramuscular booster injections of these vaccines are required to maintain sufficient immunity, and there are several concerns regarding local and systemic adverse effects [4]. Both vaccines mainly contain PA [3] but also contain EF, LF, and other unidentified components [5]. Coexistence of PA and the two exotoxins is capable of forming lethal toxin and edema toxin that may possibly contribute to unfavorable reactions.

In general, natural infection in humans is rare and mostly caused by contact with infected livestock or contaminated products [6]. However, after the attack on the US Postal Service in 2001, safer and improved human vaccines are needed [7]. In addition, the efficacy of PA-based vaccines is less effective than that of live attenuated spore vaccines [8], suggesting that components other than PA may confer better protection. Therefore, safer and easily administrable vaccines consisting of known non-toxigenic components would be desirable.

Abbreviations: AVA, anthrax vaccine adsorbed; AVP, anthrax vaccine precipitated; EA1, extractable antigen 1; EF, edema factor; ELISA, enzyme-linked immunosorbent assay; *i.n.*, intranasal; *i.p.*, intraperitoneal; LF, lethal factor; mAb, monoclonal antibody; PA, protective antigen; PBS, phosphate-buffered saline.

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The extractable antigen 1 (EA1) is a major S-layer component of *B. anthracis* [9,10]. This protein is abundant in the vegetative cell surface, but is also generally found in spore preparations [11–14]. If EA1 presents not only on the surface of vegetative form but also on that of spores, this protein could be an attractive candidate as an anthrax vaccine antigen. Immunity to EA1 could play a beneficial role in the inhibition of spore germination, the clearance of vegetative bacilli, or both.

To examine the effectiveness of EA1 as an anthrax vaccine antigen, we immunized mice with a purified recombinant protein of EA1 (rEA1) and examined its protective immunity against experimental anthrax infection with lethal spore challenge. We used nasal immunization procedures, which are non-invasive and are known to induce mucosal and systemic immunities. Furthermore, we investigated the combination effect of EA1 and PA to ensure maximum protection.

2. Materials and methods

2.1. Bacterial strain and spore preparation

The *B. anthracis* Pasteur II strain [15] carrying the $pXO1^+$ and $pXO2^+$ virulence plasmid was used in this study. Spores were prepared as described elsewhere [16]. The purified spores were heated at 80 °C for 30 min before use. The pathogen was handled in a biosafety level 3 (BSL3) facility approved by the Safety Control Committee of Obihiro University of Agriculture and Veterinary Medicine.

2.2. Preparation of recombinant EA1 and PA

The genes *eag* encoding EA1 or *pagA* encoding PA were PCR-amplified from purified genomic DNA of the Pasteur II strain using the following primers (restriction enzyme sites were underlined): EA1-F: 5′-tttggatccatgacagcaatggtagcaggta-3′; EA1-R: 5′-ccctcgagttatagatttgggttattaagaagg-3′; PA-F: 5′-attggatccgaagttaaacaggagaaccgg-3′; PA-R:5′-agagtcgacttatcctatctcatagccttt-3′. Purified PCR products were digested with restriction enzymes, and the fragments were inserted into the pGEX-6P-1 expression vector system (GE Healthcare, Uppsala, Sweden). *E. coli* BL21 (DE3) harboring the constructed vector were cultivated in 2 × YT medium (Becton Dickinson, Franklin Lakes, NJ, USA) at 37 °C until the optical density of the medium at 600 nm was approximately 0.6. A GST-tag fusion protein was induced by incubation with 1 mM isopropylthiogalactoside for another 5–6 h. Purification of recombinant proteins and the removal of the GST tag were performed according to manufacturer's instructions.

2.3. Anti-EA1 polyclonal antibody

The Animal Care and Use Committee of the university approved the animal studies. Japanese white rabbit (Charles River Japan, Kanagawa, Japan) was subcutaneously immunized with approximately 0.3 mg of rEA1 once a week for 5 weeks. Freund's complete adjuvant was used at first immunization. Serum anti-EA1 antibody titer was measured by enzyme-linked immunosorbent assay (ELISA) compared to pre-immune serum. Anti-rEA1 polyclonal IgG was purified using a Protein G MAb Trap Kit (GE Healthcare).

2.4. Immunofluorescence of B. anthracis

Spores or vegetative cells were spotted on a low-fluorescence glass slide, then fixed with 4% paraformaldehyde for 30 min. After three washes with 0.1% Tween 20 in PBS (T-PBS), slides were blocked, then added 100 μL of anti-rEA1 IgG (10 μg/mL) to each slide. After 1-h incubation, the slides were washed and subsequently incubated with appropriately diluted 100 μL of Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA) for 30 min in the dark. Slides were mounted in ProLong Gold (Invitrogen), then observed under a fluorescence microscope (Olympus BX51, Olympus, Tokyo, Japan), and images were analyzed with DP70-BSW software (Olympus). For flow cytometry, inactivated spores were incubated with anti-rEA1 IgG, followed by incubation with Alexa Fluor 488-conjugated secondary antibodies. Samples were analyzed by FACSCanto II using FACSDiva software (Becton Dickinson).

2.5. Nasal immunization

Male BALB/c mice aged 6–7 weeks (CLEA, Tokyo, Japan) were intranasally (*i.n.*) immunized with 10 μg of rEA1 once a week (10 μg) or three times a week (total of 30 μg) for three consecutive weeks, with or without 10 μg of mucosal adjuvant, a synthetic double-stranded RNA, poly (I:C) (InvivoGen, San Diego, CA, USA). Controls for each experiment received PBS and/or adjuvant according to the corresponding immunization protocol. In a separate experiment, mice were administered with rEA1 in combination with rPA (10 μg) and poly (I:C).

2.6. Immunoassays for specific antibodies

Blood, saliva and feces were collected to monitor antibody titers. Saliva samples were collected following intraperitoneal (*i.p.*) injection with 100 μL of 1 mg/mL pilocarpine (Sigma–Aldrich, St. Louis, MO, USA). Fecal samples were mixed with PBS containing 0.1% sodium azide (1 mL/100 mg) and the supernatants were assayed. Specific antibody titers were measured by ELISA. Briefly, 100 ng of rEA1 or rPA was absorbed onto ELISA plates in carbonate buffer (pH 9.6) overnight at 4 °C. After washing with T-PBS and blocking, the plates were incubated with 2-fold serially-diluted samples for 1-h at room temperature. After three washes, plates were incubated with HRP-labeled goat anti-mouse IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) or IgA (Sigma–Aldrich) for 1 h. The reaction was visualized by the addition of 50 μL of BD OptEIA TMB Substrate Reagent (Becton Dickinson) for 30 min, and then stopped by adding an equal volume of 1 N H_2SO_4. The absorbance at 450 nm was measured using a plate reader (Tecan, Maennedorf, Switzerland). End point titers were defined as the reciprocal of the highest dilution that had an absorbance value greater than or equal to means ± SD of the pre-serum.

2.7. Mouse infection model of anthrax

We employed *i.p.* route of infection in this study to establish a systemic anthrax [16,17]. A 100-μL spore suspension (approximately 5×10^4 spores per mL) per mouse was challenged. The suspension was serially diluted and plated on Luria broth (LB) agar (MP Biomedicals, Santa Ana, CA, USA) in triplicate for accurate enumeration of the challenged spores. Survival of mice was monitored more than twice a day up to 14 days after challenge. To enumerate the number of bacteria in the lungs, livers and spleens, mice were sacrificed at days 2 and 3 after challenge. The isolated organs were homogenized in sterile distilled water (100 mg of organ/mL). Serially diluted homogenates were plated on LB agar for bacterial enumeration.

2.8. Statistical methods

Differences between the experimental groups and the control group were tested using Mann–Whitney *u*-test, and Kruskal–Wallis one-way ANOVA. The statistical significance of differences in