Biochemical and Biophysical Research Communications 421 (2012) 323–328

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

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

Makoto Uchida [a], Toshihiko Harada [a], Jargalsaikhan Enkhtuya [a], Akiko Kusumoto [a], Yoshiyasu Kobayashi [b], Shiori Chiba [b], Anselme Shyaka [a], Keiko Kawamoto [a,*]

[a] Laboratory of Food Microbiology and Immunology, Research Center for Animal Hygiene and Food Safety, Obihiro University of Agriculture and Veterinary Medicine, 2-11 Inada, Obihiro, Hokkaido 080-8555, Japan
[b] Department of Pathobiological Science, Obihiro University of Agriculture and Veterinary Medicine, 2-11 Inada, Obihiro, Hokkaido 080-8555, Japan

ARTICLE INFO

Article history:
Received 16 March 2012
Available online 9 April 2012

Keywords:
Anthrax
EA1
Mucosal immunization
Vaccine

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.

* Corresponding author. Fax: +81 155 49 5890.
E-mail address: kkeiko@obihiro.ac.jp (K. Kawamoto).

0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.04.007

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

survival curves of immunized group compared to control group was determined by the log rank (Mantel-Cox) test using Graph Pad Prism 5 (Graph Pad Software, La Jolla, CA, USA). A p-value of <0.05 was considered significant.

3. Results

3.1. Localization of EA1 protein

To examine the location of EA1, immunofluorescence study was performed on *B. anthracis* spores and vegetative bacilli using anti-EA1 polyclonal antibody (Fig. 1A). EA1 localized on the surface of encapsulated vegetative cells. Fluorescence indicating the presence of EA1 was also observed on the surface of spores. The binding of anti-rEA1 IgG to spore surfaces was further analyzed using flow cytometry, resulting in a clear shift of fluorescence intensity (Fig. 1B). These results indicate that EA1 was present on the surface of *B. anthracis* spores as well as on that of vegetative cells.

3.2. Protective immunity by rEA1

We immunized mice with rEA1 alone (10 or 30 μg), or together with poly (I:C) by the nasal route. Thirty microgram of rEA1 together with mucosal adjuvant poly (I:C) stimulated significant serum anti-EA1 antibody production (Table 1). Saliva and feces were collected to determine the secretory IgA response at sites proximal and distal to the immunization site. Similar to the serum antibody responses, co-administration of the adjuvant resulted in a significant secretary IgA response, while 10 μg of the antigen alone failed to induce a distal mucosal response. There was no significance in antibody responses between 10 and 30 μg of rEA1 when immunized with the adjuvant.

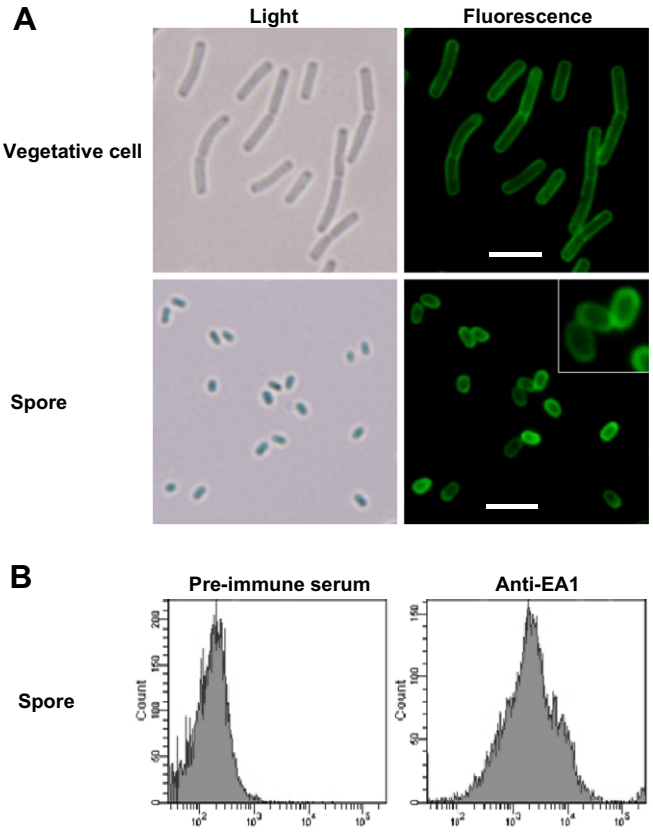

Fig. 1. Localization of EA1 on *B. anthracis*. (A) Spores and vegetative cells were immobilized with 4% paraformaldehyde, and immunostained with affinity-purified rabbit anti-rEA1 IgG, followed by Alexa Fluor 488-conjugated goat anti-rabbit IgG. Bars, 5 μm. (B) Flow cytometric analysis of EA1 on the surface of *B. anthracis* spores. Inactivated spores were immunostained using anti-rEA1 IgG. Purified IgG from pre-immune rabbit serum was used for the negative control.

Mice were *i.p.* challenged with a lethal number of *B. anthracis* spores (4.8×10^3) at 2 weeks after the last immunization (Fig. 2). Median survival time of control group, and immunized group without adjuvant was 2 and 3 days, respectively. The groups of EA1 vaccination with adjuvant notably delayed the time to death and improved survival compared with the control group ($P = 0.0128$ and $P = 0.0020$ for the survival curve of 10 and 30 μg, respectively). None of mice treated with poly (I:C) alone survived in lethal infection (data not shown). These results indicate that rEA1 immunization protects mice against lethal challenge from *B. anthracis*.

3.3. Protective effects of rEA1 combined with rPA against spore challenge

Serum and mucosal antibody responses to rEA1 and rPA were shown in Table 2. Significant ($p < 0.05$) levels of titers were detected in the saliva, fecal extract, and serum compared to that of control. Then, mice were infected with *B. anthracis* spores (4.9×10^3). As shown in Fig. 3A, the best protective effect was obtained by immunization with a mixture of both recombinant proteins ($p < 0.0001$ for the survival curve, compared to PBS control).

We enumerated bacteria in the lungs, livers, and spleens of the infected mice. Fig. 3B illustrates the results of bacteriological examination of the tissues. At 2 and 3 days after challenge, approximately 10^7–10^9 CFU/g of bacteria were present in the organs; however, the levels decreased to 10^5–10^6 CFU/g (rPA), 10^2 CFU/g (rEA1), 10^2 CFU/g or to undetectable levels (rEA1 plus rPA) in the immunized mice. The CFU/g from various organs in the rPA-immunized group were lower than that of the control group; however, substantial numbers of bacteria were detected. In contrast, the numbers of bacteria significantly decreased at day 2 in mice immunized with rEA1 or rEA1 plus rPA ($p < 0.0001$, compared to PBS or rPA). At day 3, the bacterial counts in these mice were below the detection limit (<100 CFU/g).

Histological examination showed that control mice manifested the most severe pathological condition. There was marked congestion of the marginal zone sinuses of the spleens in the control mice (Fig. 3C). Abundant karyorrhectic cellular debris was present throughout the red pulp. These pathological findings were barely observed in the mice doubly immunized with rEA1 and rPA. Large numbers of bacilli were identified by HE stain not only in the control mice, but also in the rPA-immunized mice (Fig. 3C).

4. Discussion

The present study yielded three important findings. First, nasal immunization by rEA1 with poly (I:C) not only induced both systemic and mucosal anti-EA1 antibody responses, but also protected mice against lethal challenge of *B. anthracis* spores. Second, double immunization of mice with rEA1 plus rPA by the nasal route conferred better protection against lethal anthrax infection than immunization with rEA1 or rPA alone. Lastly, *i.n.* administration of rEA1 contributed to a significant reduction of bacteria during the early phase of infection.

Ezzell and Abshire [18] have reported that immunization of guinea pigs with guanidine extracts of crude cell wall of *B. anthracis*, neither elicited protective antibody nor protected animals against anthrax. On the other hand, Zhang et al. [19] found that intradermal inoculation of a plasmid harboring *eag* (encoding EA1 protein) stimulated strong antibody production in mice, which indicates immunogenic potency of EA1. However, the protective effects of

Table 1
Effect of dose and mucosal adjuvant in specific antibody responses to EA1.[a]

Weekly dose of EA1[b] (μg)	Adjuvant[c]	EA1-specific titer (log_2)			
		Serum IgG	Serum IgA	Salivary IgA	Fecal IgA
10	–	16.6 ± 2.7	–[d]	6.4 ± 3.8	–
10	+	22.0 ± 1.5	12.5 ± 0.5	8.6 ± 1.4	9.3 ± 1.5
30	–	20.6 ± 1.3	12.5 ± 0.5	6.9 ± 1.1	7.3 ± 4.4
30	+	24.7 ± 1.1	14.5 ± 0.5	10.3 ± 1.2	10.4 ± 0.8

[a] Data is shown as mean (reciprocal log_2 endpoint titer) ± SD.
[b] Mice were *i.n.* received 10 μg of EA1 once a week (10 μg) or three times a week (total of 30 μg) for three consecutive weeks.
[c] 10 μg of poly(I:C) was used as mucosal adjuvant.
[d] Undetectable.

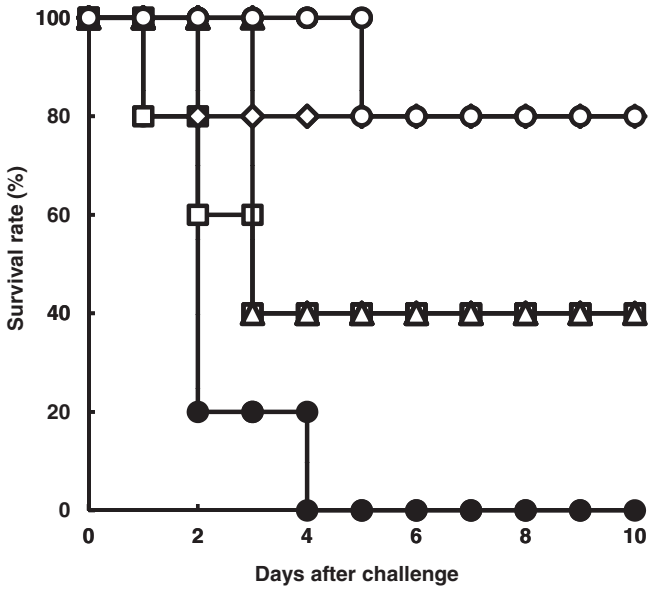

Fig. 2. Survival curves of EA1-immunized mice in *B. anthracis* infection. Challenge experiments were done at 2 weeks from last immunization. Mice (five per group) were *i.n.* immunized with rEA1 once a week (10 μg) with (open rhombus) or without adjuvant (open square), three times a week (10 μg × 3) with (open circle) or without adjuvant (open triangle). PBS was used for the controls (closed circle). Mice were *i.p.* infected with a lethal dose of virulent *B. anthracis* spores (4.8×10^3 spores/mouse).

Table 2
Antibody responses to EA1 and PA.[a]

Group[b]	Serum IgG	Serum IgA	Salivary IgA	Fecal IgA
PA-specific titer (log_2)				
PBS	–[c]	–	–	–
rPA	18.9 ± 0.9	12.8 ± 0.7	11.3 ± 1.4	8.0 ± 1.2
rEA1	–	–	–	–
rPA + rEA1	20.3 ± 1.2	16.9 ± 1.2	11.5 ± 0.9	8.6 ± 1.2
EA1-specific titer (log_2)				
PBS	–	–	–	–
rPA	–	–	–	–
rEA1	19.1 ± 0.8	15.1 ± 0.8	11.8 ± 0.7	8.5 ± 0.9
rPA + rEA1	20.5 ± 0.9	14.5 ± 0.5	11.6 ± 0.9	9.1 ± 1.5

[a] Data is shown as mean (reciprocal log_2 endpoint titer) ± SD.
[b] All mice were immunized together with mucosal adjuvant poly(I:C).
[c] Undetectable.

pure EA1 alone in an animal model of lethal spore challenge have not been demonstrated. The present study is the first report demonstrating the protective effect by EA1 against anthrax.

EA1 is a major S-layer component of *B. anthracis*, and a highly immunogenic surface antigen. However, there are conflicting reports in the literature regarding the presence of EA1 on the spores. Proteome analysis and ELISA using live attenuated spores showed that EA1 is also present as a spore protein [11–14,20,21]. However, EA1 has been reported to be difficult to remove during spore harvest; therefore, this protein is considered a contaminant during spore preparation in the studies using mAb (monoclonal antibody) SA26 [22,23]. Wang et al. generated mAbs using purified spores as an immunogen, and obtained several clones that specifically bound to the surface of *B. anthracis* spores and vegetative cells with high affinity and specificity. The proteins recognized by these mAbs were identified as EA1 by mass spectrometric analysis [21]. Love et al. [20] also demonstrated that single-chain antibodies to recombinant protein of *B. anthracis* EA1 bound to washed spores. We examined the reactivity of SA26. Although SA26 recognized purified rEA1 by immunoblot analysis, no binding of SA26 was detected on the surface of washed spores by flow cytometry and immunohistochemistry (data not shown). We interpret this discordance as a likely reflection of the differences in avidity and epitopes recognized by the antibodies.

Immunization with rEA1 greatly reduced the number of bacteria in infected organs and protected the mice from lethal infection. Since there was no significant difference in the reduced number of bacteria between rEA alone and rEA1 plus PA, rEA1-induced protective immunity may largely contribute to the rapid and effective elimination of bacteria from infected organs. Immunity to EA1 might contribute to a protective immunity at the early phase of infection, before massive multiplication and toxin production by vegetative cells take place. The mechanism of protection conferred by rEA1 immunization should be elucidated with further experiments.

We also observed a reduction in the number of bacteria in the mice immunized with rPA alone. It has been reported that PA protein is associated with *B. anthracis* spores, and anti-PA antibody enhances the phagocytic and sporicidal activities of macrophages [24]. Our observation might confirm previous findings. It has been reported that immunization with PA alone showed no or partial protection against full virulence strain [25–27]. In our results, protective effect by rPA was greater than as previously reported. It may have resulted from differences of immunization protocol such as dose of immunogens, mucosal adjuvant, and route of immunization.

Present human anthrax vaccines require intramuscular injection, which do not induce mucosal immunity at the initial site of infection in anthrax. Mucosal immunization not only elicits local immune protection, but also triggers systemic responses [28,29]. In our study, nasal delivery of rEA1 and rPA induced antigen-specific IgA responses at both proximal and other distal mucosal sites (Tables 1 and 2). Thus, this route of vaccination may be effective for not only inhalational anthrax, but also gastrointestinal and cutaneous anthrax. Poly (I:C) is shown to be effective and safe as a mucosal adjuvant when *i.n.* administered in previous study [30].

A number of animal infection models of anthrax have been described that use different routes of inoculation of *B. anthracis*

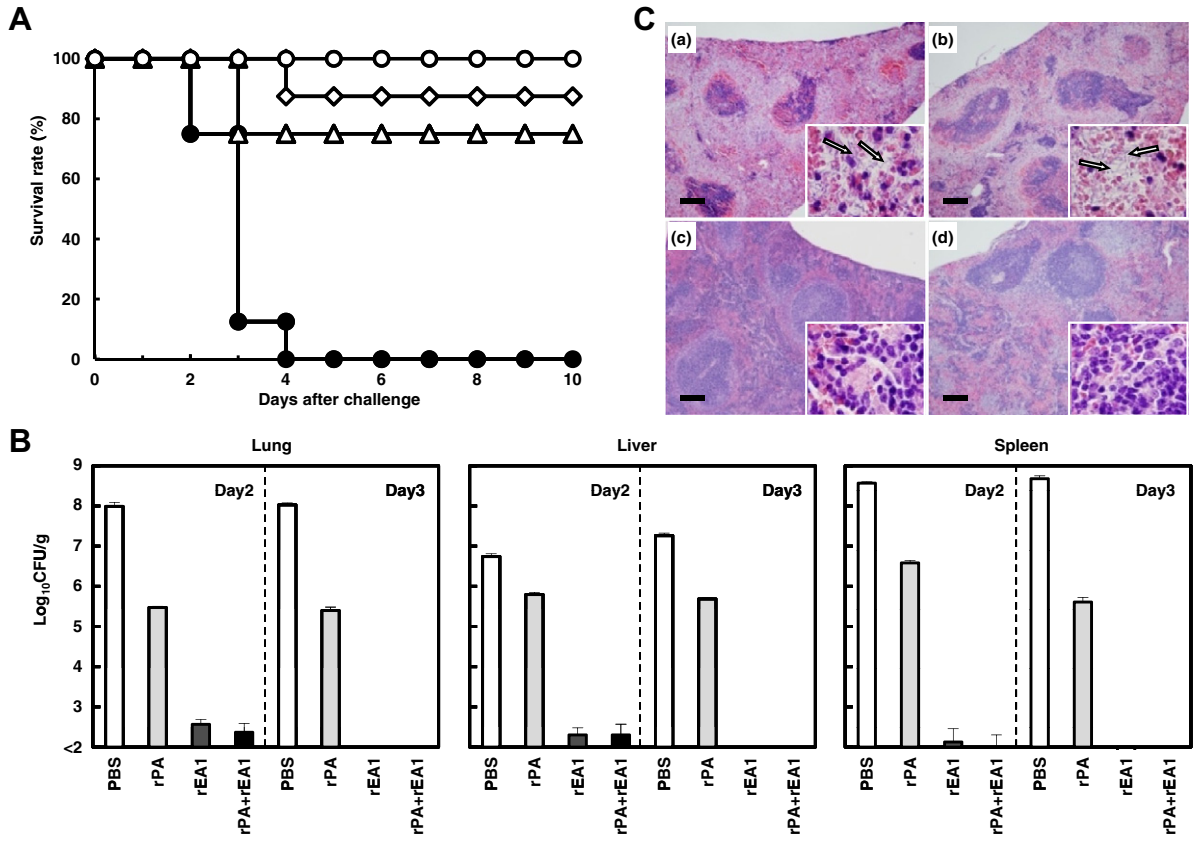

Fig. 3. Protective immunity of EA1 in combination with PA against anthrax. (A) Mice (eight per group) were *i.n.* administered with rPA (10 μg, open triangle), rEA1 (10 μg, open rhombus), and rPA and rEA1 (10 μg each, open circle) three times a week together with adjuvant. PBS with adjuvant was used for the controls (closed circle). Mice were *i.p.* challenged with virulent *B. anthracis* spores (4.9×10^3 spores/mouse). Representative results of two independent experiments are shown. (B) Clearance of bacteria in EA1-immunized mice. The livers, lungs, and spleens of mice were isolated at 2 and 3 days post-challenge. The data are presented as mean (SD). (C) Histology of spleens in immunized mice (HE stain). (a):PBS, (b):rPA, (c):rEA1, and (d):rPA and rEA1. Arrows indicated bacilli in the tissues. Bars, 200 μm.

spores. Intraperitoneal route of infection was used to establish a systemic anthrax in this study. For the study of inhalational anthrax, an aerosol challenge is a desirable model to reflect the natural route of infection. The protection effects by EA1 against inhalational anthrax by aerosol challenge should be elucidated with further experiments at appropriate specialized facilities which ensure the laboratory safety associated with fully virulent strains.

Despite early antibiotic treatment, it has been reported that inhalational anthrax develops into severe systemic illness. In our study, rEA1 immunization delayed the onset of disease. These findings suggest that immunization with rEA1 may increase the chance of successful antibiotic treatment after exposure to *B. anthracis* spores. Currently, a full 60-day course of antibiotic treatment in combination with AVA vaccination is recommended for people who have been exposed to *B. anthracis* [31]. Since rEA1 immunization effectively eliminates the spore and vegetative forms of the bacteria, it is possible that rEA1 treatment can be used in combination with current therapy to shorten the period of prolonged antibiotic use. Targeting spores, vegetative cells, and toxins may be more useful for multiple protections. Our results give insight into the design of safer mucosal component vaccines to elicit the appropriate protective immune response to *B. anthracis*.

Acknowledgments

This study was supported by a Grant-in-Aid for Scientific Research (00001987-01, 12575029, and 13576013) from the Japanese Society for the Promotion of Science and a Grant from the Ministry of Health, Labor, and Welfare (Research on Emerging and Re-emerging Infectious Diseases). We would like to thank Ms. Masumi Kagawa, Ms. Haruko Kariya and Ms. Mio Yagihashi for their technical assistance.

References

[1] T.C. Dixon, M. Meselson, J. Guillemin, et al., Anthrax, New England Journal of Medicine 341 (1999) 815–826.
[2] J.A. Young, R.J. Collier, Anthrax toxin: receptor binding, internalization, pore formation, and translocation, Annual Review of Biochemistry 76 (2007) 243–265.
[3] J.N. Tournier, R.G. Ulrich, A. Quesnel-Hellmann, et al., Anthrax, toxins and vaccines: a 125-year journey targeting *Bacillus anthracis*, Expert Rev Anti Infect Ther 7 (2009) 219–236.
[4] N. Marano, B.D. Plikaytis, S.W. Martin, et al., Effects of a reduced dose schedule and intramuscular administration of anthrax vaccine adsorbed on immunogenicity and safety at 7 months: a randomized trial, Jama 300 (2008) 1532–1543.
[5] G.C. Whiting, S. Rijpkema, T. Adams, et al., Characterisation of adsorbed anthrax vaccine by two-dimensional gel electrophoresis, Vaccine 22 (2004) 4245–4251.
[6] M. Mock, A. Fouet, Anthrax, Annu Rev Microbiol 55 (2001) 647–671.
[7] D.K. Bhalla, D.B. Warheit, Biological agents with potential for misuse: a historical perspective and defensive measures, Toxicol Appl Pharmacol 199 (2004) 71–84.
[8] S. Cohen, I. Mendelson, Z. Altboum, et al., Attenuated nontoxinogenic and nonencapsulated recombinant *Bacillus anthracis* spore vaccines protect against anthrax, Infect Immun 68 (2000) 4549–4558.
[9] S. Mesnage, E. Tosi-Couture, M. Mock, et al., Molecular characterization of the *Bacillus anthracis* main S-layer component: evidence that it is the major cell-associated antigen, Mol Microbiol 23 (1997) 1147–1155.

[10] A. Fouet, S. Mesnage, E. Tosi-Couture, et al., Bacillus anthracis surface. capsule and S-layer, J Appl Microbiol 87 (1999) 251–255.
[11] V.G. Delvecchio, J.P. Connolly, T.G. Alefantis, et al., Proteomic profiling and identification of immunodominant spore antigens of *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis*, Appl Environ Microbiol 72 (2006) 6355–6363.
[12] C. Redmond, L.W. Baillie, S. Hibbs, et al., Identification of proteins in the exosporium of *Bacillus anthracis*, Microbiology 150 (2004) 355–363.
[13] E.M. Lai, N.D. Phadke, M.T. Kachman, et al., Proteomic analysis of the spore coats of *Bacillus subtilis* and *Bacillus anthracis*, J Bacteriol 185 (2003) 1443–1454.
[14] H. Liu, N.H. Bergman, B. Thomason, et al., Formation and composition of the *Bacillus anthracis* endospore, J Bacteriol 186 (2004) 164–178.
[15] I. Uchida, S. Makino, C. Sasakawa, et al., Identification of a novel gene, dep, associated with depolymerization of the capsular polymer in *Bacillus anthracis*, Mol Microbiol 9 (1993) 487–496.
[16] J. Enkhtuya, K. Kawamoto, Y. Kobayashi, et al., Significant passive protective effect against anthrax by antibody to *Bacillus anthracis* inactivated spores that lack two virulence plasmids, Microbiology 152 (2006) 3103–3110.
[17] K. Sinha, R. Bhatnagar, GroEL provides protection against *Bacillus anthracis* infection in BALB/c mice, Mol Immunol 48 (2010) 264–271.
[18] J.W. Ezzell Jr., T.G. Abshire, Immunological analysis of cell-associated antigens of *Bacillus anthracis*, Infect Immun 56 (1988) 349–356.
[19] Y. Zhang, J. Qiu, Y. Zhou, et al., Plasmid-based vaccination with candidate anthrax vaccine antigens induces durable type 1 and type 2 T-helper immune responses, Vaccine 26 (2008) 614–622.
[20] T.E. Love, C. Redmond, C.N. Mayers, Real time detection of anthrax spores using highly specific anti-EA1 recombinant antibodies produced by competitive panning, J Immunol Methods 334 (2008) 1–10.
[21] D.B. Wang, R. Yang, Z.P. Zhang, et al., Detection of *B. anthracis* spores and vegetative cells with the same monoclonal antibodies, PLoS One 4 (2009) e7810.
[22] D.D. Williams, C.L. Turnbough Jr., Surface layer protein EA1 is not a component of *Bacillus anthracis* spores but is a persistent contaminant in spore preparations, J Bacteriol 186 (2004) 566–569.
[23] M.K. Swiecki, M.W. Lisanby, F. Shu, et al., Monoclonal antibodies for *Bacillus anthracis* spore detection and functional analyses of spore germination and outgrowth, J Immunol 176 (2006) 6076–6084.
[24] S. Welkos, S. Little, A. Friedlander, et al., The role of antibodies to *Bacillus anthracis* and anthrax toxin components in inhibiting the early stages of infection by anthrax spores, Microbiology 147 (2001) 1677–1685.
[25] U.K. Hahn, R. Boehm, W. Beyer, DNA vaccination against anthrax in mice-combination of anti-spore and anti-toxin components, Vaccine 24 (2006) 4569–4571.
[26] F. Brossier, M. Levy, M. Mock, Anthrax spores make an essential contribution to vaccine efficacy, Infect Immun 70 (2002) 661–664.
[27] Y.P. Gauthier, J.N. Tournier, J.C. Paucod, et al., Efficacy of a vaccine based on protective antigen and killed spores against experimental inhalational anthrax, Infect Immun 77 (2009) 1197–1207.
[28] M.R. Neutra, P.A. Kozlowski, Mucosal vaccines: the promise and the challenge, Nat Rev Immunol 6 (2006) 148–158.
[29] M. Jertborn, I. Nordstrom, A. Kilander, et al., Local and systemic immune responses to rectal administration of recombinant cholera toxin B subunit in humans, Infect Immun 69 (2001) 4125–4128.
[30] T. Ichinohe, I. Watanabe, S. Ito, et al., Synthetic double-stranded RNA poly(I:C) combined with mucosal vaccine protects against influenza virus infection, J Virol 79 (2005) 2910–2919.
[31] E.J. Stern, K.B. Uhde, S.V. Shadomy, et al., Conference report on public health and clinical guidelines for anthrax, Emerg Infect Dis 14 (2008).