Significant passive protective effect against anthrax by antibody to *Bacillus anthracis* inactivated spores that lack two virulence plasmids

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The protective-antigen (PA)-based cell-free vaccine is the only vaccine licensed for use against *Bacillus anthracis* infection in humans. Although the PA shows strong immunogenicity, the capsule or spore-associated somatic antigens may be important as additional vaccine targets for full protection against anthrax. In this study, the protective effect of spore-associated antigens against *B. anthracis* infection was determined. Rabbits were immunized with formalin-fixed spores of a non-toxigenic unencapsulated *B. anthracis* strain that lacked the two virulence plasmids pXO1 and pXO2, and the protective effects of the immune antibody were evaluated. Immunostaining and Western blot analysis revealed that the anti-*B. anthracis* (anti-BA)-spore IgG specifically bound to the surface of spores or endospores of *B. anthracis*, but not to vegetative cells, or closely related *Bacillus* species, such as *Bacillus cereus*, *Bacillus subtilis* and *Bacillus thuringiensis*. Passively transferred anti-BA-spore IgG protected mice from intraperitoneal challenge with a lethal dose of fully virulent *B. anthracis* spores, and increased the survival rate in a dose-dependent manner. Pre-incubation of spores with antibody also reduced their infectivity in a dose-dependent manner. The number of bacteria (c.f.u.) in spleens and livers of infected mice was significantly lower in antibody-treated mice than in untreated mice. Treatment with anti-BA-spore IgG also inhibited the germination of spores in J774.1 macrophages, suggesting that opsonization of spores promotes phagocytosis and subsequent killing by macrophages. These results indicate the usefulness of spore surface antigens as vaccine targets. In combination with major virulence factors such as the PA, spore-associated antigens may offer a safer and more effective multicomponent vaccine for *B. anthracis* infection.

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INTRODUCTION

Bacillus anthracis, a Gram-positive spore-forming bacterium, is the causative agent of anthrax. Dormant spores are highly resistant to stressful conditions, and they are able to survive for decades in the environment. Pathogenesis of inhalational and cutaneous anthrax includes the entry of *B. anthracis* spores into the host, followed by germination, bacterial multiplication, dissemination and toxin production. Macrophages play an important role in the early stages of anthrax, and constitute a primary site for spore germination (Guidi-Rontani *et al.*, 1999). However, the spore germination mechanism in the presence of host

immunity has not yet been fully elucidated. The virulence of *B. anthracis* infection is mainly mediated by two virulence plasmids, termed pXO1 and pXO2 (Okinaka *et al.*, 1999a, b). pXO1 encodes three toxin components: lethal factor (LF), (o)edema factor (EF) and protective antigen (PA). pXO2 encodes an anti-phagocytic capsule that consists of poly- γ -D-glutamic acid polymer (Makino *et al.*, 1988; Mikesell *et al.*, 1983; Uchida *et al.*, 1986). The PA binds to the surface of receptors expressed on target cells, and serves as a carrier for LF and EF to deliver toxins into the host cell cytosol (Nourez *et al.*, 2002).

The natural course of anthrax infection commonly occurs in wild and domestic animals through the uptake of spores that remain viable in contaminated soil for many years (Turnbull, 2002). Humans are an occasional host, but may also become infected upon exposure to spores from

Abbreviations: anti-BA, anti-*Bacillus anthracis*; AVA, anthrax vaccine adsorbed; EF, (o)edema factor; i.p., intraperitoneal(ly); LF, lethal factor; PA, protective antigen; p.i., post-infection.

infected animals or their tissues (Mock & Fouet, 2001). However, after the anthrax attack via the US postal system in 2001, the potential use of spores as bioweapons has been a public concern, which has resulted in a heightened interest in the pathogenesis of anthrax, and development of a vaccine against it (Inglesby *et al.*, 2002; Jernigan *et al.*, 2001, 2002).

The anthrax vaccines currently available are: anthrax vaccine adsorbed (AVA) for humans; live attenuated spore vaccines derived from encapsulated strains, for veterinary use only (Brey, 2005); and the UK anthrax vaccine, the anthrax vaccine precipitated, which has been in use for over 40 years (Williamson *et al.*, 2005). The live spore STI vaccine has been used in Russia for many years in humans (Romanov, 1980). Many researchers have demonstrated that high titres of anti-PA antibody also give passive protection in rabbits, guinea pigs and mice (Beedham *et al.*, 2001; Kobiler *et al.*, 2002; Pitt *et al.*, 2001). Antibodies to the PA may neutralize toxin activity by blocking the binding of the PA to its receptor, and/or by the formation of a toxin complex. Due to its function and immunogenicity, the PA has been an attractive target for vaccine development. Protection can also be induced by immunization with purified PA, the whole organism, or DNA plasmids carrying recombinant PA (Gu *et al.*, 1999; Iacono-Connors *et al.*, 1991; Ramirez *et al.*, 2002; Rhie *et al.*, 2005; Vodka & Leppla, 1983; Watson *et al.*, 2005). However, more effective vaccines giving full protection against anthrax may require additional bacterial components, including the capsule or somatic antigens.

In this study, we elucidate the immunogenicity of spore-associated antigens. To eliminate the possible involvement of the major virulence factors, such as the PA, LF, EF and capsule, we immunized the rabbits with formalin-fixed spores of the *B. anthracis* Pasteur II strain, which lacks the pXO1 and pXO2 virulence plasmids. Purified antibodies from the rabbit serum recognized the surface molecules expressed on the spores of both the fully virulent and the plasmidless *B. anthracis* strains, but not on those of other closely related *Bacillus* species. In immunoblot analysis, several spore-specific proteins were detected using anti-BA-spore IgG. The passive transfer of purified anti-BA-spore IgG into naive mice conferred protection against lethal doses of *B. anthracis* challenge, and the effect was dose dependent. Treatment with anti-BA-spore IgG also promoted the killing of the bacteria by macrophages, and caused a reduction in the number of germinated spores. These results suggest that somatic antigens expressed on the spore surface may be useful for the development of a new and more effective anthrax vaccine.

METHODS

Bacterial strains and spore preparation. *B. anthracis* Pasteur II (Uchida *et al.*, 1993), a fully virulent (pXO1⁺, pXO2⁺) strain, its derivative plasmidless (pXO1⁻, pXO2⁻) strain, and the Sterne (pXO1⁺, pXO2⁻) strain, were used in this study. Other *Bacillus* species, including *B. cereus*, *B. subtilis* and *B. thuringiensis* strains

maintained in our laboratory, were also used. For spore preparation, fresh cultures grown in Luria–Bertani broth (LB broth) were spread on a low-nutrient agar [0.8% nutrient broth and 1% yeast extract (Difco), 0.05% MgSO₄·7H₂O, 0.2% KCl, 1.6% agar, 0.1% 0.1 M MnSO₄, 0.1% glucose, 0.1% 1 M Ca(NO₃)₂, 0.1% 1 mM FeSO₄], and incubated at 32 °C for 7 days. After 7 days, the spores were collected, and washed 10 times with chilled sterile distilled water. To kill the vegetative cells, the suspension was incubated at 85 °C for 20 min. The suspension was then washed several times with distilled water, and the spores were examined by Gram staining. Spore numbers were enumerated using the plate counting method, and the spores were preserved at 4 °C in sterile distilled water until use.

Antibody production and purification. *B. anthracis* (pXO1⁻, pXO2⁻) spores were inactivated overnight with 4% paraformaldehyde (Sigma), and washed twice with PBS. After ensuring that spores had been inactivated, by testing for growth on LB agar at 37 °C for 24 h, they were used as an immunogen. Two Japanese white rabbits (Charles River), weighing about 2 kg each, were subcutaneously immunized four times, at 2 week intervals, with a mixture containing 10⁷ spores and Freund's complete or incomplete adjuvant (Sigma) to induce sufficient antibody production. The antibody titre was monitored by ELISA. Rabbit blood was collected 2 days after the last immunization, and IgG was purified from immune serum by using a protein G-conjugated column (Mab Trap kit; Amersham Biosciences).

Specificity of anti-BA-spore IgG. The spores prepared from *Bacillus* species were fixed overnight with 1% paraformaldehyde on microscopic glass slides until completely dry. The inactivated and immobilized spores were treated with PBS containing 0.5% Tween 20 (PBS-T) and 3% skim milk to block non-specific binding sites, and washed three times with PBS-T. The slides were incubated with 100 µl anti-BA-spore IgG (10 µg ml⁻¹) for 1 h at room temperature in a humid chamber. This was followed by washing, and 100 µl Alexa Fluor 488-conjugated goat anti-rabbit IgG at a dilution of 1:1000 (Molecular Probes) was added to each slide, which was then incubated for 30 min in a dark place. After washing, the slides were mounted in aqueous mounting medium containing an anti-fading agent (Biomedica), and sealed with nail polish. The samples were observed under a fluorescence microscope (Olympus BX51; Opelco), and the images were visualized using DP70-BSW software.

Spore protein preparation, and Western blot assay. Spore protein samples were prepared as described by Kim *et al.* (2004). Briefly, the spores were suspended in UDS buffer [6 M urea (Sigma), 50 mM DTT and 1% SDS (Wako)], and incubated at 37 °C for 20 min. The suspensions were centrifuged at 10 000 g for 6 min, and the supernatants were collected. The incubation was repeated, and the supernatants were pooled. The protein concentrations of samples were measured by spectrophotometry at A₂₈₀. The same amounts of samples (<10 µg) were mixed with sample buffer, and, after boiling, the proteins were separated in an electrophoretic cell (Mini Protean 3; Bio-Rad) at a constant current (20 mA per gel) for 1 h. The separated proteins were transferred to a PVDF membrane (Millipore) in an electrophoretic semi-dry transfer cell (Trans-Blot SD; Bio-Rad) at 15 V for 1 h. The PVDF membrane was blocked by overnight incubation in PBS-T containing 3% skim milk. After washing three times with PBS-T, the membrane was incubated at room temperature for 1 h with anti-BA-spore IgG diluted 1:40 000 in PBS-T containing 0.3% skim milk, and washed as described above. The membrane was then incubated at room temperature for 1 h with peroxidase-conjugated anti-rabbit IgG (Amersham Biosciences) diluted 1:10 000 in PBS-T containing 0.3% skim milk. The blots were washed in PBS-T, and bound peroxidase enzyme was detected with the ECL-Plus Western blotting detection reagent (Amersham Biosciences).

Mouse infection. Four-week-old female ICR mice (Clea) were separated into groups containing equal numbers, and were injected intraperitoneally (i.p.) with 200 μ l PBS containing 5×10^3 fully virulent *B. anthracis* spores, in the presence or absence of anti-BA-spore IgG. Before administration, the spores were incubated with a single dose of 0.01, 0.1 or 0.5 mg anti-BA-spore IgG per mouse, or PBS as a control, on ice for 30 min, and washed twice with sterile PBS to remove excess antibodies. The infection was performed in two separate experiments, and survival of the mice was monitored twice a day for up to 8 days post-infection (p.i.). In order to detect bacteria in the spleens and livers of mice, at 44, 68, 92, 116 and 140 h p.i. (five mice per group), the spleens and livers were isolated, and homogenized in sterile distilled water. Appropriately diluted homogenates were plated on tryptic soy agar (Difco) for enumeration of bacteria.

Passive immunization. Six-week-old female ICR mice (Clea) were injected with anti-BA-spore IgG or PBS at different times pre- and post-challenge (i.p.) of 5×10^3 fully virulent *B. anthracis* spores. For the pre-exposure prophylaxis, the mice (seven per group) were administered i.p. with a single dose of 0.01, 0.1 or 0.5 mg anti-BA-spore IgG at 30–40 min prior to spore challenge. PBS was given to the control groups. To evaluate the post-exposure efficacy, mice were challenged with the spores, and then injected i.p. with a single dose 0.01, 0.1 or 0.5 mg anti-BA-spore IgG, or PBS as a control, at 24, 36 and 48 h post challenge. All mice were observed twice daily for 8 days post-challenge.

Histopathology. The organs of mice that had been infected with pre-incubated anthrax spores, in the presence or absence of IgG (a single dose of 0.01, 0.1 or 0.5 mg per mouse), as described above, were used for histopathological examination. The mice were sacrificed at 44 h p.i., and the spleen and kidneys were removed and then fixed in 4% paraformaldehyde for 7 days. The sectioned tissues (5 μ m) were stained with haematoxylin and eosin, by using conventional protocols, and observed under the microscope (Olympus BX51; Opelco). The images were visualized using DP70-BSW software.

Effect of anti-BA-spore IgG on macrophage and spore interaction. The J774.1 cell line (ATCC TIB-106) was cultured for 3–4 days at 37 °C in 5% CO₂ in RPMI medium containing 10% fetal bovine serum (Sigma), 200 mM L-glutamine, 50 U penicillin ml⁻¹ and 50 μ g streptomycin ml⁻¹. The cells were then washed, suspended in sterile PBS, and 2×10^5 cells dispensed into a

siliconized microcentrifuge tube (Fisherbrand). The spores were incubated on ice for 30 min with 0.01, 0.1 or 0.5 mg anti-BA-spore IgG or PBS, and washed twice with sterile PBS to remove excess antibodies. Then they were allowed to interact with macrophages (m.o.i. 1:10) for 30 min at 37 °C in 5% CO₂. The cells were then washed three or four times with PBS, and suspended in RPMI medium containing 10 μ g gentamicin ml⁻¹, and incubated for 30 min at 37 °C in 5% CO₂. After each washing step, the supernatants were collected for counting of non-interacting spores. After completion of the incubation, the samples were heat inactivated at 75 °C for 30 min to kill any vegetative bacilli. Intracellular ungerminated spores were then enumerated using the plate-counting method and tryptic soy agar plates. Enumeration of spores that had interacted with macrophages was also performed.

RESULTS

Specificity of anti-BA-spore IgG

We evaluated the specificity of rabbit anti-BA-spore IgG by fluorescence immunostaining. As shown in Fig. 1, distinct fluorescence, indicating the specific binding of antibody, was observed in the spores of the pXO1⁻, pXO2⁻ strain and the pXO1⁺, pXO2⁺ strain. Fluorescence was not detected in spores of *B. cereus*, *B. subtilis* and *B. thuringiensis* (data not shown). Immunofluorescence of anti-BA-spore IgG was localized on the surface of *B. anthracis* spores and endospores, but not in the vegetative cells. These results suggest that anti-BA-spore IgG reacts specifically with *B. anthracis* spores.

To identify the molecules recognized by the anti-BA-spore IgG, spore protein samples were prepared from three strains (pXO1⁻, pXO2⁻ strain, pXO1⁺, pXO2⁺ Pasteur II strain and pXO1⁺, pXO2⁻ Sterne strain), and subjected to immunoblotting. As shown in Fig. 2, eight protein bands were found to be common to all the spores; however, the intensity of each band was different among the strains. In spores of the pXO1⁻, pXO2⁻ strain, two bands with

Fig. 1. Immunostaining of *B. anthracis* spores and endospores in a vegetative cell. Spores of two strains of *B. anthracis* (pXO1⁻, pXO2⁻ strain, and pXO1⁺, pXO2⁺ strain) were inactivated and immobilized by overnight fixation with 1% paraformaldehyde, and then immunostained with affinity-purified rabbit anti-BA-spore IgG. Fluorescence and light images were taken at $\times 100$ magnification, while merged images were enlarged. The images were visualized using DP70-BSW software. Bars, 50 μ m.

Fig. 2. Western blot analysis of the *B. anthracis* spore proteins recognized by anti-BA-spore IgG. Whole spore proteins were prepared in UDS lysis buffer, and separated on a 12% SDS gel. Lanes: 1, pXO1⁻, pXO2⁻; 2, pXO1⁺, pXO2⁺ Pasteur II; 3, pXO1⁺, pXO2⁻ Sterne.

molecular masses of >250 and 25 kDa were prominent, while the other six bands were faint (lane 1). In the pXO1⁺, pXO2⁺ Pasteur II strain, and the pXO1⁺, pXO2⁻ Sterne strain, the anti-BA-spore IgG identified essentially the same proteins as in the pXO1⁻, pXO2⁻ strain. However, the bands that showed up weakly for the pXO1⁻, pXO2⁻ strain were stronger in the Pasteur II and Sterne strains, despite the protein samples being the same concentration for all the strains. In the Sterne strain, the band corresponding to 25 kDa was weaker than in the other two strains, and additional protein bands of about 37 and 15 kDa were detected.

Protective effect of anti-BA-spore IgG against anthrax infection in mice

To examine the protective effect of anti-BA-spore IgG in mice, 12 mice in each group were injected i.p. with a lethal dose of *B. anthracis* spores (5×10^3), and various concentrations of the anti-BA-spore IgG (a single dose of 0.01, 0.1 and 0.5 mg per mouse). All the mice in the control group died within 3 days of the anthrax spore challenge (Fig. 3). In contrast, the antibody-treated mice survived longer, and treatment with the anti-BA-spore IgG resulted in a significant decrease in mortality. The experiment was performed in triplicate, and the results were combined.

Fig. 3. Survival rates of mice with *B. anthracis* infection. The data show the combined results of three independent experiments. Mice (12 per group) were infected i.p. with a lethal dose of virulent *B. anthracis* spores (5×10^3 spores per mouse) that had been pre-incubated with 0, 0.01, 0.1 or 0.5 mg anti-BA-spore IgG. The survival of mice was monitored twice daily for up to 8 days p.i.

Doses of 0.5 and 0.1 mg IgG produced survival rates of 75 and 25 %, respectively, at 8 days p.i. The results suggest that antibody specific to *B. anthracis* spores has a protective effect against anthrax in mice. The surviving mice were sacrificed, and bacteria were not detected in the liver and spleen.

We examined the numbers of bacteria in the livers and spleens of the sacrificed mice during infection (Fig. 4). When the mice were inoculated with 5×10^3 spores i.p., the number of bacilli in the livers and spleens reached levels of approximately 10^9 or 10^8 c.f.u. g⁻¹ at 44 h p.i. in the group infected with spores, and none of the mice had survived on day 3 p.i. In contrast, the numbers of c.f.u. in the organs of the antibody-treated mice were reduced in a dose-dependent manner. The difference in mortality between the control and antibody-treated mice paralleled the change in the bacterial count in the organs. In the spleens and livers of surviving mice treated with 0.1 or 0.5 mg antibody, it was found that all bacteria had been eliminated by day 6 p.i.

Histopathological examination of paraformaldehyde-fixed paraffin-embedded spleens and kidneys of infected mice was also conducted. Infected mice that had not had antibody

Fig. 4. Inhibitory effect of anti-BA-spore IgG on bacterial proliferation. Mice (five per group) were infected i.p. with a lethal dose of virulent *B. anthracis* spores (5×10^3 spores per mouse) that had been pre-incubated with 0 ($\diamond$), 0.01 ($\square$), 0.1 ($\triangle$) or 0.5 mg ($\circ$) anti-BA-spore IgG. From 44 h p.i., the spleens and livers were removed (see Methods) for bacterial counts. The data are shown as means ($\pm$ SD).

treatment showed infectious splenitis to varying degrees (Fig. 5a). In the spleen, at 44 h p.i., numerous karyorrhectic lymphocytes were diffusely observed in the splenic follicles and red pulp. Abundant starry sky figures by tingible macrophages were also detected in the splenic follicles. Furthermore, there was considerable lymphoid depletion with congestion, dilation of splenic sinuses, and neutrophil infiltration in the splenic red pulp, especially at the perifollicular areas. Numerous rod-shaped bacilli were also identified by haematoxylin and eosin stain, most often abundant in the perifollicular areas. In the kidneys, numerous bacilli were noted in congested blood capillaries at the glomerular tufts and interstitium (Fig. 5c). However, these pathological findings were not as marked as those in the organs of the antibody-treated mice, including those in the low-dose antibody-treated group (0.01 mg). Furthermore, clusters of bacilli were not observed in any of the tissues from the antibody-treated mice (Fig. 5b, d).

Passive protective effect of anti-BA-spore IgG against *B. anthracis* spore challenge in mice

The ability of the anti-BA-spore IgG to protect against anthrax was tested in mice pre- and post-challenge with *B. anthracis* spores. Pre-challenge administration of anti-BA-spore IgG was performed 30–40 min prior to anthrax spore challenge: mice ($n=7$) were given a single i.p. injection of 0.01, 0.1 or 0.5 mg IgG, and the control group ($n=7$)

received 100 μ l PBS. In the control group, all mice had died by day 3 post-challenge (Fig. 6a). In contrast, the mice that had received anti-BA spore IgG, including those that received a single dose of 0.01 mg only, survived longer than the control mice. Anti-BA-spore IgG at 0.1 or 0.5 mg prolonged the lives of the mice, and produced survival rates of up to 14.2 and 42.8 %, respectively. The post-challenge administration of anti-BA-spore IgG was performed 24 ($n=5$), 36 ($n=6$) and 48 ($n=8$) h after anthrax spore challenge: mice were given a single i.p. injection of 0.01, 0.1 or 0.5 mg IgG, and the control group received 100 μ l PBS. When anti-BA-spore IgG administration was performed 24 h post-anthrax-spore challenge, the mice in the control group had died within 3 days, while the mice in the IgG-

Fig. 5. Photomicrographs of spleens (a, b) and kidneys (c, d) from mice infected with *B. anthracis* spores (5×10^3 spores per mouse). (a, c) Tissues from a control mouse infected with spores; (b, d) tissues from a mouse infected with spores, and treated with 0.5 mg anti-BA-spore IgG. In sections from the control mouse, necrotizing splenitis (a) and dilation of glomerular capillaries (c) were evident, with clusters of bacilli [a (inset), c]. In sections from the antibody-treated mouse, there were no pathological findings (b, d), and clusters of bacilli were not observed [b (inset), d]. The sections were stained with haematoxylin and eosin, and images were visualized using DP70-BSW software.

Fig. 6. (a) Survival rates of mice injected with anti-BA-spore IgG prior to *B. anthracis* spore challenge. Mice (seven per group) were injected with a single dose (i.p.) of 0.01 (continuous line), 0.1 (dashed line) or 0.5 mg (bold continuous line) anti-BA-spore IgG, or PBS as a control (dotted line), 30–40 min prior to spore challenge (5×10^3 spores per mouse). Experiments were performed twice independently. (b) Survival rates of mice treated with anti-BA-spore IgG 24 h after exposure to *B. anthracis* spores. Mice (five per group) were challenged i.p. with a lethal dose of virulent *B. anthracis* spores (5×10^3 spores per mouse), before receiving 0.01 (continuous line), 0.1 (dashed line) or 0.5 mg (bold continuous line) anti-BA-spore IgG, or PBS as a control (dotted line).

treated groups survived a day longer (Fig. 6b). When antibody (0.01, 0.1 or 0.5 mg) administration was performed at 36 and 48 h post-anthrax-spore challenge, all mice treated with the anti-BA-spore IgG had died by day 3, regardless of which group they were in, and this was the same for the control mice (data not shown).

Inhibitory effect of anti-BA-spore IgG on germination

Macrophages may play a dual role in *B. anthracis* infection: as an intracellular niche permissive for spore germination, and as effector cells for clearing spores. Therefore, we studied the effect of anti-BA-spore IgG on the germination of spores in macrophages. J774.1 macrophage cells (2.5×10^5) were cultured with *B. anthracis* spores (m.o.i. 1:10) in the presence of various amounts of anti-BA-spore IgG (0.01, 0.1 and 0.5 mg). In the untreated cells, intracellular ungerminated spores were approximately 10% of the total interacting spores. However, in the cells treated with various amounts of anti-BA-spore IgG, numbers of ungerminated spores were significantly higher than for the untreated group. In the 0.01 mg IgG-treated group, ungerminated spores were approximately 15% of the total interacting spores, while 27 and 50% of the total interacting spores were not able to germinate in groups treated with 0.1 and 0.5 mg IgG, respectively. The results indicate that the anti-BA-spore IgG has an inhibitory effect on spore germination in a dose-dependent manner.

DISCUSSION

Studies on *B. anthracis* by other authors have demonstrated that the efficacy of PA-based vaccines is not satisfactory when compared with the live attenuated spore vaccine (Little & Knudson, 1986; Welkos & Friedlander, 1988). Vaccination studies with live or attenuated pXO1⁺, pXO2⁻ spores have shown that immune responses against the *B. anthracis* spore and PA lead to greatly improved protection in comparison with responses to PA alone (Brossier *et al.*, 2002; Cohen *et al.*, 2000; Welkos & Friedlander 1988). This could be because PA-based vaccines target toxæmia rather than bacteraemia. The capsular material itself has been shown to be poorly immunogenic, and thus not suitable as a vaccine component; however, conjugation of poly- γ -D-glutamic acid as protein carrier makes it more effective as a vaccine (Joyce *et al.*, 2006). These findings indicate the complexity of anthrax pathogenesis, and also suggest that PA, capsule and somatic antigens are essential for full protection against the disease. Chromosomally encoded molecules expressed on the spore surface have been considered as possible candidates for vaccine development; however, there have been no reports evaluating the immunogenicity of spores derived from virulence-plasmid-negative *B. anthracis* strains. Since the efficacy of a spore vaccine has been demonstrated for strains harbouring pXO1⁺ (Brossier *et al.*, 2002), it is difficult to elucidate the possible contribution of spore antigens in protective

immunity. Thus, to assess the immunogenicity of spore surface proteins, we used pXO1⁻, pXO2⁻ *B. anthracis* spores, and conducted experiments to evaluate the antibodies obtained from the animals that had been immunized with formalin-fixed, plasmidless spores. The immune antibody specifically reacted with *B. anthracis* spores or endospores, but not with vegetative cells, and it protected mice from lethal challenge with fully virulent *B. anthracis* in the passive immunization test (Fig. 6a). We also examined whether antibodies in the anti-BA-spore polyclonal IgG could bind to PA, but no reactivity was observed in an ELISA (data not shown).

The anti-BA-spore IgG specifically reacted with the surface of *B. anthracis* spores, but not with spores of related bacilli, such as *B. cereus*, *B. subtilis* and *B. thuringiensis*, as shown by immunostaining (Fig. 1). As shown in Fig. 2, the polyclonal antibody recognized some spore proteins of pXO1⁻, pXO2⁻ *B. anthracis* spores, as well as those of the fully virulent pXO1⁺, pXO2⁺ Pasteur II strain, and the pXO1⁺, pXO⁻ Sterne strain. These results indicate that these spore-associated immunogenic antigens may be encoded by genes on the *B. anthracis* chromosome, and that they may be present on the spore surface. The two spore proteins that appeared as the strongest bands in immunoblots for the strains in this study were of a similar molecular size to those identified in other reports. For example, the band a with molecular mass of 250 kDa is likely to be the collagen-like spore surface glycoprotein (BclA), which is a structural component of the filaments of the hairy nap, and is highly immunogenic (Sylvestre *et al.*, 2002). We also detected a band in the vicinity of 25 kDa that may correspond to a 24.6 kDa spore surface protein predicted to be iron/manganese superoxide dismutase, based on its amino-terminal sequence (Read *et al.*, 2002; Steichen *et al.*, 2003).

Some hypothetical proteins have been detected in dormant and germinating spores of the Sterne 34F2 strain, and these include one with a molecular mass of 25.5 kDa (Huang *et al.*, 2004). Also, in a recent investigation, *B. anthracis* spore-associated proteins have been identified in reactions with the sera of humans immunized with AVA vaccine, and the molecular masses of some of the proteins are close to 25 kDa, for example, transdolase, thiazole biosynthesis protein and rRNA adenine dimethylase (Kudva *et al.*, 2005). The other protein bands detected in the immunoblot analysis (Fig. 2) were of weak intensity for the pXO1⁻, pXO2⁻ strain, and corresponded to 50, 70, 90 and 110 kDa. However, the bands were stronger for the pXO1⁺, pXO2⁺ and pXO1⁺, pXO2⁻ virulent *B. anthracis* strains, and this result might suggest that the two virulence plasmids (pXO1 and pXO2) contain genes that control the production of these proteins. In other studies of spore-associated proteins, several identified proteins have been found to have similar sizes to those found in this study, including spoOB-associated GTP-binding protein, sensory box histidine kinase, immuno-inhibitor A, and S-layer homology domain, with molecular masses of 50, 70, 90 and

110 kDa, respectively (Huang *et al.*, 2004; Kudva *et al.*, 2005). The structure and function of these exosporium components of *B. anthracis* spores have not yet been fully elucidated, and further study, such as 2D electrophoresis in combination with MS, is required. The identification of these surface-exposed antigens may provide useful information for the development of new and more effective vaccines for human and animal use.

The exosporium molecules may play a role in the interaction of spores with the infected host. Macrophages are major effectors of the host immune system against bacterial infection. The entry of the spore into macrophages is a critical step for *B. anthracis* infection. *B. anthracis* spores are able to escape from phagolysosomes, and can germinate and multiply within the macrophage cytoplasm (Dixon *et al.*, 2000). There may be an association between macrophages and exosporium components of *B. anthracis* spores (Dixon *et al.*, 2000; Guidi-Rontani *et al.*, 2001). In this study, we demonstrated that anti-BA-spore IgG inhibited the germination of spores in macrophages, which suggests that the binding of the antibody to spores opsonizes the pathogen, and promotes phagocytosis and subsequent killing by macrophages. Our data also suggest the possibility that the spore-associated proteins detected by the anti-BA-spore IgG may have important roles in germination, and escape of spores from macrophages. The exact mechanisms by which *B. anthracis* spores germinate, and escape from macrophages, are not well characterized. The immunogenic spore proteins shown in this study are potentially involved in the germination process and/or escape from killing by macrophages. Taken together, the results indicate that the anti-BA-spore IgG may be useful in controlling the early phase of anthrax infection, and thus contribute to averting the toxemia and bacteraemia that result from the massive replication of bacteria.

Mendelson *et al.* (2005) have demonstrated that intramuscularly administered spores disperse to other organs immediately, and that clearance of viable spores from the spleen takes at least 60 days p.i. This explains the long-term protection and sustained titres of neutralizing antibodies achieved by spore vaccination. However, the persistent survival of virulent spores in the body may be a cause of concern for clinicians, and for patients who are exposed to *B. anthracis* spores. To date, antibiotics are the only treatment available for anthrax infection, but they cannot help in the late stage of anthrax infection. Anti-BA-spore IgG prolonged life in infected mice, and decreased mortality (Fig. 6). These results suggest that a combination therapy of antibiotics and anti-BA-spore IgG in cases of possible exposure may be advantageous in inhibiting germination, and clearing remaining spores from the body.

The live spore vaccine is effective; however, there are safety concerns. The vaccine can occasionally result in necrosis at the injection site, or death of treated animals (Turnbull, 1991). A PA-based vaccine is a promising strategy in anthrax prophylaxis, and anti-PA antibody has a definitive role in

protective immunity; however, the PA-based vaccine does not provide protection against all virulent strains of *B. anthracis* (Welkos & Friedlander, 1988). Accumulated evidence suggests that other virulence factors are required to optimize the efficacy of anthrax vaccines (Hahn *et al.*, 2005). Considering the pathology of *B. anthracis*, an ideal vaccine would confer protection against spores, bacilli and toxins in a comprehensive manner. Although further investigation should be carried out, the data presented here support the idea that a vaccine with combined action against the major virulent component PA and the spore-specific somatic antigens is a safer option, and may offer full protection against anthrax.

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