Effects of altering the germination potential of *Bacillus anthracis* spores by exogenous means in a mouse model

C. K. Cote,¹ J. Bozue,¹ N. Twenhafel² and S. L. Welkos¹

Correspondence

C. K. Cote

Christopher.cote2@us.army.mil

¹Bacteriology Division, US Army Medical Research Institute of Infectious Diseases, 1425 Porter Street, Fort Detrick, Frederick, MD, USA

²Pathology Division, US Army Medical Research Institute of Infectious Diseases, 1425 Porter Street, Fort Detrick, Frederick, MD, USA

Inhalational anthrax is the most severe form of anthrax. It has been shown in small-animal and non-human primate models that relatively large pools of ungerminated *Bacillus anthracis* spores can remain within the alveolar spaces for days to weeks post-inhalation or until transported to areas more favourable for germination and bacillary outgrowth. In this study, spores of the Ames strain that were exposed to germination-inducing media prior to intranasal delivery were significantly less infectious than spores delivered in either water or germination-inhibitory medium. The effect of manipulating the germination potential of these spores within the lungs of infected mice by exogenous germination-altering media was examined. The data suggested that neither inducing germination nor inhibiting germination of spores within the lungs protected mice from the ensuing infection. Germination-altering strategies could, instead, significantly increase the severity of disease in a mouse model of inhalational anthrax when implemented *in vivo*. It was shown that germination-altering strategies, in this study, were not beneficial to the infected host and are impractical as *in vivo* countermeasures.

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INTRODUCTION

Inhalational anthrax, if untreated, has a mortality rate approaching 100 % (Friedlander, 2000; Mock & Fouet, 2001). Treatment of inhalational anthrax is hampered by the pool of ungerminated spores found within the lungs post-infection (Altboum *et al.*, 2002; Barnes, 1947; Cote *et al.*, 2006; Friedlander *et al.*, 1993; Henderson *et al.*, 1956; Loving *et al.*, 2007; Pickering *et al.*, 2004; Ross, 1957). Whilst germinated *Bacillus anthracis* spores are killed by antibiotics and/or the innate immune response (Bozue *et al.*, 2005; Hu *et al.*, 2006; Kang *et al.*, 2005; Pickering *et al.*, 2004; Welkos *et al.*, 1986, 2002), ungerminated spores are largely unaffected by these strategies (Driks, 2002; Drusano *et al.*, 2008; Gut *et al.*, 2008). According to the generally accepted model of anthrax pathogenesis, inhaled spores are trafficked from the alveolar spaces by macrophages or other phagocytes to the lymph nodes (Cleret *et al.*, 2007; Ross, 1957). The spores can germinate within the phagocytes during or after their translocation; however, it has been established that spores do not readily germinate extracellularly within the alveolar spaces (Barnes, 1947; Bozue *et al.*, 2007a; Cote *et al.*, 2006; Friedlander *et al.*, 1993; Glomski *et al.*, 2007; Guidi-Rontani *et al.*, 1999; Henderson *et al.*, 1956; Heninger *et al.*,

2006; Loving *et al.*, 2007; Pickering *et al.*, 2004; Ross, 1957). Upon germination, the bacilli multiply, express anthrax toxins and capsule, and spread systemically.

Recent reports indicate that spores may germinate at the primary infection site. Spore germination has been observed in nasal-associated lymphoid tissues (Glomski *et al.*, 2007) or in lung tissues, when associated with macrophages and after delivery as a large bolus (Sanz *et al.*, 2008), and may infect without requiring transport to the mediastinal lymph nodes. These findings suggest that alternative models for the initial events in inhalational anthrax may be possible and warrant further investigation using fully virulent strains of *B. anthracis* and larger animal models. Regardless of their final role in disease pathogenesis, the lungs remain a reservoir for infectious spores (Altboum *et al.*, 2002; Barnes, 1947; Cote *et al.*, 2006; Friedlander *et al.*, 1993; Henderson *et al.*, 1956; Loving *et al.*, 2007; Pickering *et al.*, 2004; Ross, 1957).

Accordingly, the spores retained in the lungs could be targeted to facilitate more efficacious clearance of the infection. Novel therapeutics could involve strategies to alter the germination potential of the inhaled spores. Germination-altering strategies include two approaches, both intuitively beneficial to the host. One possibility presumes that inhibiting germination would slow the

Abbreviation: H&E, haematoxylin and eosin.

infection, potentially allowing more time for efficacious therapies to be implemented (Akoachere *et al.*, 2007; Alvarez & Abel-Santos, 2007). This strategy, however, also potentially leaves a larger pool of ungerminated spores that are resistant to the immune response and exogenous therapies (McKevitt *et al.*, 2007). Alternatively, spore germination could be induced, rendering the spores sensitive to macrophage killing or antibiotic therapy (Kang *et al.*, 2005; Welkos *et al.*, 2002). However, this strategy might exacerbate the infection by stimulating toxin production by the germinating spores.

Spore germination *in vitro* can be controlled with germination medium (Ireland & Hanna, 2002; Moir *et al.*, 2002). Medium composed of L-alanine, adenosine and Casamino acids (AAC medium) or L-alanine and inosine (AI medium) can initiate germination (Welkos *et al.*, 2004). These media stimulate the production of the protective antigen component of anthrax toxins; however, these media are inadequate to support bacillary replication *in vitro* (Cote *et al.*, 2005; Welkos *et al.*, 2004).

McKevitt *et al.* (2007) demonstrated that the LD₅₀ of spores of the *B. anthracis* vaccine strain Sterne delivered intratracheally in 1 M D-alanine, an inhibitor of germination, was significantly lower than that of spores delivered in PBS (McKevitt *et al.*, 2007). When the spores were introduced in germination-inducing medium containing 0.5 mM L-alanine, the LD₅₀ was higher than for spores delivered in PBS alone (McKevitt *et al.*, 2007). Our data expand upon this idea by assessing germination-altering strategies using the fully virulent Ames strain. We also examined the potential of using germination-altering strategies post-infection as potential therapies of inhalational anthrax.

METHODS

Strains and media. The wild-type toxin- and capsule-producing Ames strain of *B. anthracis* (Little & Knudson, 1986) was used for all studies. To obtain spores, cultures were prepared in Leighton–Doi sporulation medium (Leighton & Doi, 1971) as described previously (Cote *et al.*, 2006). The spores were purified by washing the sporulated culture three times in sterile water, centrifuging the suspension through a Hypaque-76 gradient (Nycomed) and washing the separated spores an additional three times (Cote *et al.*, 2006). The spores were heat-activated (65 °C for 30 min) and then held on ice before animal challenges. Unless otherwise noted, the following final concentrations of germination-altering media were used: D-alanine stock (1 mM); AAC containing L-alanine (9.5 mM), adenosine (3.1 mM) and Casamino acids (0.165%); AI containing L-alanine (0.25 mM) and inosine (1 mM); and AI-0.5 mM containing L-alanine (0.5 mM) and inosine (1 mM).

Animal infection. Pathogen-free female BALB/c mice between 6 and 10 weeks old were obtained from the National Cancer Institute (Fort Detrick, Frederick, MD, USA). For the intranasal challenge experiments, mice were anaesthetized with 100 µl ketamine (100 mg ml⁻¹), acepromazine (10 mg ml⁻¹) and xylazine (20 mg ml⁻¹) injected intramuscularly. The mice were then challenged by intranasal instillation of *B. anthracis* Ames spores. The total volumes of inocula instilled were 25–50 µl. The dose of Ames spores delivered is described in the figures. Mice were monitored several times each

day, and moribund animals were euthanized and the mortality rates recorded. The selected concentration of spores used for challenge was considered a relatively low challenge dose. These doses were selected to identify treatment differences in our sensitive BALB/c model using Ames strain spores. These lower challenge doses, delivered via intranasal instillation, resulted in some variation in the mortality rates seen in control animals. All animal challenge experiments were subjected to statistical analyses. In some experiments, mice were given germination-altering medium either before or after infection with spores. This medium was either instilled intranasally or administered as aerosols, which were generated as described previously (Cown *et al.*, 1957; Friedlander *et al.*, 1993; May, 1973). To examine the bacterial burden in the euthanized mice, lungs were harvested and homogenized, and the lung homogenate was plated as described previously (Cote *et al.*, 2006).

Histopathology. Mice were euthanized by pentobarbital overdose at specific time points post-infection. The lungs were collected from mice at 0, 24, 48 and 72 h after challenge with wild-type Ames spores for histopathological evaluation and were immersion-fixed in 10% neutral buffered formalin for 30 days. Sections prepared for examination using light microscopy were embedded in paraffin, sectioned and stained with haematoxylin and eosin (H&E). Immunohistochemistry was performed on lung sections using primary antibody (monoclonal mouse anti-*B. anthracis* capsular antibody at a 1:8000 dilution) and secondary antibody, a peroxidase-labelled polymer (EnVision Peroxidase kit; Dako). The slides were stained with substrate chromogen solution and counterstained with haematoxylin.

Histopathological sections of lung were evaluated in a blinded study and scored based on the pathological changes present in H&E sections or in immunohistochemically stained sections (i.e. oedema, haemorrhage, necrosis, inflammation and presence of bacilli). Sections were scored as follows: 0–2, minimally affected; 3–4, mildly affected; 5–6, moderately affected; 7–8, markedly affected; 9–10, severely affected.

Statistics. Survival rates were compared between each group and the control group by using Fisher's exact test with permutation adjustment for multiple comparisons. Mean times to death or euthanasia were compared between each treatment group and the control group by using *t*-tests with permutation adjustment for multiple comparisons. Analyses were conducted using SAS version 8.2 (SAS Institute). Except where noted in the figure legends, all groups for survival studies contained ten mice per group.

RESULTS AND DISCUSSION

Effect of manipulating spore germination potential before infection

Differences were noted in survival rates of mice challenged with Ames strain spores of various germination states. Whereas 70% of mice challenged with ungerminated spores administered intranasally in water succumbed to the infection, only 20% or less succumbed to the infection initiated by spores pre-exposed to germinant (Fig. 1a). Spores that were exposed to AAC before intranasal instillation were significantly less virulent when compared with ungerminated spores delivered in water ($P=0.006$ for both survival rate and survival curve). Interestingly, the germinant exposure time did not appreciably affect this phenomenon. Spores that were exposed to AAC germina-

Fig. 1. Effect of altering spore germination potential prior to infection. (a) Mice were infected with approximately 4.75×10^5 Ames spores delivered by intranasal instillation in water (filled circles), in D-alanine (triangles) or in AAC-germination medium (filled and shaded squares). Spores were either exposed to AAC briefly (filled squares) or allowed to germinate in AAC for approximately 60 min at 37 °C (shaded squares). Delivering the spores in D-alanine offered no benefit to the mice. (b) Effect of germination-altering media on the bacterial burden associated with the lungs 48 h after intranasal instillation. Approximately 3×10^6 spores were delivered in water ($n=6$), AAC medium ($n=6$) or D-alanine ($n=3$). At 48 h post-challenge, mice were euthanized and the harvested lungs were homogenized and plated for total bacterial counts. The data shown are the means $\pm$ SD from two experiments.

tion medium for less than 1 min at room temperature and then (while still in AAC) placed immediately on ice while awaiting intranasal instillation were slightly less infectious than spores that were exposed to AAC germination medium for 60 min at 37 °C prior to intranasal instillation (Fig. 1a). The spores in either case were delivered intranasally to the mice in the AAC medium. These data suggest that, with regard to spore infectivity or virulence, the extent of spore germination may not be as important as the initiation of germination. Both groups of mice receiving spores exposed to AAC were significantly more

likely to survive the infection when compared with mice receiving spores delivered in D-alanine ($P=0.0036$ for survival rate and 0.0015 for survival curve comparing spores briefly exposed to AAC with spores in D-alanine, and $P=0.042$ for survival curve comparing spores subjected to a longer exposure to AAC with spores delivered in D-alanine).

When the simpler defined germinant (AI-0.5 mM) was used, a similar survival advantage was associated with the animals receiving the spores delivered in the germination medium (Fig. 2b).

We also observed that spores delivered by intranasal instillation in the presence of D-alanine (a potent germination inhibitor) were at least as virulent as spores delivered in water. In some of our experiments, there was a trend suggesting that D-alanine may exacerbate or worsen the infection (Figs 1a and 2c). McKevitt *et al.* (2007) reported similar observations using an intratracheal model of infection with attenuated Sterne strain spores. Whilst our data are in general agreement with those presented by McKevitt *et al.* (2007), there are some differences. We showed a statistically significant impact of introducing the spores in the presence of L-alanine, but our results using D-alanine were not statistically significant. In addition to inherent differences in the models being used (i.e. *B. anthracis* strains, mouse strains, infection route, etc.), it is likely the most significant difference would be medium composition. Our L-alanine-containing media (AAC, AI and AI-0.5 mM) contained approximately 9.5, 0.25 and 0.5 mM L-alanine, respectively. McKevitt *et al.* (2007) utilized a germination-inducing medium containing 0.5 mM L-alanine. Interestingly, in both studies, this lower amount of L-alanine (0.5 mM) resulted in statistically insignificant differences in virulence, which suggested increased protection (this study and McKevitt *et al.*, 2007). However, when we increased the amount of L-alanine to approximately 9.5 mM, we did observe statistically significant protection afforded to the mice that received spores delivered in this medium (Fig. 1). Regarding D-alanine, McKevitt and colleagues used 1 M D-alanine, whilst we used approximately 1 mM D-alanine. This lower concentration of D-alanine was potentially a more therapeutically practical concentration to use *in vivo*, and we had previous experience with this concentration of D-alanine with *in vitro* studies (Cote *et al.*, 2005). Whilst McKevitt and colleagues demonstrated statistical significance (using 1 M D-alanine), our data were not significant (using 1 mM). When taken together, these data are supportive, and also underline the impact of the concentration of either exogenous L-alanine or D-alanine on the state of the spore and subsequent pathogenesis.

Quantitative cultures were performed on lung homogenates from mice challenged with spores delivered in water, AAC or D-alanine to determine the total bacterial load associated with each group 48 h post-infection. There were no significant differences noted between the bacterial

Fig. 2. Effect of administering germination-inducing media to mice after intranasal challenge with Ames spores. (a) Mice received approximately 5.2×10^5 Ames spores delivered in water (filled circles). Another group of mice received AAC medium at 5 h post-infection (open squares). (b) Mice received approximately 3.5×10^5 Ames spores delivered by intranasal instillation in water (filled circles) or in AI-0.5 mM germination medium (open circles). Another group of mice received spores in water and this was followed by an intranasal instillation of AI-0.5 mM germination media 5 h post-infection (shaded circles). (c) Mice received approximately 3.2×10^5 Ames spores delivered by intranasal instillation in water (filled circles) or in D-alanine (filled triangles, $n=9$). Another group of mice received spores in water and this was then followed by an intranasal instillation of D-alanine 5 h post-infection (shaded triangles).

burdens in mice receiving spores in water or D-alanine (Fig. 1b). As shown in Fig. 1(b), the mice challenged with spores administered in germination medium (AAC) had a significantly lower bacterial burden compared with those challenged with spores delivered in water ($P=0.026$) or in the presence of D-alanine ($P=0.008$).

Effect of manipulating spore germination potential *in vivo* after infection

As our preliminary results suggested that germinated spores or spores primed to germinate were less likely to initiate a lethal infection, the next set of experiments was performed to determine the effect of altering spore germination potential within the lungs. Mice were infected by intranasal instillation with ungerminated Ames spores, and AAC germinant was then administered intranasally approximately 5 h later (Fig. 2a). Surprisingly, the treatment group had a lower survival rate and a significantly shorter time to death ($P=0.018$) when compared with the group receiving spores alone. Administration of the less robust germinant (AI-0.5 mM) (Fig. 2b) or an inhibitor of germination (D-alanine) post-infection did not significantly alter the course of infection when compared with spores alone (Fig. 2c). However, the mice receiving spores delivered in AI-0.5 mM were more likely to survive the infection than mice receiving AI-0.5 mM delivered 5 h post-infection ($P=0.02$ for survival curve) (Fig. 2b).

One hypothesis concerning the lack of protection offered by germination induction was that intranasal administration of germination-altering medium may not have been an adequate form of delivery due to the large droplet size. To test this hypothesis, AAC, AI-0.5 mM or D-alanine were administered to groups of infected mice by small-particle nebulizers, either approximately 1 h before or approximately 5 h after intranasal challenge with ungerminated Ames spores. The nebulizer delivers particles of approximately $1 \mu\text{m}$ in size (May, 1973). These smaller particles of germination-modifying medium would reach deeper areas of the lungs most likely not reached by intranasal instillation of the medium. The survival results of mice challenged in these experiments (Fig. 3) were similar to those described in experiments involving intranasal administration of germination-modifying media (Fig. 2). The animals receiving AAC by aerosol exposure were less likely to survive the spore challenge than control animals infected with spores in water and not treated with AAC medium ($P=0.023$ for survival curve) (Fig. 3a). Interestingly, this increased mortality was observed regardless of whether the medium was administered to the mice before or after the intranasal spore challenge. The survival rates of the challenged animals that were exposed to aerosols of either AI-0.5 mM medium (Fig. 3b) or D-alanine (Fig. 3c) were not altered in a statistically significant manner, but the observed trends continued to suggest that neither strategy benefited the infected mice.

When the germination-altering medium was administered to mice after the instillation of spores, the germination medium did not ease the severity of the infection and, in most cases, actually intensified the infection. Whilst administration of AAC alone did cause minor congestion in control animals, this congestion was seemingly inconsequential when the spores were delivered while in the process of germinating (Fig. 1a). Interestingly, the AAC medium only exacerbated the infection when the mice were infected with ungerminated spores. This was observed when mice were treated with AAC 5 h after intranasal infection with ungerminated spores or if the ungerminated spores were delivered within 1 h after the AAC administration (Fig. 3). When germinated spores or spores that

Fig. 3. Effect of exposure to aerosolized germination-altering media administered before or after intranasal spore challenge. (a) Mice were infected with approximately 4.6×10^5 Ames spores by intranasal instillation either 5 h before (filled squares, $n=5$) or within 1 h after (shaded squares) exposure to aerosolized AAC germination medium. The control group received Ames spores delivered in water with no germination-altering medium (filled circles). (b) Mice were challenged with approximately 5×10^5 Ames spores by intranasal instillation either 5 h before (shaded circles) or within 1 h after (open circles) exposure to aerosolized Al-0.5 mM germination media. The control group received Ames spores delivered in water with no germination-altering medium (obscured by other data points). (c) Mice were infected with approximately 4.6×10^5 Ames spores by intranasal instillation either 5 h before (filled triangles) or within 1 h after (shaded triangles) exposure to aerosolized D-alanine germination inhibitor. The control group received Ames spores delivered in water with no germination-altering medium (filled circles, $n=9$).

were primed to germinate were administered to the mice in AAC, no detrimental effects were noted; in fact, the animals were significantly more likely to survive the infection (Fig. 1). Thus the detrimental effects could not be attributed solely to the impact of the medium on the lung tissues, but rather to the medium in conjunction with ungerminated spores. These data further emphasize the importance of the germination state of the spore in pathogenesis and also indicate that changes in the lung environment that may be potentially exploited by inhaled ungerminated spores are not necessarily exploited by germinated spores.

Both toxin and capsule production have been shown to be initiated very soon after germination initiation (Cote *et al.*, 2005; Drysdale *et al.*, 2005; Guidi-Rontani *et al.*, 1999). Russell *et al.* (2008b) demonstrated that germinated Ames strain spores are significantly less likely to adhere to the A549 bronchoepithelial cell line when compared with ungerminated spores, suggesting that early capsule production associated with the germinating Ames spores may hinder spore–cell interactions (Russell *et al.*, 2008b). Hosts may not be as affected by germinated spores because of such altered interactions, but also by the fact that germinated spores are significantly more susceptible to the sporicidal nature of the innate immune response (Hu *et al.*, 2006; Kang *et al.*, 2005; Welkos *et al.*, 2002).

Histopathology

The results of histopathological analyses of samples from infected mice supported the *in vivo* challenge data (Fig. 4). For these studies, mice received intranasal doses of spores delivered in water followed by exposure to aerosolized germination-altering medium approximately 5 h later. Lungs were harvested from euthanized mice at 0, 24, 48 and 72 h after infection with wild-type Ames spores. Sections of lung were evaluated and scored based on

pathological changes (i.e. oedema, haemorrhage, necrosis, inflammation and presence of bacilli) present in H&E-stained sections or immunohistochemically stained sections. The results are summarized below and illustrated in Figs 4 and 5.

As depicted in Fig. 4, the histopathological changes associated with the lungs of mice infected with spores delivered in water were considered to be mild to moderate during the 72 h time-course experiment. This generally included mild congestion and oedema and only rare to minimal neutrophilic inflammation.

Fig. 4. Histopathology results of a blinded study characterizing the effects of germination-altering media administered post-infection. For these studies, mice received intranasal doses of approximately 5×10^5 spores followed by exposure to an aerosolized germination-altering medium (AI or AAC) approximately 5 h later, or D-alanine approximately 5 h later. Lungs were harvested from euthanized mice at 24 h (a), 48 h (b) or 72 h (c) after infection with wild-type spores. Control (Ctl) mice were given the germination-altering media but were not infected with spores. Histopathological sections of lung were evaluated and scored based on pathological changes (i.e. oedema, haemorrhage, necrosis, inflammation and presence of bacilli) present in H&E sections or immunohistochemically stained sections. Sections were scored as described in Methods. The number of mice is indicated for each group in parentheses. The *P* values depicted are from comparing the treatment groups with the group receiving spores alone.

The histopathology was more pronounced in samples from *B. anthracis*-infected mice treated with germination-inducing medium. Neutrophilic inflammation was noted in the mice receiving spores followed by AAC as early as 24 h post-challenge, resulting in a significantly elevated lung pathology score compared with the other treated and untreated groups ($P < 0.0001$) (Fig. 4a). This trend was also observed at 48 h post-infection (Fig. 5a). The lungs of the mice receiving AAC after the spores were significantly more affected at 48 h post-challenge than the lungs of the mice receiving spores alone ($P = 0.0001$) (Fig. 4b). Infected mice treated with AAC had a detectable bacillary burden as early as 48 h post-infection. At 48 h post-infection, 3/3 of the AAC-treated mice were positive for bacilli in the lungs (Fig. 5b), whilst only 2/8 mice receiving spores alone had bacilli present in the lungs. The differences in bacillary burden were less pronounced at 72 h post-infection, when 2/5 of the mice treated with AAC were positive for bacilli in the lungs compared with 2/9 of the mice receiving spores alone. There were few differences noted in the animals receiving AI following spore challenge when compared with mice receiving spores alone, at all three time points (Fig. 4).

At 24 h post-exposure, the histopathology scores indicated that the mice that received the spores followed by D-alanine were significantly less affected than the mice that received only spores ($P = 0.018$) (Figs 4a and 5c). These observations suggested that the inhibition of germination by D-alanine may be beneficial at the very early stages of infection. The mice that were infected and then treated with D-alanine did show signs of moderate neutrophil inflammation at approximately 48 h post-infection. At 72 h post-infection, bacilli were observed in the lungs of some of the mice that received D-alanine. Interestingly, however, by 72 h, the mice that received spores followed by D-alanine were scored similarly when compared with the mice receiving spores followed by AAC (Fig. 4c); both treatment groups were significantly more affected than mice receiving spores

Fig. 5. Histological evaluation of the lungs of pathogen-free female BALB/c mice challenged by intranasal instillation of *B. anthracis* Ames spores and given germination-altering media after infection with spores. Representative micrographs are shown depicting some of the pathology associated with administering the germination-altering media after infection. (a) Spores followed by AAC; mouse euthanized at 48 h post-infection. Immunohistochemistry using anti-capsule antibodies was performed to confirm the presence of *B. anthracis* and help identify bacteria (arrows). This lung was given an overall score of 7. (b) Spores followed by AAC; mouse euthanized at 48 h post-infection. Note the numerous bacilli (arrows) present in the alveolar interstitium. This lung was given an overall score of 7. (c) Spores followed by D-alanine; mouse euthanized at 24 h post-infection. There were mild pathological changes consisting of multifocal, mildly widened alveolar septae (arrows) and few inflammatory cells. This lung was given an overall score of 2. (d) Spores followed by D-alanine; mouse euthanized at 72 h post-infection. There was necrosis of alveolar septae with haemorrhage (asterisk). Other septae were moderately expanded by oedema, congestion and myriad bacilli (arrows). This lung was given an overall score of 6. (e) Spores followed by D-alanine; mouse euthanized at 72 h post-infection. There was pronounced widening of the alveolar septae with oedema, congestion and inflammatory cells consisting primarily of viable neutrophils (arrows). This lung was given an overall score of 7. (f) Spores followed by D-alanine; mouse euthanized at 72 h post-infection. Myriad bacilli were noted (arrow) in addition to moderate congestion, oedema and neutrophilic inflammation. This lung was given an overall score of 5. All sections were stained with H&E, and the magnification is indicated.

alone ($P=0.01$ and 0.0003 , respectively) (Fig. 5d–f). At 72 h post-challenge, 2/5 of the mice receiving D-alanine were positive for bacilli in the lungs, compared with 2/9 of the mice receiving spores alone. Identical observations were made regarding the mice receiving AAC at this time point. These data suggest that administering D-alanine can impact disease severity, albeit delayed when compared with the effects observed with AAC administration.

Control experiments were performed to evaluate the effects of intranasal instillation of AAC, AI or D-alanine in the absence of spores. No gross changes were observed in the lungs, and histopathology did not identify any significant changes associated with the administration of these media.

Conclusions

Our goal was to define conditions under which *B. anthracis* spore germination could be inhibited or promoted so as to attenuate or prevent an anthrax infection. Spores of the Ames strain pre-exposed to germinants were significantly less likely to initiate a fatal infection in a mouse intranasal model of infection than ungerminated spores. We then evaluated the effects on virulence and infection of either inhibiting germination or inducing the spores to germinate within the lungs. We hypothesize that these germination-altering strategies do not benefit the infected host for several reasons. It has been demonstrated that spores that have begun to germinate will produce and secrete toxin components very soon after germination initiation (Cote *et al.*, 2005; Guidi-Rontani *et al.*, 1999). Thus it is possible that the ungerminated spores become associated with lung tissues (Bozue *et al.*, 2007b; Russell *et al.*, 2008a, b) and when germination is induced by exogenous means (i.e. administration of AAC) in the lungs, toxin production could conceivably be initiated. This toxin production could damage the pulmonary tissue (Warfel *et al.*, 2005) with which the spore is associated or in close proximity to, causing increased vascular permeability, allowing increased access of the bacteria and/or toxins to the bloodstream, and ultimately augmenting rapid disease progression.

We also found that germination inhibition (with the administration of D-alanine) was ineffective at preventing infection. Whilst germination inhibition with D-alanine is reversible, this transient state of germination inhibition may allow a greater percentage of spores to survive the hostile intracellular environments of phagocytes. This concept has been illustrated by experiments that have demonstrated that if spores have a delayed germination phenotype (Giorno *et al.*, 2007; McKevitt *et al.*, 2007) or do not germinate at all (Kang *et al.*, 2005), they are resistant to macrophage killing. Even this slight delay in germination initiation may allow greater numbers of spores to survive the translocation and innate immune response, potentially increasing the macrophage spore burden (and eventually bacilli and toxin). This increased burden could therefore overwhelm the natural ability of the phagocytes to dispose of the spores as they germinate (Banks *et al.*, 2005; Cote

et al., 2008). We conclude that the balance between bacterial burden and host survival is quite sensitive. Our previous work (Bozue *et al.*, 2007b) and that of others (Oliva *et al.*, 2008) have indicated that wild-type spores may preferably traffic towards professional phagocytes. It was also shown that these phagocytes, especially macrophages and dendritic cells, are sporocidal unless overwhelmed by a high m.o.i. of spores (Bozue *et al.*, 2005; Cleret *et al.*, 2007; Hu *et al.*, 2006; Kang *et al.*, 2005; Pickering *et al.*, 2004; Welkos *et al.*, 1986, 2002). In addition, it has been shown that germination does not readily occur in the alveolar spaces of the lungs (Barnes, 1947; Bozue *et al.*, 2007a; Cote *et al.*, 2006; Friedlander *et al.*, 1993; Glomski *et al.*, 2007; Guidi-Rontani *et al.*, 1999; Henderson *et al.*, 1956; Heninger *et al.*, 2006; Loving *et al.*, 2007; Pickering *et al.*, 2004; Ross, 1957). Thus if any of these situations are altered by the addition of exogenous medium, the balance could be shifted in favour of the infecting bacteria.

Ultimately, this report has demonstrated that, in our models, germinated spores of the Ames strain are less virulent than ungerminated spores of the Ames strain by the pulmonary route. We also noted that alterations in the lung environment that favourably change the disease outcome are not readily achievable and are essentially ineffective as predictable countermeasures.

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