

The detection of protective antigen (PA) associated with spores of *Bacillus anthracis* and the effects of anti-PA antibodies on spore germination and macrophage interactions

C.K. Cote^a, C.A. Rossi^b, A.S. Kang^c, P.R. Morrow^c, J.S. Lee^d, S.L. Welkos^{a,*}

^aUnited States Army Medical Research Institute of Infectious Disease (USAMRIID), Bacteriology Division, 1425 Porter Street, Fort Detrick, Frederick, MD 21702, USA

^bUSAMRIID, Diagnostic Systems Division, 1425 Porter Street, Fort Detrick, Frederick, MD 21702, USA

^cAvanir Pharmaceuticals, San Diego, CA 92121, USA

^dUSAMRIID, Virology Division, 1425 Porter Street, Fort Detrick, Frederick, MD 21702, USA

Received 1 November 2004; received in revised form 14 February 2005; accepted 14 February 2005

Available online 22 April 2005

Abstract

The protective antigen (PA) component of the anthrax toxins is an essential virulence factor of *Bacillus anthracis* and is the major protective immunogen. The kinetics of PA production during growth of *B. anthracis*, and the roles of anti-PA antibody in host immunity are not clearly defined. Production of PA by the vegetative organisms peaks during the shift from exponential to stationary phase of growth. Recently, PA was also found to be associated with spores. In our study, PA-specific mRNA was detected in spores by RT-PCR within 15-min of exposure to germinant. PA protein was detected by immunomagnetic electrochemiluminescence (ECL) on spores within 1 h of exposure to a germination medium and was rapidly released into the supernatant. PA was not demonstrated on ungerminated spores by RNA analysis, ECL, or spore-based anti-PA ELISA; however, it was detected on ungerminated spores by immunoelectron microscopy (immunoem). In rabbits, PA induces polyclonal antibodies (Abs) that, in addition to their anti-toxin neutralizing activities, exhibit anti-spore activities. In this study, the anti-spore effects of a human monoclonal Ab specific for PA (AVP-hPA mAb, Avanir Pharmaceuticals) were characterized. AVP-hPA mAb retarded germination in vitro, and enhanced the phagocytic and sporicidal activities of macrophages. The activities were comparable to those of the polyclonal rabbit anti-rPA Ab. Assays to detect germination inhibitory activity (GIA) in serum from vaccinated mice and guinea pigs suggested a possible role for anti-PA Abs in protection. Thus, anti-PA Ab-mediated, anti-spore activities may play a role in protection during the early stages of an anthrax infection.

© 2005 Elsevier Ltd. All rights reserved.

Keywords: Anthrax; *Bacillus anthracis*; Spores; Protective antigen; Anti-PA antibodies; Immunity

1. Introduction

Bacillus anthracis is the etiologic agent of anthrax, a disease primarily of herbivores and accidentally, or intentionally, of humans [1–5]. The infectious form of *B. anthracis* is the dormant spore [6]. To cause disease, the spore must germinate, i.e. lose its dormancy and resistance properties, reactivate its metabolism, and commence vegetative growth [6,7]. The most severe form of

the disease, inhalational anthrax, has a high mortality rate due to its initial nonspecific symptoms and rapid course. It is hypothesized that after exposure to aerosolized spores, the spores are taken up by alveolar macrophages that subsequently migrate to the regional lymph nodes [8]. There, the spores germinate within the phagocytes [8–14] and form vegetative cells that are released and begin to multiply and spread systemically. The bacilli produce three major virulence factors, an antiphagocytic capsule and the anthrax edema and lethal toxins. The toxins are composed of the cell-binding protective antigen (PA) associated with edema factor (EF) or lethal factor (LF), respectively [15]. The toxins are associated with various anti-host activities that allow the infection to proceed to a terminal outcome. If spore germination in the host and other events early in

* Corresponding author. Tel.: +1 301 619 4930; fax: +1 301 619 2152.
E-mail address: susan.welkos@amedd.army.mil (S.L. Welkos).

infection were retarded, then the subsequent toxic activities could be prevented. A better understanding of these early stages is required to facilitate the development of effective intervention and prevention strategies for anthrax.

Antibodies (Abs) to PA have an essential role in immune protection [16–19], and, in addition to their well-defined toxin-neutralizing activity [19], anti-PA Abs have also been shown to exhibit anti-spore activities [13,20]. Rabbit anti-rPA pAbs were shown to enhance the phagocytosis and subsequent killing of the phagocytosed spores by macrophages in the assays [13,14]; and they partially inhibited germination in vitro [13,20]. Human monoclonal Abs were recently generated by the Xenerex™ recall technology from AVA (BioThrax, BioPort, Lansing, MI)-vaccinated donors (Avanir Pharmaceuticals; 21). These mAbs exhibited strong toxin-neutralizing capacity in in vitro and rat challenge models [21]. We examined the anti-spore activities of one such human anti-PA mAb, AVP-hPA, to include in vitro germination inhibitory activity and the effects of Ab pretreatment on phagocytosis and killing by macrophages. The role of these antitoxin-mediated anti-spore activities in vivo is not known. Likewise, the kinetics of toxin production by the organism in vivo is not firmly established. The production of PA in vitro by the vegetative organisms in culture reaches a peak at the end of the exponential phase of growth [22–25]; however, there is evidence that PA can be detected much earlier in the transition from spore to vegetative bacillus. The findings that toxin-associated proteins are expressed early in infected cultured cells; that anti-PA Abs bind to the spore surface and inhibit spore germination; and that inactivated spores elicit protective immune responses in animals [11,13,20,26] suggest that anti-PA Abs and possibly other anti-spore Abs can impede spore germination in vivo and may enhance the ability of early responding cells to oblate the infection.

2. Results

2.1. Detection of PA associated with spores

2.1.1. Detection of PA-specific mRNA

RNA was harvested from *B. anthracis* Ames strain spores and subjected to RT-PCR analysis by using the Qiagen One-step RT-PCR kit that can detect pg/ml quantities of mRNA. The *pagA* transcript was consistently identified after just 15-min exposure to a two-fold dilution of AAC stock solution (shaking at 100 rpm and at 37 °C), (Fig. 1). The *pagA* transcript was also observed, and at possibly higher levels, upon 30-, 60-, and 120-min exposures to AAC medium. Spores incubated in the AAC medium for less than 15 min and ungerminated spores incubated in sterile water for injection did not produce the *pagA* transcript at levels detectable by this procedure. The DNA control reactions remained negative, ensuring that the resulting *pagA* PCR fragment was amplified from cDNA

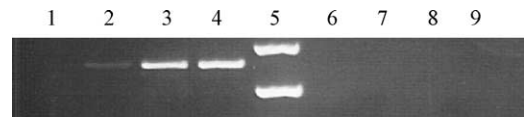


Fig. 1. Detection of *pagA* mRNA by RT-PCR performed on RNA extracted from *B. anthracis* spores. RT-PCR reactions were performed on total RNA extracted from ungerminated spores (lane 1) and from spores exposed to AAC (1:2 dilution in WFI) for 15 min (lane 2), 30 min (lane 3), and 60 min (lane 4). Lanes 6–9 are DNA control reactions to verify the absence of contaminating DNA in the RNA preparations; reactions are performed on the same total RNA as described in lanes 1–4, respectively, but without the reverse transcription step of the reaction. Lane 5 is 1 kb plus DNA ladder (Invitrogen), the top band represents 650 bp, while the lower band represents 500 bp.

generated from RNA and not the result of contaminating DNA during the RNA extraction procedure (Fig. 1).

There were never detectable levels of *pagA* mRNA identified in extracts of totally ungerminated spores (Fig. 1). To verify that this observation was due to lack of transcript and not merely due to the difficulty of disrupting ungerminated spores, a control PCR reaction was performed using the same *pagA*-specific primers used in the RT-PCR experiments. The extract resulting from the mechanical disruption of ungerminated spores did contain DNA, and the *pagA* gene fragment was amplified with standard PCR techniques (data not shown). The presence of the *pagA* fragment amplified in the PCR reaction suggested that the spores were being sufficiently disrupted as to release DNA present within the spore cortex. Accordingly, if a significant amount of *pagA* specific mRNA was present in ungerminated spores, it should have been detected, suggesting that the *pagA* mRNA (Fig. 1) is newly synthesized in the germinating spores. In addition, spores incubated for 60 min in AAC medium stock diluted 1:8 did not yield detectable levels of *pagA* transcript (data not shown). When the concentrations of L-alanine, adenosine, and casamino acids were increased (AAC medium stock diluted 1:2), the presence of the *pagA* transcript was consistently observed in spores that were at a concentration of 10^8 CFU/ml, again suggesting that the detected *pagA*-specific mRNA is newly synthesized in germinating spores.

2.1.2. Detection of PA by a spore-based anti-PA ELISA

With the spore-based anti-PA ELISA, the progressive loss of PA on spores with increasing extent of purification was demonstrated (Fig. 2). Purified ungerminated spores had no detectable surface PA when these spores were used to coat the plates, whereas spores freshly harvested and not subjected to gradient purification procedures had appreciably greater amounts of associated PA. In addition, PA was not detected on plates coated with either live or gamma-irradiated killed purified ungerminated spore suspensions; and the latter were also negative using either affinity-purified rabbit anti-rPA IgG or human monoclonal anti-PA Ab. Thus, purified ungerminated spores had no detectable surface PA, within the limits of detection of this assay.

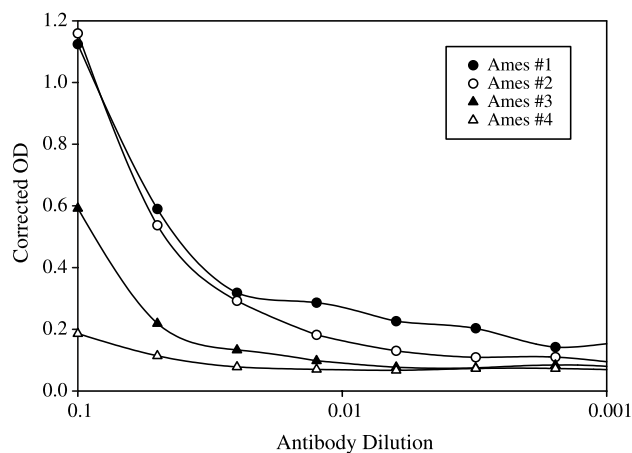


Fig. 2. Detection of PA on the surface of live *B. anthracis* spores at various stages of purification. Ames # 1 represents spores freshly harvested from Leighton Doi medium and resuspended in WFI, Ames #2 represents spores washed twice in WFI, Ames #3 represents spores that have been gradient purified and resuspended in WFI, and Ames #4 represents spores that have been gradient purified and then washed three additional times with WFI. The detection antibody used was a polyclonal rabbit anti-rPA IgG fraction that had been affinity purified over a PA column (1.18 mg/ml). Residual PA appears to be removed from *B. anthracis* spores during the purification process.

Although this assay is presumed to be a relatively insensitive technique for detecting PA, the absence by whole-spore ELISA of detectable spore-associated PA in purified spore preparations was supported by results of the other assays described below, e.g. the ECL assay. To determine whether PA was present in greater quantities on germinated spores, ungerminated spores were exposed to synthetic germination medium (AAC) and then collected at various time points. While these data suggested that PA was present in greater abundance at later stages of germination, the relatively low OD readings obtained were not considered to be significant due to the sensitivity threshold of the ELISA (data not shown). The difficulty in detecting significant amounts of surface PA on germinated spores by ELISA could also be explained by the transitory nature of PA expression on the spore surface (Section 2.1.3).

2.1.3. Immunomagnetic electrochemiluminescence detection of PA (ECL)

Ungerminated spores and spores exposed to a germinant were assayed for PA in an ECL assay using biotinylated anti-PA mAbs as capture Abs, and a purified Ruthenium (Ru)-labeled anti-PA polyclonal IgG as the detector Ab. The former consisted of either a pool of three murine mAbs or a single human mAb, as described in Section 4. In preliminary experiments to evaluate the pool of three murine mAbs, the individual human mAbs, and the rabbit anti-rPA pAb as detector and capture Ab, it was determined that maximal sensitivity was observed by using either the pool of murine anti-PA mAbs or human AVP-hPA mAb as detector and

rabbit anti-rPA pAb as capture Ab. The ungerminated spores were exposed to either the AAC or AI germinants for varying periods after which aliquots of spores and supernatants were assayed. PA was not detected on ungerminated, phase-bright spores, or in their supernatants for the duration of the experiments (Table 1 and Fig. 3A). Spore-associated PA was detected after 1 h of incubation in AAC. Both spore and supernatant samples were positive at 1 h and not at the earlier time intervals (Table 1 and Fig. 3B) at the concentrations of germinant and spores used (AAC stock diluted 1:8 in Expt. #11 and AAC stock diluted 1:2 in Expt. #15 and #16; and 10^7 spores/ml and $3\text{--}4 \times 10^8$ spores/ml, respectively). AI-exposed spores were not positive for PA until 2 h exposure, at which time PA was detected unequivocally only in the concentrate of the supernatant (Table 1). Nevertheless, nearly 90% of spores became phase-dark (i.e. similar in appearance to the spores shown in Fig. 3B) within 30-min exposure to AI (or AAC). These data (Table 1 and Fig. 3) suggest that newly formed PA reaches detectable levels on germinant-exposed spores after they have proceeded through the very early stages in germination; a very early and specific phase of germination must be reached before induction of new PA expression as detected by ECL, and a threshold level of PA must also be present. The PA appears to be secreted or otherwise released from the spore soon after it becomes detectable on the surface (Table 1).

2.1.4. Detection of spore-associated PA by microscopy

Immunoelectron microscopy (immunoem) provided additional evidence for the presence on highly purified spores of an antigen(s) recognized by anti-PA Abs. Affinity-purified rabbit polyclonal anti-rPA IgG exhibited significant binding to ungerminated spores, in contrast to the lack of binding by the normal serum IgG control (Fig. 4, panels A and B). These results confirmed those reported previously, using rabbit anti-rPA whole antisera [13]. Similarly, a human anti-PA monoclonal Ab, AVP-hPA, produced from AVA-vaccinated donors (Avanir Pharmaceuticals) and shown to have strong toxin-neutralizing capacity, reacted with ungerminated spores to a significantly greater extent than did the normal human IgG (Fig. 4, panel C and D). Both the rabbit and human anti-PA Abs reacted with the germinated spores to a reduced extent compared to that observed using ungerminated spores (Fig. 4 panels E and F compared to A and C, respectively). The germinated and ungerminated spores were prepared and inactivated under containment conditions before their analysis in the BSL2-level electron microscopy facility. Current procedures require these spores to be inactivated by incubation in 4% formaldehyde for 14 days followed by several washes in water. Given the apparent rapid release of newly formed surface PA during germination, it is not surprising that PA was detectable on germinated spores by immunoem to a lesser extent than it was on ungerminated spores. Also, for both the ungerminated and germinated spores, the anti-PA

Table 1
Detection of spore-associated PA expression by immunomagnetic ECL

Sample	ECL assay Exposure:		S/N ^a			Interpretation
	Germinant	Time (min)	#11	#15	#16	
<i>Controls</i>						
PA, 10 ng/ml	–	–	49.31	47.01	19.46	Positive
PA, 1 ng/ml	–	–	4.87	4.55	2.98	Positive
Medium, no spores	–	–	Baseline ^b	Baseline	Baseline	
<i>Test samples^c:</i>						
Spores	Water	0	1.01	0.91	1.01	Negative
		120	0.99 ^d	0.93	0.93	Negative
Supernatant	Water	0	0.99	0.99 [1.03]	nd [0.81]	Negative
		120	0.98	1.02 [0.98]	nd [0.83]	Negative
Spores	AAC	0	1.01	0.92	1.03	Negative
		30	1.00	nd	1.05	Negative
		60	1.60 ^e	nd	3.42	Positive
		120	2.93 ^f	21.58	15.89 ^f	Positive
Supernatant	AAC	0	1.01	1.01[0.99]	0.88 [0.86]	Negative
		30	1.01	nd	0.86 [0.87]	Negative
		60	1.31	nd	1.03 [2.04]	Suggestive [positive]
		120	6.60	2.45[14.40]	2.28 [17.04]	Positive [positive]
Spores	AI	0	nd	0.98	nd	Negative
		30	nd	0.94	nd	Negative
		60	nd	1.01	nd	Negative
		120	nd	1.17	nd	Suggestive
Supernatant	AI	0	nd	1.02 [0.98]	nd	Negative
		30	nd	1.05 [0.97]	nd	Negative
		60	nd	1.04 [1.05]	nd	Negative
		120	nd	1.13 [1.62]	nd	Negative [positive]

Results of three experiments (#11, #15, and #16). nd=not done.

^a Samples (50 µl) containing either washed spores or the supernatant from spores exposed to AAC or AI germination medium with 0.8% bicarbonate were added to PA-specific ECL assay (3 biotinylated monoclonal anti-PA Ab-prebound beads and Ru-tagged anti-rPA polyclonal Ab). Results are expressed as signal-to-noise (S/N) ratios, which is the sample average ECL value divided by the baseline negative control average ECL value. Samples were considered positive if the ECL signal was more than the mean plus 3 SDs or 1.2 times the mean of the negative controls (S/N=1.2), whichever was higher.

^b Medium alone (AAC, AI, or water) in which no spores were added. The AAC medium was diluted 1:8 in Experiment. 11 and 1:2 in Experiment. #15 and #16. These were used to determine baseline values and subsequently used in S/N determinations (footnote a).

^c After exposure to germinant, one aliquot of spores was immediately filter-sterilized and the supernatant saved for the ECL assay. A second aliquot of spores was immediately diluted tenfold into cold water with 0.2 M D-alanine and washed twice before being added to the ECL assay. Washed spores: In Expt. 11, spores were present at a final concentration in the assay of about 1×10^7 /ml, and in Expt. #15 and #16 at approximately $3\text{--}4 \times 10^8$ /ml. Supernatants: Supernatants from cultures with 1×10^8 /ml were tested, and the supernatants from these experiments were also concentrated 50-fold by centrifugation using CentriconYM-10™ filtration units. The S/N values of the concentrates are shown in brackets. Spore suspensions used in these assays, consisted of a freshly prepared culture of the Ames strain sporulated by incubation in Leighton-Doi broth and harvested, washed, gradient-purified two times, suspended in a final concentration of 1.4×10^{10} /ml in water-for-injection with 0.1% phenol, and stored at 4 °C for less than 2 days before use.

^d Shown in Fig. 3A.

^e Shown in Fig. 3B.

^f As seen by phase contrast microscopy, the sample contained a mixture of germinated spores and slightly elongated, possibly early vegetative forms.

Abs appeared to interact with antigen in various subsurface regions of the spore, as well as in the exosporium (Fig. 4, panel A, C, E, F). The binding to both germinated and ungerminated spores was negligible for the normal rabbit and human IgGs (Fig. 4, panels B and D, and data not shown). In some immunoem experiments, human mAbs to tetanus toxin (anti-TTTox) were also used as a negative control. Although the reactivity with spores of anti-TTTox mAbs was less than that of the human anti-PA mAbs, it was greater than that of the human IgG control (data not shown) and appears to be associated with the presence of a cross-reacting antigen (as discussed below).

2.2. Effects of anti-PA antibody on spore function and host responses: germination inhibitory effect of anti-PA antibodies

2.2.1. Rabbit and human anti-PA Abs

In addition to their toxin-neutralizing activity, anti-PA Abs have anti-spore activities, including the inhibition of spore germination in vitro [13]. The in vitro germination inhibitory activity (GIA) of rabbit polyclonal Abs to rPA was shown previously by measuring the spectrometric decline in absorbance or the increase in spore staining with dyes by light microscopy of anti-rPA Ab-treated and untreated

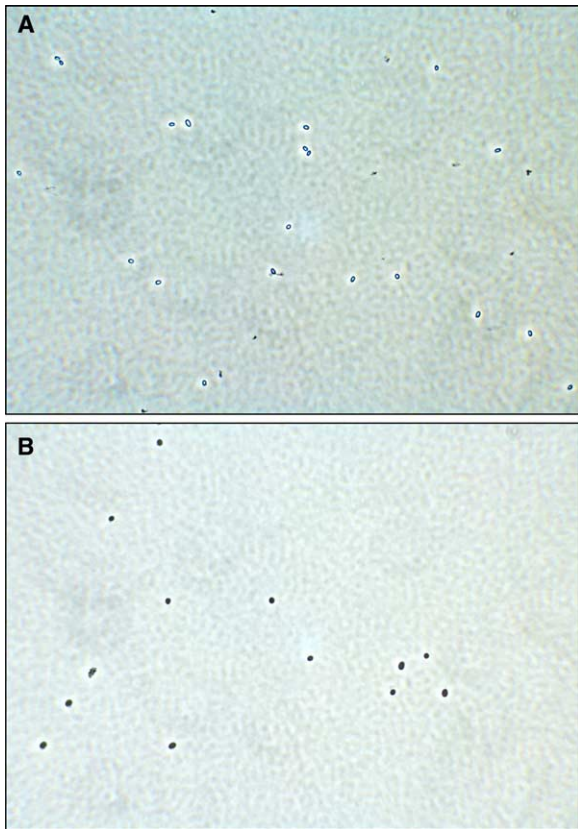


Fig. 3. Phase contrast photomicrograph of spores assayed for PA by the immunomagnetic ECL. Shown are: (A) spores incubated for 2 h in water (<0.5% germinated and >99.5% ungerminated, or phase-bright); and (B) spores incubated for 1 h in AAC germinant (99% germinated, or phase-dark).

spores [13]. More recently, microtiter spectrofluorometric assays of germination were developed based on the increase in fluorescence of spores with time during their incubation in a germinant [27]. Treating spores with affinity-purified rabbit polyclonal IgG against rPA before their exposure to germinant inhibited spore germination (Fig. 5). The GIA was manifested by significant differences in areas under the fluorescence curve and in regression parameters, i.e. the *a* coefficient [27], as illustrated in Fig. 5.

The human monoclonal AVP-hPA Ab was significantly more inhibitory than a control Ab, human anti-TTTox mAb (Avanir), in similar fluorescence tests of spores exposed to the germinant continuously during the assay, as shown in Fig. 6.

The effects of the mAbs on germination of spores exposed to germinant for defined periods were also assayed. Spores were pretreated with Ab or buffer, exposed to germinant for different defined periods of time, and then removed and evaluated by phase microscopy and fluorescence assays. Anti-PA mAb-treatment was associated with the retarded germination of spores. After 30-s exposure to germinant, 23.8% of the spores had become nonrefractile, compared to 53% treated with anti-TTTox mAb and 48%

treated with buffer alone (Fig. 7). By 2 min, approximately 90% of spores had turned phase-dark, regardless of Ab pretreatment, indicating the reversible inhibition of germination that is associated with the Ab-spore interaction.

2.2.2. Effect of strain on germination inhibition by anti-PA Abs

Anti-rPA pAbs inhibited germination of strains Ames and Sterne spores but not of strain Vollum IB (V1B) spores to a significantly greater extent than did normal rabbit serum IgG (Fig. 8). Thus, V1B might express less of the anti-PA Ab-reactive antigen on the spore surface than Ames and Sterne; this observation agrees with the absence of extractable PA from V1B spores as detected by SDS-PAGE analysis [13]. This strain-related difference in susceptibility of spores to anti-PA Ab-mediated germination inhibition was observed using both the rabbit polyclonal and human monoclonal anti-PA Abs (data not shown).

2.3. Serum germination inhibitory activity as a correlate of immunity

The association between protection, serum GIA, and serum anti-PA Ab titer was examined in mice inoculated with the PA-encoding viral replicon vaccine [18,28] or AVA. Sera were collected from individual vaccinated mice before each vaccine dose and just before s.c. challenge with a lethal dose of *B. anthracis* strain Sterne. In addition, sera collected from guinea pigs vaccinated with purified PA (either recombinant PA or PA purified from Sterne strain cultures) were also examined for GIA potential.

2.3.1. PA-replicon-vaccinated mice

Spores were pretreated with either preimmune sera or sera from PA replicon-vaccinated C57Bl/6 mice before exposure to germinant. GIA was assayed by determining the ratio of the area under the fluorescence curve of the prechallenge compared to the preimmune-treated spores. The area ratio for the serum pair from immune C57Bl/6 mice ($n=10$) correlated significantly with the anti-PA Ab ELISA titers ($p=0.0033$, $r=0.855$); i.e. high anti-PA Ab titers in prechallenge sera were associated with strong GIA (data not shown). In contrast to the C57Bl/6 mice, the inhibitory activity of PA replicon-vaccinated A/J mice ($n=16$) was not significantly correlated with the anti-PA Ab ELISA titer (data not shown).

2.3.2. AVA-vaccinated mice

The association between serum GIA and serum anti-PA Ab titer was examined in mice vaccinated with the licensed human AVA vaccine. The decreased area value for the postimmune sera collected just before challenge ('post') compared to the preimmune sera ('pre') was significant for both the A/J and C57Bl/6 mice (Fig. 9). The sera were assessed for their spore GIA and for their anti-PA Ab ELISA titers. The results again suggested that the serum

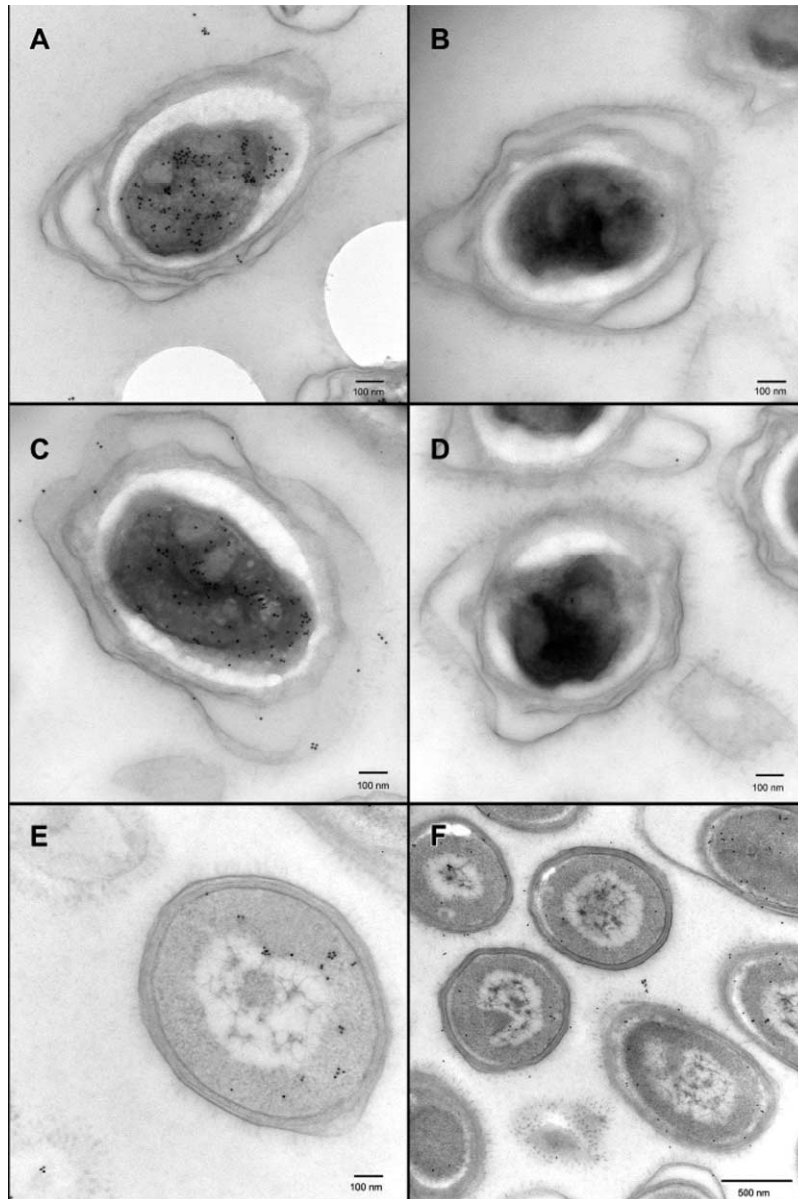


Fig. 4. Detection of PA associated with spores by immunogold labeling and electron microscopy. Ames strain spores that were ungerminated or germinated were incubated with primary rabbit or human IgG preparations and detected with goat anti-rabbit or anti-human IgG conjugated to 10 nm gold particles. All antibodies were normalized to 1 mg/ml and diluted 1:100 prior to incubation with the spores. The samples were processed as described in Section 4. The identities of the spores and of the primary antibodies used in each panel are as follows. A, Ungerminated Ames spores and rabbit affinity-purified anti-rPA IgG; B, Ungerminated Ames spores and normal rabbit serum IgG; C, Ungerminated Ames spores and human anti-rPA IgG (AVP-hPA mAb); D, Ungerminated Ames spores and normal human serum IgG/kappa; E, Germinated Ames spores and rabbit affinity-purified anti-rPA IgG; F, Germinated Ames spores and human anti-PA IgG (AVP-hPA mAb).

GIA correlated with the prechallenge anti-PA Ab titers of sera from vaccinated mice (data not shown).

2.3.3. PA-vaccinated guinea pigs

The association between serum GIA and survival after challenge was examined in guinea pigs vaccinated with purified PA. ELISA endpoint anti-PA Ab titers did not correlate with animal survival. Differences in germination of serum-pretreated Ames were determined by comparing the areas under the germination curve for spores pretreated

with sera from the survivors with those for spores pretreated with sera from the nonsurvivors. As shown in Fig. 10, sera from survivors appeared to have more inhibitory activity (i.e. smaller areas under the germination curve) than did sera from nonsurvivors, with areas of 17,681 [SEM 1125] and 20,710 [SEM 1118], respectively. Survival appeared significantly associated with serum GIA by *t*-test analysis ($p=0.04$), however the differences were not statistically significant when examined by a more rigorous logistic regression analysis.

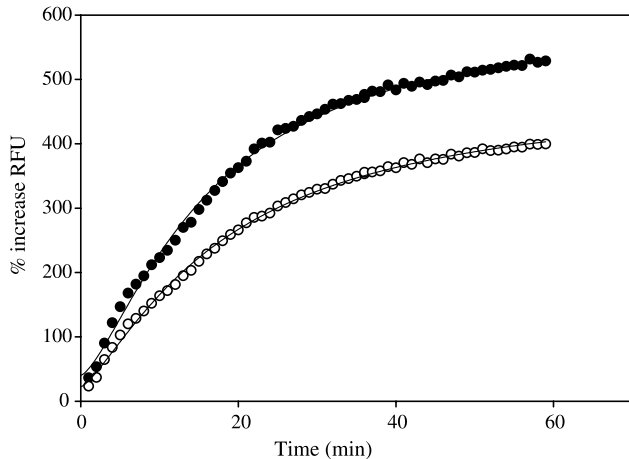


Fig. 5. Inhibition of germination of spores of the Ames strain of *B. anthracis* by pretreatment with anti-PA Abs. Ungerminated spores were pretreated with a 1:50 dilution of purified rabbit anti-rPA polyclonal IgG (○) or purified normal rabbit serum (NRS) IgG (●), and the spores then incubated in AAC. The rate and extent of germination was assayed using a fluorometric procedure that utilizes a fluorescent nucleic acid-binding dye which stains germinated spores but not ungerminated spores [27]. The increase in relative fluorescence units (RFU) was measured every minute for 1 h. The anti-PA Ab-treated spores germinated to a significantly lesser extent than did the spores pretreated with normal rabbit IgG, as shown by the smaller area-under-the-curve and *a* coefficient values ($p < 0.0001$ by Z-test) of the fluorescence plots.

2.4. Effects of anti-PA antibody on the phagocytosis and survival of spores in macrophages

In previous studies [13,14], the anti-spore activities of rabbit polyclonal anti-rPA Abs included effects on the uptake and fate of spores in murine macrophages (primary

and cell culture lines). These anti-PA Abs enhanced the phagocytosis of spores, their rate of intracellular germination, and the sporocidal activity of the macrophages.

In our study, the effects of human mAbs on the phagocytosis and survival of spores in macrophages were determined by using histology, viable counts, and FA staining. RAW 264.7 macrophages were exposed for 1 h to Ames spores that were ungerminated or were germinated for 1 h in AAC (>95% phase dark) before use. These spores were pretreated with human anti-PA mAb or control reagent (anti-TTTox mAb or medium alone) just before infection of the RAW264.7 cells. After 1 h of uptake, the macrophage cultures were then washed extensively and assayed for phagocytosis immediately (time 0), or the medium was replaced and cultures reincubated for another 4 or 8 h in fresh medium. The latter contained 10% horse serum to suppress germination by extracellular spores, as reported [13,14]; no antibiotics were used during the experiment. Including a 15-min incubation in DMEM with 2.5–5 $\mu\text{g}/\text{ml}$ gentamicin and 10% fetal bovine serum to stimulate germination (followed by replacement with the usual medium) did not affect the results (data not shown).

For the ungerminated spores, significantly more anti-PA mAb-pretreated spores were phagocytosed compared to anti-TTTox mAb-pretreated spores after 1 h incubation (time 0), as determined by counting cells stained ($p = 0.0288$) as described in Fig. 11 panel A. The staining also revealed that a greater proportion of the intracellular anti-PA mAb-treated than of the anti-TTTox mAb-treated spores were germinated by the end of the phagocytosis period (data not shown). In one experiment, for instance, whereas 63% of

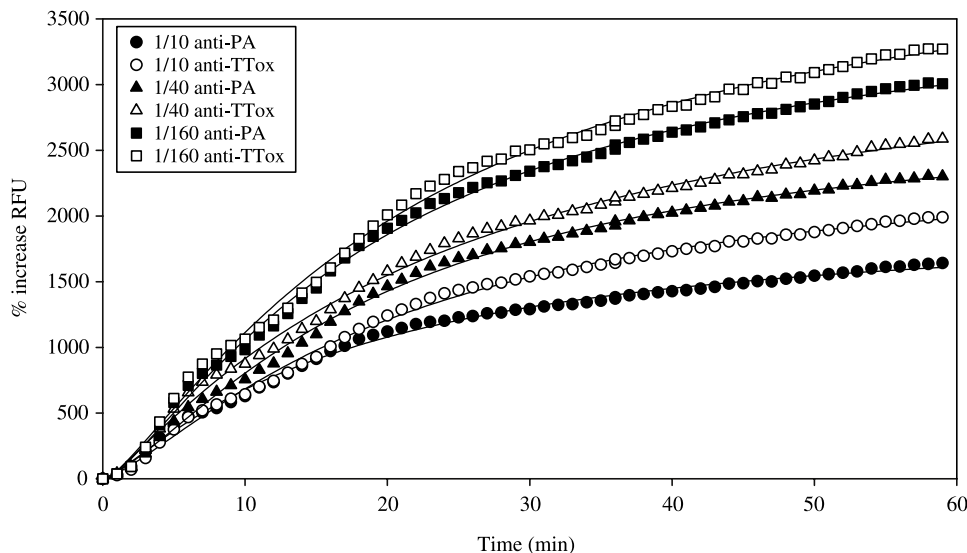


Fig. 6. Effects of human mAbs on in vitro germination of spores continuously exposed to germinant. Spores were pretreated with dilutions of either the human anti-PA mAb (anti-PA) or the control Ab (anti-tetanus toxin mAb, anti-TTTox). They were then incubated with germinant and spore-germination was assayed spectrofluorometrically, as described in Fig. 5. The anti-PA mAb was significantly more inhibitory than anti-TTTox mAb, as determined by statistical analysis of regression parameters [27]. Significances by dilution were $p = 0.0002$ (1:10), $p = 0.0002$ (1/40), and $p = 0.003$ (1:160). The 'a' coefficients of the anti-PA mAb-treated spores and anti-TTTox mAb-treated spores were not significantly different at 1:320 ($p > 0.05$).

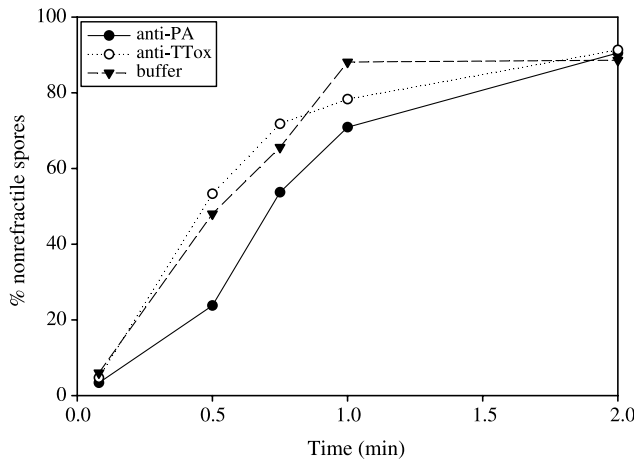


Fig. 7. Effects of human mAbs on in vitro germination of spores exposed to germinant for defined periods. Spores were pretreated with Ab or buffer, exposed to germinant for different periods, germination was stopped with the addition of D-alanine (M), and then removed and evaluated by phase contrast microscopy. The number of phase-dark (nonrefractile) germinated spores and phase-bright (refractile) ungerminated spores was counted at each sampling time. Anti-PA mAb treatment was associated with the retarded germination of spores. After 30-s exposure to germinant, 23.8% of the spores had become nonrefractile (compared to 53% treated with anti-TTox mAb and 48% treated with buffer alone). By 2 min, approx. 90% of spores had turned phase-dark, regardless of Ab pretreatment.

the anti-PA mAb-treated spores were germinated (stained blue by Diff-Quik) at time 0, 40% of the anti-TTox mAb-treated spores were germinated and 60% were ungerminated (stained with malachite green). There was a decline in the numbers of stainable intracellular spores after a further 4 h incubation in both the anti-PA mAb- and anti-TTox mAb-pretreated spore samples, but it was greater for the anti-PA mAb-treated samples (16-fold versus five-fold, respectively). This differential rate in decline of staining suggested that the spores treated with anti-PA mAbs might have been killed more efficiently from t_0 to 4 h (Fig. 11 panel A). Human anti-PA mAbs stimulated increased killing of the spores upon phagocytosis by the macrophages and during the subsequent incubation. As determined by viable counts, there was a statistically significant nine-fold loss in viability from time 0 to 4 h for the anti-PA mAb-treated spores ($p=0.014$) compared to a 3.9-fold loss in counts for the anti-TTox mAb-treated spores ($p=0.074$) (Fig. 11 panel B). Using fluorescent Abs to evaluate spore uptake, anti-PA mAb-pretreatment was again associated with a greater extent of phagocytosis than spores pretreated with non-specific Ab or with medium alone (data not shown); and with reduced numbers of stainable spores in macrophages incubated for 4 h. Changes in the number of phagocytosed spores from t_0 to 4 h as determined by FA (Fig. 11 panel C) did not correlate well with changes in the spore viable count or in the number of spores as determined by spore stain/Diff-Quik (Fig. 11 panels A and B) or by DAPI staining (data not shown; discussed below). The apparent decline as detected by FA over the 4-h period of both

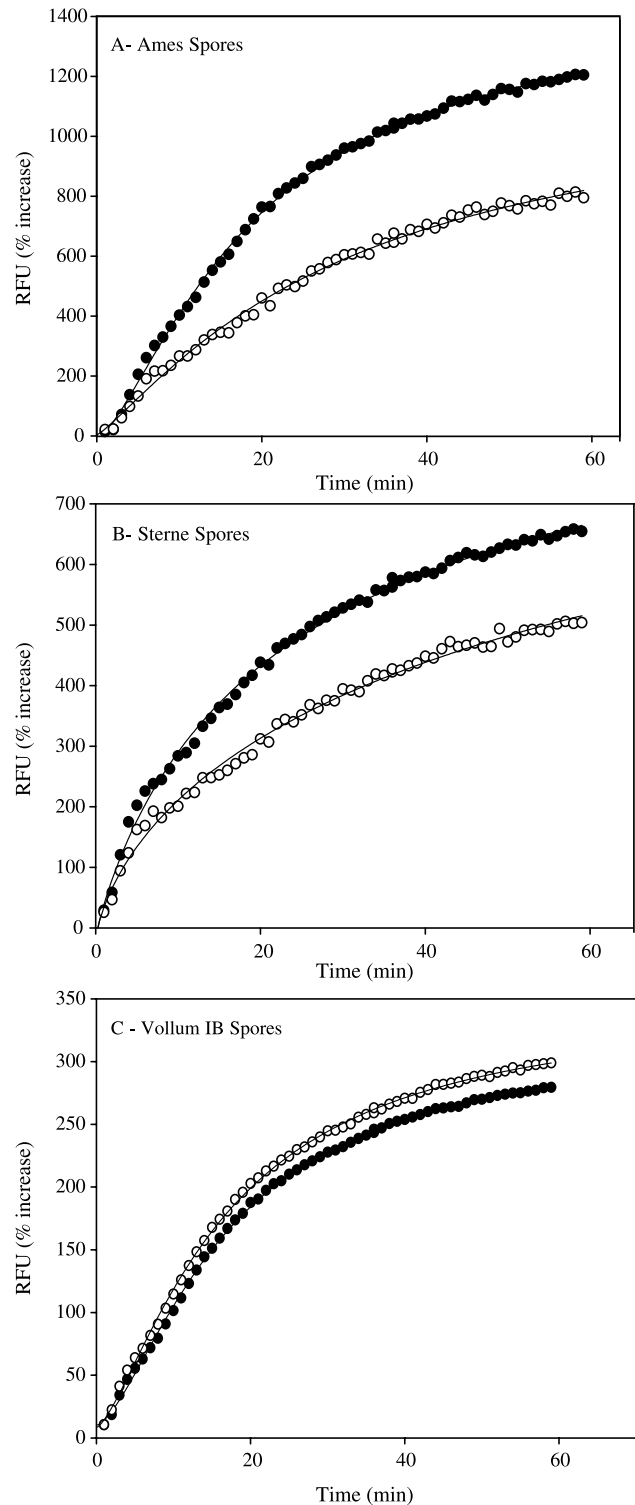


Fig. 8. Strain-related differences in effects of anti-rPA Abs on germination. Ungerminated spores of the Ames, Sterne, and V1B strains were prepared and purified under the same conditions and used at the same concentrations ($5-8 \times 10^6$ spores/well). Germination of spores pretreated with rabbit anti-rPA IgG (\circ) or normal rabbit sera (NRS) IgG (\bullet), both diluted 1:100, is shown for spores of Ames (A), Sterne (B), and V1B (C). Anti-rPA Abs inhibited germination of Ames and Sterne spores [$p=0.017$ ($n=$ eight experiments) and 0.011 ($n=$ two experiments), respectively], but not of V1B spores ($n=$ two experiments), to a significantly greater extent than did NRS IgG.

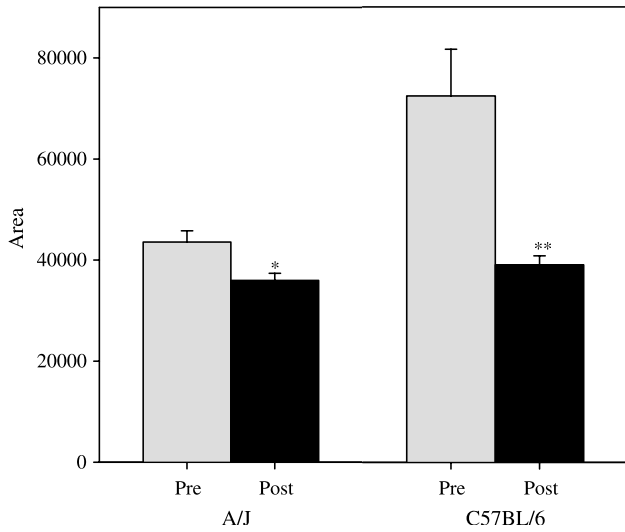


Fig. 9. Area under the fluorescence curve of spores exposed to germinant after treatment with either preimmune serum ('pre', grey bars) or the prechallenge serum ('post', black bars) from the same animal after vaccination with three to four doses of AVA (BioThrax, BioPort). Shown are the combined data from three experiments each with three A/J and two C57BL/6. The decreased GIA (mean areas) of the prechallenge sera (35,979, SEM 1399) compared to that of the preimmune sera (43,568 SEM 2211) was significant for the A/J (**p*=0.023). For the C57BL/6 mice, the decreased mean areas and thus decreased GIA was even more apparent for the prechallenge (38,827 SEM 1789) compared to preimmune (72,058 SEM 9195) values (***p*=0.024) mice.

anti-PA mAb- and anti-TTox mAb-treated spores was less than indicated in panel A. Neither the extent of phagocytosis (*t*₀ values) nor the number of stainable organisms present in the macrophages at 4 h were significantly different between the anti-PA mAb- and anti-TTox mAb-treatment groups. Nonviable spores that have yet to be digested apparently can



Fig. 10. Prechallenge sera from the vaccinated guinea pigs were assayed for their GIA. The sera were from guinea pigs inoculated i.m. with recombinant PA (closed circles) or native PA purified from the Sterne strain (open circles) and then challenged i.m. with Ames spores, 100 LD₅₀ doses or 2000 LD₅₀ doses, respectively. Differences in germination of serum-pretreated Ames were determined by comparing the areas under the germination curve for spores pretreated with sera from the survivors and the nonsurvivors. Areas were determined of the % increase in RFU compared to the *t*₀ value, with time after incubation in germination medium.

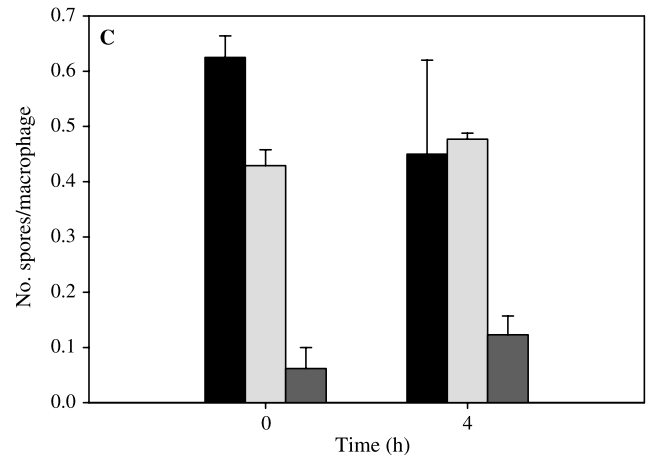
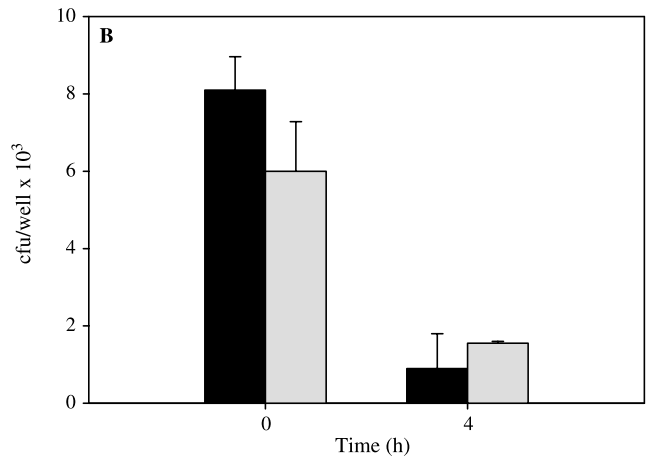
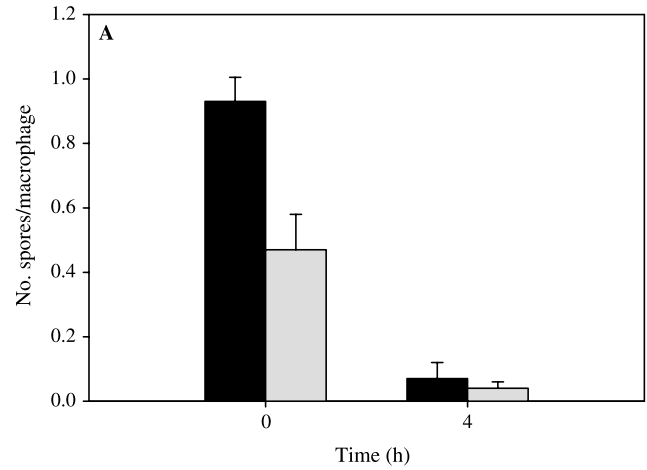


Fig. 11. The effects of human mAbs on the phagocytosis of ungerminated spores and their survival in macrophages were determined by three procedures. (A) Malachite green and Diff-Quik staining. (B) Viable counts. (C) Fluorescent antibody staining. The black bars represent spores pretreated with human anti-PA mAb, the light grey bars represent spores pretreated with human anti-TTox mAb, and the dark grey bars (C) represent data collected when Cytochalasin D (cyto) treatment controls were used to determine the extent of unphagocytosed spores that were not removed by washing. Panels A and B are representative experiments each with 3 replicates per treatment group and panel C depicts the mean data of 3 experiments each with 3 replicates per treatment.

Table 2
Effects of human anti-PA antibody on phagocytosis and fate of germinated Ames spores

Time	Viability			Phagocytosis staining	
	Treatment	CFU/well $\times 10^{2a}$	No. DAPI ⁺ /total ^b	Histologic ^c	FA ^d
T 0	Anti-PA	11.0 [1.7]	43.5	0.29 [0.41]	0.285[0.025]
	Anti-TTTox	9.7 [1.5]	83.7	0.21 [0.04]	0.12[0.007]
4 h	Anti-PA	1.58 [0.32]	<5	0.019 [0.006] ^e	0.225[0.105]
	Anti-TTTox	1.66 [0.33]	26.7	0.031 [0.009] ^e	0.217[0.03]
8 h	Anti-PA	1.58 [0.13]	nd	0.01 g	0.31
	Anti-TTTox	2.0 [0.41]	nd	0.023 ^f	0.2

The phagocytosis and intracellular viability of spores pretreated with anti-PA AVP-hPA mAb or anti-TTTox mAb before exposure to RAW264.7 cells. Results are the means of two to three experiments each.

^a Mean no. CFU/well [\pm SEM], n =three experiments.

^b No. spores DAPI⁺ divided by the total number of intracellular spores counted (%); n =two experiments.

^c No. spores per cell as counted by malachite green spore staining with DiffQuik counterstain (see Section 4). n =three experiments.

^d No. intracellular spores per cell, as detected by staining with human anti-PA mAb or anti-TTTox mAb and FITC-conjugated rabbit anti-human IgG. n =two experiments.

^e Decrease in comparison to the values at t0 was 15.3-fold (anti-PA mAb) and 6.8-fold (anti-TTTox mAb). n =three experiments.

^f Decrease in comparison to the values at t0 was 29-fold (anti-PA mAb) and 9-fold (anti-TTTox mAb). n =two experiments.

still bind the Abs and be stained by the FA conjugates, thus analysis by FA detects the total number of spores present regardless of spore viability. The cytochalasin D treated macrophages served as a control for nonspecific background levels of extracellular spores that are not efficiently removed by washing.

The effects of the human anti-PA mAb on the interaction of macrophages with germinated spores were also determined. Ungerminated spores were incubated for 1 h in the AAC medium at which time >95% spores were germinated as determined by phase microscopy and heat resistance, before their use in the experiments. The effects of human mAbs on the phagocytosis and survival of germinated spores in macrophage, as determined by the three procedures is shown in Table 2. As shown by FA staining, phagocytosis of germinated spores was again enhanced more than two-fold (t_0) by pretreatment with the human anti-PA mAb. Macrophage sporicidal activity was also stimulated, as revealed by viable counts and histology stains. There was a 15.3-fold decline in intracellular anti-PA mAb-treated spores from time 0 to 4 h, and a 6.8-fold decline in numbers of anti-TTTox mAb-treated. Corresponding values for samples collected after 8 h incubation were 29-fold decrease in intracellular anti-PA mAb-treated spores from time 0 to 8 h, and nine-fold decline for the anti-TTTox mAb-treated germinated spores (Table 2).

As was demonstrated with the rabbit anti-rPA pAbs [14], killing of the AVP-hPA mAb-treated spores began during the 1-h uptake period. Although anti-PA mAb treatment stimulated opsonization of the germinated spores, the numbers of viable spores after 1 h (t_0) were only slightly higher for anti-PA mAb-treated compared to anti-TTTox mAb-treated spores, with means [SEM] of triplicate wells from three experiments using germinated spores of 11.0×10^2 [1.7] cfu/well and 9.7×10^2 [1.5] cfu/well, respectively. The loss of viability of intracellular spores at time 0 was also observed by histologic staining of macrophages infected

with spores; there were increased numbers of poorly stained (and thus uncountable) spores in cultures infected with anti-PA mAb-treated compared to anti-TTTox mAb-treated spores ([14]; and data not shown). Results of DAPI staining also correlated with loss of spore viability. DAPI dye stains the nucleus of germinated and not that of ungerminated spores (data not shown), and unlike fluorescent-labeled Abs, appears unable to stain killed spores. As illustrated by the results shown in Table 2, a smaller percentage of the FA-stained anti-PA mAb-treated spores than anti-TTTox mAb-treated spores were positive for DAPI staining at time 0, and whereas 26.7% of the FA-labeled anti-TTTox mAb-treated spores were also DAPI positive at 4 h, nearly all the anti-PA mAb-treated spores had lost DAPI stainability.

In summary, pretreating spores with AVP-hPA mAb enhanced the phagocytosis and killing of spores by RAW264.7 macrophages to an extent similar to that obtained with rabbit polyclonal Abs. These macrophage-associated activities were demonstrated for both ungerminated and germinated spores.

3. Discussion

In laboratory cultures of vegetative *B. anthracis*, the syntheses of the three components of anthrax toxin, the cell-binding PA component and the effector moieties, LF and EF, all peak during the transition from log phase to stationary phase of growth. Both the levels of toxin gene transcripts and toxin protein are maximal at the end of exponential growth [22–24]. Recently, PA (and LF transcript) were detected in association with spores; however, the extent of their detection in vitro as well as in vivo and their role in the spore infection have not been determined [11,13,14].

The spores of *B. anthracis* were shown previously to react with anti-PA Ab [13]. This reactivity was

demonstrated as a direct interaction between the spores and anti-PA Ab as detected by immunoem with intact spores and by immunoblot analysis of spore extracts [13]; PA was also detected indirectly by functional assays to include anti-PA Ab mediated germination inhibition and anti-PA Ab-enhanced macrophage phagocytosis and killing [13,20]. However, it has been suggested that the PA is present as a passive contaminant found on the surface of spores that were washed incompletely during their purification from sporulated cultures (C. Quinn, personal communication). Thus, the source of this spore-associated PA has been obscure and was the major question examined in the present study.

We showed that PA carryover from vegetative cultures was not the probable basis of at least the majority of the spore-associated PA. As determined in three of the assays we employed, spore-associated PA was observed in association with germinating spores and not with ungerminated spores. First, the purification of spores from sporulated cultures of *B. anthracis* removed PA at levels detectable by the spore ELISA. As shown in Fig. 2, this loss of reactivity to anti-PA Ab occurred during the final steps in spore purification. Secondly, PA was not detected by immunomagnetic ECL in association with ungerminated spores or their supernatant, even in concentrated samples of the latter, before exposure to germinant (Table 1). Under the standard assay conditions used, a 1-h exposure of ungerminated spores to germinant was required to detect PA. Finally, transcription of the *pagA* gene was not detected in purified preparations of ungerminated spores, but was detected very early after exposure of the spores to germinant, i.e. mRNA levels were detected within 15-min of exposure and possibly higher levels detected after longer exposures to relatively high concentrations of germinant. While these experiments were not quantitative, the results suggest an upregulation of *pagA* expression during early stages of germination. They also suggest that spores must reach a germination threshold before PA can be detected, and that this threshold is at least partially reached by exposure to the more concentrated AAC germination medium.

These kinetics agreed with first detection of PA protein on spores by ECL under standard assay conditions after their exposure to germinant for 1 h. These results are also in agreement with the observations reported previously on toxin gene expression. Using *B. anthracis* strains carrying *lacZ-LF* gene transcriptional fusions and detection with a fluorescent β -D-galactosidase substrate, Guidi-Rontani et al. showed that LF gene (*lef*) transcription could be detected 3 h after uptake of spores by macrophages and was associated with germinated spores; it was not detected earlier, in spores that were still ungerminated. However, *pagA* gene transcription was not examined [11].

In contrast, thin sections of ungerminated spores pretreated with anti-PA Abs and examined by immunoem

revealed the presence of PA or anti-PA Ab-reactive antigen. Instead of being confined to the surface exosporium, the spore-associated PA recognized by anti-PA Abs could also be found in subsurface layers, e.g. the spore coat(s) (Fig. 4, panels A, C, E, and F). The presence of internal, as well as surface, anti-PA Ab-reactive antigen contrasts with the surface-only binding observed by immunoem of Abs directed to spore surface entities known to localize only in the outer surface layers (J. Bozue, unpublished data). The anti-PA Ab staining pattern supports the conclusion from the ECL data that PA is made or is present in the spores and is translocated to the surface upon exposure of the ungerminated spore to germinant; and that upon reaching the surface exosporium is soon released into the medium. The reduced staining observed by immunoem of the germinated (compared to the ungerminated) spores with anti-PA Ab also supports this model.

The extent of detection of spore-associated PA appeared to vary according to the technique employed. For instance, although PA was not detected by spore ELISA using germinated or ungerminated spores as coating antigen, it was detected by immunogold electron microscopy on ungerminated spores by using rabbit polyclonal or human anti-PA mAb and less so on germinated spores probed with labeled rabbit anti-rPA Ab; these results confirmed the previously reported detection by electron microscopy of PA on germinated and ungerminated spores using rabbit reagents [13]. Also, in the functional assays, PA was indirectly detected at presumably low levels in the fluorescence assay of spore germination and in the phagocytosis assays. Thus, spores pretreated with anti-PA Ab (human or rabbit) were more susceptible to Ab-mediated GIA and to the opsonizing effect of anti-PA Ab than were spores pretreated with either preimmune normal IgG or to anti-TTTox mAb. The phagocytosis enhancing effect of anti-PA Ab appeared to be at least as great for ungerminated as for germinated spores. If spores were incubated with germinant and then held for >24 h before use in macrophage assays, the opsonizing effects on spores of anti-PA Ab was lost (data not shown). Again, these observations agree with the rapid release of PA into the supernatant of germinating spores as observed by ECL.

As inferred in the discussion above, the differences exhibited by the different assays in their ability to detect spore-associated PA could be due to several factors. Assay sensitivity likely plays a major role. PA was found negligibly on the surface of ungerminated spores as assayed by the ECL and spore ELISA, a finding that conflicts with the evidence for PA by immunoem and functional assays (macrophage phagocytosis and germination inhibition assays). The spore ELISA likely lacks the sensitivity needed to detect low levels of PA present on germinating or early germinated spores, possibly due to the extensive washing of the ELISA plates, increased spatial distribution, and decreased concentration of PA that is presented to anti-PA antibodies on the spore, compared to binding of anti-PA Ab

to soluble PA. Similarly, effects of steric hindrance probably reduce the sensitivity of the ECL assay for PA in its spore-bound form. The antibody-labeled spore must bind the streptavidin-labeled beads and the complex survive subsequent rigorous washings prior to detection. Thus, this ECL assay of whole-spore PA would not be expected to be as sensitive for PA as is the corresponding assay for soluble PA ([29]; Table 1, B. Kearney et al., unpublished) which can detect PA at pg/ml levels. In contrast to these findings, in the phagocytosis assays, anti-PA Ab facilitated the ability of macrophages to take up and kill both ungerminated and germinated spores to a comparable extent, suggesting that PA or another anti-PA Ab reactive antigen is present on ungerminated spores. One explanation could be that the sensitivity of the biofunctional assays for PA is high and is comparable to the level apparently associated with immunoem. Secondly, incubation of the ungerminated spores with macrophages during the phagocytic uptake period might activate spore germination (and possibly PA expression) in response to release by the macrophages of a spore germinant [30]. However, such evidence for extracellular macrophage-associated spore germination was not detected previously in the tissue culture conditions used by fluorescence staining [14]. The basis of the in vitro GIA of anti-PA Abs for ungerminated spores as demonstrated in the in vitro fluorescence assays could be similarly explained in part by the preceding rationale. After ungerminated spores are preincubated with anti-PA Abs, they are centrifuged, resuspended, exposed to germinant and assayed immediately. The results could be explained by Ab recognition of newly expressed PA that is rapidly translocated to the surface upon exposure to germinant. Alternately, although the exosporium is currently thought to be a barrier that is impermeable to molecules larger than small solutes [14,31–35], the extent of its porosity is not certain. If the exosporium possessed pores large enough to allow passage of Abs, subsurface anti-PA Ab-reactive antigen might be detectable by the germination inhibitory Abs. Results of previous studies suggested that in a spore-vaccinated animal, exosporium-specific antibodies, only, react with the intact spore [32,33,35]. Also, because the exosporium is outermost on the spore, it would seem logical that it is not penetrated by Abs [33]. Various methods were used previously to assess the molecular sieving capacity of the exosporium in *Bacillus sp*, however the results varied greatly, and the permeability of the *Bacillus* exosporium, especially that of *B. anthracis*, remains equivocal [34]. Also, differences in the exosporium of different strains of *B. anthracis* have been observed ultrastructurally [36].

We have yet to unequivocally characterize the entity on the spore surface that is the target of the anti-PA Abs. It is homologous to PA as determined antigenically by immunoblot [13], ECL, ELISA, and electron microscopy; and functionally by the effects of purified anti-PA Abs on spore germination and phagocytosis. However, the anti-PA receptor could be a variant, e.g. truncated, form of authentic

PA or a cross-reactive but otherwise different surface protein. It was observed by immunoem that the reactivity of the human anti-TTTox mAb with spores, like that of the human anti-PA mAb, appeared to be significantly greater than that of normal human IgG, and suggests the presence on spores of antigens that are cross-reactive with tetanus toxin or common to both toxins. However, the anti-TTTox mAb-spore antigen interaction did not appear to be as functionally relevant as anti-PA mAb, as described in Results. The rabbit polyclonal anti-rPA Ab demonstrated heavier labeling by immunoem of spores than did the human anti-PA mAb, AVP-hPA. The latter prevents binding of PA to its cellular target and appears to be specific for the C-terminal domain of PA. However, both polyclonal and monoclonal Abs had similar extents of functional anti-spore activity in vitro (GIA and opsonization). It is thus possible that a portion of the Abs in the PA affinity-purified rabbit polyclonal IgG are specific for a cross-reacting antigen(s) distinct from PA. This idea is supported by the presence of bands recognized by the rabbit anti-rPA pAb on western blots of extracts from Δ Ames and other PA-negative strains [13]. Thus, the evidence en toto suggests that PA is the entity on spores that interacts in a functionally significant manner with Ab directed against purified PA, but that there are other cross-reacting spore antigens that are able to bind polyclonal anti-PA Ab. We are currently pursuing proteomics studies to identify the target of the anti-PA antibody on spores. Other recent proteomic work has demonstrated that PA is expressed very early during spore germination (A. Friedlander, personal communication).

The significance of spore-associated PA in anthrax pathogenesis, and of host anti-spore and anti-PA responses in the immune response to anthrax, are not yet known. The uptake by macrophages and the phagosomal residence of *B. anthracis* spores appears to be conducive to spore germination [9,11,12,14,30,37,38]; it has been hypothesized that the intracellular environment is required to initiate germination and outgrowth in vivo [12,30]. However, there is recent direct and indirect evidence that germination of *B. anthracis* spores in vivo can occur outside of host cells as well. Sterne spores housed in a host cell-excluding chamber were able to germinate in the guinea pig peritoneum (Piris-Giminez et al., unpublished); and mice depleted to a significant extent of macrophages succumbed to spore infection more rapidly than did untreated mice [39]. These observations suggest that: [1] the in vitro GIA of Abs that has been demonstrated (Figs. 5 and 6, [28]) might be significant in vivo; and also that [2] in addition to their facilitative role in an anthrax infection, macrophages are active in host defense against infection and exhibit sporicidal activity [14,39,40]. Both rabbit polyclonal and human monoclonal anti-PA Abs appeared to stimulate macrophage spore uptake and disposal, activities that might contribute to vaccine-induced acquired immunity to anthrax.

However, defining the extent to which anti-PA Abs that are delivered by passive transfer or induced by active vaccination can intervene in the early stages of disease will require more extensive evaluation. The effects of the anti-PA Ab-treatment on spores *in vitro* (germination inhibition, opsonization, and sporicidal activity) were consistent but not extensive. They were observed at high concentrations of antibody, and at least in the germination inhibition assay, they were temporary; i.e. germination was delayed but not prevented. Also, the tests done to detect GIA in sera from vaccinated mice and guinea pigs suggested a possible role for anti-PA Ab in protection; however, a correlation between the GIA of serum anti-PA Ab and immunity has yet to be clearly established. These observations might be linked to the apparent short-lived presence of significant quantities of surface PA on spores. Although anti-PA Abs might interfere to a significant extent in the rate of onset of the earliest stages of infection, they would have a supplemental role as an anti-spore therapeutic and prophylactic. We hypothesize that anti-spore Abs directed to well-characterized intrinsic antigens of the ungerminated and early-germinating spores could be at least as effective as anti-PA Ab in preventing disease or modifying its course, and should be evaluated for inclusion in candidate multi-component anthrax vaccines.

4. Materials and methods

4.1. Bacterial strains, spore preparation, and spore germination

Strains of *B. anthracis* used included the virulent, encapsulated, toxigenic Ames and Vollum 1B strains and the non-encapsulated toxigenic Sterne vaccine strain. Spores were prepared and purified from broth cultures of the strains, as described [13,27,41,42], and were used only if >95% were refractile (ungerminated) as determined by phase microscopy and heat-resistance. Spore preparation included two centrifugations in density gradient medium (58 ml Hypaque-76™, Nycomed into 42 ml WFI) accompanied by extensive washing in sterile water for injection (WFI). The spores were activated by heating at 65 °C for 30 min just before use in assays [27]. In some experiments (described below), germinated spores were prepared by incubation at 30 or 37 °C for various times in the presence of a germinant. The latter consisted of a defined mixture of L-alanine, adenosine, and casamino acids (AAC, [27]) or of L-alanine and inosine (AI, [27]). AAC stock solution contained equal volumes of L-alanine (5 mg/ml), adenosine (5 mg/ml) and casamino acids (1%) and was stored at -20 °C. An aliquot was thawed just before use and diluted to the desired concentration (as described in the text). AI germinant consisted of L-alanine at concentrations of 0.25 mM or higher and 1 mM inosine dissolved in a buffer [10 mM potassium phosphate, 0.1 M NaCl, pH 7.2,

[43]], as indicated in the text. Germination was terminated by rapidly diluting the spores into ice-cold sterile water containing 0.1 M D-alanine followed by centrifugation and resuspension [27]. The germinated spores were used immediately or within 24 h of germination, as detailed below. In assays requiring uniformly germinated spores, the suspensions were verified by phase contrast microscopy and heat-resistance to consist of homogeneously germinated spores (>95%); no outgrown bacilli were detected.

4.2. Antisera and antibodies

Immune sera from mice, guinea pigs, and rabbits were obtained from animals vaccinated with AVA (BioThrax, BioPort, Lansing, MI), purified PA, or PA-producing Venezuelan equine encephalitis (VEE) viral replicon particles [18,28]. Rabbit anti-rPA antisera were from animals hyperimmune to PA purified from the ΔSterne-1[pPA102]CR4 strain [13,17,18,28,44]. Affinity-purified polyclonal rabbit anti-rPA IgG (1.05 mg IgG/ml), provided by S. Little and C. Rossi, was obtained by chromatography of rPA antisera over a PA antigen column followed by a Protein A column. IgG from serum collected from a non-immune normal rabbit (2.05 mg IgG/ml, NRS) was prepared similarly, as described previously [13]. Preimmune sera were obtained from A/J or CBA/J mice vaccinated with AVA or the PA replicon vaccine as described by Lee et al. [18]. Sequential sera were then collected from individual vaccinated mice before each vaccine dose and just before s.c. challenge with a lethal dose of *B. anthracis* strain Sterne [18]. The PA-replicon vaccine contained a nonreplicating derivative of the VEE RNA virus that expresses a cloned PA gene, as described previously [28]. Preimmune sera were collected from guinea pigs vaccinated twice i.m. with recombinant PA or native PA purified from the Sterne strain and then challenged i.m. with >100 LD₅₀ doses of Ames spores; they were provided by P. Fellows and B. Ivins. The sera from the vaccinated animals were assessed for their spore germination inhibitory activity (GIA) and for their anti-PA Ab ELISA titers, to determine their association with protection from lethal challenge, as described below.

Three anti-PA murine monoclonal antibodies (mAbs), 2F9, 6H3, and 14B7, which recognize different domains of PA, were kindly provided by S. Little [16]. Four human anti-PA specific mAbs were generated by the Xenex™ recall technology from AVA-vaccinated donors (Avanir Pharmaceuticals) as described [21]; a human anti-tetanus toxin mAb (TTox) was similarly isolated and evaluated [21] and used as a control. A macrophage cytotoxicity assay and a rat anthrax toxin challenge model were used to determine the potency of anti-PA-mediated toxin neutralization [21]. The human anti-PA mAbs tested were all specific for PA, exhibited very strong binding to and neutralization of PA [21], and recognized different domains of PA. AVP-hPA and AVP-1C6 were specific for PA domain IV and prevented binding of PA to anthrax toxin receptor (ATR).

AVP-21D9 and AVP-22G12 both prevented PA63 heptamer assembly, and were specific for domains II and I, respectively. Except as indicated in the text, all experimentation was conducted using AVP-hPA.

4.3. Isolation and analysis of *pagA*-specific mRNA

Upon collection by centrifugation, the spores (ungerminated or germinated at 37 °C in AAC germinant) were treated with RNA protect bacteria reagent (Qiagen, Hercules, CA) as described by the supplier. The spores were pelleted in a microcentrifuge and resuspended in 1 ml of Trizol reagent (Invitrogen, Carlsbad, CA). The spore suspensions were then transferred to a microfuge tube containing 0.1 mm silica spheres (QBiogene, Carlsbad, CA), chilled on ice, and were shaken in the Fast Prep[®] Instrument (QBiogene) for 45 s, followed by a 5–10-min incubation on ice and an additional 45-s agitation. A 200- μ l aliquot of chloroform was added to the tube, and the contents were mixed thoroughly and spun in the microcentrifuge at top speed for 15 min at 4 °C. The aqueous layer was removed and the RNA was precipitated with an equal volume of isopropanol overnight at –20 °C. The precipitated RNA was washed with 70% ethanol and then subjected to DNase treatment with RNase free DNase (Promega, Madison, WI). The RNA was extracted again with Trizol reagent and precipitated in isopropanol overnight at –20 °C. The RNA was heated to 95 °C for 3 min and then immediately placed on ice to ensure the separation of all remaining DNA/RNA duplexes. The RNA was treated with DNase a second time and then purified with an RNeasy column (Qiagen). The resulting RNA was determined to be free of DNA by control RT-PCR reactions.

The mRNA of the *pagA* transcript was identified by reverse-transcription PCR, using the One-step RT-PCR kit (Qiagen). Primers (Invitrogen) were designed [COTE-PAGA-5'/(GTGCATGCGTTCGTTCTTTGA)- and COTE-PAGA-3' (GCCGCTATCCGCCTTTCTA)] that would amplify a 596 base pair internal fragment of the *pagA* cDNA generated during the RT reaction. The annealing temperature used for the PCR portion of the reaction was 50 °C. Amplicons were visualized on an ethidium bromide-stained 1.5% agarose gel.

4.4. Spore-based anti-PA ELISA

Spore-based anti-PA ELISAs were performed as previously described [27]. Briefly, whole *B. anthracis* spores (irradiated or live) were used as the capture antigen. Spores diluted to approximately 6×10^8 /ml were tested either completely ungerminated or at different time points during germination; spores collected at different stages during purification were also tested. Polyclonal rabbit anti-rPA affinity-purified IgG and human monoclonal anti-PA IgG were tested for their reactivity to the spores. The secondary Abs used were either HRP-conjugated anti-rabbit IgG or

anti-human IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD).

4.5. Immunomagnetic electrochemiluminescence detection of PA (ECL)

A pool of monoclonal anti-PA capture Abs, or alternately, AVP-hPA human anti-PA mAb, was biotinylated and bound onto streptavidin-coated paramagnetic beads. A polyclonal rabbit anti-rPA IgG preparation was labeled with ruthenium (Ru) and acts as a detection antibody in these assays. Antibody concentrations were optimized in checkerboard titrations of labeled antibodies and purified PA [29]. Spores were exposed to the AI or AAC germinant or water [27], with or without bicarbonate, and samples were collected after 0-, 30-, 60-, or 120-min exposure times. They were then centrifuged and washed in water with 0.1 M D-alanine. In addition, supernatants were also collected, and in some experiments concentrated approximately 50-fold by centrifugation using CentriconYM-10TM units (Millipore, Bedford, MA). The washed spores and saved supernatants were assayed separately by combining 50 μ l of sample with the appropriate prebound capture Ab bead and Ru rabbit detector antibody. After 15-min incubation in a vortexing carousel, the samples were read on the ORIGEN[®] 1.5 analyzer (BioVeris, Gaithersburg, MD) and analyzed using Excel software (Microsoft). The limit of detection of free purified PA was 100 pg/ml. For both assays, AI, AAC, and water were used to establish background counts. The average ECL value and standard deviation for the controls was determined for each assay run. Due to the high reproducibility associated with this procedure, the standard deviation is quite low, thus making it necessary to establish an alternative method for establishing a cutoff for positive values. A factor of 1.2 was established after running the assay numerous times, using known concentrations of PA as the testing material. Experimental values were considered positive if the ECL signal was greater than the average plus three times the standard deviation of the control, or 1.2 times the average of the controls, whichever was higher. Differences in ECL signals generated from using different instruments were negated by determining the signal-to-noise ratio of each sample, calculated by dividing the sample ECL value by the average ECL value of the controls.

4.6. In vitro spore germination assays

The in vitro germination of untreated and Ab-pretreated spores was characterized by several assays including the detection of changes in heat resistance, microscopic refractility [27], stainability [13], and by recently described spectrofluorometric assays of germination [27], briefly described as follows. Germination of dormant spores exposed to germinant was assayed spectrophotometrically by two procedures. In one, germination was assayed as the increase in relative

fluorescence units (RFU) over time during continuous incubation in germination medium [27]. The typical germination medium used was AAC, described above, and diluted 1:16 to the final working concentration in the assay. RFU values were recorded automatically at 60-s intervals for 30–60 min. A four-parameter logistic regression model fit the sigmoidal data generated in the fluorescence assays. The percent change in RFU was calculated by subtracting the RFU of a sample at t_0 from the RFU of that sample at a given time during germination and then dividing that value by the RFU of the sample at t_0 . The resulting value was multiplied by 100. Two statistical parameters of the response, the a coefficient (distance to the maximal plateau) and area under the regression curve, were used to compare the significance of differences in germination between antibody- or preimmune IgG-treated or untreated spores [27,43]. In the second assay procedure, spores were incubated in germinant for defined periods of time and then germination stopped by dilution into cold water containing the inhibitor D-alanine, as described previously [27]. The samples were then read spectrophotometrically as described above, except that readings were collected at 5-min intervals for 45 min. In addition to the spectrophotometric determinations, aliquots of the spores taken before spectrometry were evaluated by phase microscopy.

4.7. Macrophage phagocytosis and intracellular spore viability assays

Macrophages were cultured and used in *in vitro* phagocytosis and intracellular spore viability assays as described previously [13,14], using the macrophage cell line RAW264.7. Heat-activated, ungerminated spores of the Ames strain were used. In some assays, the spores were first germinated by incubation at 30 °C in the presence of the AAC germinant [27] for 1 h. Except where indicated, the germinated spores were used in the assays within 24 h of germination. The effects of anti-PA Abs on spore intracellular germination and viability were assayed bacteriologically by viable count determinations and by light and immunofluorescence (FA) microscopy [13,14]. Samples were stained with spore stain (malachite green) and counterstained with a Wright-Giemsa stain (Diff-Quik) [42] for light microscopy; and with secondary Abs (anti-rabbit or anti-human IgG) labeled with green and red fluorescent tags for FA, as described for rabbit reagents [13, 14]. Secondary Abs to probe cultures exposed to human Ab-pretreated spores included goat anti-Human IgG conjugated with fluorescent tags (FITC/TRITC, Molecular Probes). For the FA experiments, the Vectashield mountant also contained the DAPI nuclear stain to detect the macrophages and germinated spores.

4.8. Immunoelectron microscopy

Ungerminated spores and spores germinated by incubation for 1 h in AAC were used in experiments to detect spore-associated PA microscopically. Immunoelectron microscopy (immunoem) was performed on spores incubated in 4% (v/v) formaldehyde (Tousimis Research Co., Rockville, MD) for 14 days at 4 °C and washed in water (ungerminated spores) or PBS (germinated spores). They were then dehydrated using graded alcohol, infiltrated with 1:1 EtOH:LRWhite, and embedded in the LR White resin (Polysciences Inc., Warrington, PA). After curing under UV (long wave) for 72 h, approximately 80–85 nm thick sections were mounted on 200 mesh nickel grids and stored desiccated at 4 °C until used. All procedures were carried out at –20 °C including the curing of the resin. The sections were blocked in blocking buffer (1% ovalbumin + 0.2% cold water fish skin gelatin + 1% normal horse serum in PBS pH 7.4) for 60 min at room temperature. They were incubated in primary Abs (after normalizing to 1 mg/ml), human anti-PA monoclonal or rabbit anti-rPA polyclonal, at a dilution of 1:100, bringing the final concentration to 10 µg/ml. These procedures were done at 4 °C for 18 h or overnight. Sections were washed in Tris buffer and transferred to the secondary Abs (anti-human IgG or anti-rabbit IgG) conjugated to 10 nm gold. After incubation for 1 h at room temperature, the grids were rinsed twice in Tris buffer and three times with deionized water. Sections were stained in 2% saturated aqueous uranyl acetate for 3 min, washed, and air dried before inserted in a JEOL Jem 1010 transmission electron microscope. Images were captured with a Hamamatsu CCD camera aided with AMT 12-HR software. Primary antibodies included the rabbit and human purified anti-PA Abs; negative controls were normal nonimmune rabbit and human IgGs and human anti-tetanus toxin mAb (TTox). Additional sections were prepared to test specificity of secondary Abs, and these sections were treated with blocking buffer and gold-labeled secondary Abs.

4.9. Statistical analysis

The germination kinetics of spores treated with different sera or IgG preparations were analyzed by using a four-parameter logistic regression model (SigmaPlot®). Differences were found in two of the regression parameters and in the areas under the curve, as described previously [27]. Standard methods were used to determine statistical significance and to analyze the data and included the mean, standard error of the mean (SEM), analysis of variance, Z-tests and unpaired Student's *t*-tests. In comparing groups, a *p* value of <0.05 was considered to indicate a significant difference. The association between the *in vitro* germination inhibitory activity of sera from guinea pigs vaccinated with PA and survival after challenge was analyzed by using Cox's proportional hazards model as described elsewhere [45].

Acknowledgements

The authors thank these individuals for their invaluable contributions to our work: N. Shah and Dr Q.-C. Yu (University of Pennsylvania) and K. Kuehl (USAMRIID) for immunogold electron microscopy; P. Fellows and B. Ivins for the gift of guinea pig sera; S. Little for purified rabbit anti-rPA and normal rabbit IgG; B. Kearney, K. Rea, and J. Bashaw for technical assistance; and S. Norris for expert statistical evaluations. Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the US Army. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to the principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. The research described herein was sponsored by the Medical Biological Defense Research Program, US Army Medical Research and Materiel Command, Project #: 02-4-5C-023.

References

- [1] Friedlander AM. Anthrax 2000;20.
- [2] Lustig N. CDC: Investigation of bioterrorism-related anthrax. *Morbidity and Mortality Weekly Report* 2001;50:1077–9.
- [3] Meselson M, Guillemin J, Hugh-Jones M, Langmuir A, Popova I, Shelokov A, et al. The sverdlovsk anthrax outbreak of 1979. *Science* 1994;266:1202–7.
- [4] Mock M, Fouet A. Anthrax. *Ann Rev Microbiol* 2001;55:647–71.
- [5] Turnbull PCB. Introduction: Anthrax history, disease, and ecology. Chapter 1. In: Koehler TM, editor. *Anthrax*. Berlin: Springer; 2002. p. 1–19.
- [6] Moir A, Smith D. The genetics of bacterial spore germination. *Annu Rev Microbiol* 1990;44:5331–553.
- [7] Nicholson WL, Setlow P. Sporulation, germination and outgrowth. In: Hardwood CR, Cutting SM, editors. *Molecular biology methods for Bacillus*. Farmington, CT: Wiley; 1990. p. 391–450.
- [8] Ross JM. The pathogenesis of anthrax following the administration of spores by the respiratory route. *J Pathol Bacteriol* 1957;73:485–94.
- [9] Guidi-Rotani C. The alveolar macrophage: the trojan horse of *Bacillus anthracis*. *Trends Microbiol* 2002;10:405–9.
- [10] Guidi-Rotani C, Levy M, Ohayon H, Mock M. Fate of germinated *Bacillus anthracis* spores in primary murine macrophages. *Mol Microbiol* 2001;42:931–8.
- [11] Guidi-Rotani C, Weber-Levy M, Labruyere E, Mock M. Germination of *Bacillus anthracis* spores within alveolar macrophages. *Mol Microbiol* 1999;31:9–17.
- [12] Hanna PC, Ireland JA. Understanding *Bacillus anthracis* pathogenesis. *Trends Microbiol* 1999;7:180–2.
- [13] Welkos S, Little S, Friedlander A, Fritz D, Fellows P. The role of antibodies to *Bacillus anthracis* and anthrax toxin components in inhibiting the early stages of infection by anthrax spores. *Microbiology* 2001;147:1677–85.
- [14] Welkos S, Friedlander A, Weeks S, Little S, Mendelson I. In-vitro characterisation of the phagocytosis and fate of anthrax spores in macrophages and the effects of anti-PA antibody. *J Med Microbiol* 2002;51:821–31.
- [15] Leppla SH. Anthrax toxin. *Bacterial protein toxins*, chap. 19. vol. 145. Berlin: Springer; 2000.
- [16] Little SF, Novak JM, Lowe JR, Leppla SH, Singh Y, Klimpel KR, et al. Characterization of lethal factor binding and cell receptor binding domains of protective antigen of *Bacillus anthracis* using monoclonal antibodies. *Infect Immun* 1996;64:1807–13.
- [17] Ivins B, Welkos S. Cloning and expression of the *Bacillus anthracis* protective antigen gene in *Bacillus subtilis*. *Infect Immun* 1986;54:537–42.
- [18] Lee JS, Hadjipanayis AG, Welkos SL. Venezuelan equine encephalitis virus-vectored vaccines protect mice against anthrax spore challenge. *Infect Immun* 2003;71:1491–6.
- [19] Pitt MLM, Little SF, Ivins BE, Fellows P, Barth J, Hewetson J, et al. In vitro correlate of immunity in a rabbit model of inhalational anthrax. *Vaccine* 2001;19:4768–73.
- [20] Stepanov AV, Marinin LI, Pomerantsev AP, Staritsin NA. Development of novel vaccines against anthrax in man. *J Biotechnol* 1996;44:155–60.
- [21] Sawada-Hirai R, Jiang I, Wang F, Sun SM, Nedellec R, Ruther P. Human anti-anthrax protective antigen neutralizing monoclonal antibodies derived from donors vaccinated with anthrax vaccine adsorbed. *J Immune Based Therap Vacc* 2004;2(5–19).
- [22] Koehler TM, Dai Z, Kaufman-Yarbray M. Regulation of the *Bacillus anthracis* protective antigen gene: CO₂ and a trans-acting element activate transcription from one of two promoters. *J Bacteriol* 1994;174:370–80.
- [23] Saile E, Koehler TM. Control of anthrax toxin gene expression by the transition state regulator AbrB. *J Bacteriol* 2002;184:370–80.
- [24] Sirard JC, Mock M, Fouet A. The three *Bacillus anthracis* toxin genes are coordinately regulated by bicarbonate and temperature. *J Bacteriol* 1994;176:5188–92.
- [25] Leppla SH. Production and purification of anthrax toxin. *Methods Enzymol* 1988;165:103–16.
- [26] Ezzell JW, Abshire TG. Encapsulation of *Bacillus anthracis* spores and spore identification. In: Turnbull PCB, editor. *Proceedings of the International Workshop Anthrax*, vol. 87. Winchester, England: Salisbury Medical Society; 1996. p. 42.
- [27] Welkos SL, Cote CK, Rea KM, Gibbs PH. A microtiter fluorometric assay to detect the germination of *Bacillus anthracis* spores and the germination inhibitory effects of antibodies. *J Microb Methods* 2004;56:253–65.
- [28] Pushko P, Parker M, Ludwig GV, Davis NL, Johnston RE, Smith JF. Replicon-helper systems from attenuated venezuelan equine encephalitis virus: expression of heterologous genes in vitro and immunization against heterologous pathogens in vivo. *Virology* 1997;239:389–401.
- [29] Kijek TM, Rossi CA, Moss D, Parker RW, Henchal EA. Rapid and sensitive immunomagnetic-electrochemoluminescent detection of staphylococcal enterotoxin B. *J Immunol Methods* 2000;236:9–17.
- [30] Ireland JA, Hanna PC. Macrophage-enhanced germination of *Bacillus anthracis* endospores requires gerS. *Infect Immun* 2002;70:5870–2.
- [31] Driks A. Maximum shields: the armor plating of the bacterial spore. *Trends Microbiol* 2002;10:251–4.
- [32] Desrosier JP, Cano Lara J. Synthesis of exosporium during sporulation of *Bacillus cereus*. *J Gen Microbiol* 1984;130:935–40.
- [33] Gerhardt P. Cytology of *Bacillus anthracis*. *Fed Proceed* 1967;26:1504–17.
- [34] Gerhardt P, Scherrer R, Black S. In: Spores v, editor. *Molecular sieving by dormant spore structures*. Washington, DC: American Society for Microbiology; 1972. p. 68–74.

- [35] Tomcsik J, Bouille M, Baumann-Grace JB. Specific reaction of the exosporium of the *Bacillus cereus* and *Bacillus anthracis*. Schweiz Z Allgem Pathol Bakteriolog 1959;22:630–40.
- [36] Kramer MJ, Roth IL. Ultrastructural differences in the exosporium of the Sterne and Vollum strains of *Bacillus anthracis*. Can J Microbiol 1968;15:1297–9.
- [37] Guidi-Rontani C, Weber-Levy M, Labruyere E, Mock M. Fate of germinated *Bacillus anthracis* spores in primary murine macrophages. Mol Microbiol 2001;42:931–8.
- [38] Dixon TC, Fadl AA, Koehler TM, Swanson JA, Hanna PC. Early *Bacillus anthracis*-macrophage interactions: Intracellular survival and escape. Cellular Microbiol 2000;2:453–63.
- [39] Cote CK, Rea KM, Norris SL, Van Rooijen N, Welkos SL. The use of a model of in vivo macrophage depletion to study the role of macrophages during infection with *Bacillus anthracis* spores. Microbial Pathol 2004;37(4):169–75.
- [40] Gimenez PA, Wu YZ, Paya M, Delclaux C, Touqui L, Goossens PL. High bactericidal efficiency of type iia phospholipase A2 against *Bacillus anthracis* and inhibition of its secretion by the lethal toxin. J Immunol 2004;173:521–30.
- [41] Leighton TJ, Doi RH. The stability of messenger ribonucleic acid during sporulation in *Bacillus subtilis*. J Biol Chem 1971;246:3189–95.
- [42] Welkos SL, Trotter RW, Becker DM, Nelson GO. Resistance to the Sterne strain of *B. anthracis*: phagocytic cell responses of resistant and susceptible mice. Microb Pathogen 1989;7:15–36.
- [43] Stewart G, Johnstone K, Hagelberg E, Ellar D. Commitment of bacterial spores to germinate. Biochem J 1981;198:101–6.
- [44] Worsham P, Sowers M. Isolation of an asporogenic (spo0A) protective antigen-producing strain of *Bacillus anthracis*. Can J Microbiol 1999;45:1–8.
- [45] Welkos SL, Keener TJ, Gibbs PH. Differences in susceptibility of inbred mice to *Bacillus anthracis*. Infect Immun 1986;51(3):795–800.