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Monoclonal Antibodies for Bacillus anthracis Spore Detection and Functional Analyses of Spore Germination and Outgrowth

Melissa K. Swiecki,*† Mark W. Lisanby,*† Fengyu Shu,*† Charles L. Turnbough, Jr., † and John F. Kearney2*†

All members of the Bacillus genus produce endospores as part of their life cycle; however, it is not possible to determine the identity of spores by casual or morphological examination. The 2001 anthrax attacks demonstrated a need for fast, dependable methods for detecting Bacillus anthracis spores in vitro and in vivo. We have developed a variety of isotypes and specificities of mAbs that were able to distinguish B. anthracis spores from other Bacillus spores. The majority of Abs were directed toward BcIA, a major component of the exosporium, although other components were also distinguished. These Abs did not react with vegetative forms. Some Abs distinguished B. anthracis spores from spores of distantly related species in a highly specific manner, whereas others discriminated among strains that are the closest relatives of B. anthracis. These Abs provide a rapid and reliable means of identifying B. anthracis spores, for probing the structure and function of the exosporium, and in the analysis of the life cycle of B. anthracis. The Journal of Immunology, 2006, 176: 6076–6084.

Members of the Bacillus genus, including ubiquitous species such as Bacillus subtilis, Bacillus thuringiensis, Bacillus cereus, and Bacillus anthracis, are capable of forming endospores when replicative vegetative cells are placed under nutritional constraint (1, 2). A complex series of differentiative processes occurs under these conditions resulting in the conversion of the vegetative mother cell to a resting ametabolic spore (3). The resistance of spores to irradiation, heat and other harsh conditions allows them to persist in a dormant state for extended periods of time in the environment (4). Under appropriate conditions spores will begin the process of germination resulting in the production of replicative vegetative cells, thus completing the life cycle (5).

Although many microorganisms form spores, B. anthracis is of particular interest because the vegetative form of this organism has the ability to cause the fatal disease anthrax if the spores are acquired by the host through breaks in the skin, by inhalation; or by ingestion. Recent studies have shown that B. anthracis is genetically similar to other spore-forming organisms including B. thuringiensis and B. cereus. These studies include conventional bacteriological assays and more recently RFLP and other molecular fingerprinting methods (6–8). Identification and classification of spore species by metabolic studies and gene polymorphisms by genome analyses have focused on the vegetative form of these organisms. The nature of spores and their ability to survive harsh extraction procedures have, until recently, circumvented all but rudimentary studies on the biochemical nature and, in particular, the molecular structure of Bacillus spores.

We have concentrated our studies on the structure of the B. anthracis exosporium, the outermost surface of the spore. Spores of all pathogenic Bacillus species, which includes B. anthracis, B. cereus (capable of causing human food poisoning), and B. thuringiensis (an insect pathogen), are enclosed by a prominent exosporium. The formation of the exosporium during sporulation is a complex and energy-consuming process. As such, it is unlikely that this structure would be maintained by the bacterium unless it was important for survival. The pressure to maintain the exosporium is probably related to some aspect of infection because most non-pathogenic Bacillus spores do not possess a (well-developed) exosporium. It is through the exosporium that the initial interactions with a human or animal host occur, potentially leading to an anthrax infection.

We describe the preparation and characterization of mAbs for B. anthracis that are specific for the spore stage of its life cycle, do not react with vegetative contaminants in spore preparations, and distinguish B. anthracis spores from other Bacillus species.

Materials and Methods

Mice

Female 8- to 12-wk-old BALB/cJ mice were purchased from The Jackson Laboratory. Mice were housed under specific pathogen-free conditions and used according to the protocols approved by the University of Alabama at Birmingham (Birmingham, AL).

Spores, exosporium preparations, and recombinant proteins

Spores, exosporium preparations, and recombinant BcIA, BxpA, and BxpB were prepared as previously described (9, 10). Mutant B. anthracis spores used in this study had either a deletion of the Bacillus collagen-like protein of anthracis gene (∆bcIA), a gene deletion resulting in a strain unable to synthesize l-rhamnose (ΔrmlD), or a gene deletion resulting in a strain unable to produce the exosporium basal layer protein BxpB (∆bxpB) (9–11). Isolates shown to be close relatives of B. anthracis were obtained from K. Hill and P. Jackson (Bioscience Division, Los Alamos National Laboratory, Los Alamos, NM).
Production, labeling, and sequencing of mAbs

BALB/cJ mice were immunized s.c. in each calf region with a mix of 10^7–40,000 rad-irradiated B. anthracis Sterne and Ames spores, first in CFA and then at 4-day intervals with spores and saline. Spores were irradiated with 10,000 rads at the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID), which inactivated 100% of the spores as determined by P. Boyaka (Department of Microbiology, University of Alabama at Birmingham, Birmingham, AL) labeled with Alexa Fluor 555, and labeled with Alexa Fluorochromes according to instructions (Molecular Probes/Invitrogen Life Technologies). Sequencing of the Ig genes was done as previously described (13).

Hybridoma screening

Wild-type or mutant B. anthracis spores (10^7) were incubated with hybridoma culture supernatants or an isotype control in 1% BSA solution containing 50 μg/ml for 20 min on ice. Spores were washed twice with 1% BSA in PBS and an Alexa Fluor 488-labeled goat anti-mouse IgG (H + L) Ab from Molecular Probes/Invitrogen Life Technologies was used as the secondary Ab at a concentration of 2–5 μg/ml. Spores were washed again and resuspended in 1% BSA in PBS for FACS. Flow cytometry was done using a FACS Calibur from BD Biosciences and labeled with Alexa Fluorochromes from Amersham Biosciences and labeled with Alexa Fluorochromes according to instructions (Molecular Probes/Invitrogen Life Technologies). Sequencing of the Ig genes was done as previously described (13).

FACS

B. anthracis (wild-type, ΔbcIA, ΔbpB and Δrmd), B. thuringiensis, B. cereus or B. subtilis spores (10^7) were incubated with Alexa Fluor 488-labeled anti-BcIA, BxpA, and BxpB Abs or an isotype control in 1% BSA in PBS at 10 μg/ml for 20 min on ice. Spores were washed twice with 1% BSA in PBS and analyzed with a FACS Calibur. Histograms were generated using FlowJo software. Staining for protective Ag (PA) was done using the Ab 8C2.18 (provided by P. Boyaka, Department of Microbiology, University of Alabama at Birmingham, Birmingham, AL) labeled with Alexa Fluor 488. Spores and/or vegetative cells were also stained with spore-specific Abs, SA26 (Novus Biologicals) and SA27 (HyTest) at 1–2 μg/ml for 20 min on ice followed by staining with Alexa Fluor 488-labeled goat anti-mouse IgG (H + L) Ab at 2–5 μg/ml for 20 min on ice.

Microscopic analysis

Glass microscope slides or coverslips were coated for 30 min with a solution containing 50 μg/ml poly-l-lysine in distilled water. Slides were washed with distilled water, and 10 μl of well-washed spores at 2 × 10^6/ml in distilled water were allowed to air-dry overnight onto the poly-l-lysine-coated slides. Mixtures of vegetative cells and spores were dried onto microscope slides in the same way and fixed in ethanol at 4°C before staining. For fluorescence microscopy, the adherent spores and/or bacteria were stained with Alexa Fluorochrome-labeled Abs at 1–10 μg/ml. The purified SA27 Ab was used at 1 μg/ml, and the secondary Ab used was goat anti-mouse IgG2a-RITC at 5 μg/ml (Southern Biotechnology Associates). The high-affinity anti-PA single-chain Ab 14B7 was provided by G. Georgiou (Department of Chemical Engineering, University of Texas, Austin, TX) and labeled with Alexa Fluor 555.

Intravenous spore immunizations and sera collection

To study the Ab response to intact, viable spores, two groups of five BALB/cJ mice were injected i.v. with either 10^9 or 10^7 wild-type B. anthracis Sterne spores in 200 μl of PBS. A control group of three mice was injected with PBS. On day 28, both spore-immunized groups were given 1.5 × 10^8 spores in 200 μl of PBS i.v., and the control mice received PBS again. Mice were bled approximately every 7 days by retro-orbital bleeding and blood samples were stored overnight at 4°C. The following day, tubes were centrifuged at maximum speed (14,000 rpm) in a Spectrafuge for 30 s, and sera were collected and stored at −20°C until use.

Tissue sectioning and staining

BALB/cJ mice were injected i.v. with either unlabeled wild-type B. anthracis Sterne spores or a mixture of unlabeled spores that contained B. anthracis Sterne, B. thuringiensis Kurstaki, and B. subtilis 1A700. Mice injected with B. anthracis spores alone were sacrificed 6 days after injection, and those injected with spore mixtures were sacrificed 1 h after administration. Spleens were removed and blocked with OCT Compound (Tissue-Tek), and frozen sections were prepared with a cryostat (IEC Micronome; International Equipment) and kept at −70°C until staining. Slides were blocked for 20 min at room temperature with PBS plus 10% horse serum then stained for 20 min at room temperature with fluorescent-labeled mAbs specific for B. anthracis (BF1-AMCA or BF1-Alexa 488), B. thuringiensis (BT6-PE), and B. subtilis (D4-Alexa 488). Abs were diluted in PBS plus 10% horse serum, and slides were mounted with Fluoromount-G from Southern Biotechnology Associates. Mac-1-PE was purchased from BD Biosciences.

Quantitative ELISA

High binding 96-well ELISA plates (Costar) were coated overnight with recombinant BcIA, BxpA, or BxpB at 1 μg/ml in 0.1 M borate saline at 4°C. Plates were washed four times with PBS using an automatic plate washer. Wells were blocked for 30 min at room temperature with 1% BSA in PBS and then washed four times. Then 100 μl/well of diluted mouse sera (1 μl sera/ml) or purified anti-BcIA Ab standards (IgM, IgG1, IgG2a, IgG2b, and IgG3) were added to the first row of 96-well plates. Samples and standards were then serially diluted 1/4. Plates were incubated at 37°C for 2 h and then washed four times. Dilutions of 1/500 of goat anti-mouse IgG, IgG1, IgG2a, IgG2b, and IgG3 were added to the second row of 96-well plates. Samples and standards were then serially diluted 1/4. Wells were incubated for 2 h at 37°C and then washed 4 times. AP-substrate (Sigma-Aldrich) was dissolved in AP-substrate buffer (1 mg/ml) and added to wells (50 μl/well) for 5–10 min at room temperature. Reactions were stopped by adding 25 μl/well of 5 N NaOH. Optical density was read at 405 nm with a Titertek Multiskan Plus ELISA reader (Flow Laboratories), and concentrations of serum Ig specific for BcIA, BxpA, and BxpB were determined by Delta Soft ELISA analysis software (Bio-Tek). Sera, purified Abs, and secondary Abs were diluted in 1% BSA in PBS.

Monitoring germination and outgrowth of spores by FACS

Wild-type and/or mutant B. anthracis Sterne spores at 1 × 10^7/ml were incubated in brain-heart infusion medium (BHI; Difco), 20 g/L, at 37°C plus 10% CO_2 for 3 h. Samples of 100 μl were removed at various time points and kept on ice. Time zero samples were kept on ice in PBS for 3 h. Samples were washed and stained with an anti-BcIA mAb (JC8-5-PE) and/or an anti-vegetative cell Ab (EAI1-FITC and EALI-Alexa 488) diluted at 5–10 μg/ml for 20 min on ice. Samples were washed and analyzed by FACS. Dot plots and histograms showing spore and vegetative populations were generated with WinMDI software (J. Trotter; available at http://pauling.salk.edu/software.html) and FlowJo.

Time lapse microscopy

Spores were coated onto coverslips or slides as described above and incubated in RPMI 1640 with l-glutamine and BHI (20 g/L, pH 7.4) at 37°C and 5% CO_2 for 4 h. Spores were suspended in PBS for an additional 3 h. Spores were washed 4 times. AP-substrate (Sigma-Aldrich) was dissolved in AP-substrate buffer (1 mg/ml) and added to wells (50 μl/well) for 5–10 min at room temperature. Reactions were stopped by adding 25 μl/well of 5 N NaOH. Optical density was read at 405 nm with a Titertek Multiskan Plus ELISA reader (Flow Laboratories), and concentrations of serum Ig specific for BcIA, BxpA, and BxpB were determined by Delta Soft ELISA analysis software (Bio-Tek). Sera, purified Abs, and secondary Abs were diluted in 1% BSA in PBS.

Results

Production and analysis of mAbs to Bacillus spores

Previous attempts to make spore-specific Abs used formalin-fixed spores as Ags, presumably to prevent germination and/or loss of spore-specific Ags in the immunized animals (14). In pilot studies to determine the optimal method for making spore-specific Abs, two separate immunizations were made with B. subtilis, one with formaldehyde-fixed spores and the other with native (unfixed) spores using the protocol of s.c. immunizations followed by fusion of B cells from the local draining lymph nodes (12). Initially, the Abs were screened by ELISA, but most later primary screenings

3 Abbreviations used in this paper: AP, alkaline phosphatase; BHI, brain-heart infusion medium; PA, protective Ag.
were done on spore suspensions using multicolor flow cytometry. Only 3 of 192 (1.6%) hybridomas from fixed spore-injected mice made mAbs reactive with spores, whereas immunization with unfixed spores gave 95/384 (25%) mAbs that reacted strongly with spores of B. anthracis. In subsequent analysis, we concentrated on those that bound preferentially to B. anthracis spores.

mAbs to B. anthracis spores

We next immunized BALB/cJ mice with a mixture of heavily irradiated B. anthracis spores of the Ames and Sterne strains, generated hybridomas, and screened for anti-spore Ab production by flow cytometry. Some mAbs bound only B. anthracis spores, whereas others were cross-reactive with B. cereus or B. thuringiensis. Percentages of B. anthracis anti-spore Abs that bound to B. subtilis and B. thuringiensis were 0 and 42%, respectively. For subsequent analysis, we concentrated on those that bound preferentially to B. anthracis.

B. anthracis, B. thuringiensis, and B. cereus spores differ from B. subtilis in that their spores are encapsulated in loosely fitting
exosporium. Because morphological studies show the exosporium is a prominent feature of B. anthracis spores, we made a final panel of mAbs against purified exosporium isolated from the B. anthracis Sterne strain to ensure that the heavy y-irradiation did not alter proteins on the spore surface resulting in spurious anti-spore activity. The staining profiles of these exosporium-derived mAbs suggested that they bound to a highly homogeneous structure on the spore surface as typified by patterns of binding shown by EA4, EA2, and EG4 (Fig. 1A). The lower three rows show negative staining of B. thuringiensis (Bt Kurstaki), B. cereus (Bc T), and B. subtilis (Bs 1A700). To further test Ab specificity, mixtures of B. anthracis Sterne, B. thuringiensis Kurstaki, and B. subtilis 1A700 spores were used in three-color flow cytometric analysis with our spore-specific Abs, and high discrimination was achieved, with the three spore types readily detected as separate populations (data not shown). Similar discrimination was obtained by microscopic analysis of spore mixtures on slides and on frozen sections of mouse spleen tissue 1 h after i.v. injection (Fig. 1, B and C). Because spores have the potential to linger in the host for many days after entrance into the body (15), we next wanted to determine whether our Abs were capable of detecting spores in mice 6 days after i.v. injection of spores (Fig. 1, D and E). B. anthracis Sterne spores could be observed in the red pulp of the spleen as detected with the mAb BF1-Alexa 488.

As a final test for B. anthracis spore specificity, we screened our anti-spore mAbs on a panel of 10 B. cereus and B. thuringiensis isolates obtained from worldwide sources which were shown genetically to be the closest relatives of B. anthracis (7, 8). It can be seen in Table I that our B. anthracis Abs also bound 3 of 10 of the closely related isolates, B. thuringiensis B8, B. thuringiensis 97-27, and B. cereus D17. These strains have been shown to be particularly difficult to distinguish from B. anthracis by conventional assays. In another experiment, several anti-BclA Abs raised against irradiated spores were tested by immunofluorescence microscopy on a panel of 11 different virulent and avirulent strains of B. anthracis (data not shown). The majority of the Abs reacted with all of the B. anthracis strains, indicating that with respect to Ab reactivity, virulent and avirulent strains behave similarly.

Abs to spore components BxpA and BxpB and vegetative cell components

We also isolated and characterized hybridomas from mice immunized with recombinant BxpA, BxpB, and several other components from the exosporium (9, 10). Most of the mAbs to these components did not bind to wild-type B. anthracis (Sterne and ΔAmes) but did bind to ΔbclA and ΔrmlD spores, indicating that the target epitopes on BxpA and BxpB for these Abs were masked or hidden by the BcIA component of the exosporium (Ref. 10 and Fig. 2A). We were also unable to show binding of PA-specific Abs to wild-type or mutant ungerminated, Renograffin-purified spores. However, we could detect binding of anti-BxpB and anti-PA mAbs to exosporium remnants after the vegetative cells had ruptured and burst out of the exosporium (Fig. 2B).

The SA27 spore-specific Ab used in our studies did not bind to intact spores that had been purified with Renograffin. However, by gating the flow cytometer on debris in the initial spore preparation or in cultures where the spores had germinated, we were able to show that binding to debris occurs in unpurified preparations (Fig. 3). Similar observations were made using the spore-specific Ab SA26 (data not shown). It has been claimed by proteomic analysis (16, 17) that the S-layer proteins, specifically EA1, are associated with spores. Further analysis revealed that EA1 is actually a major contaminant found in spore preparations (18). SA27 is most likely recognizing one of these vegetative cell contaminants; therefore, these results support previous observations that products of vegetative cells may be erroneously interpreted as being associated with the spore surface (18).

Spore Ag targets and Ab heterogeneity

Table II shows that the majority of the Abs made against intact spores and also the exosporium preparations react with the recombinant expressed protein BcIA. Many of these Abs recognize epitopes in the C-terminal domain of BcIA which is 134 aa long and composes the distal end of each filament of the hair-like nap (19). BcIA has been characterized molecularly and has been shown to contain multiple tandem repeats of amino acid sequence motifs. Molecules such as these and complex carbohydrates often elicit polyclonal Ab responses of limited heterogeneity (20) or conversely can elicit diverse polyclonal responses (21). To investigate these alternatives, we sequenced 20 Ig H and 16 L chain V region genes from our panel of Ab-producing hybridomas made against irradiated spores to determine their clonal heterogeneity. Results showed that there was no pattern of prominent individual VH or VL gene expression, with most of the major VH gene families and an assortment of VL genes being represented (Table III). Examination
immunization with intact Ab response to BclA showed no evidence of oligoclonality. Similarly high degree of heterogeneity (data not shown). Thus, the panel of Abs made against purified exosporium also displayed an identical, suggesting that these hybridomas resulted from the fusion of CDR3 regions showed no examples of possible clonal identity with the exception of DE3 and AF10, the L chains of which were identical, suggesting that these hybridomas resulted from the fusion of two members of the same clone. Ig gene sequencing of a panel of Abs made against purified exosporium also displayed a similarly high degree of heterogeneity (data not shown). Thus, the Ab response to BclA showed no evidence of oligoclonality.

Ab responses to recombinant BclA, BxpA, and BxpB after immunization with intact B. anthracis spores

Because hybridomas were made by immunization with spores or purified exosporium in adjuvant, we also determined serum Ab responses to recombinant BclA as well as two other exosporium components, BxpA and BxpB (9, 10), after i.v. immunization with intact B. anthracis spores.

BALB/cJ mice were injected i.v. with intact, unmodified B. anthracis Sterne spores or with PBS and boosted with spores or PBS on day 28. Mice were bled weekly, and anti-BclA Ab concentrations were determined by quantitative ELISA. In the primary response, anti-BclA Abs of each isotype (IgM, IgG1, IgG2a, IgG2b, and IgG3) were detected in the sera of immunized mice. Large amounts of the same isotypes, particularly IgG3 and IgG2b, were produced after an i.v. boost and were detected in sera over a prolonged period (Fig. 4). The same groups of mice were also tested for their response to the exosporium proteins, BxpA and BxpB, detection of which by microscopy and flow cytometry on wild-type spores appears to be occluded by the major exosporium protein BclA. This was done to determine whether these components were immunogenic after possible spore exosporium modification in vivo. Sera from mice taken 2 wk after boost was tested for total anti-BclA, -BxpA, and -BxpB Ig. Anti-BclA Ab could be detected at sera dilutions >1:512,000, whereas Abs to BxpA and BxpB were hardly detectable at sera dilutions of 1:2000 (data not shown). This experiment, combined with the results obtained from our hybridoma panel, indicates that BclA is not only the major exosporium protein as shown by Western blot analysis (9, 22) but also the immunodominant spore surface Ag after both methods of immunization.

Detection of spore and vegetative populations of B. anthracis by flow cytometry

We next compared the binding patterns of our spore-specific mAbs with an anti-vegetative cell Ab on spores during germination and outgrowth. In the top panels of Fig. 5A, the forward scatter and side scatter of spores incubated in PBS on ice or in BHI at 37°C were detected by FACS when displayed on the x- and y-axes. Ungermiated spores in each panel are defined by the blue gate R1. Germimated spores are defined by the red gate R2 after 1 and 3 h of incubation in BHI. Vegetative cells are defined by the green gate R3 after 3 h of incubation. In the middle panels, the same populations are defined by staining with the anti-BclA Ab J8-5-PE (y-axis) and the vegetative cell-specific Ab EAIIF-FTIC (x-axis) which appears to be specific for galactose-N-acetylgalcosamine polysaccharide expressed by the vegetative cell (23). In gate R1, containing spores, only the anti-BclA Ab is bound. After 1 and 3 h, new populations of vegetative cells are stained with EAIIF. Cells expressing both markers, in gate R2 circled in purple, are emergent vegetative cells still attached to the spore exosporium. The population of J8-5-bright, EAIIF-dim particles (gray circle) in gate R3

![FIGURE 3. SA27 Ab binds to vegetative cell debris but not to spores.](image)

Samples were taken at different stages of the spore purification process and stained with SA27 or an anti-BclA Ab (EG4-4-1) and then analyzed by FACS and microscopy. Scatter profiles of unstained samples are shown in the first column. Stained samples from each preparation are shown in the second and third columns. The shaded region represents staining with an isotype control, and the white region shows staining with either SA27 or EG4-4-1. Images in the fourth column show preparations stained with SA27 (red) and EG4-4-1 (green). Prep 1, freshly harvested from sporulation medium (unwashed); Prep 2, washed three times with water; Prep 3, Renograffin-purified; Prep 4, Renograffin-purified followed by three washes with water.
is likely to be another population of cell/exosporium remnants. The correlates of these populations as determined by microscopy are displayed on the phase contrast photomicrograph (Fig. 5B). It was recently revealed by time-lapse microscopy that deleting the exosporium protein BxpB resulted in faster germination (transition of phase bright to dark) and outgrowth times compared to wild-type spores (10). Fig. 5C also shows that the vegetative form outgrows faster from /H9004 bxpB spores than wild-type, /H9004 bclA, and /H9004 rmlD spores by staining with the vegetative cell-specific Ab EAII.

**Analysis of B. anthracis spore germination, outgrowth, and elongation by phase contrast time lapse microscopy**

Time-lapse microscopy is a powerful tool for monitoring spore germination and outgrowth as demonstrated by Steichen et al. (10). There is a definite polarity in the germination process, and under the conditions we have used to germinate spores, the spores do not split along the central axis but appear to rupture at one end releasing the vegetative cell from the exosporium (Fig. 6). In the top three panels of Fig. 6A, germination occurs asynchronously in all spores by ~7 min. The black arrows are to assist the reader to follow the fate of two representative spores in the field of view. In the middle three panels, the explosive emergence of the vegetative cells from the ruptured exosporium takes place over a period of 3 s, leaving the newly emergent vegetative cell still attached to the phase-brighter exosporium remnant (white arrows). In the lower three panels, the continued growth of the vegetative cells occurs, with one of the pair still retaining the exosporium remnant. These stages can also be visualized by microscopy using spore- and vegetative cell-specific Abs (Fig. 6B). Thus, flow cytometry and microscopy can be used in concert to accurately quantitate at a single-cell level the stages of spore germination and outgrowth in vitro for wild-type and mutant spores.

**Discussion**

We have isolated and characterized panels of mAbs that are highly specific and can discriminate among spores of the *Bacillus* family, including the strategically important *B. anthracis*. To date, there are no examples of such Abs in the literature. Abs made in sheep and other animals against *B. subtilis* or *B. anthracis* do not discriminate among spores of other families (24, 25). Our reagents are likely unique for two reasons: 1) we did not chemically modify the spores before immunization; and 2) these are mAbs made by immunizing and fusing local lymph nodes, a procedure that has not been used previously with spores. Although we have a complete variety of Abs with respect to their isotypes except for IgA, this route of administration may also be the reason why we have not yet isolated Abs to carbohydrates associated with the exosporium or many IgM Abs. From our past experience, these types of Abs are almost exclusively made by mouse spleen marginal zone B cells (20). The combined results obtained from our hybridoma panels and after s.c. immunization with intact spores or exosporium in CFA and the serum Ab responses after i.v. administration of live spores in BALB/c mice are shown in Fig. 4. Anti-BclA serum Ab levels from spore-immunized mice were compared to unimmunized (PBS) mice at the intervals shown. Results are expressed as a geometric mean with error bars representing the SD (n = 5 for both immunized groups and n = 3 for unimmunized).

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**Table III. V_{HL} and V_{L} gene usage of B. anthracis anti-spore mAbs**

<table>
<thead>
<tr>
<th>Anti-Spore Ab</th>
<th>V_{HL} Family</th>
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intact spores show conclusively that BclA is not only the major exosporium protein (9, 22) but also the immunodominant spore surface Ag in mice. Only minute amounts of Ab was made against other exosporium components BxpA and BxpB, although in independent experiments large numbers of hybridomas were obtained by immunizing mice with purified recombinant versions of these exosporium proteins (10).

It has been shown that BclA has multiple tandem repeats, a feature common with some of the other exosporium proteins (9, 22). The strong and prolonged Ab response after immunization with intact spores or exosporium produced a pattern similar to that of T-independent Ab responses with IgM, IgG3 (26–28), and IgG2b (29) Abs predominating. Tandem repeats tend to be within exposed loops of proteins and have the potential of eliciting strong immune responses due to their amino acid composition and their interactions with B cell receptors (30). They also have been implicated in immune evasion by replicating eukaryotic parasites (31, 32). In the latter case, these molecules may be involved in diversification of the immune response resulting in competitive use of cytokines and other resources.

A speculative hypothesis is that a potent polyclonal Ab response to exosporium proteins such as BclA may protect the spore or germinating spore by subverting the immune system from making potential sporicidal or germination-inhibiting Abs to hidden Ags on the germinating wild-type spores. The fact that B. subtilis spores are cleared very rapidly from mice (33) suggests that highly conserved Abs (possibly existing as natural Abs) to B. subtilis spore components may be responsible for this rapid clearance. These same determinants, if found on B. anthracis, would normally be covered and may therefore prevent the functional inactivation of the B. anthracis spores until they are safely inside their cellular targets. In addition, there may be tradeoffs between exosporium properties necessary to protect the nascent vegetative cell from host innate and adaptive immune systems within or outside host cells and those that facilitate spore uptake by host phagocytic cells, proposed by some as an obligatory step in infection (34–37).

As shown by conventional bacteriological assays and more recently by RFLP and other molecular fingerprinting methods, B. anthracis is genetically similar to other spore-forming organisms including B. thuringiensis and B. cereus. Despite these similarities, it is clear that the B. anthracis strains form a monomorphic group that exhibit a distinct pattern separate from those of other species (6–8, 38). However, strains of B. cereus and B. thuringiensis have been defined that map within or close to the B. anthracis group (7, 8). We found in a sample panel of these close relatives that some reacted with the anti-BclA Abs. These strains have proved difficult.

**FIGURE 5.** In vitro flow cytometric assays for B. anthracis spore germination and outgrowth. A. The upper two panels are described in detail in the text. B. In the lower photomicrograph, examples of each life cycle stage are shown with corresponding colored arrows. Blue arrow, birefringent phase-bright ungerminated spores; red arrow, germinating phase-dark spores; purple arrow, vegetative cells with attached exosporium remnants; green arrow, vegetative cells. C. Wild-type (WT) and mutant B. anthracis spores were incubated for various amounts of time in BHI and then stained with EAII-Alexa 488.
to distinguish from *B. anthracis* by conventional diagnostic means. It is of interest that these reactive strains were isolated from infected individuals and may be more pathogenic than their normal counterparts (8, 39).

Germination of *Bacillus* spores is usually detected in vitro by changes in spore refractility, heat resistance, and stainability, all of which are done in bulk culture (40). By using flow cytometry and stage-specific Abs, spore germination and vegetative cell outgrowth can be easily and accurately quantitated at the single-cell level. Single-cell interrogation in cytometric assays and the ability to gate out debris and other contaminants help avoid some of the pitfalls of using bulk populations of organisms at various stages of the life cycle. This was exemplified by the failure to detect protective Ag and S-layer proteins on highly purified spore preparations of *B. anthracis*. In addition to these uses, anti-spore Abs can be used in a variety of platforms for the rapid detection of *B. anthracis* spores in environmental and clinical samples without the need for bacterial growth and/or the extraction of spore components for diagnostic analyses.

Previous vaccination and passive immunization studies suggest that spore-specific Abs might possess protective potential. A single vaccination with the nontoxigenic strain Δ14185 provided limited protection to guinea pigs following challenge with the fully virulent Vollum strain (41). Brossier et al. (42) demonstrated that immunization with formaldehyde-inactivated spores imparted partial protection to guinea pigs and mice following challenge with the virulent 9602 strain. It was also shown that vaccination with formaldehyde-inactivated spores provided total protection to guinea pigs and partial protection to mice challenged with the attenuated 9602P strain. The nature and identity of these potentially protective Abs and their targets have yet to be determined. In passive immunization studies, guinea pigs that received antiserum produced against the Sterne vaccine strain before challenge with fully virulent Ames spores exhibited a delayed time to death (43). In contrast, it was recorded that guinea pigs receiving anti-Sterne Abs before challenge with the Vollum strain had a statistically significant number of survivors compared with controls (44). Such discrepancies are likely the result of differences in protocols, given that the researchers were using different challenge strains, challenge doses, and routes of administration. Although it is reasonable to assume that our anti-spore mAbs might alter spore uptake by phagocytic cells after engaging Fc receptors, the significant differences in
protocols in previous passive immunization studies make it difficult to speculate about the therapeutic potential of spore-specific Abs following passive immunization. Experiments to determine the potential uses of our Abs beyond spore identification are now in progress.

Acknowledgments

We thank Jeremy Boydston, Chris Steichen, Nicole Kushner, and Lisa Jia for their help in facilitating these experiments. We also thank John Ezzell and Joany Jackman for their contributions to the initial mAb studies; Karen Hill and Paul Jackson for Bacillus isolates; Prosper Boyaka, John Ezzell, and George Georgiou for providing us with Abs; and Ann Brookshire for editorial advice.

Disclosures

The authors have no financial conflict of interest.

References


CORRECTIONS


In Results, in the penultimate sentence of the second paragraph under the heading A Gly-Gly motif is conserved within the CDR3 of lymph node T cell hybridomas, reference to Figure 7a and 7b are reversed. The corrected sentence is shown below.

The lymph node TCR contains a rigid loop with an extended planar surface (Fig. 7b), whereas the splenic Th1 TCR presents a round shape with a less extended surface (Fig. 7a).


The tenth author’s last name is incorrect. The correct name is Guillaume Darrasse-Jeze.


In Table II, the data reported for GA2–3b in column six (ΔrmlD spores) should be negative (−) not 2-log shift (++). The corrected table is shown below.

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*ND, Not determined, −, negative by Western or FACS; +, positive by Western or 1-log shift by FACS; ++, 2-log shift by FACS ABS-EF12, mAbs raised against spores; AA2-1-JC8-5, mAbs and raised against exosporium.

*Included in Ref. 9.
In Results, in the last sentence of the paragraph under the heading CCL18 is up-regulated in BAL and sera from AA patients, and in Figure 4C, the concentration of serum CCL18 is expressed incorrectly as "pg/ml" instead of "ng/ml." The corrected sentence and figure are shown below.

CCL18 was significantly elevated in AA (73.9 ± 11.2 ng/ml) compared with NA (31.7 ± 5 ng/ml) subjects (Fig. 4C).


The third author’s first name is incorrect. The correct name is Kaihong Su.


During production, the figure from an unrelated article was inadvertently inserted as the image for Figure 8. The correct figure is shown below. The error has been corrected in the online version, which now differs from the print version as originally published.