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Activation of the Classical Complement Pathway by Bacillus anthracis Is the Primary Mechanism for Spore Phagocytosis and Involves the Spore Surface Protein BclA

Chunfang Gu, Sarah A. Jenkins, Qiong Xue,1 and Yi Xu

Interactions between spores of Bacillus anthracis and macrophages are critical for the development of anthrax infections, as spores are thought to use macrophages as vehicles to disseminate in the host. In this study, we report a novel mechanism for phagocytosis of B. anthracis spores. Murine macrophage-like cell line RAW264.7, bone marrow-derived macrophages, and primary peritoneal macrophages from mice were used. The results indicated that activation of the classical complement pathway (CCP) was a primary mechanism for spore phagocytosis. Phagocytosis was significantly reduced in the absence of C1q or C3. C3 fragments were found deposited on the spore surface, and the deposition was dependent on C1q and Ca2+. C1q recruitment to the spore surface was mediated by the spore surface protein BclA, as recombinant BclA bound directly and specifically to C1q and inhibited C1q binding to spores in a dose-dependent manner. C1q binding to spores lacking BclA (ΔbclA) was also significantly reduced compared with wild-type spores. In addition, deposition of both C3 and C4 as well as phagocytosis of spores were significantly reduced when BclA was absent, but were not reduced in the absence of IgG, suggesting that BclA, but not IgG, is important in these processes. Taken together, these results support a model in which spores actively engage CCP primarily through BclA interaction with C1q, leading to CCP activation and opsonophagocytosis of spores in an IgG-independent manner. These findings are likely to have significant implications on B. anthracis pathogenesis and microbial manipulation of complement. The Journal of Immunology, 2012, 188: 000–000.

The complement system is an essential component of host immune defenses against microorganisms. Activation of the complement system results in opsonization and phagocytosis of microbes, formation of the membrane attack complex, and generation of inflammatory modulators (anaphylatoxins), all of which are important elements of immune surveillance and defense against microorganisms. Complement deficiencies result in increased susceptibility to microbial infections (1, 2). In contrast, some pathogenic microbes have evolved strategies to manipulate the complement system, tipping the defense balance in their own favor (1–3).

Some microbial pathogens evolved strategies to either avoid complement recognition or suppress complement activation. For example, Neisseria meningitidis serogroup B strains express a surface protein that is capable of binding human complement regulatory factor H, and the binding imparts serum resistance to the pathogen (4–6). Vaccines based on the factor H-binding protein showed protective efficacy in preclinical and clinical trials (7–11). Staphylococcus aureus has multiple complement evasion mechanisms that target different components of the complement cascade, such as C3, C3 convertases, C5, and C5a receptor (3, 12–21). For intracellular pathogens, gaining entry into host cells is critical for the establishment of infections. A number of intracellular pathogens directly activate the complement system to promote their entry into host cells. For example, mycobacteria are known to activate all three of the complement pathways to promote uptake by professional phagocytes (22–24). The HIV gp41 activates the classical complement pathway (CCP) by binding to complement component C1q, which is the ligand recognition subunit of C1. This leads to opsonization of HIV with C3 fragments and entry into host cells by opsonophagocytosis (25, 26). These diverse strategies used by microbial pathogens highlight the importance of the complement system in the interplays between pathogens and the host defense system.

Bacillus anthracis is a spore-forming, Gram-positive bacterium that causes anthrax infections. The infections are initiated by the entry of B. anthracis spores into the host via cuts or wounds in the skin, the gastrointestinal tract, or the respiratory system. After entry into the host, spores are taken up by macrophages (27–29), dendritic cells (30–32), and epithelial cells (33). The interactions between spores and these different types of host cells are important for the survival of spores, dissemination of spores from the initial sites of exposure to distal organs (27–31, 34), and germination of spores into vegetative bacilli that are capable of producing the anthrax toxins and other virulence factors (35–37). Thus, spore entry into host cells is critical for the development of anthrax infections. This is further demonstrated by findings that disruption of spore uptake by macrophages or epithelial cells significantly improved the survival of infected mice and reduced
bacterial burden in different tissues in mouse models of anthrax infections (28, 29, 34).

Due to the importance of spore entry into host cells, a number of studies have investigated the molecular mechanisms mediating spore adherence to and uptake by host cells. Complement receptor 3 (CR3; also known as Mac-1, integrin αMβ2, and CD11b/CD18) was found to be a receptor mediating spore phagocytosis by macrophages (29). CR3-mediated phagocytosis is dependent on Bacillus collagen-like protein of anthracis (BclA), a major glycoprotein on the surface of B. anthracis spores (29). Mice deficient in CR3 were more resistant to challenges by B. anthracis spores compared with wild-type (WT) mice (29), suggesting that spore uptake by macrophages is important for the pathogenesis of this microorganism. However, whether BclA directly interacts with CR3 or through intermediate molecules was unclear, nor was the role of the complement system in CR3-mediated phagocytosis of spores. Recently, it was demonstrated that BclA was able to directly bind C1q in a dose-dependent and saturable manner (38). Furthermore, C1q acted as a bridging molecule between BclA and integrin αMβ2 to mediate B. anthracis spore entry into epithelial cells in a complement activation-independent manner (38).

In this study, we investigated the effect of BclA and C1q on complement activation and opsonophagocytosis of B. anthracis spores. The results reveal a novel mechanism of complement manipulation by B. anthracis spores. The importance of these findings to B. anthracis pathogenesis, host defense, and microbial manipulation of complement in general is discussed.

Materials and Methods

Bacteria and preparation of spores

B. anthracis Sterne strain 7702 (39) and its isogenic bclA deletion mutant (∆bclA, provided by C. Turnbough, University of Alabama at Birmingham) (40) were used. Spores were prepared from these strains, as previously described (39). Briefly, B. anthracis was cultured in phage assay media at 30°C with shaking for 7–10 d and centrifuged. The pellet was suspended in sterile HBSS (serum-free medium [SFM]) or SFM supplemented with 10% NHS for 10 min. Spores were washed and analyzed by SDS-PAGE to determine the spore titer in each sample. The coverslips were then mounted and examined using a Zeiss LSM 510 laser-scanning confocal microscope. On average, ~1000 macrophages were counted for each experimental condition. The numbers of intracellular and extracellular adhered spores per macrophage, respectively, were calculated. In some cases, the number of associated spores (intracellular plus extracellular adhered spores) per macrophage was calculated.

Depletion of IgG from serum

IgG was depleted from NHS by passing NHS through protein G HP SpinTrap columns (GE Healthcare) following the manufacturer’s instructions. The procedure was repeated until IgG depletion was confirmed by SDS-PAGE and Western blot analysis using anti-human IgG Abs.

Expression and purification of recombinant BclA

This was done, as previously described (38), with slight modifications. Briefly, full-length BclA protein with an N-terminal His tag was expressed in Escherichia coli BL21 Rosetta 2 strain (Novagen). The recombinant protein was purified using Ni2+ affinity chromatography and ion-exchange chromatography in an AKTA prime plus FPLC system (GE Healthcare). The purified protein was analyzed by SDS-PAGE and circular dichroism to evaluate the purity and the proper folding of the recombinant protein, as previously described (38).

Binding of C1q to spores

Sporozoites (2.5 × 10^7 or 5 × 10^7) were incubated in SFM containing purified human C1q (1 μg/ml) at 37°C for 10 min in the presence of 2.5 mM α-alanine. The spores were then washed with SFM three times and resuspended in PBS. An aliquot of the spore suspension was diluted plated on Luria-Bertani agar plates to determine the spore titer in each sample. The rest of the suspension was boiled in a reducing SDS sample buffer for 10 min and stored at −20°C until ready to be analyzed by SDS-PAGE and Western blot. The volume of each sample loaded onto the SDS gels was adjusted so that samples of the same experimental conditions contain the same number of spores. Goat anti-C1q Abs (1:5,000; Complement Technology) and HRP-conjugated rabbit anti-goat secondary Abs (1:10,000; Invitrogen) were used in Western blot analysis. The intensity of the bands was determined by densitometry using Image J.

To determine whether soluble recombinant BclA (rBclA) protein could inhibit C1q binding to spores, C1q (1 μg/ml) was preincubated with various concentrations of rBclA at 4°C for 60 min before adding to the spores.

Deposition of C3 and C4 fragments on spores

This was done according to a procedure described previously (23), with some modifications. To detect C3 fragments, spores (∼2 × 10^7) were incubated in SFM containing different types of serum (10%) with or without 10 mM EGTA at 37°C for 30 min. Spores were then washed three times with SFM, boiled in a reducing SDS sample buffer, and applied to SDS-PAGE and Western blot analysis using goat anti-C3 polyclonal Abs (1:5000; Complement Technology) and appropriate secondary Abs. Purified C3, C3b, and iC3b (Complement Technology) were also included in the SDS-PAGE as standards for the different C3 fragments. To detect C4 fragments, spores (∼2 × 10^7 or 5 × 10^7) were incubated in SFM containing 10% NHS for 10 min. Spores were washed and analyzed by Western blot using anti-C4 Abs (1:5000; Complement Technology). The intensity of the bands was determined by densitometry using Image J.

ELISA

This was carried out, as previously described (38), with slight modifications. Briefly, purified C1q or OVA were coated onto the wells of 96-
well plates, blocked, and incubated with HBS (10 mM HEPES, 150 mM NaCl [pH 7.4]) containing various concentrations of rBclA with or without 10 mM EGTA or EDTA. Bound rBclA was detected with HRP-conjugated anti-His mAbs. Apparent K_D was calculated using the nonlinear regression analysis method in the GraphPad Prism (version 4) program (GraphPad).

**Macrophage viability assay**

The viability of macrophages infected with spores was measured using a lactate dehydrogenase-based CytoTox-ONE Homogeneous Membrane Integrity Assay kit (Promega). RAW264.7 cells (2 × 10^5 cells/well) were infected with 7702 or ΔbclA spores in the presence of NHS or HIS, as described above. Postinfection, cells were extensively washed and incubated in DMEM/10% FBS containing 5 mM t-alanine, which promotes spore germination, and 100 μg/ml gentamicin for 1 h to eliminate extracellular bacteria. We found that this treatment eliminated >95% of extracellular spores and bacteria. The cells were then washed and incubated with DMEM/10% FBS containing 2.5 mM t-alanine (to inhibit germination and growth of any residual extracellular spores) for 0, 6, or 24 h. Culture supernatants were collected, and lactate dehydrogenase release was measured by reading fluorescence at 560/590 nm, according to the manufacturer’s instructions.

**Intracellular survival of spores**

RAW264.7 cells (2 × 10^5 cells/well) were infected with 7702 or ΔbclA spores and incubated in DMEM/10% FBS containing 100 μg/ml gentamicin and 5 mM t-alanine for 1 h, as described above. To examine spore intracellular survival, cells were washed and incubated in DMEM/10% FBS containing 100 μg/ml gentamicin for 0, 1, 3, 6, or 24 h. The cells were washed, lysed, and dilution plated to determine the number of viable bacteria.

**C1 and C4 activation assays**

For C1 activation, rBclA protein (26 μM), heat-denatured rBclA (hrBclA; 26 μM, heated at 50°C for 30 min), IgM (10 μg/ml), or buffer only was pre-incubated with HBS-containing C1 complex (0.2 μg/ml, 1:10 dilution; Complement Technology) for 60 min at 4°C, followed by incubation at 37°C for 30 min in the presence of 0.5 mM CaCl_2. For C4 activation, the responsive proteins were incubated with HBS containing NHS (1% v/v). The samples were applied to 12% SDS-PAGE gel electrophoresis and analyzed by Western blot using sheep anti-C1s Ab (1:5000; R&D Systems) or anti-C4 Ab (1:5000; Complement Technology), followed by appropriate secondary Abs.

**Statistical analyses**

For pairwise comparisons, statistical analysis was performed using Student t test. For comparison between multiple groups, ANOVA was used. The GraphPad Prism program was used for these analyses.

**Results**

**Heat-labile serum factors are important for B. anthracis spore adherence to and phagocytosis by macrophages**

To investigate the role of complement in spore interaction with phagocytes, we first compared spore adherence to and uptake by macrophage cell line RAW264.7 and primary macrophages in SFM, SFM supplemented with 10% NHS, or 10% HIS. Compared to adherence in the presence of NHS, spore adherence to RAW264.7 cells in SFM or HIS was decreased, although the decrease was statistically insignificant (Fig. 1A). The effect of serum on spore adherence was more pronounced in BMDMs (Fig. 1B) and PMs (Fig. 1C). Spore adherence to BMDMs and PMs was increased by ∼8–9- and ∼20-fold, respectively, in the presence of NHS compared with that in SFM (p < 0.001). The increase was primarily mediated by heat-labile factors in the serum (Fig. 1B, 1C).

Spore uptake by macrophages was similarly affected by heat-labile serum factors. Compared with that in NHS, phagocytosis was significantly reduced in SFM or HIS, with the sharpest reduction seen in RAW264.7 cells (Fig. 1D–F).

Spore adherence and phagocytosis were also examined when macrophages were incubated with spores at different MOIs (1, 10, and 100). The effects of serum factors on adherence and phagocytosis were similar at the different MOIs (Supplemental Fig. 1).

**Phagocytosis of spores by macrophages is mediated by CCP in an IgG-independent manner**

To investigate the role of C1q in spore interaction with macrophages, spore adherence and phagocytosis assays were performed in SFM supplemented with 10% C1qD. We observed a ∼80–90% reduction in spore adherence to RAW264.7 macrophages and BMDMs in C1qD compared with that in NHS (Fig. 2A, 2B). Addition of purified C1q to C1qD restored spore adherence to these macrophages. Spore uptake by RAW264.7 macrophages and BMDMs in C1qD was also significantly reduced (by ∼98% and ∼80%, respectively) compared with that in NHS (p < 0.01 for RAW264.7 and p < 0.001 for BMDMs) (Fig. 2C, 2D). Addition of purified C1q to C1qD significantly increased the uptake by RAW264.7 macrophages compared with that in C1qD (Fig. 2C) and almost restored the uptake by BMDMs to the level seen in NHS (Fig. 2D). Taken together, these data suggest that C1q plays a major role in spore adherence to and uptake by macrophages.

**FIGURE 1.** Phagocytosis of B. anthracis spores by macrophages requires heat-labile serum factors. Phagocytosis assays were performed, as described in Materials and Methods. Briefly, RAW264.7 (A, D), BMDMs (B, E), and PMs (C, F) were seeded onto coverslips in 24-well tissue culture plates and incubated with spores from the Sterne strain 7702 for 30 min in SFM, SFM supplemented with 10% NHS, or 10% HIS. The germination inhibitor t-alanine (2.5 mM) was included in the assays. Extracellular spores were detected by staining unpermeabilized cells with anti-spore Abs. The cells were then permeabilized and stained again with anti-spore Abs to detect intracellular spores. Relative adherence (A–C) is the ratio of the number of extracellular adhered spores versus the number of macrophages examined, normalized to that in NHS. Relative phagocytosis (D–F) is the ratio of the number of intracellular spores versus the number of macrophages examined, normalized to that in NHS. Results were combined from two to four independent experiments. The actual numbers of spore adherence and phagocytosis are 0.38 ± 0.10 and 0.96 ± 0.18 spores per RAW264.7 cell; 1.75 ± 0.39 and 1.86 ± 0.16 spores per BMDM; 3.33 ± 1.33 and 3.56 ± 1.48 spores per PM, respectively. Approximately 1000 macrophages were analyzed for each condition in each independent experiment. **p < 0.01, ***p < 0.001; Student t test.

However, the difference in phagocytosis between NHS and SFM or HIS was smaller at a MOI of 100 than at the lower MOIs, which may suggest that the high MOI is getting close to a saturating dose. For the rest of the study, all the phagocytosis and adherence experiments reported were conducted with a MOI of ∼10.

Taken together, these results suggest that heat-labile serum factors, possibly the complement system, play a major role in spore adherence to and uptake by macrophages.
C1q may be important in spore phagocytosis by macrophage via two possible mechanisms. One is by activation of CCP, resulting in deposition of C3 fragments on spore surfaces and phagocytosis via C3 receptors. The other is by directly interacting with C1q receptors on macrophages and mediating phagocytosis in a complement activation-independent manner (42, 43), as reported for *Listeria monocytogenes* (44) and apoptotic cells (45). We investigated whether C1q-mediated phagocytosis of spores required complement activation. Purified C1q was added to SFM in the absence of other complement components. The results showed that C1q alone did not increase spore uptake by macrophages compared with that in SFM (Fig. 2E).

To further determine whether complement activation is required for spore uptake, we performed phagocytosis assays in SFM supplemented with C3D. Depletion of C3 almost abolished spore uptake by RAW264.7 macrophages (Fig. 3A) and BMDMs (Fig. 3B). (C–E) Relative phagocytosis by RAW264.7 macrophages (C), and BMDMs (D). The results were combined from at least three independent experiments. Approximately 1000 macrophages were analyzed for each condition in each independent experiment. *p < 0.05, **p < 0.01, ***p < 0.001; Student t test.

The most common way to activate CCP is by binding of C1q to Abs in immune complexes. To investigate the possibility that CCP might be activated by IgG in human serum that bound to the spores nonspecifically, we depleted IgG from NHS. IgG depletion was confirmed by SDS-PAGE and Western blot analysis of the depleted serum (IgG-depleted serum [IgG-D]) (Supplemental Fig. 2A, 2B). Results from phagocytosis assays showed that IgG depletion did not reduce spore phagocytosis by RAW264.7 macrophages (Fig. 3G) and spore association to macrophages (Fig. 3H), but rather a modest increase in phagocytosis was observed (Fig. 3G), suggesting that IgG is not required for the spore phagocytosis process. Taken together, the above results indicate that phagocytosis of spores by macrophages is dependent on C1q and C3, and independent of IgG. The results also suggest that IgG-independent activation of CCP is the primary mechanism for spore phagocytosis by macrophages.

FIGURE 2. Phagocytosis of *B. anthracis* spores requires complement component C1q. Phagocytosis assays and detection of extracellular and intracellular spores are as described in the legend for Fig. 1, except that SFM was supplemented with different types of serum or C1q. The concentration for NHS and C1qD was 10%, and for purified C1q was 10 μg/ml. (A, B) Relative adherence to RAW264.7 macrophages (A) and BMDMs (B). (C–E) Relative phagocytosis by RAW264.7 macrophages (C, E) and BMDMs (D). The results were combined from at least three independent experiments. Approximately 1000 macrophages were analyzed for each condition in each independent experiment. *p < 0.05, **p < 0.01, ***p < 0.001; Student t test.

FIGURE 3. Phagocytosis of spores requires complement component C3, but not IgG. Phagocytosis assays and detection of extracellular and intracellular spores are as described in the legend for Fig. 1, except that SFM was supplemented with 10% NHS, 10% C3D, or 10% NHS depleted of IgG (IgG-D). Relative phagocytosis (A–C, G) is the ratio of the number of intracellular spores versus the number of macrophages examined, normalized to that in NHS. Relative association (D–F, H) is the ratio of total extracellular adhered and intracellular spores versus the number of macrophages examined, normalized to that in NHS. (A, D, G, and H) RAW264.7 macrophages; (B and E) BMDMs; (C and F) PMs. The results were combined from two to four independent experiments. Approximately 1000 cells were analyzed for each condition in each independent experiment. **p < 0.01, ***p < 0.001; Student t test.

Spore surface protein BclA mediates the binding of C1q to the spore surface

Previously, we showed that rBclA bound C1q in a dose-dependent and saturable manner (38). BclA is the major protein on the surface...
of spores. We investigated whether native BclA could mediate C1q binding to the spore surface. Spores from the Sterne strain 7702 and its isogenic bclA deletion mutant (ΔbclA) were incubated in buffer containing purified C1q. C1q bound on the spore surface was detected by Western blot analysis using Abs specific for C1q (Fig. 4A) and quantified by densitometry (Fig. 4B). The results showed that the presence of BclA on the spore surface significantly increased the amount of C1q bound to the spores (*p < 0.05). Small amounts of C1q could still be detected on the ΔbclA spores, suggesting that there is a minor, BclA-independent secondary mechanism of C1q binding. We further examined whether soluble rBclA could block C1q binding to spores. Purified C1q was preincubated with increasing concentrations of rBclA or BSA before the addition to 7702 or ΔbclA spores. The results showed that C1q binding to 7702 spores was specifically inhibited by rBclA in a dose-dependent manner (Fig. 4C, 4D). As expected, rBclA had no effect on C1q binding to ΔbclA spores.

Ca²⁺ has been reported to be involved in C1q recognition of ligands such as IgG, IgM, C-reactive protein, pentraxin (46), and gp41 (47), possibly by enhancing the electrostatic stability of the binding interaction. We examined the effect of EGTA and EDTA on C1q binding to rBclA. The results showed that EGTA did not affect the binding compared with that in HBS only, whereas EDTA reduced the binding by ~9-fold (Fig. 4E). This suggests that divalent metal ions other than Ca²⁺ facilitate rBclA binding to C1q.

Taken together, these results demonstrate that BclA binds directly to C1q and is able to recruit C1q to the spore surface.

Deposition of C3 on the spore surface is initiated via IgG-independent activation of CCP and is significantly promoted by the presence of BclA

We investigated the deposition of C3 fragments on the surface of 7702 and ΔbclA spores. When 7702 spores were incubated in NHS containing media, C3 fragments were detected on the spore surface (Fig. 5A). Judging by the presence of the truncated α-chain (α'), which is indicative of iC3b, and the intensity of the ε'-chain relative to the β-chain, which represents total C3 fragments, iC3b is the dominant C3 fragment on the spore surface. The identities of the high m.w. species were unclear. Upon activation, the thiol-ester bond in C3 is exposed, allowing covalent anchorage of C3b as well as its subsequent cleavage fragments to nearby molecules. It is possible that some of the high m.w. species represent complexes between C3 fragments and spore surface components. It is also possible that some of them were the α-chain of C3b. A small amount of C3 β-chain was detected on 7702 spores incubated in media containing HIS and was most likely due to nonspecific association of C3 to spores (Fig. 5A–C). The amount of C3 deposited on 7702 spores after incubation in C1qD was similar to the background level observed in HIS (Fig. 5A, 5B), suggesting that C3 deposition on spore surfaces was mediated by CCP. Nothing was detected when spores were incubated in C3D, indicating that the C3 Abs were specific. C3 deposition was also examined in the presence of EGTA (Fig. 5D), which disrupts the activation of CCP, but not the alternative complement pathway. The results showed that EGTA abolished the deposition of C3 fragments on the surface of 7702 spores, further confirming that C3 deposition on the spores was initiated via CCP and not alternative complement pathway.

To determine whether CCP activation is initiated by C1q recognition of immune complexes, we examined C3 deposition on 7702 spores in media containing IgG-depleted serum. IgG depletion did not affect C3 deposition on 7702 spores (Fig. 5E), indicating that IgG is not involved in the initiation of CCP activation on the spore surface. These results are in agreement with the phagocytosis results described earlier (Fig. 3G, 3H).

To determine the role of BclA, C3 deposition on ΔbclA spores was examined. The amount of both total C3 fragments (repre-

FIGURE 4. Spore surface protein BclA mediates C1q binding to spores. (A) 2.5 × 10¹⁹ or 5 × 10¹⁹ spores from the Sterne strain 7702 and its isogenic mutant ΔbclA, respectively, were incubated with 1 µg/ml purified C1q at 37°C for 10 min. C1q binding to spores was determined by Western blot, as described in Materials and Methods. (B) Densitometry analysis of the C1q band using Image J. The top bands in the gel were analyzed. The results shown are the mean density ± SEM, combined from three independent experiments and normalized to that of 7702 (2.5 × 10¹⁹ spores). (C and D) rBclA inhibited C1q binding to 7702 spores, but not to ΔbclA spores in a dose-dependent manner. Purified C1q was preincubated with different concentrations of rBclA or BSA at 4°C for 1 h prior to incubation with the spores. The samples were analyzed by Western blot (G) and quantified using Image J (D). The relative intensities in (D) were combined from three independent experiments. *p < 0.05; Student t test. (E) The effect of EGTA and EDTA on rBclA binding to C1q. ELISAs were performed as described in Materials and Methods. Nonlinear regression analysis was used to calculate the apparent binding affinity (GraphPad Prism).
FIGURE 5. C3 deposition on the spore surface is dependent on activation of CCP, promoted by BclA, and independent of IgG. (A and B) C3 deposition is dependent on C1q and promoted by BclA. The 7702 and ΔbclA spores were incubated with 10% HIS, NHS, C1qD, or C3D at 37°C for 30 min. C3 deposition on the spore surface was examined by Western blot analysis using C3 Abs (A), as described in Materials and Methods. Purified C3, C3b, and iC3b were loaded as protein standards. Different chains of C3/C3b/iC3b were indicated with arrows. The intensity of the β-chain (B) and the iC3b α’-chain (C) was determined using Image J and normalized to those in the iC3b standard. The results shown are the mean density ± SEM combined from three independent experiments. *p < 0.05, ***p < 0.001 (7702 NHS versus ΔbclA NHS); Student t test. (D) C3 deposition is dependent on Ca2+. The 7702 spores were incubated in 10% NHS with or without 10 mM EGTA, and C3 deposition was examined by Western blot analysis. (E) C3 deposition is independent of IgG. The 7702 spores were incubated in 10% NHS or 10% IgG-D, and C3 deposition was examined by Western blot.

We then investigated whether rBclA alone could directly activate CCP. Upon activation, C1q undergoes conformational changes that lead to cleavage of C1r and C1s. Thus, rBclA protein, IgM, hrBclA, or buffer only (HBS) was incubated with purified C1 complex, and the samples were analyzed by Western blot using anti-C1s Abs. Cleavage of C1s was greatly enhanced in the presence of IgM; however, there was no significant difference in C1s cleavage between rBclA, hrBclA, and buffer only (Supplemental Fig. 3). We further examined the cleavage of C4 by incubating rBclA, hrBclA, or IgM with NHS. The samples were analyzed with Western blot using anti-C4 Abs. We did not observe any significant difference in C4 cleavage between rBclA, hrBclA, and buffer only, whereas IgM induced C4 cleavage (data not shown). These results suggest that rBclA alone is insufficient to activate CCP. Activation of CCP requires either other additional molecules on the spore surface or posttranslational modifications of BclA (e.g., glycosylation) that are absent in rBclA.

Taken together, our results suggest that deposition of C3 fragments on the spore surface is initiated by CCP activation in an IgG-independent manner and is significantly promoted by the presence of BclA.

BclA mediates spore phagocytosis by macrophages in a C1q- and C3-dependent manner

We investigated the role of BclA in spore adherence to macrophages and in CCP-mediated phagocytosis of spores. For spore adherence, ΔbclA spores appeared to adhere to both RAW264.7 (Fig. 7A) and BMDMs (Fig. 7B) as least as well as or better than (although statistically insignificant) 7702 spores regardless of the media. These results suggest that in the absence of BclA, other spore surface components can also mediate adherence to macrophages.

For phagocytosis, we compared the uptake of 7702 and ΔbclA spores in SFM or SFM supplemented with NHS or HIS. In the presence of NHS, phagocytosis of ΔbclA spores was significantly lower than that of 7702 spores, that is, ~90% lower (p < 0.001) in RAW264.7 cells (Fig. 7C) and ~70% lower (p < 0.01) in BMDMs (Fig. 7D). In contrast, in SFM or HIS, phagocytosis of ΔbclA spores was not significantly different compared with that of 7702 spores, suggesting that BclA’s function in the spore phagocytosis process was dependent on complement activities.
We further examined the effect of C1q and C3 depletion on phagocytosis of ΔbclA spores. Contrary to the C1q- and C3-dependent phagocytosis of WT 7702 spores shown in Figs. 2 and 3, uptake of ΔbclA spores by macrophages was not reduced, but instead was increased in C1qD and C3D compared with that in NHS (Fig. 7E). Taken together, these results suggest that the effect of BclA on spore phagocytosis is dependent on C1q and activation of CCP. The results also suggest that in the absence of BclA and complement, a distinct mechanism of spore phagocytosis comes into action. The nature of this BclA- and CCP-independent mechanism is currently unknown.

**CR3 is the primary phagocytic receptor for spores in the presence of serum**

CR3 is the phagocytic receptor for iC3b on macrophages. To determine whether CR3 is the receptor in the BclA/CCP-mediated phagocytic pathway, we examined spore uptake by BMDMs and PMs isolated from CD11b<sup>−/−</sup> mice. The results showed that in the presence of NHS, phagocytosis of 7702 spores by CD11b<sup>−/−</sup> BMDMs and PMs was significantly reduced compared with that by WT macrophages (Fig. 8A, 8B). The total number of spores associated with CD11b<sup>−/−</sup> BMDMs and PMs (extracellular adhered and intracellular spores) was also significantly lower compared with that with WT macrophages (Fig. 8C, 8D). These results indicate that in the presence of complement, CR3 is the primary macrophage phagocytic receptor for spores. In the presence of HIS, phagocytosis of 7702 spores by CD11b<sup>−/−</sup> macrophages was also lower than that by WT macrophages (Fig. 8), suggesting that CR3 is also involved in spore uptake by macrophages in the absence of complement. This latter result is consistent with the previous data by Oliva et al. (29).

**The effect of different phagocytic mechanisms on the intracellular fate of spores and the viability of macrophages**

We investigated the survival of spores phagocytosed through different phagocytic pathways. The 7702 and ΔbclA spores were phagocytosis by macrophages in the presence of NHS and HIS (complement opsonization versus no complement opsonization), and intracellular survival was examined over a 24-h time period. The results showed that 7702 spores phagocytosed in the presence of NHS survived better than that in the presence of HIS; however, the difference was not statistically significant except at 6 h postphagocytosis (p < 0.05, ANOVA) (Fig. 9). Similarly, ΔbclA spores phagocytosed in the presence of NHS survived better than those phagocytosed in the presence of HIS; however, the difference was only statistically significant at 1 (p < 0.05) and 3 h (p <

**FIGURE 7.** BclA is required for complement-mediated phagocytosis of spores. Relative adherence (A, B) and relative phagocytosis (C–E) are as described in the legend for Fig. 1. Results were combined from three independent experiments. Approximately 1000 macrophages were analyzed for each condition in each independent experiment. (A and C) RAW264.7; (B, D, and E) BMDMs. *p < 0.05, **p < 0.01, ***p < 0.001; Student t test.

**FIGURE 8.** CR3 is the major phagocytic receptor for spores in the presence of serum. Phagocytosis assays were performed, as described in Materials and Methods. Relative phagocytosis (A, B) is the ratio of the number of intracellular spores versus the number of macrophages examined, normalized to that from WT macrophages in NHS. Relative association (C, D) is the ratio of total extracellular adhered and intracellular spores versus the number of macrophages examined, normalized to that from WT macrophages in NHS. Results shown were combined from two to three independent experiments. Approximately 1000 macrophages were analyzed for each condition in each independent experiment. *p < 0.05, **p < 0.01, ***p < 0.001; Student t test. (A and C) BMDMs; (B and D) PMs.
We did not observe any significant difference in macrophage phagocytosis by other mechanisms. Spores phagocytosed via complement opsonization appeared to survive better when comparison was made between 7702 and ΔbclA spores in the presence of NHS or HIS. The biggest difference in survival was observed when comparison was made between 7702 spores phagocytosed in the presence of NHS (BclA mediated and complement opsonized) and ΔbclA spores in the presence of HIS (non BclA mediated and no complement opsonization) (p < 0.001 at 3 and 6 h, p < 0.01 at 24 h, ANOVA). Overall, spores phagocytosed via complement opsonization appeared to survive better than spores phagocytosed by other mechanisms.

The effect of the different phagocytosis pathways on macrophage viability was also investigated by measuring lactate dehydrogenase activities in the cell culture media in a 24-h time course. We did not observe any significant difference in macrophage viability when infected with the different spores in the presence of NHS or HIS (data not shown).

Discussion

The complement system is a critical aspect of host defense against microbes. Understanding the dynamic exchanges between pathogens and the complement system at a molecular level will provide important insights into microbial immune manipulation strategies and new possibilities to treat infectious diseases. In this study, we report that spores of *B. anthracis* are able to activate CCP to promote uptake by macrophages via opsonophagocytosis. CCP activation is independent of IgG and appears to be primarily initiated by recruitment of C1q to the spore surface via the spore surface protein BclA. This mechanism of complement manipulation is novel for *B. anthracis*. Complement activities are present in the blood as well as in the fluid lining the mucosal surfaces (1, 2, 48). Therefore, the mechanism elucidated in this study most likely takes place as soon as spores enter the host. In addition, spore entry into host cells is a critical event in the establishment of anthrax infections. Thus, the mechanism should be relevant and important to understanding the pathogenic mechanisms of *B. anthracis* at early stages of infections.

That phagocytosis of spores is primarily mediated by activation of CCP is supported by the following results. Spore adherence to and phagocytosis by macrophages were significantly reduced in HIS, C1qD, and C3D compared with that in NHS. Addition of purified C1q to C1qD restored spore adherence to and phagocytosis by macrophages; however, addition of C1q to SFM did not increase phagocytosis. Furthermore, C3 deposition on the spore surface was dependent on C1q and Ca²⁺.

That CCP activation is independent of IgG and is promoted by the spore surface protein BclA is supported by the following results. Neither C3 deposition on the spore surface nor spore phagocytosis was affected by IgG depletion from the serum; however, both were significantly reduced when BclA is absent from the spore surface. In addition, C4 deposition in the spore surface was significantly reduced in the absence of BclA. That BclA mediates C1q binding to spores is supported by the following evidence. BclA bound purified C1q in a dose-dependent and saturable manner and inhibited C1q binding to WT spores, but not to ΔbclA spores. In addition, ΔbclA spores bound significantly less C1q than WT spores.

Based on these findings and previous reports, we propose a model in which WT *B. anthracis* spores use a common initial step, that is, BclA–C1q interaction, in the pathways to gain entry into different types of host cells (Fig. 10). In the presence of complement (which is the in vivo situation), spore surface protein BclA interacts with C1q. The interaction mediates spore entry into epithelial cells via integrin αβ₁, as previously reported (38). The interaction also leads to activation of CCP (most likely involving additional factors), resulting in deposition of C4 and C3 fragments on the spores. Our results show that iC3b is the dominant form of C3 fragments on the spore surface. Currently, we do not know whether the conversion of C3b to iC3b on the spore surface is a result of the normal cleavage process mediated by factor I and cofactors in the serum or spores actively recruit some of these factors to promote the cleavage. Preliminary data from our laboratory suggest a possibility of active recruitment, which is currently being investigated. In any case, the deposition results in spore phagocytosis via iC3b receptors such as CR3 on macrophages. We further propose that this mechanism should also function to mediate spore uptake by other iC3b receptors such as CR4 (also known as CD11c/CD18, αβ₂) on dendritic cells. When BclA is absent, there are significantly less C1q and C3 fragments on the spore surface and consequently less efficient spore entry into host cells compared with that of WT spores. As spore entry into host macrophages, dendritic cells, and epithelial cells is thought to be critical for spore dissemination and germination into vegetative bacilli, the model described in this work is likely to play a key role during the early stages of infections.

The outer membrane proteins and LPSs of a number of Gram-negative bacteria (49–57) and the capsular polysaccharide of group B streptococci (58–60) have been reported to bind C1q and activate CCP in an Ab-independent manner. In these cases, the activation leads to increased susceptibility of the bacteria to killing by complement or phagocytes. We did not find any decrease in spore viability after the spores (both WT and ΔbclA spores) had been incubated in NHS (data not shown), indicating that activation of CCP does not lead to increased serum susceptibility of spores. This is consistent with the knowledge that spores are generally resilient and that spores are enclosed by an exosporium composed.
of proteins and glycoproteins instead of a lipid bilayer. Therefore, the mechanism described in this work primarily functions to promote spore entry into host phagocytes.

The intracellular fate of phagocytosed spores has been studied by multiple groups. Following phagocytosis, spores are trafficked to phagolysosomes, as evidenced by colocalization of spores with lysosome-associated membrane protein 1 (61). There was evidence suggesting that spores germinate in the phagolysosomes and eventually escape from macrophages (36, 62, 63). Intracellular germination appears to be important for the escape and is a complex process requiring multiple and diverse genetic loci. These loci include several germination operons as well as intergenic regions with unknown functions (37, 64–66). The anthrax toxins were shown to promote the adherence and invasion of pneumococci to respiratory epithelial cells (78). Thus, there seems to be an emerging theme that members of the bacterial collagen-like protein family are actively involved in interactions with host cells and components of the innate immune system.

The role of BclA in anthrax infections remains to be clarified. Studies using animal models of anthrax infections by different groups indicate that ΔbclA spores are either as virulent as (82) or more virulent than WT spores (29). In contrast, CR3 deficiency resulted in significantly less susceptibility of mice to anthrax infections (29), suggesting that entry into macrophages is important for B. anthracis pathogenesis. How to reconcile these observations is unclear. It is possible that the absence of BclA allows other spore surface components to be exposed and these components may interact with host factors that normally are not accessible to them in WT spores. The observations that ΔbclA spores adhere to epithelial cells (38) and macrophages as well as or better than WT spores and the increased uptake of ΔbclA spores by macrophages in the absence of C1q support this possibility. Furthermore, our recent data indicate that BclA also interacts with additional host factors (C. Gu, S. Jenkins, and Y. Xu, unpublished observations). This complicates the interpretation of the in vivo results from studies using ΔbclA spores. More targeted mutations in BclA that only affect its C1q-binding capability, but not its targeting to the spore exosporium, the assembly of the exosporium, or its interactions with other host factors will be helpful in elucidating the role of BclA–C1q interaction in anthrax infections. It will also be interesting to examine the impact of C1q deficiency on the development and outcome of anthrax infections.

In conclusion, the results presented in the current study elucidate a novel mechanism of complement manipulation by spores of B. anthracis that is likely to be significant to the pathogenesis of this microorganism. In addition, as a novel ligand of C1q, investigations to elucidate the molecular mechanisms of BclA–C1q interaction, its effect on activation of C1, and the overall complement system will provide new insights into C1q–ligand interactions as well as microbial manipulation of complement.

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Disclosures

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