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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. Recombinant BclA bound directly and specifically to C1q and inhibited 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. 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.
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 (ΔbclA, 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 phase assay media at 30°C with shaking for 7–10 d and centrifuged. The pellet was suspended in sterile RPMI 1640 supplemented with 10% heat-inactivated FBS. The cells were then washed, fixed with 2% paraformaldehyde without permeabilization, blocked, and incubated with rabbit anti-spore Abs raised against formalin-killed 7702 or ΔbclA spores (1:500 dilution; Strategic Biosolutions), followed by goat anti-rabbit secondary Abs conjugated to Alexa-Fluor 594 (1:1000; Invitrogen) to detect extracellular spores. Cells were then permeabilized with 0.1% saponin and incubated with anti-spore Abs, followed by secondary Abs conjugated to Alexa-Fluor 488 (1:1000; Invitrogen) to detect both intracellular and extracellular spores. 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**

Spores (2.5 × 10⁷ or 5 × 10⁸) 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-α-nan. The spores were then washed with SFM three times and resuspended in PBS. An aliquot of the spore suspension was diluted platted 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 gel 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 Bc1a (rBc1a) protein could inhibit C1q binding to spores, C1q (1 µg/ml) was preincubated with various concentrations of rBc1a 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 × 10⁷) 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 (2.5 × 10⁷ or 5 × 10⁷) 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.
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 release assay. Culture supernatants were collected, and lactate dehydrogenase release for 0, 6, or 24 h. 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).

**Results**

**Heat-labile serum factors are important for B. anthracis spores 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 $\sim$8–9- and $\sim$20-fold, respectively, in the presence of NHS compared with that in SFM ($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).
C1q may be important in spore phagocytosis by macrophages 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), BMDMs (Fig. 3B), and PMs (Fig. 3C). The total number of spores associated with macrophages (intracellular + extracellular adhered spores) was also significantly reduced in C3D compared with those in NHS (Fig. 3D–F). These results indicate that C3 is important for both spore adherence to and uptake by macrophages.

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) or 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.

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 the 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 (α⁰), which is indicative of iC3b, and the intensity of the α²-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 (representative of iC3b) and total C3 fragments (representative of C3b) were determined by Western blot analysis, and the relative intensities were quantified using Image J. The results showed that BclA significantly promoted C3 deposition on the ΔbclA spores (Fig. 5F). The effect of EGTA on C3 deposition was also examined. The results showed that EGTA abolished the deposition of C3 fragments on the ΔbclA spores, further confirming that C3 deposition on the ΔbclA spores was initiated via CCP and not alternative complement pathway.

**FIGURE 4.** Spore surface protein BclA mediates C1q binding to spores. (A) 2.5 × 10⁷ or 5 × 10⁷ 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 show the mean density ± SEM, combined from three independent experiments and normalized to that of 7702 (2.5 × 10⁷ spores). (C and D) rBclA inhibited C1q binding to 7702 spores, but not to ΔbclA spore 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 (C) and quantified using Image J (D). The relative intensities (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% NHS, 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.

FIGURE 6. C4 deposition on the spore surface. (A) The 7702 and ΔbclA spores (2.5 × 10⁷ and 5 × 10⁷ spores, respectively) were incubated with 10% NHS at 37°C for 10 min. C4 deposition on spores was determined by Western blot analysis using anti-C4 Abs and secondary Abs. Cleaved fragments of C4/C4b were indicated by arrows; the 94-kDa α-chain; the 84-kDa α'-chain; the 75-kDa β-chain; the 32-kDa γ-chain; and the 14-kDa α'-chain. (B) Densitometry analysis of the C4b γ-chain using Image J. The results shown are the mean density ± SEM, combined from three independent experiments and normalized to that of 7702 (2.5 × 10⁷ spores). *p < 0.05; Student t test.

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 phagocytosed by macrophages in the presence of NHS and HIS (complement opsonized 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 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 vi-
droxygenase activities in the cell culture media in a 24-h time course.

Phage viability was also investigated by measuring lactate dehy-
dytosed via complement opsonization appeared to survive better
at 3 and 6 h, (non BclA mediated and no complement opsonization) (\( p < 0.001 \) at 3 and 6 h, \( p < 0.01 \) at 24 h, ANOVA). Overall, spores phago-
cytosed via complement opsonization appeared to survive better than spores phagocytosed by other mechanisms.

The effect of the different phagocytosis pathways on macro-
phage viability was also investigated by measuring lactate dehydro-
genase activities in the cell culture media in a 24-h time course. We did not observe any significant difference in macrophage vi-
ability 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 patho-
gens 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 \textit{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 initi-
tiated by recruitment of C1q to the spore surface via the spore surface protein BclA. This mechanism of complement manipulation is novel for \textit{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 \textit{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 phagocy-
tosis by macrophages; however, addition of C1q to SFM did not increase phagocytosis. Furthermore, C3 deposition on the spore surface was dependent on C1q and Ca\(^{2+}\).

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 phagocy-
tosis 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 sig-
ificantly reduced in the absence of BclA. That BclA mediates C1q binding to spores is supported by the following evidence. rbclA bound purified C1q in a dose-dependent and saturable manner and inhibited C1q binding to WT spores, but not to rbclA spores. In addition, rbclA spores bound significantly less C1q than WT spores.

Based on these findings and previous reports, we propose a model in which WT \textit{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 \( \alpha_\text{v}\beta_1 \), 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 labor-
atory suggest a possibility of active recruitment, which is cur-
rently being investigated. In any case, the deposition results in spore phagocytosis via iC3b receptors such as CR3 on macro-
phages. 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, \( \alpha_\text{v}\beta_2 \)) 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 kill-
ing by complement or phagocytes. We did not find any decrease in spore viability after the spores (both WT and rbclA 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
However, when mice were challenged with B. anthracis spores, the effect of different phagocytic mechanisms on intracellular trafficking pathway can affect the intracellular trafficking pathway as well as the kinetics of trafficking. These differences can potentially influence the viability of spores, the rate of spore germination, the survival of germinated spores, and the expression of factors involved in intracellular survival and escape.

It was previously reported that compared with WT mice, CR3 knockout mice were more resistant to infections by WT spores. However, when mice were challenged with ΔbclA spores, the difference in survival between CR3 knockout and WT mice became statistically insignificant (29). This, combined with our finding that BclA-mediated complement-opsonized spores lead to significantly improved intracellular survival compared with non-BclA–mediated, no complement-opsonized spore phagocytosis. It is possible that distinct phagocytic mechanisms can affect the intracellular trafficking pathway as well as the kinetics of trafficking. These differences can potentially influence the viability of spores, the rate of spore germination, the survival of germinated spores, and the expression of factors involved in intracellular survival and escape.

BclA is the major glycoprotein on the surface of B. anthracis spores. It contains a short N-terminal region, followed by a collagen-like central domain and a C-terminal globular domain (40, 73). The collagen-like central domain forms collagen-like triple helices in solution, as shown by its circular dichroism spectrum (40). The x-ray crystal structure of the BclA C-terminal globular domain resembles that of the globular head of C1q even though the primary amino acid sequences of the two proteins do not show significant homology to each other. This makes BclA similar to C1q in both domain organization and tertiary structure, and thus, a potential member of the C1q family (74). In addition, rBclA binding to C1q may involve a distinct mechanism from the previously characterized C1q ligands. Thus, BclA is a novel ligand for C1q. It will be interesting to elucidate the molecular details of BclA–C1q interaction, that is, the region/motif in each protein responsible for binding and if or how the binding affects C1q recognition of immune complexes and association with C1r and C1s. Our data also indicate that rBclA is insufficient to activate CCP. The data on C1q binding to spores suggest that there is a secondary C1q binding site on the spores. It is possible that this additional site functions together with BclA in CCP activation. Alternatively, native BclA is glycosylated (75). It is possible that whereas the core protein mediates binding to C1q, some of the glycosyl moieties are important for CCP activation. Studies to elucidate the detailed mechanisms of C1q binding and CCP activation are currently underway in our laboratory.

BclA belongs to a family of recently reported bacterial surface proteins that contain collagen-like structures. Other members of the family are the group A streptococcal collagen-like proteins (76, 77) and the pneumococcal collagen-like protein (78). Scl1 was shown to mediate the internalization of group A streptococci by host cells via direct interactions with integrin αβ (79), and has the ability to bind low-density lipoprotein (80) and complement factor H and factor H-related protein 1 (81). Pneumococcal collagen-like protein was 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 or C3 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, or its assembly, 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.

The role of complement in host defense against B. anthracis has not been investigated previously except for complement C5. Compared with WT mice, mice deficient in C5 displayed increased susceptibility to B. anthracis infections (83). There was some evidence suggesting that this effect of C5 may be primarily through vegetative bacilli (84), as the increase in susceptibility appeared to be more apparent in infections caused by B. anthracis strains that were unable to produce the capsule, which is only present at the vegetative bacilli stage. These results underscore the idea that the interplays between B. anthracis and the complement system are complex. At different stages of infection, different complement components may have distinct functions and importance with respect to disease progression.

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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References
Supplemental figure S1. Phagocytosis of *B. anthracis* spores by macrophages with different MOI. RAW264.7 cells were incubated with 7702 spores at different MOI for 30min and detection of extracellular and intracellular spores were as described in the legend for Figure 1. **A**, the number of extracellular adhered spores per macrophage. **B**, the number of intracellular spores per macrophage. 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’s *t* test.
Supplemental figure S2. Depletion of IgG from normal human serum. IgG was depleted by passing the serum through a protein G column as described in the Materials and Methods section. The depletion was confirmed by SDS-PAGE (A) and western blot (B). The heavy chain and the light chain of IgG were indicated by an arrow and an arrowhead, respectively.
Supplemental figure S3. recombinant BclA (rBclA) does not activate C1. Purified C1 was incubated with rBclA (26 μM), heated treated rBclA (hrBclA 26 μM), IgM (10 μg/ml) or HBS buffer only and samples were analyzed by western blot using anti-C1s antibody and a second antibody. Full-length C1s and cleavage products A chain and B chain were indicated by arrows.