Murine Macrophages Kill the Vegetative Form of *Bacillus anthracis*

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Anti-protective antigen antibody was reported to enhance macrophage killing of ingested *Bacillus anthracis* spores, but it was unclear whether the antibody-mediated macrophage killing mechanism was directed against the spore itself or the vegetative form emerging from the ingested and germinating spore. To address this question, we compared the killing of germination-proficient (gp) and germination-deficient (ΔgerH) Sterne 34F2 strain spores by murine peritoneal macrophages. While macrophages similarly ingested both spores, only gp Sterne was killed at 5 h (0.37 log kill). Pretreatment of macrophages with gamma interferon (IFN-γ) or opsonization with immunoglobulin G (IgG) isolated from a subject immunized with an anthrax vaccine enhanced the killing of Sterne to 0.49 and 0.73 log, respectively, but the combination of IFN-γ and IgG was no better than either treatment alone. Under no condition was there killing of ΔgerH spores. To examine the ability of the exosporium to protect spores from macrophages, we compared the macrophage-mediated killing of nonsonicated (exosporium +) and sonicated (exosporium −) Sterne 34F2 spores. More sonicated spores than nonsonicated spores were killed at 5 h (0.98 versus 0.37 log kill, respectively). Pretreatment with IFN-γ increased the sonicated spore killing to 1.39 log. However, the opsonization with IgG was no better than no treatment or pretreatment with IFN-γ. We conclude that macrophages appear unable to kill the spore form of *B. anthracis* and that the exosporium may play a role in the protection of spores from macrophages.

*Bacillus anthracis*, the causative agent of anthrax, is a highly virulent gram-positive and spore-forming bacterium that is typically acquired through contact with anthrax-infected animals or animal products or atypically through intentional exposure as a biological weapon (6, 8). Virulent strains of *B. anthracis* carry two large plasmids, pXO1 and pXO2, that carry the genes encoding anthrax toxin production and capsule formation, respectively. Dormant spores are highly resistant to adverse environmental conditions but are able to reestablish vegetative growth in the presence of favorable environmental conditions (29).

Germination is the conversion of a resistant, dormant spore into a heat-sensitive bacillary form and must occur within an appropriate host environment for the appearance of disease. The germination of *B. anthracis* spores within macrophages and the outgrowth of vegetative bacilli constitute the first stage of anthrax infection (11). Spore germination enables the bacteria to proliferate actively and to synthesize their virulence factors, leading to massive septicemia (6).

The interaction between *B. anthracis* and macrophages that are responsible for the recognition and elimination of microbial pathogens (5, 19, 20, 35, 36) represents a critical early event in anthrax pathogenesis, but the mechanisms of this interaction are not clearly understood.

In susceptible mice, *B. anthracis* infection causes the alteration in function and/or death of the macrophages, which may allow the bacteria to avoid detection by the innate immune system (8). Lethal toxin induces apoptosis of susceptible macrophages (16, 17, 23), but lysis of macrophages by the accumulation of large numbers of bacilli might also occur (5). The mechanism by which macrophages ingest and kill *B. anthracis* spores is not well characterized and is the subject of the present studies.

Since the spores are likely to be the first form of *B. anthracis* to encounter host phagocytes, antibodies directed against the *B. anthracis* spore may facilitate its uptake and killing. Previously, anti-protective antigen (PA) antibody was reported to enhance macrophage killing of ingested *B. anthracis* spores, but it was unclear whether the antibody-mediated macrophage killing mechanism was directed against the spore itself or the vegetative form emerging from the ingested and germinating spore (35, 36). To address this question, we compared the killing of germination-proficient *B. anthracis* Sterne strain 34F2 (pXO1 + pXO2 −) and that of the congenic germination-deficient ΔgerH (gerH-null) strain spores by murine peritoneal macrophages (33, 34). The tricistronic gerHABC operon encodes germinant sensors in the presence of macrophages and macrophage-conditioned media and is required for endospore germination within the macrophage environment (33). Thus, the Δger mutant is a useful tool for determining whether the macrophage is capable of killing the *B. anthracis* endospore in the absence of germination.

The exosporium of *B. anthracis* is the outermost layer of the spores and contains specific glycoproteins which are highly immunogenic (24, 30). The exosporium may play a role in the interaction of the spore with the infected macrophages and

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influence spore germination within the macrophages (5, 10). Although there is an association between the exosporium of *B. anthracis* spores and macrophages, the ability of the exosporium to protect *B. anthracis* spores from the macrophage intracellular environment has yet to be elucidated fully. Therefore, we also compared the intracellular survival of exosporium-depleted *B. anthracis* Sterne strain 34F2 spores to that of Sterne strain 34F2 with the exosporium intact.

**MATERIALS AND METHODS**

Reagents. RPMI 1640 was purchased from Gibeo-BRL (Frederick, MD). Fetal bovine serum was obtained from Atlanta Biologicals (Lawrenceville, GA). Gentamicin and phosphate-buffered saline (PBS) were purchased from Biosource International (Rockville, MD). Thioglycollate Medium Brewer Modified was obtained from Becton Dickinson (Cockeysville, MD). Sulfanilamide and 2-naphthylethylenediamine dihydrochloride were purchased from Sigma Co. (St. Louis, MO). Gamma interferon (IFN-γ) was obtained from PBL Biomedical Laboratories (Piscataway, NJ).

*B. anthracis* strains and spore preparation. *B. anthracis* Sterne 34F2 and the ΔgerH (the congenic gerH-null) strain, constructed from Sterne 3F2 (34), were examined during this study. The congenic gerHΔ-null strain was constructed from *B. anthracis* 34F2 using a deletion construct in *B. anthracis* gerHA containing an Erm resistance cassette (33). Spores were prepared from the two *B. anthracis* strains, as previously described (2). Viable spore titer was determined by dilution plating before and after heat killing (65°C for 30 min) of vegetative cells.

Sonication of spores. The exosporium was removed by disruption of the spores with sonication. Spores were centrifuged at 10,000 × g for 10 min at 4°C. Pellets were resuspended to approximately 3 × 10^9^ spores/ml in 50 mM Tris-HCl, 0.5 mM EDTA buffer (pH 7.5). All subsequent manipulations were at 4°C. Spores were sonicated (Branson Sonifier 150; Branson Ultrasonics Co., Danbury, CT) with maximum power (amplitude, 12 μm; 10 min/50 W) for 7 to 10 min bursts, each separated by 2 min of cooling on ice. Exosporium fragments were separated from spores by centrifugation at 9,000 × g for 15 min at 4°C. The spore pellets were washed once in PBS, and the exosporium-containing supernatants were pooled and then centrifuged again to remove the remaining spores. Any residual endospores in the exosporium-containing supernatant were removed by filtration through 0.45- and/or 0.2-μm low-protein-binding filters (Acrodisc syringe filter; Pall Co., Timonium, MD). Comparison of viable colony counts of the sonicated spores with those of an aliquot from the same culture retained on ice showed no quantitative difference (data not shown). This suggests that the sonication treatment did not alter the viability of the spores.

Electron microscopy. Sonicated and nonsonicated *B. anthracis* Sterne strain 34F2 spores were suspended in PBS, pH 7.4, and applied to glow-discharged carbon-coated grids for negative staining in 1% (wt/vol) phosphotungstic acid. Grids were then washed extensively to remove the fixative and negatively stained with 1% uranyl acetate. Specimens were evaluated using a Zeiss 10 CA transmission electron microscope at 80-kV accelerating voltage.

Preparation of murine peritoneal macrophages and culture. Macrophages were cultured according to the method of Fortier et al. (7) with some minor modifications. Primary peritoneal macrophages were obtained from C57BL/6-129 (ICR) BR mice (Jackson Laboratory, Bar Harbor, ME) 4 days after intraperitoneal inoculation of 3 ml of 3% thioglycollate. Peritoneal fluid was drained through the abdominal wall with a 23-gauge needle. Fluid from mice was pooled and washed, and total cell counts were determined using a hemacytometer, and the remaining fluid was centrifuged at 380 × g for 10 min at 4°C. Washed cell suspensions were adjusted to 10^7^ spores/ml in culture medium containing RPMI 1640 with 10% fetal bovine serum and 50-μg/ml gentamicin and incubated in polystyrene tubes (Elkay Products, Inc., Shrewsbury, MA) in 5% CO_2_ at 37°C overnight before exposure to spores. In some experiments, macrophages were primed overnight with 100 U/ml of IFN-γ.

IgG preparation. The human serum sample used in this study was collected from an individual who had received the United Kingdom-licensed anthrax vaccine (3). Human immunoglobulin G (IgG) was isolated from the serum using a protein A column (Pierce, Rockford, IL) per the manufacturer’s instructions. The concentration of the purified IgG was determined using a total IgG assay kit (Pierce). Nosocomian (Berna Biotech, Berne, Switzerland) was used as a control, nonimmune IgG. It is an IgG preparation.

Infection of macrophages. The spores were opsonized with IgG from human immune serum, nonimmune IgG, or medium alone. Spores were mixed in 100-μl aliquots (10^6^ spores) with an equal volume of IgG (50 μg/ml) and incubated at 4°C for 30 min on a rotator. They were then added to the macrophages at a multiplicity of infection (MOI) of 1:1 to 100:1 (macrophages to spore) and incubated at 37°C in 5% CO_2 for 30 min to allow phagocytosis. The cells were washed once with fresh medium and then with medium containing gentamicin (at a final concentration of 50 μg/ml), and incubation was continued for an additional 30 min to remove the extracellular bacilli. After removal of the medium, the cells were washed once with fresh medium, resuspended to the original volume with fresh medium without antibiotic, and incubated in 5% CO_2_ at 37°C. The numbers of macrophage-associated *B. anthracis* cells were determined at 1, 3, 5, and 24 h. At each of these time points cells were washed, lysed with distilled water, diluted in PBS, and plated for viable colony counts. Aliquots from each sample also were incubated at 65°C for 30 min to assess the presence of vegetative cells. Samples were plated on L agar plates for colony counts. While the ΔgerH strain cannot germinate within the macrophage environment, it readily germinates and grows on L-agar plates.

Statistics. Student’s *t* test was used to analyze the data for statistical significance, and results were considered significant at *P* values of <0.05.

**RESULTS**

Macrophage-mediated killing effect on the viability of *B. anthracis* spores. Peritoneal macrophages were harvested from ICR mice and infected with spores of *B. anthracis* Sterne 34F2 and its ΔgerH congenic mutant strain. At the initial sampling time point of 1 h (i.e., after addition of spores to macrophage cultures), there was an initial period of 30 min to allow for uptake of spores by the macrophages and an additional 30 min during which the cultures were incubated in gentamicin to kill the extracellular *B. anthracis*. There was similar macrophage association of Sterne 34F2 and Δger spores by the macrophages (Fig. 1). The degree of spore germination within that 1 h was assessed by heat treatment of the samples obtained. Since heat treatment kills the vegetative but not spore form of *B. anthracis* within the sample, viability after heat treatment serves as an indicator of the relative amount of spores within the total sample obtained. Thus, at the initial sampling point, nearly 60% of ingested Sterne 34F2 spores had germinated (*P* < 0.05, comparison between heated and nonheated aliquots from same sample), while in contrast there was no statistically significant reduction in CFU in the heated aliquot of ΔgerH.

For each assay, the macrophage-associated CFU were determined at 3, 5, and 24 h postinfection. Bacterial counts (CFU) at these time points were compared to the CFU from the initial 1-h sample, and the difference was expressed as a log reduction in CFU. We examined the antispore activity of macrophages at multiple MOIs whereby decreasing numbers of spores were added to a constant number of macrophages (10^6^/ml). When macrophages were infected with the reduced number of Sterne 34F2 spores (i.e., lower MOI), they killed more of the administered spores. This was most evident after 24 h in culture (Fig. 2). When the MOI was decreased from 1:1 (1 × 10^6^ spores/ml) to 1:20 (5 × 10^5^ spores/ml) (Fig. 2), spore killing increased from 0.25 to 1.56 log kill at 24 h. Infecting macrophages with Sterne 34F2 spores at an MOI of 1:100 resulted in complete killing at 24 h after infection (data not shown). In contrast to the killing observed with Sterne strain 34F2, there was no killing of the Sterne 34F2 ΔgerH mutant, even at an MOI of 1:20 (Fig. 2). Since the ΔgerH mutant is unable to germinate in the macrophage environment (but can do so when plated on agar), these findings suggest that macrophages are unable to kill the spore form of *B. anthracis* but rather appear to kill the vegetative form emerging from the spore.
Effect of IgG from a human immunized with an anthrax vaccine and/or macrophage priming with IFN-γ on the viability of spores. Since there was relatively little killing of Sterne 34F2 and ΔgerH spores at an MOI of 1:1, we assessed the ability of either opsonization of spores with IgG or activation of macrophages with IFN-γ to enhance the killing at this MOI. At an MOI of 1:1 killing of the Sterne 34F2 strain by the macrophages was observed between the initial and 3-h time points, but the number of spores subsequently increased slightly between the 5- and 24-h time points (Fig. 3). While macrophages ingested both spores equally, only the Sterne 34F2 was killed at 5 h (0.37 log kill of CFU versus CFU count at 1 h) and at 24 h (0.21 log kill) in the absence of either IFN-γ pretreatment of macrophages or immune IgG. No decrease in CFU between initial and later time points was observed for the ΔgerH strain. Sterne strain 34F2 spores opsonized with immune IgG (50 μg/ml) resulted in enhanced killing by macrophages to 0.73 and 0.58 log kill at 5 and 24 h, respectively. In contrast, opsonization with nonimmune IgG showed killing of spores to 0.35 and 0.21 log kill at these same time points, respectively. There was no reduction in the viable CFU of the opsonized ΔgerH strain, however (Fig. 3). Pretreatment of macrophages with IFN-γ also enhanced the killing of Sterne 34F2 spores by macrophages: IFN-γ-primed macrophages increased their killing of administered Sterne 34F2 spores by...
0.49 and 0.46 logs at 5 and 24 h, respectively. The combination of IFN-\(\gamma\) priming and IgG opsonization of spores was no better than the opsonization with IgG alone (Fig. 3). Under no condition was there killing of \(\Delta gerH\) (\(gerH\)-null) spores, again confirming that macrophages are unable to kill the spore form of Sterne 34F2. These observations were also confirmed microscopically. During the first 5 h of incubation there were increasing numbers of Gram stain-positive bacilli in wells to which Sterne 34F2 spores were added, while such bacilli were rarely observed in wells having \(gerH\) spores (data not shown).

The role of exosporium in protection of spore from macrophage-mediated killing. The \(B.\ anthracis\) endospore is surrounded by a loose-fitting exosporium whose role in the pathogenetic cycle of \(B.\ anthracis\) is not well characterized. To examine the potentially protective role of the exosporium, we compared the macrophage-mediated killing of nonsonicated and sonicated Sterne strain 34F2 spores. Sonication of spores for 7 min caused fragmentation of the exosporium without disruption of the spores (Fig. 4). Some spores in the population did retain small portions of exosporium on their surface. Whole spores were removed by low-speed centrifugation, the supernatant was filtered (0.45 \(\mu\)m or 0.2 \(\mu\)m) to remove all remaining live spores, and the exosporium fragments were concentrated. While macrophages were able to ingest similar amounts of the two spore types, more sonicated spores than nonsonicated spores were killed: compared to nonsonicated Sterne 34F2, in the absence of either IFN-\(\gamma\) pretreatment or IgG opsonization of spores, macrophages were better able to kill sonicated Sterne 34F2 at each time point examined (0.98 and 1.11 log kill at 5 and 24 h, respectively, versus initial 1-h colony count) (Fig. 5). Pretreatment of macrophages with IFN-\(\gamma\) increased the killing of the sonicated spores to 1.39 and 1.6 log kill at 5 and 24 h, respectively. This suggests that activated macrophages are able to enhance the killing of exosporium-deficient spores. In contrast, while opsonization of spores with the immune IgG enhanced the killing of nonsonicated spores relative to untreated spores (Fig. 5), opsonization of sonicated spores with immune IgG did not improve the killing of administered spores. This suggests that the IgG targeted an antigen(s) on the exosporium surface. The combination of IFN-\(\gamma\) and IgG did not enhance the killing of either sonicated or nonsonicated spore more than either treatment alone. These results suggest that the exosporium may play a role in the protection of spores from macrophage-mediated killing.

**DISCUSSION**

The germination of ingested spores is an early event in the development of anthrax disease. For germination to occur, the spore needs to be either inside, or within high proximity to phagocytes, suggesting that spores possess a means of sensing germinants in this environment and triggering germination (5, 11, 26, 33). Events following germination are still somewhat controversial. It is generally agreed that \(B.\ anthracis\) must possess mechanisms which enable the newly germinated, relatively fragile, vegetative bacterium to survive within the harsh environment of the phagolysosome (11).

An antispore immune response that interferes early in this sequence of events, before the onset of bacteremia, could conceivably offer effective protection against lethal infection. Understanding the events in interaction between spores and host phagocytes is therefore critical for the development of new therapeutic options. Although much of the spore-host
interaction takes place within the phagocyte, the ways in which the macrophage responds to the spore infection remain unclear. Here, we investigated the killing mechanisms of spores mediated by macrophages.

Our study has two new findings that may have potential therapeutic implications. We found that (i) spores are not killed directly by macrophages and (ii) the exosporium has a protective function for the spores. While we demonstrate that the antibacterial weapons of the macrophages target the newly germinated organism rather than the spore itself, we also show that germinated spores were more efficiently destroyed by murine peritoneal macrophages after treatment with either immune IgG or IFN-γ (Fig. 3). In contrast, even after treatment with immune IgG or IFN-γ, macrophages had no effect on the viability of ΔgerH, a congenic variant of Sterne unable to germinate within murine macrophages but which can germinate on laboratory medium (33).

There are conflicting findings on the fate of phagocytosed spores. Welkos et al. (35) reported that anti-PA antibodies enhanced the rate of germination of phagocytosed spores and suggested that the more rapid germination of anti-PA antibody-treated spores was associated with the increased sporiciidal activity of macrophages. While Welkos et al. suggested
that increased germination in macrophages could be associated with increased bacterial killing by the macrophages (35), Guidi-Rontani et al. showed that germinated spores survive in macrophages but cannot multiply within them (10). These differences between the two studies might depend on the differences in the macrophages (e.g., cell line and primary cell), the strain of B. anthracis used, the MOI, or the growth condition used.

To determine if spore surface-located proteins play any role in germination and subsequent bacterial survival within the macrophage, we compared the killing profiles of wild-type and exosporium-deficient Sterne 34F2 spores. Under all conditions tested, spores lacking exosporium were killed at a higher rate than wild-type spores. Since we observed no macrophage-mediated killing in the absence of germination, these data suggest that the exosporium contains factors which regulate both germination and intracellular survival.

The events which trigger spore germination within the macrophage are as yet unclear. We previously reported that exposure of spores to physiologically relevant levels of superoxide, such as those achieved in the phagolysosome, triggered in vitro germination, and this led us to propose that the phenomenon may be due to the inactivation of negative regulators of spore germination, such as alanine racemase and inosine hydrolase, which are located at the spore surface within the exosporium (2, 13, 24, 27, 31, 32). Alanine racemase is a well-described inhibitor of the germination of B. anthracis and Bacillus cereus spores. It converts L-alanine to D-alanine, an isomer that is not recognized by germination receptors (4, 9, 31).

In addition to germination regulators, the exosporium also contains homologs to enzymes such as superoxide dismutase, alkyl hydroperoxide reductase, catalase, and the DNA repair enzyme endonuclease IV which are known to circumvent the toxic events initiated by O$_2^-$ and hydrogen peroxide (H$_2$O$_2$), produced by, and the result of, the oxidative burst (13–15, 24, 27, 32). We recently reported the ability of spores of the Sterne strain to scavenger O$_2^-$, suggesting that some or all of these enzymes may be active (2). The organism must also possess means of avoiding the attentions of nitric oxide. We observed that B. anthracis spores induced the production of NO• by macrophages and that spores lacking exosporium stimulated the production of significantly more NO• at 24 h than wild-type spores did (unpublished data). These data suggest that some of the constituents of the exosporium may subvert macrophage killing mechanisms, and in the absence of exosporium macrophages may be better able to kill the emerging vegetative form.

Recent work has demonstrated that the phagocytosis of B. anthracis spores by murine macrophages and the subsequent killing are enhanced by anti-PA antibodies from a range of animal species directed against spore surface-associated proteins, probably PA nonspecifically associated with the exosporium (35). They concluded that the mechanism by which the macrophage subsequently kills the organism, though yet unclear, is probably due to antibacterial factors present in the phagolysosome. We also found that human antibodies from an immunized individual promoted spore uptake and killing at a level comparable to that seen in IFN-γ-treated cells. As shown in Fig. 3, pretreatment of spores with immune IgG enhanced the macrophage-mediated killing of spores with exosporium (i.e., nonsonicated spores), but pretreatment with IFN-γ and the combination of IgG and IFN-γ were no better than treatment with IgG alone (Fig. 3). This is the first report of immune human IgG mediating the killing of B. anthracis. In contrast, the effect of IFN-γ was better than that of IgG in the treatment of sonicated spores (Fig. 5). Thus, these differences suggest that antispore activity by anti-PA-IgG is the result from its interaction with the surface of nonsonicated (i.e., exosporium-replete) spores but not sonicated (or exosporium-depleted) spores. The failure to enhance the killing of sonicated spores is further evidence that the antibody target is associated with the exosporium. Further, only in the absence of the exosporium can IFN-γ-activated macrophages kill B. anthracis.

PA is the principal protective immunogen of the current U.S. and United Kingdom licensed human vaccines (1). Its ability to stimulate protective immunity in a range of animal models including primates has been repeatedly demonstrated (18). Neutralization of toxin activity has been identified as a major correlate of protection but may not be the only one (21, 25). The ability of PA-specific antibodies to mediate spore uptake and killing may explain why sterile immunity was reported in two recent DNA PA vaccine studies of rabbits (12, 25). The ability to confer sterile immunity would be of considerable advantage in the context of genetically engineered strains of B. anthracis (22, 28). Since genetically engineered strains of B. anthracis could be developed that contain foreign genes encoding non-B. anthracis toxins, such as botulinus toxins, the elimination of B. anthracis from the body (i.e., sterile immunity) may be a better strategy of protection against this “Trojan horse” approach of potential bioterrorists rather than the neutralization of anthrax toxins alone, which is the current strategy.

In conclusion, the macrophage is unable to kill the spore form of the organism. We observed that immune IgG from human immune serum had significant activity in the killing of B. anthracis by macrophages, and treating spores with the immune IgG prior to phagocytosis was associated with an enhanced rate of killing. The exosporium appears to play an important role in protecting the bacterium from the antibacterial arsenal of the cell. While it is possible that anti-PA IgG in individuals vaccinated against anthrax might contribute to a protective immune response early in infection before outgrowth and toxin secretion by bacilli, identification of other antigens within the exosporium to which immune IgG may be directed could lead to improved next-generation anthrax vaccines.

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