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Poly-γ-Glutamate Capsule-Degrading Enzyme Treatment Enhances Phagocytosis and Killing of Encapsulated *Bacillus anthracis*▽

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The poly-γ-D-glutamic acid capsule confers antiphagocytic properties on *Bacillus anthracis* and is essential for virulence. In this study, we showed that CapD, a γ-polyglutamic acid depolymerase encoded on the *B. anthracis* capsule plasmid, degraded purified capsule and removed the capsule from the surface of anthrax bacilli. Treatment with CapD induced macrophage phagocytosis of encapsulated *B. anthracis* and enabled human neutrophils to kill encapsulated organisms. A second glutamylase, PghP, a γ-polyglutamic acid hydrolase encoded by *Bacillus subtilis* bacteriophage ΦNIT1, had minimal activity in degrading *B. anthracis* capsule, no effect on macrophage phagocytosis, and only minimal enhancement of neutrophil killing. Thus, the levels of both phagocytosis and killing corresponded to the degree of enzyme-mediated capsule degradation. The use of enzymes to degrade the capsule and enable phagocytic killing of *B. anthracis* offers a new approach to the therapy of anthrax.

Bacillus anthracis is the causative agent of anthrax, primarily a disease of domesticated and wild herbivores, which is also lethal to humans. While naturally occurring anthrax infection of humans is rare, the recent bioterrorist use of letters containing *B. anthracis* spores to cause disease has focused the attention of the biomedical community and generated interest in developing improved countermeasures. The virulence of *B. anthracis* is due primarily to the presence of two antihost exotoxins (lethal and edema toxins) and an antiphagocytic γ-linked poly-D-glutamic acid capsule surrounding the bacillus that are encoded on separate plasmids, pX01 (36) and pX02 (17, 54). Strains of *B. anthracis* lacking capsule have markedly reduced virulence in animal models (40), and such strains have been used successfully for more than 50 years to prevent anthrax in domesticated animals (51). Similar vaccines have also been used to control anthrax in humans in countries of the former Soviet Union and the People's Republic of China (48). Capsule synthesis is regulated by factors encoded on both of the virulence plasmids pX01 (11, 12, 20, 53) and pX02 (3, 53, 57) and is induced by the presence of serum and carbon dioxide (26, 35). The capsule is believed to enhance virulence by its antiphagocytic property. Although the mechanism by which the capsule inhibits phagocytosis is not firmly established, other bacterial capsules are known to inhibit phagocytosis by their anionic charge and by shielding potential bacterial surface adhesins (44, 45, 58). Similarly, the capsules of *B. anthracis* and *Bacillus subtilis* NAFM5 may function as a physical barrier to phage infection (24, 33).

Phagocytic cells have long been known to play an important role in anthrax pathogenesis (34). Alveolar phagocytes are thought to be involved in transporting inhaled spores to draining lymph nodes, where the spores are thought to germinate (42). Macrophages (60) and neutrophils (23, 32) are reported to phagocytize and kill *B. anthracis*, and macrophages are required for resistance to infection in mice (7). Recent work suggests that antibody to the *B. anthracis* capsule enhances phagocytosis and killing of encapsulated bacilli (6, 46, 59) and that active (6) and passive (27) vaccination with capsule protects against experimental infection. Taken together, these data suggest that methods to increase the phagocytosis of encapsulated anthrax bacilli may be valuable in the treatment of anthrax.

The strategy of using microbial enzymes as antibacterial agents has a long history, including the use of extracts of *Bacillus pyocyaneus* to treat anthrax (see reference 2 and references therein) and of *Aspergillus fumigatus* to treat tuberculosis (56). Toenniessen (see reference 2 and references therein) first reported that an encapsulated bacterium, *Klebsiella pneumoniae*, became unencapsulated when incubated with *Bacillus vulgatus*, suggesting that the latter organism secreted an enzyme that degraded the capsule. Avery and Dubos extended these ideas to demonstrate that microbial enzymes could be used to remove the capsule from the pneumococcus and successfully treat experimental infections (2). Similar approaches have been used in experimental *Escherichia coli* infections (37, 38).

Enzymes capable of degrading the capsule of *B. anthracis* have been isolated from dog liver (55) and from *B. anthracis* (4, 52). The *B. anthracis* capsule depolymerase gene, *capD*, encodes a capsule-degrading enzyme, CapD, thought to be necessary for virulence (4, 31, 52) and for anchoring the capsule to the cell wall peptidoglycan (4). Poly-γ-glutamate hydrolase (PghP) is a 25-kDa enzyme encoded by the bacteriophage ΦNIT1 that specifically cleaves D- and L-polyglutamic acid, a component of the capsule produced by several strains of *B. subtilis* (24).

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In our study, we found that recombinant CapD degraded high-molecular-weight capsule, removed capsule from the bacterial surface, and facilitated macrophage phagocytosis and neutrophil killing of encapsulated *B. anthracis* bacilli. PghP had less activity in degrading capsule and only a minimal effect on phagocytosis and killing. These results may lead to the development of a novel therapy for anthrax.

MATERIALS AND METHODS

Bacterial strains and spore preparation. *B. anthracis* Ames ($pX01^+$ $pX02^+$) (U.S. Army Medical Research Institute of Infectious Diseases collection) was cultured in brain heart infusion (BHI) broth (Becton Dickinson and Co., Sparks, MD) at 37°C with 0.8% sodium bicarbonate and 5% carbon dioxide (CO_2), except as noted. *B. anthracis* spores were generated as previously described (6). *B. subtilis* natto (U.S. Department of Agriculture) was cultured at room temperature on nutrient broth yeast extract agar with 20% CO_2.

Purification of CapD and PghP. The open reading frame of *capD*, excluding the signal sequence (amino acids 1 to 27), was amplified by PCR and cloned into pET15b (EMD Biosciences, San Diego, CA) as an XhoI-HindIII fragment (forward primer, 5′-GTC GCT CGA GTC TTT CAA TAA AAT AAA AGA CAG TGT TA-3′; reverse primer, 5′-GCG GCG AAG CTT CTA TTT ATT TGA TTT CCA AGT TCC ATT CTC TCT GCC-3′). The open reading frame of the *pghP* gene was amplified from ΦNIT1 DNA and cloned into pET15b as a BamHI-NdeI fragment (forward primer, 5′-GCG GCG CAT ATG GCA CAA ACA GAC ACA TAT CCA AAT ATT GAA GCA-3′; reverse primer, 5′-GCG GCG GGA TCC TTA GCC ATA ATA CTC TGC CTC TGC TTC TTT AAT-3′). Recombinant proteins were expressed and purified according to the manufacturer's instructions.

Capsule degradation. Capsules from *B. subtilis* and *B. anthracis* were purified as described previously (6) and digested with 10-fold dilutions of purified recombinant CapD or PghP for 1 h at 37°C. Each reaction mixture contained 1.5 μl (4 μg) of capsule, 15.5 μl of phosphate-buffered saline (PBS) (pH 7.4), 1 μl of 2 mM $ZnSO_4$, and 2 μl (1 μg) of enzyme. No $ZnSO_4$ was added to the reaction mixture containing CapD. There was no effect of $ZnSO_4$ on capsule migration. After the reaction, an equal volume (20 μl) of 2× sodium dodecyl sulfate (SDS)-Tricine sample buffer was added to each sample, and the degradation products were analyzed on a 10% SDS-Tricine polyacrylamide gel. Gels were washed three times for 3 min in water, fixed in several changes of 10% propanol with 10% formamide, and stained with StainsAll (10 mg/100 ml [Sigma, St. Louis, MO] in 10% propanol with 10% formamide).

Encapsulated *B. anthracis* Ames bacilli were prepared by culturing spores for 2 h in BHI broth containing 0.8% sodium bicarbonate at 37°C with 5% CO_2. Bacilli were washed with PBS and adjusted to 1×10^8 bacilli/ml in PBS. CapD or PghP was then added to a final concentration of 50 μg/ml, and cells were incubated for 20 min at 37°C. Controls were incubated with PBS alone. Bacterial suspensions were mixed with India ink (Becton Dickinson and Co.) and visualized by phase-contrast microscopy.

Macrophage phagocytosis. RAW264.7 murine macrophages were grown on coverslips as previously described (6). *B. anthracis* Ames spores were resuspended in BHI broth plus 0.8% sodium bicarbonate at a concentration of 2×10^7 spores/ml and were allowed to incubate at 37°C in 5% CO_2 for 90 min without shaking to generate single, encapsulated bacilli. For each assay condition, duplicate 1-ml aliquots of the newly germinated bacilli were centrifuged and resuspended in 200 μl of Dulbecco's modified Eagle medium (DMEM) containing 10% heat-inactivated fetal bovine serum and 0.1 mM $MnCl_2$, resulting in a concentration of 1×10^8 bacilli/ml. Manganese or zinc, required as a cofactor for PghP activity (24), was included in all experiments with PghP. Purified CapD or PghP was added to a final concentration of 20 μg/ml. In some experiments with PghP, erythromycin was added to a final concentration of 0.5 μg/ml in both PghP and PBS control samples. Experiments were also performed without antibiotics for comparison. The suspensions were incubated for 10 min at 37°C and then layered onto coverslips containing adhered macrophages and allowed to incubate at 37°C in 5% CO_2 for 30 min. In some experiments, opsonization of encapsulated bacilli with a high-titer anticapsule or normal mouse serum (21) at a 1:20 dilution was carried out for 30 min prior to layering onto macrophages. After the incubation, coverslips were washed extensively in PBS (pH 7.4) and stained with Wright Giemsa stain. Phagocytosis was measured by counting the number of adherent bacilli on approximately 100 macrophages from each of two coverslips. The phagocytic index was defined as the average number of bacilli that adhered to each macrophage ± standard error of the mean.

Human neutrophil bactericidal activity. Human neutrophils were isolated from healthy, unvaccinated volunteers using Histopaque 1077 and 1119 gradients according to instructions provided by the manufacturer (Sigma-Aldrich, St. Louis, MO) or by Ficoll-Hypaque density gradient centrifugation followed by dextran sedimentation (28) and resuspended in DMEM at a concentration of 2×10^7/ml. Results were the same with neutrophils purified by either method. Single, encapsulated *B. anthracis* bacilli prepared as described above were resuspended in DMEM at 1×10^7 bacilli/ml and treated with either PBS or 20 μg/ml of CapD or PghP for 10 min at 37°C. In some experiments, encapsulated bacilli were instead pretreated for 30 min with anticapsule or normal mouse serum at a 1:20 dilution. Neutrophils were adjusted to a final concentration of 5×10^6 neutrophils/ml in a 1.5-ml microcentrifuge tube in duplicate, and enzyme-treated bacilli were added to 1×10^5 bacilli/ml for a neutrophil-to-bacillus ratio of 50:1, except as noted. Normal, autologous human serum (10%) was included as a source of complement, and enzymes or anticapsule serum was added to maintain a final concentration of 20 μg/ml and 5%, respectively. The total reaction mixture volume was 550 μl. An aliquot was immediately removed at time zero to determine CFU by serial dilution in water and plating onto Luria-Bertani agar. The sample was then rotated at 37°C, and bacterial viability was measured at 2 h. To determine the effect of phagocytosis inhibition on CapD-mediated killing, cytochalasin D (Sigma-Aldrich) was added to neutrophils (10 μg/ml, 10 min, 37°C) prior to the addition of bacilli.

Statistics. Phagocytic index comparisons were evaluated for statistical significance by analysis of variance (ANOVA) with Tukey's post hoc tests between groups. ANOVA for phagocytic indices between groups was significant ($P < 0.0001$). Differences in neutrophil killing between groups over time were evaluated with repeated-measures ANOVA and shown to be statistically significant ($P < 0.0001$). Differences within a group between time zero and 2 h were tested using post hoc paired *t* tests. Differences between groups at 2 h were tested using Tukey's post hoc tests. *P* values for post hoc analysis are indicated in the text.

RESULTS

Hydrolysis of capsule with purified CapD and PghP. Encapsulated *B. anthracis* Ames bacilli were treated with recombinant CapD or PghP and visualized by phase-contrast microscopy. Capsule removal was observed as early as 5 min after treatment with either enzyme, and after 20 min, very little capsule was visible (Fig. 1). Treatment with CapD at a concentration as low as 0.035 μg/ml was sufficient to visibly decrease the size of the capsule by India ink staining.

To determine the extent of enzyme degradation, capsule purified from *B. anthracis* Ames and *B. subtilis* was digested with CapD or PghP for 1 h at 37°C and examined by SDS-polyacrylamide gel electrophoresis on a 10% SDS-Tricine gel. As seen in Fig. 2, the *B. anthracis* capsule was digested to completion with 35 μg/ml of CapD, and activity was clearly detectable at 3.5 μg/ml (lanes 4 to 6). CapD also hydrolyzed the *B. subtilis* capsule to completion at 35 μg/ml (lane 3). By contrast, PghP had significantly less activity than CapD on the *B. anthracis* capsule (Fig. 2, compare lanes 10 to 12 to lanes 4 to 6) but was more active than CapD on the *B. subtilis* capsule, with activity detected at concentrations as low as 0.035 μg/ml (lanes 7 to 9).

Phagocytosis of enzyme-treated encapsulated bacilli. We next examined the effect of enzymatic treatment of encapsulated *B. anthracis* on phagocytosis by macrophages. Encapsulated *B. anthracis* bacilli were treated with purified CapD or PghP and then incubated with RAW264.7 murine macrophages, and the phagocytic index was determined as described in Materials and Methods. Treatment with CapD resulted in a dramatic increase in phagocytosis. The phagocytic index increased from 1.3 ± 0.2 to 43 ± 4.7 (Table 1) ($P < 0.0001$ [ANOVA]; for CapD versus PBS, $P < 0.0001$ [Tukey's post hoc test]). PghP treatment did not increase the phagocytic

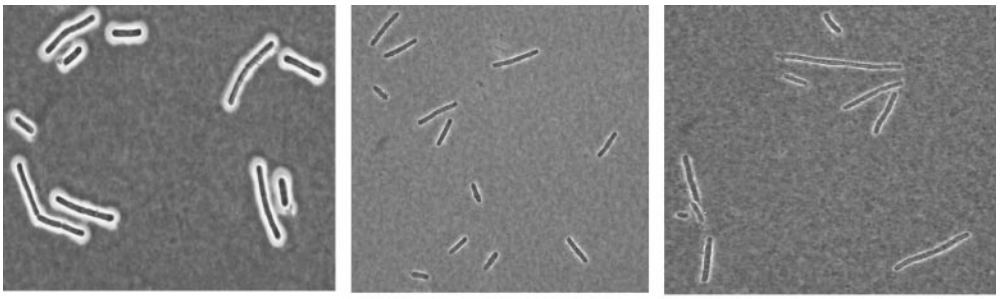

FIG. 1. Removal of capsule from encapsulated *B. anthracis* by CapD and PghP. *B. anthracis* Ames bacilli were grown under conditions to express capsule and treated for 20 min at 37°C with PBS (A), 50 μg/ml CapD (B), or 50 μg/ml PghP (C) as described in Materials and Methods and examined by India ink and phase-contrast microscopy (magnification, ×1,000).

index above that seen with PBS, although in some experiments, a slight effect was observed. The higher level of CapD-mediated phagocytosis is consistent with the greater degree of degradation of purified *B. anthracis* capsule observed with CapD than with PghP (Fig. 2). We observed that capsule regeneration occurred during incubation in 5% CO_2, which could reduce the effect of the PghP enzyme due to its relatively low efficiency of capsule degradation. To counteract this, a bacteriostatic concentration of erythromycin (0.5 μg/ml) was added to inhibit the regrowth of the capsule. Under these conditions, increased phagocytosis was observed after PghP treatment, with the phagocytic index increasing 5.6-fold from 1.2 ± 0.3 in the PBS control with erythromycin compared to 6.7 ± 2.4 in cells treated with PghP and erythromycin ($P < 0.0001$ [ANOVA]; $P < 0.0001$ for PghP versus PBS [Tukey's post hoc test]) (data not shown). Treatment with erythromycin alone in the absence of PghP had no effect on the phagocytic index compared to PBS alone (1.2 ± 0.3 versus 1.3 ± 0.2) ($P < 0.0001$ [ANOVA]; $P > 0.1$ for PghP versus PBS [Tukey's post hoc test]). There was no effect of erythromycin on CapD-mediated phagocytosis (data not shown). Adherence of enzyme-treated bacilli to macrophages is illustrated in Fig. 3. The extent of phagocytosis observed after treatment with CapD was similar to that seen with unencapsulated bacilli that germinated from spores in the absence of sodium bicarbonate and CO_2 (Table 1) ($P < 0.0001$ [ANOVA]; $P < 0.0001$ for unencapsulated bacilli versus PBS [Tukey's post hoc test]). Opsonization of encapsulated bacilli with anticapsule serum resulted in a significant increase in the phagocytic index to 6.5 ± 0.39 compared to 1.1 ± 0.1 with normal mouse serum ($P < 0.0001$ [ANOVA]; $P < 0.0001$ for anticapsule versus normal serum [Tukey's post hoc test]) (data not shown).

Enzyme-mediated neutrophil killing of encapsulated bacilli. We then determined the effect of treating bacilli with recombinant CapD or PghP on their killing by human neutrophils. Newly germinated, encapsulated *B. anthracis* bacilli that were pretreated with enzymes were incubated with neutrophils in the continued presence of enzymes, and bacterial viability was measured by serial dilution and plating. Treatment with puri-

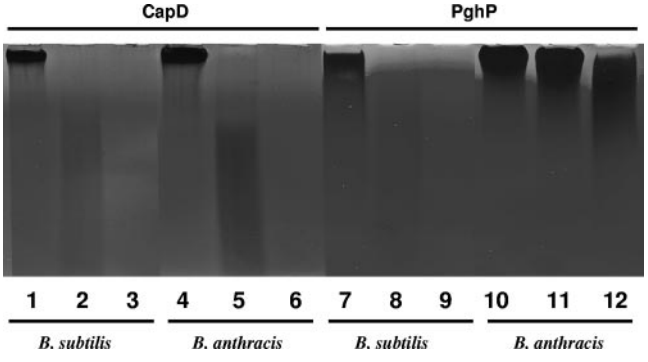

FIG. 2. Degradation of capsules from *B. anthracis* and *B. subtilis* by CapD and PghP. Capsule purified from *B. subtilis* (lanes 1 to 3 and 7 to 9) and *B. anthracis* (lanes 4 to 6 and 10 to 12) were digested with CapD (lanes 1 to 6) or PghP (lanes 7 to 12) and examined by SDS-polyacrylamide gel electrophoresis as described in Materials and Methods. Lanes 1 and 4, 0.35 μg/ml of CapD; lanes 2 and 5, 3.5 μg/ml of CapD; lanes 3 and 6, 35 μg/ml of CapD; lane 7, 0.035 μg/ml of PghP; lanes 8 and 10, 0.35 μg/ml of PghP; lanes 9 and 11, 3.5 μg/ml of PghP; lane 12, 35 μg/ml of PghP.

TABLE 1. Enzyme treatment enhances macrophage phagocytosis and neutrophil killing of encapsulated *B. anthracis* Ames

Treatment[a]	Macrophage phagocytosis (mean no. of bacilli ± SEM) (phagocytic index)	Neutrophil killing (% viable)
PBS	1.3 ± 0.2	72.0[c]
CapD	43.0 ± 4.7[b]	0.5[d]
PghP	1.2 ± 0.3	52.5[e]
CapD + cytochalasin D	ND[g]	67.9[f]
Unencapsulated bacilli	40.9 ± 2.7[b]	0.15[d]

[a] Encapsulated *B. anthracis* Ames bacilli were treated with PBS alone, CapD, or PghP before being assayed for macrophage phagocytosis and neutrophil killing as described in Materials and Methods. A control with unencapsulated bacilli grown in the absence of sodium bicarbonate and CO_2 was included. Cytochalasin D was added to neutrophils prior to the addition of CapD-treated bacilli. Results of macrophage phagocytosis experiments are expressed as the mean number of bacilli that adhered to each macrophage ± standard error of the mean. Results of neutrophil killing are expressed as the viability (percentage) of bacilli derived from measuring the CFU of duplicate samples at 2 h compared to that at time zero. Neutrophil killing data are from one experiment that is representative of seven experiments with CapD and two with PghP.

[b] $P < 0.0001$ compared with the PBS control (Tukey's post hoc test).

[c] $P = 0.0025$ compared with time zero (paired *t* test).

[d] $P < 0.0001$ compared with time zero (paired *t* test) and with PghP and PBS control at 2 h (Tukey's post hoc test).

[e] $P < 0.0001$ compared with time zero (paired *t* test) and with PBS control at 2 h (Tukey's post hoc test).

[f] $P < 0.0001$ compared with CapD-only treatment at 2 h (Tukey's post hoc test).

[g] ND, not determined.

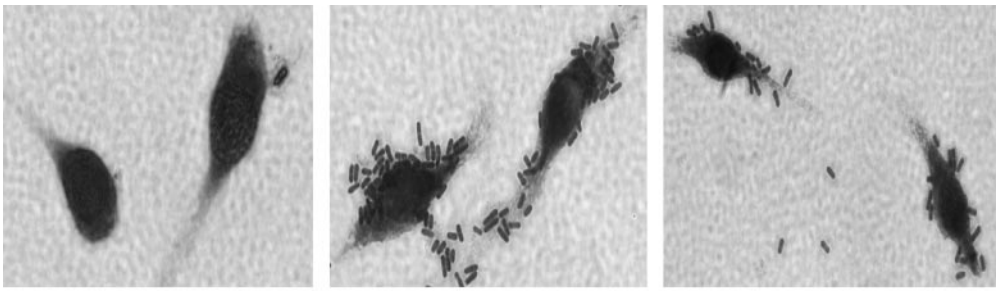

FIG. 3. Phagocytosis of encapsulated *B. anthracis* by RAW264.7 macrophages. Encapsulated *B. anthracis* Ames bacilli were treated with PBS (A), CapD (20 μg/ml) (B), or PghP (20 μg/ml) (C) and incubated with macrophages as described in Materials and Methods. The PBS and PghP samples contained 0.5 μg/ml of erythromycin.

fied CapD, and to a lesser extent with PghP, facilitated neutrophil killing of encapsulated *B. anthracis* (Table 1). CapD treatment resulted in neutrophil killing of >99% of encapsulated bacilli, with viability falling to 0.5% (Table 1) ($P < 0.0001$ [ANOVA]; $P < 0.0001$ for CapD at time zero versus that at 2 h [paired *t* test]) of the input bacilli after 2 h incubation. This extensive degree of killing is similar to that observed with unencapsulated bacilli produced by growth in the absence of sodium bicarbonate and CO_2 (Table 1) ($P < 0.0001$ [ANOVA]; $P < 0.0001$ for unencapsulated bacilli at time zero versus that at 2 h [paired *t* test]). It was also similar to the neutrophil killing observed after opsonization of encapsulated bacilli with anticapsule serum (>99% after 2 h incubation) (data not shown). In six other experiments with CapD treatment, killing after 2 h was ≥99%. Similar results were observed (>99% killing) at a neutrophil-to-bacillus ratio of 1:1 (Fig. 4). Treatment with PghP resulted in only a moderate degree of neutrophil killing, with viability at 2 h reduced to 52.5% (Table 1) ($P < 0.0001$ [ANOVA]; $P < 0.0001$ for PghP at time zero versus that at 2 h [paired *t* test]) and 29.4% ($P < 0.0001$, paired *t* test) in two experiments compared to that at time zero. The effect of PghP treatment on bacterial viability was significantly less than that of CapD (Table 1) ($P < 0.0001$ [ANOVA]; $P < 0.0001$ for CapD versus PghP [Tukey's post hoc test]). This again is consistent with the lower activity of PghP compared to CapD in degrading the purified *B. anthracis* capsule (Fig. 2). To eliminate the possibility that high-molecular-weight capsule fragments released by PghP treatment could impair neutrophil killing, we prepared the supernatant containing released capsules from encapsulated bacilli treated with PghP and added it to neutrophils incubated with unencapsulated bacilli as described in Materials and Methods. The addition of this supernatant did not impair the ability of neutrophils to kill unencapsulated bacilli (data not shown).

In the absence of enzyme treatment, a moderate decrease in bacterial viability was observed after incubation with neutrophils in some experiments, while modest growth occurred in others. In the experiment presented in Table 1, killing (28% decrease in CFU) was observed at 2 h (Table 1) ($P < 0.0001$ [ANOVA]; $P = 0.0025$ for PBS at time zero versus that at 2 h [paired *t* test]). The average percent viability at 2 h in the PBS control groups from seven experiments was 98.6 ± 26.7 (standard error of the mean). There was variability using different donors, with statistically significant ($P < 0.0001$ [ANOVA]; $P < 0.05$ for PBS at time zero versus that at 2 h [paired *t* test]) killing observed in four experiments, while statistically significant growth was seen in the three other experiments. An additional control showed that encapsulated bacilli treated with

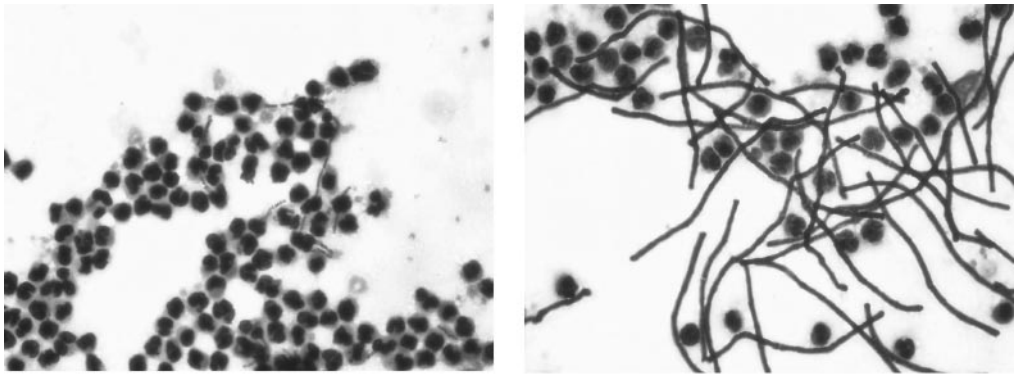

FIG. 4. CapD-mediated killing of *B. anthracis* Ames by human neutrophils. Human neutrophils (5×10^6 neutrophils/ml) were mixed with encapsulated bacilli (5×10^6 neutrophils/ml) with (A) or without (B) 20 μg/ml of CapD and incubated for 3 h at 37°C on an Eppendorf tube rotator. Cells were then concentrated 10-fold by centrifugation and stained with Wright Giemsa stain (magnification, ×1,000). Viability was reduced by CapD treatment to 0.35% after 3 h compared to that at time zero. No killing occurred in the sample (B) incubated without CapD.

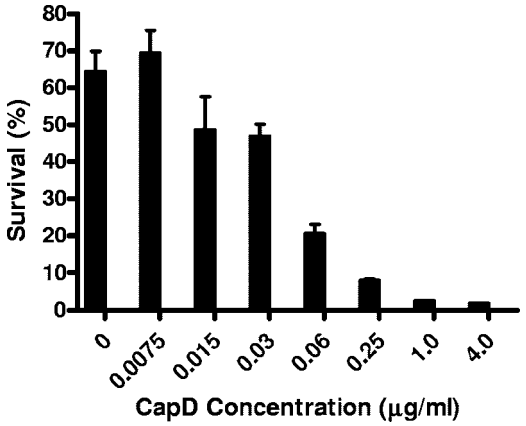

FIG. 5. CapD concentration-dependent neutrophil killing. CapD was serially diluted in PBS and used to treat encapsulated *B. anthracis* Ames bacilli before being mixed with human neutrophils as described in Materials and Methods. The bacterial viability of duplicate samples (means ± standard deviations) was measured at 2 h.

CapD alone in complete medium (DMEM containing 10% normal human serum) in the absence of neutrophils were not killed but rather doubled in CFU by 2 h (data not shown). Thus, efficient killing of encapsulated bacilli required both CapD treatment and neutrophils. Optimal killing (≥99%) occurred in the presence of normal but not heat-inactivated human serum (data not shown), indicating the likely requirement for complement. Additionally, treatment of neutrophils with cytochalasin D (10 μg/ml) almost completely blocked the killing of CapD-treated bacilli, with 67.9% survival, compared to 0.5% with CapD alone (Table 1) ($P < 0.0001$ [ANOVA]; $P < 0.0001$ for CapD versus CapD plus cytochalasin D [Tukey's post hoc test]), indicating that killing was phagocytosis dependent.

In the next experiment, we determined the minimal concentration of CapD required to mediate neutrophil killing. As shown in Fig. 5, CapD treatment enhanced neutrophil killing in a concentration-dependent manner. The concentration of CapD necessary to facilitate optimal neutrophil killing of encapsulated bacilli by 2 h was approximately 1 μg/ml (Fig. 5), and 0.25 μg/ml was sufficient to facilitate the killing of >90% of bacilli.

DISCUSSION

During the initial stages of infection by the aerosol route, spores are thought to be engulfed by macrophages or other phagocytic cells and are transported to regional lymph nodes. Some organisms are killed, likely those that germinate (22, 38a, 42, 60), while others remain viable and proliferate (43, 47). Germinated spores and bacilli are thought to eventually escape from the macrophage into the lymph nodes and then the bloodstream, where they spread systemically to most organs and replicate virtually unimpeded as short chains of phagocytosis-resistant encapsulated bacilli. Evidence suggests that the toxins (19) and capsule (15) are synthesized early after germination. The lethal and edema toxins are thought to interfere with macrophage function, although their role in intracellular killing and the fate of *B. anthracis* itself in macrophages remain unclear, with either killing (60), persistence (18), or growth (10) being reported, which is likely related to methodological differences and the use of different cells and bacterial strains, both encapsulated and unencapsulated. The toxins have also been reported to interfere with neutrophil function (1, 13, 23, 39, 61), while capsule synthesis and encapsulation renders the bacillus resistant to phagocytosis when tested in vitro (23, 30, 49) (Table 1), and in infected animals, encapsulated bacilli are essentially observed only extracellularly (9). With host innate immune defenses compromised, the bacteria grow to levels as high as 10^8 to 10^9 CFU/ml in the blood.

We demonstrated in this study that CapD can remove the capsule from the surface of encapsulated *B. anthracis* bacilli and degrade the purified capsule. Most importantly, encapsulated *B. anthracis* bacilli that are resistant to phagocytosis can be made highly susceptible to phagocytosis by macrophages and to killing after ingestion by neutrophils by enzymatically removing the capsule with CapD. While PghP treatment appeared to remove the capsule from the surface of bacilli, it had no effect on macrophage phagocytosis by itself and a minimal effect on neutrophil killing (Table 1). PghP was also much less efficient than CapD in degrading the purified *B. anthracis* capsule to lower-molecular-weight species (Fig. 2). The *B. anthracis* capsule is composed entirely of poly-D-glutamic acid, and PghP, in contrast to CapD, may have specificity for mixtures of poly-D- and L-glutamic acids and thus may only inefficiently degrade the pure D form. This is reflected in the dramatically greater activity of PghP on the poly-D- and L-glutamic acid capsule of *B. subtilis* than on the poly-D-glutamic acid capsule of *B. anthracis* (Fig. 2). Indeed, in preliminary experiments, CapD but not PghP was able to degrade a 30-mer of poly-D-glutamate (data not shown). There may be more residual capsule remaining on the bacillus that is not apparent by India ink microscopy after PghP treatment compared to CapD treatment, which may be responsible for the differences in phagocytosis and killing. Another possible explanation for the minimal effect of PghP treatment on neutrophil killing compared to CapD treatment may be a "*trans*" effect due to remaining large capsule fragments released into the medium by PghP treatment, which could impair neutrophil killing. This possibility was eliminated by showing that the addition of supernatant from PghP-treated encapsulated bacilli to neutrophils did not impair their ability to kill unencapsulated bacilli under these assay conditions.

In the absence of CapD treatment, minimal killing by neutrophils was noted in some individuals, while no killing occurred in others. This may be related to the presence of various amounts of cross-reacting anticapsule antibodies in some individuals due to prior exposure to other bacteria, as polyglutamic acid capsules are known to be made by *Staphylococcus epidermidis* (25) and other *Bacillus* species and organisms (5). Future studies will be necessary to address this point.

CapD-mediated neutrophil killing appeared to be dependent on complement, as it was reduced when heat-inactivated serum was used, similar to what has long been reported for other bacteria (16, 28, 41), and complement was shown to be necessary for neutrophil killing of an unencapsulated *B. anthracis* strain (data not shown). This is consistent with a preliminary report noting less C3 binding to encapsulated than to unencapsulated *B. anthracis* as well as less killing by human

blood cells, although few details were provided (40a). Thus, CapD-mediated enzymatic removal of the capsule may allow for greater complement deposition and subsequent neutrophil killing. Further studies will be necessary to confirm this. A lack of capsule on the bacillus surface may also render them more susceptible to the products of phagolysosomal fusion, such as reactive oxygen species and cationic peptides. Indeed, it has been reported previously that capsule can block the bactericidal activity of neutrophil extracts (23), and we have observed that purified capsule can interfere with the bactericidal activity of some cationic peptides including alpha defensins present in neutrophils and beta defensins produced by nonphagocytic cells (38b). Thus, in addition to its antiphagocytic property, the capsule may contribute to resistance to intracellular killing and resistance to nonphagocytic innate immune defenses.

Gamma glutamyltranspeptidase, encoded by *capD*, has been functionally characterized as a virulence factor (4, 31, 52). The 55-kDa protein has depolymerase activity and degrades high-molecular-weight capsule with the release of small amounts of lower-molecular-weight species that have been suggested to contribute to virulence (31). CapD was also reported to be necessary for anchoring the capsule to the peptidoglycan (4). It is possible, however, that the expression of *capD* may be tightly regulated, as overexpression could lead to a high level of depolymerization and a loss of capsule from the bacterial surface. A preliminary proteomic analysis of *B. anthracis* grown in medium containing bicarbonate and CO_2 revealed that although *capD* is on a polycistronic operon with *capB*, *capC*, and *capA* (52), the amount appeared to be significantly lower than those of the other three components (data not shown). This may indicate that *capD* is differentially regulated compared to the other components of the operon.

The strategy of targeting *B. anthracis* bacilli to phagocytes to treat anthrax infection has only recently been explored. The demonstration of protection in animals mediated by anticapsule antibodies (6, 27) supports a role for phagocytes in combating anthrax infection by controlling the encapsulated vegetative bacillus. Macrophages have traditionally been viewed as having a dual role in the disease: as carriers of *B. anthracis* spores to the lymph nodes and thus a vehicle for uptake and dissemination after inhalation (42) and as a factor in contributing to protection (7). The role of macrophages as carriers and sites of germination of spores in cutaneous infection is less well established. Polymorphonuclear neutrophils are a primary defense mechanism to control many bacterial infections, but depletion experiments have suggested that they play a limited role in natural resistance to encapsulated *B. anthracis* infection in the susceptible mouse model (8). This is consistent with the idea that once the organism becomes encapsulated, the role of neutrophils in natural resistance is diminished. In the present report, we confirmed the resistance of encapsulated bacilli to phagocytosis and killing. Most importantly, we observed that treatment with CapD promoted neutrophil bactericidal activity that resulted in a $\geq$99% decrease (2 to 3 logs) in vegetative cell viability, suggesting that efficient capsule removal may potentially lead to severe attenuation of *B. anthracis* and subsequent clearance from the host. This degree of killing is similar to what we observed with unencapsulated bacilli and with anticapsule antibody. Further experiments with infected animals will be needed to determine whether enzyme treatment will be as effective as anticapsule antibodies.

A recent study by Mayer-Scholl et al. reported that human neutrophils kill encapsulated as well as unencapsulated *B. anthracis* (32). At a neutrophil-to-bacterium ratio of 0.1:1, there was ~45% and ~70% killing of the encapsulated wild-type bacilli compared to ~90 and ~95% killing of the unencapsulated strain after 2 h and 3 h, respectively, showing a minimal effect of the capsule. At a ratio of 1:1, there was no difference in killing of the encapsulated versus unencapsulated bacilli, with ~90% of the latter killed after only 30 min. In contrast, we observed that encapsulated bacilli were resistant to killing (<30% killing in five of seven experiments), compared to $\geq$99% killing after the removal of capsule by CapD treatment, confirming the antiphagocytic activity of the capsule reported previously by others (23, 30, 49). The ~2- to 3-log killing that we observed is similar to what occurs with neutrophils incubated in suspension with other bacteria such as *Staphylococcus* (28; data not shown) and occurred at neutrophil-to-bacterium ratios of both 50:1 and 1:1. The degree of killing that we observed after capsule removal and for unencapsulated bacilli was at least 1 log greater than what Mayer-Scholl et al. previously observed for unencapsulated bacilli ($\geq$99% versus ~90 to 95%). However, there are several notable methodological differences in the neutrophil killing assays that likely explain the discrepancies. While we used neutrophils in suspension at neutrophil-to-bacterium ratios of 50:1 and 1:1, those authors used plastic adherent cells that were preactivated before exposure to bacilli centrifuged onto the neutrophils at neutrophil-to-bacterium ratios of 1:1 and 0.1:1. Furthermore, the killing that they observed was predominantly extracellular, as it was only modestly inhibited by cytochalasin D, and electron microscopy showed most bacilli to be extracellular. This is in contrast to our results, where killing was dramatically inhibited by cytochalasin D (Table 1), showing that the killing that we observed depends on phagocytosis, consistent with prior observations on neutrophil bacterial killing in suspension (29).

The development of methods to promote phagocytosis and killing of encapsulated anthrax bacilli may offer an alternative approach for the treatment of anthrax. In this regard, it has been suggested that the inhibition of CapD may result in a loss of capsule from the surface and attenuation of the organism (4). The present results suggest that enzymes such as CapD, by degrading the capsule and making the bacillus susceptible to phagocytic killing, may offer a new approach to therapy that would be both effective against naturally occurring organisms and of particular value against possible antibiotic- and vaccine-resistant strains.

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