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# Protective Role of *Bacillus anthracis* Exosporium in Macrophage-Mediated Killing by Nitric Oxide▽

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**The ability of the endospore-forming, gram-positive bacterium *Bacillus anthracis* to survive in activated macrophages is key to its germination and survival. In a previous publication, we discovered that exposure of primary murine macrophages to *B. anthracis* endospores upregulated NOS 2 concomitant with an ·NO-dependent bactericidal response. Since NOS 2 also generates $O_2^{\cdot -}$, experiments were designed to determine whether NOS 2 formed peroxynitrite ($ONOO^-$) from the reaction of ·NO with $O_2^{\cdot -}$ and if so, was $ONOO^-$ microbicidal toward *B. anthracis*. Our findings suggest that $ONOO^-$ was formed upon macrophage infection by *B. anthracis* endospores; however, $ONOO^-$ does not appear to exhibit microbicidal activity toward this bacterium. In contrast, the exosporium of *B. anthracis*, which exhibits arginase activity, protected *B. anthracis* from macrophage-mediated killing by decreasing ·NO levels in the macrophage. Thus, the ability of *B. anthracis* to subvert ·NO production has important implications in the control of *B. anthracis*-induced infection.**

Endospores of the gram-positive bacterium *Bacillus anthracis*, the causative agent of anthrax, when inhaled and deposited in the lungs, are phagocytosed by resident macrophages. Thereafter, these endospores circumvent macrophage-mediated host defenses and germinate, which is a prerequisite to replication and dissemination (2), into vegetative microbes that, following the exit from the phagocyte, spread throughout the bloodstream (7, 14, 15). Unfortunately, pathways by which endospores survive to germinate to bacilli and escape from the macrophage are poorly understood. We explore here one of the mechanisms by which *B. anthracis* circumvents microbial killing by macrophages.

Macrophages play an important role in host immune defense against bacterial infection in which these phagocytes use oxidants (14, 38), cationic proteins (26), and defensins (9, 22) as part of their microbicidal activity. Upon phagocytosing a microbe, including *B. anthracis*, expression of the immunological isozyme of nitric oxide synthase (NOS 2), the enzyme that metabolizes L-arginine to L-citrulline and nitric oxide (·NO), is upregulated (29). Nitric oxide synthase also reduces $O_2$ to $O_2^{\cdot -}$ and $H_2O_2$ (27, 34). Nitric oxide and $O_2^{\cdot -}$ react at $19 \times 10^9$ $M^{-1}$ $s^{-1}$ to generate peroxynitrite ($ONOO^-$) (19).

We have reported that, despite the presence of several superoxide dismutases (SODs) in the exosporium of *B. anthracis*, low fluxes of $O_2^{\cdot -}$ ($\leq 1$ μM/min) promote the germination of the endospore into the vegetative bacillus (1). However, at high fluxes of $O_2^{\cdot -}$, one of the exosporium-containing SODs appears to afford protection from oxidative damage (25). Most recently, our laboratory has shown that NOS 2 generated ·NO and perhaps $ONOO^-$, if it were formed, play a significant role in the killing of *B. anthracis* (29). Unanswered in that study (29) was which of the NOS 2-derived reactive oxygen species, ·NO or $ONOO^-$, was responsible for the observed bactericidal response. To elucidate the role of ·NO and $ONOO^-$ in the killing of *B. anthracis*, experiments were designed to determine which of these compounds was primarily responsible for the observed microbicidal activity. We demonstrate here that macrophage-generated ·NO, but not $ONOO^-$, is a critical intermediate in the killing of *B. anthracis*. Further, in addition to SODs, the exosporium also contains an arginase, which allows this organism, by attenuating ·NO generation, to subvert the consequences of host production of this free radical. Therefore, the ability of this bacterium to limit ·NO generation may have important implications for the virulence of *B. anthracis*-mediated infection.

## MATERIALS AND METHODS

**Reagents.** RPMI 1640 was purchased from Gibco-BRL (Frederick, MD). Fetal bovine serum was obtained from Atlanta Biologicals (Lawrenceville, GA). The murine macrophage cell line, RAW264.7, was obtained from the American Type Culture Collection (catalog no. TIB-71; Manassas, VA). Gentamicin and phosphate buffered saline (PBS) were purchased from Biosource International (Rockville, MD). Thioglycolate Medium Brewer Modified was from Becton Dickinson (Cockeysville, MD). Sulfanilamide, *N*-1-(naphthyl)ethylenediamine dihydrochloride, polyethylene glycol SOD (PEG-SOD), SOD, bovine arginase, urea, oxyhemoglobin ($HbO_2$), dihydrorhodamine 123 (DHR), and L-$N^6$-(1-iminoethyl)lysine (L-NIL) were purchased from Sigma Co. (St. Louis, MO). The

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spin trap 5-*tert*-butoxycarbonyl-5-methyl-1-pyrroline *N*-oxide (BMPO) was synthesized as described in the literature (33). Linda Roman of the Department of Biochemistry at The University of Texas Health Science Center, San Antonio, TX, provided purified NOS 2.

***B. anthracis* strain.** The Sterne 34F2 strain of *B. anthracis* (35) (Colorado Serum Co., Denver, CO), an attenuated variant ($pXO1^+$ $pXO2^-$), which is used extensively as an animal vaccine, was used in the present study. For studies in which the uptake of spores was examined independent from its killing, we used the congenic germination-deficient Δ*gerH* mutant of Sterne 34F2, which is not killed by macrophages (18). Both organisms were stored in 10% glycerol L broth at −20°C. Difco L agar and Difco L broth were both obtained from Becton Dickinson and Company (Franklin Lakes, NJ) and prepared as described in the instructions to users. Isolation agar consisted of the following: Oxoid nutrient broth No. 2 (6 g; Oxoid, Ltd., New York, NY), Oxoid Agar No. 3 (12 g), $MnSO_4$ (300 mg/liter; JT Baker), $NaH_2PO_4$ (0.25 g; Omnipur EM Science), and 1 liter of sterile distilled water. The pH was adjusted to 6.7, and the agar was sterilized by autoclaving at 121°C for 15 min. Brain heart infusion broth (Difco) was used as a general growth media.

**Endospore production.** A single colony harvested from an overnight culture grown on L agar at 37°C was used to inoculate 100 ml of L broth in a 250-ml conical flask. The culture was incubated at 37°C on an orbital shaker (200 rpm) for 6 h. At the end of this period, 3 ml of culture was transferred to a 225-$cm^2$ vented tissue flask (Corning, Inc.) containing 175 ml of isolation agar. After inoculation, flasks were incubated at 37°C until 99 to 100% of the bacilli had formed endospores (as determined from microscopic examination and phase-contrast analyses). The percent spore yield was determined by comparing colony counts of heated (70°C for 20 min) and unheated samples. Endospores were harvested by adding 20 ml of sterile PBS to the flask. The resuspended endospores from 20 flasks were pooled and centrifuged at 4,200 rpm for 10 min at 4°C. The resulting pellet was resuspended in 200 ml of sterile PBS and centrifuged again. This procedure was repeated ten times, with endospores being resuspended in a final volume of 50 ml, which was refrigerated at 4°C. The final endospore stock concentration was determined to be $10^9$ spores/ml. Endospores used for experimentation were heat shocked at 56°C for 30 min to kill any remaining bacilli.

**Sonication of endospores.** The exosporium was removed by disruption of the endospores with sonication. Endospores were centrifuged at 4,200 rpm for 10 min at 4°C. Pellets were resuspended to approximately $3 \times 10^7$ spores/ml in Tris buffer (10 mM) containing EDTA (0.5 mM) and adjusted to pH 7.5. All subsequent manipulations were at 4°C. Endospores were sonicated (Branson Sonifier 150; Branson Ultrasonics Co., Danbury, CT) with maximum power (amplitude, 12 μm; 10 min, 50 W) for 10 1-min bursts, each separated by 2 min of cooling on ice. Exosporium fragments were separated from endospores by centrifugation at 4,000 rpm for 15 min at 4°C. The endospore pellets were washed once in PBS, and the exosporium-containing supernatants were pooled and then centrifuged again to remove the remaining endospores. Any residual endospores in the exosporium-containing supernatant were removed by filtration through 0.45 and/or 0.2-μm-pore-size low-protein-binding filters (Acrodisc Syringe Filter; Pall Co., Timonium, MD). This method does not damage the endospore, and it effectively removes the exosporium from the spores as previously reported (18).

**Primary peritoneal murine macrophage preparation and PEG-SOD studies.** Murine peritoneal macrophages were cultured according to the method of Fortier et al. (8), with some minor modifications. Primary peritoneal macrophages were obtained from Crl:CD-1 (ICR) BR mice (Jackson Laboratories, Bar Harbor, ME) 4 days after intraperitoneal inoculation of thioglycolate (3 ml of 3%). Peritoneal fluid was drawn through the abdominal wall with a 23-gauge needle. Fluid from mice was pooled and washed, and total cell counts were determined by using a hemacytometer, and the remaining fluid was centrifuged at $250 \times g$ for 10 min at 4°C. Washed cell suspensions were adjusted to $10^6$ macrophages/ml in RPMI 1640, containing fetal bovine serum (10%), sodium pyruvate (1 mM), L-glutamine (2 mM), penicillin (50 U/ml), and streptomycin (50 μg/ml) and then incubated in polypropylene tubes (Elkay Products, Inc., Shrewsbury, MA) in 5% $CO_2$ at 37°C overnight before exposure to experimental conditions.

In several experiments, the primary peritoneal macrophages were pretreated with PEG-SOD (25 to 200 U/ml) for 3 to 24 h and then washed three times in PBS buffer (pH 7.4). Afterward, these cells were infected with Sterne 34F2 sonicated endospores ($10^5$ spores/ml), MOI of 1:10, for up to 24 h at 37°C in a 5% $CO_2$, 95% air-humidified incubator. After infection, extracellular cell culture media were removed at defined times and subjected to the Griess reaction assay for measurement of the nitrite accumulation. Control experiments were performed without preincubation with PEG-SOD.

**Nitrite measurement by the Griess reaction assay.** Accumulation of nitrite was determined colorimetrically after mixing 0.5 ml each of culture media and freshly prepared Griess reagent [0.1% *N*-(1-naphthyl)ethylenediamine in water and 1% sulfanilamide in 5% phosphoric acid, mixed 1:1] (6, 13). Concentrations of nitrite were estimated by using a UV-Vis spectrophotometer (Uvikon, model 940; Research Instruments, San Diego, CA) by comparing absorbance readings at 550 nm against standard solutions of sodium nitrite prepared in the same media.

**Nitric oxide measurement using the $HbO_2$ assay.** The initial rate of $^{\cdot}NO$ production by purified NOS 2 was estimated by using the $HbO_2$ assay (23). The reaction, run at 23°C, was initiated by the addition of NOS 2 (1.7 μg) to a cuvette containing 50 mM potassium phosphate buffer (containing 0.5 mM EGTA adjusted to pH 7.4), $HbO_2$ (20 μM), NADPH (150 μM), and L-arginine (100 μM). The final volume of the reaction was 0.5 ml. In several experiments, the NOS inhibitor L-NIL (100 μM) was added to the reaction mixture described above. A UV-Vis spectrophotometer (Uvikon, model 940) was used to monitor the conversion of oxyhemoglobin to methemoglobin during the course of the reaction. Specifically, the increase in absorbance at 401 nm was used to quantify the reaction, using an extinction coefficient of 60 $mM^{-1}$ $cm^{-1}$.

**Spin trapping $O_2^{\cdot -}$.** Spin trapping of $O_2^{\cdot -}$ from purified NOS 2 was conducted in the following manner. NADPH (150 μM) was added to potassium phosphate buffer (50 mM Chelexed containing 1 mM EGTA and 1 mM DTPA adjusted to pH 7.4) that included NOS 2 (6.5 μg) and BMPO (50 mM) in the absence or presence of L-NIL (100 μM) to a final volume of 0.3 ml. The reaction of BMPO with $O_2^{\cdot -}$ forms the spin-trapped adduct BMPO-OOH, which can be detected by electron paramagnetic resonance (EPR) spectroscopy (33). The reaction mixture was then transferred to a flat quartz cell and placed into the cavity of an EPR spectrometer (model E-109; Varian Associates, Inc., Palo Alto, CA). The EPR quartz cell was open to the air, allowing $O_2$ to enter. EPR spectra were recorded at room temperature. Instrument settings were as follows: microwave power, 20 mW; modulation frequency, 100 kHz; modulation amplitude, 0.5 G; sweep time, 12.5 G/min; and response time, 0.5 s.

**Peroxynitrite measurement by the DHR assay.** The initial rate of $ONOO^-$ production by purified NOS 2 was estimated by using the DHR assay (20). The reaction was initiated by the addition of NADPH (150 μM) to a cuvette containing 50 mM potassium phosphate buffer (containing 0.5 mM EGTA adjusted to pH 7.4), NOS 2 (3.3 μg), DHR (20 μM), and L-arginine (100 μM) to a final volume of 0.5 ml at 23°C. In several experiments, the NOS inhibitor L-NIL (100 μM) or SOD (60 U/ml) was added. A UV-Vis spectrophotometer (Uvikon, model 940) was used to monitor the conversion of DHR to rhodamine during the course of the reaction. Specifically, the increase in absorbance at 500 nm was used to quantify the reaction, using an extinction coefficient of 74.5 $mM^{-1}$ $cm^{-1}$.

**NOS 2-mediated macrophage killing assay.** Primary peritoneal macrophages pretreated with or without L-NIL (1 mM), PEG-SOD (100 U/ml) or exosporium (0 to 75 μg of protein/ml) were infected with Sterne 34F2 sonicated endospores at an MOI of 1:10 and incubated at 37°C in 5% $CO_2$ for 30 min to allow phagocytosis. Exosporium concentration was measured by the BCA protein assay (31). As previously reported (29), an MOI of 1:10 was found to be optimal; therefore, experiments were carried out at this MOI with endospores. Infected macrophages were washed once with fresh medium and then incubated for 30 min in media containing gentamicin (50 μg/ml), and incubation was continued for an additional 30 min to remove the extracellular bacilli. After removal of the medium, cells were washed once with fresh medium and resuspended to the original volume with fresh medium without antibiotic. Samples were obtained at 1, 5, and 24 h after initial infection and diluted in PBS for 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. Log kill was calculated utilizing the following equation: (log CFU at 1 h) − (log CFU at 3, 5, or 24 h). Culture supernatants were collected for $^{\cdot}NO$ measurement and stored at −70°C until analysis.

In order to evaluate the uptake of endospores by macrophages independently of its killing, we added a nongerminating mutant of Sterne 34F2, the Δ*gerH* mutant, to macrophages under conditions similar to those for the Sterne 34F2 spore. Since macrophages are unable to kill these spores (18), the number of colonies determined at the 1-h time point relative to the number of spores added should measure spore uptake alone.

**Measurement of arginase activity.** Arginase is a manganese metalloenzyme responsible for the hydrolysis of L-arginine to L-ornithine and urea. Arginase activity was estimated by monitoring the metabolism of L-arginine to urea using a modification of a previously developed method (5). Before enzymatic activity was determined, glycine (0.1 M) and $MnCl_2$ (10 mM) were added to the exosporium, ranging in concentration from 0 to 75 μg of protein/ml of PBS at pH 7.4. After preactivation, initiation of enzymatic activity proceeded by the addition of L-arginine (25 mM, final concentration) at 37°C. The reaction was run for 3 h and terminated with the addition of a 10% perchloric acid solution. A urea colorimetric assay was then performed with samples brought to a 2.0-ml

volume and incubated with 1 ml of concentrated phosphoric acid and sulfuric acid at a ratio of 3:1 and 2-isonitrosopropiophenone (4%) in a boiling water bath for 1 h. Absorbance readings were measured at 540 nm against standard solutions of urea. Arginase activity in the exosporium was estimated by utilizing a concentration curve for purified bovine arginase with activity of 19.3 U/mg, where 1 U of enzyme catalyzes the hydrolysis of 1.0 μmol of L-arginine/min at 37°C (pH 9.5).

**Statistics.** A Student *t* test was used to analyze the data for statistical significance, and results were considered significant at $P$ values of $<0.05$.

## RESULTS

**Purified NOS 2 generated ·NO, $O_2^{\cdot-}$, and $ONOO^-$.** It has been reported that during purified NOS 2 oxidation of L-arginine to L-citrulline and ·NO, $O_2^{\cdot-}$ is also generated, most of which is derived from the reductase domain of the enzyme (34, 36). Since in a chemical, nonenzymatic system these free radicals react at a diffusion-controlled rate to form $ONOO^-$ (19), we designed experiments to determine whether purified NOS 2 produced $ONOO^-$ when ·NO and $O_2^{\cdot-}$ were generated and, if so, whether the addition of L-NIL, a NOS 2 inhibitor, or SOD would diminish the concentration of $ONOO^-$ by inhibiting NOS 2-produced ·NO or by scavenging $O_2^{\cdot-}$ generated by this enzyme.

In our initial series of experiments, we incubated a competent purified NOS 2 with L-arginine (100 μM) in the absence or presence of L-NIL (100 μM), measuring the initial rate of ·NO production using the oxyhemoglobin assay. In the absence of L-NIL, the initial rate of ·NO production was found to be 907 ± 54 nmol/min/mg of protein. When L-NIL was added to the reaction mixture, the initial rate of ·NO formation decreased to 110 ± 37 nmol/min/mg of protein. Furthermore, with the addition of SOD (60 U/ml) to the competent NOS 2 incubated with L-arginine, the initial rate of ·NO increased to 1,273 ± 51 nmol/min/mg of protein.

In the second series of experiments, spin trapping/EPR spectroscopy was used to estimate $O_2^{\cdot-}$ production from NOS 2 (27). Superoxide can be spin trapped by BMPO, affording BMPO-OOH with its unique EPR spectrum (33). When activated NOS 2 was incubated with L-arginine in the presence of BMPO, an EPR spectrum was recorded corresponding to BMPO-OOH, indicating that $O_2^{\cdot-}$ was generated by a competent NOS 2 and spin trapped accordingly (Fig. 1). In the absence of either NADPH or NOS 2, or inclusion of SOD (60 U/ml), no EPR spectrum was obtained (data not shown). In contrast to NOS 2 generation of ·NO, $O_2^{\cdot-}$ production was not inhibited by the presence of L-NIL (100 μM) but, in fact, the flux of $O_2^{\cdot-}$ was slightly increased (Fig. 1), verifying earlier studies (34, 36).

In a third series of experiments, the initial rate of $ONOO^-$ formation from a competent NOS 2 in the presence of L-arginine (100 μM) was determined to be 29 ± 3.7 nmol/min/mg of protein using the DHR assay; however, with the addition of either SOD (60 U/ml) or L-NIL (100 μM), the initial rate markedly declined to 10.5 ± 2.5 nmol/min/mg of protein and 9.9 ± 2.4 nmol/min/mg of protein, respectively. Taken together, data from all three sets of experiments prove that during purified NOS 2 metabolism of L-arginine, ·NO and $O_2^{\cdot-}$ are produced, some of which reacted to form $ONOO^-$ (21).

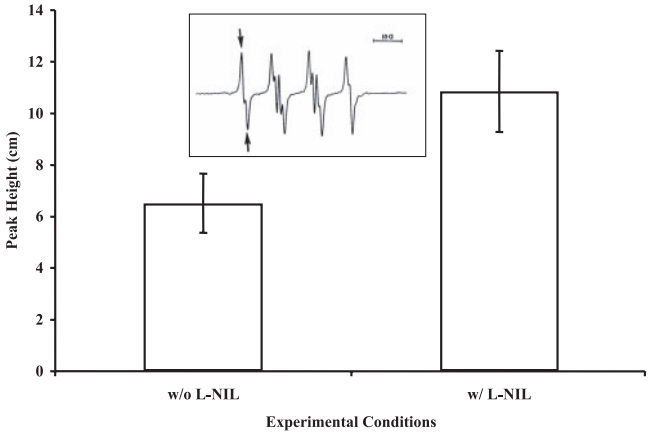


FIG. 1. Representative plot depicting spin trapped $O_2^{\cdot-}$ generated by purified NOS 2. The reaction system consisted of NOS 2 (6.5 μg), L-arginine (100 μM), BMPO (50 mM), and NADPH (150 μM) in potassium phosphate buffer (Chelexed [50 mM] containing EGTA [1 mM] and DTPA [1 mM] adjusted to pH 7.4). Each bar on the graph is the peak height of the first low-field peak of EPR spectrum of BMPO-OOH from the reaction of BMPO with NOS 2-generated $O_2^{\cdot-}$. The data are representative of three independent experiments, expressed as the means and standard deviations. The inset shows a typical EPR spectrum for the reaction of BMPO with $O_2^{\cdot-}$ generated by NOS 2 in the presence of L-arginine. EPR spectra were recorded continuously, and data presented in this figure were obtained 10 min after the addition of NADPH. Receiver gain was $10 \times 10^4$.

**Infection of PEG-SOD-pretreated primary murine peritoneal macrophage with sonicated *B. anthracis* endospores.** We have previously reported that RAW264.7 macrophages and primary murine peritoneal macrophages, when encountering native (exosporium containing) or sonicated (exosporium lacking) endospores of *B. anthracis*, upregulate NOS 2, which in the presence of L-arginine generates ·NO (29). In that study (29), native and sonicated endospores were found to induce the same level of NOS 2 protein in RAW264.7 macrophages. However, subsequent production of ·NO was significantly greater when these macrophages were infected with sonicated endospores compared to native endospores. This observation suggested that the native endospore might limit ·NO production either by masking structures beneath the endospore cortex proteins that induce optimal levels of ·NO and/or that the native endospore contains an enzyme that may limit ·NO generation. Therefore, we used sonicated endospores here to explore the importance of the exosporium in *B. anthracis* infection.

Given that purified NOS 2 produces $ONOO^-$, we were interested in determining what role, if any $ONOO^-$ might play in primary murine peritoneal macrophage-mediated microbicidal activity. Before arriving at that point, we used a nitrotyrosine enzyme-linked immunosorbent assay (37) in an attempt to measure $ONOO^-$ in lipopolysaccharide-treated and endospore-infected RAW264.7 macrophages, which we have shown induces NOS 2 (28). Using the method described in the literature (37), we were unable to detect $ONOO^-$ in either experimental paradigm, unlike in the case of purified NOS 2.

An alternative method was then sought to indirectly measure $ONOO^-$. Since SOD inhibits $ONOO^-$ formation by purified NOS 2, we decided to increase SOD levels in primary

murine peritoneal macrophages, thereby decreasing the $ONOO^-$ concentration were this reactive compound to be produced. We have previously demonstrated that cellular levels of SOD may be increased by treatment with PEG-SOD (3, 28). Thus, we estimated the change in nitrite concentration in the media described above for primary murine peritoneal macrophages ($10^6$ cells/ml) that were pretreated with PEG-SOD and then infected with endospores as described in Materials and Methods. The nitrite concentration was estimated at defined times by using the Griess assay. The Griess assay estimates ·NO production by measuring one of its oxidation products, nitrite, in the growth media and has a sensitivity limit of ~1.0 μM. We found that levels of nitrite were detectable from macrophages infected with endospores in as little as 1 h and that nitrite concentrations increased for the next 24 h. For instance, preincubating these cells with PEG-SOD (100 U/ml for 24 h) followed by infection with native endospores resulted in an increase in nitrite formation in the extracellular milieu from 4.6 ± 0.1 μM for controls to 6.1 ± 0.1 μM for PEG-SOD-treated macrophages. Since ·NO and $O_2^{\cdot -}$ were the source of $ONOO^-$, increasing SOD levels in primary murine peritoneal macrophages with a concomitant increase in ·NO suggested that these phagocytes formed $ONOO^-$ after infection with endospores of *B. anthracis*. In a separate experiment, preincubating primary murine peritoneal macrophages with the specific NOS 2 inhibitor L-NIL (1 mM for 24 h) followed by infection with native endospores resulted in minimally detectable nitrite (0.35 ± 0.02 μM) in the extracellular milieu compared to the control (4.6 ± 0.1 μM).

**Effect of PEG-SOD on primary murine peritoneal macrophage-mediated killing of *B. anthracis*.** We earlier demonstrated that while primary murine peritoneal macrophages are unable to kill the endospore of *B. anthracis*, these phagocytic cells do kill the vegetative bacilli emerging from the endospore (18) and that this microbicidal activity was dependent upon NOS 2 generated ·NO and perhaps $ONOO^-$ but not $O_2^{\cdot -}$ (28). Experiments were designed to determine whether $ONOO^-$ or ·NO mediated the observed microbicidal activity. We used a bacterial killing assay as an end point.

When primary murine peritoneal macrophages were infected with sonicated endospores (MOI of 1:10), as described in Materials and Methods, increased killing of *B. anthracis* was observed over time, which was markedly inhibited by the addition of the specific NOS 2 inhibitor L-NIL (1 mM) (Fig. 2). In contrast, when similar experiments were conducted with PEG-SOD (100 U/ml)-pretreated primary murine peritoneal macrophages, there was no significant change in microbicidal activity (Fig. 2). Given an increase in nitrite production in the PEG-SOD-treated macrophages and the data presented in Fig. 2, it is unlikely that $ONOO^-$ was responsible for the observed primary murine peritoneal macrophage-mediated killing of *B. anthracis*.

After the addition of $6.7 \times 10^5$ Δ*gerH* spores to the macrophages, we incubated the culture for 1 h in the presence of gentamicin to kill any bacilli that may be present and then washed the cells and determined the cell-associated viable spore colonies. There was a 20.9% uptake of exosporium-positive Δ*gerH* spores. Of interest, when the exosporium was removed from the Δ*gerH* spores, 49.3% of the added spores remained associated with the macrophages. This suggests that the exosporium interferes with the uptake of *B. anthracis* spores by macrophages. There was no difference in uptake of spores by macrophages treated with either L-NIL or PEG-SOD compared to untreated macrophages.

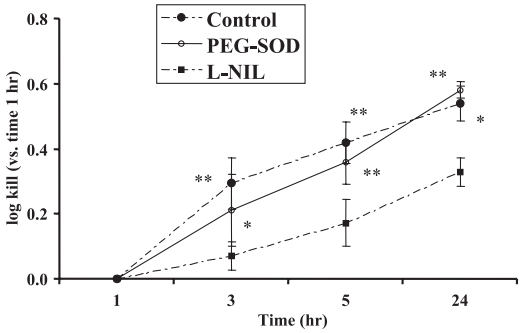


FIG. 2. Killing of *B. anthracis* in spore-infected macrophages. Primary murine peritoneal macrophages ($10^6$ cells/ml) were infected with spores ($10^5$ spores/ml) prepared from sonicated exosporium ($exo^-$) *B. anthracis* strain Sterne 34F2 and treated either with L-NIL (1 mM) (■) or PEG-SOD (100 U/ml) (○) and incubated at 37°C in 5% $CO_2$ for 30 min to allow phagocytosis. Samples of infected macrophages were obtained at 1, 3, 5, and 24 h after initial infection; washed; and lysed for viable count plating, and the CFU were determined. The data are expressed as log values. Log kill was determined as defined in Materials and Methods. The data are expressed by their differences in log values. *, $P < 0.05$; **, $P < 0.01$ (versus the L-NIL treatment group). The data are shown as means ± the standard deviation of values obtained from two independent experiments, each conducted in duplicate.

**Arginase activity in the *B. anthracis* exosporium.** In 1983, Soru (32) reported that *B. anthracis* contains the enzyme arginase, which metabolizes L-arginine to L-ornithine and urea. More recently, we showed that there was a concentration dependency in endospores and vegetative bacilli, with the latter having higher arginase activity (29). The results of that study (29) suggested a possible functional role of *B. anthracis* exosporium in ·NO-mediated killing and that arginase in the *B. anthracis* exosporium may compete with NOS 2 for L-arginine. Given the importance of ·NO in primary murine peritoneal macrophage killing of *B. anthracis*, we designed experiments to determine what role, if any, arginase located in the exosporium plays in the control of *B. anthracis* infection.

Arginase activity in the exosporium, which was removed by sonication of the Sterne strain, was estimated as detailed in Materials and Methods. The exosporium has been reported to contain at least 20 different enzymes, one of which is arginase (30). Even with this crude exosporium preparation, we observed significant arginase activity (Fig. 3). In the absence of cofactors or L-arginine, however, there was no formation of urea, independent of the concentration of the exosporium added to the reaction mixture. Moreover, not surprisingly, by increasing the concentration of the exosporium, an increase in enzyme activity was noted (Fig. 3). We next sought to determine what impact, if any, an incremental increase in crude exosporium, which contains a functional arginase, would have on the killing of *B. anthracis* by primary murine peritoneal macrophages.

**Role of the exosporium in primary murine peritoneal macrophage-mediated killing of *B. anthracis*.** As described previ-

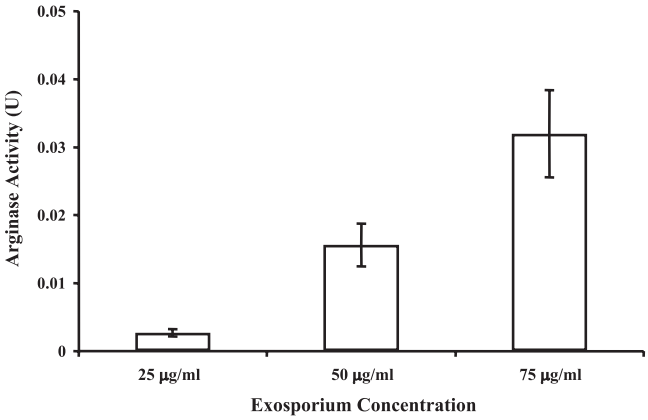


FIG. 3. Arginase activity from Sterne exosporium samples of various concentrations. Arginase activity assay was used to determine enzyme activity from various concentrations of exosporium isolated from Sterne 34F2 strain *B. anthracis* (25 to 75 μg/ml). Initiation of enzyme activity began with the addition of L-arginine (25 mM), and the reaction was allowed to proceed for 3 h before being terminated with perchloric acid. Each bar on the graph is the average of three independent experiments, expressed as means and standard deviations.

ously (18), primary murine peritoneal macrophages kill the vegetative bacterium emerging from the endospore, and this killing is dependent on NOS 2-generated ·NO (29). To further understand the role of the exosporium-containing arginase, the ability of primary murine peritoneal macrophages to kill sonicated Sterne 34F2 at an MOI of 1:10 was estimated by increasing the concentration of crude exosporium from 0 to 75 μg of protein/ml (Fig. 4). With sonicated endospores, 0.33- and 0.70-log bacterial kills were observed at 5 and 24 h, respectively (Fig. 4). To determine the physiologic relevance of the observations shown in Fig. 4, primary murine peritoneal macrophages were infected with native, nonsonicated Sterne 34F2 endospores at an MOI of 1:10. Under these experimental conditions, microbicidal activity decreased to 0.15- and 0.42-log bacterial kills at 5 and 24 h, respectively, a result comparable to that of sonicated endospores to which 25 μg/ml of crude exosporium was added (Fig. 4). As the concentration of the exosporium increased, the bactericidal property of the macrophage decreased. Of particular importance was the finding that at 75 μg of protein/ml of exosporium, the microbicidal activity was essentially abolished (Fig. 4).

In a parallel series of studies, we measured nitrite in the extracellular milieu at 24 h after the experiment was initiated. We found that nitrite concentration decreased with increasing concentration of added exosporium (0 to 75 μg of protein/ml) (Fig. 5.) For instance, the addition of 75 μg of protein/ml of exosporium to sonicated endospores resulted in 50% decrease in nitrite concentration in the extracellular milieu from 8.5 ± 0.2 μM for sonicated endospores with no added exosporium to 4.3 ± 0.3 μM in the presence of 75 μg of protein/ml of exosporium. Apparently, at this concentration of crude exosporium, the flux of ·NO generated by NOS 2 was insufficient to support bacterial killing (34). Finally, the concentration of nitrite in the extracellular milieu after macrophages were infected with nonsonicated endospores was comparable to that when 25 μg of protein/ml of exosporium was added to macrophages infected with sonicated endospores (Fig. 5). These findings parallel the log kill data shown in Fig. 4.

These results presented here suggest that ·NO is crucial to NOS 2-mediated killing of Sterne 34F2 in primary murine

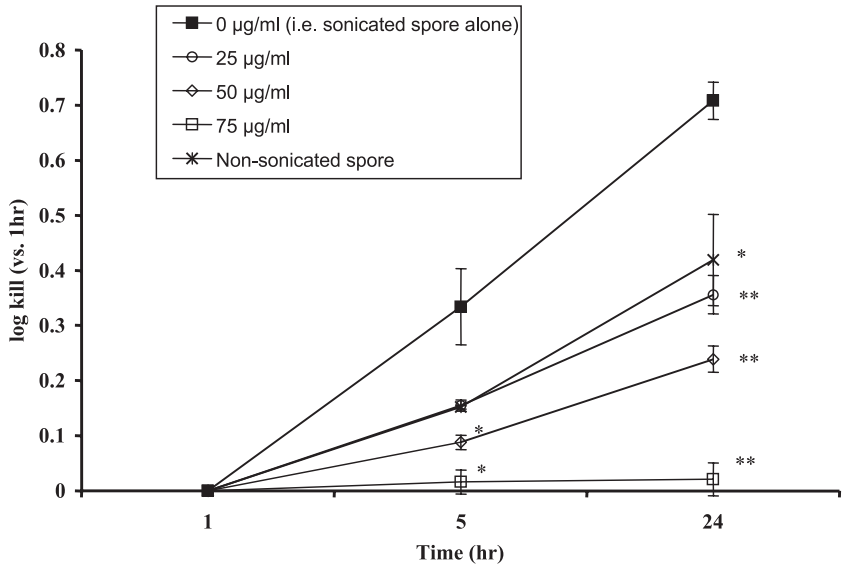


FIG. 4. Exosporium-dependent killing of *B. anthracis* in spore-infected macrophages. Primary murine peritoneal macrophages ($10^6$ cells/ml) were infected with either native ($exo^+$) or sonicated endospores ($exo^-$) ($10^5$ spores/ml) or isolated exosporium at indicated concentrations (25, 50, and 75 μg/ml) to sonicated endospores and incubated at 37°C in 5% $CO_2$ for 30 min to allow phagocytosis. Samples of infected macrophages were obtained at 1, 5, and 24 h after initial infection; washed; and lysed for viable count plating, and CFU were determined. The data are expressed as log values. Log kill was determined as defined in Materials and Methods. The data are expressed by their differences in log values. *, $P < 0.05$; **, $P < 0.01$ (versus exo 0 μg/ml, i.e., sonicated). The data are shown as means ± the standard deviation of values obtained from two independent experiments, each conducted in duplicate.

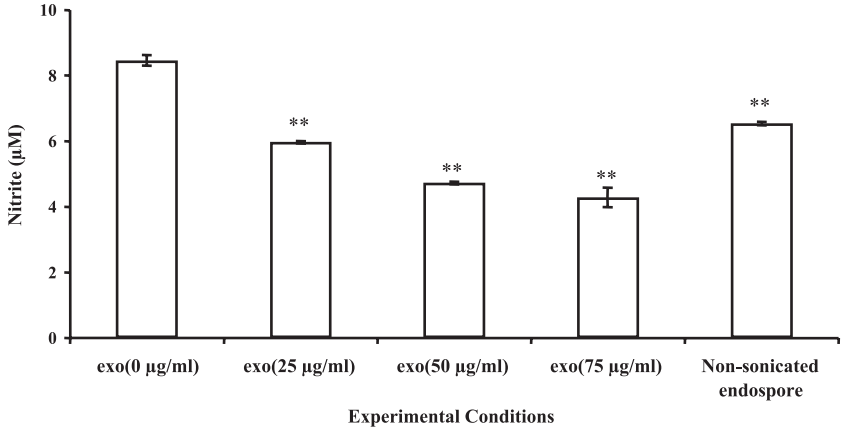


FIG. 5. Differential nitrite production by primary murine peritoneal macrophages infected with *B. anthracis* in the presence of exosporium. Primary murine peritoneal macrophages ($10^6$ cells/ml) were infected with either native ($exo^+$) or sonicated endospores ($exo^-$ or exo 0 μg/ml) ($10^5$ spores/ml) or isolated exosporium at the indicated concentrations (25, 50, and 75 μg/ml) to sonicated endospores and incubated at 37°C in 5% $CO_2$ for 30 min to allow phagocytosis. The Griess assay was used to determine the concentration of nitrite, the oxidation product of ·NO, in the extracellular milieu at 24 h after primary murine peritoneal macrophages were infected with sonicated endospores. **, $P < 0.01$ (versus exo 0 μg/ml). The data are representative of three independent experiments.

peritoneal macrophages and that an active arginase, by limiting the availability of L-arginine to be metabolized by NOS 2 to ·NO, affords a certain degree of protection against ·NO-mediated killing of *B. anthracis*.

## DISCUSSION

Macrophages play an important role in host immune defense against bacterial infection, possessing the $O_2^{\cdot -}$-generating enzyme, NADPH-oxidase, as well as NOS 2, which produces ·NO and $O_2^{\cdot -}$ (24). Upon phagocytosing a microbe, macrophages release $O_2^{\cdot -}$ and ·NO. These free radicals, along with the products derived from them, e.g., $H_2O_2$ and $ONOO^-$, become part of the microbicidal arsenal aimed at killing the microorganism. However, questions still remain as to how these species are involved in microbial killing, specifically with respect to *B. anthracis*.

In the case of endospores of *B. anthracis*, the environment within this phagocyte supports germination of these endospores to vegetative bacilli, some of which escape and eventually enter the bloodstream, where they multiply to high levels, promoting toxicity to the host (17). We have shown that *B. anthracis* endospores, when phagocytosed by macrophages, upregulate the enzyme NOS 2. Earlier data from our laboratory demonstrate that macrophages use NOS 2-derived intermediates as part of their microbicidal activity toward *B. anthracis* (28). Since NOS 2 generates ·NO and $O_2^{\cdot -}$ and these two free radicals react at a diffusion-controlled rate, affording $ONOO^-$ (19), we designed experiments to determine whether ·NO or $ONOO^-$ is responsible for the observed killing of *B. anthracis*, as documented in Fig. 2.

Although we have been able to estimate the rate of purified NOS 2-generated ·NO, $O_2^{\cdot -}$, and $ONOO^-$ by using different experimental methods (20, 27, 34); using isolated macrophages, we were unable to detect $ONOO^-$ by protocols reported in the literature (37). In fact, many of the previously published studies added synthesized $ONOO^-$ to their cell preparation (4, 11, 12, 16). Alternatively, we reasoned that by enhancing SOD levels through pretreating primary murine peritoneal macrophages with PEG-SOD, $O_2^{\cdot -}$ concentrations in these phagocytes would be significantly diminished, thereby enhancing the cellular level of ·NO and decreasing the $ONOO^-$ concentration. In fact, data presented demonstrate that by enhancing cellular levels of SOD, ·NO concentrations in growth media were markedly increased. Despite the nearly 25% increase in ·NO by pretreating primary murine peritoneal macrophages with PEG-SOD, there was no corresponding change in the killing of *B. anthracis*. These findings suggested that ·NO but not $ONOO^-$ is responsible for NOS 2-mediated microbicidal activity. Although ·NO is a critical effector molecule in murine macrophages, its role in human macrophages is less clearly defined, and therefore caution must be used in extrapolating our data to the situation in humans.

The endospore of *B. anthracis* has a number of proteins that are tightly associated with the exosporium, including alanine racemase, inosine hydrolase, GroEL, ExsF, CotY, ExsY, CotB, and a novel protein named ExsK (30). Although the functional role of many of these proteins is currently unclear, the finding that a family of SODs is in the exosporium suggests the importance of preventing oxidative injury to *B. anthracis* by host macrophages (25). The research described here demonstrates the significance of another endospore-containing protein, arginase, since this enzyme effectively subverts host defenses by decreasing the concentration of L-arginine available to be oxidized by NOS 2 to ·NO by the host macrophage.

Our finding that arginase activity within the exosporium may disarm a principal microbicidal mechanism by which murine macrophages kill *B. anthracis* suggests an important role for the exosporium in the pathogenesis of anthrax infection. For example, in a recently published study, Giorno et al. report that *cotE* mutant *B. anthracis* spores that are unable to form an exosporium are fully virulent in a mouse model, indicating that the exosporium is dispensable in the control of infection (10). We previously have proposed that the exosporium is involved

in regulating both germination and intracellular survival (1). In addition to arginase, the exosporium has at least 20 other proteins on its surface, including two types of SODs, alanine racemase and inosine hydrolase, each of which has been shown to affect spore germination (1, 30). *B. anthracis* spores require proximity to macrophages to trigger germination, and we hypothesized that macrophage-generated free radicals inhibit the activity of enzymes such as alanine racemase and inosine hydrolase located on the exosporium that negatively regulate spore germination. Removal of the exosporium would remove these negative regulators of germination and, as a consequence, one would no longer expect the spore to require the presence of macrophages to germinate in vivo. Further, the exosporium masks the ability of antigens on the endospore surface to elicit a robust cytokine response: exosporium-negative spores induce significantly greater tumor necrosis factor alpha and interleukin-1β levels in macrophages than their exosporium-positive counterparts (unpublished data). Finally, exosporium-negative spores become more avidly associated with macrophages in vitro and, once taken up, are more easily killed, as we demonstrate here and in an earlier publication (18). Thus, whether the exosporium has a role in pathogenesis or virulence is highly dependent on the measure of virulence. Clearly, further studies are warranted to evaluate the role of the exosporium in the pathogenesis of anthrax infection.

Finally, our studies show that while NOS 2 is capable of generating $^{\cdot}NO$, $O_2^{\cdot -}$, $H_2O_2$, and $ONOO^-$, it appears that only $^{\cdot}NO$ plays a crucial role in *B. anthracis* killing. We are currently exploring what signaling events in the macrophage promote the observed microbicidal activity toward *B. anthracis*.

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