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Role of superoxide in the germination of *Bacillus anthracis* endospores

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Abstract

The spore forming Gram-positive bacterium *Bacillus anthracis*, the causative agent of anthrax, has achieved notoriety due to its use as a bioterror agent. In the environment, *B. anthracis* exists as a dormant endospore. Germination of endospores during their internalization within the myeloid phagocyte, and the ability of those endospores to survive exposure to antibacterial killing mechanisms such as superoxide ($O_2^{\bullet-}$), is a key initial event in the infective process. We report herein that endospores exposed to fluxes of $O_2^{\bullet-}$ typically found in stimulated phagocytes had no effect on viability. Further endospores of the Sterne strain of *B. anthracis* were found to scavenge $O_2^{\bullet-}$, which may enhance the ability of the bacterium to survive within the hostile environment of the phagolysosome. Most intriguing was the observation that endospore germination was stimulated by a flux of $O_2^{\bullet-}$ as low as 1 μM/min. Data presented herein suggest that *B. anthracis* may co-opt $O_2^{\bullet-}$ which is produced by stimulated myeloid phagocytes and is an essential element of host immunity, as a necessary step in productive infection of the host.

Keywords: Anthrax; Endospores; Superoxide; Germination

1. Introduction

The ability to form an impervious spore is the key survival strategy of members of the bacillus family [1]. Once formed the spore enables the organism to remain dormant until the local environment has improved to a level that will support active growth. The spore also confers considerable resistance to chemical and thermal insult. There is also evidence to suggest that the spore may play a direct role in virulence. For instance, spore coat extract from two serovars of *Bacillus thuringiensis* have been reported to be toxic for the Indian meal moth [2]. *B. thuringiensis* belongs to a group of genetically related organisms which include *B. cereus* and the mammalian pathogen *Bacillus anthracis* [3]. All members of this group form endospores with a structurally similar outer layer called the exosporium. This layer is chemically complex, consisting of protein, amino and neutral polysaccharides and lipids [4]. More recently, it has been shown that glycoproteins, which appear to be specific to the outer surface layers of the

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spore, are synthesized by *B. thuringiensis* [5], *B. cereus* [6] and *B. anthracis* [7].

Preliminary studies have identified proteins in the exosporium that play a role in insect pathogenicity (i.e., immune inhibitor A, a zinc metalloprotease, which degrades components of the insects immune system), regulate germination (i.e., alanine racemase, inosine preferring nucleoside hydrolase) and control intracellular survival of *B. anthracis* (i.e., iron/manganese superoxide dismutase), the key early event in anthrax [8–11].

In their quiescent state, macrophages are metabolically subdued. Yet upon encountering a microorganism, these cells become stimulated, resulting in sequestration of the invading microbe into an enclosed vacuole, the phagosome, into which $O_2^{\bullet-}$ is secreted and proteins and proteases are released following fusion of lysosomes to form the phagolysosome [12,13]. Studies with mouse alveolar macrophages have demonstrated that for anthrax spores germination occurs within the phagolysosome [14].

Thus to survive within this harsh environment the bacterium must possess mechanisms, such as superoxide dismutase (SOD) and catalase, to circumvent the toxic events initiated by $O_2^{\bullet-}$ and hydrogen peroxide (H_2O_2), produced by and the result of the oxidative burst, by scavenging these oxidants.

Microorganisms have developed a number of strategies to deal with oxidants: avoidance, neutralization and prevention of production [15]. For example, the identification of a spore surface located SOD raises the possibility that endospores can control the availability of $O_2^{\bullet-}$, generated by stimulated macrophages, which would otherwise mediate cell killing [16]. Thus, prior to germination the spore represents a target against which antibacterial factors can expend their efforts without having any adverse effect on the dormant organism.

This study addresses two critical questions. First, can the spore protect the organism from physiologically relevant levels of $O_2^{\bullet-}$ enabling germination to proceed? Second, what impact does the oxidant environment, typically found within the phagolysosome have on the rate of spore germination? To address these questions, endospores of the Sterne strain of *B. anthracis* were exposed to a continuous flux of $O_2^{\bullet-}$ at a rate typically found in stimulated phagocytes [12,13]. The degree of $O_2^{\bullet-}$ scavenging, spore survival, and subsequent rate of germination was determined.

2. Materials and methods

2.1. The bacterial strain and media

The Sterne, 34F2 strain of *B. anthracis* (Colorado Serum Company, Denver, CO, USA) an attenuated variant employed extensively as an animal vaccine, was examined during this study. The organism was stored in 10% glycerol L broth at −20 °C. Difco L agar and Difco L broth were obtained from Becton Dickinson and Company (Becton Drive, Franklin Lakes, NJ) and made up as per the manufacturers instructions. Isolation Agar was formulated as follows; 6 g Oxoid Nutrient broth No. 2 (Oxoid Ltd, NY), 12 g Oxoid Agar No. 3, 300 mg/L $MnSO_4$ (JT Baker, NJ) 0.25 g NaH_2PO_4 (Omnipur EM Science) and 1 L 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. To examine the role of specific germinants the following mixtures were used: L-alanine solution contained 100 mM L-alanine (Calbiochem), 100 mM NaCl (Omnipur EM Science) and 10 mM NaH_2PO_4 at pH 7.2 [17]. The inosine mixture contained 5 mM inosine (Calbiochem), 100 mM NaCl and 10 mM NaH_2PO_4. The combined alanine/inosine mixture contained 100 mM L-alanine, 5 mM inosine, 100 mM NaCl and 10 mM NaH_2PO_4 at pH 7.2 [17].

2.2. Spore 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 Duran. 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^3 vented tissue flask (Corning Inc) containing 12 mL isolation agar. Following inoculation flasks were incubated at 30 °C until 99–100% of the organisms had formed endospores (microscopic examination/phase contrast). The % spore yield was determine by comparing colony counts of heated (70 °C for 20 min) and unheated samples. Endospores were harvested by adding 20 mL of sterile phosphate buffered saline (PBS) to the flask. The re-suspended endospores from 20 flasks were pooled and centrifuged at 4200 rpm for 10 min at 4 °C. The resulting pellet was resuspended in 200 mL of sterile PBS and centrifuged again. To determine the effect of repeated washings on spore activity, endospores were washed either as total of 3 or 10 times with the endospores being resuspended in a final volume of 50 mL, which was refrigerated at 4 °C until required. The final endospore concentration was determined to be 1×10^9 endospores/mL.

2.3. Spin trap experiments

Prior to the commencement of each experiment, endospores were heat activated at 56 °C for 30 min. This relatively low temperature was employed through out the study to minimize heat-induced inactivation of surface located enzymes such as SOD and alanine racemase.