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Short communication

Inhaled non-capsulated *Bacillus anthracis* in A/J mice: nasopharynx and alveolar space as dual portals of entry, delayed dissemination, and specific organ targeting

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Abstract

Bacillus anthracis virulence is dependent on toxins and capsule. Encapsulation is associated with dissemination. We hypothesized that eliminating capsule would modify the portal of entry and the spread of bacteria. Using a bioluminescent model of inhalational anthrax, we demonstrated that aerosolized spores of a capsule-deficient strain administered at moderate doses initiated infection in the nasopharynx. Dissemination beyond the nasopharynx was delayed for at least 24 h and then targeted the kidneys. Interestingly, high intranasal doses led to spore germination in the alveoli. We conclude that eliminating capsule while maintaining toxin production alters dissemination, but allows infection initiation in the lungs.

Keywords: Anthrax; Inhalational infection; Mice; Toxin; Lung; Kidney

1. Introduction

Bacillus anthracis is a Gram positive sporulating bacterium that is associated with disease in livestock, but can also infect humans. The primary virulence factors of *B. anthracis* include a tri-partite toxin; lethal and edema toxins that share the same cell-binding subunit named protective antigen; and a poly-gamma-D-glutamic acid capsule [1]. The toxins alter host cell signaling, thereby modulating the immune response of the host, and can cause death [2]. The capsule hinders phagocytosis and is a non-immunogenic surface [3]. It is associated with bacterial dissemination [4] and is vital for virulence [1].

In previous studies, we have demonstrated that in vivo bioluminescent imaging (BLI) is a powerful tool to visualize *B. anthracis* infection kinetics in real time and track dissemination to unpredicted tissues [5–7]. During a cutaneous infection, encapsulated non-toxinogenic (Cap+) strains disseminate rapidly to the spleen and lung [5]; in contrast, toxinogenic non-encapsulated (Tox+) strains disseminate more slowly to the kidneys and stomach, but not the spleen [6,8,9]. Modeling inhalational anthrax is often performed in the A/J mouse with Tox+ strains, such as the Sterne strain [7–9]. In this study, using this murine model of inhalational anthrax and a bioluminescent Tox+ strain that produces light during vegetative cell growth [6,10], we questioned whether the absence of capsule in Tox+ strains could markedly alter the entry and dissemination of the bacteria in comparison to previously described Cap+ strains [5].

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2. Materials and methods

2.1. Bacterial strains

Luminescent 9602R bacteria were constructed as previously described [5,6]. Briefly, the *luxABCDE* operon was inserted in the conjugative shuttle vector pAT113 [11] under the control of the *pagA* promoter, which is highly expressed under in vivo conditions. 9602R is a derivative of the highly virulent natural human isolate 9602 [12], that has been cured of plasmid pX02 [13]. It therefore does not produce a capsule, but maintains toxin production − a phenotypic equivalent to the Sterne strain. Spores were produced and purified as previously described [11].

2.2. Mouse infection

Six- to 10-week-old female A/J mice (Harlan Labs, UK) were maintained under specific pathogen free condition at the Institut Pasteur in compliance with European animal welfare regulations. Aerosol infections were performed by delivering spores for 20 min using a Raindrop® nebulizer (Tyco, Mansfield, MA, USA) − 5×10^8 spores/ml in water in an all-glass nose-only aerosol chamber [5]. There was an average of 4.6 ± 0.1 $\log_{10}$ (mean ± SD) spores in the lungs 2 h post-infection. Intranasal (IN) inoculation was performed in anesthetized mice by introducing 8.3 ± 0.1 $\log_{10}$ or 7.3 ± 0.1 $\log_{10}$ spores in 20 µl PBS into the nostrils. An average of 7.4 ± 0.1 $\log_{10}$ and 5.8 ± 0.1 $\log_{10}$ spores (for high and low dose infection respectively) were found in the lungs 2 h post-infection. For CFU determination, organs were removed and immediately placed in ice-cold saline, then homogenized in a chilled glass tube and dilutions were plated on BHI plates.

2.3. Image acquisition and analysis

Images were acquired using an IVIS100 system (Xenogen Corp., CA, USA) according to instructions. Analysis and acquisition were performed using Living Image 2.5 software (Xenogen Corp.). Mice were anesthetized using a constant flow of 2.5% isoflurane mixed with oxygen using a XGI-8 anesthesia machine (Xenogen Corp.). Images were acquired with a binning of 16. Luminescent signals from the exterior of mice were acquired for 1 min, whereas luminescence of internal organs during dissection was integrated for 10 s. All other photographic parameters were held constant. Quantification of the photons/s emitted by each organ was performed by defining regions of interest corresponding to the organ of interest.

2.4. Histology

The lung lobes, either displaying luminescence or not, were removed during necropsy, immediately fixed in 4% buffered formalin and embedded in paraffin. Serial 5-µm sections were cut and stained with hematoxylin–eosin and Gram stain [14].

2.5. Statistical analysis

Statistical analysis was performed with Student's unpaired *t*-test and Graphpad Prism 4 software.

3. Results

3.1. Aerosol infection

A/J mice exposed to an aerosol of Tox+ spores had measurable luminescence in the nasopharynx at an average of 18 h post-infection, and in the submandibular lymph node at approximately 45 h post-infection (Fig. 1A). When bioluminescence was first visible in the nasopharynx, it was not found in the lung and tracheobronchal mediastinal lymph nodes (Fig. 1B), despite the effective delivery of spores into the lungs (see Section 2). Small quantities of heat-resistant spores were also found in the tracheobronchal lymph nodes. In both lungs and lymph nodes, vegetative bacteria could not be detected (Fig. 1D). After 78 h of increasing intensity of luminescence in the nasopharynx and lymph nodes, luminescence spread to the kidneys and lungs (Fig. 1C). Occasionally, focal points of luminescence were observed in the pyloric region of the stomach (Fig. 1A,C), as previously seen in cutaneous infections [6], and the heart, and/or the gallbladder (not shown). No luminescence was observed in the spleen (Fig. 1A,C). Approximately 24 h lapsed between the first detection of bioluminescence in the nasopharynx and spread to the draining lymph node, while it took an additional 24 h for the luminescence to be detected in the kidneys.

3.2. Intranasal (IN) inoculation

Bacterial infection patterns after IN inoculation were dependent on dose size. A dose of 7.3 $\log_{10}$ spores initiated an infection in the nasopharynx (Fig. 2A), as observed after aerosol infection (Fig. 1A). In contrast, when a dose of 8.3 $\log_{10}$ spores was delivered, three different patterns of bioluminescence were observed at the beginning of infection: (i) initiation in the nasopharynx (27% of the animals) (not shown), as observed after aerosol (Fig. 1A) and low dose IN infections (Fig. 2A); (ii) initiation in the lungs as focal points (36% of the animals) (Fig. 2B); and (iii) concomitant initiation of infection at both sites (36% of the animals) (Fig. 2C). All types of infection ultimately became lethal systemic infections with high levels of bioluminescence in the kidneys. No luminescence and few CFU were detected in the spleen (Fig. 2E,F), in contrast to lungs and kidneys.

Necropsy after detection of bioluminescence in the lungs showed that the infection started in the post-caval lobe (Fig. 2D). Histological analysis of the lung was carried out in three mice. The first two were analyzed immediately after bioluminescence was detected as localized luminescent points emanating from the thorax. One lung had low luminescence (2×10^5 photons/s), while the other had a high level (2×10^6 photons/s). The third mouse that was analyzed displayed initiation of infection in the nasopharynx, subsequent