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Research paper

Effects of age and macrophage lineage on intracellular survival and cytokine induction after infection with *Rhodococcus equi*

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ABSTRACT

Rhodococcus equi, a facultative intracellular pathogen of macrophages, causes life-threatening pneumonia in foals and in people with underlying immune deficiencies. As a basis for this study, we hypothesized that macrophage lineage and age would affect intracellular survival of *R. equi* and cytokine induction after infection. Monocyte-derived and bronchoalveolar macrophages from 10 adult horses and from 10 foals (sampled at 1–3 days, 2 weeks, 1 month, 3 months, and 5 months of age) were infected *ex vivo* with virulent *R. equi*. Intracellular *R. equi* were quantified and mRNA expression of IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12 p40, IL-18, IFN-γ, and TNF-α was measured. Intracellular replication of *R. equi* was significantly ($P < 0.001$) greater in bronchoalveolar than in monocyte-derived macrophages, regardless of age. Regardless of the macrophage lineage, replication of *R. equi* was significantly ($P = 0.002$) higher in 3-month-old foals than in 3-day old foals, 2-week-old foals, 1-month-old foals, and adult horses. Expression of IL-4 mRNA was significantly higher in monocyte-derived macrophages whereas expression of IL-6, IL-18, and TNF-α was significantly higher in bronchoalveolar macrophages. Induction of IL-1β, IL-10, IL-12 p40, and IL-8 mRNA in bronchoalveolar macrophages of 1–3-day old foals was significantly higher than in older foals or adult horses. Preferential intracellular survival of *R. equi* in bronchoalveolar macrophages of juvenile horses may play a role in the pulmonary tropism of the pathogen and in the window of age susceptibility to infection.

1. Introduction

Rhodococcus equi, a Gram-positive facultative intracellular pathogen that primarily infects macrophages, is one of the most important causes of disease in foals between 3 weeks and 5 months of age. *R. equi* has also emerged as a common opportunistic pathogen in immunosuppressed people, especially those infected with the human immunodeficiency virus or receiving immunosuppressive chemotherapy (Arlotti et al., 1996; Donisi et al., 1996; Harvey and Sunstrum, 1991; Yamshchikov et al., 2010). Infection in either species is most commonly characterized by life-threatening pyogranulomatous cavitating pneumonia although systemic infections occur occasionally (Reuss et al., 2009; Yamshchikov et al., 2010). As opposed to foals, adult horses are typically immune to *R. equi* infections and the reasons for the peculiar susceptibility of foals to infection with *R. equi* are unknown.

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The ability of *R. equi* to survive and replicate in macrophages is at the basis of its pathogenicity. Multiple studies have confirmed that survival and replication of *R. equi* in macrophages concurs with virulence studies in both mice and foals (Coulson et al., 2010; Giguère et al., 1999a; Jain et al., 2003). *R. equi* has been shown to survive in various lineages of macrophages from several species including murine or equine monocyte-derived macrophages, murine peritoneal macrophages, and equine or human alveolar macrophages (Hondalus and Mosser, 1994; Ramos-Vivas et al., 2011; Ryan et al., 2010). Some facultative intracellular pathogens are known to replicate preferentially in certain lineages of macrophage over others (Gangadharam and Pratt, 1983; Polsinelli et al., 1994). To date, a direct comparison of intracellular survival and replication of *R. equi* in different lineages of macrophages across ages from birth to adulthood has not been reported. As a result, it is unknown if the macrophage plays a role in the pulmonary tropism of *R. equi* and in the window of age susceptibility to infection.

Studies in mice have clearly shown that a type 1 response, characterized by IFN-γ production by T helper lymphocytes, is sufficient to effect pulmonary clearance of *R. equi* while a type 2 response, characterized by IL-4 production, is detrimental (Kanaly et al., 1995, 1996). The cytokine environment that is present as T helper cells differentiate is important in determining the subset that will subsequently develop (Del Prete and Romagnani, 1994). Because of its importance in innate defense against *R. equi*, the macrophage may be one of the keys to the development of either a type 1 or a type 2 response by the nature of the cytokines produced shortly after infection. The ability of some strains of intracellular pathogens to survive in macrophages *in vitro* has been associated with their capacity to induce particular cytokines (Furney et al., 1992; Kuhn and Goebel, 1994; Sarmento and Appelberg, 1995).

As a basis for this study, we hypothesized that macrophage lineage and age would affect intracellular survival of the pathogen and cytokine induction in response to *ex vivo* infection of equine macrophages with *R. equi*. The present study demonstrates that intracellular replication of *R. equi* is significantly greater in bronchoalveolar than in monocyte-derived macrophages regardless of age. Regardless of macrophage lineage, replication of *R. equi* is significantly higher in 3-month-old foals than in younger foals or adult horses. Furthermore, both age and macrophage lineage had profound effects on cytokine induction.

2. Materials and methods

2.1. Animals and sample collection

Ten foals and 10 adult horses were used. Animals were considered healthy on the basis of physical examination, complete blood count, and IgG concentration at 24 h of age (foals only). Adult horses were sampled once and foals were sampled at 1–3 days, 2 weeks, 1 month, 3 months, and 5 months of age. At each sampling time, blood (180 mL) was collected by jugular venipuncture in 60 ml syringes containing EDTA as the anticoagulant. Alveolar macrophages were obtained by bronchoalveolar lavage. Animals were sedated intravenously with xylazine (0.5 mg/kg) and butorphanol (0.04 mg/kg) prior to BAL fluid collection. A 1.8 m bronchoalveolar lavage catheter (Jorvet, Loveland, CO) was passed *via* nasal approach until wedged into a bronchus. The lavage solution consisted of 4 aliquots of 50 mL physiologic saline (0.9% NaCl) solution infused and aspirated immediately. The study was approved by the Institutional Animal Care and Use Committee of the University of Georgia.

2.2. Isolation of monocyte-derived and bronchoalveolar macrophages

Mononuclear cells were harvested from blood by density gradient centrifugation (Ficoll-Paque, Amersham Biosciences, Pittsburgh, PA), washed 3 times with phosphate buffered saline (PBS), and counted. Monocyte-derived macrophages were obtained using the procedure previously described by Raabe et al. (1998). Gelatin-coated plates were incubated for 1 h at 37 °C in 6% CO_2 with 15 ml of donor horse serum, and washed 3 times with PBS prior to plating the blood mononuclear cells. Blood mononuclear cells were suspended at a concentration of 4×10^6 cells/ml in Minimum Essential Medium-alpha (MEMα) containing 10% horse serum (HS), penicillin G (100 U/ml), streptomycin (80 μg/ml) and amphotericin B (25 μg/ml), and incubated for 18 h at 37 °C in 6% CO_2. After incubation, non-adherent and loosely adherent cells were removed by a series of washes and the remaining cells were harvested by eluting with a 1:1 mixture of 10 mM EDTA and MEMα + 10% HS medium for 5–10 min at 37 °C. Cells were pelleted by centrifugation at $200 \times g$ for 10 min at 4 °C. Monocyte-derived macrophages were suspended at a concentration of 1×10^6 macrophage/ml in Minimum Essential Medium-alpha (MEMα) containing 10% HS and amphotericin B (25 μg/ml).

Total nucleated cell count in bronchoalveolar lavage fluid was determined by use of a cell counter (Cellometer Auto T4, Nexelom Bioscience, Lawrence, MA). Bronchoalveolar cells were washed 3 times with phosphate buffered saline (PBS), and counted. Bronchoalveolar cells were suspended at a concentration of 1×10^6 macrophage/ml in Minimum Essential Medium-alpha (MEMα) containing 10% HS and amphotericin B (25 μg/ml).

2.3. Infection of cells for intracellular survival and replication assay

0.5 ml of the monocyte-derived or 1 ml of the bronchoalveolar macrophage cell suspension was added to each well of a 24 well plate (Nunc, ThermoFisher Scientific, Rochester NY) and of chamber slides (Lab-Tek, ThermoFisher Scientific Rochester NY). Cells were incubated for 3 h at 37 °C in 6% CO_2. After incubation and subsequent washing, approximately 5×10^5 cells remained attached to each well. More than 97% of the adherent cells were macrophages as determined by Wright-Giemsa stain of duplicate monolayers. Media was removed and macrophages were then infected with virulent *R. equi* (ATCC #33701, Rockville, MD). Bacterial cultures were