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Short communication

Early development of cytotoxic T lymphocytes in neonatal foals following oral inoculation with *Rhodococcus equi*

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ABSTRACT

Rhodococcus equi is an important respiratory pathogen of young foals for which a vaccine has long been sought. Two major impediments to effective vaccination are the functionally immature type I immune responses of neonatal foals and early exposure to the bacterium via the environment. Despite these obstacles, it appears that under specific circumstances foals can develop a protective immune response. In this study we investigated the protective mechanisms behind oral inoculation of foals with virulent *R. equi* bacteria. Two foals receiving an oral inoculum demonstrated accelerated development of *R. equi* specific cytotoxic T lymphocytes (CTL) as evidenced by significant lysis of *R. equi* infected, ELA-A mismatched cells at 3 weeks of age. As in a previous study, CTL were not detected until 5–6 weeks of age in two control foals. At each time point the ability of foal peripheral blood mononuclear cells (PBMC) to produce IFN-γ following stimulation with live *R. equi* or extracted cell wall lipids was similar to that of an adult horse control and between foals, regardless of treatment. These results provide a potential mechanism of protection which has previously been shown to occur following oral inoculation, and suggest that the early detection of CTL may be a useful marker for induction of protective immunity.

1. Introduction

Rhodococcus equi is an important bacterial pathogen of foals between 1 and 5 months of age. The organism, which produces life threatening pyogranulomatous pneumonia, is ubiquitous in equine environments (Cohen et al., 2008; Hondalus, 1997; Takai, 1997). As a result, foals are exposed and possibly infected in the first weeks of life. In contrast, adult horses are immune and this protection is associated with a type 1 memory response that operates throughout life (Hines et al., 2001, 2003; Patton et al., 2004). The protective response involving both CD4+ and CD8+ T lymphocytes was originally demonstrated in mice (Kanaly et al., 1993, 1996); later experiments suggest a similar mechanism is occurring in adult horses. *R. equi*-specific CD4+ Th1 cells activate macrophages though the production of IFN-γ, while CD8+ cytotoxic T lymphocytes (CTL) recognize and lyse *R. equi*-infected cells in an MHC class I unrestricted fashion (Hines et al., 2001, 2003; Patton et al., 2004). Antibody may also play a role in immunity, although it is likely insufficient without type 1 cellular responses.

The unique age restricted susceptibility of foals to *R. equi* is postulated to reflect their lack of immunologic memory (i.e. their naïve status) and the diminished immunologic capacities that characterize early life in virtually all mammals. In general, neonates and perinates have decreased abilities to mount Th1 responses and to generate CTL compared to adults (Adkins et al., 2004; Boyd et al., 2003; Breathnach et al., 2006; Liu et al., 2009). In some species, there is an initial Th2 bias that is likely an outcome

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of maternal conditions necessary to maintain pregnancy (Bengt, 1999; Raghupathy, 2001). A number of similar immunologic deficiencies have been identified in young foals, including an apparent decrease in their ability to produce IFN-γ and the absence of *R. equi*-specific CTL during the first 3–6 weeks of life (Breathnach et al., 2006; Liu et al., 2009; Patton et al., 2005). The observations that foals may be infected shortly after birth during a time when the immune system is both naïve and relatively immature has led some veterinarians to propose that active immunization will not be an effective strategy for prevention of rhodococcal pneumonia.

There is, however, evidence to suggest that young foals are capable of mounting protective immune responses to *R. equi*. For example, even on endemic farms where morbidity is high, a majority of foals resist infection and/or clear bacteria to become immune (Chaffin et al., 2003). Likewise, a recent study showed that intrabronchial challenge of neonatal foals with *R. equi* resulted in a strong Th1 response as evidenced by IFN-γ mRNA expression that exceeded that in adults receiving a similar challenge (Jacks et al., 2007). The authors suggested that similar to human neonates immunized with *Mycobacterium bovis* BCG, foals have the ability to mount adult-like type 1 immune responses so long as the stimulus is appropriate. Variables such as the nature of the antigen, antigen dose, route, adjuvant, etc. are likely critical. In support of this idea, oral inoculation of foals with virulent *R. equi* during the first 2 weeks of life was shown to provide strong protection against a subsequent intrabronchial challenge (Chirino-Trejo et al., 1987; Hooper-McGrevy et al., 2005). These immunization trials are the most promising to date. Although inoculation of virulent bacteria is not an acceptable real world practice, the studies suggest that the use of an oral route and live bacteria may be the keys. Unfortunately the only immunologic parameter measured was antibody responses.

In the pilot study reported here, we revisited the oral inoculation model to characterize the immune responses produced. Our first hypothesis was that the protective oral immunization protocol accelerates the appearance of *R. equi*-specific CTL. Recent work in our laboratory has shown that the MHC class I unrestricted CTL which lyse *R. equi* infected cells recognize lipid antigens found in the unique cell wall of *R. equi* (Harris et al., 2010). To further investigate the role of lipid antigens in immunity to *R. equi*, we also examined the ability of *R. equi* cell wall lipids to stimulate transcription of IFN-γ and IL-4. Our second hypothesis was that oral immunization of foals with live bacteria induces adult-like levels of IFN-γ expression in lymphocytes stimulated with *R. equi* lipid and live *R. equi*.

2. Materials and methods

2.1. Oral inoculation

Four Arabian foals were screened at birth with a physical exam, complete blood count, and IgG level, and were determined to be healthy. Two foals were controls and two were orally infected with virulent *R. equi* using previously published methods (Hooper-McGrevy et al., 2005). Briefly, at 2, 7, and 14 days of age a single inoculum containing 1×10^{10} CFU of *R. equi* ATCC 33701 diluted in 100 ml phosphate buffered saline (PBS) or 100 ml of PBS alone (negative control) was administered at each time point through a nasogastric tube. The tube was then flushed with 100 ml of distilled water, kinked, and removed. Bacterial concentration was estimated with an optical density reading and confirmed by plating serial dilutions on brain heart infusion agar. All horse use was approved by the Washington State University institutional animal care and use committee.

2.2. Cytotoxicity assay

At 1, 3, 5, and 7 weeks of age, 250 ml of blood was collected from alternating jugular veins and the peripheral blood mononuclear cells (PBMC) were harvested for CTL assays using previously published methods (Harris et al., 2010; Patton et al., 2004). Briefly, effector cells were derived by stimulating 10^8 PBMC (average 20% monocytes and 80% lymphocytes) with 6×10^6 *R. equi* 33701 (approximately 0.3 multiplicity of infection in monocytes; MOI) for 5 days at 37 °C with 5% CO_2, followed by resting for 2 days without antigenic stimulation. ELA-A (equine MHC class I) mismatched target cells were obtained from a single Hanovarian horse (H68) by eluting adherent peripheral blood adherent cells (PBAC). The effector cells were then added to target cell wells containing PBAC which were previously labeled with ^{51}Cr (PerkinElmer, Waltham, MA). Lysis of target cells infected with 5 MOI of virulent *R. equi* in comparison to uninfected control target cells was calculated by chromium release assay using the following formula: $[(E-S)/(T-S)] \times 100$, where E is the mean of three test wells, S is the mean spontaneous release from three target cell wells without effector cells, and T is the mean total release from three target cell wells with 2% Triton X-100 (Sigma–Aldrich, St. Louis, MO). As in previous equine CTL assays, significant lysis was defined as 3 standard errors above the uninfected negative control target cell value, and greater than 10% total lysis (McGuire et al., 1994; Patton et al., 2004). To increase the sample size for statistical analysis between the foals at the 3 week time point, CTL assays from a previous season's foals (Patton et al., 2005) were added and compared in a Fisher's exact test. These foals had no experimental inoculation with *R. equi* during the first 3 weeks of life and so were included as additional controls.

2.3. Cytokine PCR

A total of 3×10^7 PBMC in antibiotic-free complete medium were stimulated with 2×10^6 live *R. equi* 33701, 800 μg of *R. equi* lipid extract, or unstimulated (negative control) using previously published methods (Harris et al., 2010). Following an 18 h incubation RNA was extracted using the RNeasy plus mini kit (Qiagen, Valencia, CA). Expression of equine IFN-γ, IL-4, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cells was determined by performing real-time PCR with previously described primers (Lopez et al., 2002). Transcript levels were determined for each sample by comparing the threshold cycle values of IFN-γ, IL-4, and GAPDH to the corresponding plasmid standard curves. Amplification of plasmid DNA also