Immunity to *Rhodococcus equi*

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The development of disease involves pathogen, host, and environmental factors. When looking specifically at the foal and its susceptibility to *Rhodococcus equi* infection and disease, age-dependent limited function of the immune system and its naïve state at birth seem to be plausible explanations. Nevertheless, natural and experimental infections of the foal have been shown to induce robust humoral and Th1-type immune responses superior or at least comparable with the adult horse, which is considered protective. The challenge is that these types of responses require time and effective signaling interactions between antigen-presenting cells, lymphocytes, phagocytes, and antibodies within a positive-feedback loop model. Perhaps some foals do require a longer warm-up period of the immune system, and this time creates a window of greater susceptibility. A better understanding of the pathogenic mechanisms in the early stages of infection in the airways could bring new information to light about how the immune system of the foal coordinates these interactions for effective bacterial removal and killing before disease establishment and how this elaborates into long-life immunity. Author’s address: College of Veterinary Medicine, Cornell University, Ithaca, New York 14882; e-mail: mbf6@cornell.edu. © 2010 AAEP.

1. Introduction

Foals are exposed to *Rhodococcus equi* in the first few days to weeks of life.1,2 *R. equi* disease manifests before 4–6 mo of life and not in older foals and adult horses, unless immunodeficiency is present.3,4 Several studies using experimental infection of foals revealed that the respiratory tract is the most likely route of exposure for infections resulting in pulmonary disease, whereas intragastric inoculation with large numbers of organisms does not induce the typical pulmonary lesions.5–7 During the establishment of infection, hypercellularity of the alveolar septa develops, and the alveoli and terminal bronchioles become infiltrated with neutrophils, macrophages, giant cells, and numerous cell-associated bacteria.8 Subsequently, pyogranulomatous inflammation and necrosis progress into the destruction of lung parenchyma. Experimental infections of piglets and mice with doses greater than those lethal to foals fail to produce pulmonary abscesses, unless immunosuppressive therapy is instituted.9–11 Therefore, incompletely understood host-specific natural conditions exclusive to the foal are necessary for *R. equi* to establish disease.

The unique susceptibility of young foals to *R. equi* disease is still puzzling, despite many studies investigating their innate and acquired immune systems. Although age-dependent developmental limitations of the immune system of the foal are suspected to be involved, recent studies have shown that foals can elaborate a Th1-type immunity in response to *R. equi* infection. This is the type of protective immunity developed by adult horses on experimental infection. However, acquired immunity takes time to establish, and this window of susceptibility could favor pathogen replication to overwhelming levels.
and tissue damage. Perhaps effective phagocytic function could control pathogen expansion and disease development in the foal before acquired immunity is developed. However, the mechanisms of appropriate activation of phagocytes against *R. equi*, a key process to control infection in the airway environment, are poorly understood.

2. The Pathogen

*R. equi* is a Gram-positive, obligate aerobic, facultative intracellular coccobacillus capable of multiplying in macrophages, including alveolar macrophages. Of the 40 genera in the actinomycete group, *Rhodococcus* is placed among the mycolata taxon, along with *Mycobacterium, Corynebacterium, Nocardia, Gordonia, Dietzia, Turicella, Tsukamurella, Williamsia*, and *Shermania*. Mycolic acids are long fatty acids found in the lipid-rich cell-wall envelope of these bacteria, which form a protective barrier that increases resistance to chemical damage, dehydration, oxidative stress, and low pH. Ellenberger et al. suggested that a component of the *R. equi* cell wall is capable of inhibiting bactericidal mechanisms of phagocytes and allowing intracellular survival. Surprisingly, recent studies by Sydor et al. have shown that the bacterial capsule of *R. equi* is not an essential virulence factor. Nevertheless, *R. equi* has evolved a mechanism to escape bactericidal activity in macrophages. Inside macrophages, maturation of phagosomes containing virulent *R. equi* does not progress into a late endocytic organelle, which does not acidify and lacks the proton-pumping vacuolar adenosine triphosphate (ATP)-ase. The phagosome membrane remains intact during the period of bacterial replication. In foal alveolar macrophages, viable intracellular bacteria were observed 24 h after infection. In vitro macrophage infection with virulent *R. equi* revealed intracellular bacterial replication starting beyond 6–12 h in culture, reaching a five-fold increase in bacterial numbers by 48 h. *R. equi* infection of macrophages is cytotoxic, and cell degeneration occurs because of necrosis.

The virulent *R. equi* depends on the presence of a large plasmid, which encodes a family of eight thermoregulated virulence-associated proteins (VapA and VapC to VapI). This plasmid is critical for intracellular replication within macrophages and the development of disease in the foal. *R. equi* depleted of the virulent plasmid replicates poorly inside macrophages and does not induce disease. In vitro, *R. equi* binding to and invasion of macrophages can be mediated by complement receptor CR3 (CD11b/CD18 or MAC-1), mannose receptor (which binds lipoarabinomannan), and Toll-like receptor 2. When opsonized with specific antibodies, *R. equi* is internalized through Fc receptor on both neutrophils and macrophages, and this mechanism increases phagolysosome formation and bacterial killing.

3. The Innate Immune System

Early events in the pathogenicity and *R. equi* removal in the Airways are poorly described, and most of our understanding comes from studies in vitro (using infected macrophages or neutrophils alone) and mouse models. Phagocytes participate in the early stages of immune response by removing and killing pathogens and play a critical role in the protection of the immunologically naive neonate.

Neutrophils are important cells of the innate immune system and inhabitants of the upper airways of horses. They are the first cells to be recruited to a site of infection in response to tumor necrosis factor (TNF)α and interleukin (IL)-6 cytokines and the chemokines IL-8, macrophage inflammatory protein (MIP)-2, and keratinocyte chemoattractant. Immunocompetent mice are resistant to intrabronchial challenge with *R. equi*; high doses of inoculation result in slower but effective pulmonary clearance, starting at 24 h with neutrophilic influx, followed by macrophages at 48–72 h, and peribronchial and perivascular mononuclear infiltrate. Indeed, using an anti-neutrophil monoclonal antibody, mice deficient in neutrophils developed more severe disease and greater *R. equi* replication than control mice. In contrast to macrophages, foal neutrophils have been shown to be bactericidal to the opsonized bacteria. Despite the presence of large numbers of neutrophils in the Airways in response to *R. equi* infection, bacterial replication and disease may still progress in the foal. In contrast, adult horses have not shown a significant increase in bronchoalveolar lavage neutrophils 7 days post-experimental infection. Opsonization of bacteria with *R. equi*-specific antibodies is critical for the efficient bacterial killing in neutrophils. In vitro, neutrophils of foals less than 7 days of life revealed similar *R. equi* killing capacity in comparison to 35-day-old foals and adult horses, particularly in the presence of opsonic elements. Yet, some neonates in those studies showed decreased efficiency, which could partially explain individual susceptibility to disease. Although neutrophils are competent in the neonate, disease process can alter their function. In addition, *R. equi* preparations (pellet or supernatant fractions) have been shown to inhibit oxidative metabolism of neutrophils, including myeloperoxidase-dependent peroxide production. Besides bacterial removing and killing, neutrophils may influence airway immunomodulation and the type of adaptive immune response to *R. equi*, because they serve as a source of many pro-inflammatory cytokines and chemokines. Importantly, in foals, the expression of certain pro-inflammatory cytokines by neutrophils in response to *R. equi* is influenced by age, which may limit important cellular signaling and activation during *R. equi* infection.
**R. equi** evolved a mechanism to escape bacterial activity in macrophages, which, paradoxically, are important cells of the immune system that perform surveillance, removal, and killing of microorganisms. *R. equi* readily invades alveolar macrophages, multiplies, and causes their destruction, favoring its replication and spreading in the respiratory tract.19 Virulent *R. equi* replicated efficiently in macrophages, starting beyond 6–12 h of culture and affecting macrophage viability by 48 h; in contrast, bacterial numbers of avirulent (plasmid-cured) *R. equi* decreased during the same period.20 Although macrophages fail to impede intracellular bacterial replication, *R. equi* infection still induces an immune response in macrophages characterized by translocation of nuclear factor κB (NF-κB) into the nucleus and subsequent secretion of cytokines.41,42 Both virulent and avirulent *R. equi* strains induce IL-1β, IL-10, IL-12p40, and TNFα cytokine response in murine macrophages by 4 h of infection.43 Osporonization of *R. equi* with antibody against capsular components increases phagocytosis and intracellular killing in foal alveolar macrophages.12 Macrophages also become activated when cell-surface receptors (e.g., Toll-like receptors [TLRs]) recognize highly conserved structural motifs only expressed by microbial pathogens (so-called pathogen-associated molecular patterns [PAMPs]). Darrah et al.42 and Aderem and Ulevitch44 suggested that TLR-2 is involved in *R. equi* recognition, similar to the response to *Mycobacterium tuberculosis*.45 In addition, macrophage activation is also enhanced by cytokines, in particular interferon γ (IFNγ) and TNFα.46,47 It is possible that macrophage activation before intracellular infection results in efficient *R. equi* killing. Kanaly et al.58,49 showed that mice treated with IFNγ monoclonal antibodies (to block IFNγ activity) failed to clear pulmonary infection. Therefore, this cytokine plays an important role in resistance to *R. equi* pneumonia. Indeed, macrophages from *R. equi*-exposed foals showed a 100% increase in their killing capacity when co-cultured with autologous lymphocyte factors (pre-conditioned medium derived by in vitro co-culture of lymphocytes and *R. equi* surface antigens), whereas macrophages from non-exposed foals showed only a 50% increase.12 The exposure of macrophages to cytokines, importantly IFNγ, results in enhanced microbicidal activity through the secretion of both reactive oxygen intermediates (ROIs) and reactive nitrogen intermediates (RNIs). Interferon regulatory factors (IRFs) augment the production of ROIs, RNIs, and cytokines (e.g., TNFα). Individually, ROIs and RNIs promote negligible microbicidal activity; in fact, hydrogen peroxide alone has been shown to induce the expression of *R. equi* virulence-associated genes.50 However, their combination (e.g., peroxynitrite ONOO−) brings greater efficiency in killing of *R. equi*.51,52 Antigen-presenting cells including macrophages and dendritic cells potentially direct the type of immune response (Th1 or Th2) on encounter with *R. equi*. Similar to macrophages, dendritic cells encounter pathogens in the tissues through TLRs, phagocytose, and process them for presentation to effector cells belonging to the acquired immune system (lymphocytes) in the regional lymph nodes. Ultimately, a Th1-type immune response with IFNγ production and the development of cytotoxic T cells (CTLs) specific for *R. equi* would have to derive from antigen-presenting signals (i.e., IL-12 production) in the initial stages of infection. Therefore, in immunocompetent individuals, *R. equi* induces a protective response that starts from the innate immune system. The effect of *R. equi* on dendritic cells has been poorly studied in vivo. In vitro, Darrah et al.42 showed that purified *R. equi* virulence-associated protein VapA induced murine dendritic cell maturation. Additionally, the newborn foal monocyte-derived dendritic cells have been shown to respond to *R. equi* infection comparably with adult horse cells, with the expression of IL-12.53 Although TLR-mediated immune response to ligands has been implicated in immature immunity in neonates, dendritic cells from the foal seem to recognize, process, and respond to *R. equi*.54 One concern is the age-dependent limited expression of major histocompatibility complex class II (MHC class II) molecules on the surface of foal dendritic cells, which are used as markers of maturation and competence for antigen presentation to lymphocytes.53,55,56 Although both macrophages and neutrophils patrol the airway surface to eradicate bacteria, recent studies of lung inflammation have suggested that airway epithelial cells are the first cell type to respond to environmental stimuli. Respiratory epithelial cells play a key role in the recruitment and activation of inflammatory cells and perhaps, coordinate the type of inflammatory process.57 Consequently, airway epithelial cells not only form a barrier for protection against inhaled pathogens and toxins but also are an integral part of the innate immune system for inflammatory response. Bronchial epithelial cells of the horse express TLR-2 and can potentially recognize and bind to *R. equi*.58,59 Mathew et al.60 showed that NF-κB activation in mouse tracheal epithelial cells generated signals that contributed to activation of macrophages. Bronchial epithelial cells incubated in the presence of endotoxin revealed a significant increase in the expression and production of TNFα, IL-8, integrin CD11b/CD18 (Mac-1), and the adhesion molecules ICAM-1, which are important activation signals to phagocytes.28 In addition, the presence of bacteria directly induces mucin production by the airway epithelial cells.61 Mucins are the key structural components of mucus, and they form a barrier to protect underlying cells from inhaled microorganisms and irritants. A wide variety of infectious and non-infectious equine respiratory diseases are associated with increased volumes of respiratory secretions and a change in the physical and chemical
nature of the mucus.\textsuperscript{62,63} Whereas the mucus gel in the respiratory tract is vital for the protection and normal function of the airways, the overproduction and change in its composition significantly affects the pathologic feature of these conditions. Indeed, phagocytic function may be reduced in mucus secretions.\textsuperscript{64} Limited information is available about these gel-forming glycoproteins in the horse airway mucus, and much less is known about their role in \textit{R. equi} pneumonia. Our laboratory is currently examining how airway epithelial cells interact with phagocytes and \textit{R. equi} in a biologically relevant in vitro three-dimensional culture system of equine bronchial epithelium that fully differentiates into ciliary beating and mucus-producing cells.\textsuperscript{65}

4. The Acquired Immune System—Cellular Immunity

Specific host factors that determine protection or susceptibility in \textit{R. equi} infection in foals have not been fully identified. In mice, protective immune response against \textit{R. equi} is mediated by CD4\textsuperscript{+} T cells, and the role of CD8\textsuperscript{+} T cell is disputed.\textsuperscript{48,49,66,67} The clearance of \textit{R. equi} in mice depends mainly on IFN\textsubscript{\gamma} production by lymphocytes, whereas biased production of IL-4 (Th2-biased) increases risk of disease.\textsuperscript{48,49} Experimentally infected adult horses rapidly clear the organism without developing disease; the immune response includes an increase in both CD4\textsuperscript{+} and CD8\textsuperscript{+} T lymphocyte counts in the bronchoalveolar lavage fluid, an increase in the number of CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells producing IFN\textsubscript{\gamma}, and a greater post-infection proliferative response to \textit{R. equi} antigen or recombinant VapA in vitro.\textsuperscript{15,33,68,69} The latter suggests an antigen-specific recall response in the lungs of adult horse. Therefore, it seems that, in the horse, both CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells are present for clearance of the organism. Although \textit{R. equi} pneumonia is observed in human patients with immunodeficiency and CD4\textsuperscript{+} T cell lymphopenia, foals that develop \textit{R. equi} pneumonia have comparable CD4\textsuperscript{+} and CD8\textsuperscript{+} T cell distribution in peripheral blood with healthy foals.\textsuperscript{70} Importantly, T lymphocytes and plasma cells are virtually absent in lung tissues in the first week of life, and bronchus-associated lymphoid tissue (BALT) is only observed by 12 wk of age.\textsuperscript{71} In addition, the concentration of leukocytes in bronchoalveolar lavage fluid in foals less than 3 wk of age is one-half the value for adult horses.\textsuperscript{72}\textsuperscript{7} The percentage of CD4\textsuperscript{+} T cells increases markedly in the first 3 wk of life, and B cell counts in bronchoalveolar lavage fluid (BALF) are almost undetectable in the first 4 wk of life, reaching values comparable with adult horses by 8 wk.\textsuperscript{72} Therefore, the pulmonary leukocyte population is quite limited during the first several weeks of life, and it is unlikely that these cells would control substantial airway infection during this period. Adult horse and >6-wk-old foal CTLs efficiently kill \textit{R. equi}-infected cells; however, the same is not observed with CTLs from foals younger than 3 wk.\textsuperscript{73} Patton et al.\textsuperscript{74} have shown that CD8\textsuperscript{+} T cells recognize and kill \textit{R. equi}-infected macrophages in a major histocompatibility complex (MHC class I) unrestricted fashion in adult horses, perhaps involving the CD1 molecule. Importantly, foal monocyte-derived macrophages express lower levels of CD1 than adult horse cells, and \textit{R. equi} induces down-regulation of CD1b on equine monocyte-derived macrophages.\textsuperscript{75}

Despite a reported age-dependent limited IFN\textsubscript{\gamma} production in foals in response to mitogens,\textsuperscript{76,77} \textit{R. equi} infection in foals is characterized by a significant increase in the percentage of CD4\textsuperscript{+} T lymphocytes in bronchoalveolar lavage fluid and a robust IFN\textsubscript{\gamma} response.\textsuperscript{78,79} Compared with adult horses, experimentally infected foals are capable of mounting equivalent or superior lymphoproliferative responses and high IFN\textsubscript{\gamma} expression and IFN\textsubscript{\gamma}/IL-4 ratio by regional bronchial lymph-node lymphocytes.\textsuperscript{80} Altogether, these data suggest that foals can elaborate a Th1-type immune response to \textit{R. equi} at least equivalent to adult horses, which is considered protective.

5. The Acquired Immune System—Humoral Immunity

Antigen-specific antibodies can directly neutralize pathogens before they reach the intracellular space. Antibodies also play an essential role as opsonins for efficient phagocytosis. In the foal, humoral protection would be useful during the initial exposure to the pathogen, before \textit{R. equi} reaches the intracellular environment. Therefore, in the neonate, the passive transfer of immunoglobulins through colostrum or plasma transfusion could be helpful in providing the initial protection in the airways until its own cellular and humoral immunity are developed. Few studies have investigated the immunization of dams with \textit{R. equi} and the efficiency of antibodies transferred through colostrum in preventing \textit{R. equi} pneumonia in foals.\textsuperscript{81–83} Although results were somewhat favorable in controlled conditions, individual differences in humoral response to vaccines, quality of colostrum (antibody concentration), and factors involved in adequate passive transfer of immunoglobulins would put a percentage of foals at risk of disease. A more effective means to standardize the transfer of \textit{R. equi}-specific antibodies to each foal is through intravenous transfusion of hyperimmune plasma; however, some studies also indicate disparity in the efficiency of intravenous hyperimmune plasma transfusion in the prevention of disease. In equine neonates infected experimentally with \textit{R. equi}, intravenous plasma transfusion containing specific antibodies against \textit{R. equi} or VapA administered before infectious challenge aided significantly in the recovery of disease compared with placebo.\textsuperscript{35,84,85} Perkins et al.\textsuperscript{86} suggested that survival and severity of disease in foals experimentally infected with \textit{R. equi} was comparable when foals were pre-treated with intravenous transfusion of plasma with low \textit{R. equi} antibody titers or hyperimmune plasma; however, this study
did not use a placebo control group to evaluate spontaneous recovery from infection. In naturally infected foals and herd studies, intravenous administration of hyperimmune plasma showed a decrease in the incidence of pneumonia,\textsuperscript{91} a reduction in the risk of developing disease,\textsuperscript{87} or no significant effect,\textsuperscript{88,89} which suggests the participation of other risk factors (e.g., pathogen exposure or competence of different arms of the immune system) in the development of disease in natural conditions. It is possible that \textit{R. equi}-specific antibodies and other non-specific immunomodulators present in plasma may synergistically enhance bacterial removal and killing.

When instituted as prophylaxis and as a means to standardize humoral protection, intravenous transfusion of hyperimmune plasma would be the most beneficial in the first few hours of life before the foal is exposed to \textit{R. equi}. The mechanism of protection of the hyperimmune plasma has not been fully described. In vitro experiments using a commercially available hyperimmune plasma enriched with \textit{R. equi}-specific antibody for the opsonization of \textit{R. equi} revealed an increase in the oxidative burst activity of neutrophils and macrophages and the release of the pro-inflammatory cytokine TNF-\alpha from macrophages compared with non-opsonized bacteria.\textsuperscript{90} In addition, bacterial viability and growth in broth was reduced in the presence of the hyperimmune plasma. The viability of intracellular bacteria may be affected by the opsonization, but results suggest the need for additional activation of macrophages to elicit effective bacterial killing. If \textit{R. equi}-specific antibodies transferred through intravenous administration of hyperimmune plasma effectively diffuse into the airways of the foal, it is possible that they improve bacterial clearance at least before \textit{R. equi} reaches the intracellular space.

In natural conditions, serum \textit{R. equi}-specific immunoglobulin G (IgG) is detected in all foals, and concentrations correlate with quantitative changes of fecal \textit{R. equi}, suggesting a role for oral antigenic stimulation.\textsuperscript{91} Thus, foals are capable of producing a robust humoral response against \textit{R. equi} antigens; therefore, the presence of \textit{R. equi}-specific antibodies in serum reflects exposure to the pathogen and not exclusively disease. In another study, the antibody response to \textit{R. equi} virulence proteins VapA and VapC was comparable among healthy foals, foals with clinical pneumonia, and healthy adult horses, with higher concentrations of IgG\textsubscript{4/7} isotype in pneumatic foals.\textsuperscript{84} \textit{R. equi}-specific IgA is also detected in tracheal aspirates of foals with pneumonia.\textsuperscript{92} Experimental infection induces rapid humoral response in foals and adult horses.\textsuperscript{15} Three doses of oral administration of virulent \textit{R. equi} at 2, 7, and 14 days of life induced protection against intratracheal challenge and specific humoral response characterized by higher concentrations of IgG\textsubscript{4/5} isotype.\textsuperscript{93} In foals <10 days of life, intratracheal experimental infection induced a marked increase in serum IgG\textsubscript{1-} and IgG\textsubscript{4/7}-specific antibody concentrations against antigens of \textit{R. equi}, with concentrations higher than control adult horses.\textsuperscript{78} Interestingly, the size of inoculum modulates the immunoglobulin isotype response and possibly, the cytokine profile of foals.\textsuperscript{94} \textit{R. equi} IgG\textsubscript{1-} and IgG\textsubscript{4/7}-specific antibodies are effective in opsonization of the pathogen and complement activation, which favor bacterial removal and killing. Altogether, these data indicate the foal’s competence in elaborating a robust humoral response to \textit{R. equi} antigens.

6. Summary

The development of disease involves pathogen, host, and environmental factors. \textit{R. equi} evolved resistance to escape mechanisms of bacterial killing. Naïve foals are exposed to high numbers of virulent bacteria on farms endemic with \textit{R. equi}. However, most foals do not develop disease, suggesting individual risk factors. Taken together, these data suggest that susceptibility to \textit{R. equi} infection and disease in the foal is not because of their inability to produce a Th1-type immune response, although this type of response requires time and effective signaling interaction between antigen-presenting cells and lymphocytes. Perhaps susceptibility to disease relates to an early phase of infection when phagocytes fail to remove and kill the pathogens during the establishment of infection. Although foal phagocytes are competent at birth, \textit{R. equi} is killed more efficiently by phagocytes after primary exposure to the organism, which suggests the involvement of a recall response and activation of macrophages, perhaps involving IFN\textgamma from lymphocytes. Therefore, a positive-feedback interaction between the innate and acquired immune system is required for protection of the foal against \textit{R. equi}. The effect of colostrum- or hyperimmune plasma-derived \textit{R. equi}-specific antibodies in the neutralization and opsonization of the bacterium before it reaches the intracellular space is not clear, and passive transfer of immunoglobulins through intravenous plasma transfusion helps but does not prevent disease completely. Passive transfer of \textit{R. equi}-specific antibodies through colostrum may vary according to individual colostrum quality and foal factors that result in efficient or deficient antibody absorption. The administration of hyperimmune plasma may add to a positive outcome in the prophylaxis of \textit{R. equi}, but its effectiveness may depend on the timing of administration (before pathogen exposure) and quality of the product (standardized concentration and quality of antibodies relevant to virulent strains). Many current studies investigate the pathogenic mechanisms in the early stages of infection to design better prophylactic methods, including the development of immunomodulators and vaccines and improved herd-management practices.
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