Review
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Rhodococcus equi: Clinical Manifestations, Virulence, and Immunity

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Pneumonia is a major cause of disease and death in foals. *Rhodococcus equi*, a Gram-positive facultative intracellular pathogen, is a common cause of pneumonia in foals. This article reviews the clinical manifestations of infection caused by *R. equi* in foals and summarizes current knowledge regarding mechanisms of virulence of, and immunity to, *R. equi*. A complementary consensus statement providing recommendations for the diagnosis, treatment, control, and prevention of infections caused by *R. equi* in foals can be found in the same issue of the *Journal*.

Key words: Bacterial; Bacterial species; Bacterial virulence mechanisms; Cellular immunity; Immunology; Microbiology; Parenchymal disease; Pneumonia; Respiratory tract.

Clinical Manifestations

Pyogranulomatous Pneumonia

The most common clinical manifestation of disease caused by *Rhodococcus equi* infection is pyogranulomatous bronchopneumonia with abscessation (Fig 1).[1] Because ultrasonographic screening for early detection has become a routine practice at many farms endemic for pneumonia caused by *R. equi* (see consensus statement), the most frequently recognized form of *R. equi* infection on those farms is a subclinical form in which foals develop sonographic evidence of peripheral pulmonary consolidation or abscessation without manifesting clinical signs.[2,3] On those farms, the cumulative frequency of sonographically visible areas of focal pulmonary consolidation or abscessation considerably exceeds the historical frequency of clinical pneumonia attributed to *R. equi*[2] suggesting that many subclinically affected foals might spontaneously recover without treatment. The proportion of such subclinically affected foals that progress to clinically apparent disease is currently unknown, and might vary by farm, geographical region, and age at which foals are examined.[3] When respiratory disease does become clinically apparent, disease is frequently initially insidious, becoming chronic and progressive. A small proportion of affected foals develop a severe, subacute form of pneumonia; these foals might be found dead or develop severe, acute respiratory distress.

Abbreviations:

CDSs	coding sequences
CTL	cytotoxic T lymphocytes
EPDs	extrapulmonary disorders
LAM	lipoarabinomannan
ORFs	open reading frames
PAI	pathogenicity island
Vap	virulence-associated protein

Usually, foals first manifest clinical signs of *R. equi* pneumonia between 3 and 24 weeks of life, with most foals showing signs before 16 weeks of age. Infections are uncommon among horses older than 6 months of age. Clinical signs of pneumonia are variable and are dependent upon the stage and severity of pulmonary lesions. Initial clinical signs might include fever, lethargy, and cough.[4] As pneumonia progresses, clinical signs might include anorexia, tachycardia, tachypnea, flared nostrils, and increased effort and abdominal excursion during respiration.[4] Tachypnea and coughing are sometimes exacerbated by exertion during exercise or handling. Bilateral nasal discharge is an inconsistent finding.[5] Early in the course of disease, body condition is generally normal; however, in foals with chronic disease, weight loss or failure to grow might be apparent. In a recent report of 161 foals affected with *R. equi* pneumonia, the most common clinical signs were cough (71%), fever (68%), lethargy (53%), and increased respiratory effort (43%).[5]

Extrapulmonary Disorders (EPDs)

There are numerous EPDs that are associated with *R. equi* infections, including extrapulmonary sites of infection and immune-mediated disorders.[6] In a study of 150 foals with *R. equi* infections admitted to a teaching hospital, at least 1 of 39 different EPDs were recognized in 74% of foals, although some EPDs were recognized only during necropsy examination.[6] Survival was lower among foals with EPDs (43%; 48/111) than among foals without EPDs (82%; 32/39).[6] EPDs might occur concurrent with or independent of pneumonia,

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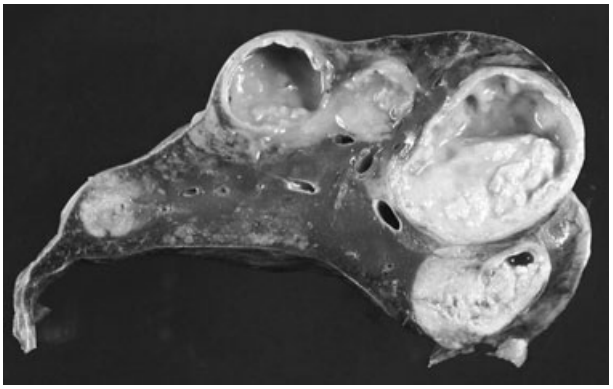

Fig 1. Cross-section of a lung from a foal with pyogranulomatous pneumonia caused by *Rhodococcus equi*. Courtesy of Dr William Castelman.

and some foals have multiple EPDs concurrently.[1,6] Some EPDs are subclinical and can be difficult to recognize antemortem.[6] Some EPDs (eg, polysynovitis) might be the 1st clinical manifestation of *R. equi*-associated disease, detected before signs of pneumonia. Some EPDs (eg, abdominal abscesses) might negatively affect prognosis, even in foals whose pneumonia responds to treatment.[6]

Diarrhea might occur in foals infected with *R. equi* either as an EPD caused by pyogranulomatous typhlocolitis or as a result of antimicrobial treatment.[1,6–8] Abdominal lesions are identified in approximately 50% of foals with *R. equi* pneumonia that are presented for necropsy,[1] and include any of the following, alone or in combination(s): pyogranulomatous enterotyphylocolitis (Fig 2); pyogranulomatous lymphadenitis of the mesenteric or colonic lymph nodes; large intra-abdominal abscesses; and peritonitis.[1,6] Intestinal lesions can be difficult to detect antemortem, but lymphadenitis and abdominal abscesses can be detected sonographically in some foals.[6] Interestingly, of 31 foals with ulcerative enterotyphlocolitis identified at necropsy, only 12 foals had diarrhea whereas 9 foals had diminished growth.[6] Abdominal abscesses are uncommon, but typically are large and contain mucopurulent material, sometimes with caseous centers, that presumably originate from abdominal lymph nodes infected with *R. equi*.[6,9] Abscesses are frequently adhered to other abdominal organs, including the intestinal tract, liver, spleen, or body wall.[6] Of 25 foals with abdominal abscesses, clinical signs included diarrhea (9 foals), diminished growth (8 foals), and colic (1 foal).[6] Some foals with abdominal lymphadenitis will have lymphatic obstruction resulting in lymphangiectasia.[10] Foals with abdominal abscesses have a poor prognosis.[6]

Polysynovitis occurs in approximately one fourth to one third of foals with *R. equi* infections.[6,11] Clinical signs include effusion of one or more synovial structures, generally without apparent lameness. Multiple joints are often affected, most commonly the stifles, tarsocrural, carpal, and fetlock joints. Immunoglobulin

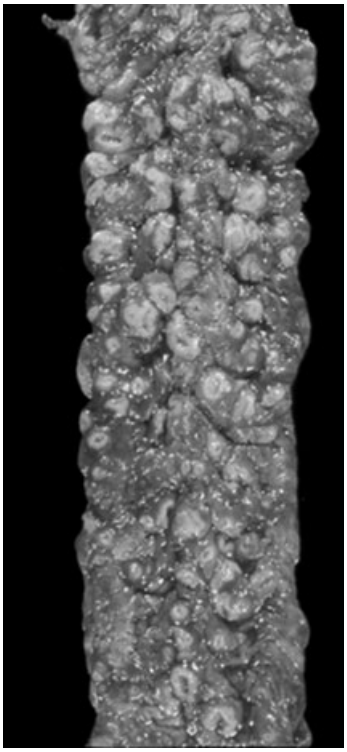

Fig 2. Mucosal surface of colon of foal with pyogranulomatous colitis caused by *Rhodococcus equi*.

was detected within the synovial membrane of 3 affected foals and antibodies directed against autologous or heterologous Fc portion of immunoglobulin were identified in the synovial fluid of 1 foal.[12,13] These findings suggest an immune-mediated process. However, heavy intrabronchial challenge with virulent *R. equi* also results in polysynovitis without lameness.[14] Culture of the synovial fluid of affected foals within a few days of the onset of synovial effusion yields growth of *R. equi* and histological examination of the synovial membrane reveals suppurative inflammation.[14] Therefore, an alternative and more likely explanation is that septic polysynovitis results from bacteremia, but that the infection is rapidly cleared from the synovial structures resulting in chronic nonseptic inflammation at the time of diagnosis.

Some foals with *R. equi* infections develop ocular lesions including uveitis, keratouveitis, and panophthalmitis.[6] Affected foals might display epiphora, photophobia, aqueous flare, hypopyon, iris discoloration, miosis, and other ophthalmic abnormalities. The pathogenesis of uveitis is often unknown, but might include dissemination of bacteria to the eye or immune-mediated mechanisms. In 1 study, foals with uveitis were less likely to survive than foals without uveitis.[6]

Rhodococcus equi can cause osteomyelitis, septic synovitis, or both.[6] Septic synovitis is differentiated from polysynovitis (described above) by the severity of lameness and by cytological evidence of septic synovial fluid. Septic synovitis can involve only 1 joint, whereas polysynovitis by definition involves more than 1 site.

Vertebral osteomyelitis caused by *R. equi* has been described in foals.[6] Clinical signs might include stiff gait, reluctance to move, palpable pain, and sometimes soft-tissue swelling associated with paravertebral abscessation.[6] If the infection or associated swelling spreads to the epidural space, neurological signs of spinal cord disease or nerve root compression might be apparent.[6] Specific neurological signs are dependent upon the specific region of the spinal cord that is affected. Foals with vertebral osteomyelitis generally have a poor prognosis, although a foal with diskospondylitis involving 3 sacral vertebrae responded to surgical debridement of infected bone and long-term antimicrobial treatment.[15]

Pyogranulomatous mediastinal lymphadenopathy can result in compression of the trachea, and occasionally an outward swelling of the pectoral region[6]; tracheal compression might cause respiratory distress or a respiratory noise. Few foals with *R. equi* pneumonia develop pleural effusion.[6,9] Pyogranulomatous *R. equi* lesions also can develop in the liver, kidney, spleen, or nervous tissue.[6] Disease caused by such lesions is often subclinical and only recognized during postmortem examination. A foal with a brain abscess (recognized via computed tomography) associated with *R. equi* infection has been described.[16]

Intermittent or persistent bacteremia with *R. equi* might be more common than recognized, and might result in metastatic spread of infection. The proportion of foals that are blood culture-positive and the stages of disease during which bacteremia is most likely to occur are ill-defined. *Rhodococcus equi* was isolated from the blood of 11 of 19 foals;[6] foals with positive blood culture results were less likely to survive than foals that were culture-negative.[6]

Other less common EPDs associated with metastatic spread of *R. equi* include pericarditis, endocarditis, cellulitis, dermatitis, subcutaneous abscesses, peripheral lymphadenopathy, guttural pouch empyema, pleuritis, sinusitis, myositis, stomatitis, pyometra and omphalitis.[6] Other immune-mediated EPDs include immune-mediated hemolytic anemia, immune-mediated thrombocytopenia, and telogen effluvium.[6]

Pathogenesis and Virulence

Pathogenesis

Inhalation of virulent *R. equi* is the major route of pulmonary infection. The incubation period after experimental intrabronchial challenge varies from approximately 9 days after administration of a heavy inoculum to approximately 2–4 weeks when a lower inoculum is administered.[14,17] Lung consolidation can be detected as early as 3 days after heavy intrabronchial challenge.[14] The incubation period under field conditions is unknown and likely depends on several factors including the number of virulent bacteria in air samples in the environment, age of the foal, and host defense mechanisms. Ingestion of the organism is an important route of exposure, and likely also of immunization, but rarely leads to hematogenously acquired pneumonia unless a foal has multiple exposures to large numbers of bacteria.[18]

Epidemiological evidence suggests that most foals on endemic farms become infected early in life.[19] The median age at the time of diagnosis is approximately 35–50 days on most endemic farms.[5,20] Given the fairly long incubation period of the disease, this finding would also support the fact that many foals become infected early in life. In 1 study, foals aged between 3 and 13 days (mean 6.4 days) were more susceptible to experimentally induced *R. equi* pneumonia than foals aged between 14 and 36 days (mean 25 days).[87] Collectively, these findings indicate that many foals on endemic farms become infected at a young age and that younger foals are more susceptible to infection caused by *R. equi*. However, these findings do not necessarily indicate that foals are only susceptible to *R. equi* during the neonatal period. Older foals are also susceptible to experimental infection with *R. equi*. In 1 study, intratracheal administration of *R. equi* to 10 foals between 27 and 67 days (mean 49 days) of age resulted in disease in all foals including those receiving a low dose challenge.[21]

Virulence

Twenty years ago, independent laboratories reported the seminal finding that strains of *R. equi* isolated from foals with pneumonia contained a plasmid of 80- to 90-kb in size.[22,23] Subsequently, the association between plasmid possession and virulence was established by demonstrating that plasmid curing or loss yielded a bacterial strain unable to cause disease in mice and foals.[14,21,24] Furthermore, the plasmid was shown to enable intracellular replication in macrophages.[14,25]

Sequencing and annotation of the virulence plasmid revealed 73 coding sequences (CDSs),[26,27] and that it was divisible into 4 discrete areas based upon open reading frame (ORF) amino acid sequence similarity and predicted protein function. The "backbone" sequence of the plasmid is highly similar to that of a plasmid found in the environmental organism *Rhodococcus erythropolis* and consists of regions for replication/partitioning, conjugation, and unknown functions.[26] The 4th plasmid region is an approximately 21-kb pathogenicity island (PAI) that is crucial for virulence of this bacterium for foals.[26,27] The PAI likely was acquired through horizontal gene transfer from a bacterial source of unknown origin, an insertion event which probably took place in the soil and dramatically increased the in vivo survival capabilities of the host bacterium.

The 26 CDSs of the PAI include the unique and *R. equi*-specific family of proteins, the *v*irulence-*a*ssociated *p*rotein family (Vap family).[27] There are 6 full-length *vap* genes, (*vapA*, *-C*, *-D*, *-E*, *-G*, *-H*) and 3-truncated *vap* pseudogenes (*vapF*, *-I*, and *-X*).[26] To date, *vapA*, which encodes an immunodominant, temperature-inducible, and surface-expressed lipoprotein,[28,29] is the

only *vap* gene with a demonstrated role in virulence,[30] whereas Vaps C, D, E, F, G, I, and X appear to be dispensable.[30,31] VapA is required for establishment of a persistent infection in severely immunodeficient mice and for intracellular growth in macrophages,[30] where it aids in preventing maturation of the phagosome to the stage of fusion of *R. equi*-containing vacuoles with lysosomes.[32] The functions of the other Vap proteins are unknown. Interestingly, *R. equi* strains isolated from pigs with lymphadenitis also contain a plasmid with a similar backbone, but distinct PAI-encoded *vap* genes, suggesting that the specific Vaps present somehow dictate host species tropism.[26]

Although *vapA* is necessary for virulence, it is not sufficient.[14] Additional virulence determinants reside within the PAI.[31] Two of these, *virR* and *orf8*, each encode a regulatory protein.[33,34] Loss of either regulator results in decreased *vapA* transcription and also in attenuation of the organism.[33,34]

A 2nd major advance in the understanding of *R. equi* virulence was the recent sequencing and annotation of the genome of *R. equi* strain 103.[35] The 5.04-Mb *R. equi* genome is most similar to that of the environmental bacterium *Rhodococcus jostii* (RHA1) and then to *Nocardia farcinica* and *Mycobacterium tuberculosis*.[35] As with these other Actinomycetes, a large number of the 4,598 genes of *R. equi* appear to be involved in lipid metabolism (both anabolism and catabolism). Like *M. tuberculosis*, lipids are a key component of the outer cell envelope of *R. equi*.[36] This mycolic acid-containing glycolipid barrier might serve to protect the peptidoglycan and plasma membrane from the damaging effects of host-generated enzymes and immune-mediated reactive intermediates. Mycolic acid carbon chain length varies among *R. equi* isolates and, notably, it was observed that strains with longer mycolic acids were more lethal to mice.[37] Furthermore, it has been established that host lipids are a preferred in vivo carbon source for *M. tuberculosis*[38] and such also is likely to be true for *R. equi*. The finding that a mutant of *R. equi* possessing a defective glyoxylate shunt enzyme activity, isocitrate lyase, necessary for growth on fatty acids, was unable to multiply in macrophages and was attenuated in mice and foals, is consistent with this contention.[39]

Analysis of the genome sequence reveals that *R. equi* has 23 complete 2-component regulatory systems (TCS) apart from the PAI-encoded orphan response regulator (*orf8*). TCS represent the most common type of regulation system found in bacteria. The more complicated an organism's lifestyle, the greater is the number of TCS needed to allow for genetic adaptation to specific environmental changes. Recently, it has been determined that the TCS sensor kinase MtrB of *R. equi* is needed for adaptation to and growth within the intramacrophage environment.[40] It is likely that other TCS also participate in the expression of virulence traits.

Some of the most exciting recent data in the *R. equi* field is the recognition of molecular "crosstalk" between the virulence plasmid and the *R. equi* chromosome. Microarray analysis of chromosomal gene expression patterns of the virulence plasmid-containing strain 103 and a plasmid-free derivative were compared under *vap* gene-inducing (37°C; pH 6.5) and noninducing conditions (30°C; pH 8.0) showed that the virulence plasmid enhanced the expression of a number of chromosomal genes when the bacteria were cultured under *vap* gene-inducting conditions.[35] This suggests that a gene or genes on the plasmid directly or indirectly regulate chromosomal gene expression patterns, and that the 2 regulators in the PAI region could be involved. Two chromosomal genes, REQ23860 and REQ23850, encoding a chorismate mutase and a bifunctional anthranilate synthase, respectively (enzymatic components of the aromatic amino acid biosynthesis pathway), were most strongly co-induced with the *vap* genes.[35] Independent mutation of each of these genes decreased the capacity of the bacterium to replicate in macrophages. Interestingly, the *R. equi* genome contains 4 chorismate mutase genes, 1 of which is located in the virulence plasmid PAI region. In general, aromatic amino acids are likely limiting in the macrophage intracellular vacuolar environment, and thus an enhanced ability to synthesize aromatic amino acids via increased utilization of the chorismate precursor could translate to improved intracellular survival.

Although *R. equi* is considered an obligate aerobe, mutation of *narG*, encoding nitrate reductase, was found to be severely attenuating to the pathogenicity of the bacterium in infected mice. [41] The latter finding might indicate an inability of the mutant to assimilate nitrate in vivo in the absence of NarG, or might suggest that *R. equi* uses nitrate as a terminal electron acceptor in the hypoxic and possibly anaerobic granulomatous environment it faces in vivo.

Immunity

Rhodococcus equi-*Phagocytic Cell Interactions*

Once inhaled, *R. equi* is taken up by alveolar macrophages through a process of receptor-mediated phagocytosis. One of the receptors used by macrophages to engulf complement opsonized *R. equi* is complement receptor 3 (CR3 or Mac-1).[42] In addition, *R. equi* might utilize the macrophage mannose receptor for entry which might recognize lipoarabinomannan (LAM), an outer surface component of the bacterium, either directly or via mannose binding protein or surfactant molecules adhered to LAM.[43] Once engulfed by resident macrophages, virulent *R. equi* are able to modify the phagocytic vacuole to prevent acidification and subsequent fusion with lysosomes.[32,44–46] Bacterial gene expression patterns are altered to accommodate survival in the intracellular environment and allow acquisition of essential nutrients such as iron, as well as to promote resistance to host-derived reactive oxygen intermediates.[47,48] Uncontrolled intracellular replication of *R. equi* leads to necrosis of the macrophage.[49] If opsonized with *R. equi*-specific antibody, presumably promoting bacterial entry via the

macrophage Fc receptor, the fate of the *R. equi*-containing phagosome is altered and lysosome fusion occurs.[50] This might explain how the presence of *R. equi* antibody might aid in the prevention of infection. Killing of *R. equi* by mouse macrophages is dependent upon the presence of interferon (IFN)-γ, which activates macrophages to produce both reactive oxygen and reactive nitrogen intermediates. These 2 radicals combine to form peroxynitrite, which efficiently kills *R. equi*.[51] Neither reactive oxygen nor reactive nitrogen intermediates alone are sufficient to mediate killing of *R. equi*.[51] Additional cytokines such as TNF-α might have similar effects on macrophages, because both IFN-γ and TNF-α are both required for clearance of virulent *R. equi* in mice.[52] Neutrophils play an important role in early host defense against virulent *R. equi*.[53] As opposed to macrophages, neutrophils from foals and adult horses are fully able to kill *R equi*.[54–56] As seen with macrophages, killing of *R. equi* by neutrophils is considerably enhanced by specific opsonizing antibody.[57,58]

Adaptive Immunity

Immunity to *R. equi* pneumonia in foals probably depends on both the antibody and cell-mediated components of the immune system, but its exact basis remains to be determined. The strongest evidence for a role of antibody in protection against *R. equi* is the partially protective effect of passively transferred anti-*R. equi* hyperimmune equine plasma (summarized in the consensus statement in this issue). Because of the facultative intracellular nature of *R. equi*, cell-mediated immune mechanisms are thought to be of major importance in resistance to infection (Fig 3). A large part of the knowledge of cell-mediated immunity to *R. equi* infections comes from infection of mice. Deficiencies in the complement component C5 and NK cells in mice do not impair the pulmonary clearance of virulent *R. equi*.[59] In contrast, functional T lymphocytes are absolutely required for the clearance of virulent *R. equi* in mice.[60–62] However, mice lacking functional T lymphocytes clear plasmid-cured derivatives from their lungs within 1 week of infection, suggesting that clearance of avirulent plasmid-negative strains in mice does not require functional lymphocytes and depends mainly on innate defense mechanisms.[61]

The 2 major mechanisms by which T lymphocytes mediate clearance of intracellular pathogens are secretion of cytokines and direct cytotoxicity. Although both $CD4^+$ (helper) and $CD8^+$ (cytotoxic) T cells contribute to host defense against *R. equi* in mice,

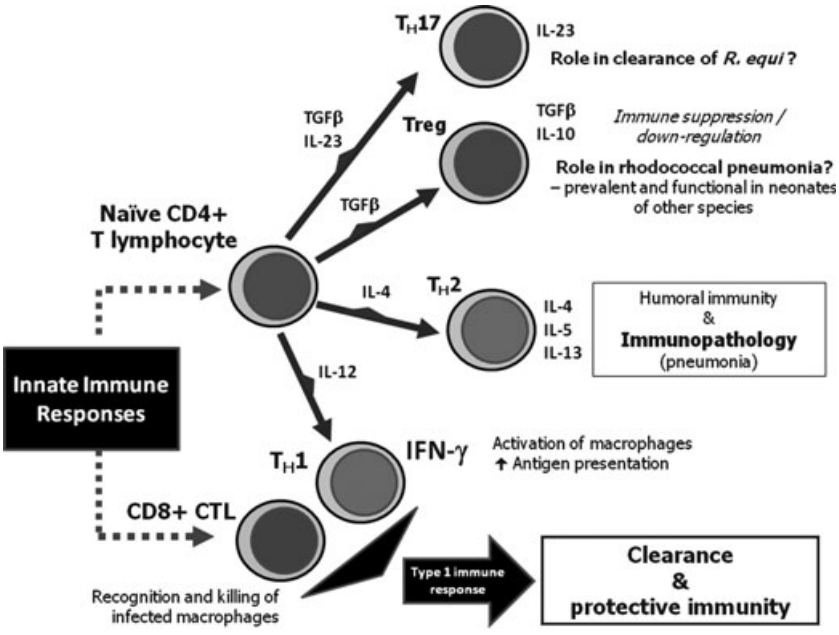

Fig 3. Paradigm for adaptive immunity: The current working hypothesis is that an effective vaccine to prevent rhodococcal pneumonia will need to drive a foal's immune response to *Rhodococcus equi* toward a protective Type 1 response. Type 1 responses are characterized by the production of antigen-specific Th1 lymphocytes, which allow for clearance of intracellular *R. equi* via the production of interferon (IFN)-γ and the activation of macrophages, and by antigen-specific cytotoxic T lymphocytes, which recognize and kill *R. equi*-infected cells. In contrast, foals that respond to infection with a Th2 response are predicted to develop potentially life-threatening pulmonary lesions. As shown via dashed lines, innate immune responses, including the responses of dendritic cells, strongly influence the ensuing adaptive response. The role of suppressive regulatory T cells (Tregs) remains unknown. However, these cells are prevalent and functional in neonates of other species. Likewise, there has been little work to investigate the role of the Th17 subset of T lymphocytes. Th17 cells develop under the influence of interleukin (IL)-23 and TGF-β and are defined by the signature cytokine IL-17. Th17 cells and IL-17 are known to play roles in autoimmune diseases and immune clearance of other intracellular pathogens, and so are likely to be involved in immunity to *R. equi* as well.

$CD4^+$ T lymphocytes probably play the major role and are absolutely required for complete pulmonary clearance.[62–64] 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* whereas a Type 2 response, characterized by IL-4 production, is detrimental.[60,65]

As opposed to foals, adult horses are typically resistant to *R. equi* infections. Immune adult horses have been used as a relevant model to better understand what responses are necessary for immunologic protection. Clearance of virulent *R. equi* in immune adult horses is associated with lymphoproliferative responses to *R. equi* antigens, development of *R. equi*-specific cytotoxic T lymphocytes (CTL), and IFN-γ induction.[66–68] Interestingly, *R. equi*-specific CTL, which are apparently present in all immune adults, are major histocompatibility complex (MHC) class I-unrestricted and appear to recognize unique bacterial lipids from the cell wall.[69,70] The current thought is that these lipid antigens might be presented to T lymphocytes via the CD1 system, as has been well described in *M. tuberculosis*.[71] How these findings in mice and adult horses relate to the foal is an area of active research.

Immunity to R. equi *in Foals*

Virtually all newborn mammals are immunologically immature and have an assortment of immunologic deficits compared to older animals. In general, neonates and perinates have diminished innate immune responses, decreased antigen-presenting cell function, and are less able to mount type-1 immune responses.[72] As a result, neonates of various species demonstrate an increased susceptibility to certain infections.

A number of relative immunologic deficits have been demonstrated in foals. In conjunction with the lack of immunologic memory in newborns, these are postulated to account for the unique age-associated susceptibility of foals to rhodococcal pneumonia. Age-related deficiencies in *R. equi*-specific CTL activity has been documented in 3-week-old foals.[70] Activity of CTLs is improved by 6 weeks of age and is similar to that of adult horses by 8 weeks.[70] Antigen-presenting cells from foals have significantly lower CD1 and MHC class II expression compared to that from adult horses.[71,73] In addition, several studies have demonstrated that the ability of equine lymphocytes and neutrophils to produce or upregulate various cytokines, or both, is strongly influenced by age.[72–77] In particular, the finding that young foals are deficient in their ability to produce IFN-γ in response to mitogens has led to the hypothesis that an IFN-γ deficiency and Th2 bias might be at the basis of their peculiar susceptibility to *R. equi* infections.[74,75] However, recent data demonstrate that foals are also deficient in their ability to produce IL-4 in response to stimulations with mitogens and after vaccination with a killed adjuvanted vaccine, suggesting that a clear polarization toward a Th2 response is unlikely in neonatal foals.[78–80] Consistent with these findings, experimental infection of young foals with virulent *R. equi* results in IFN-γ induction and antibody responses similar to or greater than that of adult horses undergoing the same experimental challenge.[81]

Recent studies have investigated various immunostimulants that might enhance host defense mechanisms during the relatively narrow period of susceptibility to *R. equi*. Inactivated *Parapoxvirus ovis*, *Propionibacterium acnes*, and unmethylated CpGs enhance ex vivo or in vitro phagocytic cell function or cytokine induction in foals.[82–84] However, despite successfully enhancing IFN-γ production in foals, inactivated *P. ovis* failed to decrease the cumulative incidence of pneumonia at an *R. equi*-endemic farm. In the same study, IFN-γ and IL-4 secretion at birth was not associated with subsequent development of pneumonia.[85]

Spontaneous resolution of *R. equi* pneumonia after experimental challenge has been recognized.[86–88] Many foals on farms where the disease is endemic do not develop disease or develop subclinical disease that resolves without intervention. In addition, intragastric administration of live, virulent *R. equi* to newborn foals confers complete protection against subsequent heavy intrabronchial challenge.[89,90] Oral inoculation with virulent *R. equi* results in accelerated development of *R. equi*-specific CTL,[91] providing a potential mechanism for the protection conferred by oral inoculation. Collectively, these findings unequivocally demonstrate that most foals have the ability to mount protective immune responses to *R. equi*. The basis for the peculiar susceptibility of foals to infection with *R. equi* is likely complex and multifactorial rather than involving a simple and single explanation. Similarly, it is unknown why some foals develop EPDs and others do not.

Attempts at Active Immunization

It would be convenient to control *R. equi* pneumonia on endemic farms by active immunization of mares or foals with a protective antigen. To date, however, this approach has been largely unrewarding. The role of antibody in partial protection against *R. equi* infection would suggest that vaccination of mares could confer at least some degree of protection. However, in both a field study and an experimental challenge, vaccination of mares did not provide protection against *R. equi* pneumonia despite a significant increase in colostral *R. equi*-specific antibody and transfer of these antibodies to foals.[92,93] More recently, vaccination of a small number of mares with VapA associated with a water-based nanoparticle adjuvant led to high anti-VapA IgG concentrations in mares and foals and might have conferred protection against natural challenge compared to nonvaccinated controls.[94] Large-scale studies at endemic farms will be necessary to confirm these preliminary findings before widespread vaccination of mares can be recommended.

Because cell-mediated immunity is of paramount importance for protection against *R. equi*, active

immunization of foals probably will be required for complete protection. Oral immunization with *Salmonella enterica* Typhimurium expressing the VapA antigen protects mice against *R. equi* infection.[95] Recent studies in mice indicate that DNA immunization with *vapA* protects against *R. equi* infection and that the IgG subisotype response is consistent with a Th1-based immune response.[96] A similar DNA vaccine containing the *vapA* gene has been shown to induce strong cell-mediated immune responses in adult horses, but responses were poor in foals.[97] Intrabronchial immunization of neonatal foals with a live, fully attenuated, riboflavin auxotrophic strain of *R. equi* stimulated immune responses but did not confer protection against subsequent intrabronchial challenge with live *R. equi*.[98] The fact that live, avirulent plasmid-cured *R. equi* does not elicit adaptive immunity and is cleared rapidly indicates that replication is needed for induction of strong cell-mediated immune responses.[66] Intrabronchial immunization with a deletion mutant of *R. equi* lacking the chromosomal genes isocitrate lyase (*icl*) and cholesterol oxidase (*choE*) conferred protection against subsequent challenge in 3 foals.[99] However, 2 foals developed pneumonia caused by the mutant strain.[99] Collectively, the aforementioned studies indicate that there is a fine line between sufficient replication of *R. equi* for induction of strong cell-mediated immune responses and disease from the vaccine strain. Additional challenges are that immunization in foals will need to be initiated very early in life and an effective vaccine will have to overcome the relative immaturity of the naive neonatal immune system.

There are several critical areas in need of further study to develop a vaccine against *R. equi*, including (1) determining immunologic correlates of protection; (2) defining limitations of foal immunity; and (3) specifying antigens that confer protective immunity. These 3 issues are considered below.

Although much has been learned from mice and immune adult horses, the immunologic correlates of protection against *R. equi* in foals (ie, "the responses an effective vaccine must induce in foals") are only vaguely defined. Moreover, foals are clearly different from both mice and adult horses in their inherent susceptibility, immunologic capabilities (discussed above), and lack of immunologic memory. An important need that remains is a broad and cost-effective repertoire of standardized immunologic tests to measure the responses of horses to infection and immunization. With these tools, veterinary scientists could better define what constitutes a protective response and measure the ability of new vaccines to induce those responses in foals. Immunogens could then be designed or modified to induce better protection.

Researchers have begun to define the immunologic capabilities of neonatal and perinatal foals. The primary focus has been on immunologic deficits that might contribute to susceptibility to *R. equi* infection or that are likely obstacles to vaccination of horses early in life. A problem with these studies is that most examine transcription of cytokine mRNA, rather than protein expression. Moreover, differences among studies relative to the nature of the immune stimuli, timepoints examined, and methods make comparison difficult. As a result, the relevance of these data to what occurs *in vivo* is not always clear. As noted above, improved tools to measure relevant immune responses in standardized ways would be a significant step forward. Perhaps more importantly, there needs to be more research focused on methods to induce strong innate and Type 1 immune responses in neonatal foals. In other words, we must devise means to overcome the inherent age-associated immune limitations of early life. This might involve investigation of new immunostimulants, improved adjuvants, and novel approaches such as targeting the common mucosal immune system via the gut.

We still have little knowledge of which bacterial antigens are targets of protective immune responses. Although much work has focused on proteins encoded by the virulence plasmid, it remains to be determined whether or not a subunit approach can be successful. Given the complicated life-style of this bacterium and the complexity of the organism, a modified live vaccine might prove to be more appropriate. For example, evidence exists that immune adult horses recognize secreted antigens of *R. equi*.[100] Moreover, all immune adult horses have CTL that appear to recognize unique lipid antigens in the bacterial cell wall.[68,69] A live attenuated vaccine could provide for at least limited intracellular replication, expression of selected *vap* genes, translation of secreted proteins, and immune presentation of bacterial lipids. Newer methods for genetic manipulation of *R. equi* are providing a means to strategically produce mutants that can be tested for their ability to induce protective responses in foals.[98,101] The goal will be to generate strains that are attenuated enough to be safe and yet immunogenic enough to protect against challenge. In summary, active immunization to prevent rhodococcal pneumonia remains a very real possibility, but additional research is needed.

Summary

Rhodococcal infection in foals is a complicated problem. An ill-defined proportion of foals with pulmonary infection will remain free of clinical signs and eventually clear the infection, whereas other foals might have either an insidiously progressive pneumonia or an acute onset of severe respiratory distress that is generally fatal. A wide array of EPDs can occur either alone or concurrent with pneumonia. Some EPDs (eg, intra-abdominal abscesses, osteomyelitis) markedly worsen the prognosis for survival, and can develop in the face of successful management of pneumonia. There is great need to develop means for preventing disease. Understanding the mechanisms of virulence of *R. equi* is critical for developing preventive strategies. It is clear that interactions occur between gene products of the virulence-associated plasmid and chromosomal gene products that modulate virulence.

The picture that has emerged is one of a soil bacterium whose disease-causing capabilities were dramatically expanded through the acquisition of foreign DNA that provided critical novel virulence factors and further altered the expression of pre-existing chromosomal genes to promote adaptation as a pathogen of foals. These virulence factors represent target candidates for developing live attenuated vaccines. Finally, development of a vaccine against *R. equi* is dependent upon stimulating an effective immune response to key antigens of the organism. Unfortunately, an effective vaccine remains elusive because of the complexity of the immune response to *R. equi* and the challenges posed by limitations of some elements of the immune response of young foals. Despite these challenges, there are observational and experimental data that allow hope for the development of an effective vaccine. A recent manuscript indicates that genes involved in the steroid catabolic pathway are promising targets for the development of a live-attenuated vaccine against *R. equi* infections.[102]

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