**Rhodococcus equi:** Pathogenesis and Virulence

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Inhalation of the facultative intracellular bacterium, Rhodococcus equi, can result in a severe pyogranulomatous pneumonia in young horses and immunocompromised people. This organism is able to resist innate immune defenses and establish residence within the intracellular environment of the alveolar macrophage. Virulence determinants of *R. equi* have yet to be fully elucidated, but all equine isolates possess a large virulence-associated plasmid and express the plasmid-encoded surface lipoprotein, VapA. Furthermore, experimental evidence has confirmed that an intact cellular immune response is necessary for clearance of the organism. Author's address: Howard Hughes Medical Research Institute, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461. © 1997 AAEP.

1. Introduction

*Rhodococcus equi* was originally isolated from pulmonary lesions of foals by Magnusson in 1923. It has since become a well-established veterinary pathogen, and recently it has become familiar to physicians as an opportunistic pathogen of immunocompromised people. This facultative intracellular bacterium inhabits the alveolar macrophages (Fig. 1) of infected animals and humans, replicates there, and can cause a potentially life-threatening pyogranulomatous pneumonia. Although much research remains to be done, considerable progress has been made in the past few years regarding the understanding of the pathogenesis of rhodococcal disease. Much of the research contributing to that understanding is discussed in this review.

2. Epidemiological and Clinical Aspects of *R. equi* Infection

Foals younger than 6 months of age are susceptible to the development of rhodococcal disease, with the majority of cases occurring in those animals younger than 3 months. The occasional report of *R. equi* disease in the adult horse has occurred primarily in immunocompromised animals or those with concurrent systemic illness. Most cases of *R. equi* pneumonia occur during the summer months, a time that corresponds with the peak of foal susceptibility and with optimal conditions for environmental bacterial multiplication.* R. equi* is a common inhabitant of the soil, and inhalation of dust is believed to be the primary route of exposure for both animals and man. Experimental aerosolization and intrabronchial inoculation of bacteria in foals produce pulmonary lesions that mirror those of natural infection. *R. equi* disease is insidious in its onset, ultimately leading to extensive bronchopneumonia and the development of multiple lung abscesses of varying sizes. Initial lung lesions are characterized by macrophage, multinucleate giant cell, and to a lesser degree, a neutrophilic influx into the alveolar spaces. In addition, morphologically intact bacteria can be commonly found within the macrophages and giant cells. As the
disease advances, necrosis and destruction of the lung parenchyma occur and numerous degenerative bacteria-laden macrophages can be observed at this time.

Ulcerative colitis or mesenteric lymphadenitis occurs in approximately half of all foals with clinical R. equi pneumonia.\(^4\) The intestinal disease, which manifests as diarrhea, rarely occurs in the absence of pneumonia, and it probably stems from the ingestion of large amounts of bacterially contaminated sputum. The disease originates in the Peyer’s patches, which become ulcerated and destroyed, and with time there is significant mesenteric lymph node involvement. Repeated experimental intragastric inoculation of large numbers of bacteria results in intestinal lesions characterized by pyogranulomatous inflammation of the bowel.\(^10\) However, in cases of natural infection, the ingestion of smaller numbers of R. equi most likely leads to subclinical colonization and infection of the intestinal tract.\(^11\) Intraluminal intestinal bacterial replication in foals has been reported\(^11\) and may give rise to subsequent measurable immune responses.\(^12,13\)

R. equi may disseminate from the lung to other body sites; septic arthritis, serositis, and intervertebral abscessation have been observed.\(^4\) Occasionally, R. equi infection can present as cutaneous ulcerative lymphangitis, a condition likely stemming from bacterial contamination of a pre-existing wound.\(^14,15\)

In swine, R. equi can cause chronic cervical lymphadenitis,\(^16,17\) but the organism can also be isolated with similar frequency from the lymph nodes and tonsils of otherwise healthy pigs.\(^16\) R. equi infection of additional species has been described, but generally the infection is considered extremely rare in these species, and lesions are typically confined to lymph node abscessation or wound infection. Pneumonia is almost never a manifestation of disease in the nonequine animal species.

Under most circumstances, R. equi poses no danger to humans. However, in people with a compromised immune function, such as those individuals with AIDS or cancer, the organism can be fatal.\(^2,18,19\) The first reported human infection involved a patient who developed R. equi pneumonia while undergoing corticosteroid therapy for chronic hepatitis.\(^20\) Since then, steadily increasing numbers of cases have been documented; almost all occurred in individuals with compromised immunity, particularly those with HIV infection.\(^2,18\) As in foals, pneumonia is the predominant clinical manifestation of R. equi infection in humans,\(^18\) and histopathological findings are similar in both species.\(^21,22\) Rarely can the source of exposure for these individuals be deter-
mined, and most often these people do not report contact with horses or to areas upon which they have grazed. However, because *R. equi* is a common soil inhabitant, environmental exposure must be considered likely in cases of human disease.

3. Experimental in vivo Infection Studies

The establishment of an ideal laboratory animal model of *R. equi* disease has been hampered by the fact that an experimental infection of animal species other than the foal does not reproduce the lesions typical of natural infection in the horse and human. For example, although an experimental infection of piglets results in the development of interstitial pneumonia characterized by macrophage infiltration, the lesions never progress to abscessation, and they resolve quickly. Likewise, the immunocompetent mouse is fairly resistant to *R. equi* challenge, but nevertheless studies in this species have revealed differences in virulence among strains of the bacteria and have furthered our understanding of the specific immune system components essential in the protection against *R. equi* disease. Bacterial clearance and lesion development following intrabronchial challenge in immunocompetent mice have been observed to be dose dependent, with larger bacterial inocula resulting in slower pulmonary clearance and more extensive bronchopneumonia. However, the bronchopneumonia will resolve and ultimately the infection is completely cleared. In contrast, cyclophosphamide treatment of *R. equi*-infected mice results in an increased bacterial burden and mortality. In addition, immunosuppression of mice by either cyclophosphamide or cortisone acetate treatment, followed by intranasal challenge with *R. equi*, has led to the development of lesions analogous to those found in natural infection in foals. These studies show that a compromised immune function is associated with an increased lethality of *R. equi* in mice, a hypothesis substantiated by findings that demonstrate that T-cell deficient nude (nu/nu) mice and severe combined immunodeficient (SCID) mice are exceptionally susceptible to progressive *R. equi* disease. Compromised cell mediated immunity is an important factor in human cases of *R. equi* disease, and it may play a role in the development of the disease in foals as well.

4. Cell Biology of Infection

The typical pulmonary lesion associated with *R. equi* disease is one of pyogranulomatous bronchopneumonia with abscessation, in which a heavy neutrophilic infiltration occurs. Both foal and adult equine polymorphonuclear leukocytes have been shown to effectively phagocytize and kill virulent *R. equi* in vitro. In addition, specific opsonizing antibody has been demonstrated to enhance the killing capacity of this phagocyte.

In contrast, macrophages are often less able to cope with virulent *R. equi*. Several independent researchers have provided evidence that *R. equi* can persist within macrophages in vitro. Zink and colleagues performed in vitro infectivity studies by using alveolar macrophages of foals and discovered that a large percentage of bacteria associated with the macrophages were viable 4 h postinfection. Precisely how *R. equi* is able to resist killing by macrophages remains a mystery. Brumbaugh et al. reported that the phagocytosis of *R. equi* by equine macrophages is not associated with a functional respiratory burst. Two groups have used electron microscopy to demonstrate a correlation between intracellular persistence and an absence of phagosome–lysosome fusion. Both groups could observe replicative forms of the bacteria within primary unfused phagosomes. The implication of these studies is that *R. equi* is able to affect the maturation process of the phagosome in a manner similar to that of the intracellular organisms, *Mycobacterium* spp., *Nocardia asteroides*, and *Legionella pneumophila*. Of particular note, prior antibody opsonization was associated with an enhancement of phagosome–lysosome fusion, suggesting that the mechanism of cellular entry could affect the subsequent fate of the bacteria.

It has been demonstrated that virulent *R. equi* isolates can replicate in vitro in nonactivated macrophages of both equine and murine origin (Fig. 2). Following an initial lag phase of several hours, the intracellular doubling time of *R. equi* was determined to be approximately 6–8 h, and replication appeared to be confined within membrane enclosed vacuoles. In contrast, avirulent strains fail to replicate intracellularly within cultured macrophages in vitro. Though able to persist, the avirulent organism does not give rise to the large numbers of bacteria that are observed with the virulent equine isolates. Consistent with these results is the report that virulent human isolates of *R. equi* are more resistant to in vitro macrophage killing than are avirulent strains.

It is likely that the activation status of the macrophage can influence whether *R. equi* will grow intracellularly or not. Tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) are two cytokines whose actions serve to activate macrophages and to increase the microbicidal function of the phagocyte. It has been shown that supernatants of foal lymphocyte cultures stimulated with *R. equi* antigens enhance macrophage killing of intracellular *R. equi*. In addition, spleen cells from noninfected mice were demonstrated to produce TNF-α in vitro in a dose-dependent manner when incubated with *R. equi*, and the amount produced was greater in response to live versus killed organisms. Furthermore, treatment of immunocompetent mice with anti-IFN-γ or anti-TNF-α antibodies significantly compromised *R. equi* clearance mechanisms, yielding higher bacterial organ counts postchallenge. Thus the experimental evidence would seem to indicate that an intact cellular immune response includ-
Fig. 2. Fluorescence microscopy of a methanol-fixed murine peritoneal macrophage monolayer, following infection with virulent *R. equi*. Murine peritoneal macrophage monolayers at (a) 1 h, (b) 48 h postinfection with *R. equi*. Bacteria were immunofluorescently stained with rabbit polyclonal anti-*R. equi* antiserum and fluorescein isothiocyanate-conjugated goat antirabbit immunoglobulin G. Bacteria appear as bright spots. Magnification 1000×, oil emersion.
ing efficient activated macrophage function is necessary to prevent intracellular replication and promote host clearance of R. equi.

R. equi infection ultimately proves toxic for macrophages with many cells sustaining irreversible damage. Macrophage degeneration in vitro is apparent by 8 h postinfection and is marked by 24 h.35,38 Hietala and Ardans35 speculated that nonspecific degranulation of lysosomes, concomitant with the release of lysosomal contents into the cellular cytosol, may result in the reduced macrophage viability observed. Nordmann and colleagues44 stated that cellular toxicity was a property confined to virulent strains, and furthermore they reported that supernatants from cultures of the virulent isolates were toxic for epithelial and fibroblast cell lines.

5. Bacterial Virulence Determinants

Despite widespread environmental distribution, R. equi disease is not recognized on most horse farms; the infection is endemic to some and occurs occasionally in others,48 a finding that may perhaps relate to bacterial strain virulence. Generally, clinical isolates are more virulent than environmental isolates,49 and strains originating from the soil of farms with endemic rhodococcal disease are more likely to be virulent than those isolated from the soil of farms without a history of R. equi pneumonia.50 In addition, experimental infectivity studies have identified differences in virulence among isolates of R. equi.8,43,51,52

Putative virulence factors of R. equi include capsular polysaccharide, the exoenzyme cholesterol oxidase, cell wall mycolic acids, and the products encoded by a large virulence-associated plasmid. The polysaccharide capsule of R. equi may contribute to its virulence by interfering with the ability of leukocytes to phagocytose the bacterium,53 as has been reported for other encapsulated organisms.34,55 There is extensive heterogeneity of capsular antigens (serotypes 1–27) among strains of R. equi,56,57 with capsular serotype 1 exhibiting the most prevalence worldwide. There is no apparent relationship between capsular serotype and the source of a bacterial isolate; identical serotypes can be identified from a variety of sources.56 Currently, it is believed that the variability in serotypes found on farms more likely reflects geographic differences rather than virulence differences.57 A recent study demonstrated that a particular capsular serotype was not directly associated with virulence, as both avirulent and virulent isolates of the same serotype were identified,58 and thus the polysaccharide capsule may be necessary but not sufficient for virulence.

The exoenzyme cholesterol oxidase produced by R. equi has been termed the equi factor, and it is another candidate virulence determinant. In the early 1980’s, Linder and Bernheimer59 were able to demonstrate in vitro synergistic hemolysis of sheep erythrocytes by using the cholesterol oxidase produced by R. equi coupled with phospholipase D made by Corynebacterium pseudotuberculosis. This membranolytic activity may bestow some advantage to the bacterium, or perhaps it contributes to the macrophage degeneration that has been observed both with in vitro35,38,43 and in vivo infection.9 However, as both virulent and avirulent strains of R. equi secrete this enzyme, its direct role in disease pathogenesis is speculative at present.60

Recent studies have suggested that mycolic acid-containing glycolipid, a major cell wall component of Rhodococcus spp., may influence virulence. Mice infected with strains of R. equi expressing longer carbon chain mycolic acids developed more granulomas and were more likely to die from the infection than mice infected with isolates of shorter carbon chain mycolic acid.61 Furthermore, intravenous inoculation of purified glycolipid yielded similar results.61 This granuloma-inducing capacity of R. equi glycolipid is similar to that found with the glycolipids of Nocardia spp., in which granuloma formation and disease severity correlates quite well with mycolic acid chain length.62,63 Furthermore, it is of particular interest that strains of R. equi isolated from the abscessed lymph nodes of infected pigs were found to contain longer chain mycolic acid, whereas strains isolated from normal lymph nodes or tonsils of healthy pigs expressed the shorter carbon chain mycolic acids.64 Thus the synthesis of long chain mycolic acids is a feature that may increase the virulence of the organism.

It had been observed that many clinical equine isolates of R. equi expressed 15-kd and 17.5-kd proteins, which were found to be absent in a non-pathogenic type of strain.64 Subsequently, Takai and colleagues58 discovered that all isolates that were virulent for mice expressed these protein antigens. Furthermore, they showed that sera obtained from foals naturally infected with R. equi reacted to proteins of this molecular weight, whereas sera from healthy foals did not. Both Takai et al.65 and Tkachuk-Saad and Prescott66 independently published reports establishing that the equine isolates of R. equi that expressed these antigens also possessed a large plasmid of approximately 85 kilobases. Further studies demonstrated that strains cured of the plasmid by repeated passage in culture no longer expressed the antigens and were rendered avirulent for both mice65 and foals.67 The gene encoding the 15- to 17-kd antigens has been cloned (termed VapA), and its location on the plasmid has been confirmed.68 The product of this single gene gives rise to at least three differentially lipid-modified, antigenically related proteins of different molecular weight.

It has been recently demonstrated through studies performed in mice that R. equi replication and granuloma formation in vivo is correlated with plasmid presence.69,80 In addition, through the examination of isogenic strain pairs of bacteria, differing only with regard to plasmid possession, it has been established that intramacrophage replication is restricted to the plasmid-positive strains. The plas-
mid cured, VapA-negative isogenic strains do not exhibit any perceptible growth in macrophages in vitro.\textsuperscript{43,a} At the present time, the function of the VapA protein is unknown, and whether it has any true role in virulence or serves merely as a marker for virulence must be established by specific VapA gene knockout experiments. However, because VapA is expressed during intracellular infection, its putative role in virulence is even more enticing (Fig. 3).

From the limited number of studies centering on an analysis of strains of \textit{R. equi} isolated from humans, it has become apparent that the pathogenesis of \textit{R. equi} infection in immunocompromised people may be different from that of foals. The importance of the virulence-associated plasmid in human infection is less well defined. In a survey of 39 isolates of \textit{R. equi} from immunocompromised human patients with and without AIDS, only eight strains were found to contain a plasmid of approximately 85 kilobases, to express the 15- to 17-kd virulence-related VapA antigens, and be virulent in mice.\textsuperscript{67} Twelve did not carry a detectable plasmid, but 19 isolates possessed cryptic plasmids of varying size. A further analysis of these 19 strains\textsuperscript{69} demonstrated that the majority of these plasmids shared DNA homology at the plasmid level and produced a plasmid-associated 20-kd protein. These strains were of intermediate virulence in mice, because their \(\text{LD}_{50}\) values were tenfold greater than VapA-expressing isolates. In addition, plasmid DNA cross-hybridization studies indicated a relatedness with that of VapA-encoding plasmids,\textsuperscript{69} suggesting the possibility of shared virulence determinants. Thus, in contrast to the foal, in which all clinical isolates contain a large plasmid, express VapA antigens, and are virulent for mice, \textit{R. equi} strains that infect humans display greater diversity and exhibit a broader range in virulence. Therefore, it is certainly plausible that the severely immune-deficient status of the patient permits lesser virulent organisms to persist.

Nordmann and colleagues\textsuperscript{47,70} have studied a small number of non-plasmid-containing \textit{R. equi} strains isolated from humans, and they have reported that \(\beta\)-lactam antibiotic resistance is linked to virulence. The mechanism of \(\beta\)-lactam resistance is unclear, as the organisms do not exhibit \(\beta\)-lactamase activity; nor are their penicillin-binding protein profiles different from susceptible phenotypes. Interestingly though, the \(\beta\)-lactam-resistant strains possess cell surface-associated appendages that appear to resemble the tails of bacteriophage, structures not observed in the \(\beta\)-lactam susceptible strains. In addition, bacteriophage-like particles (PLP's) were detected in the culture supernatants of all appendage-producing strains.\textsuperscript{70} However, attempts to use culture filtrates to transfer \(\beta\)-lactam resistance to the \(\beta\)-lactam-susceptible strains have been unsuccessful, and thus it is currently unknown whether the PLP's actually confer \(\beta\)-lactam resistance. Obviously further research is needed to identify the nature of the characteristic surface material in these strains. It is of interest that two animal strains of \textit{R. equi} were also found to be \(\beta\)-lactam resistant, exhibit cell surface-associated appendages, produce PLP, and cause chronic infection in nude mice.\textsuperscript{70} However, these two strains also carried a large plasmid and produced VapA proteins.\textsuperscript{65} At present, it is unclear whether distinct species-specific virulence determinants exist or whether simply the severely immunocompromised status of the majority of people with \textit{R. equi} disease allows even relatively avirulent organisms to endure and produce disease.

6. Future Research

The emergence of \textit{R. equi} as an opportunistic pathogen of humans is somewhat of a double-edged sword. We in the veterinary field have long realized the pathogenic potential of this bacterium and have often been frustrated by its continued presence on certain horse farms. Its recent appearance as a cause of chronic granulomatous pneumonia in immunocompromised people, although tragic in the immediate sense, has fostered an expanded awareness of and interest in this pathogen. Through increased research, the veterinary profession and the equine industry will surely be the benefactors of this en-

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**Fig. 3.** Expression of VapA during intracellular infection. Virulent \textit{R. equi} were incubated with murine peritoneal macrophages; nonphagocytosed organisms were removed by subsequent washing of the monolayer. At 24 h postinfection, the monolayers were fixed with methanol and stained by indirect immunofluorescence by using monoclonal antibody against VapA protein. Monolayers were analyzed by confocal microscopy. Macrophages appear red; bacteria are green. (Photograph taken by T. Darrah.)

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hanced interest. The need for an equine vaccine is clearly evident. However, in order to develop an effective vaccine, we must identify the bacterial factors responsible for promoting virulence and allowing the intracellular survival of the bacterium, and we must determine the specific host components of a protective immune response. Thus future research on R. equi will focus on (1) defining the role of VapA in virulence, (2) identifying additional proteins encoded by the virulence plasmid and determining their function, (3) evaluating whether the virulence determinants are specific to equine isolates, (4) evaluating both human and animal isolates for the presence of PLP and determining whether they truly have any role in pathogenesis, and (5) establishing whether any of the putative virulence factors are protective antigens and therefore worthy of consideration as vaccine candidates.

References and Footnotes