Effects of opsonization of Rhodococcus equi on bacterial viability and phagocyte activation

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**Objective**—To investigate the effect of opsonization of Rhodococcus equi with R equi-specific antibodies in plasma on bacterial viability and phagocyte activation in a cell culture model of infection.

**Sample**—Neutrophils and monocyte-derived macrophages from 6 healthy 1-week-old foals and 1 adult horse.

**Procedures**—Foal and adult horse phagocytes were incubated with either opsonized or nonopsonized bacteria. Opsonization was achieved by use of plasma containing high or low concentrations of R equi-specific antibodies. Phagocyte oxidative burst activity was measured by use of flow cytometry, and macrophage tumor necrosis factor (TNF)-α production was measured via an ELISA. Extracellular and intracellular bacterial viability was measured with a novel R equi–luciferase construct that used a luminometer.

**Results**—Opsonized bacteria increased oxidative burst activity in adult horse phagocytes, and neutrophil activity was dependent on the concentration of specific antibody. Secretion of TNF-α was higher in macrophages infected with opsonized bacteria. Opsonization had no significant effect on bacterial viability in macrophages; however, extracellular bacterial viability was decreased in broth containing plasma with R equi-specific antibodies, compared with viability in broth alone.

**Conclusions and Clinical Relevance**—The use of plasma enriched with specific antibodies for the opsonization of R equi increased the activation of phagocytes and decreased bacterial viability in the extracellular space. Although opsonized R equi increased TNF-α secretion and oxidative burst in macrophages, additional factors may be necessary for effective intracellular bacterial killing. These data have suggested a possible role of plasma antibody in protection of foals from R equi pneumonia. (Am J Vet Res 2011;72:1465–1475)

Foals are often exposed to Rhodococcus equi in the first few days to weeks after birth.1,2 A common cause of bacterial pneumonia in foals, R equi infection has also been implicated in the development of osteomyelitis, mesenteric abscesses, enteritis, and infections of other anatomic sites.3 Rhodococcus equi is a gram-positive facultative intracellular bacterium that belongs to the mycolata taxon, along with Mycobacterium, Coryne-

**ABBREVIATIONS**

- HBSS: Hank’s balanced salt solution
- IL: Interleukin
- MOI: Multiplicity of infection
- OD: Optical density
- TNF: Tumor necrosis factor

bacterium, Nocardia, Gordonia, and Tsukamurella spp.4 Mycolata are named for the mycolic acids (long fatty acids) in the lipid-rich cell wall envelope of these bacteria. These mycolic acids form protective barriers that increase resistance to chemical damage, dehydration, oxidative stress, and conditions of low pH.5 Similar to several other mycolata, R equi has evolved a mechanism to escape bactericidal activity in macrophages and instead establish a replicative niche in cells that function successfully in the destruction of other microbes.6 Phagosomes containing virulent R equi do not progress into endocytic organelles that fuse with lysosomes and fail to acidify because of a lack of acquisition of the proton-pumping vacuolar ATPase.7,8 Colonization of host macrophages by R equi depends on the presence of a thermoregulated large plasmid, which encodes a family of 8 virulence-associated proteins (VapA and VapC to VapI).9–12 This plasmid is critical for intracellular
Replication of *R. equi* within macrophages and for the development of *R. equi*-associated disease in foals. *Rhodococcus equi* that lack the virulent plasmid replicate poorly inside macrophages and consequently do not colonize and induce disease. *Rhodococcus equi*-associated disease typically appears within 4 to 6 months after birth and rarely develops in older foals and adult horses unless immunodeficiency is present. Results of several studies involving experimental *R. equi* infection of foals indicate that the respiratory tract is the most likely route of exposure for infections resulting in pulmonary disease. Intragastric inoculation with large numbers of organisms does not induce the typical pulmonary lesions and may result in protective immunity. During the establishment of an *R. equi*-associated pulmonary infection, hypercellularity of the alveolar septa typically develops, and alveoli and terminal bronchioles become infiltrated with neutrophils, macrophages, giant cells, and numerous cell-associated bacteria.

*Rhodococcus equi* affects immunocompromised or immunologically naive individuals, but immunocompetent individuals are spared, and much research has focused on elucidating the reasons for this predilection. Although age-dependent developmental limitations of the immune system of foals are suspected to be involved, recent studies have revealed that foals can elaborate a T helper 1-cell–type immune response against *R. equi* infection. This is the type of protective immunity that adult horses and mice develop following experimental infection. However, this acquired immunity requires time to become established and a longer period of susceptibility in foals may favor pathogen replication.

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 cytokines and chemokines. Removal of neutrophil function has been shown to result in more severe disease and greater *R. equi* replication in mice experimentally infected with that organism. In contrast to macrophages, foal neutrophils are bactericidal against opsonized bacteria. *Rhodococcus equi* affects immunocompromised or immunologically naive individuals, but immunocompetent individuals are spared, and much research has focused on elucidating the reasons for this predilection. Although age-dependent developmental limitations of the immune system of foals are suspected to be involved, recent studies have revealed that foals can elaborate a T helper 1-cell–type immune response against *R. equi* infection. This is the type of protective immunity that adult horses and mice develop following experimental infection. However, this acquired immunity requires time to become established and a longer period of susceptibility in foals may favor pathogen replication.

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Under natural conditions following pathogen exposure, serum *R. equi*-specific IgG is detected in healthy foals, foals with clinical signs of pneumonia, and healthy adult horses. Yet the passive transfer of immunoglobulins via colostrum or plasma transfusion could be helpful in providing initial protection in the airways before *R. equi* reaches the intracellular environment and until a foal’s own cellular and humoral immunity develop. Few studies have investigated the effectiveness of antibodies transferred through colostrum ingestion in preventing development of *R. equi* pneumonia in foals. Although results of the studies performed in controlled conditions were somewhat favorable, individual differences in humoral response to vaccines, quality of colostrum (antibody concentration), and factors involved in adequate passive transfer of immunoglobulins put a percentage of foals at risk for disease. A more effective means to standardize the transfer of antibodies is via IV transfusion of hyperimmune plasma; however, there appears to be disparity in the apparent efficiency of plasma transfusion in disease prevention.

In equine neonates infected experimentally with *R. equi*, an IV plasma transfusion containing antibodies against *R. equi* or VapA administered before infectious challenge aided significantly in the recovery from disease. Perkins et al suggested that IV transfusions of plasma with low *R. equi* antibody titers were comparable to IV transfusions of hyperimmune plasma with regard to decreasing the severity of disease in experimentally infected foals, although a placebo-treated control group was not evaluated. However, in naturally infected foals, IV administration of hyperimmune plasma is associated with a decrease in the incidence of pneumonia or no effect. The mechanism of protection associated with IV administration of hyperimmune plasma has not been fully described. It is possible that *R. equi*-specific antibodies diffuse into the airways and opsonize the microorganisms before they reach the intracellular environment.

The purpose of the study reported here was to investigate the effect of opsonization of *R. equi* with *R. equi*-specific antibodies on bacterial viability and phagocyte activation in a cell culture model of infection. On the basis of results of previous studies, we proposed that opsonization with *R. equi*-specific antibodies would increase the phagocytic response to *R. equi*, thereby decreasing intracellular bacterial viability. More specifically, our intent was to investigate the effects of opsonization with a plasma preparation containing a high concentration of *R. equi*-specific antibodies that is currently used prophylactically in foals. The effects of opsonization on respiratory burst activity in neutrophils and macrophages, the release of TNF-α from macrophages, and the degree of bacterial viability within macrophages were measured. We also proposed that *R. equi*-specific antibodies would directly decrease bacterial viability prior to phagocytosis, which would provide new insights about the pathogenicity of this bacterium, the role of humoral immunity in protection, and the use of this method of prophylaxis in foals.

**Materials and Methods**

Blood samples from foals and adult horses—The study was conducted following a protocol approved by the Cornell University Center for Animal Resources and Education and regulations mandated by the Institutional Animal Care and Use Committee. Six healthy
foals (3 females and 3 males) of various breeds (2 Thoroughbreds and 1 each of Bavarian, Westphalian, Oldenburger, and Holsteiner) and 1 adult horse (18-year-old Thoroughbred gelding) belonging to the Cornell University Equine Park were used for blood sample collection. All parturitions were observed, and the successful absorption of colostral immunoglobulins was assessed by use of a test for circulating IgG within 14 hours after birth. Physical examination, including measurements of rectal temperature, heart rate, and respiratory rate, was performed daily during the study period to evaluate potential inflammatory or infectious conditions. Mares and foals received conventional pasture management and feeding. The mares had been vaccinated against Eastern and Western equine encephalitis, tetanus, and influenza 30 days prior to the expected foaling date. From each foal, a 60-mL blood sample was obtained via jugular venipuncture once and collected into an evacuated tube containing sodium heparin and kept cold until cell isolation. Blood sample collection from 6 foals was performed between 5 and 7 days after birth once and from the adult horse as needed for replicates (n = 4 to 6).

Isolation of equine neutrophils and monocyte-derived macrophages—Neutrophils were isolated from blood samples obtained from each foal and from the adult horse; only adult horse monocyte-derived macrophages were isolated in culture. Adult horse cell experiments were prepared in replicates. For neutrophil isolation, 10 mL of whole heparinized blood was layered over density gradient solution and centrifuged at 150 ×g most RBCs. Plasma containing leukocytes was then diluted in cold HBSS, counted by use of a hemocytometer, and stored on ice pending the oxidative burst activity determinations.

For monocyte-derived macrophage isolation, 50 mL of whole heparinized blood was kept at room temperature for 20 minutes to allow sedimentation of most RBCs. Plasma containing leukocytes was then centrifuged at 150 × g at 4°C for 10 minutes, and pelleted cells were resuspended in PBS solution. The cell solution was layered over density gradient solution and centrifuged at 700 × g for 15 minutes. The cloudy interface layer was collected, and cells were washed 3 times with PBS solution at 150 × g for 10 minutes. The cell pellet was resuspended in a mixture of Dulbecco modified Eagle medium and F12 nutrient medium with 10% bovine serum and 1× antimicrobials-antimycotics (penicillin, streptomycin, and amphotericin B) combination. Monocytes were counted, and approximately 5 × 10^6 cells/well were allowed to adhere in 24-well culture-treated plates for 2 hours at 37°C in 5% CO₂. Loosely adherent and nonadherent cells were removed by use of warm PBS solution washes. Numbers of adherent cells were estimated by counting the nonadherent cells removed with PBS solution washes; in general, 15% to 20% of cells adhered to the plates. One milliliter of the mixture of Dulbecco modified Eagle medium and F12 nutrient medium with 10% bovine calf serum without the antimicrobials-antimycotics combination was added to each well, and cells were incubated at 37°C in 5% CO₂ for 3 days prior to experimental R equi infections.

R equi culture and opsonization—Virulent R equi 103S, a strain originally obtained from a foal with pneumonia (and for which a genome sequence is available at the Sanger Institute), was grown in Mueller-Hinton broth and subsequently cultured on trypticase soy agar at 37°C for 48 hours. One colony was initially inoculated in 10 mL of trypticase soy broth and incubated at 37°C. When the cultures reached an OD value (measured at 600 nm) between 0.4 and 0.5 (ie, exponential growth phase), 100-μL aliquots were frozen as 10% glycerol stocks at –80°C. For each of the experiments described, an aliquot was thawed and spread on soy agar plates and incubated at 37°C for 48 hours. One colony from each cultured aliquot was then transferred to 5 mL of soy broth and used as inocula when ODs reached between 0.4 and 0.5. The bacterial concentrations were estimated from OD values by use of an equation previously determined from a polynomial curve fit of CFUs versus OD. Virulence plasmid retention was verified by use of PCR with primers for vapA.

Because R equi are known to be difficult to enumerate by assessment of CFUs because of bacterial cell clumping, a strain in which combined bacterial numbers and metabolic activity could be monitored through the expression of luciferase evaluated by use of a luminescence assay (R equi–LUX assay) was constructed. Plasmid pLou5 consists of a PCR-generated fragment containing the Vibrio harveyi luxAB region obtained from plasmid pSM1 cloned in place of the firefly luciferase reporter gene, which contains the HindIII fragment of mycobacterial vector pLuc10. Stability of the plasmid and bacterial luciferase expression in R equi 103S was verified through subculturing and testing individual colonies for multiple generations (data not shown). Bacteria containing the plasmid were cultured as described with the inclusion of kanamycin sulfate (400 μg/mL).

For opsonization, a bacterial suspension (OD, 0.4 to 0.5) was divided into 3 microtuge tubes (600 μL each) with either 10% or 40% (vol/vol) commercially available equine plasma product enriched with immunoglobulins against R equi (opsonized) or PBS solution (nonopsonized) to a final volume of 1 mL. Also, a sample of this same plasma was heat inactivated at 56°C for 30 minutes to compare immunoglobulins without heat-labile opsonins. Similar opsonizations included a plasma product with low amounts of R equi–specific immunoglobulins, R equi–absorbed hyperimmune plasma to remove all antibodies against R equi, and serum obtained from a healthy newborn foal before it was allowed to suckle. The amount of R equi–specific antibodies in each product was determined by the manufacturer by use of a validated ELISA based on ODs. When concentrations of R equi–specific antibody in plasma were compared, the following classifications were used:
commercially available plasma enriched with *R. equi*-specific immunoglobulins (OD, 1.05), plasma containing lower amounts of *R. equi*-specific immunoglobulins (OD, 0.61), and absorbed plasma (OD, 0.28). Opsonization was performed at 37°C for 30 minutes with gentle rotation. Opsonized bacteria were washed twice with PBS solution and centrifuged at 1,000 × g for 5 minutes. The final cell pellet was resuspended in 1 mL of PBS solution and for use as inocula.

A series of pilot experiments involving various numbers of bacteria and volumes of plasma solutions were performed to determine differences in inhibition of bacterial growth. A solution of bacteria in soy broth (2 × 10⁶ bacteria/mL) was mixed with the various plasma volumes (0%, 25%, 40%, 50%, 75%, 80%, and 100% [vol/vol] plasma) and incubated at 37°C for 30 minutes in a tube rotator. Tenfold dilutions of opsonized bacteria were then plated on soy agar at 37°C for 48 hours for CFU counts. The proportions of plasma and bacteria used in this study (ie, 10% and 40% [vol/vol] opsonization) were selected on the basis of the results of these experiments.

**Oxidative burst activity**—Production of peroxynitrite and reactive oxygen species by phagocytes was assayed by following the conversion of dehydrodorhamine 123 to intracellular green fluorescent rhodamine 123. Infected phagocytes (1 × 10⁶ cells/150 µL of HBSS) inoculated with opsonized or nonopsonized bacteria were incubated with 2.5 µg of dehydrodorhamine 123/reaction for 15 (neutrophils) or 30 (monocyte-derived macrophages) minutes at 37°C. Stimulation of phagocytes with 100 ng of phorbol myristate acetate was used as a posi-

**Figure 1**—Flow cytometric dot plots of light side scatter (SSC-H) versus oxidative burst activity (relative fluorescence intensity height [FL1-H]) for neutrophils (Np) obtained from an adult horse following culture under various conditions with or without *Rhodococcus equi* that had or had not been opsonized with *R. equi*-specific antibodies. The SSC-H provides information relative to cell granular content and allows data collection from the phagocytes specifically. The fluorescent channel FL1-H detects the light emitted by fluorescent rhodamine 123 indicative of oxidative burst activity. From left to right by column (top to bottom), the experimental conditions were as follows: neutrophils alone (negative control), neutrophils plus dehydrorhodamine (DHR) 123 (baseline neutrophil oxidative burst activity), neutrophils plus phorbol myristate acetate (PMA [positive control]), neutrophils infected with nonopsonized *R. equi* (NONOPS), neutrophils infected with *R. equi* opsonized (OPS) with 10% fresh hyperimmune plasma, neutrophils infected with *R. equi* with 40% fresh hyperimmune plasma, neutrophils infected with *R. equi* opsonized with 40% antibody-absorbed (ABS) hyperimmune plasma, neutrophils infected with *R. equi* opsonized with 40% serum obtained from a newborn foal before it was allowed to suckle, neutrophils infected with *R. equi* opsonized with 40% heat-inactivated (heat-inact) hyperimmune plasma, or neutrophils infected with *R. equi* opsonized with 40% heat-inactivated antibody-absorbed (heat-inact ABS) hyperimmune plasma. Quadrant analysis was based on phagocyte distribution (horizontal line within each plot) and baseline oxidative burst activity (vertical line in each plot). Increased oxidative burst activity of neutrophils is indicated by higher fluorescence intensity of intracellular rhodamine 123 (ie, shift to the right on the x-axis).
Flow cytometric analysis of fluorescent rhodamine 123 was immediately performed, and data were analyzed as histograms and dot plots, with median fluorescence intensity determined on the gated cells by use of software. The phagocytes (neutrophils or monocyte-derived macrophages) were gated according to size on the basis of forward light scatter and granularity determined by use of 90° side light scatter and previous labeling with myeloid markers.

TNF-α ELISA—The concentration in medium of TNF-α produced by phagocytes upon infection under the various conditions and by noninfected control cells was measured at 24 hours of culture by use of a sandwich ELISA for the equine protein according to the manufacturer's protocol. Experiments were performed in duplicate in a series of 2-fold dilutions, and values were calculated by use of a standard curve.

R equi survival—Monocyte-derived macrophages were infected with opsonized or nonopsonized luciferase expressing R equi 103S at an MOI of 5 bacteria/cell for 1 hour at 37°C in 5% CO₂. Noninfected cells were cultured under the same conditions for comparison. Gentamicin was added at a final concentration of 50 µg/mL to prevent any extracellular growth of bacteria. After 24 hours of culture, supernatants were harvested and centrifuged at 1,000 × g. Cell-depleted supernatants were frozen at −20°C for TNF-α concentration determinations. Cells remaining on plates were removed with 100 µL of EDTA, pelleted at 500 × g to discard supernatant, and lysed with 100 µL of 1X gene assay lysis buffer. Lysates were transferred to flat-bottom, solid-white polystyrene 96-well plates for extracellular bacterial viability and growth were measured by luciferase activity (in relative light units) in a plate luminometer according to size on the basis of relative mean fluorescence intensity (Figure 2). Intracellular bacterial viability and growth were measured by luciferase activity (in relative light units) in a plate luminometer after adding 100 µL of decyl aldehyde/well as a substrate. Luciferase activity was measured with the equipment software. For extracellular bacterial viability, luciferase-expressing R equi 103S (initial OD, 0.4 to 0.5) were cultured in independent tubes in triplicates with 1X gene assay lysis buffer. Lysates were transferred to flat-bottom, solid-white polystyrene 96-well plates and immediately placed at 20°C until analysis. Luciferase activity of each aliquot was measured by use of the luminometer as described for the intracellular experiments, and median values were used for statistical analysis.

Statistical analysis—The distribution of data was non-Gaussian; hence, measures of central tendency were expressed as medians and variability as 75th and 25th percentiles and ranges. Graphically, these measures were displayed in box plots. Comparisons among treatments with bacteria that were nonopsonized, opsonized with 10% hyperimmune plasma, and opsonized with 40% hyperimmune plasma were performed by use of a Wilcoxon rank sum test. Phagocytes alone or stimulated with phorbol myristate acetate were used as negative and positive assay internal controls and not included in the analysis. The acceptable type 1 error risk was set at 0.05, and central tendency measures were allowed a 10% type 1 error risk. Statistical analyses were performed by use of a commercial software package.

Results

Effect of opsonization on phagocyte oxidative burst activity—Flow cytometric dot plots (Figure 1) were made of side scatter versus mean fluorescence intensity that corresponds to oxidative burst activity of adult horse neutrophils under various conditions: neutrophils alone (negative control), neutrophils plus dehydrorhodamine 123 (baseline neutrophil oxidative burst activity), neutrophils plus phorbol myristate acetate (positive control), and neutrophils infected with nonopsonized R equi or R equi opsonized with 10% or 40% fresh hyperimmune plasma, 40% heat-inactivated hyperimmune plasma, 40% fresh or heat-inactivated antibody-absorbed hyperimmune plasma, or 40% pre-suckle serum. The data indicated that enhancement of oxidative burst activity of adult horse neutrophils was dependent on R equi–specific antibodies in plasma used for bacterial opsonization.

The effect of plasma opsonization of R equi on the oxidative burst activity of adult horse neutrophils was determined (Figure 2) in 3 conditions: nonopsonized, opsonized with 40% plasma containing lower amounts of R equi–specific immunoglobulins, or opsonized with

![Figure 2: Box-and-whisker plots of oxidative burst activity](image-url)
40% commercially available plasma enriched with *R equi*–specific immunoglobulins. Neutrophils infected with nonopsonized bacteria had a significant (*P* = 0.03) decrease in oxidative burst activity, compared with bacteria opsonized with 40% plasma containing lower amounts of *R equi* immunoglobulins or 40% commercially available hyperimmune plasma. The latter treatment resulted in greater (*P* = 0.03) oxidative burst activity than did the former. Adult horse neutrophils infected with bacteria opsonized with 40% heat-inactivated plasma had greater (*P* = 0.009) oxidative burst activity than neutrophils infected with bacteria opsonized with 40% fresh commercially available hyperimmune plasma (data not shown).

The effect of opsonization of *R equi* on foal and adult horse neutrophils was assessed (Figure 3). There were no differences in the oxidative burst activity of foal neutrophils infected with nonopsonized *R equi* or *R equi* opsonized with 10% or 40% fresh hyperimmune plasma (*P* > 0.06); however, oxidative burst activity generally increased with increased concentration of fresh hyperimmune plasma used for opsonization. In contrast, adult horse neutrophils that were infected with *R equi* opsonized with 10% or 40% fresh hyperimmune plasma had greater (*P* = 0.03) oxidative burst activity than those that were infected with nonopsonized bacteria; there was also a difference (*P* = 0.03) between oxidative burst activities of adult horse neutrophils that were infected with *R equi* opsonized with each of the 2 concentrations of fresh hyperimmune plasma. In addition, foal neutrophils had increased (*P* = 0.009) oxidative burst activity, compared with adult horse neutrophils, when infected with nonopsonized bacteria.

A beneficial effect of opsonization on the oxidative burst activity of adult horse macrophages was detected (Figure 4). Similar to adult horse neutrophils, adult horse macrophages that were infected with *R equi* opsonized with 10% or 40% fresh hyperimmune plasma had greater (*P* = 0.008) oxidative burst activity than those infected with nonopsonized bacteria; there was also an increase (*P* = 0.008) in oxidative burst activity with increasing concentrations of plasma containing *R equi*–specific antibody (10% and 40%).

**Effect of antibody opsonization on macrophage TNF-α secretion**—After 24 hours of culture, secretion of TNF-α by adult horse macrophages infected with *R equi* opsonized with 10% or 40% fresh hyperimmune plasma was higher (*P* = 0.008) than secretion of TNF-α by macrophages infected with nonopsonized bacteria (Figure 5). Secretion of TNF-α by macrophages infected with *R equi* opsonized with each of the 2 concentrations did not differ (*P* = 0.8).

**R equi viability**—To measure the effect of opsonization on intracellular and extracellular bacterial viability, the luciferase activity of *R equi* following phagocytosis and in standard medium samples was determined by use of the *R equi*–LUX assay. Overall, *R equi* luciferase activity in adult horse macrophages at 24 hours after infection with bacteria opsonized with 40% hyperimmune plasma was decreased, albeit not significantly (*P* = 0.1), compared with findings after infection with nonopsonized bacteria.
Macrophages alone without bacteria were tested in parallel to provide a background value. Luciferase activity (extracellular) decreased when bacteria were grown in broth with hyperimmune plasma containing *R. equi*–specific antibodies (Figure 7). After incubation for 0.5 hours, there was a decrease in luciferase activity when bacteria were opsonized with 30% (*P* = 0.003) or 80% (*P* < 0.001) hyperimmune plasma, compared with nonopsonized bacteria. A similar difference (*P* < 0.001) was evident after incubation for 5.5 hours. After 23 hours, the difference (*P* < 0.001) was maintained only between nonopsonized bacteria and bacteria opsonized with 80% hyperimmune plasma. At 0.5 and at 5.5 hours, there was a difference (*P* = 0.01) in luciferase activity between bacteria opsonized with 30% and 80% hyperimmune plasma.

**Discussion**

*R. equi* infection of immunocompromised or immunonaïve foals has a complex pathogenesis, and effective prophylaxis, including the use of passively transferred *R. equi*–specific antibodies via IV transfusion of hyperimmune plasma, has proved elusive. Results of the present study confirmed that opsonization of *R. equi* with *R. equi*–specific antibodies in a commercially available plasma product increases phagocytic function of neutrophils and macrophages, including increases in oxidative burst activity and TNF-α production and a decrease in bacterial viability. This effect was dependent on the *R. equi*–specific antibodies because nonopsonized bacteria and *R. equi* opsonized with fresh antibody-absorbed hyperimmune plasma containing *R. equi*–specific antibodies (Figure 7). After incubation for 0.5 hours, there was a decrease in luciferase activity when bacteria were opsonized with 30% (*P* = 0.003) or 80% (*P* < 0.001) hyperimmune plasma, compared with nonopsonized bacteria. A similar difference (*P* < 0.001) was evident after incubation for 5.5 hours. After 23 hours, the difference (*P* < 0.001) was maintained only between nonopsonized bacteria and bacteria opsonized with 80% hyperimmune plasma. At 0.5 and at 5.5 hours, there was a difference (*P* = 0.01) in luciferase activity between bacteria opsonized with 30% and 80% hyperimmune plasma.
plasma or serum obtained from a foal before it was allowed to suckle induced lower oxidative burst activity in phagocytes, compared with bacteria opsonized with fresh hyperimmune plasma. Luciferase activity was decreased, although not significantly, within macrophages 24 hours after infection with bacteria opsonized with hyperimmune plasma, compared with the effect of infection with nonopsonized bacteria. Importantly, bacteria had lower luciferase activity over time when the culture broth contained hyperimmune plasma.

In contrast to adult horse neutrophils, there was no difference in oxidative burst activity of foal neutrophils infected with nonopsonized *R. equi* or bacteria opsonized with 10% or 40% fresh hyperimmune plasma. Nevertheless, the data indicated that foal neutrophils are endogenously activated, and there was no statistical difference between foal and adult horse neutrophil oxidative burst activity associated with *R. equi* opsonized with 10% or 40% fresh hyperimmune plasma. It is possible that the increased innate activity of foal phagocytes (ie, greater in vivo activity in response to natural pathogens) impaired the statistical evidence of an effect. The high level of activity of foal neutrophils has been previously described for other types of microbes. Therefore, we used adult horse cells (neutrophils and macrophages) to evaluate the effect of *R. equi* opsonized with fresh hyperimmune plasma on phagocytic activation to eliminate age-dependent variables naturally observed in foals.

The binding to and invasion of macrophages by *R. equi* 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 *R. equi*-specific antibodies, *R. equi* is internalized via Fcγ receptors on neutrophils and macrophages and this mechanism increases phagolysosome formation and bacterial killing. Therefore, the increase in oxidative burst activity of phagocytes detected after hyperimmune plasma opsonization of *R. equi* is consistent with this mechanism and confirms findings with different sources of *R. equi*-specific antibodies. In the present study, opsonization of *R. equi* with plasma that contained a lower concentration of antibodies also promoted greater oxidative burst activity in phagocytes than did nonopsonized bacteria, indirectly supporting the findings from the in vivo study by Perkins et al. Higher concentrations of antibodies in plasma resulted in significantly greater stimulation. This may have been due to an increased number of opsonized bacteria phagocytosed by the neutrophils by binding Fcγ receptors.

Inhaled *R. equi* are able to invade resident alveolar macrophages and can replicate within the phagosomes of nonactivated phagocytes until lysis of the host cell allows spreading into the bloodstream and respiratory tract. Infection of macrophages in cell culture by virulent *R. equi* involves intracellular bacterial replication that starts between 6 and 12 hours of culture and reaches macrophage-compromising levels (3-fold increase) by 48 hours. In contrast, avirulent bacterial numbers decrease during the same period. In the present study, expression of bacterial luciferase activity within macrophages during the initial 24 hours after infection was generally decreased, although not significantly, when 40% hyperimmune plasma was used to opsonize virulent *R. equi*. Although opsonization with *R. equi*-specific antibody is important for macrophages to kill intracellular *R. equi* by oxidative burst, macrophage killing is also enhanced by cytokines, in particular interferon-γ and TNF-α, which may be required for intracellular killing in vivo. Kanaly et al demonstrated that mice treated with interferon-γ monoclonal antibodies (to block interferon-γ activity) failed to clear pulmonary *R. equi* infection. Macrophages from *R. equi*-exposed foals have a 100% increase in killing capacity when cocultured with autologous lymphocyte factors (preconditioned medium derived by in vitro incubation with *R. equi* surface antigens), whereas macrophages from nonexposed foals have only a 50% increase. The exposure of macrophages to cytokines, most importantly interferon-γ, results in enhanced microbicidal activity via the secretion of reactive oxygen and nitrogen intermediates. Individually, reactive oxygen intermediates promote negligible microbicidal activity; in fact, hydrogen peroxide alone has been shown to induce the expression of *R. equi* virulence-associated genes. However, their combination with nitrogen species (eg, peroxynitrite [ONOO⁻]) results in greater efficiency in killing of *R. equi*. It is possible that, when stimulated...
with interferon-γ, equine macrophages may be capable of even more efficient killing of opsonized *R equi* than that achieved by cells without stimulation in the present study.

In the present study, we used a novel *R equi*-luciferase construct to measure bacterial viability as a means to improve assay sensitivity and minimize the inaccuracy of bacterial counts due to clumping with the CFU method. Another luciferase gene, that from the American firefly (*Photinus pyralis*), has previously been applied in studies of intracellular growth and immune response to *Mycobacterium* spp. Our use of a bacterial luciferase reporter construct in *R equi* allowed objective, rapid, and repeatable measurements of intracellular bacterial viability to be obtained. In addition, because the emission of light is dependent on a cofactor such as ATP or reduction of flavin mononucleotide (FMNH2), only live bacteria produce luminescence. Decreased numbers of viable bacteria result in decreased luminescence. In contrast to the labor-intensive counting of CFUs, the use of a luminometer requires no dilutions or additional time for growth of the colonies. As illustrated by results of the present study, this new construct retains in vitro bacterial replication in broth and within normal adult horse macrophages. However, additional in vivo studies would need to be performed to confirm that full bacterial virulence has been retained. This new construct may facilitate future *R equi* research.

In the experiments we conducted, it was not possible to differentiate between a decrease in rate of bacterial replication and an increase in intracellular killing. Although the initial infective dose of *R equi* was controlled among the experimental groups, kinetic assessments of the growth curve were not performed. Therefore, at the 24-hour time point, the decreased number of viable bacteria that had been opsonized with hyperimmune plasma could have been attributable to decreased replication, increased intracellular death, or a combination of both. Further studies are needed to understand this antibody effect on bacterial viability.

The finding that intracellular bacterial viability was compromised after opsonization motivated us to further evaluate the direct effect of opsonization on the bacteria. Bacterial viability in the presence of 30% and 80% hyperimmune plasma was decreased after 5.5 hours of culture, compared with the viability of nonopsonized bacteria. After 23 hours of culture, decreased bacterial viability in the presence of 80% hyperimmune plasma was evident. Importantly, the effect of *R equi*-specific antibody on the growth of *R equi* in broth was dose dependent over time because higher plasma concentration had a more significant effect than the lower concentration. The fact that extracellular growth of *R equi* was inhibited by opsonization with *R equi*-specific antibody suggests an important effect of timing in the benefits of IV hyperimmune plasma transfusion. The initial step in the pathogenesis of *R equi* is inhalation into the airways, followed by phagocytosis by alveolar macrophages. If high concentrations of *R equi*-specific antibodies are present on the pulmonary mucosa at the time of infection, bacterial viability may be reduced, and phagocytosis and intracellular bacterial killing may be improved.

Although macrophages that are not activated fail to impede intracellular bacterial replication, *R equi* infection induces an immune response in macrophages that is characterized by translocation of nuclear factor κB into the nucleus and subsequent secretion of cytokines. Virulent and avirulent *R equi* strains can induce IL-1β, IL-10, IL-12p40, and TNF-α cytokine response in murine macrophages within 4 hours after infection. In the present study, secretion of TNF-α was higher in macrophages infected with *R equi* opsonized with fresh hyperimmune plasma than it was in macrophages infected with nonopsonized bacteria, indicating that opsonization also increases macrophage activation when bacteria are phagocytosed via Fcγ receptors.

Results of the present study suggested that plasma containing high concentrations of *R equi*-specific antibody may be beneficial in vivo. In terms of protective mechanisms, administration of this type of enriched plasma may limit the viability of bacteria on the mucosal surface, may increase the intracellular killing by airway phagocytes, and may increase the oxidative burst activity and the release of TNF-α from airway phagocytes, which may promote activation of local phagocytes.

The in vitro use of plasma enriched with *R equi*-specific antibody for the opsonization of *R equi* increased the oxidative burst activity of neutrophils and macrophages and the release of the proinflammatory cytokine TNF-α from macrophages and decreased bacterial viability in the extracellular space. The viability of intracellular bacteria was affected by opsonization with hyperimmune plasma, but results were not significantly different from control findings, suggesting the need of additional activation of macrophages for effective bacterial killing. The data obtained in the present study provide new insights into the pathogenesis of and immunity against *R equi* infection that require further investigation. Future studies to evaluate the effect of opsonization with or without prior activation of macrophages on intracellular bacterial viability may help elucidate the synergism between antibodies and cytokines for successful bacterial clearance. In addition, performing more frequent measurements of intracellular bacterial viability during culture of macrophages with opsonized *R equi* may elucidate the growth curve and explain whether the decrease in bacterial viability is due to decreased replication, increased intracellular killing, or a combination of both.

a. SNAP Foal IgG Test, Idexx, Westbrook, Me.
b. Intervet, DeSoto, Kan.
c. Becton Dickson Vacutainer Systems, Rutherford, NJ.
d. Ficoll Histopaque-1077, Sigma Diagnostics, St Louis, Mo.
e. Hyclone Laboratories Inc, Logan, Utah.
f. Invitrogen, Carlsbad, Calif.
g. Becton Dickinson Labware, Franklin Lakes, NJ.
h. Provided by J. A. Vázquez-Boland, Centre for Infectious Diseases, University of Edinburgh, Edinburgh, Scotland.
i. Sigma-Aldrich, St Louis, Mo.
j. BBL tryptophic soy agar, Becton, Dickinson and Co, Sparks, Md.
k. BBL tryptophic soy broth, Becton, Dickinson and Co, Sparks, Md.
l. SmartSpec Plus Spectrophotometer, Bio-Rad, Hercules, Calif.
m. Amresco, Solon, Ohio.
n. NYS Animal Health Diagnostic Laboratory, College of Veterinary Medicine, Cornell University, Ithaca, NY.
References


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