Received: 15 September 2020 | Accepted: 2 December 2020

DOI: 10.1111/jvim.16002

STANDARD ARTICLE

Journal of Veterinary Internal Medicine Open Access

ACVIM American College of Veterinary Internal Medicine

Opsonization but not pretreatment of equine macrophages with hyperimmune plasma nonspecifically enhances phagocytosis and intracellular killing of *Rhodococcus equi*

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Funding information
Link Equine Research Endowment of Texas A&M University College of Veterinary Medicine Infectious Disease Epidemiology Program

Abstract

Background: Evidence regarding the efficacy of equine hyperimmune plasma to prevent pneumonia in foals caused by *Rhodococcus equi* is limited and conflicting.

Hypothesis: Opsonization with *R. equi*-specific hyperimmune plasma (HIP) will significantly increase phagocytosis and decrease intracellular replication of *R. equi* by alveolar macrophages (AMs) compared to normal plasma (NP).

Animals: Fifteen adult Quarter Horses were used to collect bronchoalveolar lavage cells.

Methods: In the first experiment, AMs from 9 horses were pretreated (incubated) with either HIP, NP, or media only (control) and then infected with nonopsonized *R. equi*. In a second experiment, AMs from 6 horses were infected with *R. equi* either opsonized with HIP or opsonized with NP. For both experiments, AMs were lysed at 0 and 48 hours and the number of viable *R. equi* quantified by culture were compared among groups using linear mixed-effects modeling with significance set at $P < .05$.

Results: Opsonization with either HIP or NP increased phagocytosis by AMs ($P < .0001$) and decreased intracellular survival of organisms in AMs ($P < .0001$). Pretreating AMs with either HIP or NP without opsonizing *R. equi* had no effects on phagocytosis or intracellular replication.

Conclusions and Clinical Importance: Opsonizing *R. equi* with either NP or HIP decreases intracellular survival of organisms in AMs, but the effect does not appear to be enhanced by using HIP. Mechanisms other than effects on AMs must explain any clinical benefits of using HIP over NP to decrease the incidence of *R. equi* pneumonia in foals.

Abbreviations: AMs, alveolar macrophages; BAL, bronchoalveolar lavage; CFU, colony-forming units; CI, confidence interval; HIP, *R. equi*-specific hyperimmune plasma; MDM, monocyte-derived macrophages; MEMα, minimum essential media alpha; NP, normal plasma; OHIP, *R. equi* opsonized with *R. equi*-specific hyperimmune plasma; ONP, *R. equi* opsonized with normal plasma; PBS, phosphate-buffered saline; PNAG, poly-*N*-acetyl glucosamine.

KEYWORDS
alveolar macrophages, bacteria, foals, in vitro killing capacity, intracellular survival, pneumonia, serum

1 | INTRODUCTION

Rhodococcus equi is a gram-positive, facultative, intracellular pathogen that is ubiquitous in the environment and causes severe pneumonia in foals.[1] Extrapulmonary manifestations, such as uveitis, tenosynovitis, ulcerative enterocolitis, and abdominal abscessation also occur as a result of infection with *R.equi*.[2] Widespread distribution of the bacterium, insidious onset of pneumonia, limited treatment options, reported antimicrobial resistance,[3] and prolonged antimicrobial treatment make it a costly disease for the equine industry.[4-6] No effective vaccine is currently available. To date, the only commercially available product determined to decrease the incidence of *R. equi* pneumonia at affected farms is prophylactic transfusion of *R. equi*-specific hyperimmune plasma (HIP) to foals shortly after birth.[7-10] Results of field and experimental studies, however, have provided conflicting evidence regarding the efficacy of HIP in preventing clinical pneumonia in foals.[7-15]

The precise mechanism of protection provided by HIP is unclear but is presumed to be antibody-mediated. The alveolar macrophage (AM) is the natural host cell of *R. equi*, and the ability of *R. equi* to survive and replicate within macrophages is the basis of its pathogenicity. Although the effects of opsonization of *R. equi* on phagocytosis and intracellular survival in phagocytes such as peripheral blood neutrophils and monocyte-derived macrophages (MDMs) have been investigated,[16-19] no studies have examined effects of plasma on phagocytosis and survival of *R. equi* in AMs. This is important because functional responses of macrophages vary by lineage (ie, MDMs vs AMs) in foals.[20] Moreover, evidence is limited and conflicting regarding whether *R. equi*-specific antibodies in HIP mediate clinical benefits.[9,15,21,22] Evidence exists that activation of antibody receptors by antibody–antigen complexes nonspecifically targets bacteria for phagolysosomal killing.[23] A small experimental study suggested that HIP was not superior to hyperimmune plasma from donors not immunized against *R. equi*.[11] Conversely, immunization of mares with antibodies that target *R. equi* antigens has been determined to protect foals against *R. equi*,[15,24,25] and administration of *R. equi* plasma hyperimmune against the bacterial surface polysaccharide poly-*N*-acetyl glucosamine (PNAG) was shown in a small experimental study to be superior to normal plasma (NP) for protecting foals against experimental infection with *R. equi*.[25] These conflicting results and variable clinical efficacy from experimental studies, observational studies, and field trials[7-15,21,24,26] raise questions about whether HIP directly or indirectly modulates intracellular replication of virulent *R. equi* in AMs. Thus, our objectives were to compare the effects of HIP and NP on phagocytosis and intracellular survival of virulent *R. equi* either as a pretreatment of AMs (in the absence of opsonization) or when used as opsonins.

2 | MATERIALS AND METHODS

2.1 | Plasma source

The following commercially available plasma products were used: HIP (ReSolution *Rhodococcus equi* Antibody, MG Biologics, Inc., Ames, Iowa) and NP (ImmunoGlo 3700, MG Biologics Inc.).

2.2 | Animals and sample collection

All procedures were approved by the Texas A&M University Institutional Animal Care and Use Committee. Fifteen adult Quarter horses (1 gelding and 14 mares) from the Department of Large Animal Clinical Sciences research and teaching herd were used. A sample size of 6 horses was calculated on the basis of paired comparisons, power of 80%, significance of $P < .05$, and an expected difference of an at least ½ log reduction of colony-forming units (CFU) in intracellular replication. Because we had not previously studied pretreatment effects of plasma using nonopsonized bacteria, we increased our sample size to 9 for the first experiment. All animals had physical examination and blood collection performed before bronchoalveolar lavage (BAL). For the blood collection, 8 mL of whole blood was collected by jugular venipuncture into a tube containing ethylenediaminetetraacetic acid (EDTA Vacutainer, BD Biosciences, Oakville, Ontario). For the BAL, animals were sedated with romifidine hydrochloride (Sedivet 1% Injection, Boehringer Ingelheim Inc., St. Joseph, Missouri; 0.05 mg/kg IV) and butorphanol tartrate (Torbugesic, Zoetis Inc., Kalamazoo, Missouri; 0.2 mg/kg IV) before BAL. A silicone BAL catheter (Jorgensen Labs Inc., Loveland, Colorado) that was 2.4 m long and with an outer diameter of 10 mm was passed intranasally into the airways and wedged in a bronchus. Two 60-mL syringes containing 120 mL of sterile saline solution (0.9% NaCl) were infused through the catheter and immediately aspirated, and then the process was repeated 60 mL per lavage until a total of 300 mL of saline was infused. The recovered sample was placed in a cooler with ice packs for transportation to the laboratory.

2.3 | Isolation of AMs

After BAL fluid was collected, AMs were separated from the lavage sample by centrifugation within 30 minutes. The BAL fluid was centrifuged at 400*g* for 10 minutes at 4°C, the supernatant discarded, and the resulting pellet washed once with phosphate-buffered saline (PBS) solution (Gibco, Grand Island, New York). The pellet was resuspended in minimum essential media alpha (MEMα),

supplemented with 10% heat-inactivated horse serum (Gibco), 1% penicillin/streptomycin/glutamine (Gibco) and amphotericin B (5 mg/mL; Gibco). Cells were counted using an automated cell counter (Cellometer Auto T4, Nexcelom Bioscience LLC, Lawrence, Massachusetts), and 1×10^6 cells/mL were placed in each well of a 24-well plate in triplicate (Costar multiwell cell plate, Corning Inc., Corning, New York) and incubated for 24 hours at 37°C in 5% CO_2 to facilitate cell adherence.

2.4 | Pretreatment and infection of AMs

To compare the effects of pretreatment of plasma, media was removed from wells and the AMs were washed with PBS twice, and resuspended in 1 mL of each of the following: (a) MEMα media only (negative control), (b) NP, or (c) HIP. The cells were incubated for 1 hour at 37°C in 5% CO_2 and then infected with nonopsonized virulent *R. equi* (ATCC 33701, Rockville, Maryland). A duration of 1 hour was selected on the basis of our prior experience and publications.[16,26,27]

After pretreatment with either HIP or NP, media supernatant was removed from the wells and the cells washed twice with PBS and infected with nonopsonized virulent *R. equi* (ATCC 33701) at a multiplicity of infection (MOI) of 10 bacteria per macrophage suspended in 1 mL of MEMα (without serum supplementation). The cells were incubated at 37°C in 5% CO_2 for 40 minutes to allow macrophage phagocytosis. After incubation, the supernatant containing *R. equi* was discarded and the monolayers were carefully washed twice with PBS. The experiment was conducted in triplicate for each condition. Macrophages then were lysed at time 0 (immediately after infection) to determine the numbers of *R. equi* phagocytosed (T0). The remainder of the cells were suspended in 2 mL of fresh media (MEMα, 10% heat-inactivated donor horse serum supplemented with amikacin [8 μg/mL] and amphotericin B [0.25 μg/mL]), and incubated at 37°C in 5% CO_2 for 48 hours. After 48 hours of incubation,[20] supernatant was removed and cells were lysed as described below to determine *R. equi* replication (T48).

To compare the effects of opsonization of HIP with NP, virulent *R. equi* (ATCC 33701) were opsonized with either HIP or NP for 45 minutes; *R. equi* that were not opsonized were used as a negative control. The AMs were cultured as described above, and then infected with *R. equi* (opsonized with HIP or NP, or nonopsonized) at an MOI of 10 as described above.

2.5 | Quantification of viable *Rhodococcus equi*

At T0 and T48, the bacterial supernatant was removed from the wells, and the monolayers were washed twice with PBS. Sterile water (1 mL/well) was added and incubated at 37°C for 45 minutes. A pipette tip was used to scrape the wells and the contents were transferred to 1.5-mL tubes (Eppendorf North America, Inc., Enfield, Connecticut). The tubes were vortexed for 5 minutes at 3200 rpm, sonicated for 5 minutes, and vortexed again for 5 additional minutes. To determine the number of CFU per well, serial 10-fold dilutions (ie, 10^{-3}, 10^{-2}, 10^{-1}) were performed using the contents of each tube. Brain heart infusion (BHI, BD Biosciences, Oakville, Ontario) agar plates were inoculated with 100 μL of each dilution and incubated for 48 hours at 37°C in 5% CO_2. Triplicate cultures were performed to improve validity of counts. Forty-eight hours after incubation, the numbers of CFUs per plate were counted and recorded. Intracellular growth of *R. equi* was represented by the ratio of the intracellular concentration of *R. equi* in AMs after 48 hours (T48) relative to values at T0.[20]

2.6 | Statistical analysis

For each experiment, data were analyzed using linear mixed-effects modeling with outcomes of CFU at T0 (phagocytosis), CFU at T48 (intracellular replication), and the ratio of CFU at T48:T0 (growth inhibition/killing) as the primary outcomes, with treatment modeled as a fixed dependent effect and individual horse modeled as a random effect. Data were $\log_{10}$-transformed to meet distributional assumptions of modeling. Confidence intervals (CIs) were estimated using maximum likelihood methods, and estimated means and 95% CIs were reported back as back-transformed values. Post hoc pairwise comparisons were made using the method of Sidak.[29] For all analyses, significance was set at $P < .05$.

3 | RESULTS

3.1 | Effects of pretreatment of AMs with HIP or NP on phagocytosis and killing of nonopsonized *R. equi*

Intracellular concentrations of *R. equi* at T0 were interpreted as representing phagocytosis. Pretreatment with plasma did not enhance phagocytosis. Concentrations of *R. equi* at T0 were significantly lower for AMs treated with HIP ($P = .03$) or NP ($P = .04$) than those treated

TABLE 1 Pretreatment with *Rhodococcus equi*-specific hyperimmune plasma (HIP) or normal plasma (NP) significantly decreased phagocytosis of virulent *R. equi*, but had no significant effect on replication of *R. equi* within alveolar macrophages (AMs)

	Phagocytosis	Replication
Treatment	CFU T0 (95% CI)	Ratio T48:T0 (95% CI)
Control	2964 (844-10 404)[a]	67 (24-183)[a]
HIP	1394 (714-2772)[b]	64 (28-147)[a]
NP	1494 (775-2882)[b]	65 (29-147)[a]

Note: Model-based estimates of means (95% confidence intervals [CIs]), back-transformed from $\log_{10}$ transformations, of phagocytosis (colony forming units [CFU] after 40 minutes [T0] of co-cultivation with AMs) and replication (CFU recovered from AMs after 48 hours of incubation divided by the CFU at T0) of virulent *R. equi*. Within a column, values with different superscripted alphabets differ significantly ($P < .05$).

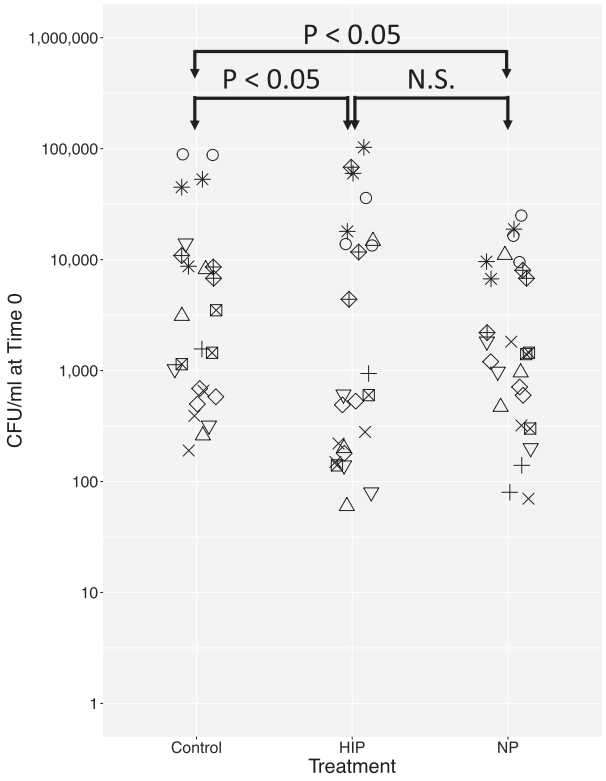

FIGURE 1 Pretreatment with *Rhodococcus equi*-specific hyperimmune plasma (HIP) or normal plasma (NP) modestly but significantly decreased phagocytosis by equine alveolar macrophages from 9 horses relative to media only (Control). Each horse was tested in triplicate, and individual symbol shapes represent individual horses. Brackets indicate significant difference ($P < .05$) between treatment relative to Control; HIP did not differ significantly from NP

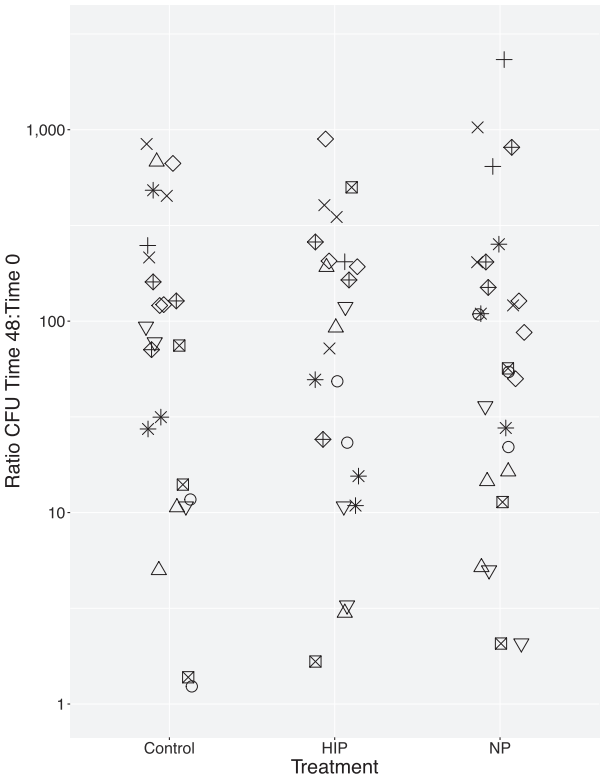

FIGURE 2 Pretreatment with *Rhodococcus equi*-specific hyperimmune plasma (HIP) or normal plasma (NP) had no significant effect on replication of virulent *R. equi* in equine alveolar macrophages from 9 horses relative to media only (Control). Each horse was tested in triplicate, and individual symbol shapes represent individual horses. There were no significant differences ($P > .05$) among treatments

with media only (Table 1; Figure 1), but concentrations of *R. equi* for HIP did not differ significantly from NP ($P = 1.0$). Pretreatment with plasma did not enhance intracellular killing. Virulent *R. equi* replicated in AMs (ie, all ratios were >1) but ratios were not significantly lower for AMs treated with HIP ($P = .91$) or NP ($P = .95$) than those treated with media only (Table 1; Figure 2): ratios of *R. equi* for HIP did not differ significantly from NP ($P = 1.0$).

3.2 | Effects of opsonizing with either HIP or NP on phagocytosis and killing of *R. equi* by AMs

Infection with both *R. equi* opsonized with HIP (OHIP) and *R. equi* opsonized with NP (ONP) *R. equi* resulted in significantly ($P < .0001$ for each) increased phagocytosis by AMs (Table 2; Figure 3), but no significant difference ($P = .08$) was found in phagocytosis induced by OHIP vs ONP. Replication ratios were significantly lower for *R. equi* opsonized with either HIP or NP ($P < .0001$ for each) than for AMs infected with nonopsonized *R. equi* (Table 2; Figure 4), but no significant difference was found in the ratios for AMs infected with *R. equi* opsonized by HIP or NP ($P = .74$).

TABLE 2 Opsonization with *Rhodococcus equi*-specific hyperimmune plasma (HIP) or normal plasma (NP) significantly increased phagocytosis and significantly decreased replication of virulent *R. equi*, within alveolar macrophages (AMs)

	Phagocytosis	Replication
Treatment	CFU T0 (95% CI)	Ratio T48:T0 (95% CI)
Control	144 241 (94 868-219 309)[a]	2.3 (1.8-2.8)[a]
HIP	245 836 (190 565-317 138)[b]	0.5 (–0.1 to 1.1)[b]
NP	332 707 (257 904-429 204)[b]	0.8 (0.2-1.3)[b]

Note: Model-based estimates of means (95% confidence intervals [CIs]), back-transformed from $\log_{10}$ transformations, of phagocytosis (colony forming units (CFU) after 40 minutes (T0) of co-cultivation with AMs) and replication (CFU recovered from AMs after 48 hours of incubation divided by the CFU at T0) of virulent *R. equi*. Within a column, values with different superscripted alphabets differ significantly ($P < .05$).

4 | DISCUSSION

Our results indicate that pretreatment of AMs with either HIP or NP did not enhance either phagocytic capacity or killing capacity of AMs for virulent *R. equi*. This finding suggests that any clinical benefits of

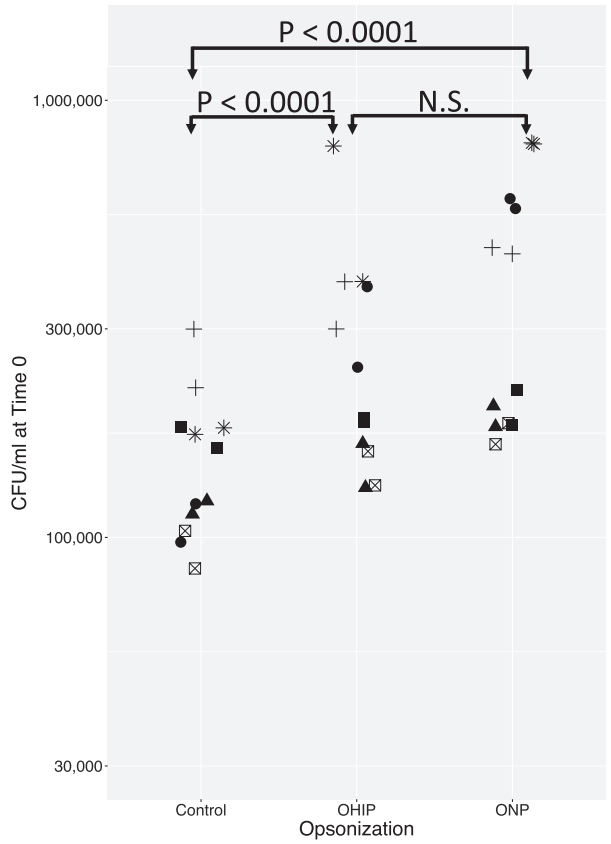

FIGURE 3 *Rhodococcus equi* opsonized with *R. equi*-specific hyperimmune plasma (OHIP) and *R. equi* opsonized with normal plasma (ONP) significantly increased phagocytosis by equine alveolar macrophages from 6 horses relative to alveolar macrophages (AMs) infected with nonopsonized *R. equi* (Control). Each horse was tested in duplicate, and individual symbol shapes represent individual horses. Brackets indicate significant difference ($P < .0001$) between treatment relative to Control; HIP did not differ significantly from NP

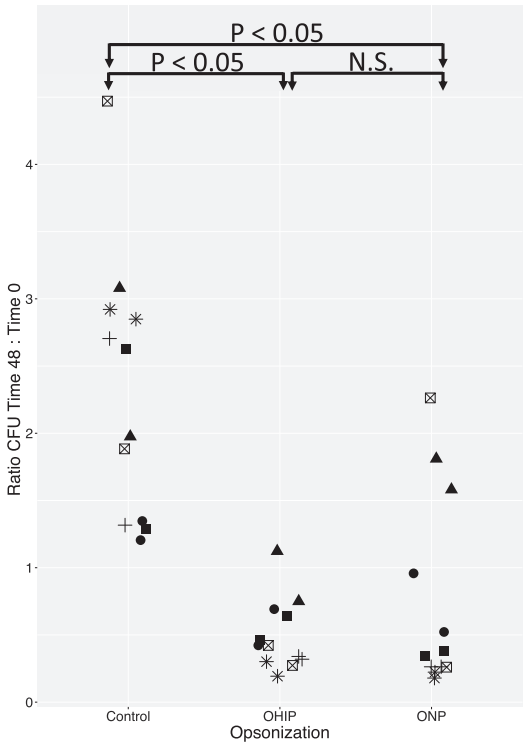

FIGURE 4 *Rhodococcus equi* opsonized with *R. equi*-specific hyperimmune plasma (OHIP) and *R. equi* opsonized with normal plasma (ONP) significantly reduced intracellular replication of virulent *R. equi* in equine alveolar macrophages from 6 horses relative to alveolar macrophages (AMs) infected with nonopsonized *R. equi* (Control). Each horse was tested in duplicate, and individual symbol shapes represent individual horses. Brackets indicate significant difference ($P < .05$) between treatment relative to Control; HIP did not differ significantly from NP

HIP or NP are not mediated by nonspecific activation of AMs by plasma components before infection. Surprisingly, a modest but significant decrease in phagocytosis of nonopsonized *R. equi* occurred as a result of pretreatment with either HIP or NP. Monomeric IgG in plasma may mediate signaling pathways that inhibit phagocytosis,[30] but the cause of the observed effect was not determined.

Opsonization with either HIP or NP enhanced phagocytosis and intracellular killing of *R. equi* by AMs. A number of possible factors may explain this result. First, antibodies may result in cross-linking of multimeric Fc receptors (ie, FcγRIII) on AMs to mediate phagocytosis and killing, as has been described for nonspecific killing of other intracellular pathogens that replicate in macrophages.[23,31] Alternatively, evidence exists that even NP plasma from the manufacturer we used has antibodies that recognize virulent *R. equi*.[14] It is thus possible that the concentrations of these anti-*R. equi* antibodies were sufficient to mediate phagocytosis and killing of *R. equi* similar to that of HIP.

Our results should not be interpreted as indicating that NP is clinically equivalent to HIP. Because AMs are the natural host cell of *R. equi* and serve a critical role as the first line of defense in the lung parenchyma, they play a crucial role in innate immunity to *R. equi*.[32] Nevertheless, recent studies indicate that antibodies targeting PNAG, in combination with complement and neutrophils, mediate protection in foals from *R. equi*, and protection of foals against *R. equi* pneumonia was significantly better with anti-PNAG hyperimmune plasma than with NP.[25,33,34]

Our study had a number of limitations. Including an isogenic avirulent isolate of *R. equi* during the experiment could have served as a control for functional responses of AMs and to document the expected decreased replication of avirulent *R. equi* in macrophages relative to the virulent isolate. We chose to focus on virulent *R. equi* for these experiments because these strains are of primary clinical importance. Using purified immunoglobulins rather than plasma would have better enabled us to make inferences about the role of antibodies, and we cannot exclude the possibility that other constituents of plasma contributed to the observed effects. We did not study foals, but evidence exists that functional responses of adult horses are not superior to those of foals.[20] We only included 1 gelding because we had more female horses available for the study. Thus, we have limited data regarding differences between female

horses and geldings or stallions and cannot extrapolate our results to male horses. Finally, we did not directly compare AMs to MDMs. Comparing the responses of these 2 lineages of macrophages to *R. equi* after exposure to both types of plasma potentially would have supported the findings of a previous study that showed that replication of *R. equi* was higher in AMs than MDMs.[20] We targeted AMs because evidence was lacking for these cells, and AMs represent the target cells for *R. equi* infection.

Despite these limitations, results of our in vitro experiment indicated that opsonization with HIP was not superior to opsonization with NP for enhancing phagocytosis and killing of *R. equi* by equine AMs. Thus, therapeutic effects of HIP are not explained by enhanced phagocytosis or killing of *R. equi* relative to NP. Importantly, this does not mean that NP in vivo would be an adequate substitute for HIP in the prophylactic treatment and prevention of *R. equi* pneumonia in foals because phagocytosis and killing by AMs might not translate to clinical efficacy. Evidence exists that transfusion of plasma hyperimmune to PNAG was superior to NP for protecting foals, [25] indicating that antigen-specific hyperimmune plasma can improve clinical outcomes relative to NP. Further evaluation of the clinical efficacy of HIP relative to NP in a well-designed, randomized, controlled trial is warranted.

ACKNOWLEDGMENTS

Funding provided by the Link Equine Research Endowment of Texas A&M University College of Veterinary Medicine Infectious Disease Epidemiology Program. The authors thank the members of the Equine Infectious Disease Laboratory at Texas A&M University for technical assistance, and MG Biologics, Inc. for providing the plasma products used in this study.

CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

The broncho-alveolar and blood collection procedures were approved by the Texas A&M University Animal Care and Use Committee (AUP# 2016-0212).

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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How to cite this article: Harvey AB, Bordin AI, Rocha JN, Bray JM, Cohen ND. Opsonization but not pretreatment of equine macrophages with hyperimmune plasma nonspecifically enhances phagocytosis and intracellular killing of *Rhodococcus equi*. *J Vet Intern Med*. 2021;35:590–596. https://doi.org/10.1111/jvim.16002