Evaluation of equine immunoglobulin specific for *Rhodococcus equi* virulence-associated proteins A and C for use in protecting foals against *Rhodococcus equi*-induced pneumonia

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Objective—To determine whether purified equine immunoglobulin specific for *Rhodococcus equi* virulence-associated proteins A and C (VapA and VapC) can confer passive protection against *R equi*-induced pneumonia in foals.

Animals—Twenty-eight 3-week-old mixed-breed pony foals.

Procedure—7 foals received IV injections of equine hyperimmune plasma (HIP) against whole-cell *R equi*, and 7 received purified equine immunoglobulin specific for VapA and VapC 1 day prior to intrabronchial infection with *R equi* strain 103+. Eleven foals were not treated prior to infection, and 3 control foals were neither treated nor infected. Heart rate, respiratory rate, and rectal temperature were recorded twice daily, and serum fibrinogen concentration and WBC count were determined every other day following infection. Foals were euthanatized 14 days following infection, and lung lesions and concentration of *R equi* in lungs were assessed.

Results—The onset of clinical signs of pneumonia was significantly delayed in the HIP- and immunoglobulin-treated groups, compared with the untreated infected group. Moreover, pulmonary lesions were less severe in the treated groups, and significantly fewer *R equi* organisms were cultured from the lungs of treated foals.

Conclusions and Clinical Relevance—Degree of protection against *R equi*-induced pneumonia provided by purified immunoglobulin specific for VapA and VapC was similar to that provided by commercially available HIP. Results not only suggest that immunoglobulin is the primary component of HIP that confers protection against *R equi*-induced pneumonia in foals but also indicate that antibodies against *R equi* VapA and VapC are protective. (*Am J Vet Res* 2001;62:1307–1313)

Rhodococcus equi, a gram-positive facultative intracellular organism, is one of the most important causes of disease in foals between 1 and 6 months of age. The infection results in the development of chronic and often severe pyogranulomatous bronchopneumonia.[1] The combination of erythromycin and rifampin is currently the standard treatment for *R equi* infections.[2] Although an effective vaccine is not yet available, passive immunization of foals with **hyperimmune plasma (HIP)** has become a standard and beneficial practice on many farms with endemic *R equi* infections.

The prophylactic value of HIP in experimentally challenged foals was first demonstrated by Martens et al[3,4] and was subsequently confirmed in foals naturally exposed to *R equi*.[5,6] The protective mechanism of HIP against *R equi*-induced pneumonia has not been identified. The list of possible effector molecules in HIP includes, but is not restricted to, specific antibodies and nonspecific factors such as fibronectin, complement, collectins, and cytokines. Antibodies are a likely candidate, because the development of pneumonia in infected foals coincides with a decrease in maternally derived antibody concentrations, which reach a minimum in foals between 5 and 10 weeks of age.[7,8] Results of in vitro studies[3,9,10] reveal the importance of opsonization by antibody in enhancing uptake and killing of *R equi* by macrophages and neutrophils.

Immunity to *R equi* in mice has been shown to depend on $CD4^+$ lymphocytes expressing a **T-helper 1 (Th1)** profile of cytokines. Antibodies only slightly enhance clearance of infection in mice.[11,12] Although cell-mediated immunity is of major importance in protection against infection with several intracellular pathogens, an effector role for antibody has also been demonstrated against *Listeria monocytogenes*,[13] *Rickettsia* spp,[14] *Salmonella* spp[15,16] and *Chlamydia* spp.[17,18] There are a number of mechanisms by which antibodies may prevent the progression of infection with intracellular organisms, including activation of complement-mediated bacterial lysis,[16,19] neutralization of bacterial lipopolysaccharide,[18] interference with bacterial adhesion or cell entry,[15] opsonization,[9,10] inhibition of intracellular growth,[20] and alteration of cytokine production by other immune cells via interaction with Fc receptors.[21,22]

Virulent strains of *R equi* are characterized by surface expression of the thermoregulated **virulence-associated protein (Vap) A**,[12,23] the gene for which is found on a large virulence plasmid.[24,25] Recently, 6 other Vap that are closely related to VapA, designated VapC through VapH, have been identified.[26] Extensive homology has been detected among the C-termini of the 7 Vap-family proteins. Strains of *R equi* that do not carry the virulence plasmid and, hence, do not express Vap lose virulence for both mice and foals and

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lose the ability to replicate in murine and equine macrophages in vitro.[27-29] We hypothesized that antibodies against Vap may protect foals from *R equi*-induced pneumonia. The purpose of the study reported here was to determine whether immunity against *R equi* conferred by HIP is mediated by antibodies against VapA and VapC.

Materials and Methods

Animals and experimental design—Twenty-eight 3-week-old mixed-breed pony foals (age range, 18 to 23 days) were used in this study. To ensure adequate transfer of colostral immunoglobulin, serum immunoglobulin concentration was determined in foals by use of a semiquantitative ELISA[a] when foals were between 12 and 24 hours old. The criteria used for selecting foals for infection with *R equi* have been described.[30]

As individual foals became available, they were randomly assigned to 1 of 4 groups and moved to an isolation facility 1 or 2 days prior to treatment. Seven foals received 1 L of commercially available HIP[b] intravenously 24 hours prior to infection with *R equi*. A second group of 7 foals received purified immunoglobulin against VapA and VapC 24 hours prior to infection, and a third group of 11 foals received no treatment prior to infection. Foals were infected by administration of 25 ml of *R equi* strain 103+ (5×10^7 CFU/ml) into each of the major bronchi as described.[30] The final group of 3 foals served as challenge controls. This latter group received sterile PBS solution instead of *R equi*.

Serum antibody titers against a **VapA-enriched antigen preparation (APTX)** were determined by use of an ELISA[31] before and after infusion of HIP or immunoglobulin and 7 and 14 days after infection. Titers were expressed as the natural log ($\log_2$). There were no significant differences in titers among groups prior to administration of HIP or immunoglobulin.

The day of infection with *R equi* was designated as day 0. Following infection, foals were assessed twice daily, and heart rate, respiratory rate, and rectal temperature were recorded. Blood samples were collected every second day for determination of WBC count and fibrinogen concentration.[c] Foals were euthanatized by IV injection of a lethal dose of pentobarbital sodium 14 days after infection. Complete postmortem examinations were performed. All experiments were approved by the University of Guelph Animal Care Committee.

Hyperimmune plasma—Hyperimmune plasma was prepared from plasma obtained from horses immunized with a suspension of clinical isolates of *R equi* according to a published protocol.[4,5] The anti-APTX titer in HIP, determined by use of an ELISA,[31] was 10.6.

Production of recombinant VapA and VapC—To produce equine immunoglobulin against *R equi* VapA and VapC, we first purified the recombinant proteins. Genes encoding VapA and VapC were amplified from the *R equi* 103+ plasmid by use of polymerase chain reaction. Primers for *VapA* amplification were as follows: 5'-GCCGGATCCACTAATGCGACCGTTCTT-3' and 5'-CATGAATTCCTAGGCGTTGTGCCA-3', whereas primers for *VapC* amplification were: 5'-GCCGGATCCGCCAATGTAGTCGCTCCGTC-3' and 5'-CATGAATTCGCGAGCGTTTACCTTCCGAC-3'. Both sets of primers contained 5'-extensions encoding restriction enzyme recognition sites for *Bam*HI or *Eco*RI. Amplified products were digested with *Bam*HI and *Eco*RI, and the digestion products were ligated to a similarly digested plasmid vector.[d] Resultant plasmids were transformed into *Escherichia coli*.[e] These plasmids allowed for the in vitro production of recombinant VapA and VapC fused to glutathione-S-transferase. Fusion proteins were purified, using glutathione beads,[f] and VapA and VapC were cleaved from glutathione-S-transferase by use of thrombin.

Production of purified equine immunoglobulin against VapA and VapC—Three adult horses with low serum antibody titers (≤ 5.7 in $\log_2$) against VapA and VapC were identified by use of an ELISA. These horses were vaccinated intramuscularly 3 times at 2-week intervals with recombinant VapA and VapC (1.5 mg of each protein); the adjuvant was 1 ml of aluminum hydroxide gel[g] in a final volume of 4 ml. Serum anti-VapA and -VapC titers were determined periodically. Three weeks after the third vaccination, 4 L of blood was obtained from each horse, and the plasma was separated. Immunoglobulin was precipitated from plasma by addition of 7.6 ml of caprylic acid[h]/100 ml of plasma, followed by a second precipitation, using 50% (vol/vol) ammonium sulfate.[i] After dialyzing for 36 hours against saline (0.9% NaCl) solution, the immunoglobulin fraction was further dialyzed against ultra pure-water until a slight precipitate developed. Purified immunoglobulin was then lyophilized and assessed by use of **sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)** and western blot analysis. To determine immunoglobulin purity, western blot analysis was performed, using alkaline phosphatase-conjugated goat anti-horse IgG (heavy and light chains)[j] diluted 1:10,000 in skim-milk blocking buffer. To determine IgG isotypes in whole plasma and isolated immunoglobulin (ie, pre- and postpurification samples), additional western blot analyses were performed, using monoclonal antibodies against IgGa (CVS 45), IgGb (CVS 39), IgGc (CVS 52), and IgGT (CVS 38)[32,k] followed by alkaline phosphatase-conjugated goat anti-mouse IgG (heavy and light chains).[j]

To determine whether antibodies against VapA and VapC predominated in the purified immunoglobulin preparation, compared with HIP, whole-cell *R equi* strain 103+ and recombinant VapA and VapC were separated by use of SDS-PAGE. Proteins were transferred to nitrocellulose, and western blot analyses were performed, using purified immunoglobulin and HIP followed by alkaline phosphatase-conjugated goat anti-horse IgG[j] diluted 1:10,000 in skim-milk blocking buffer. To prepare whole-cell *R equi*, *R equi* strain 103+ was cultured in brain heart infusion broth[l]

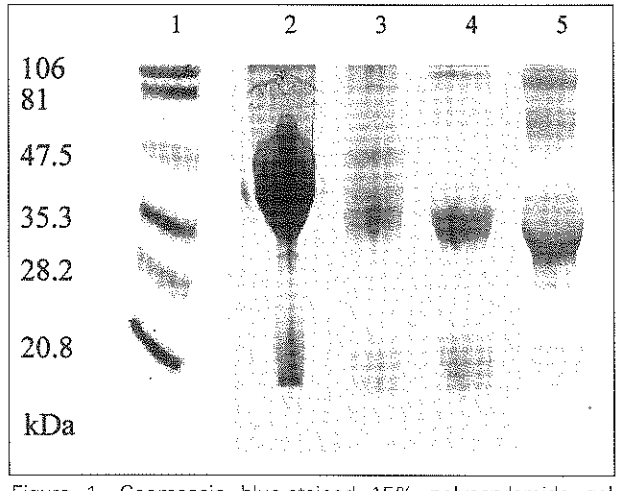

Figure 1—Coomassie blue-stained 15% polyacrylamide gel (lanes 1–4) and western blot (lane 5) depicting whole plasma from horses immunized with recombinant virulence-associated proteins (Vap) A and C of *Rhodococcus equi* (lane 2), the crude immunoglobulin fraction precipitated from whole plasma by use of caprylic acid (lane 3), and the purified immunoglobulin fraction precipitated from whole plasma by use of caprylic acid and ammonium sulfate (lanes 4 and 5). Low-range molecular weight markers are in lane 1. Immunoglobulin in lane 5 was detected by use of alkaline phosphatase-conjugated goat anti-horse IgG antibody after transfer of separated proteins to nitrocellulose. kDa = Kilodalton.

at 37 C for 48 hours. Bacterial pellets were collected and frozen at –20 C prior to SDS-PAGE.

Anti-APTX activity of purified immunoglobulin was found to be 2-fold less than that in the original plasma. Thus, prior to use, purified immunoglobulin was rehydrated in a volume of saline solution that resulted in a titer approximately equivalent to that in the original plasma. Anti-APTX titer of the reconstituted immunoglobulin, determined by use of an ELISA,[31] was 9.9, and foals received 1 L of this preparation intravenously 1 day before infection.

Necropsy—After foals were euthanatized, body and lung weights were determined. Tissue specimens for bacteriologic culture were collected from 4 evenly distributed preselected sites in each lung. For each site, number of **colony-forming units** (CFU) of *R equi* per gram of tissue was determined and expressed as $\log_{10}$.

Statistical analyses—A 1-tailed Student *t*-test was used to analyze the differences in means between treatment groups. Significance was set at $P \leq 0.05$.

Results

Immunoglobulin purification—Only minor contaminating proteins were detected in the final immunoglobulin preparation by use of SDS-PAGE and

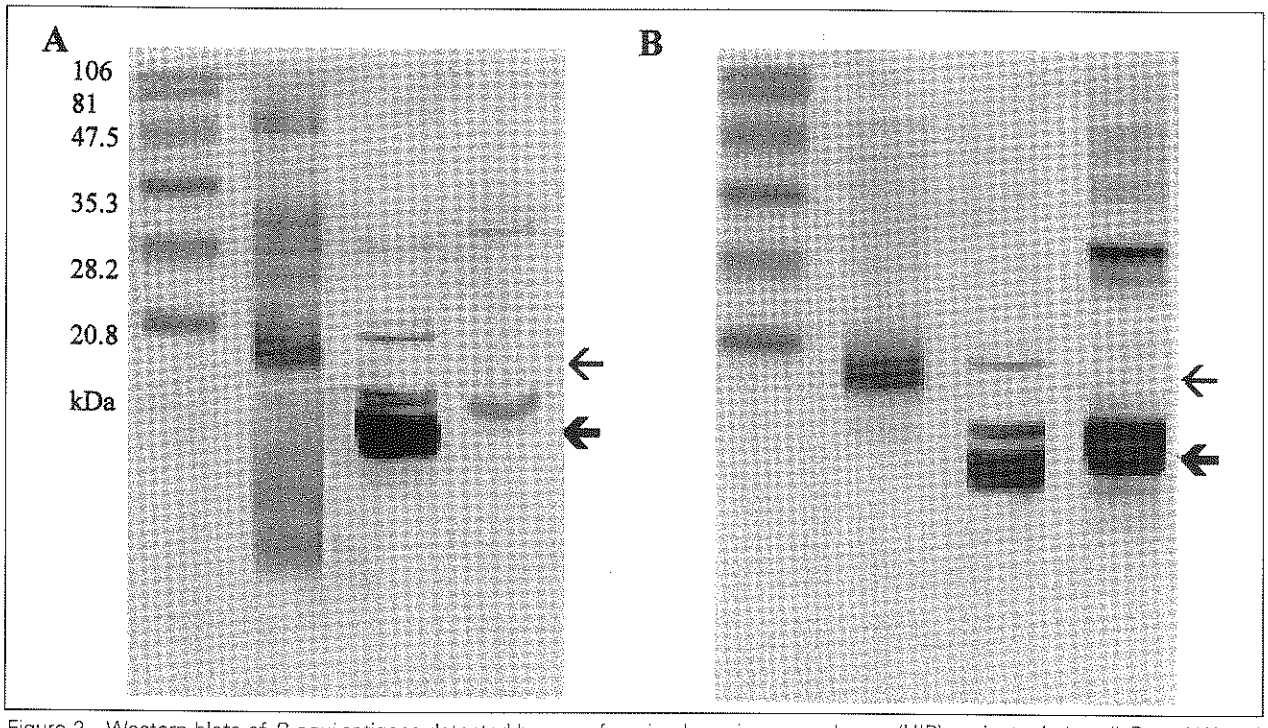

Figure 2—Western blots of *R equi* antigens detected by use of equine hyperimmune plasma (HIP) against whole cell *R equi* (A) and purified equine immunoglobulin against *R equi* VapA and VapC (B). Lane 1, low-range molecular weight markers; lane 2, whole-cell *R equi*; lane 3, purified recombinant VapA; lane 4, purified recombinant VapC. Virulence-associated proteins in whole-cell *R equi* (lane 2, thin arrow) are larger than the recombinant proteins (lanes 3 and 4, thick arrow), probably because the native protein is lipid modified.

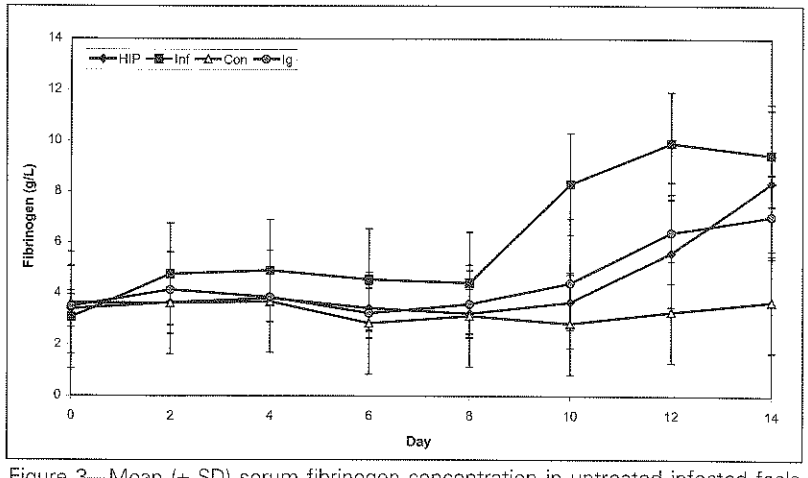

Figure 3—Mean (± SD) serum fibrinogen concentration in untreated infected foals (Inf; n = 11) and in foals treated with HIP (7) or purified immunoglobulin against *R equi* VapA and VapC (Ig; 7) 1 day prior to intrabronchial infection with *R equi* on day 0. Control horses (Con; 3) were not treated and received PBS solution instead of *R equi*.

western blot analysis (Fig 1). The predominant isotype in all samples of immunoglobulin analyzed was IgGT followed by IgGb and IgGa. Western blot analyses performed with HIP and purified immunoglobulin revealed that antibodies in the immunoglobulin preparation were predominantly against VapA and VapC, whereas antibodies in HIP were directed against a number of *R equi* antigens (Fig 2).

Clinical and hematologic evaluation—Serum fibrinogen concentrations on days 10 and 12 were significantly ($P = 0.02$) less in foals that received HIP or immunoglobulin prior to infection, compared with foals that did not (Fig 3). Moreover, rectal temperature of untreated infected foals was significantly greater than that of HIP-treated foals on days 9 through 11 and of immunoglobulin-treated foals on days 9 through 13 (Fig 4). Mean heart rate in the HIP-treated group was significantly less on days 9 through 13, compared with untreated infected foals. However, heart rate of foals treated with immunoglobulin was significantly less than that of the untreated infected group only on days 9 and 10 (Fig 5). Respiratory rate was less in the immunoglobulin-treated group on days 4 through 10, compared with the untreated infected group; however, this difference was only significant on day 9. In addition, respiratory rate was less in HIP-treated foals on days 5 through 13, compared with untreated infected foals. Differences between these groups were significant on days 5, 6, 9, 10, and 13 (Fig 6).

Serology—Serum antibody titers against APTX were > 6.46 at the time of infection (day 0) in foals that received HIP or immunoglobulin and remained high until at least day 7 after infection (Table 1). In contrast, titers in untreated foals were low or not detectable on day 0.

Necropsy—Lung abnormalities were not detected in control foals inoculated with PBS solution. Infected foals all developed pyogranulomatous lung lesions, and to objectively assess the severity of lesions, lung-to-body weight ratios were determined. Lung-to-body weight ratios were significantly greater in the untreat-

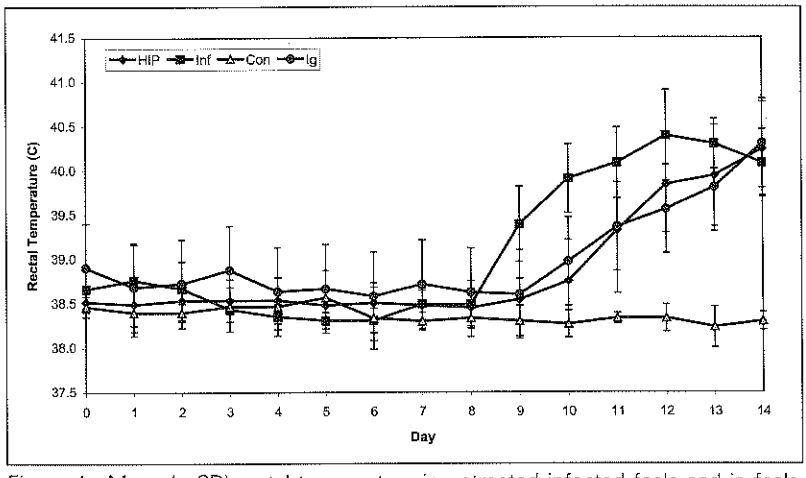

Figure 4—Mean (± SD) rectal temperature in untreated infected foals and in foals treated with HIP or immunoglobulin against *R equi* VapA and VapC 1 day prior to intrabronchial infection with *R equi* on day 0. Control horses were not treated and received PBS solution instead of *R equi*.

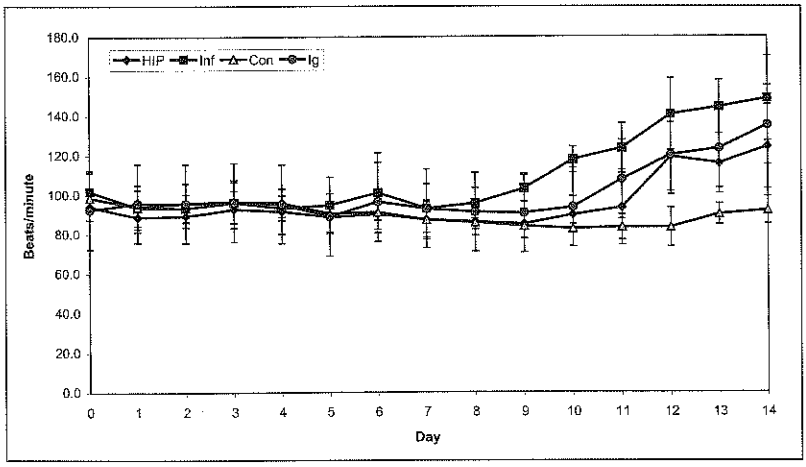

Figure 5—Mean (± SD) heart rate in untreated infected foals and in foals treated with HIP or immunoglobulin against *R equi* VapA and VapC 1 day prior to intrabronchial infection with *R equi* on day 0. Control horses were not treated and received PBS solution instead of *R equi*.

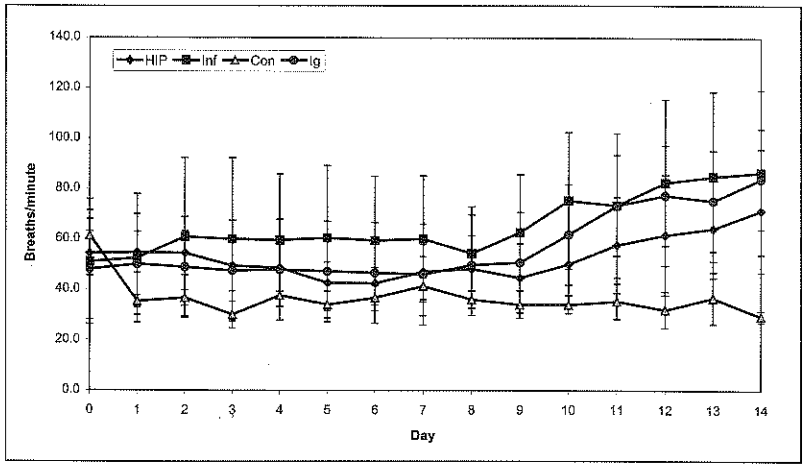

Figure 6—Mean (± SD) respiratory rate in untreated infected foals and in foals treated with HIP or immunoglobulin against *R equi* VapA and VapC 1 day prior to intrabronchial infection with *R equi* on day 0. Control horses were not treated and received PBS solution instead of *R equi*.

Table 1—Mean (± SD) serum antibody titers* against a *Rhodococcus equi* virulence-associated protein (Vap) A-enriched antigen preparation (APTX) in foals before and after intrabronchial infection with *R equi* on day 0

	Day			
Treatment† (n)	**–1**	**0**	**7**	**14**
HIP/infected (7)	3.79 ± 1.35	7.25 ± 0.84	7.25 ± 0.74	5.87 ± 1.47
Ig/infected (7)	2.40 ± 2.37	8.44 ± 0.84	8.05 ± 0.77	4.88 ± 2.32
Infected (11)	ND	2.22 ± 1.98	1.03 ± 1.47	4.53 ± 2.79
Control (3)	ND	0.00 ± 0.00	1.46 ± 2.53	1.69 ± 2.93

*Titers expressed as the natural log (log_2). †Foals in the HIP/infected group received equine HIP (1 L, IV) on day –1; foals in the Ig/infected group received purified equine Ig (1 L, IV) on day –1; foals in the control group received saline (0.9% NaCl) solution instead of *R equi* on day 0.

HIP = Hyperimmune plasma against *R equi*. Ig = Immunoglobulin specific for *R equi* VapA and VapC. ND = Not done.

ed infected group (mean ± SD, 0.042 ± 0.007), compared with the HIP- and immunoglobulin-treated groups (0.026 ± 0.008 and 0.025 ± 0.006, respectively). Additionally, mean bacterial counts (log_{10}) in lung specimens from HIP- and immunoglobulin-treated foals were significantly less (7.59 ± 0.089 and 6.91 ± 1.35 CFU/g of tissue, respectively), compared with untreated infected foals (8.98 ± 0.88 CFU/g of tissue).

Discussion

Compared with untreated infected foals, severity of disease that developed following experimental infection of foals with *R equi* strain 103+ was reduced by administration of commercially available HIP against *R equi* as well as by administration of immunoglobulin purified from plasma of horses vaccinated with *R equi* VapA and VapC. Our results not only support results of other studies[3-6,33] indicating that humoral factors are important in altering the clinical progression of *R equi*-induced pneumonia but also extend those previous results by suggesting that the protective effects of HIP are within the immunoglobulin-enriched fraction and are likely antibodies against VapA and VapC.

Although severity of disease varied among foals in each group, group size allowed for the detection of significant differences between groups. Development of clinical signs of disease was delayed in the HIP- and immunoglobulin-treated groups, compared with the untreated infected group. Most importantly, lung damage and bacterial counts were reduced in treated infected foals. These results suggest that the protective effect of HIP is mediated by the immunoglobulin fraction. We effectively purified the immunoglobulin fraction of plasma from VapA- and VapC-vaccinated horses by use of a double precipitation procedure, but we also acknowledge that the final purified immunoglobulin preparation may have contained small quantities of contaminating proteins that contributed to protection. However, results of a previous study[32] indicate that nonimmune equine serum does not provide protection against *R equi* infection in foals. In the present study, western blot analysis clearly revealed that antibody in the purified immunoglobulin preparation was almost exclusively against VapA and VapC, whereas HIP contained antibodies against other *R equi* antigens as well. We concluded that *R equi* VapA and VapC are protective antigens, because antibodies against these 2 proteins provided partial protection against *R equi*-induced pneumonia in foals equivalent to that mediated by HIP.

Analysis of whole-cell *R equi* antigens indicated that the native Vap antigens are larger than the recombinant proteins, probably because of lipid modification.[12] Interestingly, SDS-PAGE of recombinant VapA and VapC revealed the characteristic multiple forms and diffuse banding pattern typical of Vap antigens.[12] The additional protein bands in recombinant VapC may represent aggregate forms of the purified protein. Hyperimmune plasma reacted less strongly to VapC than VapA, which suggests VapC is less prominently produced by *R equi* than is VapA.

Because *R equi* is an intracellular pathogen, it is assumed that cell-mediated immunity contributes to resistance to infection. This assumption is supported by results of studies indicating that adoptive transfer of an *R equi*-specific Th1 cell line to immunodeficient mice increased clearance of bacteria from the lungs and prevented pneumonia. In contrast, adoptive transfer of a T-

helper 2 cell line did not result in bacterial clearance but did result in development of pulmonary lesions similar to those observed in *R equi*-infected foals.[34-36] Studies of the role of antibodies in protection of mice against *R equi* have resulted in conflicting conclusions. Fernandez et al[37] demonstrated protection of immunocompromised BALB/c mice against fatal intraperitoneal challenge with *R equi* after administration of immunoglobulin from horses vaccinated with partially purified VapA. In contrast, Nordmann et al[11] did not observe protection when immune serum was transferred into athymic (ie, *nu/nu*) BALB/c mice. Results of the present study indicate that, in foals, passive transfer of antibody reduces the severity of pneumonia caused by *R equi*. However, the protective effect of antibody is only partial and is in contrast to the complete protection of foals immunized orally with live virulent *R equi*.[38] The latter procedure would be expected to result in effective cell-mediated immunity. Further studies are required to develop active immunization procedures that effectively protect foals from infection with *R equi*.

Rhodococcus equi invades macrophages, and several mechanisms that may contribute to the ability of this bacterium to avoid killing have been described. Phagocytosis of *R equi* by equine macrophages is not associated with a functional respiratory burst.[39] In addition, intracellular persistence of *R equi* is correlated with absence of phagosome-lysosome fusion.[7,40] Opsonization with *R equi*-specific antibody, however, increases phagosome-lysosome fusion and significantly enhances killing of *R equi* by alveolar macrophages.[7] Hondalus et al[41] demonstrated that binding of *R equi* to macrophages requires complement and is mediated by the complement receptor Mac-1 (CR3). This route of entry into macrophages may allow bacteria to avoid induction of an oxidative burst, as has been observed for *Mycobacterium tuberculosis*.[42] It is likely that entry of *R equi* into macrophages mediated by macrophage Fc receptors following antibody binding to surface-expressed VapA or VapC leads to enhanced bacterial killing, whereas entry via the CR3 receptor in the absence of opsonizing antibody may lead to survival within macrophages.

Although the function of VapA and the other members of the Vap family is currently not known, VapA is highly immunogenic.[31] The results presented here suggest that prior to administering HIP against *R equi* to foals, anti-VapA and -VapC titers should be determined to ensure HIP quality. The failure of HIP to induce protection against natural *R equi* infection in foals, which has been described in at least 1 report,[43] may be explained by lack of relevant antibodies.

[a]Cite Test, Idexx Laboratories, Westbrook, Me.
[b]Veterinary Dynamics Inc, Templeton, Calif.
[c]Animal Health Laboratory, University of Guelph, ON, Canada.
[d]pGEX-2T, Amersham Pharmacia Biotech, Baie d'Urfé, QC, Canada.
[e]XL1Blue *E coli*, Stratagene, La Jolla, Calif.
[f]Glutathione Sepharose 4B, Amersham Pharmacia Biotech, Baie d'Urfé, QC, Canada.
[g]Reheis Inc, Berkeley Heights, NJ.
[h]Aldrich, Oakville, ON, Canada.
[i]Fisher Scientific, Nepean, ON, Canada.
[j]Bio/Can Scientific, Mississauga, ON, Canada.
[k]Courtesy of Holmes MA, Department of Veterinary Clinical Studies, University of Cambridge, Cambridge, UK.
[l]Difco Laboratories, Detroit, Mich.

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