Rhodococcus equi is a facultative intracellular pathogen that causes pneumonia in foals but does not induce disease in adult horses.Virulence of R. equi depends on the presence of a large plasmid, which encodes a family of seven virulence-associated proteins (VapA and VapC to VapH). Eradication of R. equi from the lungs depends on gamma interferon (IFN-γ) production by T lymphocytes. The objectives of the present study were to determine the relative in vivo expression of the vap genes of R. equi in the lungs of infected foals, to determine the recall response of bronchial lymph node (BLN) lymphocytes from foals and adult horses to each of the Vap proteins, and to compare the cytokine profiles of proliferating lymphocytes between foals and adult horses.

vapA, vapD, and vapG were preferentially expressed in the lungs of infected foals, and expression of these genes in the lungs was significantly (P < 0.05) higher than that achieved during in vitro growth. VapA and VapC induced the strongest lymphoproliferative responses for foals and adult horses. There was no significant difference in recall lymphoproliferative responses or IFN-γ mRNA expression by bronchial lymph node lymphocytes between foals and adults. In contrast, interleukin 4 (IL-4) expression was significantly higher for foals than for adults for each of the Vap proteins. The ratio of IFN-γ to IL-4 was significantly higher for foals than for adult horses for most Vap proteins. Therefore, foals are immunocompetent and are capable of mounting lymphoproliferative responses of the same magnitude and cytokine phenotype as those of adult horses.

Because of the facultative intracellular nature of R. equi, cell-mediated immune mechanisms are thought to be of major importance in resistance. Most knowledge of cell-mediated immunity to R. equi infections comes from studying infection of mice. In mice, functional T lymphocytes are required for the clearance of virulent R. equi. Although both CD4+ and CD8+ T cells contribute to the host defense against R. equi in mice, CD4+ T lymphocytes play the major role and are required for complete pulmonary clearance (21). Studies with mice have also clearly shown that a Th1 response, characterized by gamma interferon (IFN-γ) induction, is sufficient to effect complete pulmonary clearance of R. equi, whereas a Th2 response, characterized by interleukin 4 (IL-4) induction, is detrimental (22, 23). How these findings in mice relate to the foal remains to be determined. As opposed to foals, adult horses completely clear an intrabronchial challenge with virulent R. equi, whereas a Th2 response is not functional (31). In a recent study, an R. equi mutant lacking a 7.9-kb DNA region spanning five vap genes (vapA, -C, -D, -E, and -F) was attenuated for virulence in mice and failed to replicate in macrophages (20). Only complementation with vapA could restore full virulence, whereas complementation with vapC, vapD, or vapE could not (20). All vap genes are expressed during in vitro growth and are upregulated when R. equi is grown in equine macrophage monolayers (32).

In Vivo Expression of and Cell-Mediated Immune Responses to the Plasmid-Encoded Virulence-Associated Proteins of Rhodococcus equi in Foals

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Rhodococcus equi, a gram-positive facultative intracellular pathogen, is one of the most important causes of pneumonia in foals between ages 3 weeks and 5 months. R. equi has also emerged as a significant opportunistic pathogen in immunosuppressed people, especially those infected with the human immunodeficiency virus (3, 9, 14). In foals, the course of the disease is insidious and pathology is often extensive by the time the disease is diagnosed. Unlike environmental R. equi, isolates from pneumatic foals typically contain an 80- to 90-kb plasmid. Plasmid-cured derivatives of virulent R. equi strains lose their ability to replicate and survive in macrophages (12). Plasmid-cured derivatives also fail to induce pneumonia and are completely cleared from the lungs of foals, confirming the absolute necessity of the large plasmid for the virulence of R. equi (12, 37).

A 27.5-kb region of the virulence plasmid bears the hallmark of a pathogenicity island and contains the genes for a family of seven closely related virulence-associated (Vap) proteins, designated VapA and VapC to VapH (35). Although a recent study has proposed the designation vapf for another gene of the pathogenicity island, vamp is not functional (31). In a recent study, an R. equi mutant lacking a 7.9-kb DNA region spanning five vap genes (vapA, -C, -D, -E, and -F) was attenuated for virulence in mice and failed to replicate in macrophages (20). Only complementation with vapA could restore full virulence, whereas complementation with vapC, vapD, or vapE could not (20). All vap genes are expressed during in vitro growth and are upregulated when R. equi is grown in equine macrophage monolayers (32).

Because of the facultative intracellular nature of R. equi, cell-mediated immune mechanisms are thought to be of major importance in resistance. Most knowledge of cell-mediated immunity to R. equi infections comes from studying infection of mice. In mice, functional T lymphocytes are required for the clearance of virulent R. equi. Although both CD4+ and CD8+ T cells contribute to the host defense against R. equi in mice, CD4+ T lymphocytes play the major role and are required for complete pulmonary clearance (21). Studies with mice have also clearly shown that a Th1 response, characterized by gamma interferon (IFN-γ) induction, is sufficient to effect complete pulmonary clearance of R. equi, whereas a Th2 response, characterized by interleukin 4 (IL-4) induction, is detrimental (22, 23). How these findings in mice relate to the foal remains to be determined. As opposed to foals, adult horses completely clear an intrabronchial challenge with virulent R. equi and do not develop clinical signs. Clearance of R. equi in adult horses is associated with a significant increase in bronchoalveolar lavage fluid CD4+ and CD8+ lymphocytes, lymphoproliferative responses to R. equi antigens, including VapA, development of R. equi-specific cytotoxic T lymphocytes, and IFN-γ induction (15–17, 24, 30). A few studies have examined antibody responses of foals and adult horses to the
Vap proteins of R. equi (18, 24). However, cell-mediated immune responses and cytokine profiles of foals and adult horses for each of the Vap proteins have never been evaluated. Knowledge of gene products preferentially induced during infection in the natural host would provide important insight into the pathogenesis of the disease and may prove important for vaccine development. Despite the documented importance of some of the vap genes in the virulence of R. equi, relative expression of these genes during active infection in foals has not been studied.

In regard to the above, the objectives of the present study were to determine the relative in vivo expression of the functional vap genes of R. equi in the lungs of infected foals, to determine the recall lymphoproliferative responses of foals to each of the functional Vap proteins and compare them to those of resistant adult horses, and to determine the cytokine profiles of proliferating lymphocytes for both foals and adult horses.

**MATERIALS AND METHODS**

**Animals and intrabronchial challenge.** Five foals between 7 and 10 days of age and five adult horses between 2 and 12 years of age were used in this study. Adequate transfer of passive immunity was confirmed in foals at 12 to 24 h of age by measurement of the plasma immunoglobulin G concentration using a commercial immunoassay (DVM Stat; Corporation for advanced Applications, Newburg, WI). Foals together with their dams were moved to individual stalls in an isolation facility the day after birth. Adult horses were moved to the isolation facility at least 2 days prior to the beginning of the study. Prior to initiation of the study, all animals were determined to be healthy on the basis of a thorough physical examination, complete blood count, biochemical profile, cytology and bacterial culture of a tracheobronchial aspirate, and thoracic radiographs.

R. equi ATCC 33701, a strain containing an 80-kg virulence plasmid, was used to infect foals (35). Aliquots of R. equi were grown on tryptose soy agar plates for 48 h at 37°C. Bacteria were harvested with 4 ml of sterile phosphate-buffered saline (PBS) per plate. The bacterial concentration was determined from CFU counting. Each animal was infected intrabronchially with an inoculum of \(2 \times 10^8\) CFU per kg of body weight. This corresponded to a total inoculum of approximately \(1 \times 10^7\) for each foal and \(1 \times 10^6\) for each adult horse. The inoculum was diluted in 50 ml of sterile PBS. Prior to infection, animals were sedated with 0.5 mg/kg of xylazine hydrochloride and 0.07 mg/kg of butorphanol tartrate given intravenously. A flexible fiber-optic endoscope was used to deliver 25 ml of the bacterial suspension into each of the main bronchi. Animals were clinically assessed based on daily complete physical examinations as well as twice-daily heart rate, respiratory rate, and temperature recording. Euthanasia was performed 15 days postinfection by intravenous administration of a lethal dose of pentobarbital sodium.

Bronchial lymph nodes (BLN) were collected aseptically from a cranioventral lung lobe. This area corresponded to the most severely affected location in all foals. One sample was immediately frozen in liquid nitrogen for subsequent bacterial RNA isolation, and the other was placed in a sterile bag for quantitative bacterial enumeration.

**Preparation of BLN cells, cell stimulation, and lymphocyte proliferation assay.** Vap proteins (VapA, -C, -D, -E, -F, -G, and -H) for use in proliferation assays were collected from BLN. Briefly, BLN were cut into 125-mm³ pieces and the cells were separated in glass culture. RNA isolation, and the other was placed in a sterile bag for quantitative bacterial enumeration.

**RNA isolation, cDNA synthesis.** Total RNA from BLN cells was performed using the QIAGEN RNaseasy kit (QIAGEN, Valencia, CA) according to the manufacturer’s instructions. The RNA concentration was measured by determining the optical density at 260 nm. All RNA samples were treated with amplification-grade DNase I (Gibco BRL, Rockville, MD) to remove any trace of genomic DNA contamination. Briefly, 1 U of DNase I and 1 ml of 10× DNase I reaction buffer were added to 1 ml of RNA solution. This mixture was incubated for 10 min at room temperature and then inactivated by addition of 1 ml of 25 mM EDTA and heating at 65°C for 10 min.

cDNA was synthesized with the Advantage RT-PCR kit (Clontech, Palo Alto, CA) by using the protocol of the manufacturer. Briefly, 1 μg of total RNA was mixed with 1 μl of oligo(dT)₁₇ primer (20 μM) and heated at 70°C for 2 min. After cooling to room temperature, the following reagents were added: 4 μl of 5× first-strand buffer, 1 μl of 100 mM dNTPs, 10 μM of each of the reverse primers (10 mM each), 1 (10 μg/ml) of RNase inhibitor (40 U/ml), and 1 μl of Moloney murine leukemia virus reverse transcriptase (200 U/ml). The mixture was incubated at 42°C for 1 h, heated at 94°C for 5 min, diluted to a final volume of 100 μl, and stored at −70°C until used for PCR analysis.

**Quantification of cytokine mRNA.** Gene-specific primers and internal oligonucleotide probes for equine glycyeroldehyde-3-phosphate dehydrogenase, IL-4, and IFN-γ have been described previously (11). The internal probes were labeled at the 5′ end with the reporter dye 6-carboxyfluorescein and at the 3′ end with the quencher dye 6-carboxytetramethylrhodamine. Amplification of 2 μl of cDNA was performed in a 25-μl PCR mixture containing 900 nM of each primer, 250 nM of TaqMan probe, and 12 μl of TaqMan Universal PCR Mastermix (Applied Biosystems, Foster City, CA). Amplification and detection were performed using the ABI Prism 7700 sequence detection system (Applied Biosystems) with initial incubation steps at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Serial dilutions of cDNA from 24-h concanavalin A-stimulated equine blood mononuclear cells were used to generate a standard curve for relative quantification of each gene of interest. Each sample was assayed in triplicate, and the mean value was used for comparison. Samples without cDNA were included in the amplification reactions to determine background fluorescence and check for contamination. To account for variation in the amount and quality of starting material, values obtained for IFN-γ and IL-4 transcripts were normalized by dividing by the glycyeroldehyde-3-phosphate dehydrogenase transcript level for the same sample. The sample with the lowest level of gene expression was designated 1, and relative quantification between samples was reported as the n-fold difference relative to cytokine mRNA expression in that sample.

**Bacterial RNA isolation, DNase treatment of bacterial RNA samples, and cDNA synthesis.** Bacterial RNA extraction for determination of in vivo expression of the vap genes was performed on lung tissue from the five infected foals. For determination of in vitro expression of the vap genes, one colony of R. equi ATCC 33701 was placed in five separate tubes, each containing 5 ml of brain heart infusion broth with 1% yeast extract. The suspensions were grown in a shaking incubator at 37°C for 18 h. A 125-mm³ section of lung tissue and pellets from in vivo-grown bacteria were independently pulverized in cold Trizol reagents (Invitrogen Corporation, Carlsbad, CA) using glass tissue grinders. Total RNA was extracted using the Trizol reagents by following the instructions from the manufacturer. RNA from each source was further purified independently by passing the resulting sample through MinElute spin columns (QIAGEN, Valencia, CA).

**Relative quantification.** The sample with the lowest level of gene expression was designated 1, and relative quantification between samples was reported as the n-fold difference relative to cytokine mRNA expression in that sample.
TABLE 1. Oligonucleotide primer and probe sequences for amplification of vap genes from R. equi

| Gene   | Primer or probe | Sequence (5’ to 3’) |
|--------|-----------------|---------------------|
| vapA   | Forward         | CATCAACTTCCTCGATAGCTGTCAGGTA |
|        | Reverse         | GAGCCGCCACCACTGACATGTC |
|        | Probe           | TCCCTGGGCATACCTCCGCTG |
| vapC   | Forward         | GGGTGACTTGGTGAGG |
|        | Reverse         | CCGTATAGCTGTCCGGCCA |
|        | Probe           | TCCGAGTTCTACGGCGCACATAA |
| vapD   | Forward         | CAGAGGCTTTGCGG |
|        | Reverse         | CATTAGACGCTGCTGCA |
|        | Probe           | TTATTCCTTCTTGGCTCAGGTGCT |
| vapE   | Forward         | TGAGTACAAAGGTGTCG |
|        | Reverse         | ACCGTCGCCAGCAAC |
|        | Probe           | TACTGAGAACATCAAATC |
| vapF   | Forward         | CATCACTGCTGCAAGACT |
|        | Reverse         | CCCATGAAAGCGCATATTG |
|        | Probe           | CGCAATACTAAATAATG |
| vapG   | Forward         | CACTGAACCCCNGGA |
|        | Reverse         | GGCGGCGGAAAGACGT |
|        | Probe           | TCGAATCCGCGCAATCCACA |
| vapH   | Forward         | GTCAACTTCCTCGACTG |
|        | Reverse         | ACCGAGTCTACCCCTC |
|        | Probe           | CGCAATACTCGGTCATAGCACA |

RESULTS

Disease process and macroscopic findings. All animals maintained normal vital signs during the study and showed no evidence of disease. All five infected foals had macroscopic pulmonary lesions that consisted of mild to moderate areas of consolidation in the ventral lung lobes, along with multiple small nodular lesions up to 1 cm in diameter. The mean number of R. equi bacteria (± standard deviation [SD]) was 9.63 × 10⁶ ± 1.21 × 10⁷ (range, 6.23 × 10⁶ to 2.33 × 10⁷) CFU/g of lung tissue. The lungs of adult horses were free from lesions, and bacterial culture was negative.

In vivo expression of R. equi vap genes. Real-time PCR was used to quantify the relative in vivo expression of the vap genes in the lung tissue of infected foals, and the expression was compared to that of in vitro-grown bacteria. Expression levels of vapA, vapD, and vapG were significantly higher during in vivo growth than during in vitro growth (Fig. 1). Expression of vapG in lung tissue was significantly higher than that of all other vap genes. Expression of vapA and vapD in lung tissue was significantly higher than that of vapC, vapE, vapF, and vapH (Fig. 1). The expression of the vap genes during in vitro growth followed a similar pattern, with significantly greater expression of vapA, vapD, and vapG (Fig. 1).

Vap-specific proliferative responses and cytokine expression. To determine whether differences in lymphoproliferative responses and cytokine profiles may be at the origin of the peculiar susceptibility of foals to infection with R. equi, recall responses of susceptible, infected foals was compared to those of R. equi-resistant adult horses. Proliferation of BLN mononuclear cells following stimulation with each recombinant Vap protein was measured. There were no significant differences in lymphoproliferative responses to each of the Vap proteins.
between infected foals and adult horses (Fig. 2). For foals, lymphoproliferative responses to VapA were significantly greater than those to all of the other Vap proteins, and lymphoproliferative responses to VapC were significantly greater than those to all Vap proteins except VapA (Fig. 2). In adult horses, lymphoproliferative responses to VapA and VapC were significantly greater than those to the other Vap proteins (Fig. 2).

Induction of IFN-γ and IL-4 in proliferating cells was quantified by real-time reverse transcription-PCR. There was no significant difference in IFN-γ expression by BLN cells between foals and adult horses for any of the Vap proteins (Fig. 3A). In contrast, IL-4 mRNA expression for each of the Vap proteins was significantly greater for adult horses than for foals (Fig. 3B). The ratio of IFN-γ to IL-4 was significantly higher with foals than with adult horses for VapA, VapC, VapD, VapF, and VapH (Fig. 3C).

**DISCUSSION**

Despite a central role for cell-mediated immune responses in protection against *R. equi*, most studies have focused on antibody responses against the Vap proteins. This is the first study to examine the expression of vap genes in the lungs of *R. equi*-infected foals and to measure lymphoproliferative responses and the cytokine profiles of infected foals and adult horses for each of the functional Vap proteins. It complements an earlier study by Hooper-McGrevy et al. (18) that examined the serum immunoglobulin G subisotype responses to each of the Vap proteins for foals and for adult horses, as well as a study evaluating the expression of the pathogenicity island genes in equine macrophages (32).

VapA is expressed on the bacterial surface, and its expression is temperature regulated, occurring between 34 and 41°C (34). In contrast, VapC, -D, and -E are secreted proteins concomitantly regulated by temperature with VapA, being produced at 37°C but not at 30°C (7). The cellular locations of VapF, VapG, and VapH are unknown. In one study, all vap genes were upregulated when *R. equi* was grown in equine macrophage monolayers compared to expression during in vitro growth at 30°C (32). In the same study, vapA and vapC

![FIG. 2. Recall lymphoproliferative responses of BLN mononuclear cells from foals and adult horses to recombinant *R. equi* VapA, VapC, VapD, VapE, VapF, VapG, or VapH. The results are expressed as the fluorescence of stimulated cells minus that of unstimulated cells. The results are displayed as means ± SD. Different letters (a, b, and c) indicate significant differences in lymphoproliferative responses to Vap proteins for foals (P < 0.05). Different numbers (1 and 2) indicate significant differences in lymphoproliferative responses to Vap proteins for adult horses (P < 0.05).](http://cvi.asm.org/)

![FIG. 3. Relative IFN-γ (A) or IL-4 (B) mRNA expression or IFN-γ/IL-4 ratio (C) following stimulation of BLN mononuclear cells of foals (striped bars) or adult horses (dotted bars) with recombinant *R. equi* VapA, VapC, VapD, VapE, VapF, VapG, or VapH. The results are displayed as means ± SD. *, statistically significant difference between results for foals and those for adult horses (P < 0.05).](http://cvi.asm.org/)
were the most highly upregulated of the \textit{vap} genes (32). In the present study, only \textit{vapA}, \textit{vapD}, and \textit{vapG} were expressed at significantly higher levels in vivo than during in vitro growth. The discrepancy between findings of these studies may result from the fact that culture in macrophage monolayers does not accurately reflect all the conditions experienced by the pathogen in the lungs of foals. In addition, relative expression of the \textit{vap} genes may depend on the stage of infection. The present study evaluated expression in the lungs of foals 2 weeks after infection, whereas the macrophage study evaluated expression 4 h after the monolayers were infected with \textit{R. equi}. Finally, differences in temperatures for in vitro growth between the two studies may at least partially explain some of the inconsistencies. The macrophage study grew \textit{R. equi} in vitro at 30°C, a temperature known to decrease expression of the Vap proteins (7, 32). In contrast, the present study used the more physiological temperature of 37°C to maximize \textit{vap} gene expression during in vitro growth, hence minimizing the likelihood of identifying differences due only to dissimilar growth conditions.

The exact role of each Vap protein remains to be determined, but the proteins are probably involved in preventing acidification and late endosomal maturation of \textit{R. equi}-containing vacuoles within macrophages (10, 36). Regulation of expression of the \textit{vap} genes is complex and depends on at least five environmental signals, temperature, pH, oxidative stress, magnesium, and iron (4, 5, 32, 34). The \textit{vapA} and \textit{vapD} genes are the major acid-inducible determinants carried by the virulence plasmid (4). In another study, \textit{vapA} and \textit{vapG} were predominantly induced by H$_2$O$_2$ treatment (5). These findings have led to the hypothesis that VapA, VapD, and VapG may play a dominant role in protection against macrophage-related stresses (5). That \textit{vapA}, \textit{vapD}, and \textit{vapG} were expressed at significantly higher levels than the other \textit{vap} genes in the lungs of infected foals in the present study is consistent with this hypothesis. These three genes were also significantly more expressed in vivo than in vitro.

Several lines of evidence suggest that immune responses against VapA and potentially other Vap proteins confer a role in protection (17, 24). A recent study with mice has shown that DNA immunization with \textit{vapA} protects against \textit{R. equi} infection and that the immunoglobulin G subisotype response is consistent with a Th1-based immune response (13). A similar DNA vaccine containing the \textit{vapA} gene has been shown to induce strong cell-mediated immune responses in adult horses (25). In foals, production of antibody to VapA and VapC but not of that to other Vap proteins increases following natural exposure to \textit{R. equi} (18). The present study shows that VapA and VapC are also the proteins inducing the strongest lymphoproliferative recall responses following experimental challenge for both foals and adult horses. A prior study had demonstrated strong lymphoproliferative recall responses to VapA in association with clearance of \textit{R. equi} in adult horses (24). The present study confirmed this finding and also demonstrated that lymphocytes from foals and from adult horses proliferate similarly in response to each Vap protein.

The documented importance of IFN-\(\gamma\) in protection against infection with \textit{R. equi} (22) and the recognized Th2 bias in immune responses of neonates from many species (1) have led to the widespread hypothesis that a similar Th2 bias may be at the basis of the peculiar susceptibility of foals to infection by \textit{R. equi}. The recent finding that young foals are deficient in their ability to produce IFN-\(\gamma\) in response to mitogens led to the conclusion that neonatal foals may also be predisposed to develop a Th2-like immune response (6). In one study, pulmonary lymphocytes from adult horses collected 7 days following challenge with \textit{R. equi} expressed predominantly IFN-\(\gamma\), but they also expressed IL-4 mRNA in response to in vitro stimulation with VapA (24). Similar results were obtained following stimulation of BLN lymphocytes with VapA in the present study. In addition, the present study extends these findings to the other Vap proteins of \textit{R. equi}. The present study also demonstrates that BLN lymphocytes from foals can express IFN-\(\gamma\) in response to stimulation with each Vap protein just as well as those from immune adult horses. In fact, as a result of the significantly lower level of IL-4 expression for foals than for adult horses, foals showed a significantly higher IFN-\(\gamma\)/IL-4 ratio than adult horses in response to stimulation with most Vap proteins. The findings in the present study, therefore, do not support the conclusion that neonatal foals are predisposed to develop a Th2-like immune response. The reason for the peculiar susceptibility of foals to \textit{R. equi} must be more complex and also must take into account the unique association of the VapA-containing virulence plasmid with disease in foals.

This strong predominating IFN-\(\gamma\) response in a neonate is not unprecedented. Many studies have shown that despite an apparent Th2 bias, neonates can mount strong Th1 responses to some antigens (1, 27). For example, \textit{Mycobacterium bovis} BCG, a pathogen closely related to \textit{R. equi}, triggers a Th1 response in human neonates of a magnitude similar to that seen when it is given later in life (26). Virulent \textit{R. equi} is widespread in the environment of horse breeding farms (28, 33), yet development of clinical disease is the exception rather than the rule. The fact that oral administration of live virulent \textit{R. equi} to newborn foals confers almost complete protection against subsequent heavy intrabronchial challenge (8, 19) and the fact that most foals on farms where it is endemic do not develop disease or develop subclinical disease and eventually clear the infection are consistent with the results of the present study, which demonstrate that foals develop adequate cell-mediated immune responses to key antigens of \textit{R. equi}.

In conclusion, the data presented here indicate that \textit{vapA}, \textit{vapD}, and \textit{vapG} are the most biologically relevant \textit{vap} genes because they are preferentially induced during infection in the natural host. All the Vap proteins are immunogenic, with VapA and VapC providing the strongest lymphoproliferation stimulus. The peculiar susceptibility of foals to infection by \textit{R. equi} cannot be explained by a failure to mount Th1 immunoreactivity to the Vap proteins. Further work is required to identify the fundamental host basis of the susceptibility of foals to \textit{R. equi} pneumonia.

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