Identification of Pulmonary T-Lymphocyte and Serum Antibody Isotype Responses Associated with Protection against *Rhodococcus equi*

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***Rhodococcus equi* infects and causes pneumonia in foals between 2 and 4 months of age but does not induce disease in immunocompetent adults, which are immune and remain clinically normal upon challenge. Understanding the protective response against *R. equi* in adult horses is important in the development of vaccine strategies, since those mechanisms likely reflect the protective phenotype that an effective vaccine would generate in the foal. Twelve adult horses were challenged with virulent *R. equi* and shown to be protected against clinical disease. Stimulation of cells obtained from bronchoalveolar lavage fluid with either *R. equi* or the vaccine candidate protein VapA resulted in significant proliferation and a significant increase in the level of gamma interferon (IFN-γ) expression by day 7 postchallenge. The levels of interleukin-4 expression were also increased at day 7 postchallenge; however, this increase was not antigen specific. Anamnestic increases in the levels of binding to *R. equi* and VapA of all immunoglobulin G (IgG) antibody isotypes [IgGa, IgGb, IgG(T)] examined were detected postchallenge. The levels of *R. equi*- and VapA-specific IgGa and IgGb antibodies, the IgG isotypes that preferentially opsonize and fix complement in horses, were dramatically enhanced postchallenge. The antigen-specific proliferation of bronchoalveolar lavage fluid cells, the levels of IFN-γ expression by these cells, and the anamnestic increases in the levels of opsonizing IgG isotypes are consistent with stimulation of a memory response in immune adult horses and represent correlates for vaccine development in foals.**

Rhodococcus equi is a gram-positive bacterium that infects alveolar macrophages and causes rhodococcal pneumonia in foals less than 6 months old. In contrast, immunocompetent adult horses are immune and remain clinically normal. Pulmonary challenge of adult horses with virulent *R. equi* triggers an antigen-specific recall response with clearance of the bacteria (10). We propose that a better understanding of the correlates of immunity to *R. equi* in adult horses can be used to develop strategies to protect foals, since those correlates likely reflect the protective phenotype that an effective vaccine would need to generate in a naïve animal.

Most of what is known of immunity to *R. equi* has been derived from experiments with mouse models. Several studies have shown that protection is dependent on the induction of T lymphocytes and is mediated by gamma interferon (IFN-γ) (13, 14, 15, 23, 24). Adoptive transfer of *R. equi*-specific CD4$^+$ T-lymphocyte lines to *R. equi*-susceptible nude mice demonstrated that a Th1 response is sufficient to effect pulmonary clearance, whereas a Th2 response is detrimental (15). Furthermore, blockage of IFN-γ enhances disease in normally resistant mice (14). While these studies have defined basic regulatory and effector mechanisms, their relevance in horses is unknown. A previous study from our laboratory analyzing the T-lymphocyte recall response in bronchoalveolar lavage (BAL) fluid (BALF) of immune adult horses identified CD4$^+$

and CD8$^+$ antigen-specific T cells (10). CD4$^+$ T cells are postulated to effect clearance via enhancement of B-cell secretion of opsonizing antibodies, in concert with macrophage activation via IFN-γ. Importantly, CD4$^+$ T cells, through both cognate and cytokine-mediated interactions with B lymphocytes, are essential for the induction of high-affinity immunoglobulin G (IgG) antibodies. In addition to macrophage activation, expression of IFN-γ by CD4$^+$ T cells enhances secretion of specific opsonizing IgG isotypes. Passive transfer experiments have demonstrated that hyperimmune plasma administered before *R. equi* challenge can confer at least partial protection against infection (18, 20). However, the equine antibody isotypes that are associated with protection remain unknown.

In the study described in this report we used both whole *R. equi* and a specific vaccine candidate, virulence-associated protein A (VapA), to evaluate antibody responses and antigen-specific proliferation and IFN-γ expression of BALF cells in immune adult horses. VapA is a surface-exposed protein encoded by a virulence-associated plasmid of *R. equi*. Importantly, sera from adult horses immune to *R. equi* have anti-VapA-specific IgG (S. A. Hines, unpublished data). Likewise, purified equine immunoglobulin specific for VapA and a related protein, VapC, had a protective effect when it was passively transferred to foals prior to experimental challenge (12). Induction of high-affinity IgG, which requires CD4$^+$ T cells, to VapA indicates that VapA is an appropriate antigen target for assessment of the anamnestic response.

We hypothesized that a protective immune response against *R. equi* infection in horses is associated with IFN-γ expression

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and production of specific antibody isotypes associated with macrophage opsonization. To test the hypothesis, cells from BALF of adult horses challenged with virulent *R. equi* were stimulated and analyzed for expression of IFN-γ and interleukin-4 (IL-4) by real-time reverse transcription-PCR. Antigen-specific antibody isotypes were analyzed by enzyme-linked immunosorbent assays (ELISAs) with VapA and *R. equi* as target antigens.

MATERIALS AND METHODS

Preparation of *R. equi* for challenge. *R. equi* ATCC 33701 is a virulent strain that possesses the 82-kb plasmid and expresses the 15- to 17-kDa protein VapA, which is associated with virulence. Bacteria were kept as frozen stabilates. After reconstitution and selection of a single colony, the bacteria were grown in brain heart infusion (BHI) medium (Difco Laboratories, Detroit, Mich.) for 16 h at 37°C with shaking. A bacterial pellet was obtained after centrifugation of the culture at $800 \times g$ and washed twice with phosphate-buffered saline (PBS). *R. equi* was resuspended in PBS to a final concentration of 2×10^7 bacteria/ml.

BAL and pulmonary challenge. All animal experiments were conducted in compliance with relevant federal guidelines and the Animal Care and Use Program of Washington State University. BAL was performed on each horse as described previously (10). Briefly, horses were mildly sedated with xylazine and butorphenol. An endoscope was passed nasally and directed into the right cranial lobar bronchus. A solution of sodium chloride (0.9%)–sodium bicarbonate (0.06%) (pH 6.5) was instilled into the right lung as nine 60-ml aliquots. Following instillation of 180, 360, and 540 ml of saline, BALF was aspirated for analysis. At the end of the first BAL procedure (day 0), the right lung was inoculated with 2×10^7 *R. equi* ATCC 33701 organisms in 1 ml of PBS and the endoscope was flushed with 15 ml of air just before removal. BAL was repeated 7 days following challenge by using the identical procedure, except that no *R. equi* was instilled. After challenge and after each BAL procedure, the horses were placed in a stall and monitored daily for changes in rectal temperature, respiration, and pulse as determined by physical examination and auscultation of the lungs. Blood was obtained via jugular venipuncture on the day of each BAL procedure, and samples were submitted to the Washington State University Clinical Pathology Laboratory for determination of complete blood counts and fibrinogen concentrations.

Preparation of *R. equi* antigen. *R. equi* ATCC 33701 was grown in BHI for 72 h at 37°C with agitation. The bacteria were harvested by centrifugation at $3,750 \times g$ for 10 min and washed with sterile PBS. Two milliliters of the bacterial pellet was resuspended in 10 ml of PBS. The bacteria were disrupted by three freeze-thaw cycles at −20°C. After the third cycle, the bacterial homogenate was centrifuged at $12,000 \times g$ for 15 min to separate the pellet of intact bacteria and debris. The supernatant was centrifuged at $25,000 \times g$ for 20 min at 4°C to obtain the soluble *R. equi* antigen used to stimulate T cells. Soluble antigen derived from *Corynebacterium pseudotuberculosis* was prepared similarly for use as a negative control. All preparations were stored at −70°C until use.

Preparation of native VapA antigen. VapA antigen was prepared as described previously (30). Briefly, *R. equi* ATCC 33701 was grown in 1 liter of BHI medium for 60 h at 37°C with agitation. The bacteria were collected by centrifugation at $5,000 \times g$ for 10 min and washed with TBS (10 mM Tris [pH 7.4], 150 mM NaCl). Fifty milligrams of bacteria was resuspended per milliliter of TBS containing 2% Triton X-114 and 1 mM phenylmethylsulfonyl fluoride. Following shaking overnight at 4°C, insoluble material was removed by centrifugation at $5,000 \times g$ for 15 min at 4°C. The supernatant was recovered, warmed at 37°C for 10 min, and centrifuged at $5,000 \times g$ for 15 min at 25°C. The upper aqueous layer was discarded. The lower detergent phase was washed with a volume of TBS equivalent to the volume of the discarded aqueous layer, to a final solution of 2% Triton X-114, warmed at 37°C for 10 min, and centrifuged at $5,000 \times g$. This wash was repeated twice. Proteins of the detergent phase were precipitated with 10 volumes of acetone overnight at −20°C and centrifuged at $9,000 \times g$ for 10 min. The pellet was dissolved in Tris (pH 7.4). The proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by Coomassie blue staining and immunoblotting, and the proteins were quantified by the bicinchoninic acid method.

Recombinant VapA. The *vapA* gene from *R. equi* was amplified by PCR and cloned into the vector pTrcHis2 A (Invitrogen, Carlsbad, Calif.). This vector allows expression of recombinant proteins containing a C-terminal His$_6$ tag in *Escherichia coli*. The sequences of the primers used to amplify *vapA* were as follows: primer AHISF1 (forward primer), 5′-CCGTTCTGGATCCCGGTAGC

AGCAGTGCGA-3′; primer AHISR1 (reverse primer), 5′-TGCTAGGAATTC TGCCAGCTACCAGAGCCG-3′. The forward primer has a *Bam*HI site (underlined), and the reverse primer has an *Eco*RI site (underlined). The primers amplified a fragment of 567 bp of *vapA*, which does not contain the VapA leader sequence that promotes self-cleavage in *E. coli*. The PCR product was cloned into the *Bam*HI and the *Eco*RI sites of pTrcHis2 A. The resulting insert was confirmed by DNA sequencing. Expression of the recombinant fusion protein of the expected size in *E. coli* lysates was demonstrated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by Coomassie blue staining and immunoblotting, by using anti-*R. equi* serum and monospecific antiserum to VapA. The recombinant protein was purified by nickel affinity chromatography (ProBond resin; Invitrogen) under denaturing conditions by using the recommendations of the manufacturer.

Analysis of BALF cells. BALF was centrifuged at $400 \times g$ for 10 min, and pelleted cells were washed twice with RPMI 1640 medium containing 0.2 mM L-glutamine, 0.1 mM HEPES, 10,000 U of penicillin G per ml, 100 μg of streptomycin sulfate per ml, and 0.25 μg of amphotericin B per ml. If erythrocytes were abundant, the cells were incubated with 0.87% ammonium chloride–1 M Tris for 5 min at room temperature to lyse the erythrocytes, and then a final wash was performed. The cells were resuspended at 2×10^6 cells/ml in complete RPMI 1640 medium with 10% fetal calf serum, placed into 75-cm^2 plastic tissue culture flasks to facilitate macrophage adherence, and kept in a humidified CO$_2$ incubator (5% CO$_2$, 95% air) at 37°C for 1 h. Nonadherent cells were collected, centrifuged, and resuspended at 2×10^6 cells/ml in RPMI 1640 medium containing 10% fetal calf serum.

Proliferation assays. The proliferative responses of cells obtained from BALF and treated as described above were evaluated by measurement of the uptake of [^{3}H]thymidine. Briefly, cells from BALF were cultured in triplicate wells of 96-well flat-bottom plates. Each well contained 2×10^6 cells/ml in a total volume of 0.2 ml of complete RPMI 1640 medium. Soluble *R. equi* antigen and native VapA were added at 5, 10, 25, 50, and 100 μg/ml. Optimal stimulation was achieved with 10 μg of antigen/ml (data not shown). *C. pseudotuberculosis* antigen was prepared identically for use as a negative antigen control, and cells stimulated with pokeweed mitogen (PWM) or concanavalin A (ConA) (1 μg/ml) were used as positive controls for stimulation. The cells were stimulated for 5 days by incubation of the plates in a humidified CO$_2$ chamber (5% CO$_2$, 95% air) at 37°C. Eighteen hours before termination of the culture, the cells were pulsed with 0.5 μCi of [^{3}H]thymidine. The cells were harvested onto filter paper with a Tomtec cell harvester, and the level of incorporation of [^{3}H]thymidine was assessed by liquid scintillation counting in a β plate reader. The assays were conducted independently with two separate groups of horses ($n = 6$) by using different lots of [^{3}H]thymidine and serum. The data for each group of six horses was considered a separate data set.

Quantification of cytokine mRNA. For analysis of antigen-specific cytokine expression, cells from BALF were incubated as described above for the proliferation assays. After 24 h of incubation, the cells were harvested and frozen at −70°C. RNA was extracted by using an RNAeasy kit (Qiagen, Valencia, Calif.) according to the recommendations of the manufacturer. Contaminating DNA was eliminated by DNase treatment for 30 min at 37°C. Total RNA (1 μg) of each sample was used to generate cDNA in a reaction containing murine leukemia virus reverse transcriptase and random hexamers in a final volume of 20 μl at 42°C for 20 min. The products obtained were used in real-time PCR to detect expression of cytokines. The standard curves for cytokine transcription were generated by PWM stimulation of BALF cells obtained from a control horse not challenged with *R. equi*. These standard curves were linear for both cytokines, and the values for all samples fell within the linear range of dilutions (data not shown). To confirm the absence of genomic DNA contamination, aliquots of each DNase-treated RNA sample also were used in the cDNA reaction mixtures to which reverse transcriptase was not added (no reverse transcriptase control). To control for contamination of reagents, PCRs with no cDNA were performed and were consistently negative.

Expression of equine IFN-γ, IL-4, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in cells obtained from BALF was determined by real-time PCR. The fluorescent dye SYBR Green, which binds to double-stranded DNA, was used to measure the amplified products. All the primers used for SYBR Green PCR were designed by using Primer Express software (version 1.0; PE Applied Biosystems, Foster City, Calif.) and are described in Table 1. The amplifications were carried out in a thermocycler (GeneAmp 5700; PE Applied Biosystems) by the following procedure: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s. The reactions were performed in 96-well plates, and the 25-μl reaction mixtures contained 50 ng of cDNA, 1× SYBR Green buffer, 1.5 mM MgCl$_2$, 1 mM deoxynucleoside triphosphates, 0.04 U of uracil-*N*-glycosylase Amperase, and 0.2

TABLE 1. Primers used for real-time reverse transcription-PCR detection of GAPDH, IFN-γ, and IL-4 in BALF cells of adult horses

Target gene	Primer name	Sequence
GAPDH	GAPDH F	5′-GTAATCAACGGAAAGGCCATCA-3′
	GAPDHR	5′-CCTTTTGGCTCCACCCTTCAAG-3′
IFN-γ	IFN-γ F	5′-AGGTCCAGCGCAAAGCAATAAG-3′
	IFN-γR	5′-TTGCAGGCAGGATGACCACTAT-3′
IL-4	IL-4 F	5′-CCGAAGAACACAGATGGAAAGGA-3′
	IL-4R	5′-TCACAGTACAGCAGGTCCCGTTT-3′

U of Amplitaq Gold polymerase. In each 96-well plate, a dilution series of the cDNA control containing primers for amplification of IFN-γ, IL-4, and GAPDH was used to generate a standard curve for each gene. These curves were used as standards to compare the real-time PCRs between plates. All samples were analyzed in triplicate. After PCR amplification, data acquisition was performed with the GeneAmp 5700 sequence detection system (version 1.3). For each sample, the threshold cycle values for the three genes expressed were compared to the value from the respective standard curve to generate a transcript level. The values obtained for IFN-γ and IL-4 transcripts were normalized by using the GAPDH transcript level for the same sample.

Determination of antibody isotype titers. R. equi- and VapA-specific antibodies in serum were analyzed by ELISA. Ninety-six-well Immulon II plates were coated overnight with 1 μg of either recombinant VapA (rVapA) or R. equi soluble antigen per ml in carbonate-bicarbonate buffer (pH 9.6) (total volume, 50 μl/well) at 4°C. The plates were washed four times with PBS containing 0.05% Tween and 2.5% sodium chloride (PBST). Serum samples were serially diluted in PBST (total volume, 50 μl/well) and incubated at room temperature for 30 min. To detect the total IgG antibodies bound, the plates were washed with PBST and incubated for 30 min with 50 μl of peroxidase-conjugated caprine antibodies (1:10,000; Kirkegaard & Perry Laboratories, Gaithersburg, Md.) directed against horse IgG per well. To detect the specific antibody isotypes bound, the plates were washed with PBST before being incubated for 30 min with 50 μl of anti-equine IgGa, IgGb, IgG(T), IgA, or IgM murine monoclonal antibody (0.5 μg/ml; generously provided by Paul Lunn, University of Wisconsin) per well. These plates were washed with PBST and incubated with a peroxidase-conjugated anti-mouse IgG. The plates were again washed, and bound antibodies were detected with enhanced chemiluminescence Western blotting detection reagents (Amersham Pharmacia Biotech Inc, Piscataway, N.J.). Optical densities were read at 450 nm in an ELISA reader. Serum from a known high responder and normal horse serum were used as standard positive and negative controls on each plate, respectively, and were used to generate a standard curve and to correct for interplate variability.

Statistical analysis. Statistical analyses were performed by using Prism software (version 2.0; GraphPad Software Inc., San Diego, Calif.). Significant differences in proliferative responses and cytokine expression between antigen-stimulated cells and nonstimulated cells were analyzed by the Friedman test for analysis of variance, followed by the Dunn's multiple-comparison test. Significant differences in titers in serum and levels of cytokine expression between samples before and after challenge were detected by the Wilcoxon matched paired test. Resulting P values of less than 0.05 were considered significant.

RESULTS

Clinical response. Adult horses ($n = 12$) were challenged with live R. equi ATCC 33701 by intrabronchial inoculation with an endoscope. In the 7 days postchallenge, during which horses were examined daily, no clinical signs of upper or lower respiratory disease were observed. Two of the 12 horses developed a transient fever (38.3 to 39.4°C) which returned to normal 48 h after challenge. Complete blood counts taken at day 0 and day 7 revealed that all values were within the normal ranges for adult horses. Fibrinogen concentrations, the most commonly used serum marker of inflammation in horses, ranged from 263.6 ± 180 mg/dl at day 0 to 291.7 ± 99.6 mg/dl

at day 7. These concentrations were within the established normal ranges for the Washington State University Veterinary Teaching Hospital Clinical Pathology Laboratory. Prior to challenge, the total number of leukocytes in BALF was $10.5 \times 10^8 \pm 2.6 \times 10^8$/ml. As expected, there was considerable individual variation in both the number of cells recovered and the percentage of individual cell types, which consisted of macrophages (43.3% ± 31.5%) and lymphocytes (54% ± 29.5%). Six of 12 horses had neutrophils (3.2% ± 3.9% of the BALF cells recovered) in their BALF prior to challenge. At 7 days postchallenge the total number of leukocytes found in the BALF increased significantly ($P < 0.01$). The total number of leukocytes in BALF at day 7 was $20.4 \times 10^8 \pm 9.8 \times 10^8$/ml. There was a slight increase in the percentage of lymphocytes (61% ± 26.1%) and a slight decrease in the percentage of macrophages (38.3% ± 22.3%) compared with the values before challenge, although these differences were not statistically significant. The enhanced lymphocytic influx after challenge is consistent with a recall response (10). Neutrophils (2.3% ± 1.2% of the BALF cells recovered) were found in the BALF of 3 of 12 horses at day 7. These numbers are within the reported limits found in BALF of healthy adult horses (11).

Antigen-specific proliferative responses. The antigen specificities of the cells recovered from BALF of 12 adult horses were evaluated in proliferation assays at day 0 and day 7 after challenge. Cells were stimulated with a mitogen (PWM or ConA), VapA native protein, R. equi soluble antigen, or C. pseudotuberculosis soluble antigen. Proliferation was evaluated as a function of the level of incorporation of [³H]thymidine and expressed in counts per minute. Prior to challenge, there was no significant difference in the levels of proliferation between antigen-stimulated BALF cells and nonstimulated BALF cells (Fig. 1). This result also indicates that none of the antigens used had a mitogenic effect. In contrast, BALF cells obtained at day 7 postchallenge specifically proliferated in response to both VapA and whole R. equi, and these responses were significantly greater than the responses prechallenge (day 0). These responses to R. equi and VapA on day 7 were significantly greater ($P < 0.01$) than the responses of BALF cells stimulated with the C. pseudotuberculosis negative control as well as those of nonstimulated cells on day 7. As a positive control, BALF cells of all the horses proliferated when stimulated with a mitogen (data not shown).

Cytokine expression of BALF cells. Prior to and on day 7 after challenge with R. equi, BALF cells were analyzed by real-time PCR to detect the levels of expression of IL-4 and IFN-γ transcripts. Cells were stimulated for 24 h with PWM, VapA native protein, R. equi soluble antigen, or C. pseudotuberculosis soluble antigen. The efficiency of the real-time PCR was ≥0.93 (data not shown). The cytokine transcript levels were normalized by determining the levels of GAPDH transcripts for each sample. The normalized values of the IFN-γ and IL-4 transcripts in cells obtained from horses 1 to 6 on day 0 were low regardless of the stimulating antigen used, and no significant differences were detected between antigen-stimulated cells and nonstimulated cells (Fig. 2A and B). On day 7 postchallenge, all antigen-stimulated cells, as well as nonstimulated cells, expressed higher levels of IL-4 transcripts than before challenge. However, these increases were not R. equi specific or statistically significant compared to the values for
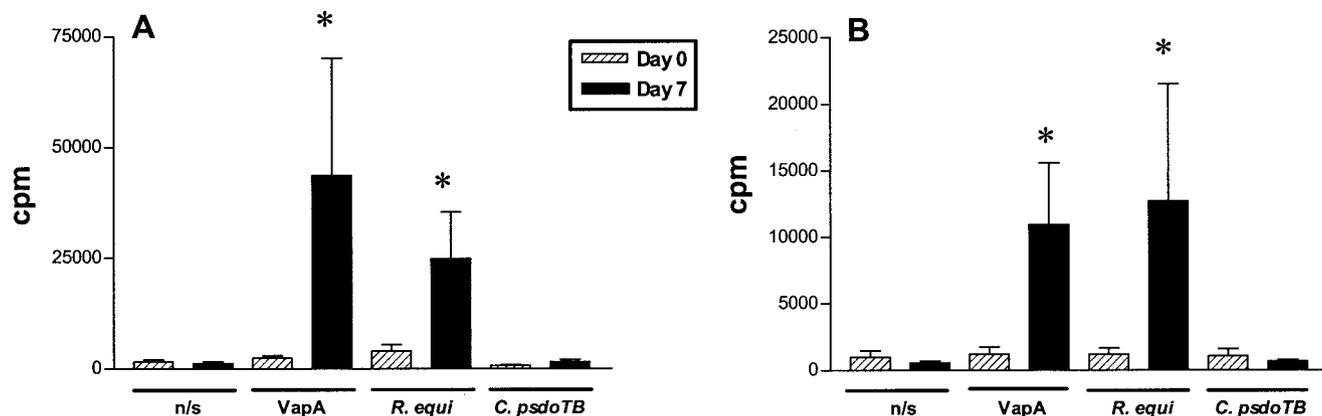
FIG. 1. Proliferative responses of BALF cells of adult horses recovered before (day 0) and after (day 7) challenge with *R. equi* ATCC 33701. BALF cells were either not stimulated (n/s) or stimulated with *R. equi*, VapA, or *C. pseudotuberculosis* (*C. psdoTB*). The data represent the mean counts per minute of three replicate cultures of BALF cells from six horses. The standard errors of the mean are indicated by error bars. (A) Horses 1 to 6; (B) horses 7 to 12. Asterisks indicate a statistically significant difference ($P < 0.05$) compared to the results for the antigen controls. The scale on the *y* axis differs between panels A and B as the assays were conducted independently with different lots of [^{3}H]thymidine and serum.

day 0. Notably, there were no statistically significant differences between the levels of IL-4 expression in VapA- or whole *R. equi*-stimulated cells and the levels in either nonstimulated cells or cells stimulated with the *C. pseudotuberculosis* negative control antigen. Prior to challenge, the level of IFN-γ expression was also low in all cells. Similar to the levels of IL-4 expression, at day 7 postchallenge, all antigen-stimulated cells and nonstimulated cells from BALF expressed increased levels of IFN-γ mRNA. In contrast to the level of IL-4 expression, however, BALF cells stimulated with either VapA or *R. equi* antigen expressed significantly higher levels ($P < 0.05$ and $P < 0.01$, respectively) of IFN-γ than *C. pseudotuberculosis*-stimulated cells or nonstimulated cells did, indicating that antigen-specific IFN-γ expression was enhanced by day 7 postchallenge. The level of IFN-γ expression in response to VapA or *R. equi* antigen at day 7 was also increased compared to that on day 0 ($P < 0.05$). Examination of IL-4 and IFN-γ transcript levels at day 7 postchallenge in the second group of six horses revealed the same pattern of no antigen-specific enhancement of IL-4 expression and an antigen-specific increase in the level of IFN-γ expression (data not shown).

Antibody responses against whole *R. equi* and VapA. The serum samples of the 12 horses were tested for anti-*R. equi* and anti-VapA total IgG by ELISA on day 0 and day 14 postchallenge. The titers of both anti-*R. equi* total IgG and anti-VapA total IgG increased significantly ($P < 0.001$ and $P < 0.01$, respectively) at day 14 (Fig. 3A and B). To test whether specific antibody isotypes were expanded postchallenge in immune horses, *R. equi* and VapA antibodies were detected by using anti-equine IgGb, IgGa, IgG(T), IgM, or IgA. There was a significant increase postchallenge in all anti-*R. equi* antibody isotypes analyzed, with the exception of IgA (Fig. 4A). The titers of all anti-VapA isotypes increased significantly by day 14 postchallenge (Fig. 4B). The titers of anti-*R. equi* IgGb and IgGa antibodies were dramatically enhanced after challenge, increasing 4.9 and 3.7 times, respectively. These increases were greater than the increases in IgM, IgA, and IgG(T) titers (Fig. 4A). Similarly, the IgGb and IgGa isotypes of anti-VapA an-
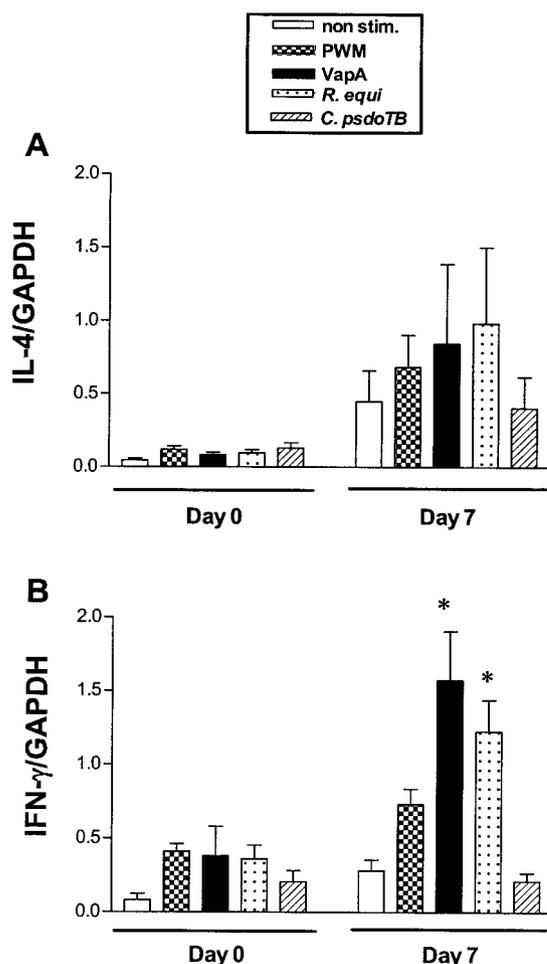

FIG. 2. IL-4 and IFN-γ expression in cells recovered from BALF of adult horses at day 0 and day 7 postchallenge. BALF cells either were not stimulated (non stim.) or were stimulated with PWM, VapA, *R. equi*, or *C. pseudotuberculosis* (*C. psdoTB*). The data represent the mean normalized values (cytokine and GAPDH) of cytokine expression in BALF cells from horses 1 to 6. (A) IL-4 expression; (B) IFN-γ expression. Asterisks indicate a statistically significant ($P < 0.05$) difference compared with the results for the negative controls (nonstimulated cells and *C. pseudotuberculosis*-stimulated cells).
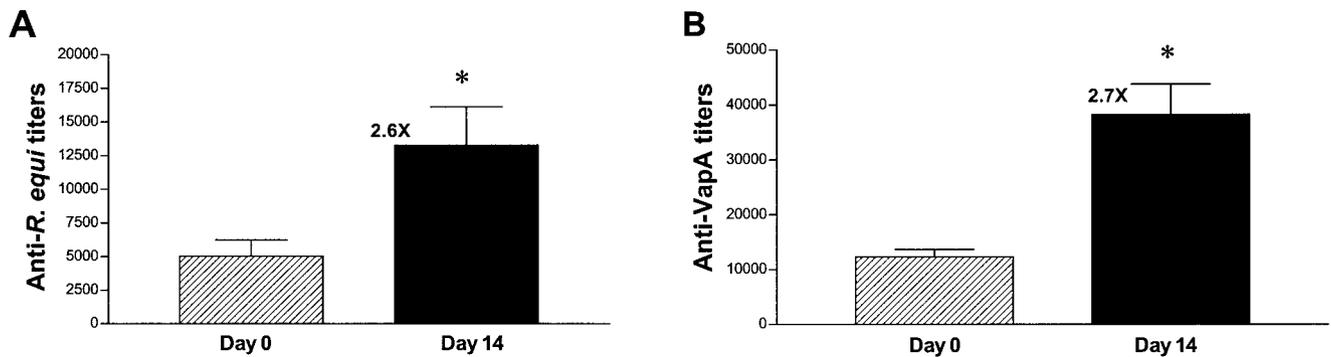
FIG. 3. Titers of total IgG antibodies in sera of adult horses at day 0 and day 14 postchallenge. The data represent the mean values for 12 horses, and the standard errors of the mean are indicated by error bars. The numbers at the top of each column on day 14 represent the fold increase in titer from day 0. (A) Anti-R. equi IgG titers; (B) anti-VapA IgG titers. Asterisks indicate a statistically significant ($P < 0.05$) increase in the titers of R. equi-specific and VapA-specific antibodies between day 0 and day 14.

tibodies predominated when the sera were examined for these isotypes at day 14 postchallenge (Fig. 4B).

DISCUSSION

An understanding of protective immune responses is essential for the development and clinical testing of effective new
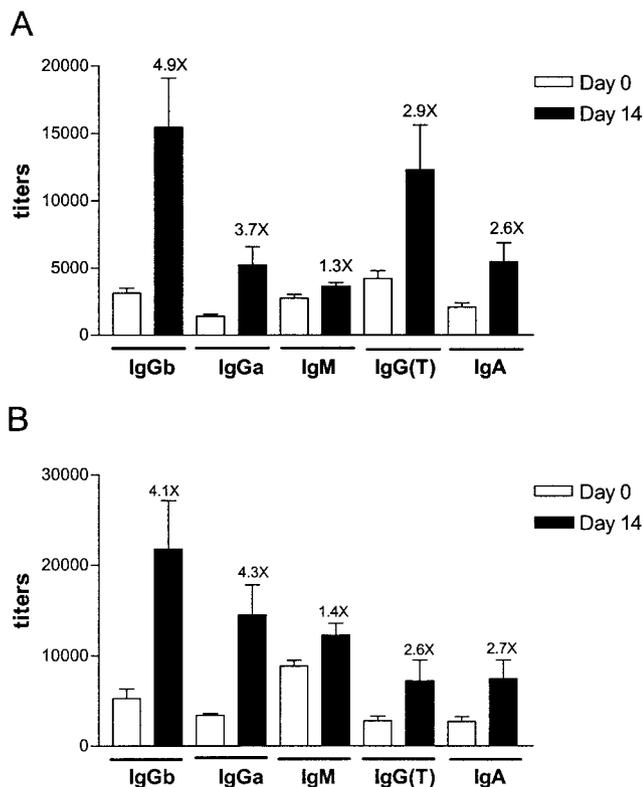

FIG. 4. Titers of immunoglobulin isotypes in sera from adult horses at day 0 and day 14 postchallenge. (A) Anti-R. equi antibody isotype titers; (B) anti-VapA antibody isotype titers. The data represent the means for 12 horses, and the standard errors of the mean are indicated by error bars. The numbers at the top of each column for day 14 represent the fold increase in titer from day 0.

vaccines. Key advantages of using mouse models to study infectious diseases and define protective responses are the ability to control for genetic variation among individuals and the wide availability of mouse-specific reagents. In research on R. equi, data from mouse studies has indicated the importance of CD4+ T cells and IFN-γ in protection against pneumonia (13, 14, 15, 23, 24). However, defining protective mechanisms in species other than inbred laboratory animals is much more difficult. As with many pathogens, the pathogenesis of R. equi infection in mice does not mimic the pathogenesis of infection that occurs in the natural host. For example, R. equi infection in immunocompetent mice is normally cleared, unlike the replication and progression to pneumonia that occurs in naïve immunocompetent foals. In addition, the T-lymphocyte responses in outbred species such as the horse do not fit a strict Th1-Th2 dichotomy as originally defined in mice. Since our goal is development of a vaccine for horses, we set out to characterize the immune responses associated with effective pulmonary clearance of R. equi in the natural host.

Our initial characterization of the immune responses in adult horses showed that the predominant cells in BALF following R. equi challenge were T lymphocytes, both CD4+ and CD8+ (10). These data support the premise that protective responses in horses resemble those in mice infected with R. equi and that both CD4+ and CD8+ T lymphocytes can play a role (13, 15, 23). CD4+ and CD8+ T cells secrete IFN-γ, which activate macrophages, the host cells of R. equi. In addition, CD4+ T cells, through both cognate and cytokine-mediated interactions with B lymphocytes, are essential for the induction of high-affinity IgG antibodies. We hypothesized that a protective immune response against R. equi infection in horses is associated with IFN-γ expression and production of specific antibody isotypes that participate in opsonization and complement fixation.

To address our hypothesis, we characterized the pulmonary T-lymphocyte responses to R. equi and VapA and the antibody responses in sera. In agreement with the results of Hines et al. (10), our results show that adult horses effectively clear a pulmonary challenge with virulent R. equi. All horses analyzed developed R. equi- and VapA-specific immune responses by day 7 postchallenge. The marked increase in IgG titers, with-

out a corresponding increase in IgM titers, further supports the assertion that these animals are mounting protective recall responses rather than a primary immune response. At day 7 postchallenge, *R. equi*-stimulated cells from BALF of all horses showed higher levels of IFN-γ transcripts than cells stimulated with medium alone or with a negative control antigen. In contrast, the *R. equi*-stimulated cells did not express a significantly increased level of IL-4. These data are consistent with our hypothesis.

R. equi is closely related to *Mycobacterium tuberculosis*, the causative agent of tuberculosis. Both are members of the nocardioform actinomycetes group of bacteria, characterized by mycolic acid-rich cell walls and high G+C contents. In addition, both *R. equi* and *M. tuberculosis* persist within infected macrophages and cause pneumonia. In tuberculosis, a Th1-like response and IFN-γ production, in particular, have been considered the most reliable markers for the induction of protective immunity (2). Although the adjuvant and the delivery system influence the response, specific antigens that give rise to high levels of IFN-γ are most likely to protect the host against mycobacterial challenge. Little is known about the antigens of *R. equi* that induce protective immune responses in horses; however, VapA is conserved among virulent strains, expressed in the lungs, and highly immunogenic (17, 27, 28, 29, 30). Although the function of VapA is unknown, previous data suggest that this protein participates in the pathogenesis of *R. equi* in foals, including the observation that VapA is encoded by a virulence plasmid (7). This plasmid is required for survival within macrophages and to produce disease in horses (7). As with BALF cells stimulated with *R. equi*, our data showed that VapA predominantly stimulates IFN-γ expression. This is the first report indicating that VapA induces IFN-γ production and further supports the contention that VapA is an antigen targeted by protective pulmonary T-lymphocyte responses in horses.

VapA is also a surface protein that is accessible to the host immune system, including antibody (30). Passive transfer experiments have provided good evidence that antibody can play a role in preventing rhodococcal pneumonia in vivo (6, 18, 20). Likewise, passive administration of immunoglobulin from horses immunized with VapA has a protective effect against experimental *R. equi*-induced pneumonia in foals, as well as in mice (5, 12). The mechanism of antibody protection has not been definitely established; however, there is evidence that opsonizing antibodies are important. Studies by Hietala and Ardands (9) demonstrated that opsonization with *R. equi*-specific antibody increased the level of phagosome-lysosome fusion and significantly enhanced the killing of *R. equi* by alveolar macrophages from foals. In addition, antiserum from a horse immunized with a VapA preparation was opsonizing when tested with a murine macrophage cell line (25). Importantly, equine immunoglobulin isotypes, like those of other species, differ in their cellular interactions and in their binding to complement. Previous studies have shown that equine IgGab and IgM fix complement after interaction with specific antigen and participate in opsonization, whereas IgG(T) and IgGc do not fix complement and have the capacity to inhibit complement fixation by IgGab (3, 22). Complement fixation contributes to opsonization of microbes and has been correlated with the concentrations of IgGb in the horse (8). Isotype responses may also be an indirect indicator of T-cell responses, reflecting the role of Th1 and Th2 cytokines in class switching by B cells (4). As shown in the present study, the titers of both IgGb and IgGa anti-*R. equi* antibodies were dramatically enhanced in association with clearance of a virulent pulmonary challenge. Similarly, IgGb and IgGa were the predominant VapA-specific isotypes induced by challenge.

The waning of maternally derived antibody has been implicated, in part, in the unique susceptibility to *R. equi* of foals between 2 and 4 months of age. IgGa and IgGb are the most abundant isotypes in equine serum and colostrum (21, 26). However, equine fetuses and foals have a limited ability to produce IgGb. In one study, the early immunoglobulin repertoire of neonatal foals comprised IgGa, IgG(T), and IgA (26). Endogenous synthesis of IgGb was not detected until 63 days of age. This limited isotype repertoire may indicate an inherent defect in young foals that is overcome as the animal and its immune system mature. Alternatively, the immunoglobulin isotype response may reflect a Th2 versus Th1 bias, as has been reported in neonates of other species (1, 16, 19). Regardless, our results raise the question as to whether a relative deficiency of *R. equi*-specific and/or VapA-specific IgGb due to low maternal antibody levels, a restricted isotype repertoire early in life, or a combination of these factors plays a role in the development of rhodococcal pneumonia. If so, an effective vaccine for foals must be designed to induce IgGb and/or the T-lymphocyte responses that production of this isotype reflects.

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