

## Clearance of Virulent but Not Avirulent *Rhodococcus equi* from the Lungs of Adult Horses Is Associated with Intracytoplasmic Gamma Interferon Production by CD4<sup>+</sup> and CD8<sup>+</sup> T Lymphocytes

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***Rhodococcus equi* is a gram-positive bacterium that infects alveolar macrophages and causes rhodococcal pneumonia in horses and humans. The virulence plasmid of *R. equi* appears to be required for both pathogenicity in the horse and the induction of protective immunity. An understanding of the mechanisms by which virulent *R. equi* circumvents protective host responses and by which bacteria are ultimately cleared is important for development of an effective vaccine. Six adult horses were challenged with either virulent *R. equi* or an avirulent, plasmid-cured derivative. By using a flow cytometric method for intracytoplasmic detection of gamma interferon (IFN- $\gamma$ ) in equine bronchoalveolar lavage fluid (BALF) cells, clearance of the virulent strain was shown to be associated with increased numbers of pulmonary CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes producing IFN- $\gamma$ . There was no change in IFN- $\gamma$ -positive cells in peripheral blood, suggesting that a type 1 recall response at the site of challenge was protective. The plasmid-cured strain of *R. equi* was cleared in horses without a significant increase in IFN- $\gamma$ -producing T lymphocytes in BALF. In contrast to these data, a previous report in foals suggested an immunomodulating role for *R. equi* virulence plasmid-encoded products in downregulating IFN- $\gamma$  expression by equine CD4<sup>+</sup> T lymphocytes. Intracytoplasmic detection of IFN- $\gamma$  provides a method to better determine whether modulation of macrophage-activating cytokines by virulent strains occurs uniquely in neonates and contributes to their susceptibility to rhodococcal pneumonia.**

The nocardioform actinomycete *Rhodococcus equi* is an important pulmonary pathogen in young horses. It is also an opportunistic infection in immunocompromised humans, especially those with AIDS (25, 42). Like *Mycobacterium tuberculosis*, a closely related actinomycete, *R. equi* is a facultative intracellular bacterium that persists and replicates within macrophages (8, 10). Intracellular survival is considered the critical event in development of disease, which is characterized in horses and humans by severe and sometimes fatal pyogranulomatous pneumonia (29). Adoptive transfer experiments in mice suggest that a Th2 response to *R. equi* results in lesion development, whereas successful immune clearance of bacteria from the lung represents a Th1 response and is mediated by gamma interferon (IFN- $\gamma$ ) (13, 15).

Essentially all equine clinical isolates of *R. equi* and the majority of human isolates carry a large virulence plasmid (36, 37). The plasmid is 80 to 90 kb in equine isolates and is required for survival within equine and murine macrophages. Plasmid-negative strains cannot produce disease in foals and are effectively cleared even in immunodeficient strains of mice (14, 21, 40). Plasmid curing of virulent strains by repeated in vitro cultivation eliminates both the virulent phenotype and the ability to persist

within macrophages (7). Although the function of the *R. equi* virulence plasmid is unknown, one report has suggested that plasmid encoded proteins modulate the host cytokine response (6). Specifically, challenge of foals with a virulent, plasmid-bearing strain resulted in decreased IFN- $\gamma$  mRNA expression in bronchial lymph node CD4<sup>+</sup> T lymphocytes when compared to an avirulent control strain that did not carry the virulence plasmid.

The goal of this study was to more closely examine the immunologic effects of plasmid-bearing versus plasmid-cured *R. equi*. These initial studies were done in adult horses, which are immune to *R. equi*. Previous work has shown that adult horses effectively clear an intrabronchial challenge with virulent *R. equi*. Immune clearance occurs in adult horses without clinical signs of pneumonia and in association with a pulmonary recall response (9, 20). The recall response is characterized, in part, by a local accumulation of CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes, presumably representing expansion of antigen-specific memory cells in the lung and/or migration from the draining lymph nodes. We hypothesized that clearance of *R. equi* would also associate with local IFN- $\gamma$  secretion in the lung, primarily by CD4<sup>+</sup> T lymphocytes. To test this hypothesis, we produced monoclonal antibodies to equine IFN- $\gamma$  and developed a method to detect intracytoplasmic IFN- $\gamma$  protein production in response to challenge with virulent and avirulent *R. equi*.

### MATERIALS AND METHODS

**Bacteria.** *Rhodococcus equi* ATCC 33701, a virulent plasmid-bearing strain, and *R. equi* ATCC 33701PC, a plasmid-cured derivative of the ATCC 33701

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strain, were stored as frozen stabulates and reconstituted by streaking on brain heart infusion agar. A single colony was then selected for overnight growth in brain heart infusion broth. Prior to inoculation into a horse, a fresh dilution of the overnight culture was grown in log phase in brain heart infusion broth until a concentration of  $2 \times 10^7$  bacteria per ml was reached (about 2 h). The bacteria were washed twice and diluted in 10 ml of saline for inoculation.

**Bronchoalveolar lavage and pulmonary challenge.** The horses used in this study were normal adult horses free of clinical signs of infection. Six horses were challenged with *R. equi* as described below. Of these, three were challenged with *R. equi* ATCC 33701 containing the virulence plasmid, and three were challenged with the plasmid-cured *R. equi* ATCC 33701PC strain. Weekly bronchoalveolar lavage was performed on each horse as described previously (9). Briefly, horses were mildly sedated with xylazine and butorphanol. An endoscope was passed nasally and directed into the right accessory lobar bronchus. Then 180 ml of phosphate-buffered saline was instilled into the right lung as three 60-ml aliquots, and bronchoalveolar lavage fluid (BALF) was aspirated. The lavage was repeated twice.

Immediately after the first complete bronchoalveolar lavage procedure (day 0), the right lung was inoculated with  $2 \times 10^7$  *R. equi* suspended in 10 ml of phosphate-buffered saline. The endoscope was then flushed with 15 ml of air and removed from the airway. Bronchoalveolar lavage was repeated 1 week (day 7) and 2 weeks (day 14) following challenge. The procedure at these times was identical to that on day 0 except that no *R. equi* was instilled. At each time, the endoscope was placed at the same site in the lung and BALF was collected. Blood was collected for lymphocyte isolation and culture at the same time points as bronchoalveolar lavage by venipuncture into 20% acid citrate dextrose. Importantly, the use of heparin as an anticoagulant was subsequently found to be detrimental to detection of intracytoplasmic IFN- $\gamma$  by flow cytometry.

**Evaluation of clinical response.** Following each bronchoalveolar lavage procedure, horses were placed in a stall for 1 week and monitored daily for changes in rectal temperature, respiration, and pulse as determined by physical exam and auscultation of the lungs. Blood was obtained via jugular venipuncture on the day of each bronchoalveolar lavage procedure for complete blood counts and determination of fibrinogen concentrations. Any horse with clinical abnormalities, including fever or increased fibrinogen, was assessed further, including auscultation of the lungs with a rebreathing bag.

**Production of anti-equine IFN- $\gamma$  monoclonal antibody EIP-53.** Hybridomas were produced with standard immunization and cell fusion techniques. (4) Briefly, a 40-amino-acid synthetic peptide containing a terminal cysteine, CQAAFFKEIENLKEYFNASNPDVGDGGLFLDKLKNWKED, was synthesized by the Washington State University Laboratory for Biotechnology and Bioanalysis and conjugated to keyhole limpet hemocyanin (Pierce Chemicals, Rockford, Ill.) according to the manufacturer's instructions. This peptide corresponds to the predicted amino terminus of equine IFN- $\gamma$  and, based on homology to other species, includes the putative extracellular binding site for the IFN- $\gamma$  receptor.

BALB/c mice were immunized with the keyhole limpet hemocyanin-conjugated peptide mixed with Ribi adjuvant (Corixa Corp., Hamilton, Mont.) by subcutaneous injection (25  $\mu$ g) at two sites. These mice were boosted by the same method at 28 days and monitored for the appearance of specific antibodies in serum by peptide enzyme-linked immunosorbent assay (ELISA). The peptide ELISA consisted of 96-well plates coated with the N-terminal peptide conjugated to ovalbumin. When specific antibodies were detected, mice were boosted with an intravenous injection of antigen without adjuvant. Three days later, spleen cells were harvested, mixed with X63 Ag8.653 myeloma cells at a ratio of 2.5 spleen cells/myeloma cell, exposed to 50% polyethylene glycol (Sigma Chemicals, St. Louis, Mo.) for 1 min, and then plated with mouse thymocytes in flat-bottomed 96-well cell culture plates. Hypoxanthine-aminopterin-thymidine selective medium (Sigma Chemicals) was added after 24 h of culture to prevent proliferation of unfused myeloma cells.

Supernatant fluid from wells containing hybridomas was screened by peptide ELISA to identify colonies secreting antibodies of interest. All hybridomas secreting antibodies reacting with the peptide were subjected to two rounds of cloning by limiting dilution, and culture supernatants were tested in the ELISA format for binding to both the N-terminal peptide and recombinant equine IFN- $\gamma$  (see below).

**Recombinant equine IFN- $\gamma$ .** Equine IFN- $\gamma$  was amplified from concanavalin A-stimulated equine peripheral blood mononuclear cells by reverse transcription-PCR. *Hind*III restriction sites (italic) were added to the PCR product with the following primers: 5'-GTTCTTCTACCTAAAGCTTCCAGG and 5'-AAATCAAAAGCTTGACGCAGG. A single band was amplified and ligated into a TA cloning vector (Invitrogen Life Sciences, Carlsbad, Calif.). The insert was subsequently excised with *Hind*III and subcloned into pTrcHis (Invitrogen). This

vector provides for expression of a recombinant fusion protein which includes a 6xHis purification moiety and has a calculated molecular mass of 23.4 kDa. The fidelity of amplification and cloning was confirmed by sequencing the insert in the clone selected for expression. Recombinant protein was purified with a ProBond nickel column (Invitrogen).

**Western blot.** For Western blot analysis, purified recombinant equine IFN- $\gamma$  protein was subjected to electrophoresis in a 4 to 20% Tris-HCl-sodium dodecyl sulfate-polyacrylamide gel (Bio-Rad Laboratories, Hercules, Calif.) and electroblotted onto nitrocellulose (Osmonics, Minnetonka, Minn.). The nitrocellulose membranes were probed with mouse serum collected both before and after immunization with the IFN- $\gamma$  peptide and with monoclonal antibody or the appropriate isotype control at 4  $\mu$ g/ml. These blots were incubated with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) as the secondary antibody (Kirkegard and Perry Laboratories, Inc., Gaithersburg, Md.), and reactivity was detected with SuperSignal West Pico (Pierce Biotechnology, Rockford, Ill.), an enhanced chemiluminescent substrate reagent. Enhanced chemiluminescence protein molecular size markers were loaded on the end lane of the gel and stained according to the manufacturer's instructions (Amersham Biosciences, Piscataway, N.J.).

**Cell isolations and culture.** Peripheral blood mononuclear cells (PBMC) were isolated from the leukocyte-rich buffy coat layer obtained from whole blood by centrifugation at  $1,250 \times g$  for 30 min. The buffy coat was diluted in Hanks' balanced salt solution without magnesium and calcium, and mononuclear cells were collected from the interface band by density gradient centrifugation with 1.077 Lymphoprep (Amersham Biosciences, Piscataway, N.J.). The PBMC were washed twice in Hanks' balanced salt solution with 10% acid citrate dextrose and resuspended in growth medium consisting of 10% fetal bovine serum in RPMI 1640 containing Glutamax-I (Gibco BRL Life Technologies, Grand Island, N.Y.) with 10  $\mu$ M HEPES, 0.05 mM 2-mercaptoethanol, 100 U of penicillin per ml, and 100  $\mu$ g of streptomycin per ml.

Cells were collected from BALF as previously described (9) and resuspended in growth medium. For intracytoplasmic staining of IFN- $\gamma$ , both BALF cells and PBMC were plated in 96-well round-bottomed plates at  $5 \times 10^5$  cells per well. Cells were cultured without stimulation (medium alone) or with stimulation (25 ng of phorbol 12-myristate 13-acetate [Sigma, St. Louis, Mo.] and 1  $\mu$ M ionomycin [Calbiochem, San Diego, Calif.] and with 2.5  $\mu$ M monensin [Sigma, St. Louis, Mo.]) for 4 h at 37°C and 5% CO<sub>2</sub>. Cells were then harvested and stained for flow cytometric analysis of lymphocyte phenotype and intracellular IFN- $\gamma$ .

**Surface staining for flow cytometry.** Equine cell populations analyzed by two-color analysis were surface stained with monoclonal antibodies to equine CD2 (HB88A, IgG1), CD4 (HB61A, IgG1), or CD8 (HT14A, IgG1) (VMRD Inc., Pullman, Wash.). Either phycoerythrin-conjugated goat anti-mouse IgG1 (Southern Biotechnologies Associates, Birmingham, Ala.) or Tricolor-conjugated goat anti-mouse IgG1 (Caltag Laboratories, Burlingame, Calif.) were used as the secondary reagent for detection of IgG1 isotypes. Cells to be analyzed for three-color staining were simultaneously surface stained with anti-equine CD2 monoclonal antibody HB88A (IgG1) and anti-equine CD8 monoclonal antibody 73/6.9.1 (IgG3) or the appropriate isotype control. Tricolor-conjugated goat anti-mouse IgG1 (Caltag Laboratories, Burlingame, Calif.) was used as the secondary reagent for detection of IgG1 isotype monoclonal antibodies, and phycoerythrin-conjugated goat anti-mouse IgG3 (Southern Biotechnology Associates, Birmingham, Ala.) was used as the secondary reagent for detection of IgG3 isotype monoclonal antibodies. The following monoclonal antibodies were used as isotype controls for surface staining and do not recognize any equine antigens; Colis69 (IgG1) and Colis42 (IgG3). (See the following section for antibodies used to detect intracellular IFN- $\gamma$ .)

All flow cytometry staining and washing procedures were carried out in the same 96-well round-bottomed plate in which they were cultured. After the 4-h culture time, the pelleted cells were stained for surface antigens according to standard protocols (4). Briefly, 50  $\mu$ l of each monoclonal antibody at a concentration of 15  $\mu$ g/ml was added per well. The 15-min ice-cold incubation with the monoclonal antibodies was followed by three washes with phosphate-buffered saline containing 10% acid citrate dextrose, 2% gamma globulin-free horse serum (Gibco BRL Life Technologies, Grand Island, N.Y.), 10 mM EDTA, and 0.2% sodium azide, designated the first wash buffer.

**Intracellular IFN- $\gamma$  staining for flow cytometry and analysis.** Following surface staining, the cells were fixed in 4% paraformaldehyde in phosphate-buffered saline for 10 min in the dark, washed twice with first wash buffer, resuspended in first wash buffer, and stored overnight on ice protected from light. The following day cells were permeabilized by washing twice in saponin staining buffer (phosphate-buffered saline containing 0.1% saponin [Sigma, St. Louis, Mo.] and 0.1% sodium azide). Cells were then incubated for 30 min on ice and in the dark with 50  $\mu$ l of the anti-IFN- $\gamma$  monoclonal antibody EIP-53 (IgG2b) at 40  $\mu$ g/ml or with

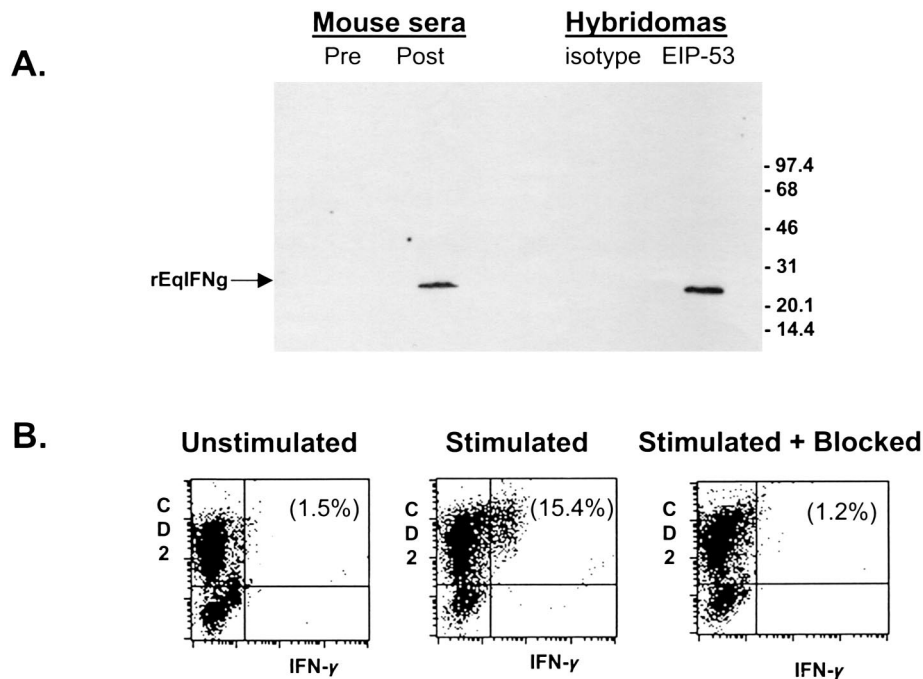


FIG. 1. (A) Immunoblot demonstrating reactivity of murine serum and monoclonal antibody EIP-53 with recombinant equine IFN- $\gamma$ . The controls were serum collected prior to immunization with synthetic peptide (Pre) and an irrelevant IgG2 monoclonal antibody (isotype). Neither antibody bound an irrelevant recombinant protein purified from *Escherichia coli* (not shown). The positions of molecular size markers are indicated (in kilodaltons). (B) Monoclonal antibody EIP-53 detects native IFN- $\gamma$  in equine peripheral blood T lymphocytes by flow cytometric analysis. PBMC were cultured with and without phorbol myristate acetate and ionomycin stimulation, followed by surface staining with anti-CD2 monoclonal antibody and internal staining with EIP-53. To demonstrate specificity, the binding of EIP-53 to stimulated cells was blocked by competitive inhibition with synthetic IFN- $\gamma$  peptide. The percentage value in the upper right quadrant of each dot plot indicates the percentage of CD2<sup>+</sup> cells that were IFN- $\gamma$  positive.

the IgG2b isotype control monoclonal antibody (3G9F3) diluted in saponin staining buffer. The cells were washed three times in saponin staining buffer and then incubated with the secondary reagent diluted in saponin staining buffer for 30 min on ice in the dark, followed by two washes in saponin staining buffer, and finally resuspended in phosphate-buffered saline with 0.9% azide for analysis.

Fluorescein-conjugated goat anti-mouse IgG2b (Caltag Laboratories, Burlingame, Calif.) was used as the secondary reagent for detection of the anti-IFN- $\gamma$  monoclonal antibody and its isotype control. Data were collected on a FACScan flow cytometer with CellQuest software (Becton Dickinson Biosciences Immunocytometry Systems, San Jose, Calif.). For three-color analysis, 10,000 events were collected after cell debris was gated out on forward scatter versus side scatter dotplots. Otherwise, 5,000 events were collected. For three-color analysis, the CD2 cells were gated first on the FL-3 channel with the isotype control to set the gates. Subsequently, the CD2-positive cells were analyzed for CD8, non-CD8, and IFN- $\gamma$  staining on FL-2 and FL-1 dotplots, with the unstimulated samples to set the quadrant markers for intracellular IFN- $\gamma$  expression.

**Blocking IFN- $\gamma$  staining with synthetic peptide.** To demonstrate that the EIP-53 monoclonal antibody staining was specific for IFN- $\gamma$ , EIP-53 was incubated at 40  $\mu$ g/ml with 100  $\mu$ g/ml of the equine IFN- $\gamma$  N-terminal peptide at 37°C for 30 min and microcentrifuged for 5 min. The blocked monoclonal antibody was then incubated with cells at 50  $\mu$ l/well in the above intracellular IFN- $\gamma$  staining procedure.

**Statistical analysis.** A one-tailed Wilcoxon rank sum, nonparametric comparison of data collected from horses inoculated with the virulent *R. equi* ATCC 33701 strain and its avirulent, plasmid-cured derivative was done with the SAS statistical package, version 6.12. Significance was determined at the  $P < 0.05$  level.

## RESULTS

**Flow cytometric detection of intracytoplasmic IFN- $\gamma$  in the horse.** A majority of the monoclonal antibodies raised against the N-terminal peptide of equine IFN- $\gamma$  reacted with recom-

binant equine IFN- $\gamma$  when tested by ELISA. In immunoblots, these antibodies recognized a single band migrating at a relative molecular mass of 21 kDa in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 1A). To identify an antibody that would bind native IFN- $\gamma$  in a flow cytometry format, subsets of the monoclonal antibodies were screened with PBMC from a normal horse. PBMC were cultured with phorbol myristate acetate and ionomycin for stimulation and with monensin to block Golgi transport, followed by permeabilization with saponin. One antibody, EIP-53, gave a strong positive signal, staining 15.4% of CD2<sup>+</sup> T lymphocytes in peripheral blood (Fig. 1B, middle panel). Importantly, the signal was not present in unstimulated PBMC (Fig. 1B, left panel), where the number of positive cells (1.5% of CD2<sup>+</sup> T lymphocytes) was identical to the number of cells stained with the isotype control (not shown). In stimulated cells, the binding of monoclonal antibody EIP-53 to positive cells could be blocked by preincubating EIP-53 with the IFN- $\gamma$  synthetic peptide prior to reacting it with cells (Fig. 1B, right panel).

To validate the flow cytometric assay and identify the cells producing IFN- $\gamma$ , a two-color system was developed with PBMC from normal horses ( $n = 5$ ) and monoclonal antibodies to T-lymphocyte surface markers CD2 (Fig. 1B), CD4, and CD8 (Fig. 2). CD2 is a surface molecule previously characterized as a T-lymphocyte marker in the horse (39). In these normal horses, there was considerable variation within the sample population in the percentages of cells that produced IFN- $\gamma$ .

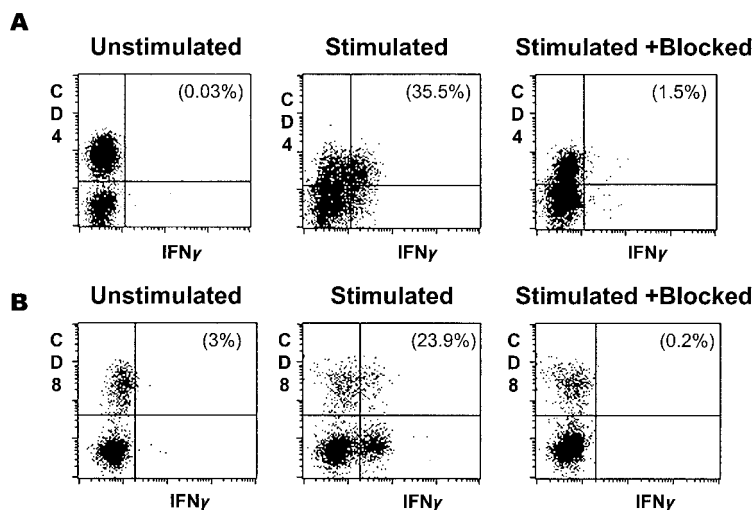


FIG. 2. Flow cytometric analysis allows detection of intracytoplasmic IFN- $\gamma$  in equine CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes. PBMC were cultured with and without stimulation, followed by surface staining with either anti-CD4 (panel A) or anti-CD8 (panel B) monoclonal antibodies and internal staining with anti-IFN- $\gamma$  monoclonal antibody EIP-53. The percentage value in the upper right quadrant of each dot plot indicates the percentage of CD4 (panel A) or CD8 (panel B) lymphocytes that were IFN- $\gamma$  positive. Note the downregulation of CD4 surface expression in response to lymphocyte stimulation (panel A, middle dot plot). To demonstrate specificity, the binding of monoclonal antibody EIP-53 in stimulated cells was blocked by competitive inhibition with synthetic IFN- $\gamma$  peptide (last dot plot of panels A and B).

For the two-color flow cytometric assay, the percentage of IFN- $\gamma$ -positive cells within each subset ranged from 13.5% to 45.7% for CD2<sup>+</sup> cells, 18.1% to 47.2% for CD4<sup>+</sup> cells, and 17.6% to 35.2% for CD8<sup>+</sup> cells. These ranges are similar to what has been reported in human PBMC (11, 19). In every experiment, the specificity of the monoclonal antibody binding to intracytoplasmic IFN- $\gamma$  in stimulated cells was demonstrated for each animal by blocking EIP-53 antibody binding with synthetic peptide (Fig. 2A and B, right dot plots). As previously described in other species, stimulation with phorbol myristate acetate and ionomycin also resulted in downmodulation of the CD4 surface marker (Fig. 2A) (2, 43). This complicated the identification of IFN- $\gamma$ -positive T lymphocytes in the CD4 subset by blurring the normally clear demarcation between CD4<sup>+</sup> and CD4<sup>-</sup> cells and, as described below, necessitated an alternative strategy for identifying CD4<sup>+</sup> IFN- $\gamma$ -positive cells (24, 28).

**PBMC response to pulmonary challenge of horses with virulent and plasmid-cured *R. equi*.** To examine the effects of *R. equi* infection on IFN- $\gamma$  production in the lung, six adult horses were challenged by intrabronchial inoculation of live bacteria. Three horses received the virulent ATCC 33701 strain of *R. equi*, while three received the plasmid-cured derivative ATCC 33701PC. BALF was collected immediately prior to challenge and at days 7 and 14 postchallenge. As reported previously (9), the *R. equi* challenge was effectively cleared. No horses developed clinical signs of respiratory infection or fever, and no abnormal lung sounds were heard.

Fibrinogen, the most commonly used measure of inflammation in the horse, was within the normal range for all horses throughout the study. For both plasmid-bearing and plasmid-cured strains, pulmonary challenge induced no detectable changes in PBMC, including no increase in the number or percentages of CD4<sup>+</sup> or CD8<sup>+</sup> T lymphocytes expressing IFN- $\gamma$ . Thus, as noted previously, peripheral blood provided

an insensitive measure of the protective immune responses involved in clearing *R. equi* from the lung, suggesting that the responses to pulmonary challenge were relatively compartmentalized to the lung (9).

**BALF cell response to pulmonary challenge with virulent and plasmid-cured *R. equi*.** Since our goal was to study the effects of *R. equi* infection on IFN- $\gamma$  expression at the site of clearance, it was important to analyze BALF cells as soon as possible after acquisition (18). Studies in other species have demonstrated that cytokine production profiles assessed in freshly obtained cells that have been activated in vitro for several hours are a fairly accurate representation of the in vivo situation (17, 41). In contrast, the cytokine expression properties of cells propagated in culture can be significantly influenced over time by cultivation conditions.

Preliminary experiments demonstrated that freshly obtained BALF cells were more heterogeneous than PBMC, and BALF lymphocytes could not always be identified with confidence based on size as assessed by forward and side scatter. Accordingly, we utilized a three-color method for flow cytometric analysis of BALF cells with monoclonal antibodies to CD2, CD8, and IFN- $\gamma$ . By first gating on CD2<sup>+</sup> cells in BALF, we were able to examine pulmonary T lymphocytes for simultaneous expression of surface CD8 and intracellular IFN- $\gamma$  (Fig. 3). To account for downregulation of the CD4 marker, CD2<sup>+</sup> CD8<sup>-</sup> T lymphocytes were defined as CD4<sup>+</sup> T lymphocytes, a method previously used in humans (24, 28).

Prior to challenge on day 0, a mean of 10.7% of CD2<sup>+</sup> T lymphocytes collected from the lungs expressed IFN- $\gamma$  when stimulated immediately ex vivo (Table 1,  $n = 6$ ). Similarly, a mean of 11.4% of the CD8<sup>+</sup> and 9.8% of CD4<sup>+</sup> BALF cells were positive for IFN- $\gamma$ . As reported previously (9), clearance of virulent, plasmid-bearing *R. equi* was associated with a significantly increased number of T lymphocytes in BALF at day

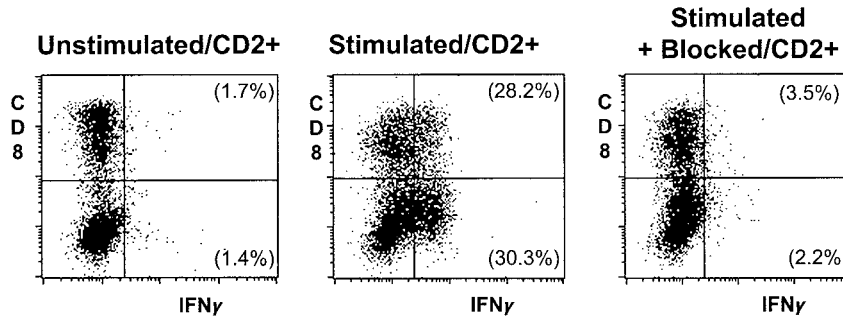


FIG. 3. Three-color flow cytometric analysis of equine BALF cells allows evaluation of intracytoplasmic IFN- $\gamma$  expression in pulmonary T-lymphocyte subsets. BALF cells were cultured with and without stimulation, followed by surface staining with both anti-CD2 and anti-CD8 monoclonal antibodies and internal staining with anti-IFN- $\gamma$  monoclonal antibody EIP-53. CD2<sup>+</sup> cells were gated and analyzed for CD8 and IFN- $\gamma$  staining. To demonstrate specificity, the signal in stimulated cells was blocked by competitive inhibition with synthetic IFN- $\gamma$  peptide. The percentage value in the upper right quadrant of each dot plot indicates the percentage of CD2<sup>+</sup> CD8<sup>+</sup> lymphocytes that were IFN- $\gamma$  positive, and the percentage value in the lower right quadrant of each dot plot indicates the percentage of CD2<sup>+</sup> CD8<sup>-</sup> lymphocytes that were IFN- $\gamma$  positive. CD2<sup>+</sup> CD8<sup>-</sup> cells were defined as putative CD4 T lymphocytes.

7 and/or 14 postinfection (Fig. 4). Both CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes were increased (data not shown).

When these T lymphocytes were examined for expression of IFN- $\gamma$  by flow cytometry, clearance of the plasmid-bearing strain was also associated with increased numbers of both CD4<sup>+</sup> and CD8<sup>+</sup> BALF T lymphocytes that were IFN- $\gamma$  positive (Fig. 5a and 5c). The increased numbers of both CD8<sup>+</sup> IFN- $\gamma$ -positive and CD4<sup>+</sup> IFN- $\gamma$ -positive lymphocytes were statistically significant compared to day 0 and to the avirulent control ( $P < 0.05$ ). As previously reported in other species, cytokine was not detected in unstimulated BALF cells, even after bacterial challenge.

Challenge with the avirulent, plasmid-cured *R. equi* induced significantly less CD2<sup>+</sup> T lymphocytes in BALF than the virulent strain (Fig. 4). In contrast to the virulent strain, there was no significant difference in IFN- $\gamma$ -positive T lymphocytes in the lung of horses challenged with plasmid-cured *R. equi* compared to prechallenge numbers (Fig. 5b and 5d).

**DISCUSSION**

Previous work has demonstrated in mice that both CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes can be involved in immune clearance of virulent *R. equi* from the lung (14, 26, 30). However, CD4<sup>+</sup> T lymphocytes are required for clearance. Moreover, adoptive transfer of *R. equi*-specific CD4<sup>+</sup> Th1 lymphocytes to T-cell-deficient *nu/nu* mice was sufficient to protect against a pulmonary challenge (15). In vivo neutralization of IFN- $\gamma$  with monoclonal antibody supported the hypothesis that activation of macrophages by type 1 cytokines is the mechanism of clearance (13). It has been unclear, however, how the murine paradigm

applies to protective immunity in the horse, which is the natural host. For example, there is good evidence in foals that passively acquired antibody can also have protective effects, whereas antibody alone has not been protective in mice (22, 23, 26).

In contrast to foals, immunocompetent adult horses effectively clear a pulmonary challenge with virulent strains of *R. equi* (9). Clearance occurs in conjunction with local, antigen-specific recall responses. Since *R. equi* is widespread in horses and their environment (37), these immune adults were presumably exposed to bacteria early in life and successfully mounted protective responses. Therefore, immune adult horses provide an opportunity to identify the correlates of protective immunity, including the phenotype of the memory response that an effective vaccine would be expected to induce.

In this report, detection of intracytoplasmic equine IFN- $\gamma$  by flow cytometry was used to show that immune clearance of

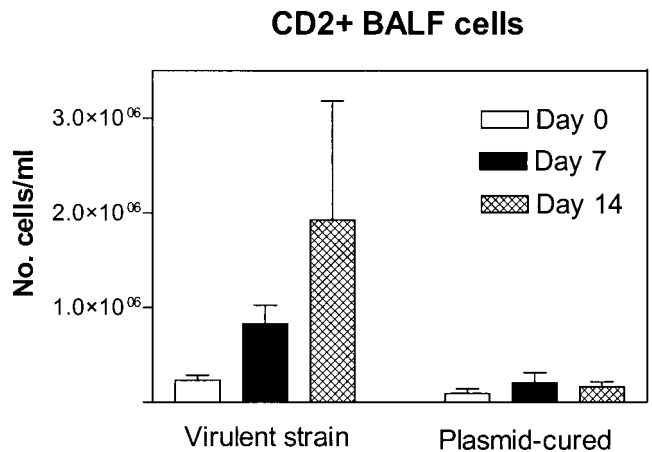


FIG. 4. Clearance of virulent, plasmid-bearing strain but not plasmid-cured derivative is associated with increased numbers of pulmonary CD2<sup>+</sup> T lymphocytes ( $P < 0.05$ ). BALF was collected prior to intrabronchial challenge (day 0, open bar) and at day 7 (solid bar) and day 14 (hatched bar) following challenge. There were three horses in each group.

TABLE 1. IFN- $\gamma$ -positive cells in BALF prior to challenge

Subset	Mean no. of IFN- $\gamma$ -positive cells (10 <sup>3</sup> /ml) (SEM)	Range (10 <sup>3</sup> /ml)	Mean % IFN- $\gamma$ positive (SEM)	Range (% positive)
CD2 <sup>+</sup>	15.0 (4.1)	4.1–28.1	10.7 (1.8)	3.5–14.9
CD4 <sup>+</sup>	8.0 (2.7)	2.0–18.9	9.8 (1.5)	3.8–14.1
CD8 <sup>+</sup>	7.2 (2.2)	2.1–17.1	11.4 (2.5)	3.3–20.1

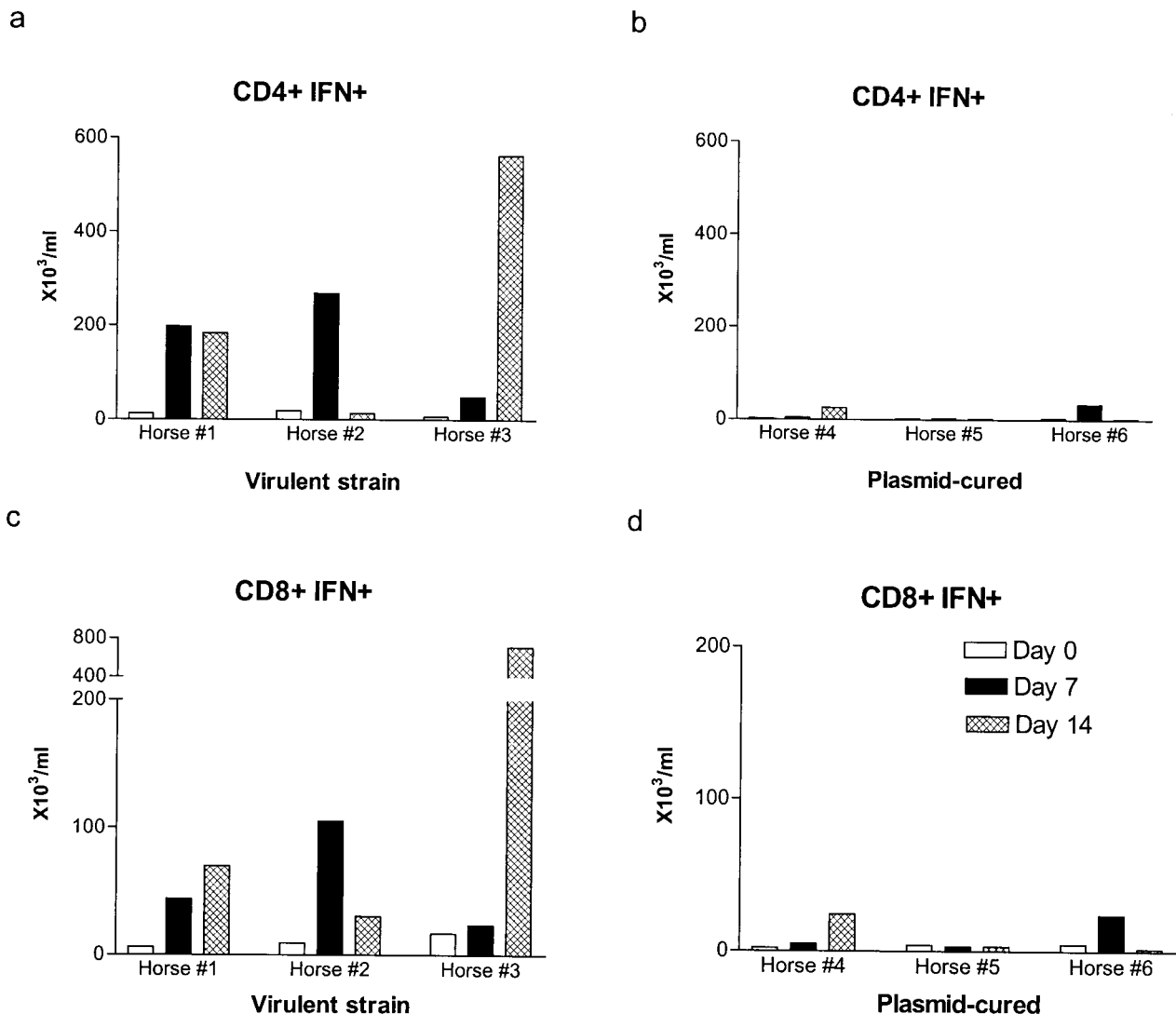


FIG. 5. Clearance of virulent, plasmid-bearing strain of *R. equi* is associated with locally increased numbers of IFN- $\gamma$ -producing cells in BALF as measured by multiparameter flow cytometry. Panels a and b depict numbers of IFN- $\gamma$ -positive cells within the CD4<sup>+</sup> subset for the plasmid-bearing and plasmid-cured strains, respectively. Panels c and d depict numbers of IFN- $\gamma$ -positive cells within the CD8<sup>+</sup> subset for the plasmid-bearing and plasmid-cured strains, respectively. Cells were examined prior to intrabronchial challenge (day 0, open bar) and at day 7 (solid bar) and day 14 (hatched bar) following challenge.

virulent *R. equi* in adult horses also associates with local production of IFN- $\gamma$  in the lung. Importantly, this technique, which has not previously been used in the horse due to a lack of reagents, allows simultaneous identification of the cytokine-producing cells based on cell surface markers. Likewise, analysis of IFN- $\gamma$  expression within hours of collection of BALF cells provides a relevant approximation of expression in vivo at the site of bacterial clearance (17, 18, 41). Both CD8<sup>+</sup> and CD4<sup>+</sup> T lymphocytes from the lung expressed IFN- $\gamma$ . These results support roles for IFN- $\gamma$  and macrophage activation in immunity to *R. equi* in the horse. The results also suggest that an effective vaccine will need to induce in the lung and/or regional lymph nodes memory cells that upon encounter with *R. equi* rapidly expand into an effector population secreting type 1 cytokines (27, 12).

CD4<sup>+</sup> T lymphocytes may also have other roles in immunity

to *R. equi*, including the production of cytokines that affect antibody production and/or antibody class switching. Likewise, CD4<sup>+</sup> T lymphocytes may provide help for CD8<sup>+</sup> T lymphocytes, which could serve as important sources of IFN- $\gamma$  and as cytotoxic effector cells that recognize *R. equi*-infected macrophages (5, 31, 33, 38).

Interestingly, the plasmid-cured derivative of *R. equi* ATCC 33701 was cleared from the lung with only mild increases in CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocyte numbers and no significant increase in IFN- $\gamma$ -positive cells. This finding differs from a previous study in foals (6). At 14 days postinfection, foals challenged with an avirulent, plasmid-cured derivative of *R. equi* strain 103 expressed high levels of IFN- $\gamma$  mRNA in bronchial lymph node CD4<sup>+</sup> T lymphocytes compared to foals infected with the virulent, wild-type strain. On day 14, the plasmid-cured *R. equi* had been completely cleared from the

lung with no lesions produced, whereas foals that received the virulent, plasmid-bearing wild-type strain had rhodococcal pneumonia. Based on these results, the investigators hypothesized that bacterial products encoded by the *R. equi* virulence plasmid are capable of downregulating IFN- $\gamma$  production by CD4<sup>+</sup> lymphocytes.

The conflicting findings in these two studies may simply reflect differences in the bacterial strains used or the site at which cells were collected (bronchial lymph node versus BALF). The discrepancy could also reflect the difference between *R. equi* encountered in a naïve foal versus those encountered in a previously primed adult. In addition to lacking memory responses, foals are initially exposed to *R. equi* at a time when their immune systems are relatively immature. There is now a body of work demonstrating important differences in the immune systems of humans and animals early in life compared to adults (1, 3, 32). These include quantitative and qualitative differences in antigen-presenting cells and a neonatal Th2 bias. Therefore, the initial interaction between foals and virulent *R. equi* may be unique and not represented in previously exposed adult horses. Importantly, though, clearance of *R. equi* in foals may ultimately require the IFN- $\gamma$ -mediated immunologic mechanisms operative in immune adults.

Observations in the mouse model also support the finding of differences associated with clearance of plasmid-bearing versus plasmid-negative strains of *R. equi*. Similar to adult horses, intravenous inoculation of mice with the virulent ATCC 33701 strain resulted in significant IFN- $\gamma$  production, whereas only minimal cytokine production was detected in mice infected with a plasmid-cured derivative (16). In addition, the virulence plasmid is required to induce protective immune responses in mice (35). Clearance of a sublethal dose of *R. equi* ATCC 33701 resulted in protection against a lethal challenge, whereas clearance of an equivalent number of plasmid-cured bacteria had no such protective effect. Innate immune responses alone appeared to be sufficient for clearance of plasmid-negative strains (14, 21). Immunodeficient mice, including SCID and athymic *nu/nu* strains that lack T lymphocytes, effectively cleared plasmid-negative *R. equi* and developed neither clinical signs nor pulmonary lesions. These data provide little evidence that virulent strains down regulate IFN- $\gamma$  production or that plasmid-negative *R. equi* strains significantly stimulate CD4<sup>+</sup> T lymphocytes as reported in foals.

In summary, a flow cytometric technique was developed to identify IFN- $\gamma$ -secreting lymphocytes in horses. Use of this technique provided evidence that clearance of virulent, but not avirulent *R. equi* from the lungs of adult horses was associated with increased numbers of IFN- $\gamma$  secreting, CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes in bronchoalveolar lavage fluid. The technique provides a relatively noninvasive method to further examine immunomodulation by products of the *R. equi* virulence plasmid in foals and, more importantly, to determine whether those effects can be overcome with an improved vaccination strategy.

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