Early Induction of Humoral and Cellular Immune Responses during Experimental *Mycobacterium avium* subsp. *paratuberculosis* Infection of Calves

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Johne's disease (paratuberculosis) of cattle is widespread and causes significant economic losses for producers due to decreased production and poor health of affected animals. The chronic nature of the disease and the lack of a reproducible model of infection hinder research efforts. In the present study, instillation of *Mycobacterium avium* subsp. *paratuberculosis* into the tonsillar crypts of neonatal calves resulted in peripheral colonization as detected by antemortem culture of feces and postmortem (320 days postchallenge) culture of intestinal tissues. Antigen-specific blastogenic, gamma interferon (IFN-γ), and nitric oxide responses by blood mononuclear cells from infected calves exceeded prechallenge responses beginning 194 days postchallenge. Upon in vitro stimulation with paratuberculosis antigens, CD4+ cells from infected calves proliferated, produced IFN-γ, and increased expression of CD26 and CD45RO (indicative of an activated memory phenotype). Utilizing a lipoarabinomannan-based enzyme-linked immunosorbent assay, specific serum immunoglobulin was detected as early as 134 days postchallenge and generally increased after this time point. Two antigens of ~50 and ~60 kDa were particularly immunodominant early in infection, as shown by immunoblot with serum collected within 2 weeks postchallenge. Findings indicate that the intratonsillar inoculation route will prove useful as an experimental model for paratuberculosis infection. Additionally, this study confirms that mycobacteria-specific antibody is detectable early in the course of experimental Johne's disease, even preceding the development of specific cell-mediated responses.

*Mycobacterium avium* subsp. *paratuberculosis* infection of cattle is widespread, with estimates of 20 to 40% of U.S. dairy herds affected and costs of $220 million per year to the dairy industry (7, 57). Considering the poor sensitivity and specificity of present paratuberculosis diagnostic tests (reviewed in reference 25), negative effects of this disease may be even greater than present estimates indicate. These diagnostic deficiencies result from, among other things, the absence of a consistent experimental infection model for diagnostic test development and by confounding responses to other closely related mycobacteria. Furthermore, the discovery that *M. avium* subsp. *paratuberculosis* colonizes rabbits, elk, deer, foxes, bighorn sheep, and other mammals that may serve as reservoir hosts adds another significant obstacle for implementation of an effective eradication campaign (5, 6, 24, 58). Developed countries with wildlife reservoirs of *Mycobacterium bovis* (i.e., bovine tuberculosis) have been unable to eradicate tuberculosis from their domestic herds and are exploring control measures, such as vaccination, as alternatives to traditional test and slaughter campaigns (3, 13). A consistent calf model of experimental *M. avium* subsp. *paratuberculosis* infection would facilitate development and validation of new diagnostic tests and evaluation of candidate vaccines.

Oral inoculation of goats with multiple doses of *M. avium* subsp. *paratuberculosis* results in consistent infection (46). Cellular and humoral immune responses follow similar kinetics in experimentally infected goats. In contrast, it is often stated that cellular and humoral immune responses of *M. avium* subsp. *paratuberculosis*-infected cattle are divergent, with gamma interferon (IFN-γ) responses detected early and antibody responses detected late in infection (reviewed in references 10 and 43). This dogma, however, has recently been questioned (29). Detection of specific antibody responses is highly dependent on the isotypes and antigens used in the assay. Additionally, many conclusions on the immune response to paratuberculosis infection come from evaluation of responses in naturally infected cattle. The dose of *M. avium* subsp. *paratuberculosis*, exposure to other mycobacterial species, housing conditions, number of lactations, exposure to other pathogens (e.g., bovine leukemia virus), administration of antibiotics and other therapies, and age of the naturally exposed animal, however, are often unknown. Experimental inoculation of calves housed in a controlled environment should minimize these variable influences.

Intratonsillar inoculation of cattle with *M. bovis* results in consistent infection with associated lesions and host responses similar to those of natural infection (37). Mycobacteria that
Three castrated male Holstein calves were challenged with *M. avium* subsp. *paratuberculosis* by instillation of 0.2 ml of challenge inoculum into each of the two tonsillar crypts weekly from 2 to 5 weeks of age. Challenge inoculum consisted of ~1.6 x 10^8 CFU of mid-log-phase *M. avium* subsp. *paratuberculosis* (i.e., four weekly doses of ~4 x 10^7 CFU/2 tonsillar crypts) grown in Middlebrook’s 7H9 medium (Becton Dickinson, Cockeysville, Md.) supplemented with 2 mg of mycobactin J (Allied Monitor Inc., Fayette, Mo.)/liter and 1% oleic acid-containing borage oil (Sigma)/liter. For pathogens infecting mucosal tissue, tonsillar tissue offers one of the first opportunities for intracellular invasion and interaction with specific host cell defenses. The primary objective of the present study was to determine if instillation of *M. avium* subsp. *paratuberculosis* into the tonsillar crypts of young calves results in a detectable host response and/or colonization of the bacterium. A secondary objective was to determine and compare the kinetics of host cellular and humoral responses.

**MATERIALS AND METHODS**

**Animals, bacterial culture, antigens, and challenge and necropsy procedures.** Three castrated male Holstein calves were challenged with *M. avium* subsp. *paratuberculosis* by instillation of 0.2 ml of challenge inoculum into each of the two tonsillar crypts weekly from 2 to 5 weeks of age. Challenge inoculum consisted of ~1.6 x 10^8 CFU of mid-log-phase *M. avium* subsp. *paratuberculosis* (i.e., four weekly doses of ~4 x 10^7 CFU/2 tonsillar crypts) grown in Middlebrook’s 7H9 medium (Becton Dickinson, Cockeysville, Md.) supplemented with 2 mg of mycobactin J (Allied Monitor Inc., Fayette, Mo.)/liter and 10% oleic acid–dextrose complex (Difco, Detroit, Mich.) plus 0.05% Tween 80 (Sigma Chemical Co., St. Louis, Mo.). Bacilli were harvested from the culture media by centrifugation at 10,000 x g, were washed with cold phosphate-buffered saline solution (PBS; 0.15 M, pH 7.2), and were diluted to the appropriate cell density for use as a challenge inoculum in PBS. Enumeration of bacilli was by serial dilution plating on Herrold’s egg-yolk slants containing mycobactin J (2 mg/liter). Whole-cell sonicates (WCS) of *M. avium* subsp. *paratuberculosis* strains K10 and 19698 were prepared for use as antigens in immunoreactions. *M. avium* subsp. *paratuberculosis* organisms were cultured in 500 ml of Middlebrook’s 7H9 medium at 37°C to an optical density at 540 nm (OD540) of 0.2 to 0.4. Mycobacteria were pelleted (10,000 x g for 20 min) and washed twice with cold PBS. The pellet was resuspended in PBS and was sonicated on ice with a probe sonicator. Sonication consisted of three cycles of 10-min bursts at 18 W on ice with 10-min chilling periods between sonications. Debris was removed by centrifugation (12,000 x g for 5 min), and supernatants were harvested and stored at ~20°C. Protein concentration was determined by using the Bio-Rad protein assay (Richmond, Calif.).

Animals were euthanized 320 days after inoculation with intravenous sodium pentobarbital. A thorough postmortem examination was done, and the following tissues were collected for microscopic examination and bacteriologic isolation of *M. avium* subsp. *paratuberculosis*: palatine tonsil; medial retropharyngeal; mandibular and parotid lymph nodes; mid-duodenum and associated lymph node; one section each of proximal, middle, and distal jejunum and associated lymph nodes; proximal, middle, and distal ileum and associated lymph nodes; ileocecal valve and ileocecal lymph nodes; cecum; spiral colon and associated lymph node; transverse and descending colon; and hepatic and ileac lymph nodes. Tissue specimens were fixed by immersion in neutral buffered 10% formalin. Tissues were routinely processed, embedded in paraffin, cut at 4 to 6 μm, and stained with hematoxylin and eosin and acridine orange/auromine O. Adjacent sections were cut from blocks containing tissues with lesions suggestive of *paratuberculosis* and were stained by the Ziehl-Neelsen technique to visualize acid-fast bacteria.

**Culture of *M. avium* subsp. *paratuberculosis* and PCR analyses.** Culture of *M. avium* subsp. *paratuberculosis* was performed on fecal samples by a double centrifugation, double decontamination procedure (45). Tissues were homogenized in 0.75% hexadecylpyridinium chloride solution (Sigma) with a stomacher for 20 min) and washed twice with cold PBS. The pellet was resuspended in PBS and was sonicated on ice with a probe sonicator. Sonication consisted of three cycles of 10 min at 107 PBMC were centrifuged (10 min, 400 g), supernatants were harvested, and cells were resuspended in 1 ml of medium was added to each well. Cells were incubated for 5 days at 37°C in 5% CO2 in air. After 5 days, 0.5 μCi of [methyl-3H]thymidine (specific activity, 6.7 Ci mmol-1; Amer sham Life Science, Arlington Heights, Ill.) in 50 μl of medium was added to each well, and cells incubated for an additional 20 h. Well contents were harvested onto fiber filters with a 96-well plate harvester (EG & G Wallace, Gaithersburg, Md.), and the incorporated radioactivity (counts per minute [cpm]) were measured by liquid scintillation counting. Treatments were run in triplicate. Data are presented as stimulation indices (i.e., mean cpm for stimulated samples/mean cpm for samples receiving medium alone).

**PKH67 proliferation assay.** Staining of PBMC with PKH67 was performed according to manufacturer instructions (Sigma) and as described previously (55). Briefly, 2 x 10^5 PBMC were incubated with 10 μg of PKH67 (Invitrogen, Carlsbad, Calif.) for 5 min, washed (twice) with RPMI 1640, and wells of 96-well round-bottom microtiter plates (Falcon; Becton-Dickinson) were seeded with 2 x 10^6 PHK67-stained mononuclear cells in a total volume of 200 μl per well (six replicates for each treatment). Cells were stimulated as described in the cell culture procedure, incubated for 7 days at 37°C in 5% CO2 in air, and harvested according to treatment for flow cytometric analysis.

**Flow cytometry.** Mononuclear cells (~2 x 10^6/ml) in 100 μl of balanced salt solution with 1% FBS and 0.1% sodium azide (fluorescence-activated cell sorter [FACS] buffer) were stained with 100 μl of primary antibody to leukocyte surface antigens ([CAC138A, immunoglobulin G1 [IgG1], anti-bovine CD4], [BAQ111A, IgM, anti-bovine CD8α], [CAC616A IgM, anti-bovine γδ T-cell receptor (TCR)], specific for the delta chain; [BAQ4A, IgG1, anti-bovine WCl, a subset of bovine γδ T cells]; [PIG45A, IgG2b, anti-bovine IgM], [CACT14A, IgG2b, anti-bovine CD20], [GC44A, IgG3, anti-bovine CD45R0]). All primary antibodies were obtained from Veterinary Medical Research and Development, Pullman, Wash. After a 15-min incubation, cells were centrifuged (400 x g, 2 min) and resuspended in 100 μl of appropriate secondary antibody (fluorescein isothiocyanate [FITC]; 1 μg/well-conjugated goat anti-mouse IgG5 [Caltag, Burlingame, Calif.], phycoerythrin [PE; 1 μg/well-conjugated goat anti-mouse IgG2a, or IgG1] or allophycocyanin (APC), or peridinin chlorophyll protein [12 μl/well-conjugated rat anti-mouse IgG1 [Caltag], TRICOLOR-conjugated goat anti-mouse IgG1 [Caltag]).
Cells were then incubated an additional 15 min, centrifuged (400 × g, 2 min), resuspended in 200 µl of FACS buffer, centrifuged again (400 × g, 2 min), and resuspended in 200 µl of FACS buffer. Cells were then analyzed by using a Becton Dickinson FACSscan flow cytometer (10,000 events, live gate, 3-color analysis). Cells were permeabilized and stained with Streptav (VMRD; GB21A, IgG2b, anti-bovine CD4 [VMRD]; GB21A, IgG2b, anti-bovine CD8 [Serotec, Kidlington, Oxford, United Kingdom] or appropriate isotype control antibodies for 60 min at 4°C. Cells were washed (FACS buffer), stained with biotinylated anti-mouse IgG2a/2b (0.06 g/10^6 cells; Becton Dickinson), washed again (FACS buffer), and incubated with streptavidin cy-chrome (Neutra Avidin Biotin-Binding Protein, Pierce, Rockford, Ill.) diluted 1:2,000, incubation for 1 h at 4°C. 

Antigen-antibody reactions were detected with an indirect ELISA that used the following conditions for all steps: 100 µl reagent per well, reagent dilutions prepared in blocking buffer, nine washes between each reagent, and incubations at RT. The reaction steps were the following: biotinylated secondary antibody (biotinylated anti-goat IgG heavy and light chains [rabbit origin] [Vector Laboratories, Burlingame, Calif.]) diluted 1:700, incubation for 1 h at 4°C; avidin (Neutra Avidin Biotin-Binding Protein, Pierce, Rockford, Ill.) diluted 1:100 and 1:400, respectively, were included in each plate for determination of test sera sample/protein dilution (S/P) ratios. Each serum was tested in duplicate by using 100 µl per well. Incubation was carried out at 4°C overnight, after which the plate was washed nine times in wash buffer.

**RESULTS**

**Bacterial culture and clinical status.** Two-week-old calves received four weekly doses (~4 × 10^7 CFU/dose) of *M. avium* subsp. *paratuberculosis* by direct instillation of the inoculum into the tonsillar crypts. Prior to necropsy, fecal culture and IS900 PCR confirmed *M. avium* subsp. *paratuberculosis* colonization in each of the three *M. avium* subsp. *paratuberculosis*-inoculated calves. Fecal shedding was intermittent and light (1 to 4 colonies/colony, with an average of 4 replicate slants). *M. avium* subsp. *paratuberculosis* was first detected at 146, 167, or 271 days postchallenge for individual calves, respectively. Clinical signs of *M. avium* subsp. *paratuberculosis* infection were not observed for any of the calves over the course of the study. Infected calves were euthanized 320 days postchallenge, and macroscopic lesions were not detected. However, *M. avium* subsp. *paratuberculosis* was isolated from tonsil, duodenum,
ileum, and jejunum as well as ileal, jejunal, and spiral colon lymph nodes. While *M. avium* subsp. *paratuberculosis* was isolated from each calf, *M. avium* subsp. *paratuberculosis* was not detected in all tissues from each calf. As reported previously for animals with early *M. avium* subsp. *paratuberculosis* infection (42), neither histologic lesions nor acid-fast bacteria were detected. Collectively, these data demonstrate that calves did not enter the clinical stage of infection as defined by Cocito et al. (12).

Recall lymphocyte proliferative responses. Blood mononuclear cells from naturally infected cattle with subclinical *paratuberculosis* often exhibit mycobacterium-specific T-cell in vitro proliferative responses (53). At 194 and 286 days postchallenge, antigen-specific DNA synthesis by PBMC from *M. avium* subsp. *paratuberculosis*-infected calves exceeded (*P < 0.05*) prechallenge responses (Fig. 1 and 2). Antigen-specific lymphocyte proliferation was confirmed by a flow cytometric method with significant (*P < 0.05*) responses by CD4+ and γδ TCR+ cell subsets (Table 1). Percentages and actual numbers of CD4+ cells, but not CD8+ or γδ TCR+ cells, increased in PBMC cultures from infected calves upon mycobacterial antigen stimulation (data not shown). Additionally, stimulation of

FIG. 1. Cellular immune response kinetics to *M. avium* PPDa. Mean (± SEM) DNA synthesis (A), IFN-γ (B), and NO (C) responses by PBMC from *M. avium* subsp. *paratuberculosis*-infected calves (closed squares) (*n* = 3). DNA synthesis was measured by [3H]thymidine uptake, IFN-γ was measured by ELISA, and nitrite (as an indication of NO synthesis) was measured by Griess reaction. *, Differs (*P < 0.05*) from response at day 0 (i.e., prechallenge response).

FIG. 2. Cellular immune response kinetics to *M. avium* subsp. *paratuberculosis* strain 19698 WCS. Mean (± SEM) DNA synthesis (A), IFN-γ (B), and NO (C) responses by PBMC from *M. avium* subsp. *paratuberculosis*-infected calves (closed squares) (*n* = 3) are depicted. DNA synthesis was measured by [3H]thymidine uptake, IFN-γ was measured by ELISA, and nitrite (as an indication of NO synthesis) was measured by Griess reaction. *, Differs (*P < 0.05*) from response at day 0 (i.e., prechallenge response).
PBMC cultures from infected animals resulted in increased percentages of CD4\(^+\) cells expressing CD26 and CD45RO (Table 2), indicative of activation of memory T cells.

Recall IFN-\(\gamma\) and NO responses. Both IFN-\(\gamma\) and NO are essential for profilloking and killing of very virulent mycobacteria (14, 18, 19, 22, 32, 35). The role for IFN-\(\gamma\) and particularly NO in the killing of less virulent mycobacteria, including \(M. avium\), is less clear (1, 17, 56, 59). As with DNA synthesis, antigen-specific IFN-\(\gamma\) responses by PBMC from infected calves exceeded \((P < 0.05)\) prechallenge responses beginning 194 days postchallenge (Fig. 1 and 2). Antigen-specific NO exceeded \((P < 0.05)\) prechallenge responses at 160 and 194 days postchallenge (Fig. 1 and 2). DNA synthesis, IFN-\(\gamma\), and NO responses to strain K10 WCS stimulation (data not shown) were similar to responses to strain 19698 WCS and PPDa. In general, DNA synthesis, IFN-\(\gamma\), and NO responses followed similar kinetics and magnitude of response (Fig. 1 and 2). As determined for calves receiving \(M. avium\) subsp. \(paratuberculosis\) by an oral route (4), CD4\(^+\) cells were the predominant subset of T cells producing IFN-\(\gamma\) (7 days poststimulation) in response to soluble mycobacterial antigens (Table 3 and Fig. 3).

Serum Ig response. In contrast to previous dogma, it has recently been determined that humoral responses to \(M. avium\) subsp. \(paratuberculosis\) are detectable early after infection (29). By using an ELISA-based assay to detect antibody to LAM antigen, \(Mycobacterium\)-specific antibody was detected as early as 134 days postchallenge (Fig. 4), and this response followed kinetics similar to those of the cellular response (Fig. 1 and 2). A commercially available ELISA kit (IDEXX Laboratories) did not detect serum antibody responses in these calves at any time point during the study. Analysis of serial serum samples from each calf by immunoblot showed reactivity to a ~50-kDa protein in K10 WCS protein lysates beginning 7 to 14 days postchallenge and continuing throughout the study (Fig. 5). A ~60-kDa protein was also detected by day 35 in animals 5902 and 5904. Reactivity to the 60-kDa antigen decreased over time in animal 5902 and was variable in intensity in animal 5904. Immunoblot analysis of serum samples from four \(M. bovis\)-infected cattle (housed in a manner similar to that of \(M. avium\) subsp. \(paratuberculosis\)-infected calves, intratonsillarily inoculated, and ~1 year of age) did not react to the 50- and 60-kDa antigens (data not shown), demonstrating the specificity of these two antigens.

**DISCUSSION**

The primary objective of this study was to determine if inoculation of \(M. avium\) subsp. \(paratuberculosis\) into the tonsillar crypts of neonatal calves would result in infection and/or a detectable immune response, as previously demonstrated with \(M. bovis\) (37). Colonization of gastrointestinal tissues and fecal shedding of \(M. avium\) subsp. \(paratuberculosis\) was indicative of infection. Both humoral and cellular immune responses were also detected. Specific CD4\(^+\) cell proliferative and IFN-\(\gamma\) responses occurred concurrently with humoral responses to a LAM-enriched antigen preparation. Both cellular responses and LAM-specific antibody were indicative of infec-

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ungated(^b)</th>
<th>CD4(^+)</th>
<th>CD8(^-)</th>
<th>(\gamma&amp;\delta) TCR(^+)</th>
<th>IgM(^+)</th>
</tr>
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<tbody>
<tr>
<td>No stimulation</td>
<td>1.07 (0.49)</td>
<td>0.51 (0.22)</td>
<td>0.34 (0.22)</td>
<td>0.11 (0.11)</td>
<td></td>
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<tr>
<td>WCS stimulation</td>
<td>12.16 (4.57)</td>
<td>6.95 (3.27)</td>
<td>0.92 (0.44)</td>
<td>0.63 (0.16)</td>
<td></td>
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<tr>
<td>PPDa stimulation</td>
<td>7.10 (2.65)</td>
<td>3.92 (1.10)</td>
<td>0.97 (0.71)</td>
<td>0.68 (0.32)</td>
<td></td>
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\(^a\) At 313 days postchallenge, PBMC were cultured with either medium alone, 5 \(\mu g\) of PPDa/ml, or 10 \(\mu g\) of strain 19698 WCS (WCS/ml) for 7 days (ionomycin, PMA, and brefeldin A added for the terminal 5 h), harvested, and analyzed by flow cytometry for phenotype and intracellular IFN-\(\gamma\) by flow cytometry. Data are presented as mean (±SEM; \(n = 3\)) percent of cells staining positive for IFN-\(\gamma\).

\(^b\) Ungated refers to the response of the total live PBMC population. Differs (*, \(P = 0.07\); **, \(P < 0.05\)) from nonstimulated samples, same subset (i.e., vertical comparisons within group). Responses were not detected for samples obtained from noninfected animals of the same sex, age, breed, and herd of origin as infected animals (data not shown; \(n = 3\)).
tion and not simply sensitization without colonization, as they were not detected until 4 to 5 months after the final *M. avium* subsp. *paratuberculosis* inoculation. Despite detection of a specific immune response and intestinal colonization, intestinal lesions were not detected in inoculated calves. Natural progression of cattle to clinical disease and associated lesion formation generally occur after a prolonged subclinical period of at least 3 to 5 years. More prolonged studies are under way to determine if this route of inoculation will induce clinical disease.

Sera from inoculated calves reacted with at least two *M. avium* subsp. *paratuberculosis* antigens by immunoblot analysis within 2 weeks after the initial intratonsillar challenge, and this antibody response persisted to the termination of the experiment. While the identity of these antigens is unknown, efforts to identify them from a *M. avium* subsp. *paratuberculosis* genomic expression library are being pursued. In contrast to prevailing dogma (10, 43), present findings (in conjunction with another recent report by Koets et al. [29]) indicate that tests for *M. avium* subsp. *paratuberculosis*-specific antibody may provide an early diagnosis of *M. avium* subsp. *paratuberculosis*-infected cattle. A serologic assay for detection of recently infected calves would greatly enhance paratuberculosis eradication efforts and likely prove more practical than cell-based assays.

Present findings confirm the predominant contribution of CD4+ cells to the peripheral IFN-γ (4) and proliferative response during the early, subclinical course of infection. Responding CD4+ cells also upregulated CD26 and CD45RO expression, indicating conversion to an effector-memory phenotype. Progression of cattle from subclinical to clinical Johne's diseases is associated with a decreased ability of mononuclear...
cells to produce IFN-γ, both specifically and nonspecifically, at the site of infection and in the blood (30, 44, 46, 51 and reviewed in references 10 and 43). Unlike tuberculous mycobacteria (20), *M. avium* subsp. *paratuberculosis* is relatively resistant to the killing actions of IFN-γ and NO (56, 59, 60). Additionally, the ability of *M. avium* subsp. *paratuberculosis* to induce IL-10 secretion, both in vitro and at the site of infection, likely suppresses macrophage activation and subsequent intracellular killing ability (15, 27, 56). Compartmentalization and loss of specific effector cells, both host and pathogen mediated, also limit containment of *M. avium* subsp. *paratuberculosis* (11, 28). Additional studies are under way to further characterize the sustainability of this response as experimentally infected cattle progress from subclinical to clinical disease.

LAM-based ELISA assays have demonstrated utility for diagnosis of mycobacterial diseases, including *M. avium* subsp. *paratuberculosis* infection (23, 26, 29, 33, 48–50). It is speculated that selective binding of the lipid portion of LAM to the test plate leaves the more immunogenic carbohydrate portion of the molecule free to react with antibody in the sample. In the present study, sensitivity of the LAM-based assay was maximized through use of a very low *M. phlei* concentration for preabsorption of sera to remove cross-reactive antibodies against antigenically similar bacteria. Failure of the commercial ELISA to detect an antibody response may have been due to preabsorption with a higher *M. phlei* concentration, or the commercial test may have been designed to detect only antibodies against protoplasmic protein antigens expressed later in the course of the disease. Utilization of comparative genomics to uncover unique *M. avium* subsp. *paratuberculosis* genes (especially in comparison to other *M. avium* species) will be necessary to develop antigens specifically recognized by samples from *M. avium* subsp. *paratuberculosis*-infected animals. Experimental models of infection, as described in this study, should prove useful for the evaluation of such unique antigens.

Two compelling research issues concerning *M. avium* subsp. *paratuberculosis* are (i) the mechanisms responsible for progression of cattle from subclinical to clinical disease and (ii) the potential for early diagnosis. An experimental model(s) of infection, as described in this report, will be necessary to fully address these two issues. Present findings indicate that mycobacteria-specific antibody is detectable early in the course of disease and concurrently with a robust CD4+ IFN-γ response. Further studies are under way to evaluate and compare responses of cattle experimentally infected with *M. avium* subsp. *paratuberculosis*, *M. avium* subsp. *avium*, or *M. bovis* to develop tools for specific mycobacterial disease diagnosis and to evaluate specific immunopathogenesis.

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